Differences in Inflammation, Apoptosis and Tissue Hypoperfusion Indicators Caused by Using Two Different Gauges in Cecal Ligation and Puncture-induced Rat Models of Sepsis

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Abstract

Background: Rodent Cecal Ligation and Puncture (CLP) models, which mimic sepsis in humans. Gauges needle size during CLP related to cytokine storm, inflammation and organ failure. This study focus on precise and low cost various biochemical markers to trace sepsis severity in cardiopulmonary system and blood after CLP with gauge needle size -18 (G-18) and -21 (G-21) in rats.

Results: For the purpose of this study, 18 male Wistar rats were assigned into three groups: CLP procedure with G-18 needle, CLP procedure with G-21 and sham. Following the procedure, oxidative stress markers, blood markers, apoptosis indicators, gene expression of autophagy and cellular hypoxia and histopathological assessments were carried out in the cardiopulmonary tissue and blood 24 hours after CLP. All measured factors were increased in both G-18 and G-21 gauge groups in comparison with the sham. Moreover, 24 hours after CLP, inflammatory markers, blood profile, lactate level, pro-inflammatory cytokines, caspases, gene expression of cellular homeostasis and autophagy in samples were more pronouncedly increased in the G-18 group in comparison with the G-21.

Conclusions: This experiment provides a significant association of inflammation and cytokine storm with anti-oxidant property, apoptotic condition and gene expression of tissue and blood infection related to severity organ damage were increased with G-18 compare to G-21. Our study has identified cost-effective indicators to evaluate organ failure during sepsis. This should be considered by all investigators using G-18 in comparison with G-21 to induce severe sepsis.

Introduction

Sepsis is a common cause of inpatient mortality in both genders and all age groups, particularly in the intensive care units (ICU) of hospitals. Annually, more than 19 million people are affected by sepsis (with a mortality rate as high as 18%) worldwide, and approximately 50 % ICU services. (1, 2) This deadly condition ensues an infection-caused inflammatory process in the body. In sepsis, the cause of death is generally multi-organ dysfunction, and in about 50% of the affected individuals, the cardiovascular system is inflicted. (3) Despite the recent decrease in its mortality rate though advancements in antibiotics and intensive care equipment (1, 2), the incidence of sepsis is increasing worldwide, possibly due to ageing populations and the increased incidence of co-morbid conditions in advanced ages. (4)

Sepsis-induced shock is associated with multi-organ failure with a mortality rate of 40%, and the most frequently affected organs are the cardiovascular and respiratory systems. (3) Although the precise mechanisms of sepsis-induced cardiomyopathy are not yet fully elucidated, it suggested that severe inflammation, metabolism insufficiencies, and defective beta-adrenergic response may be involved in this process. (4) Moreover, during sepsis, mainly due to hyperlactatemia, both systolic and diastolic cardiac impairment occur with the consequence of mitochondrial damage in the cardiac tissue (5). Pulmonary complications of sepsis develop as a result of the cumulative effect of circulating cellular elements, soluble inflammatory mediators, and cytokines on the pulmonary tissues. (6) The main underlying mechanism of lung injury during sepsis is demonstrated to be acute alveolar infiltration and changes in respiration ratio (7).

Considering its high fatality rate, sepsis leaves little opportunity for clinical research in the field. Therefore, CLP procedure which is considered as the gold standard model of human sepsis provides an invaluable opportunity for investigation into different aspects of sepsis. In this procedure in the animal model of the disease, an induced results in the secretion of intestinal poly-microbial flora into the abdomen, a contamination followed by vascular dissemination of the micro-organisms. This leads to systemic presentations very similar to those of sepsis in humans. (8)

CLP-induced damages (and sepsis in humans) stimulate the immune system, activate oxidative stress pathways, increase caspase levels (due to apoptosis), and lead to the expression of autophagy and hypoxia-related genes (9). Reactive oxygen species (ROS) are major signaling molecules involved in the oxidative stress response. In sepsis, the excessive accumulation of ROS impairs cellular homeostasis, and this leads to oxidative stress and mitochondrial dysfunction. This oxidative stress process also promotes autophagy, a defensive cytoprotective process for recycling waste organelles and other intracellular material following cellular damages.(10) Consequently, indicators of oxidative stress and autophagy processes have been widely used to investigate the intensity of sepsis induced the CLP procedure.(11) Similarly, measurement of the enzyme AMP-activated protein kinase is extensively employed for assessment of the severity of sepsis (12).

