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Study of the effects of Escherichia coli lipopolysaccharide on innate immunity: The expression profile of TLR4 and CD14 genes in rat liver

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Abstract

Background: Bacterial endotoxin [lipopolysaccharide (LPS)] is essential for bacterial virulence as it has a biphasic effect which is either harmful and leads to aseptic shock and death or assists the body defense mechanisms as it stimulates B-cells activation. Many studies have noted that LPS do their action through activation of CD14/ TLR4 pathways, which occur mainly in liver cells, including Kupffer cells, hepatocytes, and liver sinusoidal endothelial cells, which are responsible for cytokines releases and shows the good or bad LPS effect.

Aim: The current study aimed to disclose the expression changes in the profile of innate immunological receptors TLR4 and CD14 in rats' livers after stimulation with LPS.

Methods: Ten groups of male Wistar albino rats were used to study the effects of two types of LPS [extracted LPS from the local strain of *Escherichia coli* (ELPS) and standard *E. coli* (SLPS)]; these were given by using different doses (5 mg/kg and 100 µg/kg); the LPS were injected either intravenously or intraperitoneally. The TLR4 and CD14 mRNA expression patterns were estimated using qPCR after 6, 12, and 24 hours postinjection.

Results: The results show that there is a negative effect of ELPS on liver CD14 and TLR4, regardless of the dose and route of administration. On the other hand, the SLPS has an upregulatory impact on the liver gene expression. Also, different times show no effect on the gene expression of the two genes.

Conclusion: This study concludes that both LPS types used were able to stimulate the CD14 and TLR4 gene expression in the liver in different doses and routes of injection. Also, this study showed the possibility of using ELPS as an immunomodulator in rats.

Keywords: *Escherichia coli* LPS, Rat liver, Innate immunity, TLR4, CD14.

Introduction

The lipopolysaccharide (LPS) chemical structure is conserved in all Gram-negative bacteria (Ebbensgaard *et al.*, 2018), which consist mainly of three-part lipid A molecules, oligosaccharide core, and the O-antigen (Erridge *et al.*, 2002; Mazgaeen and Gurung, 2020). The LPS associates with bacterial virulence, resists the phagocytic effect, plays a role in antigenic variation (Sampath, 2018), and also plays a role in bacterial resistance to complement action and bacteriophage (Putker *et al.*, 2015; Bertani and Ruiz, 2018), as well as antimicrobial substances (May and Grabowicz, 2018), especially cationic antimicrobial peptides (Ebbensgaard *et al.*, 2018). Finally, LPS is capable of inhibiting the immune host response (Bertani and Ruiz, 2018). The researchers noted that the exact effect of LPS is dose-dependent. In high doses, the LPSs have a wide range of bad effects such as coagulation (Stief, 2009), inflammation, capillary leak, tissue toxicity aseptic shock, and lethality (Sampath, 2018) in low doses. LPSs act as a pathogen-associated molecular patterns (PAMP), which is a potent stimulator of innate and cellular immunity through the activation of TLR4/CD14/LPS complex pathway (Erridge *et al.*, 2002; Steimle *et al.*, 2016; Tsukamoto *et al.*, 2018; Mazgaeen and Gurung, 2020). The activation of TLR4 pathways leads to the activation to more than 1,000 gene transcriptions (Steimle *et al.*, 2016). Especially, interleukin 6 (IL6), tumor necrosis factor α (TNFα), and IL1 and IL12 during an inflammatory cytokines storm are responsible for septic shock (Mazgaeen and Gurung, 2020), but they also have some beneficial effects such as anti-tumor (Gonçalves *et al.*, 2016), radioprotection, activation of homoeotic (Schuettpelz and Link. 2013), activation of complement and B-cells (Kaca *et al.*, 2009), and, finally, as an adjuvant to assist the immunity system's defense (Kuznetsova *et al.*, 2020).

The immune system's responses against LPS occur mainly in the liver, which regulates the cytokines released from Kupffer cells (liver resides microphage), hepatocytes, and liver sinusoidal endothelial cells (antigen-presenting cells resemble) after recognition and modulates of LPS through TLR4 and CD14 (Jirillo *et al.*, 2002). This study aimed to disclose the expression changes in the profile of innate

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immunological receptors TLR4 and CD14 in rats' livers after stimulation with LPS.

