NFκB mediates the anti-inflammatory actions of liraglutide and sitagliptin in experimentally model of colitis in mice

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Abstract

Inflammatory bowel disease (IBD) is a serious illness that negatively affects the human health due to its chronic course and serious complications. Glucagon like peptide (GLP-1) and its degradation enzyme inhibitor; dipeptidyl peptidase (DPP)-IV inhibitor, are used primarily as anti-diabetic drugs in patients with type 2 diabetes mellitus. However, current evidence suggests that both; GLP-1 (e.g. liraglutide) and the DPP-IV inhibitor (e.g. sitagliptin) may have a potential anti-inflammatory effect on various organ systems. This study was aimed to evaluate the potential of liraglutide and sitagliptin to improve colitis induced experimentally in mice using intra-rectal acetic acid, in comparison with sulfasalazine. Intra-rectal acetic acid was used to induce colitis in mice. The degree of inflammation was assessed using disease activity index, histopathological scoring, colonic length measurement as well as the colonic tissue expression of: the transcription factor; nuclear factor kappa B (NFκB), tumor necrosis factor alpha (TNFα), the oxidative stress marker; malondialdehyde and the inflammatory parameter; C-reactive protein. Moreover, random blood glucose was measured to ensure the safety of the tested drugs. Our results showed the positive impact of both liraglutide and sitagliptin on the assessed inflammatory parameters and their tolerability compared with sulfasalazine. Further clinical studies are needed to investigate the possibility to consider GLP axis as therapeutic adjuvants for IBD in the future.

1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are the main two types of inflammatory bowel disease (IBD) [1]. A disturbed intestinal immune response to the luminal microbiota is a well-recognized theory for IBD pathogenesis [2].

The ingested food stimulates the release of Glucagon-like peptides (GLPs); GLP-1 and GLP-2 from the intestinal enteroendocrine cells; L cells. GLP-1 is mainly concerned with lowering blood glucose by: stimulating the release of insulin from islet β cells and delaying gastric emptying [3-5]. In the same context, GLP-2 has the ability to restore intestinal homeostasis through its trophic effects on the intestinal epithelium, so it is currently used for treatment of short bowel syndrome (SBS) [6,7].

Both GLPs are degraded by dipeptidyl peptidase-IV (DPP-IV) enzymes [5], therefore DPP-IV inhibitors as well as GLP-1 are used to treat patients with type 2 diabetes mellitus (T2DM), as a monotherapy or in combination with other anti-diabetic drugs [8,9]. However, many experimental animal studies have proved that GLPs-based therapy has pivotal anti-inflammatory actions on various organs [10,11].

The aim of this work was to investigate the possible beneficial actions of the GLP-1; liraglutide and the DPP-IV inhibitor; sitagliptin, in treatment of UC induced experimentally in mice, in comparison with sulfasalazine, and to determine their tolerability and the possible underlying mechanism of action.

2. Materials and methods

2.1. Drugs and Chemicals :

1. Glacial acetic acid (A.A) is obtained from Elshark Al-Awsat trade, Egypt.
2. Liraglutide (Victosa pen): 6mg/ml (Novo nordisk , Denmark).
3. Sitagliptin (Januvia®): 100mg/Tablet (MERCK, Germany). Tablets were crushed and suspended in distilled water forming a suspension that was given orally by gavage.
4. Sulfasalazine powder (Alexandria Company for pharmaceutical and chemical industries, Egypt).
5. Formalin: Formaldehyde solution. 38-40 %(Al-Nasr Pharma. Chemicals, Egypt).

2.2. Kits and instruments:

1. Mouse tissue Tumor necrosis factor- α (TNF-α) ELISA Kits. (Gamma trade, Egypt).
2. Mouse Tissue nuclear factor kappa B (NFκB) ELISA Kits. (Gamma trade, Egypt).
3. Colorometric Kits for Mouse tissue Malondialdehyde (MDA) (Gamma trade, Egypt).
4. Coulter analyzer for complete blood picture (CBC) (Faculty of Medicine, Cairo University, Egypt).
5. Mouse C Reactive Protein (CRP) ELISA Kit. (Gamma trade, Egypt).
6. Glucometer to measure blood glucose (Gluco Dr O2).

