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ORIGINAL ARTICLE

Induction of Chronic Subclinical Systemic Inflammation in Sprague–Dawley Rats Stimulated by Intermittent Bolus Injection of Lipopolysaccharide

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Abstract

Chronic subclinical systemic inflammation has a key role in stimulating several chronic conditions associated with cardiovascular diseases, cancer, rheumatoid arthritis, diabetes, and neurodegenerative diseases. Hence, developing in vivo models of chronic subclinical systemic inflammation are essential to the study of the pathophysiology and to measure the immunomodulatory agents involved. Male Sprague–Dawley rats were subjected to intraperitoneal, intermittent injection with saline, or lipopolysaccharide (LPS) (0.5, 1, 2 mg/kg) thrice a week for 30 days. Hematological, biochemical, and inflammatory mediators were measured at different timepoints and at the end of the study. The hearts, lungs, kidneys, and livers were harvested for histological evaluation. Significant elevation in peripheral blood leukocyte includes neutrophils, monocytes, and lymphocytes, as well as the neutrophils-to-lymphocyte ratio. The pro-inflammatory mediator levels [C-reactive protein, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-8] along with the biochemical profile (alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, creatine kinase, creatinine, and urea) were increased significantly ($P < 0.05$) and increased the expression of monocyte chemoattractant protein-1 and TNF-β. The histopathological changes of heart, lung, kidney, and liver tissues revealed degeneration, cellular infiltration of leukocyte in the inflammatory foci and interstitial space, edema, early signs of fibrosis, apoptosis, and necrosis. In conclusion, these results indicate that intermittent exposure to LPS produces chronic subclinical systemic inflammation in multiple organs leading to chronic conditions and supports this model to be a useful preclinical tool for developing immunotherapeutic agents that could prevent, or reduce, chronic inflammatory diseases associated with, or without, bacterial translocation.

Keywords Animal model · Chronic diseases · Cytokines · Lipopolysaccharide · Multiple organs · Systemic low-grade inflammation

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Abbreviations

LPS	Lipopolysaccharide
CSSI	Chronic subclinical systemic inflammation
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TGF	Tumor growth factor
IL	Interleukin
CRP	C-reactive protein
MCP	Monocyte chemoattractant protein

Introduction

Chronic subclinical systemic inflammation (CSSI) is defined as an elevation of inflammatory cytokines in serum because of the failure to resolve acute inflammation, oxidative stress,

or metabolic malfunction. CSSI has recently been considered a key role in pathological tissue destruction which subsequently leads to common chronic diseases. Of those diseases are cardiovascular diseases, cancer, rheumatoid arthritis, type 2 diabetes, and neurodegenerative diseases (Ranneh et al. 2017). The prevalence of chronic diseases has a huge economic and physiological burden on society and patients. At the same time, classical anti-inflammatory modalities [e.g., anti-tumor necrosis factor (anti-TNF)-α monoclonal antibodies] remain ineffective in curing those who are suffering from systemic, chronic, inflammation-related diseases. Therefore, seeking alternatives and developing promising medications to relieve and prevent CSSI consequences requires a clinically appropriate relevant experimental model to understand the therapeutic actions of these medications.

Rodents' models have attracted a great attention as crucial tools in the study of inflammation because of the physiological similarities with humans, and the variety of ways in which it can be induced (Popov and Pavlov 2013). A variety of studies have developed animal models to mimic acute or chronic inflammation in a particular organ using chemical or biological stimuli (Hamesch et al. 2015; Webb 2014). CSSI is slightly different from local or acute inflammation in the pathophysiology that results in multiple biological responses simultaneously. In addition, the characteristic response of CSSI has a characteristic profile in several chronic diseases, since their triggered pathways are common. Thus, a viable rodent model of CSSI should present characteristics common to CSSI diseases, which are: elevation of pro-inflammatory cytokines (CRP, IL-6, and TNF-α), activation of monocytes and neutrophils, and tissue destruction in multiple organs (Guo et al. 2016; Murakami and Hirano 2012).

Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria, has been commonly and extensively used to model inflammation-related diseases through activating innate immunity in rodents (Lipcsey et al. 2016). The infusion of LPS, either intravenously or intraperitoneally, in the short term, with different dose regimes to investigate anti-inflammatory agents has been well-documented in various studies (Balasubramaniam et al. 2016; Hong et al. 2012; Kowalczyk et al. 2016). Moreover, the frequency, administration route, dose, and duration of LPS contribute to physiological changes in the animal such as: increase body temperature, loss of body weight, and reduction in food intake (Kaplanski et al. 2000; O'Reilly et al. 1988; Valles et al. 2000). In some cases, a repeated bolus injection or continuous infusion of LPS could develop a tolerance, where the inflammatory response is no longer activated (West and Heagy 2002). Thereby, LPS tolerance could overlap with the results obtained from anti-inflammatory modulators studied in rodents. At the same time, the duration and dose of LPS-treated rodents have varied from a few hours to a few days and ranged from 1 ng/kg to 20 mg/kg, respectively (West and Heagy 2002).

The presence of LPS, as an endotoxin, in the blood circulation has been attributed to developing chronic inflammatory diseases (Stoll et al. 2004). Recently, coadministration of LPS and a high fat diet has been examined as a new model of non-alcoholic steatohepatitis (Guo et al. 2016). Developing a systemic bone loss rat model with coronary vessel disease was established using an LPS timed-release pellet (Smith et al. 2006). The previous research team has added a new model of cardiovascular pathology using the same technique (timed-release pellet) (Smith et al. 2009). On the other hand, injecting subclinical doses of LPS intraperitoneally into C57Bl/6 mice in a frequent time regimen has induced cardiac fibrosis (Lew et al. 2013). Thus, standardizing LPS dosage, administration route, frequency, and duration to develop chronic inflammatory model in multiple organs simultaneously would provide a more acceptable informative comparison across future studies and enhance the quality of scientific studies. Since CSSI could lead to multiple organ dysfunction, there is a strong need to develop such a rat model that presents its biochemical and histological manifestations in various organs to develop a novel drug that would attenuate CSSI.

In this current research, we hypothesized that intermittent treatment of LPS through intraperitoneal (i.p.) injection would induce a CSSI animal model with inflammatory pathological changes in the heart, lungs, kidney, and liver. To establish an optimized dosing regimen that would successfully induce novel CSSI model, three comparable concentrations of LPS have been used. The optimal dose of LPS would induce CSSI in rats with a minimum burden, retaining the inflammatory response of immune system, and mimicking the pathological features seen in a large pool of conditions predisposed to inflammatory chronic diseases. Therefore, the objective of this study was to determine whether intermittent exposure of LPS via i.p. injection produced similar pathophysiological characteristics to CSSI.

Materials and Methods

General Animal Procedures

Animal ethics approval was obtained from the Institutional Animal Care and Use Committee (IACUC) of the Universiti Putra Malaysia prior to the initiation of the study (UPM/IACUC/AUP-R007/2017). A total of 48 pathogen-free Sprague–Dawley (SD) male rats at the age of 7 weeks, weighing 260–275 g, were obtained from Universiti Putra Malaysia, Faculty of Veterinary. SD rats were housed in plastic cages in pairs under standard conditions with a light–dark cycle of 12 h/12 h maintained at 22–24 °C

and humidity 40–60%. A 3-cm layer of aspen chips was used as bedding to avoid odors and to keep the rats clean. Untreated tap water from local pipes was presented in polycarbonate bottles, replenished when necessary, and cleaned twice a week. The cages were thoroughly cleaned twice a week using water and anti-bacterial liquid detergent, and then sterilized with 70% alcohol swabs. The rats had free access to standard rodent chow and water *ad libtium* for 2 weeks of acclimatization before the beginning of the experiment.

Experimental Design

We designed a dose response study to determine the most effective and suitable dose of LPS administration to induce CSSI with a minimal burden for the rats. It has been reported that rats are insensitive to low concentration of LPS, which should be calculated based on milligram of LPS per kilogram of animal weight. LPS derived from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, USA) was dissolved in sterile, pyrogen-free phosphate-buffered saline 1X and stored at 4 °C until use. After acclimatization, the SD rats were assigned randomly into six groups ($n = 8$); Group I (control), Group II (1% saline solution only), Group III (LPS 0.5 mg/kg), Group IV (LPS 1 mg/kg), Group V (LPS 2 mg/kg), and Group VI (baseline). This last group was sacrificed on the first day of the experiment to obtain the baseline data of white blood cells (WBC), inflammatory markers, and biochemical profile. Group II received i.p. 1% saline injections in a volume 2 ml/kg body weight, while Groups III, IV, and V were intraperitoneally injected with LPS at the doses of 0.5, 1, or 2 mg/kg thrice per week in a volume 1 ml/kg body weight for 28 days. The injection course of LPS doses and saline was conducted during the light-phase (08:30–10:00 am) and lasted for 4 weeks continuously. Before starting the injection course, the rats had experienced the i.p. injection position for adaptation and stress reduction. LPS or saline was warmed to room temperature using a water bath to avoid changes in body temperature due to injecting cold substances.

