The Effects of Sub-Chronic Treatment with Pioglitazone on the Septic Mice Mortality in the Model of Cecal Ligation and Puncture: Involvement of Nitric Oxide Pathway

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Abstract- Sepsis is a systemic inflammatory response syndrome caused by an infection and remains as a major challenge in health care. Many studies have reported that pioglitazone may display anti-inflammatory effects. This study was designed to evaluate the effect of subchronic treatment with pioglitazone on high-grade septic mice survival and nitrergic system involvement. Diffused sepsis was induced by cecal ligation and puncture (CLP) surgery in male NMRI mice (20-30 g). Pioglitazone (5,10 and 20 mg/kg) was administered by gavage daily for 5 days prior to surgery. Nitric oxide involvement was assessed by sub-chronic administration of a non-selective nitric oxide synthase inhibitor, L-NAME and a selective inducible nitric oxide synthase inhibitor, aminoguanidine. TNF-α and IL-1β plasma levels were measured by ELISA. Pioglitazone (10 and 20 mg/kg) significantly improved survival rate in septic mice. The chronic intraperitoneally co-administration of L-NAME (0.5 mg/kg, daily) or aminoguanidine (1 mg/kg, daily) with a daily dose of pioglitazone, 5 mg/kg, significantly increased the survival rate. This survival improving effect was accompanied by a significant reduction in pro-inflammatory cytokines TNF-α and IL-1β plasma levels. In conclusion, sub-chronic pioglitazone treatment can improve survival in mouse sepsis model by CLP. Inhibition of nitric oxide release, probably through inducible nitric oxide synthase at least in part is responsible for this effect. Suppression of TNF-α and IL-1β could be another mechanism in pioglitazone-induced survival improving effect in septic mice.

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Keywords: Sepsis; Pioglitazone; Nitric oxide; Cecal ligation and puncture; Cytokines; Mice

Introduction

Sepsis is defined as a severe disorder caused by a systemic and dysregulated inflammatory response to an infection (1). This response may result in septic shock and multiple organs dysfunction syndromes that are associated with a high mortality rate in the clinic (2). Nitric oxide is a diffusible and highly reactive intercellular signaling molecule which is produced by various mammalian tissues such as vascular endothelium, macrophages, and neutrophils. Nitric oxide is synthesized by nitric oxide synthase (NOS, a family of heme proteins) along an NADPH-dependent pathway from L-arginine. There are three recognized isoforms of NOS including one inducible (iNOS) and two constitutively expressed (endothelial (eNOS) and neuronal (nNOS)) forms (3,4).

Based upon the previous studies, nitric oxide may exert both pro-inflammatory and anti-inflammatory effects with biphasic regulation of transcription factor NF-κB activity (5). Studies suggest that nitric oxide is a potentially major mediator in the pathogenesis of sepsis (6,7), with both beneficial and deleterious effects (4), which make it an important therapeutic target (8). Nitric oxide production by different isoforms of NOS will play different roles in sepsis; the expression of iNOS is up-regulated while the cNOS expression is down-regulated

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(9). Excessive production of nitric oxide by iNOS following an increase of circulating pro-inflammatory cytokines (such as tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6)) makes a major contribution to sepsis symptoms (4,10). The excessive production of inflammatory cytokines and chemokines by endocrine/immune cells will lead to the majority of damages induced during sepsis (11). Previous experimental studies have shown that non-selective NOS inhibitors increase mortality in septic animals, but aminoguanidine which is a selective inducible NOS inhibitor prevents organ failure and improves survival. Moreover, the studies showed that nitric oxide supplementation is beneficial in sepsis (12-14).

Pioglitazone, a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist of the thiazolidinedione class is used in the treatment of type 2 diabetes mellitus as an insulin sensitizer. Besides the antidiabetic properties, pioglitazone has been shown to inhibit inflammation (15,16). The studies show that PPAR-γ ligands reduce iNOS expression and down-regulate pro-inflammatory cytokines in macrophages (17). It is reported that PPAR-γ ligands are effective for severe sepsis treatment in animal models (18) but may act as a double-edged sword (19).