Materials and Methods

One hundred and twenty male Wistar albino rats, with weights ranging from 220 to 280 g, were divided into 10 groups, each containing 12 rats randomly (total $n = 120$).

The animals were injected with SLPS or ELPS using two different doses of LPS 5 mg/kg and 100 µg/kg. Animals in the groups G1–G4 were used for ELPS. However, the groups G5–G8 were used for SLPS, and the last two groups G9 and G10 were used as control. G1 and G5 groups received a high LPS dose (5 mg/kg) using the intraperitoneal route (IP), G2 and G6 received a low LPS dose (100 µg/kg) using IP; G3 and G7 groups received a high LPS dose using the intravenous route (IV); G4 and G8 received a low LPS dose by IV. Finally, G9 and G10 served as a control and received distal water. Rats were euthanized humanely by cervical dislocation at 6, 12, and 24 hours, respectively; the livers were collected from different animal groups. All liver samples were stored at −80°C in the deep freezer until used for detection of TLR4 and CD14 gene expressions using two steps qPCR using Promega system/USA. Isolation of RNA was carried out by using extraction and purification SV total RNA isolation system (Promega/USA). The GoScript™ Reverse Transcription System (Promega/USA) with Oligo(dT) 15 primer was used to synthesize the first strand of cDNA. Five microliters of cDNA were mixed with 20 µl reaction mixture, GoTag ® qPCR master (Promega/USA) 10 µl, forward primer 2.5 µl, reverse primer 2.5 µl, and nuclease-free water 5 µl, for detecting all gene expressions (Table 1). The qPCR system (PCRmax/UK) was used to amplify all genes, the reaction conditions were initial denaturation at 95°C, 2 minutes, 40 cycles with the second denaturation at 95°C, 15 seconds, finally, annealing and extension at 60°C, 1 minute. The gene expression was normalized with the GAPDH housekeeping gene, and then the fold changes were calculated using ΔΔCT (Livak and Schmittgen, 2001). Finally, all qPCR products were migrated in 1.5% agarose gel electrophoresis and visualized using a transilluminator and photographic using a digital camera.

The result data were statistically analyzed using IBM SPSS program statistics version 24. All data were tested using the analysis of variance, Duncan's test , and t-test, which recorded significant differences in experimental parameters.

Ethical approval

All animals were treated with the ethical rule of animal care and sample collection (University of Melbourne Animal Care and Use Standards Committee, 2019). The study design and the animal experiments were approved by the Mosul University/Local College of veterinary medicine/Institutional Animal Care and Use Committee under approved ID: UN.VET.2021.008.

Results

The CD14 mRNA gene expression showed an upregulation after the animals were injected with either LPS types, which differed between groups compared to the control group. The existing study results revealed an increase in gene expression in G5 with a rise in fold change variation from other groups (Fig. 1). Statistical analysis showed a similar expression of CD14 mRNA, which was observed at different experiment times. The effects of ELPS on CD14 gene expression were low regardless of the dose and route of administration compared with SLPS; however, the differences regarding doses and routes of injection were not significant statistically (Table 2).

The TLR4 mRNA gene expression showed upregulation after the animals were injected with either LPS type, which differed between groups compared to the control group. The current study (Fig. 2) explains the fold changes of TLR4 mRNA gene expression

Overall, the injection route and type of LPS show the reduction of TLR4 gene expression in G1 rats compared to other groups, excluding G2 and G3 animals. The statistical analysis of TLR4 mRNA exhibits a similar expression at different experimental times. SLPS demonstrates more elevation on the regulatory effect of the TLR4 gene on

Table 1. The specific primers used for gene expression.

Primer name	Primer	Sequence	Product size	Reference
TLR4	F	5′- TGCTACAGTTCATCTGGGTTTCTG - 3′	78 bp	Kocak *et al*. (2019)
	R	5′-CTGTGAGGTCGTTGAGGTTAGAAG- 3′		
CD14	F	5′-GTGCTCCTGCCCAGTGAAAGAT- 3′	268 bp	Zhishang *et al*. (2020)
	R	5′-GATCTGTCTGACAACCCTGAGT- 3′		
GAPDH[a]	F	5′-AGATCCACAACGGATACATT- 3′	309 bp	Pal *et al*. (2019)
	R	5′-TCCCTCAAGATTGTCAGCAA- 3′		

([a]): Glyceraldehyde 3-phosphate dehydrogenase.