It is demonstrated that the size of the needle used in the CLP procedure can be influential in different septic outcomes such as mortality, cytokines' concentrations, apoptosis, lactic acidosis, and autophagy. (13)

It is demonstrated that the size of the needle used in the CLP procedure can be influential in different septic outcomes such as mortality, cytokines concentrations, apoptosis, lactic acidosis, and autophagy. Previous studies showed that G-18 and G-21 induced sepsis more efficiently compared to other gauge groups(6, 11, 12).

There have been little experiments and limited set of factors affecting multi-organ dysfunction syndrome (MODS), with G-18 and G-21. New inexpensive and simple indicators have required to modify needle size related to MODS during CLP. In this study, we aimed to find and compare precise, inexpensive and new biomarkers in heart, lung and blood profile after CLP induced sepsis with G-18 and G-21.

In this study, on the presumption that these processes may underlie differences in organ dysfunction and death, we aimed to measure and compare inflammatory markers and expression of hypoxia and autophagy-related genes in cardiac and pulmonary tissues of CLP models of sepsis induced by two different gauges (18 and 21).

Materials And Methods

Animals

Adult male Wistar rats (3–4) months old (220–310 g) were procured from the Animal Breeding House of Faculty of Pharmacy of TUMS. Animals were kept according to the animal standard care facility under controlled temperature ($23 \pm 10^\circ\text{C}$), 12 h light/dark cycle, $55 \pm 10\%$ humidity and ad libitum feed. The protocol of the study was approved by the institute ethical committee under code number IR.TUMS.VCR.REC.1396.2341 and Reporting of in Vivo Experiments (ARRIVE) Guideline.

Reagents

For High mobility group box 1 (HMGB1) and TNF- α , ELISA kits were procured from ZellBio GmbH (Ulm, Germany) and Diacel (France), respectively. Lactate isolation kit we used was produced from ZellBio GmbH (Ulm, Germany). RNase solution, iScript cDNA synthesis kit, and propidium iodide were manufactured by Sigma-Aldrich GmbH (Munich, Germany). Other chemicals not specifically mentioned were all purchased from Sigma-Aldrich GmbH (Munich, Germany).

Animal groups

Eighteen rats were randomly allocated into 3 groups consisting of:

Group 1 (n=6); Sham group (which underwent exactly the same operation without the CLP procedure)

Group 2 (n=6); CLP group with G-18 (which underwent CLP procedure and two punctured with gauge 18)

Group 3 (n=6); CLP group with G-21 (which underwent CLP procedure and two punctured with gauge 21)

The procedure

Adult male Wistar rats first underwent CLP as described in details elsewhere (14). Rats were anesthetized with an intra-peritoneal dose of ketamine (80 mg/kg) and xylazine (10 mg/kg). Then, a 3-cm midline laparotomy was performed in order to expose the cecum and adjoining intestine. The cecum was ligated with a 3.0 silk suture at its base right below the ileocecal valve, and was perforated two times using 18- or 21-gauge according to the group animals were assigned to. Dimensions of the needles are as follows: Gauge 18=1.270mm, Gauge 21=0.8192mm. Next, the cecum was replaced in the peritoneal cavity and the incision was closed using 4.0 silk sutures. Following the operation, animals were hydrated through intravenous administration of an isotonic saline solution (50 mL/kg s.c.), returned to a cage in a period of 24 hours (15). CLP procedure was assessed with Murine Sepsis Score (MSS) by an experienced technician (16) .

Sample preparation

24 hours following the procedure, the animals were anesthetized with overdoses of ketamine and xylazine (80- 100 mg/kg and 5-10 mg/kg IP), and were sacrificed. Blood specimens were then collected from the hearts of the rats and subsequently divided into serum separator and ethylenediaminetetraacetic acid (EDTA) tubes for analysis of related blood markers. The tissues were harvested and washed with saline then put into two parts for further histopathological and biochemical assessments. Biochemical samples were stored as frozen samples at -80 °C refrigerator, and pathological specimens were deposited and fixed in 10 mL of 10% formalin.

