Anti-inflammatory effect of metformin against an experimental model of LPS-induced cytokine storm

IBRAHIM TAHER[1], EMAN EL-MASRY[1,2], MOHAMED ABOUELKHEIR[3,4] and AHMED E. TAHA[1,5]

[1]Microbiology and Immunology Unit, Department of Pathology, College of Medicine, Jouf University, Sakaka 72388, Saudi Arabia; [2]Department of Medical Microbiology and Immunology, College of Medicine, Menoufia University, Shebin El Koum 32511, Egypt; [3]Department of Pharmacology and Therapeutics, College of Medicine, Jouf University, Sakaka 72388, Saudi Arabia; [4]Department of Pharmacology, Faculty of Medicine, Mansoura University, Mansoura 35516; [5]Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt

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Abstract. Cytokine storm is one of the leading causes of death in patients with COVID-19. Metformin has been shown to inhibit the action of a wide range of proinflammatory cytokines such as IL-6, and TNF-α which may ultimately affect cytokine storm due to Covid-19. The present study analyzed the anti-inflammatory effect of oral and intraperitoneal (IP) metformin administration routes in a mouse model of lipopolysaccharide (LPS)-induced cytokine storm. A total of 60 female BALB/c mice were randomly assigned to one of six groups: i) Control; ii) LPS model; iii) oral saline + LPS; iv) oral metformin + LPS; v) IP saline + LPS; and vi) IP metformin + LPS. Metformin or saline were administered to the mice for 30 days, after which an IP injection of 0.5 mg/kg LPS induced a cytokine storm in the five treatment groups. Mice were sacrificed and serum cytokine levels were measured. Pretreatment of mice with either oral or IP metformin significantly reduced the increase in IL-1, IL-6 and TNF-α following LPS injection. Both metformin administration routes significantly reduced IL-1 and TNF-α levels, although IP metformin appeared to be significantly more effective at reducing IL-6 levels compared with oral metformin. Neither the oral or IP route of administration of metformin demonstrated a significant effect on IL-17 levels. Based on its ability to suppress the proinflammatory LPS-induced cytokine storm, metformin may have future potential benefits in ameliorating human diseases caused by elevated cytokine levels.

Correspondence to: Dr Ibrahim Taher, Microbiology and Immunology Unit, Department of Pathology, College of Medicine, Jouf University, 5404-Qunitra Street, Sakaka 72388, Saudi Arabia
E-mail: itaher@ju.edu.sa

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was the causative agent of the Coronavirus disease 2019 (COVID-19) pandemic. A number of drug treatments such as antiviral drugs; monoclonal antibodies; convalescent plasma and cytokine therapy targeting the SARS-CoV-2 immunopathological process have recently been either approved or tested to treat COVID-19 (1,2). While a majority of the cases of SARS-CoV-2 infection are asymptomatic or associated with mild symptoms, 10-20% of patients with COVID-19 may encounter acute respiratory distress syndrome (ARDS), particularly patients who are elderly or have co-morbidities (3). Recent studies have reported a fatal immunopathological process defined as a 'cytokine storm', which is a component of the macrophage activation syndrome, also known as secondary hemophagocytic lymphohistiocytosis that can lead to ARDS (4,5). Several cytokines, such as: IL-1; IL-5; IL-7; IL-9; IL-10 and TNF-α, were detected at higher concentrations in the serum of patients with a severe case of COVID-19, compared with those who had milder infections (6-10). Further research assessing the cytokine profile changes and their mechanisms are necessary to comprehend how COVID-19 infections can become severe, in addition to providing information that could be used to develop treatment options to control disease pathogenesis (11).