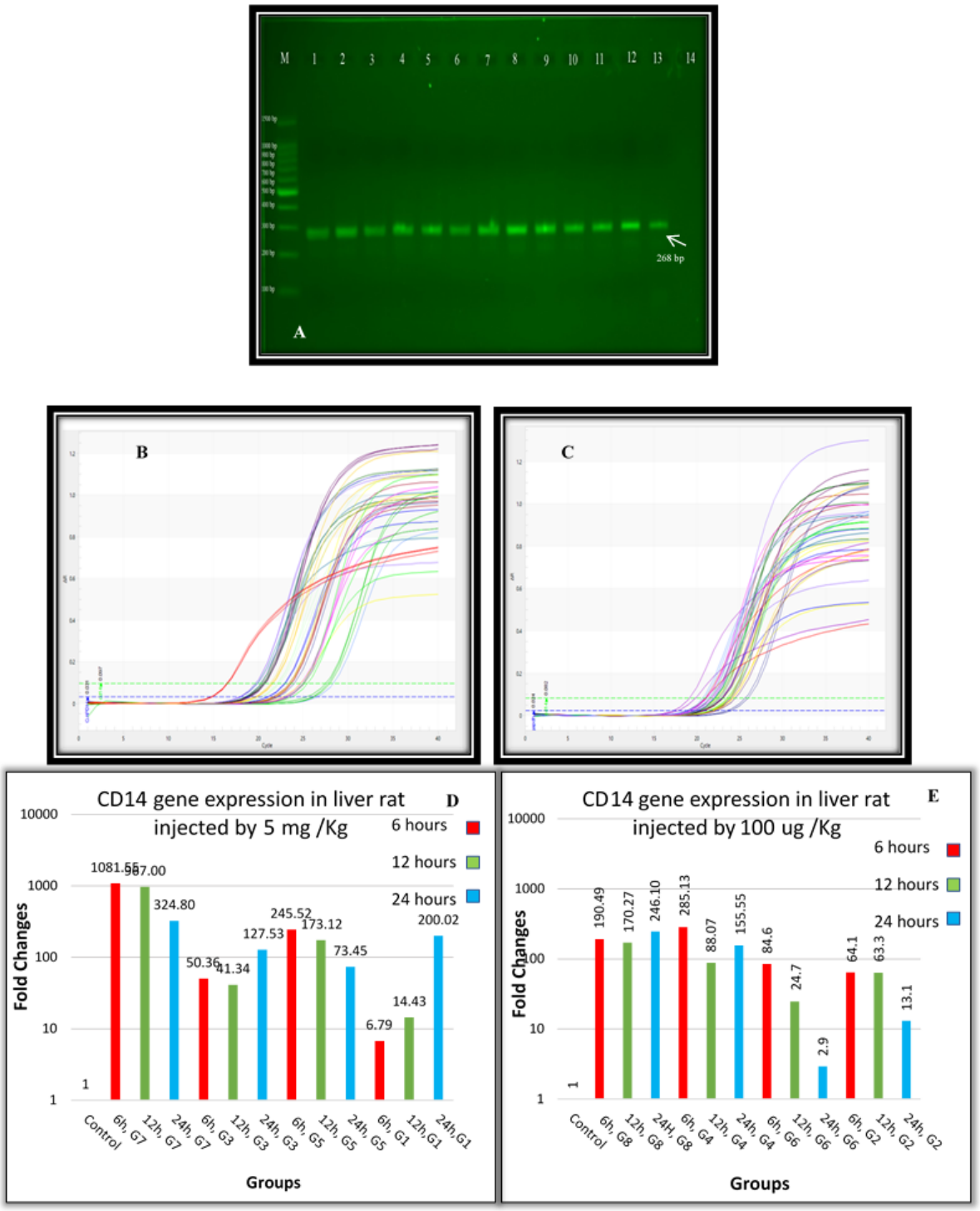

Fig. 1. (A) Gel electrophoresis of CD14 (M: kilobase marker; well 1, 2, 3: G7 at 6, 12, 24 hours; well 4, 5, 6: G3 at 6, 12, 24 hours; well 7, 8, 9: G5 at 6, 12, 24 hours; well 10, 11, 12: G1 at 6, 12, 24 hours; 13: control positive; and 14: control negative). (B) Amplification curve of CD14 using qPCR (5 mg/kg). (C) Amplification curve of CD14 using qPCR (100 µg/kg). (D) Gene expression of CD14 of rat groups that received high doses (5 mg/kg) and different routes of injections. (E) Gene expression of CD14 of rat groups that received high doses (100 µg/kg) and different routes of injections.

Table 2. Analysis of CD14 gene expression induced by ELPS and SLPS.

Dose	Route of injection	Experimental groups	Time			Average of dose, route of injection, LPS type	Average LPS type standard versus extract	Average of route of injection effect Ip versus IV	Average dose effect
			6 hours	12 hours	24 hours				
High	IP	G1	24.91 ± 0.17^{ab}	23.82 ± 0.71^{abc}	20.03 ± 6.40^{fg}	22.92 ± 3.91^{abc}	23.27 ± 2.33*	22.33 ± 2.52	22.25 ± 2.68
		G5	19.73 ± 0.14^{g}	20.24 ± 0.41^{efg}	21.47 ± 0.12^{cdefg}	20.48 ± 0.80^{d}			
	IV	G3	24.87 ± 3.40^{ab}	25.15 ± 0.23^{ab}	23.53 ± 0.13^{abcd}	24.52 ± 1.86^{a}			
		G7	20.45 ± 0.16^{efg}	20.61 ± 0.16^{efg}	22.18 ± 0.11^{bcdefg}	21.08 ± 0.84^{cd}			