Measurement of Body Weight, Body Temperature, and Food Intake

Body weight and food intake were measured for each animal immediately after LPS or saline injection using an electric digital scale 3 days per week. Pre-weighed foods were given to the rats on the first day of the experiment, and then, food intake was measured daily by subtracting the weights of the food bin from the previous day. Body temperature was obtained using a rectal probe.

Mortality Rate Observation

The survival study was conducted on all the animal groups. The mortality and survival rate after LPS or saline administration were investigated through close observation and follow-up. Kaplan–Meier survival curves were used to analyze the estimated probability.

Blood Sampling and Harvesting

During the whole experimental period, blood samples were collected after 7, 15, and 30 days. The rats were euthanized with an i.p. injection of ketamine (80 mg/kg) combined with xylazine (9 mg/kg) by which anesthesia lasted approximately 30–35 min. Then, the blood was collected via a cardiac puncture on the 7th, 15th, and 30th. Two types of tubes were used to keep the blood: an EDTA tube (BD Vacutainer Bioscience) was utilized to determine the WBC counts (Automated blood cell analyser, CellDyn, 1800). Blood film slides were made from the reminder of the EDTA tube blood and subjected to manual hematological evaluation. A serum tube (BD Vacutainer Bioscience) was used to determine the inflammatory mediators and clinical biochemistry of blood from days 15 and 30. Blood smears were performed from the EDTA blood and stained with a mixture of eosin and methylene blue dyes (Wright-Giemsa stain) for hematological evaluation. Serum was separated by centrifugation at 4000 rpm for 10 min and stored at − 80 °C for further analysis.

Determination of Inflammatory Markers

After collecting blood samples from the rats on the 15th and 30th days, sera samples were obtained by centrifuging blood samples at 3500 rpm for 15 min, and then, the sera were kept at − 80 °C. CRP, tumor growth factor (TGF)-β, TNF-α, IL-1β, IL-6, and IL-8 levels in sera were quantified by a specific sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instruction. The commercial kits of Melsin (China) are specific for detecting inflammatory mediators in rat serum, while monocyte chemoattractant protein (MCP)-1 was determined using ELISA following the manufacturer's instructions of Fine Biotech (Shanghai, China). The results of inflammatory mediators in sera were expressed as pg/ml except for CRP which was expressed as ng/ml. Hepatic function tests including alkaline phosphatase (ALP), aspartate aminotransferase (ALT), alanine aminotransferase (AST), gamma-glutamyl transferase (GGT), a cardiac function test (creatine kinase), and renal function tests (creatinine, urea) were determined using a Hitachi 900 Auto Analyzer (Roche Diagnostics, Switzerland) according to manufacturer's protocol.

Termination Procedures and Histological Evaluation

After completing 30 experimental days, rats were anesthetized using i.p. injection of ketamine (80 mg/kg) combined with xylazine (9 mg/kg) and sacrificed by exsanguination via cardiac puncture. Then, the lungs, heart, kidneys, and liver were collected and washed in phosphate-buffered saline three times. Half of the organs were preserved directly at − 80 °C for further analysis, while the rest were kept in 10% buffered formalin for histological evaluation. The organs were sliced from different lobes, and after preservation in 10% formalin, the tissues were embedded in paraffin blocks to process for routine histological evaluation using hematoxylin and eosin stain (McManus and Mowry 1960). A skilled pathologist who was blind to the study, evaluated the pathological changes, and scored the inflammatory level using Microscope Imaging Cell[f] Software connected to an Olympus light microscope BX40 (Olympus Optical Co., Japan). Inflammatory scoring was conducted based on the following criteria: score 0 = normal tissue (absence of inflammation), score 1 = the inflammatory cells and tissue damage are present in less than 25% of the field of view, score 2 = inflammation and tissue damage involved in 25–50% of the field of view, score 3 = inflammation and tissue damage involved in 50–75% of the field of view, and score 4 = inflammation and tissue damage involved in more than 75% of the field of view.

Statistical Analysis

Quantitative results were expressed as means ± standard errors of the mean (SEM). The analysis of data was performed using SPSS software program (SPSS version 12.0, Claritas Inc., USA) and GraphPad Prism software for creating the graphs (Prism 7.0, GraphPad Software Inc., CA, USA). The percentage of survival was analyzed using the log-rank test. The normality of the data (Kolmogorov–Smirnov test) and homogeneity of variance were performed. Then, identifying the significance level between groups and within groups was tested using one-way and two-way analyses of variance (ANOVA) followed by a post hoc Tukey's honest significant difference (HSD). The results were considered significant when $P < 0.05$ for all statistical tests.

Results

Intermittent Injection of LPS Induces Changes in Body Weight, Food Intake, and Body Temperature

In the first 10 days, there were no significant changes in body weight. On day 11, all LPS-treated groups (0.5, 1, 2 mg/kg—LPS 0.5, LPS 1, and LPS 2, respectively) lost weight significantly compared with saline-treated group and control. The rat's groups that had received 0.5 or 1 mg/kg LPS regained their weight by days 17 and 22, respectively. However, after 20 days, the body weight of the group treated with 1 mg/kg LPS increased to a similar significance level ($P < 0.05$) as the saline-treated, control, and LPS 0.5 groups, while the LPS 2 group maintained their weight reduction. On day 30, the body weight of saline, control, and LPS 0.5 and 1 groups was similar, whereas the LPS 2 group's weight increased slightly (Fig. 1). Overall, the results of body weight change post-treatment with saline or LPS (0.5, 1, and 2 mg/kg) thrice a week for 28 days indicated that LPS 0.5- and LPS 1-treated rats did not remain reduced in weight, as the rats recovered from the prolonged exposure to LPS after 20 days. Rats injected with 2 mg/kg LPS did not recover their body weight during the same period.

On the first day of the experiment, the mean food intake for saline-treated and LPS injection groups was its lowest in the whole experimental period comparing with the control group. Rats treated with LPS 0.5 or 1 or 2 mg/kg consumed around 22, 18, and 12 g, respectively ($P < 0.05$) (Fig. 2). On day 5, rats treated with LPS showed a steady increase in food intake, indicating that the anorexic effect had lessened despite the intermittent exposure to LPS. On day 20, the LPS 1 group demonstrated a similar food intake to the LPS 0.5 group until the last day of the experiment to reach 42 g/day, resulting in no significant difference with control or saline-treated groups. The food intake of the LPS 2 group returned to 40 g/day ($P < 0.05$) compared to control and saline-treated rats (Fig. 2).

The average body temperature post saline or LPS (0.5, 1, 2 mg/kg) injection is presented in Fig. 3. The saline-injected group did not show any statistical significance to controls during the whole experimental period, while the LPS-injected groups revealed higher levels of body temperature.