2.3. Animals and grouping:

42 adult male Swiss albino mice, obtained from the Cairo university animal house, weighing between 35-40 g. Mice were housed and placed in a room; with controlled temperature of 22 ± 3°C and a 12 hour light/dark cycle. Animals had free access to food and water throughout the study. All experiments followed the guidelines for the Care and Use of Laboratory Animals 8th Edition (2011), that is adopted by the Institutional Animal Care and Use Committee (IACUC) of Cairo University, Approval number:(CU, III, F, 81, 18), and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Before conducting experiments, animals were acclimatized to laboratory conditions for 7 days and observed to exclude any mouse with prominent gastrointestinal dysfunction (i.e. Diarrhea, mucus secretion). Mice were randomly divided into 7 groups equally. **Group 1** is control group. After fasting for 24 hours, and under isoflurane 2% anesthesia, mice in control group underwent colonic lavage with normal saline using a 4cm-polypropylene trocar cannula inserted through the rectum then left untreated all through the experiment. While groups 2-7 received 0.1 ml of acetic acid 4% intra-rectally that was left in contact with colonic tissue for 30 seconds to induce colitis, followed by colonic lavage with saline [12]. After 24 hours, all six groups continued treatment regimen for 10 days as follows: - **Group 2**: Acetic-acid/ A-A group: received saline by oral gavage. - **Group 3**: Liraglutide group: Mice in this group received liraglutide at a starting dose of 0.3 mg/kg/day S.C injection, increased to 0.6 mg/kg/day on day 2 then 1mg/kg/d from day 3 to day 10 [13]. - **Group 4**: Sitagliptin group: Mice in this group received sitagliptin 100 mg/kg/d by oral gavage once daily [14]. - **Group 5**: Sulfasalazine group: Mice in this group received sulfasalazine in a dose of 100 mg/kg/d by oral gavage once daily [15]. - **Group 6:** Combined Liraglutide-Sulfasalazine group/ CLS: Mice in this group received a combination of both drugs (liraglutide as mentioned in Liraglutide group) and (sulfasalazine 100 mg/kg/d) by oral gavage. - **Group 7**: Combined Sitagliptin-Sulfasalazine group/ CSS: Mice in this group received a combination of both drugs (sitagliptin 100 mg/kg/d) and (sulfasalazine 100 mg/kg/d) by oral gavage. Random blood glucose (RBG) was measured daily using a glucometer. Colitis activity was evaluated daily using a disease activity index (DAI) by recording weight loss, the incidence of diarrhea and rectal bleeding and scoring the findings in a range of 0-3, (0= unaffected, 1= mild, 2= moderate and 3= severe) to estimate the overall DAI [16]. After a 10 day course of treatment, mice were euthanized by cervical dislocation under 2% isoflurane inhalation. Length of colon segments were measured, dissected and cut into 2 parts each was about 2 cm in length. One part was immersed in 10% formalin for histopathological examination and the other part was frozen at -80°C, to be prepared as a tissue homogenate for testing the colonic tissue expression of NFκB, TNF-α, MDA and CRP.

2.4. Histopathological analysis

Colonic segments were immersed in paraffin wax and stained with haematoxylin and eosin (H&E). For histopathological evaluation of colitis, each segment was evaluated as regards: architectural distortion, inflammatory infiltration by (neutrophils, eosinophils, lymphocytes and macrophages), the incidence of cryp-

titis and mucosal ulceration. Each parameter was then scored in a range of 0-3, (0= unaffected, 1= mild, 2= moderate and 3= severe).

2.5. Biochemical assays

The anti-inflammatory actions of the used drugs were evaluated by measuring colonic expression of the NF-ϰB, TNF-α, MDA as a marker of lipid peroxidation, and CRP as an inflammatory index. To compare the possible incidence of hypoglycemia, RBG levels were measured in all groups.