Low	IP	G2	21.67 ± 0.47^{cdefg}	21.69 ± 0.33^{cdefg}	23.96 ± 0.32^{abc}	22.44 ± 1.18^{bc}	21.98 ± 1.74	22.93 ± 1.67	23.01 ± 1.36
		G6	21.27 ± 0.29^{cdefg}	23.04 ± 0.76^{bcdef}	26.12 ± 0.64^{a}	23.48 ± 2.18^{ab}			
	IV	G4	22.37 ± 0.23^{bcdefg}	24.06 ± 0.33^{abc}	23.24 ± 1.61^{abcde}	23.22 ± 0.95^{ab}			
		G8	22.95 ± 0.09^{bcdef}	23.11 ± 0.85^{bcde}	22.58 ± 0.54^{bcdefg}	22.88 ± 0.56^{abc}			
Time average			22.28 ± 2.08A	22.72 ± 1.71A	22.89 ± 2.59A				

All data are represented as the mean of the C_T value ± standard deviation of amplified expression of mRNA to CD14 gene. Upregulations occur when the C_T value of the tested gene is lower than the C_T value of the control gene.

The G1 and G5 groups received a high LPS dose using IP; G2 and G6 received a low LPS dose using IP method; G3 and G7 groups received a high LPS dose using IV; G4 and G8 received low LPS dose using IV route. The average of LPS type (Standard *vs*. Extract), an average of the route of injection effect IP vs. IV, average dose effect, and time-average indicates the mean of all groups that represented Standard LPS, IP, dose, times vs. the mean of all groups that represented Extracted LPS, IP, and dose time.

The different letter indicates significant difference when $p \leq 0.05$.

*Significant difference when $p \leq 0.05$.