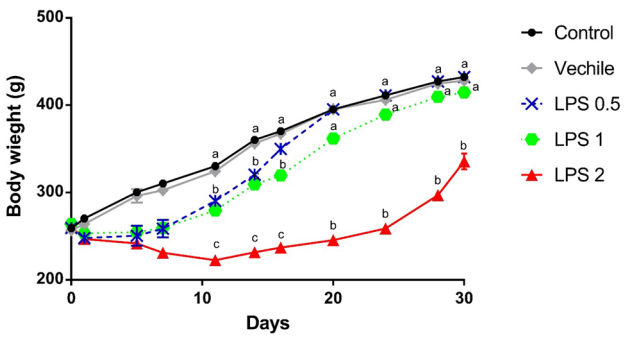

Fig. 1 Average weight gains of rats injected intraperitoneally with saline and/or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days compared with control. Each point of represented data is mean ± SEM, total $n = 8$. Significantly, different values are indicated by different superscripts at each measured timepoint ($P < 0.05$)

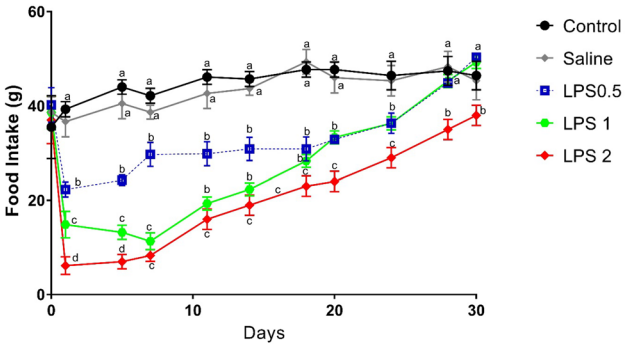

Fig. 2 Average food intake of rats injected intraperitoneally with saline and/or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days compared with control. Each point of represented data is mean ± SEM, total $n = 8$. Significantly, different values are indicated by different superscripts at each measured timepoint ($P < 0.05$)

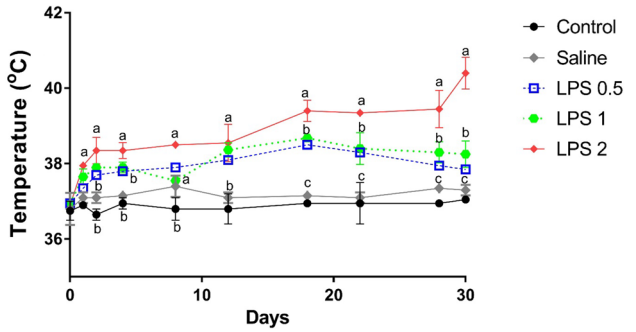

Fig. 3 Average body temperature of rats injected with saline or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. Each point of represented data is mean ± SEM, total $n = 8$. Significantly, different values are indicated by different superscripts at each measured timepoint ($P < 0.05$)

LPS 0.5 and LPS 1 groups had similar, non-significant, body temperatures ranging 37.7–38 °C ($P < 0.05$), whereas the rectal temperature of the LPS 2 group was significantly higher, reaching 38.4 °C in the first 10 days, and 40 °C on day 30 compared to control and saline-treated rats ($P < 0.05$).

Survival Rate Affected by Intermittent Injection of LPS

The survival curves of all five groups are shown in Fig. 4. The mortality rate in the control and saline-treated groups was 0% (Fig. 4). In contrast, the survival rate of the LPS 0.5 and LPS 1 groups was 75% and was significantly higher than that of the LPS 2 group 50% ($P < 0.05$). Surprisingly, there was no statistical significance observed between groups LPS 0.5 and LPS 1. However, half of the rats in the LPS 2 group died at varying intervals. The data indicate that rats treated intermittently with 2 mg/kg LPS had decreased survival rate.

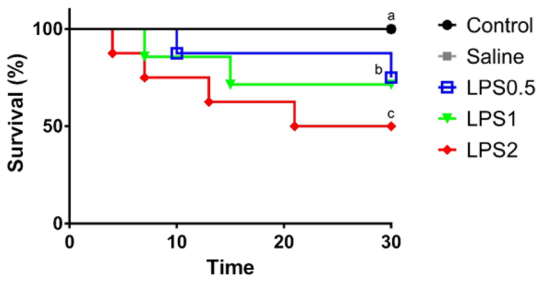

Fig. 4 Survival curves of Kaplan–Meier for the rats treated with saline and/or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. Each group consist of eight rats and the survival percentage was followed for 30 days and was 100% for the control group and saline group, 75% for the rats treated with 0.5 and/or 1 mg/kg LPS, 50% for the rats treated with 2 mg/kg LPS. The data were analyzed using log-rank test and Graphpad Prism software was used to generate the survival curve. Significantly, different values are indicated by different superscripts ($P < 0.05$)

Hematological Changes Post-intermittent LPS Injections

Absolute white blood count (WBC) counts were significantly and steadily increased at different intervals in LPS-treated groups (Fig. 5a). The LPS 2 group had the highest WBC counts ($P < 0.05$) after 7 and 15 days compared with the baseline, control, and saline groups, but on day 30, the WBC count of the LPS 1 group became similar in significance to that of the LPS 2 group. Notably, neutrophils counts were raised in all the LPS-treated groups with different levels of significance ($P < 0.05$) during the experiment (Fig. 5b). Absolute lymphocyte counts showed a notable elevation for LPS-treated groups at days 15 and 30 compared with baseline, control, and saline-treated groups, while on day 7, all groups approached a similar significant level except rats challenged with 2 mg/kg LPS ($P < 0.05$), as shown in Fig. 5c. In contrast, monocyte counts were similar in all LPS-treated groups throughout the period of study. The neutrophil/lymphocyte (N/L) ratio increased in all LPS-treated rats, but without any statistically significant differences except with respect to the control and saline-treated groups. On day 15, the N/L ratio of the LPS 2 group was more significant ($P < 0.001$) than the LPS 1 and LPS 0.5 groups. However, the LPS 1 group reached in the same significance level of the LPS 2 group on day 30.

Microscopic Evaluation of Stained Blood Smears Post-intermittent LPS Injections

Microscopic examination of blood smears demonstrated the morphologic and numerical changes in neutrophils and lymphocytes post-intermittent LPS injection at days 15 and 30 (Fig. 6). Overall, neutrophils were more

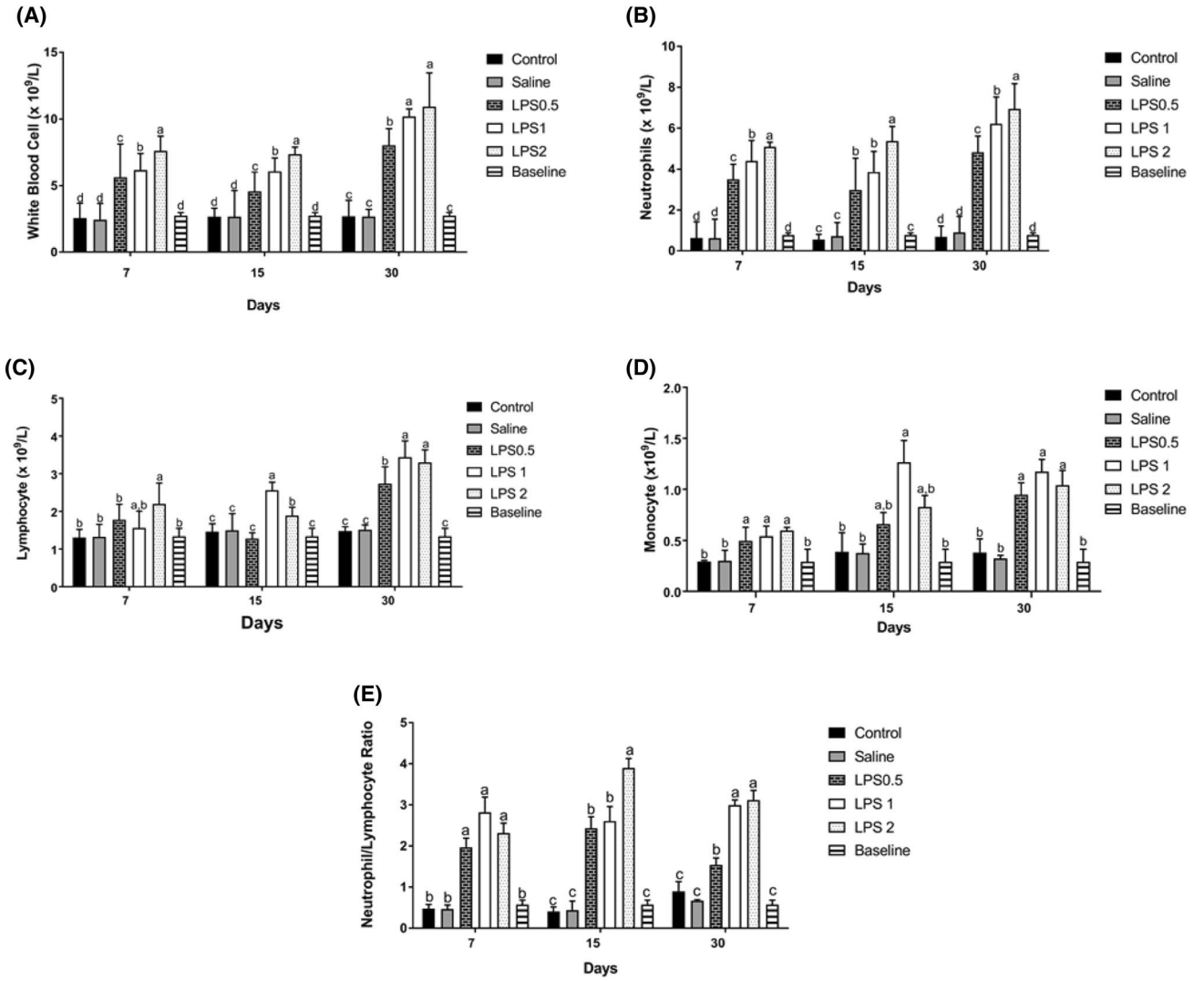

Fig. 5 Alterations in white blood cells (**a**), neutrophils (**b**), lymphocytes (**c**), monocyte levels (**d**), and neutrophil-to -lymphocyte ratio (**e**) on the 7th, 15th, and 30th days in rats injected with saline and/or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. Automated blood cell analyser (CellDyn, 1800) was used for complete count followed by manual calculations. The presented results are mean ± Standard Error of the mean ($n = 8$). One-way ANOVA followed by Tukey's multiple comparison test was used to calculate the statistical differences. Bars that have different superscript are significantly different from each other ($P < 0.05$)