In order for the immune system to target an invading virus, antigen-presenting cells (APCs) process and present viral antigens to other cells of the immune system. Viral antigens are recognized by $CD8^+$ cytotoxic T lymphocytes and natural killer (NK) cells, activating both the innate and adaptive branches of the immune system. Apoptosis is induced by NK cells and $CD8^+$ cytotoxic T cells to kill virus-infected cells. To avoid the unnecessary initiation of cell death, APCs and $CD8^+$ cytotoxic T cells are being eliminated through apoptosis after antigenic reactivity has ceased. However, defects in the cytotoxic activities of lymphocytes, whether acquired or genetic, can result in a failure of $CD8^+$ cytotoxic T cells and NK cells to lyse infected cells and activate APCs, which leads to exaggerated and prolonged interactions between adaptive and innate immune cells. High levels of serum proinflammatory cytokines, such

as IL-1, IL-6, IL-17 and TNF-α, are then released uncontrollably, resulting in a cytokine storm. Cytokine storm, ARDS, thrombotic tendency, disseminated intravascular coagulation, hepatic dysfunction and multi-organ failure can result from this immunopathologic process (5,11,12). This life-threatening phenomenon is regarded as the leading cause of death in patients with COVID-19 (13,14).

Metformin is an anti-diabetic medication with a well-characterized safety profile. Previous studies have reported that metformin may have other physiological effects in addition to lowering blood glucose levels (15). It may influence the AMP kinase (AMPK)/mTOR signaling pathway, which in turn regulates a variety of proinflammatory cytokines, such as IL-1; IL-2: IL-6; IL-12 and TNF-α (15). Metformin, an immunomodulatory drug with high tolerability, is an add-on drug in the treatment of certain malignant, autoimmune and aging-related conditions, such as antiproliferative and antioxidant effects, T2D, obesity associated inflammation, autoimmune diseases and cardio and nephro-protection (16-23). In patients with chronic inflammation, metformin can be used as a protective or therapeutic option (24,25), including conditions such as colitis-associated colon cancer (26), otitis media (27) and airway inflammation (28). Metformin has previously been reported to aid in the treatment of sepsis-related brain injury by inhibiting neuroinflammation, oxidative stress and apoptosis (29).

The aim of the present study was to evaluate the anti-inflammatory effect of oral and intraperitoneal (IP) metformin in a mouse model of lipopolysaccharide (LPS)-induced cytokine storm.

Materials and methods

Animal model. The present study included 60 female wild-type BALB/c mice aged 5-6 weeks and weighing ~25 g. The animal cages were well ventilated, and subjected to a 12-h light/dark cycle. The relative humidity was kept at around 45-55%. The rooms temperature was adjusted to a range of 22-24˚C. Food and water were freely available to the animals. After 2 weeks of acclimation, mice were randomly assigned to one of six groups (n=10/group): i) Control; ii) LPS model; iii) oral saline + LPS; iv) oral metformin + LPS; v) IP saline + LPS; and vi) IP metformin + LPS. The control group received no intervention other than the collection of blood samples at the end of the experiment to estimate the basal levels of serum cytokines. For 30 days, metformin or saline were administered to the mice via either the oral or IP route. Mice were administered metformin at a concentration of 250 mg/kg body weight diluted in 100 μl normal saline once daily via oral gavage or the IP route (28). Saline-treated groups of mice were administered an equivalent volume of saline (100 μl) daily, either orally or via the IP route. The Jouf University Local Committee of Bioethics approved all experimental procedures (approval no. 07-08-42; Sakaka, Saudi Arabia).

Murine model of LPS-induced cytokine storm. After 30 days of metformin or saline administration, LPS was administered to the five intervention groups of mice through IP injection at a concentration of 0.5 mg/kg body weight (from O111:B4 *Escherichia coli*; MilliporeSigma) to induce a cytokine storm. LPS was prepared in sterile PBS before use. At one hour following LPS was administration, mice were sacrificed by halothane at a lethal dose (5%) for 2-3 min after which animals were confirmed to be dead based on their physical status (unable to walk and lacking response to manipulations). Then blood was collected from the heart (30). Serum separator tubes were used to collect blood samples. Blood samples were allowed to clot for 2 h at room temperature, then centrifuged at 3.74 x g for 15 min at 4˚C, before being stored at -80˚C.