2.6. Statistical method:

The SPSS version 25 is the statistical package used to code and enter the data. For quantitative data, variables were summarized as mean and standard deviation. For comparison between groups, analysis of variance (ANOVA) with post hoc test for multiple comparisons was applied. Data with P-values less than 0.05 were considered statistically significant [17,18].

3. Results

3.1 Disease activity index:

DAI is an applicable guide to quantify the severity of intestinal inflammation. A-A group showed a significant increase in DAI compared to the control group. All treated groups showed significant lower DAI compared to A-A group. Liraglutide, Sitagliptin, Sulfasalazine, CLS and CSS groups showed a mean DAI of (2.3 ± 0.5), (2.0 ± 0.3), (2.6 ± 0.4), (1.9 ± 0.5) and (1.9 ± 0.2) respectively, compared to A-A group (5.2± 0.4).

3.2 Colonic Length

A-A treated group had a highly significant shortening in colon length (9.7± 0.3) as compared to the control group (12.32 ± 1.45). Contrarily, Liraglutide, Sitagliptin, Sulfasalazine, CLS and CSS treated groups showed a non-significant increase in colon length compared to the A-A group as shown in **figure 1** with the results respectively are (11.2 ± 0.82, 10.81± .078, 11.04± 0.45, 11.46± 1.02, 11.04±0.46).

3.3 Histopathological analysis

Intra-rectal injection of A-A induced a significant colonic inflammation characterized by architectural distortion, inflammatory infiltration by (neutrophils, eosinophils, lymphocytes and plasma cell), cryptitis and mucosal ulceration in comparison to the control group (P value 0.04) as shown in **figure 2** . However, there were no statistically significant differences for overall colonic inflammation between the diseased groups.

3.4 Inflammatory and oxidative markers

A-A-induced a significant increase in colonic NF-ϰB and TNF-α compared with the control group as seen in **figure 3A and figure 3B** . Furthermore, Liraglutide, Sitagliptin, Sulfasalazine, CLS and CSS significantly reduced NF-ϰB and TNF-α levels as compared to A-A group. However, CLS and CSS produced a more significant reduction of NF-ϰB level, while CSS showed a more significant reduction of TNF-α level as compared to A-A group.

As regards the oxidative stress marker, MDA level was significantly increased in A-A group in comparison to the control group. Liraglutide, Sitagliptin, CLS and CSS groups had significant lower levels as compared to A-A group with the best results are observed in CLS and CSS groups that showed no significant difference than the control group (Figure 3C and Figure 3D).

3.5 Random blood glucose(RBG)

While A-A group showed non-significant lower values of RBG than the control group. All other treated mice groups showed significant improvement in RBG values than A-A group with no significant differences than the values of the control group (Figure 3E).

4. Discussion

Intra-rectal administration of 4% acetic acid (A-A) is a well-known experimental model to induce acute colitis chemically in mice. A-A simulates mucosal injury and ulcerations, enhances vaso-permeability and neutrophil infiltration and up-regulates inflammatory mediators similarly to those seen in human IBD [19, 20].

NFκB is a transcription factor controlling the transcription of DNA, cytokine production, and cell survival. In inactivated immune cells, most of the NFκB dimers are inactivated and retained in the cytoplasm associated with small inhibitory molecules (IκBa, IκBb and IκB) [21, 22]. If there is a defect in the intestinal barrier, bacterial antigens can get access to the antigen-presenting cells (APC) in the intestinal lamina propria [23, 24]. These cells then present the antigens to $CD4^+$ lymphocytes and macrophages that will start the NFκB signaling pathway. Activation of the NFκB signalling pathway results in the release of NFκB from its inhibitory molecules and its nuclear localization, thereby inducing the expression of NFκB target genes [25, 26]. The increased NF-κB expression in the intestinal mucosa results in an increased capacity of these cells to produce and secrete pro-inflammatory cytokines such as TNF-α, IL-1β, IL-13, IL-21, IL-22, IL-6. NFκB is also able to regulate the expression of IL-12 and IL-23 that are directly involved in the mucosal damage typically seen in IBD. NFκB is simultaneously activated by TNF-α thereby providing a kind of positive feedback effect, which maintains the chronicity of the IBD [27, 28].