abundant in all LPS-treated rats compared with saline-treated and control groups on day 15 and exhibited toxic changes; cytoplasm of the basophil, Dohle bodies, cytoplasmic vacuolation and toxic granulation, while on day 30, neutrophils dead shape appeared in the LPS 1 and LPS 2 groups and were fewer in numbers. In contrast, lymphocytes were plentiful in the LPS 1 and LPS 2 groups on day 30 compared with control and saline-treated groups. The increased presence of lymphocytes indicates unresolved inflammation induced by intermittent i.p injection of LPS.

Inflammatory Markers and Biochemical Serum Changes Post-intermittent LPS Injections

LPS treatment caused several alterations in serum inflammatory markers along with biochemical parameters at 15 and 30 days (Fig. 7). The level of C-reactive protein (CRP) serum was significantly higher ($P < 0.05$) in the LPS 1 and LPS 2 groups than in the LPS 0.5 group at 15 and 30 days (Fig. 7a). As presented in Fig. 7b, g, TGF-β and TNF-α serum levels in all LPS-treated rats with (0.5, 1, 2 mg/kg) increased in a dose–response manner compared with

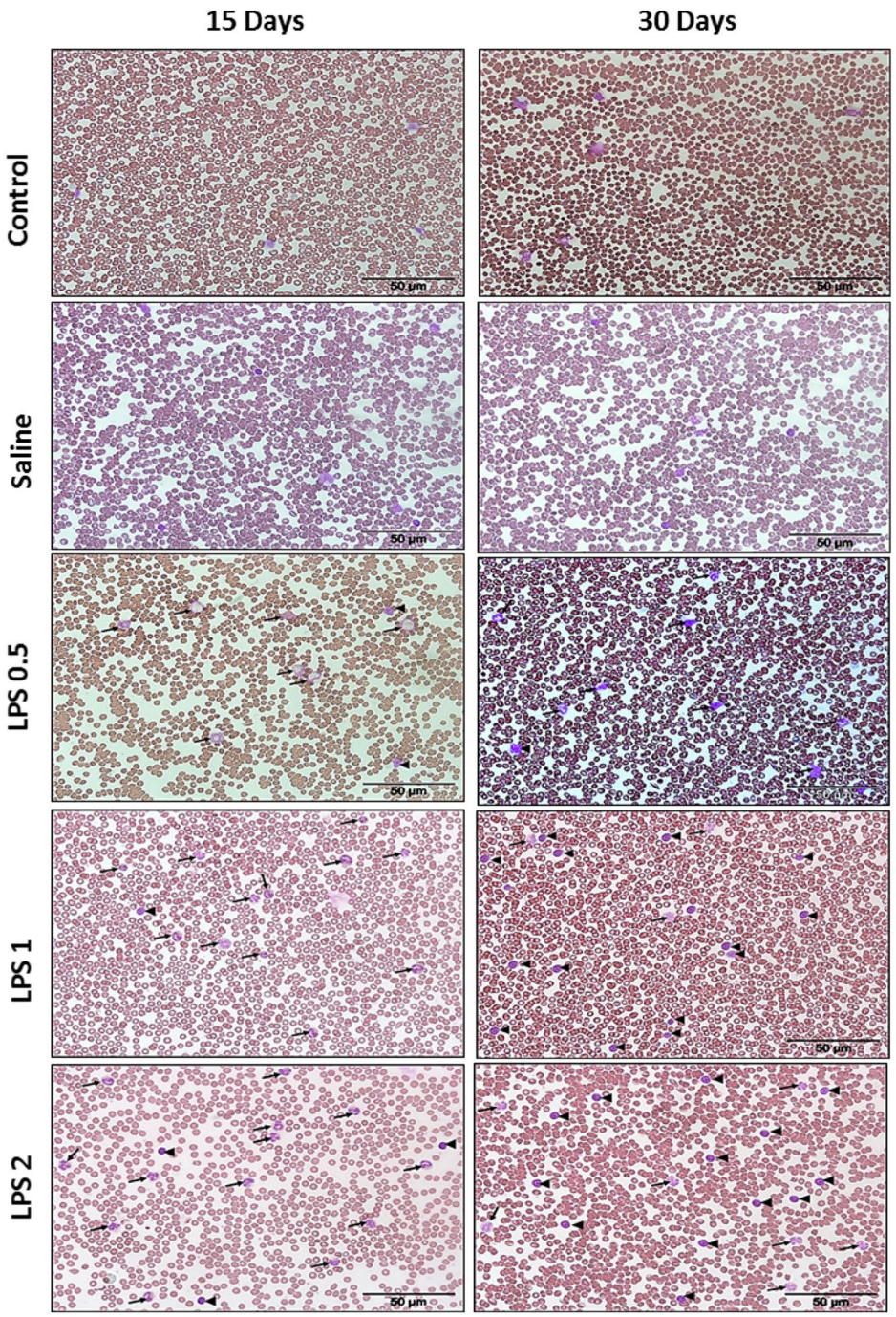

Fig. 6 Representative blood smear slides using Wright-Giemsa staining of control rats or treated with saline or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. Blood was collected from all the groups on the 15th and 30th days. Neutrophils (arrow) are abundant in LPS-treated rats after 15 days, while monocytes (head arrow) increased after 30 days

baseline, control, and saline-treated groups, but LPS 1 and LPS 2 groups' significance levels were the same ($P < 0.05$). The elevation of IL-1β (Fig. 7c) in all LPS groups ranged 200–350 ng/ml on day 15 and 280–450 ng/ml on day 30. In addition, serum IL-6 levels were lower in LPS 1 than LPS 2 and LPS 0.5 groups, while IL-8 levels were around 718 ng/ml in both LPS 1 and LPS 2 groups on day 30. Figure 7g shows that MCP-1 levels were significantly higher in LPS 2 group on day 15, but this value was slightly reduced on day 30. Overall, inflammatory markers and cytokines were increased following intermittent LPS injection in a dose–response manner; however, the significance level of the LPS 2 and LPS 1 groups was similar for most of the values. Overall, the inflammatory markers' levels were high especially in rats treated with 1 or 2 mg/kg LPS.