The current investigation aimed to evaluate sub-chronic pioglitazone effects on high-grade sepsis, induced by cecal ligation and puncture (CLP) method. To elucidate the mechanism of action of pioglitazone on survival, we investigated the role of nitrergic system, IL-1β and TNF-α in pioglitazone effects.

Materials and Methods

Experimental animals

This study was performed on male NMRI mice (20-30 g body weight; Tehran University of Medical Sciences, Iran). They were housed in standard polycarbonate cages and maintained under controlled laboratory conditions (temperature: 24 ± 1ºC, humidity: 55 ± 10%, lighting: 12-h light/dark cycle). Mice were acclimatized to the experimental room for at least two days before performing the experiments with free access to both standard laboratory pellet chow and tap water. All procedures were conducted in compliance with institutional Guideline for the Care and Use of Laboratory Animals with the approval of local Research and Medical Ethics Committee. Because of circadian rhythm effects, experiments carried out at the same time of the day. Each mouse was used only once in this study and each group consisted of at least eight animals.

Chemicals

The following drugs were used throughout this study: pioglitazone, a PPAR-γ agonist; L-NAME [L-NG-Nitro-L-arginine methyl ester hydrochloride], a non-specific inhibitor of NOS; aminoguanidine, a selective inhibitor of iNOS; ketamine and xylazine as the anesthetic/analgesic agents. All drugs were purchased from Sigma (USA). Pioglitazone suspension was prepared in carboxymethyl cellulose (CMC, 0.5%) and was administered once daily by oral gavage for five consecutive days before CLP. A mixture of ketamine and xylazine was given intraperitoneally prior to surgery. L-NAME and aminoguanidine were dissolved in sterile isotonic saline solution and were administered intraperitoneally in a volume of 10 ml/kg of the mice body weight. Mouse specific TNF-α and IL-β ELISA kits were purchased from Sigma (USA).

CLP as a sepsis model

Polymicrobial sepsis was induced by cecal ligation and puncture method, which is considered as a gold standard procedure in sepsis studies, and described by Wichterman and coworkers in 1980 (20). In brief, mice were anesthetized with a mixture of ketamine and xylazine (60 mg/kg and 5 mg/kg respectively, i.p.). After a 1.5 cm longitudinal midline abdominal incision, cecum was exposed and subjected to ligation (75% of the cecum) to induce high-grade sepsis, which was followed by two perforations with a 21-gauge needle, then cecum was relocated into abdominal cavity and incision was closed in layers. Pre-warmed normal saline was injected subcutaneously to all animals to prevent dehydration. Sham-operated mice (control for CLP operation) were submitted to laparotomy only. Survival was monitored for seven days in each group.

Measurement of TNF-α and IL-1β levels

Quantitative detection of TNF-α and IL-1β serum levels were performed with the use of mouse-specific TNF-α and IL-1β ELISA kits according to the manufacturer's instructions. Data were expressed as pg/ml.

Experimental protocol

Before starting the main study, a pilot was designed to set up CLP method. Mice were gavaged with pioglitazone (5,10 and 20 mg/kg) or CMC (0.5%) for 5 days before CLP. The doses were chosen based on previous studies (9,21,22) and pilot experiments. To determine the role of nitric oxide in survival and

improving effect of pioglitazone, we injected L-NAME (0.5 mg/kg) and aminoguanidine (1 mg/kg), 30 min before each non-effective dose of pioglitazone (5 mg/kg) during 5 days. For measurement, the serum levels of TNF-α and IL-1β, blood samples were removed by heart puncture 12 h and 24 h post-CLP in vehicle and pioglitazone-treated groups. Samples were centrifuged at 1000 × g for 10 min at 4°C to separate the serum and then stored at −20°C until the assay.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Survival rates were expressed as percentages using the Kaplan-Meier method and log-rank test. The one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons was used to indicate the statistical significance of differences between the experimental means. *P* value<0.05 was considered significant for all analyzes.