Determination of oxidative stress biomarkers

ROS assay

Tissue samples were all homogenized and centrifuged accordingly. Subsequently, they were stored in 75 µL extraction buffer, mixed with 80 µL of assay buffer, and kept for 30 min at 37 °C. Production of ROS was absorbed by 2', 7'-dichlorofluorescein diacetate (DCF-DA) as fluorogenic reagent. Identification of the absorbance change of DCF-DA was identified by means of ELISA fluorometer (Biotec, Tecan U.S.) with maximum excitation (488 nm) and emission (529 nm) spectra for an hour.

LPO assay

Malondialdehyde (MDA) is generally considered as the final by-product of lipid peroxidation (LPO) during generation process of ROS. In the current study, Thiobarbituric acid reactive substances (TBARS) assay were employed for detection of the complex of MDA and Thiobarbituric Acid (TBA). MDA concentration were reported at 532 nm with spectrophotometer. As described in details elsewhere, the amount of MDA formed by tissue samples was reported as µM/mg protein (17).

Total antioxidant power (TAP) assay

FRAP test is widely used for measurement of antioxidant potential of samples. Accordingly, for deoxidizing the Fe³⁺ - TPTZ (2, 4, 6-tris-(2-pyridyl)-s-triazin) to Fe²⁺ , 1 mL of Tris-EDTA buffer was added to 50 µL of samples, and, consequently, the absorbance was measured at 593 nm (18).

Assay of total thiol molecules (TTM)

Total thiol molecules is defined as antioxidant content of samples (19). For thiol assessment, interaction of TTM with Ellman's reagent was estimated in maximum peak at 412 nm (20).

Myeloperoxidase (MPO) activity

The homogenization of tissue samples were carried out in 50 mM potassium buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). Subsequently, the samples were centrifuged (30000 g, 15 min, 4 °C) and 100 µl of the supernatant was mixed with phosphate buffer (pH 6, 50 mM) containing 0.167 mg/ml of o-dianisidine dihydrochloride and 0.0005% H₂O₂. Change of absorbance was recorded through spectrophotometric analysis at 460 nm five minutes later. The expression of MPO activity was defined as the change of enzyme absorbance during conversion of 1 µmol of H₂O₂ to H₂O/ 1 min at 37⁰c, as described in details elsewhere (21). Unit per mg of tissue protein was considered as the index of MPO activity.

Caspase-3 and -9 activation in the cardiac and lung tissues

Colorimetric assays were utilized for measurement of the caspases-3 and -9 through specific identification of particular amino acid sequences. Briefly, the application of chromophore p-nitroaniline (pNA) produces a yellow color which can be detected by spectrophotometry at 405 nm. For this purpose, tissue samples were degraded by lyses buffer and incubated for 10 min on ice. Moreover, the caspase buffer which consisted of 100 mM of caspase-3 and -9 specific substrate along with total cell lysates were incubated at 37 °C for 4 h. The standard absorbance of caspase-3 and -9 was detected at 405 nm (22).

Lactate levels in tissue and serum samples

Tissue samples

According to the Manufacture's protocol lactate assay was performed for analysis of all samples. In short, tissue samples (100 mg) were homogenized in 8% perchloric acid and lactate level evaluated with using a standard curve.

Serum samples

After allowing the serum separator tube to clot for 10 minutes at 37⁰c, samples centrifuged (at 3000 g for 20 minutes), then supernatants were collected. The lactate levels of serum samples were determined using a standard curve and reported as mmol/L of serum protein.

Assessment of pro-inflammatory cytokines

Measurement of TNF-α levels

The quantity of TNF-α in test samples was examined by a rat-specific TNF-α ELISA kit (Diacclone, France). According to instruction of the kit, test samples and standards were added to the wells containing antibodies. After washing, biotinylated anti-rat TNF-α antibody and HRP conjugated streptavidin were added to each well, samples were rewashed, and TMB and stop solutions were mixed to the wells respectively. The conversion of blue to yellow color, which is demonstrated to be proportional to the TNF-α level, was estimated at 450 nm.

Assessment of HMGB1 levels

The quantity of HMGB1 in test samples was examined by means of a rat-specific HMGB1 ELISA kit. Test and standards samples were added to the wells containing immobilized antibodies. Following washing, biotinylated anti-rat HMGB1 antibody and HRP conjugated streptavidin were added to the wells. Then samples were rewashed and chromogen and stop solutions were added to the wells. A color change (from blue to yellow) in proportion with the HMGB1 levels was assessed at 450 nm.