The results of liver function tests showed an increase in all LPS-treated groups at 15 and 30 days compared with the baseline, control, and saline-treated groups (Fig. 8). As shown in Fig. 8a, ALP levels were significantly higher in the LPS 2 rats, reaching 214 U/L and 277 U/L on the 15th

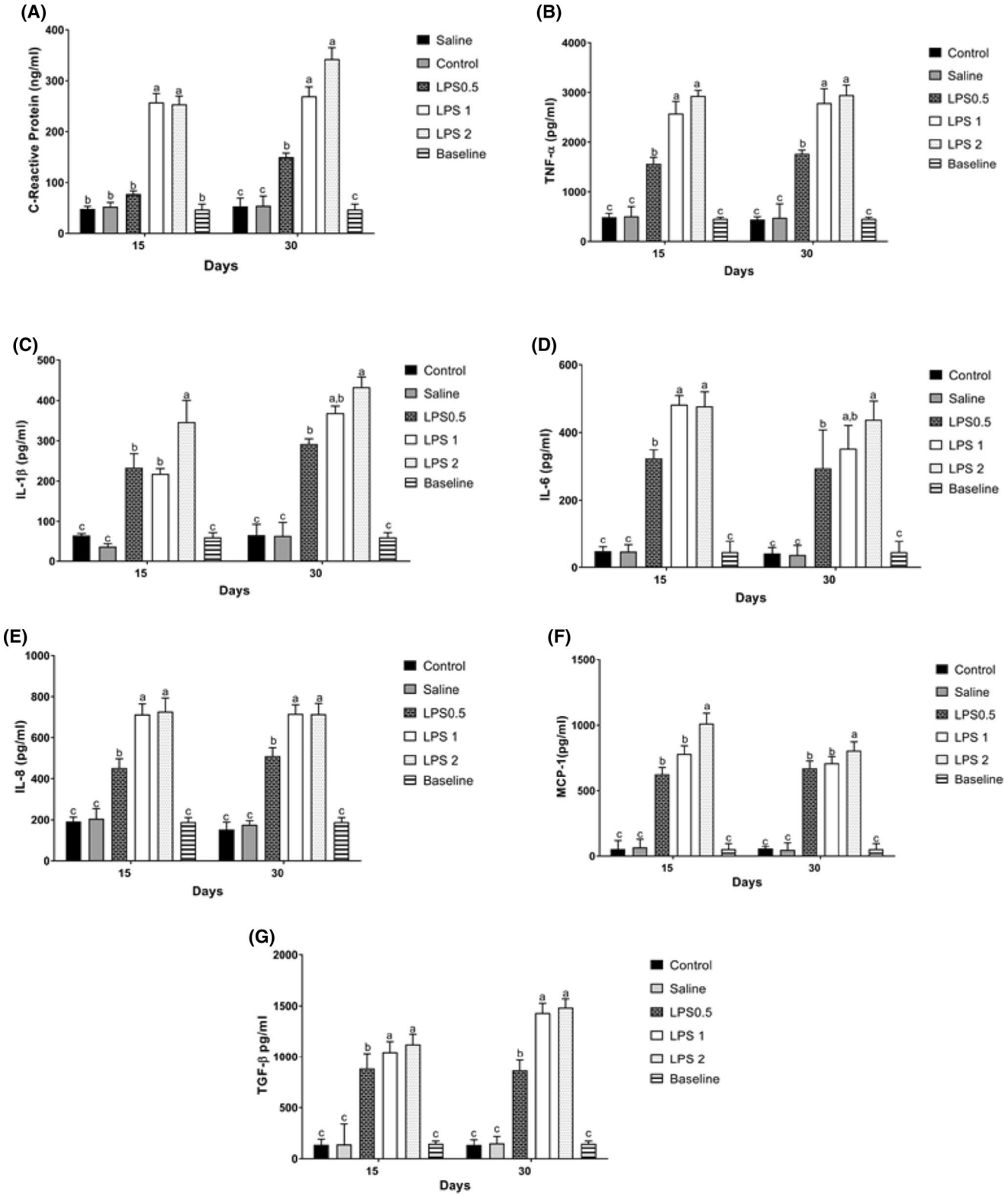

Fig. 7 Serum changes of inflammatory mediators and cytokines CRP (**a**), TNF-α (**b**), IL-1β (**c**), IL-6 (**d**), IL-8 (**e**), MCP-1 (**f**), and TGF-β (**g**) on the 15th and 30th days in rats injected with saline and/or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. The inflammatory cytokines and mediators were measured using specific ELISA. The presented results are mean ± standard error of the mean ($n = 8$). One-way ANOVA followed by Tukey's multiple comparison test was used to calculate the statistical differences. Bars that have different superscript are significantly different from each other ($P < 0.05$)

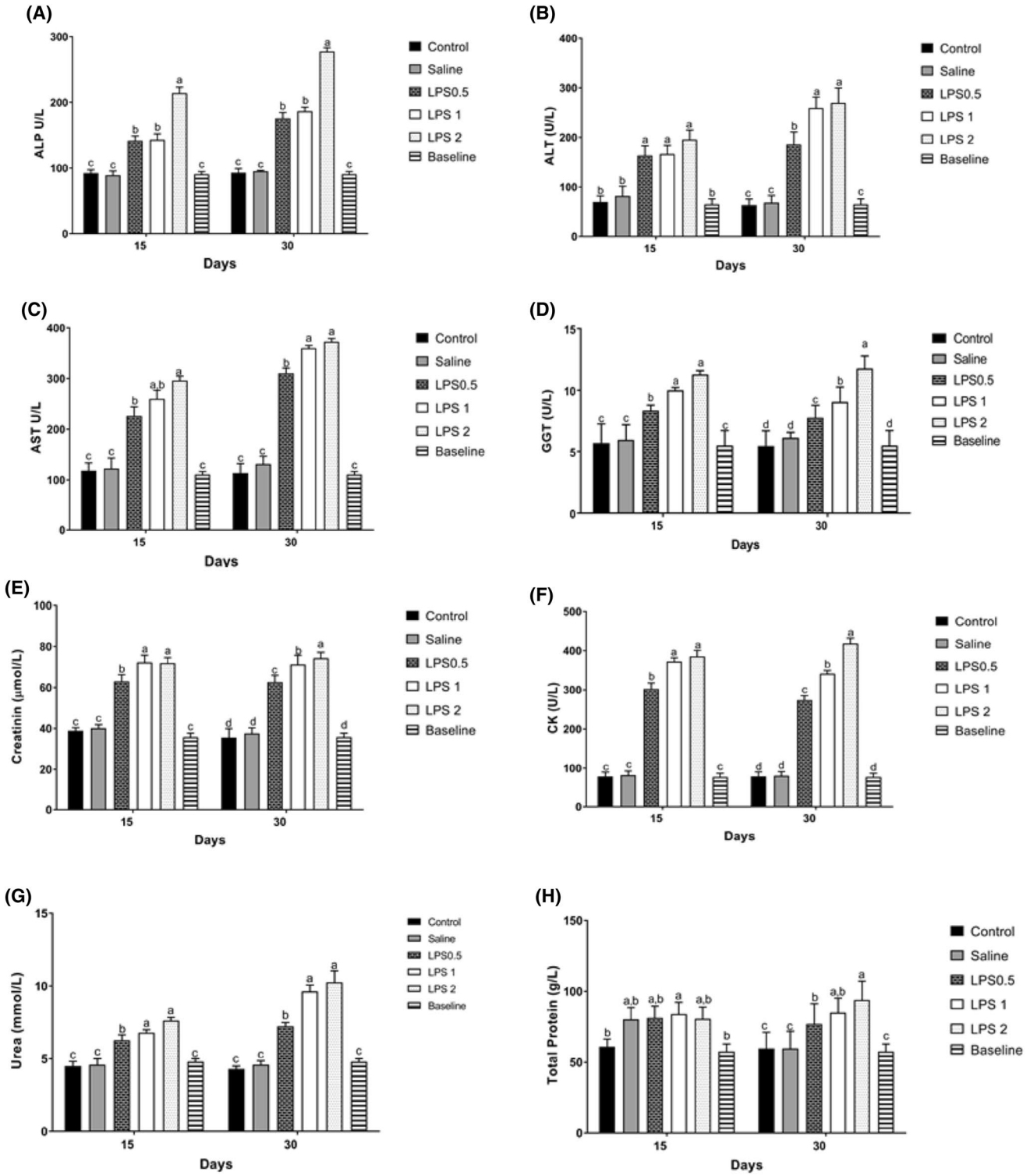

Fig. 8 Serum alkaline phosphatase (ALP; **a**), alanine aminotransferase (ALT; **b**), aspartate aminotransferase (AST; **c**), gamma-glutamyl transferase (GGT; **d**), creatinine (SCR; **e**), creatinine kinase (CK; **f**), urea (**g**), and total protein (TP; **h**) on the 15th and 30th days in rats injected with saline and/or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. The serum biochemistry data were obtained using Hitachi 900. The presented results are mean ± standard error of the mean ($n = 8$). One-way ANOVA followed by Tukey's multiple comparison test was used to calculate the statistical differences. Bars that have different superscript are significantly different from each other ($P < 0.05$)

and 30th days, respectively. ALP values in the LPS 0.5 and LPS 1 groups were equally significant at 15 and 30 days. However, all LPS-treated groups had similar serum levels of AST and ALT at both timepoints. In contrast, total protein had a significant increment in the LPS 1 group compared with other groups. The increased levels of GGT confirmed hepatic injury in all LPS-treated groups despite the mildly significant differences among them (Fig. 8d). On the other hand, intermittent LPS injection caused an elevation in renal function parameters (creatinine, creatine kinase, and urea) (Fig. 8e–g). Creatine kinase (CK) serum values were equally significant similar ($P < 0.05$) for LPS 1 and LPS 2 groups, reaching 372 U/L and 385 U/L, respectively, on day 15. The LPS 2 group exhibited significantly higher levels of CK (418 U/L) than the LPS 1 and LPS 0.5 groups at 30 days. In terms of creatinine levels, LPS 1 and LPS 2 groups had similar values at 15 and 30 days to render both values significant ($P < 0.05$) compared with control and saline-treated groups. Likewise, urea values were high in all LPS-treated rats ($P < 0.05$) compared with the baseline, control, and saline-treated groups at 15 and 30 days (Fig. 8g).