Results

Effects of interventions on survival rates after CLP

To determine the effects of interventions on CLP-induced inflammatory responses, survival rates were calculated post-CLP. All sham-operated mice survived for seven days. The control (CMC) group resulted in no survival. The 7-day survival rate was 20% in pioglitazone 5 mg/kg-CLP group, 54.7% in pioglitazone 10 mg/kg-CLP group and 70% in pioglitazone 20 mg/kg-CLP group. The survival rates were significantly higher in the pioglitazone 10 mg/kg-CLP group and pioglitazone 20 mg/kg-CLP group than in vehicle-CLP group (*P*<0.01 and *P*<0.001) (Figure 1). Next intervention showed that administration of pioglitazone (5 mg/kg) with non-effective dose of L-NAME (0.5 mg/kg, 7-day survival rate: 8.5%), significantly increased the survival rate to 86% in septic mice (*P*<0.001 vs. vehicle-CLP group) (Figure 2). Treatment with pioglitazone (5 mg/kg) and non-effective dose of aminoguanidine (1 mg/kg, 7-day survival rate: 28.5%), improved the survival rate to 90% (*P*<0.001 vs. vehicle-CLP group) after CLP (Figure 3).

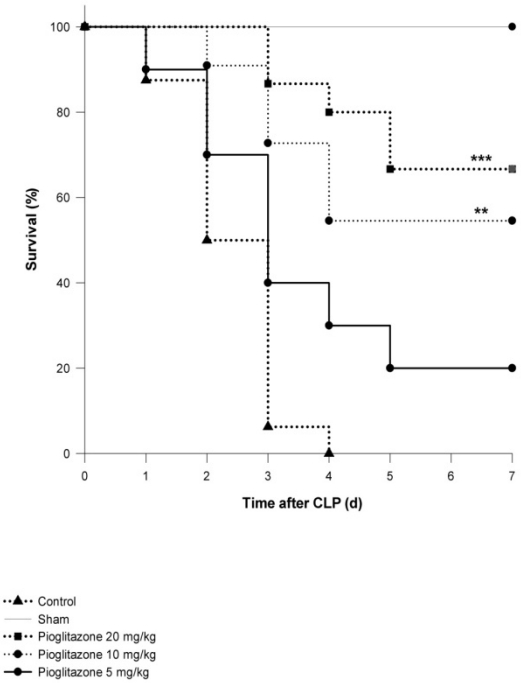

Figure 1. Effect of subchronic treatment with different doses of pioglitazone on survival after cecal ligation and puncture (CLP). Pioglitazone was administered five days by gavage. Survival rates were monitored for 7 days after CLP and the curves obtained by Kaplan-Meier method and log-rank test in at least 10 mice. **P<0.01 and ***P<0.001 compared to vehicle-CLP group

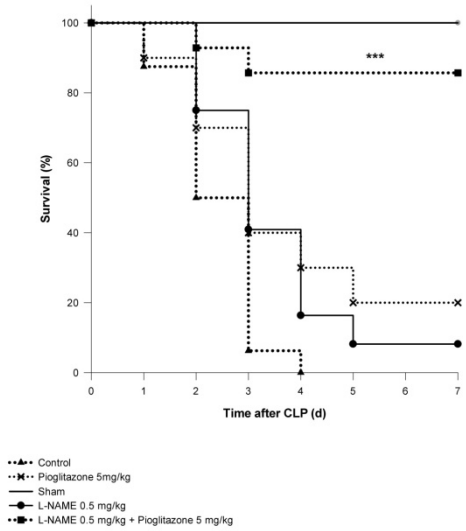

Figure 2. The effect of L-NAME administration on the survival rate after cecal ligation and puncture (CLP) in animals received pioglitazone for 5 days. L-NAME (0.5mg/kg) injected sub-chronically for 5 days, 30 min before each dose of pioglitazone. Survival rates were monitored for 7 days post-CLP, and the survival curves were obtained by Kaplan-Meier method and log-rank test in at least 10 mice.
***$P < 0.001$ compared to vehicle-CLP group.