Gene expression evaluation

For Quantitative Real-time PCR (qRT-PCR), 1 µg of total RNA was reverse transcribed to cDNA using a Primescript RT reagent kit (TAKARA, Japan). qRT-PCR was performed using the Step one plus ABI system (Applied Biosystems). qRT-PCR was carried out under the following conditions: 30 s at 95 °C for 1 cycle, 40 cycles of 95 °C for 5 s, 60 °C for 34 s and 72 °C for 45 s. One cycle of 30 s 72 °C was done finally to allow final extension (23). The sequences of primers used for qRT-PCR are presented below;

For Glyceraldehyde-3-phosphate dehydrogenase (gene symbol: GAPDH)

sense primer (5'- AGTCTACTGGCGTCTTCACC -3')

antisense primer (5'- CCACGATGCCAAAGTTGTCA -3')

For AMP-activated catalytic subunit alpha 1 (AMPK) (gene symbol: Prkaa1)

sense primer (5'- CCGTCTTATAGTTCAACCAT-3')

antisense primer (5'- TTCGTTTCATTATTCTCCTGTT-3')

For Microtubule-associated protein 1 light chain 3 beta (LC3Ib) (gene symbol: Map1lc3b)

sense primer (5'- CAGTGATTATAGAGCGATACA-3')

antisense primer (5'- GCCTTCTAATTATCTTGATGAG -3')

Blood samples parameters

Whole blood assessment

Peripheral blood samples were collected for complete blood count (CBC). The CBC blood samples were collected into EDTA tubes. Hematologic parameters were analyzed 30 mins following sample collection using a hematology analyzer (Sysmex). The counts for lymphocyte ($10^3/\mu\text{L}$) and platelets ($10^3/\mu\text{L}$) and mean platelet volume (MPV) (fL) were recorded and platelet to lymphocyte ratio (PLR) were calculated, using the results.

Blood glucose levels

24 hours after the CLP procedure, blood samples were collected by skilled personnel using the routine technique of tail-tip amputation. Then the ACCU-CHECK Compact Plus® (Roche Diagnostics, Japan) glucometer were employed for measurement of blood glucose levels in 5 seconds.

Histopathological studies

The animals were euthanized 24 hours after the procedure, and heart and lung tissues were isolated and fixed in the 10% neutral buffered formalin (NBF, pH 7.26) for 48 hours, and then were embedded in paraffin. The 5-µm thick

sections were then made ready for staining with hematoxylin and eosin (H&E). Then the histological slides were observed using light microscope (Olympus BX51, Japan). Any suspected changes such as acute or chronic inflammatory response, congestion, hemorrhage or hyperemia, necrosis was investigated in different samples.

Statistical analysis

The results were presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey's multi-comparison tests were applied with the degree of significance set at (P< 0.05).

Results

Oxidative stress and anti-oxidant parameters

ROS levels were raised significantly in lung tissue in CLP-G18 and CLP-G21 compared to the sham group (P<0.01 and P<0.05, respectively). Moreover, ROS production in heart tissue was increased significantly in CLP-G18 compared to sham group (P<0.001). Likewise, another important finding was that CLP-G21 significantly decreased the amount of ROS compared to CLP-G18 (P<0.001).

A significant increase in the levels of MDA in the lung and heart tissues were observed in CLP-G18 in comparison the sham group (P<0.01). Moreover, the LPO level was diminished in the CLP-G21 group compared to the CLP-G18 (P<0.01 and P<0.05, respectively) in two different tissues.

Tissue FRAP in lung samples was decreased significantly in CLP-G18 compared to sham group (P<0.001), but significant reduction of FRAP was only observed in heart tissue with both gauges (CLP-G18 and CLP-G21) compared to sham group (P<0.001 and P<0.01, respectively). Likewise, CLP-G21 significantly enhanced FRAP Levels in lung and tissue compared to CLP-G18 group (p<0.05 and p< 0.001, respectively).

TTM level in lung tissue was significantly reduced in CLP-G18 and CLP-G21 groups compared to sham group (P<0.01 and P<0.05, respectively), TTM level in heart tissue was significantly declined in CLP-G18 and CLP-G21 compared to sham group (P<0.001 and P<0.01, respectively). The oxidative stress parameters are presented in Table 1.