Measurement of cytokines levels. Serum levels of IL-1β, IL-6, IL-17 and TNF-α were determined using ELISA kits according to the manufacturer's instructions (cat. nos. CSB-E08054m, CSB-E04639m, CSB-E04608m and CSB-E04741m, respectively; Cusabio Technology LLC). All experimental measurements were repeated in triplicates.

Statistical analysis. Data are presented as the mean ± standard error of the mean and were analyzed using SPSS (v22; IBM Corp.). One-way ANOVA followed by Tukey's post hoc test was used to determine statistical significance between treatment groups. The correlation among the cytokine levels within each treatment group of mice was analyzed using Spearman's rank correlation coefficient analysis, and the results were presented as P- and rho values. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Serum IL-1β levels. There were no significant differences in the body weight of the treatment groups of mice compared with the control group. LPS injection significantly increased the serum levels of IL-1β in all treatment groups compared with the control ($P<0.05$; Fig. 1). Pretreatment with metformin, either orally or IP, significantly reduced this rise in IL-1β levels ($P<0.05$), with no significant difference demonstrated between the efficacy of the two methods of metformin administration.

Serum IL-6 levels. Compared with the control group, injection of LPS caused a significant elevation of IL-6 levels in all the treatment groups ($P<0.05$; Fig. 2). Pretreatment with either oral or IP metformin significantly attenuated this increase in IL-6 levels ($P<0.05$). IP metformin significantly decreased serum IL-6 levels compared with the oral route of metformin administration ($P<0.05$).

Serum IL-17 levels. Injection of LPS caused a significant increase in serum IL-17 levels in all treatment groups compared with the control group (Fig. 3). However, serum levels of IL-17 were not significantly impacted by the route of metformin administration.

Serum TNF-α levels. Compared with the control, LPS injection caused a significant increase in TNF-α levels across all treatment groups ($P<0.05$; Fig. 4). Metformin pretreatment significantly decreased the TNF-α levels in serum compared with the LPS + saline groups. There was no significant difference demonstrated by the route of metformin administration on the reduction of serum TNF-α levels.

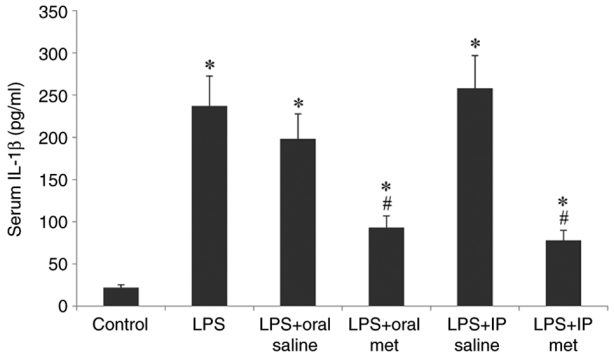

Figure 1. Serum IL-1β levels of BALB/c mice (n=10/group) pretreated with either oral or IP met for 30 days. Mice were treated daily with met (250 mg/kg body weight). LPS (0.5 mg/kg body weight) was administered to initiate a cytokine storm. Data are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control; #P<0.05 vs. corresponding LPS-treated group. LPS, lipopolysaccharide; IP, intraperitoneal; met, metformin.

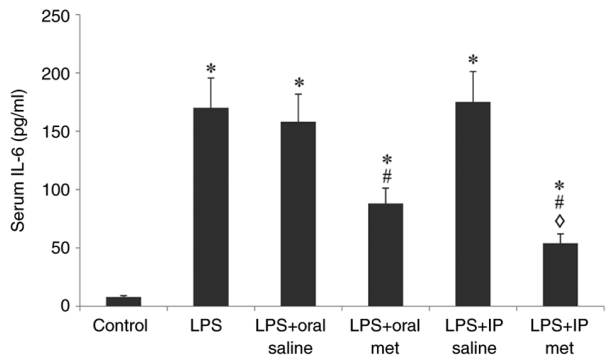

Figure 2. Serum IL-6 levels of BALB/c mice (n=10/group) pretreated with either oral or IP met for 30 days. Mice were treated daily with met (250 mg/kg body weight). LPS (0.5 mg/kg body weight) was administered to initiate a cytokine storm. Data are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control; #P<0.05 vs. corresponding LPS-treated group; ◊P<0.05 vs. LPS + oral met-treated group. LPS, lipopolysaccharide; IP, intraperitoneal; met, metformin.