Histopathological Assessment of Lung, Heart, Kidney, and Liver After Intermittent LPS Injections

The micrograph results demonstrate the morphological changes of lung, heart, kidney, and liver (Figs. 9 and 10) in rats inoculated with serial LPS concentrations (0.5, 1, and 2 mg/kg) and/or saline thrice per week for 28 days. The repeated, chronic exposure to LPS resulted in an unresolved systemic inflammation which subsequently developed the histopathological features of chronic inflammation in the above-mentioned organs. First, there was no sign of myocardia injury in control and saline-treated groups (Fig. 9a, b), while severe injuries, inflammatory infiltration, and necrosis were highly visible in the LPS 2 and LPS 1 groups (Fig. 9d, e).

Statistically, the inflammatory scores in the hearts of the LPS group rats had the same level of significance (Fig. 11) and were comparable with the saline-treated and control groups. Furthermore, histological examination of lung tissue sections showed intensive aggregation of neutrophils and macrophages which were observed in the majority of the lungs, especially in the alveolar epithelial layers of the LPS-treated groups (Fig. 9h–j). As illustrated in Fig. 9i, j, the wall thickness of the alveolar layers was directly proportional to the LPS doses. Surprisingly, these inflammatory changes were not observed in the saline-treated and control rats. However, rats treated with 2 and 1 mg/kg LPS had inflammatory scores which were of similar significance compared with the LPS 0.5, saline-treated and control groups (Fig. 11). Hematoxylin and eosin (H&E) staining of kidney tissue revealed that tubular cells underwent significant

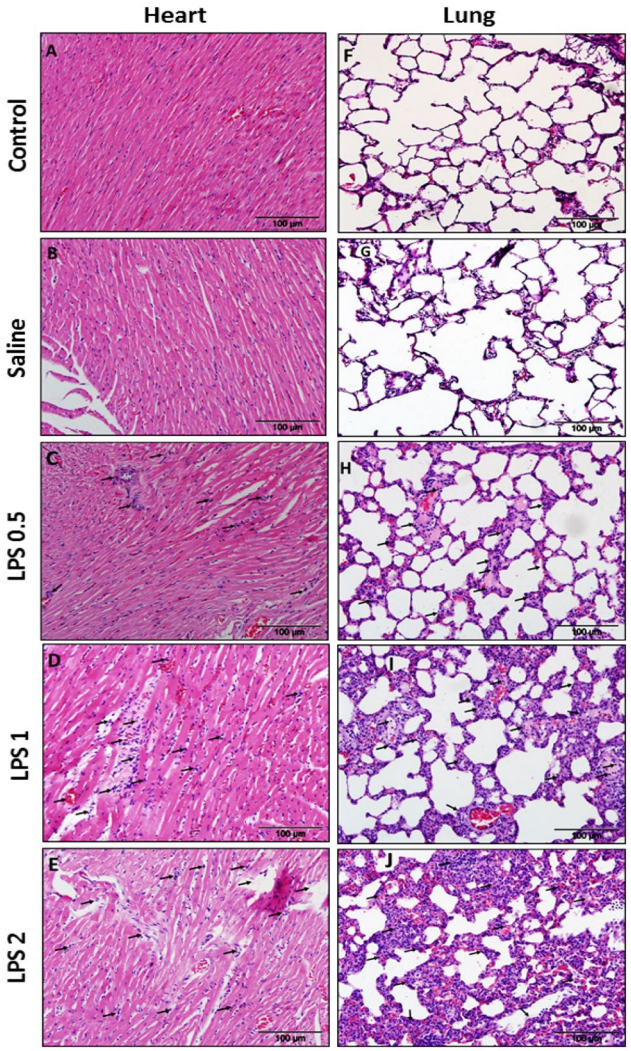

Fig. 9 Representative histopathological micrographs result of heart and lung sections stained with haematoxylin and eosin from rats treated with LPS and/or saline at × 200 magnification. **a**, **b** normal heart architecture is clearly observed without any necrosis or infiltration of leukocytes. **c**–**e** Degeneration and cellular infiltration in the inflammatory foci post-intraperitoneal injection of LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days is observed (arrows). **f**, **g** no histological changes were observed in the alveolar capillaries after no treatment and/or injection with saline intraperitoneally thrice per week for 28 days. **h**–**j** Onset of inflammatory process post-intraperitoneal injection of LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days is gradually observed; infiltration of leukocytes into alveolar and interstitial space, edema, alveolar wall thickness, and accumulation of macrophage (arrows)

damage with inflammatory tubulointerstitial and glomerular cell infiltration in LPS-treated rats (Fig. 10h–j). By increasing the dosage of LPS, tubule dilation and extensive macrophage infiltration were observed, compared with control and saline-treated rats. Concurrently, kidney injury scores confirmed the pathological changes and showed that the LPS 2 group had the highest score followed by the LPS 1 group

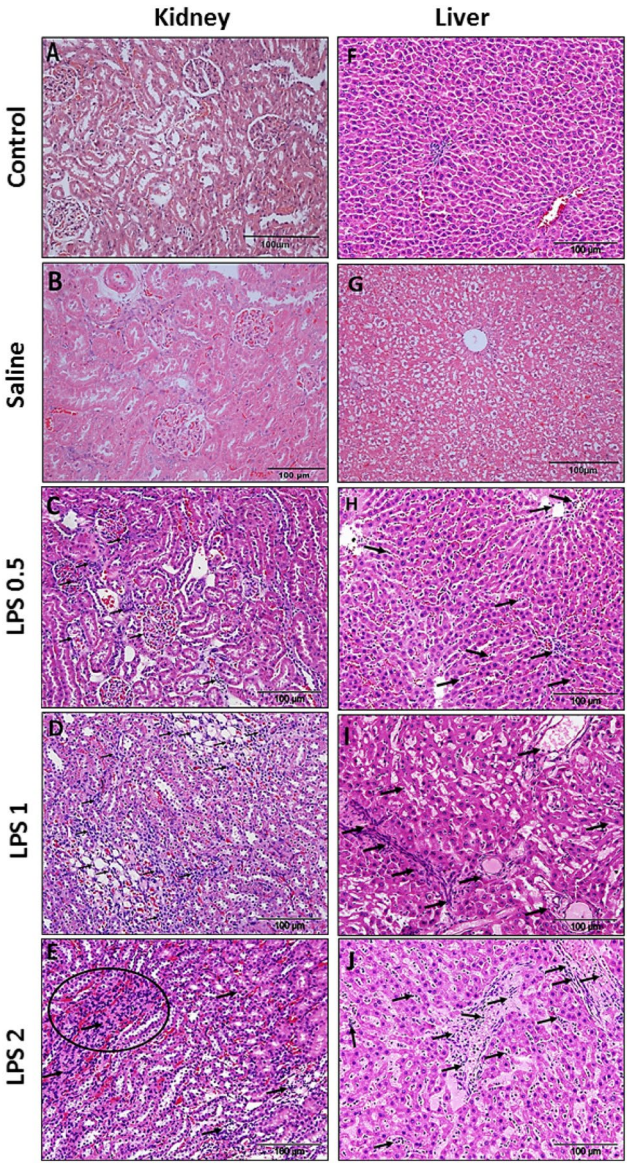

Fig. 10 Representative histopathological micrographs result of kidney and liver sections stained with haematoxylin and eosin from rats treated with LPS and/or saline at × 200 magnification. **a**, **b** no morphological changes observed, proximal tubular which is lined with simple cubic epithelium and distinguished capsular space after no treatment with LPS. **c**–**e** Affected areas were marked with vacuolar damage of tubular cells (arrows), edema, and tubulointerstitial inflammatory cells infiltration (circle) post-intraperitoneal injection of LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. **f**, **g** no pathological changes observed after no treatment with LPS. **h**–**j** Hepatocyte and hepatic lobular deformation, edema, neutrophils and macrophages infiltration (arrows), early signs of fibrosis, and necrosis (arrows) post-intraperitoneal injection of LPS (0.5, 1, and 2 mg/kg) thrice per week times for 28 days