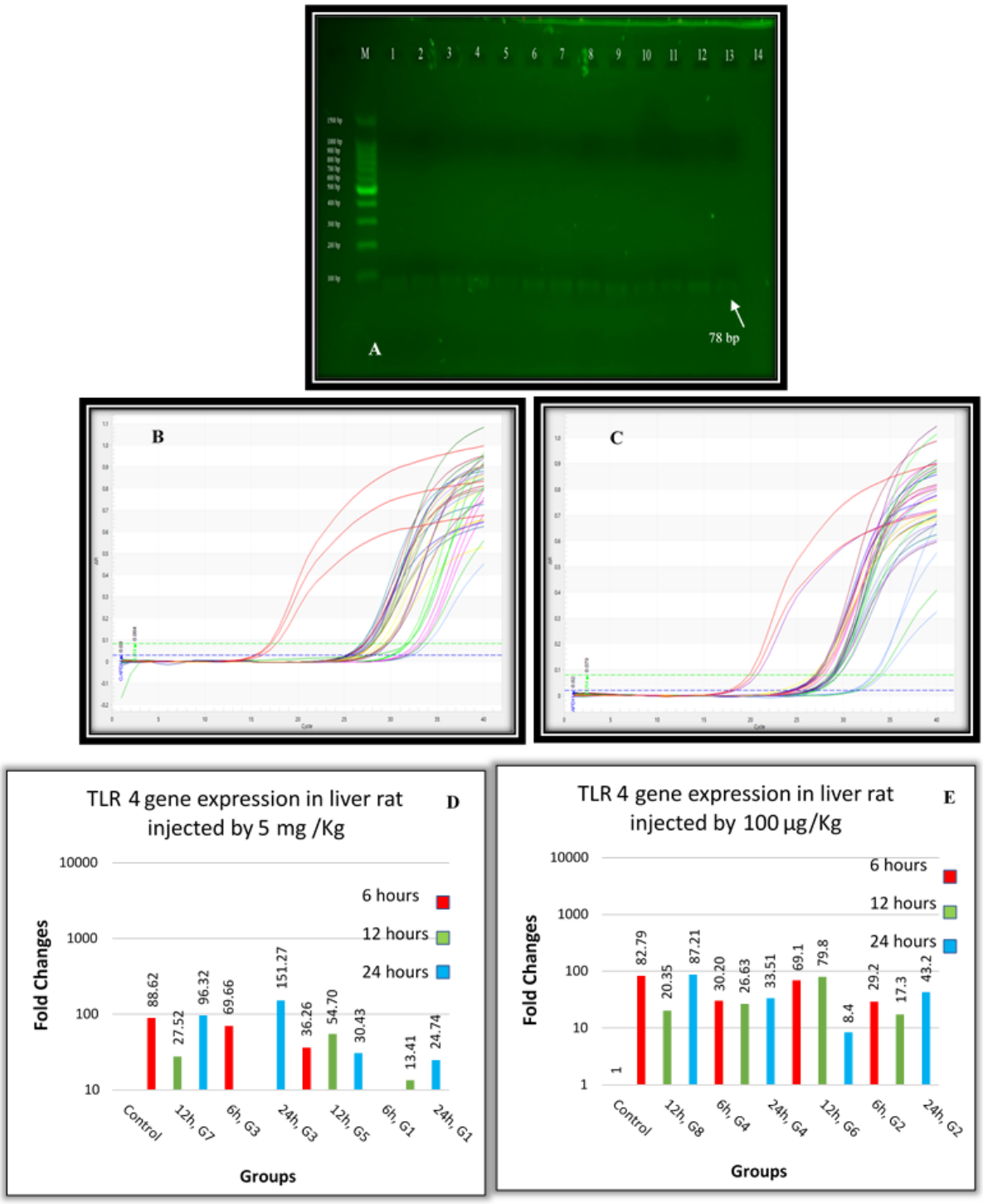

Fig. 2. (A) Gel electrophoresis of TLR4 (M: kilobase marker; well 1, 2, 3: G7 at 6, 12, 24 hours; well 4, 5, 6: G3 at 6, 12, 24 hours; 7, 8, 9: G5 at 6, 12, 24 hours; well 10, 11, 12: G1 at 6, 12, 24 hours; 13: control positive; and 14: control negative). (B) Amplification curve of TLR4 using qPCR (5 mg/kg). (C) Amplification curve of TLR4 using qPCR (100 µg/kg). (D) Gene expression of TLR4 of rat groups that received high doses (5 mg/kg) and different routes of injections. (E) Gene expression of TLR4 of rat groups that received high doses (100 µg/kg) and different routes of injections.

Table 3. Analysis of TLR4 gene expression induced by ELPS and SLPS.