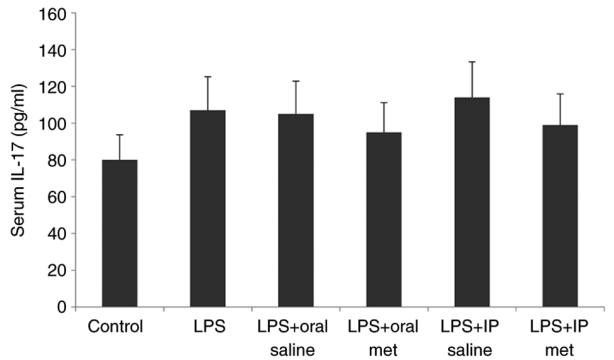

Figure 3. Serum IL-17 levels of BALB/c mice (n=10/group) pretreated with either oral or IP met for 30 days. Mice were treated daily with met (250 mg/kg body weight). LPS (0.5 mg/kg body weight) was administered to initiate a cytokine storm. Data are presented as the mean ± standard error of the mean (n=3. LPS, lipopolysaccharide; IP, intraperitoneal; met, metformin.

Correlation analysis. The results of the Spearman's correlation analysis among measured proinflammatory cytokines within each of the different subgroups demonstrated that certain correlations were statistically significant (Table I). The magnitude of the correlation coefficient indicated the strength of the relationship between the variables, with larger absolute values indicating stronger relationships.

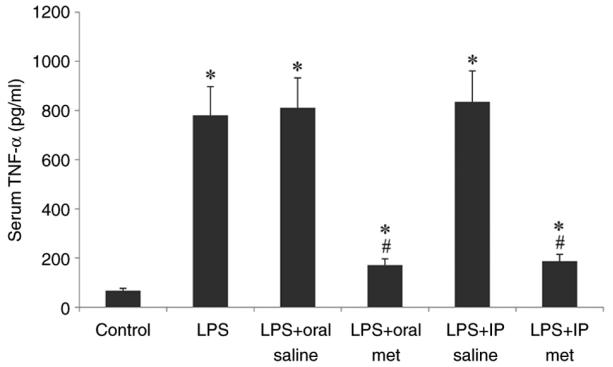

Figure 4. Serum TNF-α levels of BALB/c mice (n=10/group) pretreated with either oral or IP met for 30 days. Mice were treated daily with met (250 mg/kg body weight). LPS (0.5 mg/kg body weight) was administered to initiate a cytokine storm. Data are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control; #P<0.05 vs. corresponding LPS-treated group. LPS, lipopolysaccharide; IP, intraperitoneal; met, metformin.

Discussion

Effective drug treatments targeting the SARS-CoV-2 immunopathological process are of particular interest to reduce the disease burden caused by these infections (1). Metformin, a widely available drug treatment for diabetes, was previously reported to have a cytokine-lowering effect in both diabetic and non-diabetic patients (24). In patients with COVID-19, this effect could be critical as the development of cytokine storm can lead to very lethal outcomes such as ARDS (31). The effect of metformin on the production of cytokines is reported to be caused by blocking the AMPK/mTOR cytokine receptor pathway, which results in a decrease in the expression of certain proinflammatory genes, such as IL-1α, IL-1β, IL-2, IL-6, IL-12 and TNF-α (15). Additional studies suggest that AMPK-independent mechanisms, such as altering the gut microbiota, may also be involved in this process (32-34).