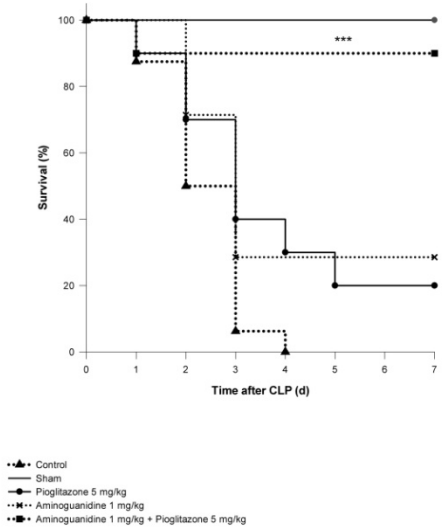

Figure 3. The effect of aminoguanidine administration on the survival rate after cecal ligation and puncture (CLP) in animals received pioglitazone for five days. Aminoguanidine (1 mg/kg) injected sub-chronically for 5 days, 30 min before each dose of pioglitazone. Survival rates were monitored for 7 days post-CLP and survival curves of mice subjected to CLP with aminoguanidine/pioglitazone were obtained by Kaplan-Meier method and log-rank test in at least 10 mice. ***$P < 0.001$ compared to vehicle-CLP group

Changes in serum levels of TNF-α and IL-1β

TNF-α and IL-1β serum levels were assessed in septic mice 12 h and 24 h after the operative procedures. Sub-chronic treatment with different doses of pioglitazone decreased TNF-α serum concentrations in septic mice, which was significant only in pioglitazone 20 mg/kg both after 12 h and 24 h after CLP surgery by comparison with vehicle-CLP group (P<0.05) (Figures 4A and 4B).