Sulfasalazine is a well-known drug used in treatment of IBD [29]. Wahl et al. (1998) studied the effect of sulfasalazine on NFκB and found that sulfasalazine can inhibit both activation and nuclear translocation of NFκB in response to three different stimuli (TNF-α, lipopolysaccharide (LPS), or phorbol ester). These data suggest that the anti-inflammatory activity of sulfasalazine may rely on its ability to modulate the actions of NFκB signalling [30].

In this study, experimentally-induced colitis led to a significant increase in the expression of the transcription factor, NFκB and the pro-inflammatory marker, TNF-α in the colon of mice as compared to the control group. This is in consistent with study of Schottelius and Baldwin (1999) that pointed out that the increased expression of NFκB is crucial for the initiation and conserving of chronic intestinal inflammation [31]. In the same context, Daneshmand et al. (2009) and Abdel-Daim et al. (2015) concluded that the exaggerated release of TNF-α in A-A model of colitis mediates the intestinal damage observed in IBD [19, 20].

Moreover, in the present study, liraglutide and sitagliptin, either alone or combined to sulfasalazine reduced colonic levels of NF-κB and TNF- α in mice. This can be explained by the ability of GLP-1R agonist to down-regulate NF-κB phosphorylation and nuclear translocation that results in reduction in the expression of the pro-inflammatory cytokines, such as TNF- α that is influential in IBD pathophysiology [25]. Similarly, DPP-IV inhibitors can partially ameliorate the IBD pathology through increasing the endogenous levels of GLP-1 and GLP-2. Tang et al. (2016) reported that the elevated levels of GLP-1 seen with DPP-IV inhibitors, subsequently suppressed the NF-κB/IκBα set-up that is responsible for NF-κB activation [32]. Furthermore, the correlation between GLP-2 and inflammation was investigated in a study by Xia et al. (2014) that studied the effect of GLP-2 on LPS-induced inflammation in macrophages. GLP-2 was found to inhibit LPS-induced NFκB translocation, IκB-degradation and IκB-α phosphorylation and thus attenuates the inflammatory cascade [33]. Moreover, Broxmeyer et al. (2012) showed that DPP-IV inhibitors have important immunomodulatory actions through the recruitment of immune cells (especially T lymphocytes) and the inhibition of the NFκB-dependent transcription of pro-inflammatory cytokines [34].

Lipid peroxidation evidenced by the high MDA level in UC patients is one of the pathogenic mechanisms of UC. Oxidants play an important role in the chronicity of IBD by increasing the number of neutrophils and macrophages that induce a self-sustaining activation loop [35]. It is famed that sulfasalazine and its metabolites are highly effective reactive oxygen metabolite scavengers that decrease MDA levels [36]. On the other hand, cytokines associated with IBD activity (IL-6, TNF-α, and IL-1 β) stimulate the production of CRP over baseline levels, which are typically less than 1 mg/L [37]. Additionally, Huh et al. (2019) mentioned in a study that evaluated some risk factors and predictors for hospitalization of patients with IBD, that in UC, a serum CRP level > 0.5 mg/dL was the only independent risk factor to predict hospitalization [38]. Therefore, CRP is considered a good predictor of disease remission and response.