Cytokine storm causes severe illness in patients with COVID-19, thereby significantly increasing the morbidity and mortality rates by ~5% (31). The present study sought to assess the anti-inflammatory effect of metformin in a mouse model of LPS-induced cytokine storm. The present results demonstrated that LPS injection caused a significant increase in serum IL-1β levels when compared with the control group. Oral and IP metformin significantly reduced the elevated IL-1β levels in BALB/c mice 1 h after IP LPS injection, with no significant difference demonstrated in efficacy between the two routes of metformin administration. These findings support previous reports that metformin is a potent inhibitor of the chronic inflammatory response. For example, metformin therapy has been reported to reduce reactive oxygen species and hypoxia-inducible factor-1 (HIF-1) levels, which in turn reduce the IL-1β expression levels after prolonged exposure to proinflammatory LPS stimuli (29,35). Additionally, it has

Table I. Spearman's correlation coefficient analysis between production of serum cytokines in a mouse model of LPS-induced cytokine storm pretreated with oral or IP metformin.

A, Control			
Cytokine	IL-6	IL-17	TNF-α
IL-1	0.027 (0.633)[a]	0.103 (0.438)	0.028 (0.632)[a]
IL-6		0.006 (0.774)[a]	0.210 (0.287)
IL-17			0.067 (0.515)
B, LPS			
Cytokine	IL-6	IL-17	TNF-α
IL-1	0.037 (0.596)[a]	0.049 (0.559)[a]	0.156 (0.358)
IL-6		0.125 (0.399)	0.021 (0.663)[a]
IL-17			0.171 (0.334)
C, LPS + oral saline			
Cytokine	IL-6	IL-17	TNF-α
IL-1	0.027 (0.636)[a]	0.116 (0.418)	0.077 (0.491)
IL-6		0.033 (0.612)[a]	0.214 (0.285)
IL-17			0.089 (0.467)
D, LPS + oral metformin			
Cytokine	IL-6	IL-17	TNF-α
IL-1	0.041 (0.584)[a]	0.033 (0.612)[a]	0.006 (0.770)[a]
IL-6		0.221 (0.274)	0.035 (0.602)[a]
IL-17			0.002 (0.830)[a]
E, LPS + IP saline			
Cytokine	IL-6	IL-17	TNF-α
IL-1	0.005 (0.782)[a]	0.005 (0.782)[a]	0.000 (0.915)[a]
IL-6		0.008 (0.758)[a]	0.015 (0.697)[a]
IL-17			0.002 (0.830)[a]
F, LPS + IP metformin			
Cytokine	IL-6	IL-17	TNF-α
IL-1	0.0014 (0.851)[a]	0.0008 (0.879)[a]	0.005 (0.782)[a]
IL-6		0.003 (0.815)[a]	0.003 (0.815)[a]
IL-17			0.003 (0.818)[a]

Data are presented as P- and (rho) values. [a]P<0.05 (statistically significant). Spearman's rho values range from -1 to +1, where a value of -1 indicates a perfect negative correlation, 0 indicates no correlation, and +1 indicates a perfect positive correlation. LPS, lipopolysaccharide; IP, intraperitoneal.

been previously established that the IL-1β mediated inflammatory response is involved in COVID-19 pathogenesis (36). Therefore, lowering IL-1β levels could reduce inflammation and mortality in patients with COVID-19 (37).

The present study demonstrated that LPS injection resulted in a significant increase in serum IL-6 levels in all treatment groups of animals tested. Pretreatment with either oral or IP metformin significantly reduced this increase in IL-6 levels. IP metformin was significantly more effective at reducing IL-6 levels compared with oral metformin. Hyun *et al* (38) reported that metformin reduces IL-1, IL-6 and TNF-α production at both the protein and mRNA level in a dose-dependent manner. Similarly, Chao *et al* (36) reported that metformin reduces the LPS-induced release of IL-6 in mouse livers. Additionally, metformin inhibits the acute inflammatory response in two macrophage-like cell lines by activating AMPK, but not HIF-1 or IL-10 (39).

TNF-α is an inflammatory cytokine produced in response to bacterial and viral infections and can elicit tissue damage and fibrosis (27). The present study demonstrated that serum TNF-α levels were significantly increased after mice were injected with LPS compared with the controls. Oral or IP metformin were equally effective in attenuating the significantly elevated TNF-α levels, with no significant differences demonstrated between either route of administration. Kim *et al* (30) previously reported that oral administration of metformin to mice treated with LPS reduced the plasma, spleen and lungs tissue levels of both TNF-α and IL-6 leading to a reduction in the effect of LPS-induced inflammation.