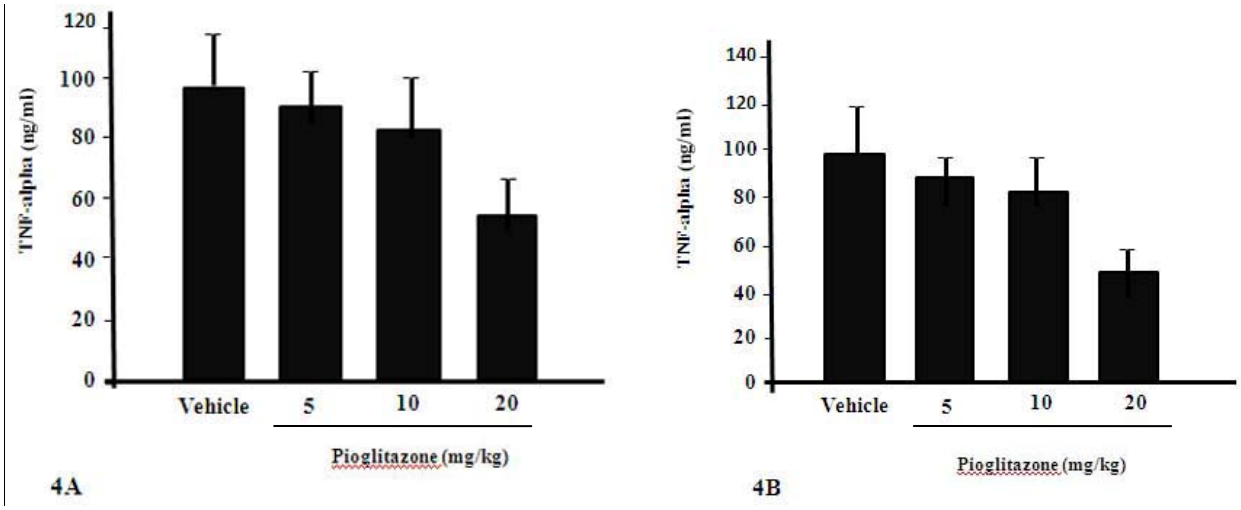

Figure 4. The effect of subchronic treatment with pioglitazone (5-10 and 20 mg/kg) in serum TNF- α levels of CLP-induced septic mice. **A:** 12 h after CLP, **B:** 24 h after CLP. Pioglitazone was administered orally for five days. Results are means ± S.E.M; n = 8-10 mice/group. **P* < 0.05 and compared to the vehicle-CLP group.

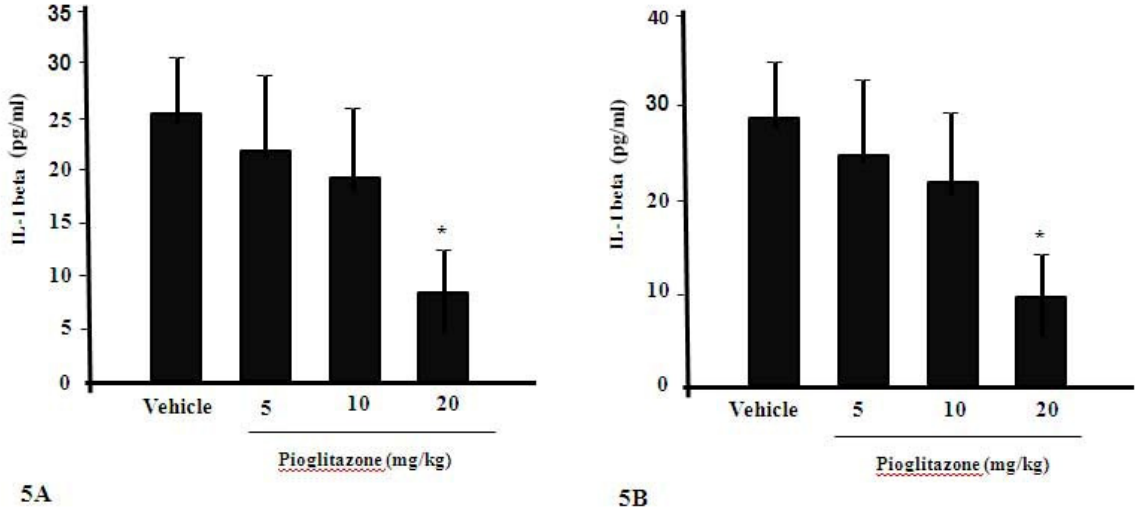

Figure 5. The effect of subchronic treatment with pioglitazone (5-10 and 20 mg/kg) in serum IL-1β levels of CLP-induced septic mice. **A:** 12 h after CLP, **B:** 24 h after CLP. Pioglitazone was administered orally for 5 days. Results are means ± S.E.M; n = 8-10 mice/group. **P* < 0.05 and compared to the vehicle-CLP group.

Serum levels of IL-1β in animals treated with pioglitazone 20 mg/kg were significantly lower than that in vehicle-treated group after 12 h and 24 h post-CLP (*P*<0.05) (Figures 5A and 5B).

Discussion

In the present study, we indicated that sub-chronic administration of pioglitazone exerted survival improving effects in the mouse sepsis model, CLP. Sub-chronic administration of L-NAME, a non-selective inhibitor of NOS or aminoguanidine, a selective inhibitor of iNOS improved the survival rates in groups of animals pretreated with pioglitazone. We also showed that the survival improving effects of pioglitazone is associated with a reduction in proinflammatory cytokines serum levels.