As shown in **figure 3C** and **figure 3D** , our data demonstrates that Liraglutide, CLS and CSS groups showed significant reduction in MDA levels. While Sitagliptin, CLS and CSS groups decreased the CRP levels in comparison to A-A group with no significant difference than the control group. In a study investigating the effect of liraglutide on peripheral neuropathy in diabetic rats, treatment with liraglutide normalized MDA levels and increased superoxide dismutase level in sciatic nerve [39]. Similarly Varanasi et al. (2012) documented a decline in the mean CRP concentration in patients with T2DM treated with liraglutide [40]. On the other hand, various studies have investigated the anti-oxidant effect of DPP-IV inhibitors. Mega et al. (2011) and Omolekulo et al. (2019) supported the anti-oxidant effect of DPP-IV inhibitors, particularly by sitagliptin in animal models of diabetic nephropathy and insulin resistance respectively that showed significant reduction in plasma MDA levels [41, 42]. While Tremblay et al., (2014) has reported that Sitagliptin, most likely by increasing plasma GLP-1 levels and improving glucose-insulin homeostasis, can down-regulate CRP concentration [43] which may explain the lower CRP levels observed with GLP based therapy [35].

Additionally, several preclinical studies have reinforced the potential anti-inflammatory effects of GLP-1 as regards IBD through regulating invariant natural killer T cells (iNKT) activity, decreasing macrophage propagation, and suppressing lymphocyte maturation and differentiation [44]. In support of these data, Yusta et al. (2015) has reported that GLP-1 receptor agonists can significantly cause a reduction in the expression of epidermal growth factor receptor (EGF), transforming growth factor (TGF)-β1, keratinocyte growth factor (KGF) and the interleukins; IL-6, IL-1 β, and IL-2, that are major constituents of the innate immunity and are involved in mucosal repair [45]. Moreover, Anbazhagan et al. (2017) showed that treatment of dextran sodium sulfate (DSS)-induced colitis with GLP-1 coated with sterically stabilized phospholipid micelles has significantly ameliorated the progress of colitis with subsequent improvement in the epithelial architecture [46]. In parallel, Bang-Berthelsen et al. (2016) found that liraglutide can improve IBD activity endpoints that include colon length and weight as well as colonic tissues histological changes [8].

Moreover, sitagliptin can potentiate the intestine-tropic effects of the endogenous GLP-2. The later was found to improve the intestinal mucosal tight junctions, decreases the internalization of enteric microbiota and to decrease plasma LPS concentration together with a significant reduction in macrophage migration and the production of oxidative stress markers; iNOS and NADPHox. While lacking of GLP-2 effects was associated with increased liability to gastrointestinal inflammation [47-49].

In the same context, Moran et al. (2012) hypothesized that the reduced activity of DPP-IV enzymes reported in patients with active CD may represent the body's venture to increase the intestino-trophic and anti-inflammatory effects of endogenous GLP-2 and GLP-1 respectively [50]. Furthermore, in an experimental model of colitis in mice, EMDB-1, a novel DPP IV inhibitor showed remarkable anti-inflammatory effect that may be explained by the upregulation of endogenous GLP-1 and GLP-2 levels [51]. Moreover, Higashijima et al. (2015) has reported that DPP-IV inhibitors can adjust the immune response in a rat model of nephritis by reducing macrophage infiltration [52]. Yazbeck et al. (2010) as well, disclosed that sitagliptin has altered the secretion of pro-inflammatory cytokines (IFN-c and IL-6), besides having the ability to regulate the production of TGF-β which modulates immune cells differentiation, maturation, apoptosis and actions [53].

Contrarily Abrahami et al. (2018) suggested a probable alliance between inhibition of DPP-IV enzyme and IBD sequel [54]. However, a meta-analysis carried out by Radel et al. (2019) has invalidated Abrahami's findings and pointed out that DPP-IV inhibitors are not correlated with IBD incidence [55], but the need for long-term clinical trials designed to identify the role of DPP-IV inhibitors in IBD is a limitation to end this argument.

Nevertheless, while hypoglycemia is one of the adverse effects reported for sulfasalazine, (possibly through its sulfapyridine component which is structurally similar to glyburide which is a member of the hypoglycemic sulfonylurea group) [56, 57], this study has supported the euoglycemic effect of GLP-1 and DPP-IV based therapy. As liraglutide and sitagliptin are well-known to have a glucose-dependent action, therefore they are essentially well tolerated and are not familiar to cause hypoglycemia unless combined with other oral hypoglycemic drugs or insulin [58].