Cho *et al* (27) investigated the anti-inflammatory effects of metformin on LPS-induced inflammation in human middle ear epithelial cell lines. LPS was found to elevate TNF-α and cyclooxygenase-2 levels. However, pretreatment with metformin reduced the production of these inflammatory factors. Metformin also decreased the production of sepsis-induced brain injury in mice inflammatory cytokines, such as IL-6, IL-1 β and TNF-α (29). These findings are consistent with the findings of the present study.

The present study demonstrated a non-significant increase in serum IL-17 levels in mice pretreated with LPS. Neither IP nor oral metformin had a significant effect on serum IL-17 levels. One possible explanation for these findings is that the effect of LPS is likely to be dose- or time-dependent. Sun *et al* (40) reported that IL-17A is upregulated in LPS-induced neuroinflammation and cognitive impairment in aged rats. Differences in the findings of the present study regarding IL-17 levels could be due to the time frame of sample collection following LPS injection. It could be possible that 1 h may not be sufficient time to demonstrate a significant increase in serum IL-17 levels. A previous study reported that whilst IL-6 and TNF-α levels peak within 1 h of LPS injection in mice, changes in IL-17 levels are slower and peak over 8 h (41).

Age-related increases in serum levels of IL-17A, IL-17F, IL-21 and IL-6 can be prevented by metformin treatment (42). In a mouse model of scleroderma treated with metformin, Moon *et al* (42) reported a decrease in the production of the proinflammatory cytokine IL-17 in dermal tissues and lymphocytes in a mice model of bleomycin-induced scleroderma. The pathophysiology of IL-17 has been studied in mice challenged with LPS, and it was reported that increased amounts of IL-17 are associated with excessive lung injury and inflammation (43). Neutralizing increased IL-17 production using anti-IL-17 antibodies improves survival and reduces lung injury (43).

Low-grade inflammation caused by microbiota dysbiosis is thought to promote the occurrence of metabolic syndrome (44,45). The gut microbiota may be involved in the regulation of immunity, inflammation and autoinflammatory diseases such as multiple sclerosis and osteomyelitis (46,47). Several studies have reported that metformin can influence the composition of the intestinal microbiota in certain clinical situations such as dysbiosis, intestinal inflammations disorders, and T2D (48-50). It has been reported that the ability of metformin to alleviate certain inflammatory diseases, such as inflammatory bowel disease is linked to its ability to modify the diversity of gut microbiota (51). By contrast, it has also been proposed that the ability of metformin to reduce indices of low-grade inflammation in metabolic syndrome is independent of its effect on the gut microbiota (52,53).

A limitation of the present study is that further studies are required to determine whether metformin has a direct or indirect effect on cultured immune system cells, such as macrophages and T cells. Investigating a potential dose-dependent response of both LPS and metformin and longer time scales between LPS injection and sample collection could also further elucidate the impact of metformin in the mouse model used in the present study.

In the present study, a mouse model of LPS-induced cytokine storm was pretreated with metformin, which was demonstrated to suppress the release of the pro-inflammatory cytokines IL-1β, IL-6, IL-17 and TNF-α. Certain serum cytokine levels demonstrated a positive correlation with other cytokines in the mouse model. These findings may potentially suggest a future role for metformin in the treatment of human diseases associated with the cytokine storm.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All of the authors have made substantial contributions towards the completion of the present study. MA and AET conceived the present study, performed the experiments, were project administrators and prepared the draft manuscript. IAT, EAEM, MA and AET collected the data, obtained resources, performed data analysis and critically reviewed and edited the manuscript. IAT and EAEM acquired funding. IAT, MA and AET supervised the project. IAT and AET confirm the authenticity of the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Local Committee of Bioethics of Jouf University (approval no. 07-08-42; Sakaka, Saudi Arabia).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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