Table 1. Effect of gauge 18 and gauge 21 on Cecal ligation and puncture (CLP) on biochemical markers in heart and lung tissues.

	Heart tissue			Lung tissue		
	Sham	CLP-G18	CLP-G21	Sham	CLP-G18	CLP-G21
ROS(unit/mg protein)	0.66±0.05	1.21±0.08***	0.77±0.04###	0.27±0.03	0.98±0.16**	0.84±0.17*
LPO (μM)	63.71±2.10	96.51±5.95**	83.58±6.25*	76.51±6.86	108.39±5.68**	80.308±3.40##
FRAP (μM)	275.9±6.31	213.57±4.28***	251.9±1.84** ###	284.23±6.16	239.57±7.30***	268.74±5.14#
TTM (μmol/mg protein)	0.96±0.11	0.35±0.08***	0.41±0.06*	1.13±0.20	0.42±0.10**	0.61±0.06*
MPO (unit/gr tissue)	77.83±3.64	97.64±6.07*	89.41±4.96	75.97±7.50	111.7±7.66**	103.16±3.78*
Caspase-3 activity(% of content)	99.85±4.26	99.85±4.26	80.07±6.59#	100.12±2.52	138.29±8.97**	107.31±5.96##
Caspase-9 activity(% of content)	99.82±7.02	124.88±3.16*	103.77±5.79#	99.85±4.62	131.44±11.10*	139.45±7.82**

ROS (reactive oxygen species), LPO (lipid peroxidation), FRAP (ferric reducing antioxidant power), TTM (total thiol molecules), MPO (myeloperoxidase), Caspase-3 and -9 assay. Results are expressed as mean ± SEM for six animals in each group.

***: significant difference from sham group at P < 0.001,

** : significant difference from sham group at P < 0.01,

*: significant difference from sham group at P < 0.05,

###: significant difference from CLP-gauge18 group at P < 0.001,

##: significant difference from CLP-gauge18 group at P < 0.01,

#: significant difference from CLP-gauge18 group at P < 0.05.

Myeloperoxidase activity

The result of MPO assay showed that a significant elevation was increased in lung tissue of CLP-G18 and CLP-G21 compared to sham group (P<0.01 and P<0.05, respectively). However, the MPO level of CLP-G18 group in heart tissue was elevated significantly (P<0.05) compared to sham and CLP-G21 groups (Table 1).

Caspase 3 and Caspase 9

The result of enzyme activities of caspase-3 and caspase-9 in lung tissue indicate that there was a significant difference between CLP-G18 and sham group (P<0.01 and p<0.05, respectively). This figure shows a decrease of caspase 3 activity in CLP-G21 compared to CLP-G18 (P<0.01). The results obtained from the caspase activity in heart tissue had no effect on the level of caspase-3 in CLP-G18 and CLP-G21 in comparison with the sham group, but the

activity of caspase-9 was increased in CLP-G18 compared to sham group ($P<0.05$). A significant reduction in the level of caspase-9 activity was observed in CLP-G21 compared to CLP-G18 group ($P<0.05$) (Table 1).

Lactate levels

Lactate production, a reliable marker of tissue hypoperfusion was increased in CLP-G18 and CLP-G21 groups in lung ($P<0.001$ and $P<0.01$) and heart tissues ($P<0.001$) compared to the sham group. The blood lactate level raised in CLP-G18 compared to sham groups ($P<0.001$). Moreover, no significant differences were found between CLP-G21 and sham group (Fig. 1).

Proinflammatory cytokines

TNF- α which is an early modulator of inflammation during sepsis was found to be significantly increased in CLP-G18 and CLP-G21 ($P<0.001$) in comparison with the sham group in lung samples. The levels of TNF- α in heart tissue was increased in CLP-G18 and CLP-G21 groups compared to the sham group ($P<0.001$ and $P<0.05$, respectively) (Fig. 2A).

The amount of HMGB1, a late phase inflammatory biomarker, was significantly elevated in CLP-G18 and CLP-G21 groups in comparison with the sham group ($P<0.001$) in both lung and heart samples (Fig. 2B).