5. Conclusion

These findings indicate the possible potential of GLP based therapy, as an add-on therapy to sulfasalazine in ameliorating IBD in A-A model of IBD in mice through regulating the secretion of the transcription factor NFκB- dependent inflammatory cytokines, decreasing the oxidative stress injury, promoting tissue repair of injured epithelium with better tolerated side effects. However, further long-term studies to evaluate the tolerability and efficacy of these drugs are required and the decision to choose any of these drugs should be tailored according to the general status of each patient and the presence of any compelling indication or contraindication.

6. Declaration

Ethical Approval: All experiments followed the guidelines for the Care and Use of Laboratory Animals 8th Edition (2011), that is adopted by the Institutional Animal Care and Use Committee (IACUC) of Cairo University, Approval number: (CU, III, F, 81, 18).**Consent to Participate**: Not applicable**Consent to Publish:** Not applicable**Availability of data and materials:** available upon request**Code availability:** not applicable**Author contributions:** OAN, MIA, NEE and WMH have made substantial contributions to conception and design. OAN has made substantial contributions to conduction of the research, acquisition of data, their analysis, their interpretation and wrote the manuscript. All authors have given final approval of the version to be published. The authors declare that all data were generated in-house and that no paper mill was used.**Funding**: This study was financially supported by the Department of medical pharmacology, Cairo University, Egypt. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.**Acknowledgements** The authors wish to thank Dr Laila Rashed, Professor of Biochemistery, Faculty of Medicine, Cairo University, Egypt, for the biochemical results in this study.**7. References**