(Fig. 11). Finally, the pathological lesions of livers from LPS-treated rats were compared with the control and saline-treated groups, as shown in Fig. 10f–j. After administrating 0.5 mg/kg LPS, hepatic lesions showed minimal infiltration by neutrophils and macrophages (Fig. 10h). In contrast, rats treated with 1 and/or 2 mg/kg LPS demonstrated marked multifocal neutrophilic infiltration, hepatocellular apoptosis/necrosis accompanied with macrophage infiltration, and early signs of fibrosis (Fig. 10i, j). The hepatocellular inflammation scores of the LPS 1 and LPS 2 groups confirmed the similarity in hepatic injury, as both groups had the same level of significance ($P < 0.05$) followed by LPS 0.5 group. Taken together, the previous data demonstrate that intermittent long-term exposure to LPS engenders CSSI along with pathologic changes in heart, lung, kidney, and liver, mainly characterized by infiltration with mononuclear cells such as lymphocytes, early signs of fibrosis, and tissue destruction.

Discussion

Non-resolving inflammation has been assumed to be the prime cause of CSSI, which subsequently leads to a progressive shift of inflammatory cells and elevation of pro-inflammatory markers in the systemic circulation along with tissue destruction (Renz et al. 2011). The excessive, irreparable damage increases the potential occurrence of chronic inflammatory diseases such as atherosclerosis (Lew et al. 2013), cancer (Aggarwal et al. 2006), inflammatory bowel disease (Jones et al. 2014), rheumatoid arthritis (Robinson et al. 2016), and neurodegenerative disorders (Fu et al. 2014). The pathophysiological onset of the above-mentioned diseases has commonalties that have been termed recently as CSSI (Song et al. 2016). Although the potentially high prevalence of CSSI and lack of optimized intervention have been documented, the mechanism of developing CSSI is poorly understood. The reason behind this is due partially to the shortage of a suitable rodent model to mimic CSSI (Calder et al. 2013). Thus, developing a model of CSSI would provide an appropriate opportunity to illustrate the mechanisms involved in its molecular pathogenesis which are complicated to define in man and to anticipate the promising approach of future interventions.

Previously, there are studies that have attempted to induce low-grade inflammation using LPS either by bolus injection or by continuous infusion. Normal subjects have been treated repeatedly with subclinical doses of LPS without considering the consequences and the ethical issues (Taudorf et al. 2007). In addition, Smith et al. (2006, 2009) induced low-grade inflammation to observe cardiovascular pathology and bone disease using LPS infusion delivered by time-release pellets for at least 60 days. The latter technique in delivering LPS for 90 days to induce low-grade chronic inflammation did not produce any significant changes in lymphocyte or neutrophil numbers and/or in the alveolar walls of the lungs, compared with a placebo (Arimura et al. 2012). However, chronic infusion of LPS via osmotic minipump failed to

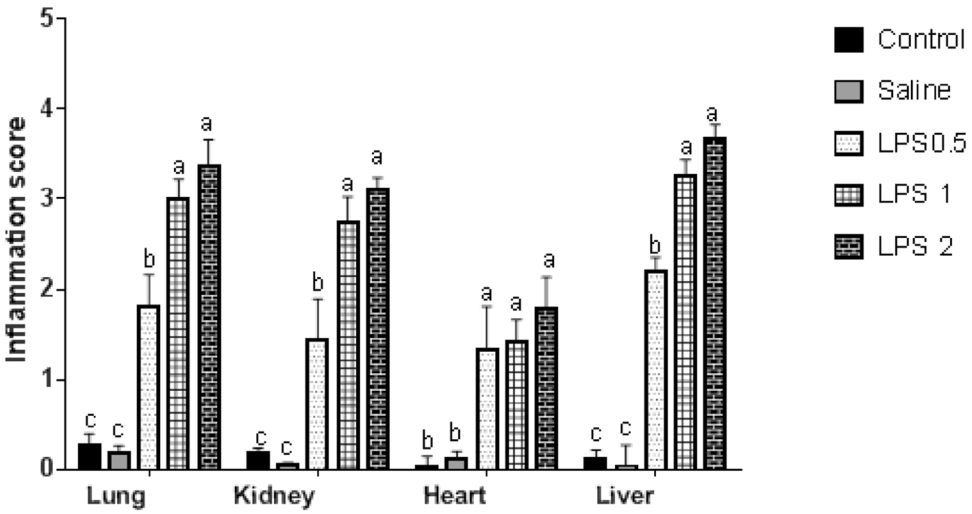

Fig. 11 Bar diagram showing inflammation score quantification for non-treated and treated rats with saline and/or LPS (0.5, 1, and 2 mg/kg) thrice per week for 28 days. Significantly, different values are indicated by different superscripts ($P < 0.05$)

induce low-grade chronic inflammation in SD rats, suggesting that intermittent injection of LPS at different days could be more effective (Fischer et al. 2015). Furthermore, two elements should be considered in the previous models which are time and cost. In the current study, LPS bolus injections were selected to be 3 days per week with a 1-day interval between each injection.

LPS treatment resulted in body weight changes due to the effect of LPS on appetite that was reflected in food intake. Even though the food intake did not recover to the normal range rapidly, body weight maintained a consistent increase, especially in rats treated with 0.5 and 1 mg/kg LPS. Probably, the tolerance to those two concentrations was sufficient to recover body weight and food intake. These observation were consistent with other studies despite differences in the methods used (Dudele et al. 2015; Valles et al. 2000). Rectal temperature was used to monitor the increase caused by post-injection endotoxemia (Chu et al. 2005). The results showed a slight increment in rectal temperature in the 0.5 and 1 mg/kg LPS-treated groups, unlike, 2 mg/kg of LPS which induced a higher temperature that could be due to an elevation in metabolic rate as an encounter response to the reduction in food intake and body weight. However, measuring the body temperature in rectal can potentially cause stress-induced fever (Dilsaver et al. 1992). In terms of mortality, the chronic bolus injection of 2 mg/kg LPS reduced the survival rate to 50% compared with the other groups; thus, this dose may not be suitable for inducing CSSI. The body weight, food intake, and rectal temperature changes of the LPS 2 group could reflect the high percentage of mortality. Therefore, selecting the proper dose of LPS to develop CSSI, based on the physiological changes and survival rates, has been considered ethically to minimize the animal burden. Overall, rats treated frequently with 0.5 or 1 mg/kg of LPS showed fewer physiological changes and a lower mortality rate, suggesting that those doses could be tolerated and be suitable for studying chronic administration of LPS. The rodents' ability to tolerate frequent LPS challenges without using lethal doses facilitates the conduct of chronic studies related to inflammation (West and Heagy 2002).

Diagnosing low-grade inflammation has been associated usually with measuring two elements: leukocyte counts and serum biochemical changes. The hematological evaluation of low-grade inflammation associated with chronic diseases has been related to the classic risk factors of these diseases, i.e., hypertension, and high levels of triglycerides (Facchini et al. 1992). The experimental protocol of the current study allowed the observation of changes in WBC counts after 7, 15, and 30 days. The toxic environment of LPS caused a consistent elevation in neutrophils along with lymphocytes despite the differences in life span. Probably, the early response of neutrophils exposed to LPS leads to an increase in the production of leukocytes from bone marrow (Nicolás-Ávila et al. 2017). On the other hand, the presence of macrophages and lymphocytes in the bloodstream is due to the increased production of chemokines and cytokines by immune cells and capillary endothelia, which finally facilitate immune cell infiltration into the tissues (Cinkajzlová et al. 2017). Furthermore, stimulation of toll-like receptors (TLR)4 by LPS leads to macrophage and lymphocyte activation that subsequently become dominant in chronic inflammation (Seki et al. 2007). Thus, measuring the neutrophil–lymphocyte ratio (NLR) has been recommended as a prognostic tool for CSSI and as a predictor of survival in different conditions (Forget et al. 2017). NLR has increased during the experimental period except in the LPS 0.5 group, while in the rats treated with 1 or 2 mg/kg of LPS reached in the same level of significance. These results were consistent with another experiment, where NLR predicted the level of distress of SD rats mediated by chronic stress (Swan and Hickman 2014).