Quantitative Real-time PCR

The expression of AMPK as the stress protein in lung tissue was significantly increased in CLP-G18 and CLP-G21 groups compared to the sham group (1.77-fold and 2.23-fold, respectively). In addition, the mRNA expression level of AMPK in CLP-G18 and CLP-G21 groups of heart tissue increased significantly compared to the sham group (3.66-fold and 1.79-fold, respectively). The level of AMPK expression has been downregulated in CLP-G21 compared to CLP-G18 in lung and heart samples ($P<0.01$ and $P<0.001$, respectively) (Fig. 3A).

The level of LC3IIb gene expression as autophagy gene in lung tissue raised in CLP-G18 and CLP-G21 groups compared to the control group (4.38-fold and 2.36-folds, respectively). The analysis of lung tissue data showed that the expression level of LC3IIb in the CLP-G21 group has been significantly reduced compared to the CLP-G18 group ($P<0.001$). Likewise, the mRNA expression level of LC3IIb in CLP-G18 and CLP-G21 groups significantly elevated compared to the sham group (5.86-fold and 5.3-fold, respectively) in heart tissue sample (Fig. 3B).

Mean platelet volume (MPV) and platelet to lymphocyte ratio (PLR)

Overall platelet function and inflammation in blood were measured in MPV and PLR, respectively. The levels of MPV and PLR were raised significantly in CLP-G18 compared to the sham group ($P<0.01$) (Fig. 4A, 4B). Also, there was a significant reduction in MPV and PLR in CLP-G21 compared to CLP-G18 group ($P<0.05$ and $P<0.01$, respectively).

Blood glucose levels

Blood glucose analysis revealed a significant reduction in CLP-G18 and CLP-G21 compared to the sham group ($P<0.001$ and $P<0.05$, respectively) (Fig. 4C).

Histopathological analysis

All the samples were visualized by an independent reviewer. The results of each sample have reported as below:

Micrographs of lung and heart in sham group showed normal structure without any histopathological findings. Histopathological evaluation of lung and heart tissues in CLP-G21 group showed a close similarity to sham group with

normal structure and organization of lung and heart tissue. Micrographs of the lung in CLP-18 group showed the various degree of lung injury such as pneumocyte type II (hyperplasia), peribronchiolar inflammation and hyperemia. The thickness of alveolar septa significantly increased in comparison to normal area. However, lung inflammation was verified by the presence inflammatory cells (lymphocyte) and edema. The inflammatory response was confirmed by lymphocyte counts in five random high-magnification ($\times 1,000$) fields per slide. 7.5 ± 0.12 lymphocyte/field was present in CLP samples. Moreover, the histopathological evaluation of the heart sample in CLP-G18 group showed myocardial cell necrosis (Fig. 5).

Discussion

We designed and carried out the current study to illuminate differences in cardiac and pulmonary tissues damages following CLP procedure in rats. To our best knowledge, this is the first study investigating the differences two needle sizes, namely 18 and 21, cause in cellular and molecular sepsis mechanisms such as oxidative stress, expression of homeostatic-autophagic related genes, proinflammatory cytokines, apoptosis, tissue perfusion and hematologic parameters in male Wistar rats. Findings of our study demonstrated that 24 hours after the CLP procedure, we observed that inflammatory markers, blood markers, blood and tissue lactate levels, pro-inflammatory cytokines, caspases, and gene expression of cellular homeostasis and autophagy in samples were more pronouncedly increased in the G-18 group in comparison with the G-21. This should be taken into consideration for development and execution of related protocols to increase the accuracy of the results and avoid waste of resources.

Oxidative stress is a major indicator of sepsis induced by CLP procedure. Oxidative stress pathways and ROS generation are consisted of several components such as: increased LPO levels, alternated metabolic gene expression, and increased MPO levels during the procedure (24, 25). In this regard, many consider MPO as a major indicator of the neutrophil infiltration process. (25-30) It is demonstrated that sepsis induced by both gauge 18 and gauge 21 can elevate ROS levels in the liver, colon and kidney 16 to 24 hours following the procedure (31-34). In addition, 6 to 48 hours after the procedure, LPO level, increases in the lung, ileum, diaphragm, heart, brain and kidney (regardless of the gauges used) (26-30, 34-41). Congruently, the findings of our study demonstrated that 24 hours after the CLP procedure, in both cardiac and pulmonary tissues, oxidative stress markers increased, whereas the levels of antioxidant indicators reduced in comparison with the sham group. Interestingly, we observed that this effect was more accentuated when gauge 18 needles were used in comparison with gauge 21. Similarly,