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8. Figure legends

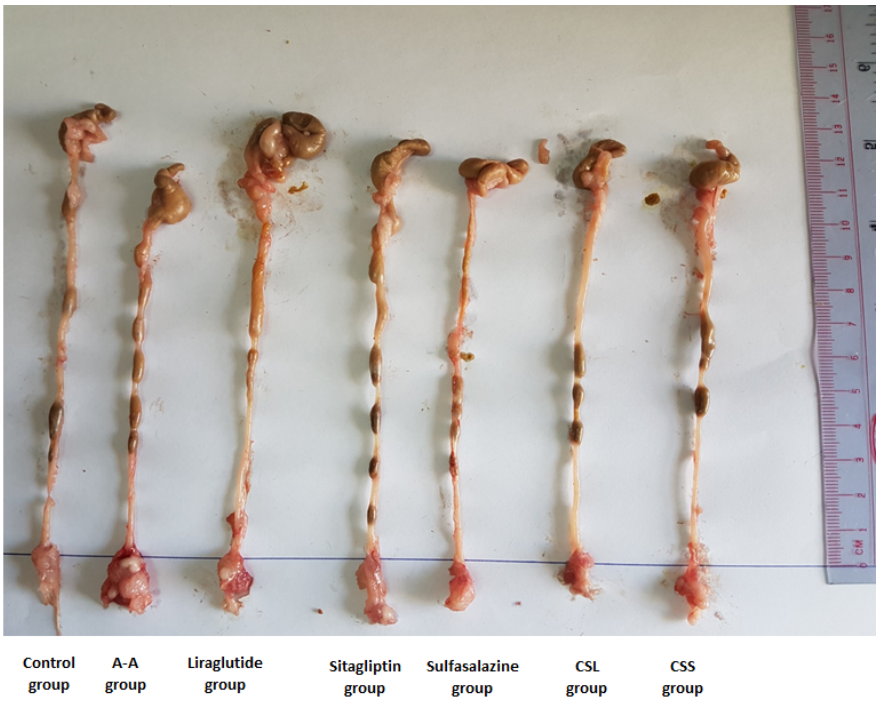

Fig I. Colonic length measurement in different studied groups

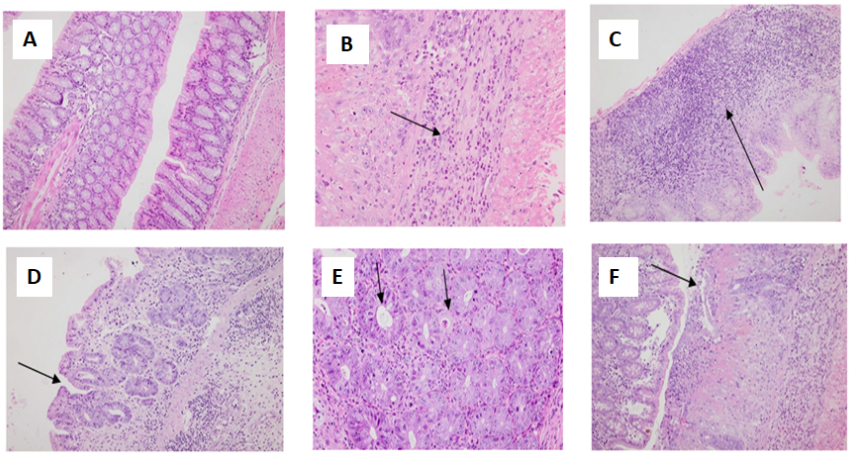

Fig II: **(A)** Microscopic picture showing: Normal histopathology of mice colon, **(B)** Microscopic picture showing: Submucosal marked acute inflammatory infiltrate, **(C)** Microscopic picture showing: Marked chronic inflammatory infiltrate, **(D)**Microscopic picture showing: Cryptitis, **(E)** Microscopic picture showing: Crypt abscess, **(F)** Microscopic picture showing: Mucosal ulceration

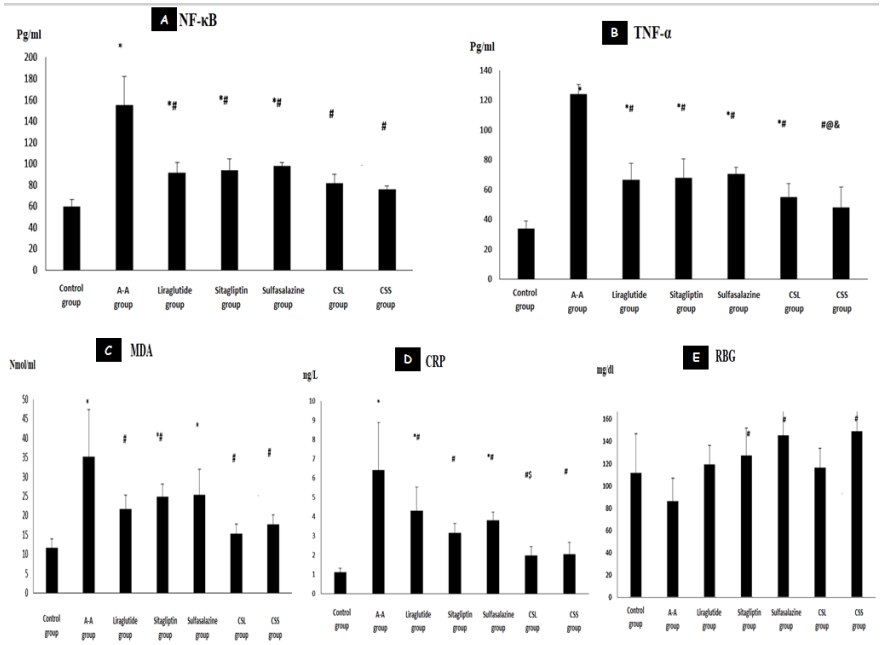

Fig III. Bar graph showing **(A)** colonic tissue concentrations of NFκB, **(B)** colonic tissue concentrations of TNF-α, **(C)** colonic tissue concentrations of Malondialdehyde (MDA), **(D)** Blood level of C-reactive protein (CRP),**(E)** Random blood glucose (RBG) concentration. Values are presented as mean ±SD. *: P<0.05 compared to control group, #: P<0.05 compared to acetic-acid group, @: P<0.05 compared to Sitagliptin group. &: P<0.05 compared to Sulfasalazine group, $: P<0.05 compared to corresponding value in liraglutide group.