Inflammatory cytokines released from the immune cells play a prime role in the onset of CSSI (Robinson et al. 2016). Despite being used as a marker of ongoing inflammation in various diseases, CRP is involved in the activating classical complement pathway that prevents autoimmunity and defends against infection (Peisajovich et al. 2008). Currently, CRP levels in serum have a predictive potential of chronic low-grade inflammation (Pepys and Hirschfield 2003). In this present study, CRP levels after 15 and 30 days increased in dose-dependent manner in all rats treated with LPS. CRP binding to the apoptotic cell surface enhances phagocytosis by macrophages and up-regulates the expression of TGF-β, which initiates fibrosis in multiple organs (Verrecchia and Mauviel 2007). Consequently, TGF-β levels in the present study could have been elevated either directly via LPS exposure to immune cells or indirectly via inflammatory cytokines. The heart, lungs, kidneys, and liver of rats treated with 1 or 2 mg/kg LPS demonstrated fibrotic signs, indicating the development of CSSI.

Once LPS binds to LPS-binding protein in the bloodstream, the CD14 membrane receptor of immune cells forms a complex with LPS-binding protein. The latter complex activates TLR4 resulting in up-regulation of two transcription factors: nuclear factor κB and activated protein-1. As a result, several inflammatory cytokines are released to regulate the inflammatory response (Seki et al. 2007). Similar to CRP, the cytokine levels of TNF-α, IL-1β, IL-6, and IL-8 are involved in the pathophysiology of chronic inflammation and autoimmune disorders (Langhans 2006). In this study, recurrent bolus injection of LPS has maintained TNF-α, IL-1β, IL-6, and IL-8 at high levels after 15 and 30 days. However, there was no observed difference between two doses of LPS, namely, 1 and 2 mg/kg. Similarly, Alonso-Castro et al. (2015) induced chronic inflammation in mice using i.p. injection of 1 mg/kg LPS twice weekly, which elevated the inflammatory mediators in a similar manner. However, continuous infusion of LPS via a time-release pellet or osmotic pump might initiate negative feedback mechanism by which a down-regulation of cytokine expression might occur. Subsequently, TNF-α and IL-18 which play a pivotal role in attracting and activating leukocytes into the inflamed site, have been suppressed (Langhans 2006). Despite suppressing the production of TNF-α, IL-6 activates lymphocytes and triggers the acute phase reactant response (Marin et al. 2001). Therefore, the stable IL-6 levels may be part of increasing the recruitment of lymphocytes in blood and tissues. At the same time, IL-6 and IL-1β are pyrogenic cytokines which could explain the increment in body temperature. MCP-1 is secreted by different types of cells, especially macrophages to attract mainly inflammatory monocytes and to stimulate the production of other cytokines (Deshmane et al. 2009). Inflammatory monocytes have been involved in low-grade inflammation and altered lipid metabolism through releasing various pro-inflammatory mediators (Geng et al. 2016).

Interestingly, the expression of TLR4 exists in various cell systems: immune cells, cardiomyocytes, bronchial endothelial cells, alveolar septal cells, tubular epithelium, glomeruli, Küpffer cells, hepatocytes, and hepatic stellate cells (Anderberg et al. 2017; Janardhan et al. 2006; Soares et al. 2010; Zeuke et al. 2002). Thus, the potential to release a large range of mediators including cytokines, chemoattractant, and matrix proteins in the lung, heart, kidney, and liver tissues is possible. The release of inflammatory mediators promotes leukocyte infiltration into the studied organs. More profoundly, the high levels of pro-inflammatory cytokines may account for the pathological changes that occurred in the previously mentioned tissues. These changes include: degeneration, cellular infiltration of leukocytes in the inflammatory foci and interstitial spaces, edema, early signs of fibrosis, apoptosis, and necrosis. In heart, LPS has been shown to increase the expression of IL-6, which plays a key role in differentiating fibroblasts into myofibroblasts and increasing collagen production (Meléndez et al. 2010). In the current study, high expression of TNF-α, IL-6, and MCP-1 contributed to the development of myocarditis, where infiltration of neutrophils along with leukocytes is present. To support that, CK elevation pointed indirectly to myositis that had been listed as an inflammatory autoimmune disease. Comparatively, the thickness wall of the alveolar in lung tissue exposed to LPS was increased in a dose–response manner. Previously, it has been reported that i.p. injection of LPS thickens the alveolar wall (Li et al. 2015). As a result, transporting oxygen into blood may have to travel a longer distance which inevitably leads to a higher rate of mortality, as is evident in LPS 2 group. The front-line organs affected by low-grade systemic inflammation are the kidney and liver (Spapen 2008). In the present research, the persistence of LPS injection induces a phenotype of kidney injury characterized by vacuolar damage of tubular cells, edema, early signs of fibrosis, and tubulointerstitial inflammatory cell infiltration. These findings were consistent with Chen et al. (2015), where chronic kidney injury developed via i.p. injection of 1 mg/kg LPS once every other day for 14 days continuously. Surprisingly, low-grade inflammation has been strongly associated with strongly chronic kidney diseases and the persistence of subclinical inflammation is considered as a risk factor of chronic kidney disease progression (Yilmaz et al. 2007). More importantly, the activation of Küpffer cells along with pro-inflammatory cytokines post-LPS stimulation serves a significant role in developing low-grade inflammation which subsequently may induce non-alcoholic fatty liver disease (Tomofuji et al. 2007). Our data revealed that hepatocyte and hepatic lobular deformation, edema, neutrophil and macrophage infiltration, early signs of fibrosis, and necrosis were present after rats had

been treated with 1 or 2 mg/kg of LPS. These inflammatory manifestations were coupled with elevation in serum levels of ALP, AST, GGT, and ALT. Indeed, the elevation of liver function enzymes has been reported to be an indicator tool for cholestasis (blocked bile flow) (Hamesch et al. 2015). Although GGT has been released mainly from hepatocytes and is considered the most sensitive marker for cholestasis, determination of bilirubin levels and specific histological analysis are required to confirm the diagnosis of cholestasis (Li and Apte 2015). In contrast, non-alcoholic fatty liver disease, which induces low-grade inflammation, promotes the elevation of liver enzymes (Obika and Noguchi 2012; Robinson et al. 2016).

Overall, the results of the current investigation have demonstrated that the intermittent exposure of LPS with three different concentrations (0.5, 1, and 2 mg/kg) induces a CSSI model with multiple organ injury. In this model, 1 mg/kg of LPS is considered the most effective dosage in inducing CSSI with a minimum burden on rodents. Circulating LPS has been thought to cause insulin resistance, diabetes mellitus, heart failure, and chronic diseases (Lew et al. 2013). In the current study, the intermittent bolus injections of LPS develop systemic inflammation gradually in multiple organs and are sustained for several days, which could be exploited to understand an effect of therapeutic agents. This would improve the opportunity of treatment for patients with chronic inflammatory diseases before CSSI insult. However, there are a few limitations in the present study. First, this study was designed to characterize CSSI associated with multiple organ injury, but not to study the underlying mechanisms of chronic inflammation-mediated changes. Second, the number of rodents may not have been sufficient for statistical power to notice the difference between groups in this study. Moreover, despite evaluating the inflammatory mediators that support potential pathways for developing CSSI, various other factors such as adhesion molecules, inflammatory transcription factors, and anti-inflammatory cytokines should be studied in further studies. Finally, with the exception of the white blood cell and cytokine results at 15 and 30 days, the histopathological findings demonstrate a single snapshot at a specific time, limiting the capability to evaluate the inflammatory changes in serial events.

In conclusion, intermittent bolus injections of LPS thrice per week for 28 days induce CSSI with multiple organ injury. The hematological and biochemical changes observed in this model are relatively similar to those seen in chronic diseases. Simultaneously, this model is presumed to be preclinical tool and to provide a novel chance for developing immunotherapeutic agents which could reduce CSSI-associated diseases with, or without, bacterial translocation.

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Author Contributions YR has designed research, performed experiments, interpreted data and wrote the manuscript. AMA has interpreted the data and supervised the work and helped in editing the manuscript. HAH, HK, and NM supervised the project and designed research. AF and MHKA edited the manuscript and interpreted data. All authors read and approved the final manuscript.

Data Availability Access to these data will be considered by the author upon request.

Compliance with Ethical Standards

Conflict of Interest The authors have no competing interests to disclose.

Research Involving Human Participants and/or Animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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