As regards blood markers (due to our limited resources), we applied low cost, quick and available hematologic tests to assess MPV, PLR and glucose level of blood samples to determine sepsis. MPV is an indicator of platelet function and endothelial disruption which is increased in the early and late phase of sepsis (42-45). Canine model of sepsis have demonstrated higher levels of MPV in the septic group in comparison with the control animals (46). In line with such observations, our findings demonstrated that MPV levels were elevated in the late phases of the procedure with the G18 needles. It is widely reported that blood glucose levels are elevated in early phase of CLP, whereas they decrease in the in the late phases (47-50). In this regard, we noticed that blood glucose levels were more pronouncedly reduced in the CLP-G18. This observation is in line with those reporting that PLR is elevated in hospitalized patients with end stage renal disease and nosocomial infection (51, 52). Thus, it can be claimed that current study was the first to report that the raise on PLR levels is more prominent in the CLP-G18 murine models. This should be seriously considered in design and development of related protocols in this field.

Lactate level is generally considered as a reliable indicator of tissue hypo-perfusion, hypoxia and altered microcirculation in CLP models of sepsis. Previous studies have separately reported raised lactate levels following CLP-G18 in blood, ileum and liver, and noted the elevation of the lactate levels in lung and colon following CLP

procedure with G22 needles (25, 27, 49, 53-55). In this regard, we observed that the blood and tissue lactate levels increased more intensely following G18 CLP procedure in comparison with CLP-G21.

Regarding proinflammatory cytokines, it is demonstrated that the blood levels of these indicators of inflammation, particularly TNF- α and HMGB1, is altered during CLP procedure. (56-59) Previous reports have demonstrated high levels of TNF- α in different organs of rats between 6 to 72 hours after CLP-G18 procedure (27, 28, 30, 31, 36, 55, 60). In this regard, we observed that in cardiopulmonary system, the levels of both aforementioned cytokines were more intensely increased when sepsis was induced using G-18 needles in comparison with the CLP-G21 group. This needs to be taken into consideration if future studies.

Mitochondrial membrane disturbance during ROS elevation results in formation activates caspase 9 and 3 with the natural consequence of cellular apoptosis (55). This programmed cell death process during CLP procedure is observed and reported in different tissues (mainly with gauge 18) such as the kidney, lung, and brain (35, 39, 40). Correspondingly, findings of our study revealed that the levels of the aforementioned caspases increased in the lung and heart with the higher intensity in CLP-G18 compared to CLP-G21.

A combination of ROS elevation and enhanced autophagy are responsible for elimination of abnormal proteins and promotion of organ failure-related apoptosis during sepsis. (61-65) Several studies have demonstrated that LC3-II/LC3-I ratio increased in the liver, kidney, spleen and mesenteric nodes 6 to 72 hours after CLP procedure (with gauges 22 to 25) (66-69). Moreover, Hsaio et al showed an increase in LC3-II levels in the renal tissues of rats after 3 hours after CLP-G18. Correspondingly, Escobar concluded that 8 hours after CLP-G22 procedure, LC3-II was increased in parallel with phosphorylated AMPK in kidney and liver (61). In this regard, we observed that the gene expression levels of mRNA of LC3-IIb and AMPK in both CLP-G18 and CLP-G21 was more pronouncedly increased in the heart and lung tissues of the animals in comparison with the sham group. Moreover, histopathological changes we observed confirmed the superiority of CLP-G18 in terms of provoking inflammation through demonstration of edematous and thickened lung tissues and myocardial cell necrosis. It is noteworthy however, that micrographs of CLP-G21 were similar to the sham group. These observations may support the hypothesis that in comparison with CLP-G21, CLP-G18 provokes oxidative stress reactions and cellular damage more pronouncedly in the cardiopulmonary system.