Dose	Route of injection	Experimental groups	Time			Average of dose, route of injection, LPS type	Average LPS type standard versus extract	Average of route of injection effect IP versus IV	Average dose effect
			6 hours	12 hours	24 hours				
High	IP	G1	32.99 ± 0.35^{a}	29.19 ± 0.32^{cdef}	28.31 ± 1.42^{defghi}	30.16 ± 2.28^{a}	$29.11 \pm 1.73^{*}$	28.75 ± 1.82	28.81 ± 1.83
		G5	27.75 ± 0.23^{fghi}	27.16 ± 0.65^{hi}	28.01 ± 0.14^{efghi}	27.64 ± 0.51^{b}			
	IV	G3	28.23 ± 1.54^{defghi}	31.73 ± 0.20^{ab}	27.11 ± 0.08^{hi}	29.02 ± 2.15^{ab}			
		G7	27.88 ± 0.24^{fghi}	29.57 ± 0.16^{cd}	27.76 ± 0.05^{fghi}	28.40 ± 0.88^{b}			
Low	IP	G2	28.72 ± 0.24^{defg}	29.48 ± 1.50^{cde}	28.16 ± 1.34^{defghi}	28.79 ± 1.16^{ab}	28.03 ± 1.25	28.38 ± 1.32	28.34 ± 1.29
		G6	27.48 ± 0.25^{ghi}	27.27 ± 1.17^{ghi}	30.53 ± 2.17^{bc}	28.43 ± 2.01^{b}			
	IV	G4	28.46 ± 0.19^{defghi}	28.64 ± 0.23^{defgh}	28.31 ± 0.16^{defghi}	28.47 ± 0.22^{b}			
		G8	27.01 ± 0.23^{i}	29.03 ± 0.26^{def}	26.94 ± 0.14^{i}	27.66 ± 1.04^{b}			
	Time average		$28.57 \pm 1.82A$	$29.01 \pm 1.50A$	$28.14 \pm 1.36A$				

All data represented as the mean of the C_T value ± standard deviation of amplified expression of mRNA to TLR4 gene. Upregulations occur when the C_T value of the tested gene is lower than the C_T value of the control gene.

The G1 and G5 groups received a high LPS dose using IP; G2 and G6 received a low LPS dose using IP method; G3 and G7 groups received a high LPS dose using IV; G4 and G8 received low LPS dose using IV route. The average of LPS type (Standard Vs. Extract), an average of the route of injection effect IP vs. IV, average dose effect, and time-average indicates the mean of all groups that represented Standard LPS, IP, dose, times vs. the mean of all groups that represented Extracted LPS, IP, and dose time.

The different letter indicates significant difference when $p \leq 0.05$.

*Significant difference when $p \leq 0.05$.

the rats' model than ELPS, which exhibits a lower effect on the same model. At the same time, different routes and doses appear to have little impact on TLR4 mRNA expression (Table 3).

Discussion

The innate immune responses are responsible for rapid detection, removal of foreign materials, and induction of inflammatory response by recognition of PAMP found in many bacterial infections. LPSs are capable of inducing innate immune inflammatory response associated with inflammatory cytokines (Mazgaeen and Gurung, 2020). So, the study of this cytokines gives a good indication about the health and ability of innate immunity to invading microbes. The most important receptors and cytokines are CD14, TLR4, IL1, and TNFα, which increase during LPS recognition and immune responses against it.

The liver plays a pivotal role in the responses of the immune system against LPS, Kupffer cells (liver resides microphage), hepatocytes, and liver sinusoidal endothelial cells (antigen-presenting cells resemble) and can recognize and modulate the response to LPS through TLR4, CD14, and regulation of cytokines releases (Jirillo *et al.*, 2002). The studies of the gene expression of CD14 with LPS showed an upregulation of the CD14 gene in all liver cells to reach a peak at 3–6 hours after using different doses with ELPS or SLPS injected by different methods; this result agrees with Li *et al.* (2003), who recorded an increase in hepatic CD14 protein level at 3 hours to reach a peak at 12 hours. After 24 hours of LPS administration, the expression of CD14 in the liver was decreased in some groups. This is due to gradually downregulating CD14 mRNA to baseline level in liver cells after 24 hours.