PPAR-γ is a member of the nuclear hormone receptor superfamily which is expressed in different

cells such as T and B lymphocytes, monocytes, macrophages, dendritic cells and endothelial cells (16, 23). Originally PPAR-γ was identified as a regulator of adipocyte differentiation and glucose metabolism (24). Recent studies implied a key role for the nuclear hormone receptor PPAR-γ in the treatment of many diseases based upon the anti-inflammatory effects (16).

PPAR-γ agonists exert anti-inflammatory properties in many situations such as rheumatoid arthritis (25), allergic reactions (26,27), inflammatory bowel disease (28), atherosclerosis (29), neuroinflammation, (22,30) and sepsis (31,32). Thiazolidinediones (TZDs) are insulin sensitizers, acting as agonists of PPAR-γ (33). There are many studies on rodent models of inflammation with TZDs treatment including murine asthma (34), caerulein-induced pancreatitis (35), carrageenan-induced edema or pleurisy (36) and epidermal hyperplasia (16,37).

Pioglitazone, a thiazolidinedione derivative agent, is a useful glucose-lowering agent for type 2 diabetes with anti-inflammatory properties (38). Tsujimura et al. examined the effects of pioglitazone on mortality and omental adipocyte function in mice with CLP, and showed that the anti-inflammatory properties of pioglitazone mediated in part by PPAR-γ activation which down-regulated the inflammatory mediators production (32). Haraguchi *et al*., indicated that pioglitazone improved survival of apolipoprotein E knockout mice after the onset of septic shock via suppression of the inflammatory responses (39). Araújo et al. reported that PPAR-γ activation is protective against cerebral microvascular dysfunction in sepsis (40).

Zingarelli and Cook provided an overview of the PPAR-γ roles in the regulating the inflammatory response and potential efficacy of PPAR-γ agonists as a novel therapeutic target in sepsis, inflammation and reperfusion injury (41). In this study, we indicated that oral subchronic administration of pioglitazone for five days improved the survival rates in mice undergone the CLP procedure, which is in line with these previous results.

In the course of sepsis, PPARγ acts as a double-edged sword (16). On the one hand, PPAR-γ inhibits pro-inflammatory gene expression such as iNOS, TNF-α or IL-1β, mediated by the inhibition of NF-kappaB activity (41). The studies showed that PPAR-γ ligands rosiglitazone, troglitazone, and 15d-PGJ2 repressed the expression of iNOS, TNF-α, gelatinase B and inducible COX-2 in activated macrophages (42,43). During the onset of sepsis activation of PPAR-γ can prevent the sepsis progression by reducing the hyperinflammatory response (44). On the other, hand PPAR-γ activation during the later phase of sepsis may increase immune paralysis and lead to sepsis outcome worsening (16). In the present study, we showed that pioglitazone-treated septic mice exhibited a fall in serum TNF-α levels 12 h and 24 h after induction of sepsis by CLP. There was also a significant reduction in serum IL-1β concentrations in the groups pre-treated with pioglitazone 12 h and 24 h post-CLP, showing the anti-inflammatory function of PPAR-γ ligand. The data of this study did not indicate the pro-inflammatory function of pioglitazone during sepsis. There is a complexity in the effects of PPAR-γ ligands on the inflammatory response, depending upon many factors such as the ligand used in the study (41).

Sepsis is a heterogeneous class of clinical syndromes caused by the systemic host response to an infection (44). Sepsis is a major health concern throughout the world, related to a complex pathophysiology. In the early 1990s, nitric oxide appeared as an important mediator in sepsis pathogenesis (44). During sepsis, nitric oxide production is augmented via iNOS overexpression, acting with both beneficial and deleterious effects (45). Several researches have highlighted the role of nitric oxide in septic shock and inflammation. Bucher *et al*., have shown the up-regulation of eNOS mRNA gene expression in the liver of rats treated with lipopolysaccharide and lipoteichoic acid. They suggested that eNOS is an even more potent source of nitric oxide than iNOS in the liver in this model of sepsis (46).