Conclusion

In conclusion, findings of this study demonstrated that CLP-G18 is superior to CLP-G21 in terms of the severity of sepsis induced in a rat model, and both the rise of inflammatory markers and the extent of the ensuing organ damage is greater in the group undergone the procedure with G18 needles. This study has identified cost-effective indicators to evaluate organ failure during sepsis. This should be considered by all investigators using G-18 in comparison with G-21 to induce severe sepsis. It can be concluded that the choice of the needle size can have a great influence in the research outcomes, and this should be considered in design and implementation of CLP studies so that researchers can inflict the precise level of sepsis they intend to induce without waste of resources.

Abbreviations

CLP: Cecal Ligation and Puncture ICU: Intensive Care Unit ROS: Reactive Oxygen Substances AMPK: 5' Adenosine Monophosphate-Activated Protein Kinase LC3IIb: Microtubule-associated protein 1A/1B-light chain 3b MPO: Myeloperoxidase HMGB1: High Mobility Group Box 1 TNF- α : Tumor Necrosis Factor alpha TUMS: Tehran University of Medical Science LPO: Lipid Peroxidation TBARS: Thiobarbituric Acid Reactive Substances MDA: Malondialdehyde FRAP: Ferric Reducing Antioxidant Power TTM: Total Thiol Molecules qRT-PCR: quantitative Real-Time Reverse

Transcription Polymerase chain reaction CBC: Complete Blood Count MPV: Mean Platelet Volume WBCs: White Blood Cells Hb: Hemoglobin PLR: Platelet to Lymphocyte Ratio

Declarations

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Authors' contributions

T.D. performed all parts of the experiment and wrote the manuscript. S.M. participated in drafting and editing the article. M.B, M.G., H.H.A. helped to perform and design the experimental part of the study. A.N. and M.B., M.R., was involved in data analysis and interpretation. M.M. contributed to the concept, design, and financial support of the manuscript. All authors read and approved the final manuscript.

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Consent for publication

Not applicable.

Competing interests:

The authors declare that they have no competing interests.

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Figures

Figure 1

Effect of gauge 18 and gauge 21 on Cecal ligation and puncture (CLP) on lactate levels in heart, lung tissues and blood. Results are expressed as mean $\pm$ SEM for six animals in each group. ***: significant difference from sham group at $P < 0.001$, **: significant difference from sham group at $P < 0.01$, ###: significant difference from CLP-gauge18 group at $P < 0.001$, #: significant difference from CLP-gauge18 group at $P < 0.01$, #: significant difference from CLP-gauge18 group at $P < 0.05$.

Figure 2

Effect of gauge 18 and gauge 21 on Cecal ligation and puncture (CLP) on proinflammatory cytokines. A: Tumor Necrosis Factor- α (TNF- α), B: High Mobility Group Box 1 (HMGB1) in myocardial and pulmonary tissues. Results are expressed as mean $\pm$ SEM for six animals in each group. ***: significant difference from sham group at $P < 0.001$, *: significant difference from sham group at $P < 0.05$, ###: significant difference from CLP-gauge18 group at $P < 0.001$, #: significant difference from CLP-gauge18 group at $P < 0.05$.

A: AMPK

B: LC3IIb

Figure 3

Effect of gauge 18 and gauge 21 on Cecal ligation and puncture (CLP) on mRNA expression pattern of LC3IIb (Microtubule-associated protein 1 light chain 3) and AMPK (AMP-activated protein kinase) genes in myocardial and pulmonary tissues. Results are expressed as mean $\pm$ SEM for six animals in each group. ###: significant difference from CLP-gauge18 group at $P < 0.001$, #: significant difference from CLP-gauge18 group at $P < 0.01$.

A: MPV

B: PLR

C: Blood glucose levels

Figure 4

Effect of gauge 18 and gauge 21 on Cecal ligation and puncture (CLP) on blood markers. A: MPV (mean platelet volume), B: PLR (platelet to lymphocyte ratio), C: Blood glucose levels. Results are expressed as mean $\pm$ SEM for six animals in each group. ***: significant difference from sham group at $P < 0.001$, **: significant difference from sham group at $P < 0.01$, *: significant difference from sham group at $P < 0.05$, ##: significant difference from CLP-gauge18 group at $P < 0.01$, #: significant difference from CLP-gauge18 group at $P < 0.05$.

Figure 5

The histopathology of the lung and heart in different groups. Arrow heads: hyperplasia of pneumocyte type A, Thick arrow: perivascular inflammation and infiltration of inflammatory cells, Thin arrows: myocardial necrosis.