The animals in the G3 and G1 groups that were injected with high doses of LPS intraperitoneally revealed a decrease in CD14 mRNA gene expression at 6 hours and a reincrease at 24 hours; this may be due to the delay in response of liver cells in those suffering from intensive injury with low CD14 expression, and this result agrees with Hozumi *et al.* (2013). In this research, no significant difference was noted between the overall time used, and this may be related partially to the time of samples collection. The collection of samples started after 6 hours of LPS injection. This may have led to missing the early expression which normally occurs within minutes or hours.

The ELPS causes less elevation in CD14 mRNA gene expression level contrary to SLPS. This may result from differences in the chemical structure which reflected different ligands that reacted differently with the binding site of CD14 (Cunningham *et al.*, 2000). Both routes of injection show a similar effect because the LPS concentration in the liver raised gradually after LPS injection, especially the smooth form of LPS (Jacque *et al.*, 2006). Furthermore, a similar effect was seen in both high and low doses on CD14 mRNA expression, and this may result partially from the presence of sCD14 in plasma, which expresses as acute phase protein and may compete with mCD14 to bind and neutralize the LPS-induced response *in vitro* or *in vivo* (Bas *et al.*, 2004).

The assay of TLR4 mRNA expression shows biphasic upregulation after 6 and 24 hours in most rat groups. A similar pattern was reported by Huang *et al*. (2017), who noted that the mRNA of TLR4 expression was "increased, decreased, then increased again" the significant increases occurred at 6 and 12 hours in chicken liver stimulated with *Salmonella* LPS.

The rapid increase in mRNA of TLR4 in the early phase reveals the early immune stimulation against the LPS. Under normal conditions liver, Kupffer cells process a low baseline of TLR4 mRNA, and under the LPS stimulation, these cells respond by upregulation of TLR4 mRNA. Hepatocytes also produce remarkable TLR4 mRNA and protein under the same condition (Huang *et al.*,2017). Increase in mRNA of TLR4 occurs to overcome the LPS effect after activation of TLR4 downstream signaling to produce pro-inflammatory cytokine (IL1, TNFα, and IFNδ) through NF-kB pathways (Mazgaeen and Gurung, 2020; Ciesielska *et al.*, 2021). The second phase of increase in TLR4 mRNA expression occurs after 24 hours is interesting and result from LPS acute injury to liver cells results in TLR4 response through NLRP3 (Gong *et al.*, 2019) or by damage-associated molecular pattern to initiate tissue repair (Ciesielska *et al.*, 2021).

The ELPS produces lower induction on TLR4 mRNA gene expression in contrast to SLPS; this reflects different LPS structures, which affect the binding site of CD14 (Cunningham *et al.*, 2000), and expand the effect on TLR4 mRNA expression; meanwhile, different effects on TLR4 mRNA expression by different *Escherichia coli* LPS strains and/or organs investigated were found (Grasa *et al.*, 2017).

The time of TLR4 expression shows no difference in TLR4 mRNA expression pattern. Studies have showed that the expression time pattern was different, depending on tissue affected, for example, the heart increased TLR4 mRNA expression at 3 hours, followed by a decrease at 6 hours and increased at 24 hours which reflects tissue macrophage/monocyte effect on TLR4 mRNA expression (Matsumura *et al.*, 2000).

TLR4 expression showed a similar effect on both routes or doses used, and this may relate to the effect of both soluble and membrane-bound CD14 molecules, which mediated delivery and internalization of TLR in both low and high doses and stimulate downstream signaling for production of TNFα (Schwabe *et al.*, 2006; Ciesielska *et al.*, 2021). Liu *et al*. (2018) showed a positive correlation between TLR4 mRNA expression and TNFα and IL12 concentration. This led us to propose that CD14 may partially regulate

TLR4 expression through TNFα production by its LPS delivery to TLR4 receptors.

Conclusion

The existing study concluded that both LPS types used were able to stimulate the CD14 and TLR4 gene expression in the liver in low and high doses with different injection routes. The statistical analysis reveals that ELPS produces less elevations of CD14 and TLR4 gene expression levels in contrast to SLPS. Furthermore, no effect of doses or time was noted on the expression of the two genes, so this study shows the possibility of using *E. coli* (local strain) as an immunomodulator in rats.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contributions

All authors have designed, written, reviewed, discussed, agreed, and contributed to this article before and during the submission of their article.

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