Araújo *et al*., demonstrated that augmented nitric oxide production in sepsis syndrome is due to the up-regulation of eNOS and not to iNOS during cecal ligation and perforation (45). Conversely, the studies showed that excessive production of nitric oxide following the cytokine-dependent induction of iNOS resulted in the development of septic or endotoxic shock. Moreover, an increase in the nitric oxide by itself may be insufficient, and generation of other mediators such as reactive oxygen intermediates play a role in tissue dysfunction during sepsis (4). Price *et al*., reported that iNOS was cardioprotective in the heart in sepsis and explained why its inhibition in man led to increased mortality in a subpopulation of patients (47).

Xie *et al*., showed that combination treatment of arginine and an iNOS inhibitor in an early phase of sepsis was a beneficial approach in sepsis and septic shock (14). Gianotti *et al*., reported that arginine-supplemented diets improve survival in gut-drived

sepsis and peritonitis by modulating bacterial clearance via the arginine-nitric oxide pathway (48). There are several papers reporting the nitrergic system involvement in pioglitazone effects. Moezi *et al*., showed that one of the mechanisms of enhanced anti-ulcer activity of pioglitazone is probably due to cNOS induction and iNOS inhibition (21).

It has been shown that troglitazone up-regulates NOS expression in vascular endothelial cells (49). Shafaroodi *et al*., pointed out that nitric oxide release through cNOS might involve in pioglitazone-induced anti-convulsant effects (22). Huang *et al*., indicated that pioglitazone significantly ameliorates endothelial dysfunction and enhances blood flow recovery after tissue ischemia in diabetic mice. Activation of eNOS appears to be important for pioglitazone to promote angiogenesis in ischemic tissue (50). Adabi Mohazab *et al*., showed the possible involvement of PPAR-gamma receptor and nitric oxide pathway in the anticonvulsant effect of acute pioglitazone on pentylenetetrazole-induced seizures in mice (51).

Babaei *et al*., investigated the possible interaction of pioglitazone with morphine in memory-impaired mice and the probable role of nitric oxide in this effect (52). Konturek *et al*., concluded that pioglitazone accelerates the healing of preexisting gastric ulcers due to the hyperemia at ulcer margin and the anti-inflammatory action including suppression of iNOS (53). PPARγ agonists inhibit the production of nitric oxide in monocytes of human peripheral blood (42).

Regarding several papers indicating the role of nitric oxide in different pioglitazone effects, we investigated the possible involvement of nitrergic system in survival improving effects of pioglitazone. Therefore, we utilized two different substances: L-NAME, a non-selective NOS inhibitor and aminoguanidine, a selective iNOS inhibitor. Simultaneous sub-chronic treatment with pioglitazone 5 mg/kg and L-NAME increased the survival rate compared to the control group. These data illustrated that inhibition of nitric oxide release through NOS might be involved in survival improving effects of pioglitazone, which is consistent with previous results, showing the role of nitric oxide in different pioglitazone effects.

We also examined aminoguanidine in the pioglitazone survival improving effects. Current data showed that sub-chronic co-administration of pioglitazone 5 mg/kg and aminoguanidine increased the survival rate compared to the control group. These data imply that iNOS might be involved in survival improving the activity of pioglitazone. Since pioglitazone protects against polymicrobial sepsis in different ways (the impairment of MyD88 responses (54) and modulating adipose inflammation (55)), more investigations are required to clarify the probable mechanisms of action. To ascertain the role of nitric oxide signaling in pioglitazone-induced protection, it would be valuable to measure nitric oxide metabolites, NOS activity/expression and perhaps cyclic guanosine monophosphate (cGMP) levels in future studies.

Taken together these data indicated that sub-chronic administration of pioglitazone exerted survival improving effects. The mechanism of this enhanced survival rate by pioglitazone is probably via inhibition of nitric oxide release through iNOS and reduction in proinflammatory cytokines TNF-α and IL-1β production.

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