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Time-dependent expression patterns of inflammatory markers in rat model of lipopolysaccharide-induced acute systemic inflammation

Eman Mohammad Albataineh[1], **Saad Abdulrahman Hussain**[2], **Sinan Subhi Farhan**[3], **Heba M Abd El Kareem**[4], **Samir Saad Mahgoub**[5]✉

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Author Affiliation:
[1]Department of Microbiology and Pathology, Faculty of Medicine, Mutah University, Al-Karak, Jordan, Email: emanbatayneh@yahoo.com
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Rafidain University College, Baghdad, Iraq, Email: saad.hussain@ruc.edu.iq
[3]Department of Basic Sciences, Faculty of Pharmacy, Al-Rafidain University College, Baghdad, Iraq, Email: sinansubhi@gmail.com
[4]Department of Biochemistry and Molecular Biology Faculty of Medicine, Mutah University, Jordan, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Benha University, Egypt, Email: hebakareem@ymail.com
[5]Department of Biochemistry and Molecular Biology Faculty of Medicine, Mutah University, Jordan, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Al-Minia University, Egypt, Email: samir_mhgb@yahoo.com

✉**Corresponding author**
Department of Biochemistry and Molecular Biology Faculty of Medicine, Mutah University, Jordan, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Al-Minia University, Egypt,
Email: samir_mhgb@yahoo.com

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ABSTRACT

Objectives: The study aimed to assess the relationship between various inflammatory markers in an induced systemic inflammation by lipopolysaccharide (LPS) after 6 and 24-hr intervals, and to compare their sensitivity and specificity. *Materials and methods*: Two hundred and seventy rats were allocated into two groups; 150 rats (control group) and 120 rats (test group). The control group was injected i.p with saline, while the test group was injected with 100 μg/kg of LPS i.p. A sample of blood was collected from each rat at 6 and 24 hrs intervals to evaluate hsCRP, TNF-α, IL-6, and IL-10. Rats were sacrificed to get their livers for estimating myeloperoxidase, COX-2, caspase-3, and ALT. *Results*: A significant difference in the levels of the studied mediators after 6 and 24 hrs was revealed in the test group versus the control group. The correlation analysis showed positive values at both time intervals. ROC analysis demonstrated that the AUC was the highest for TNF-α and hsCRP after 6 and 24 hours, respectively. hsCRP was the only marker that showed an increase in AUC after 24 hrs compared with 6 hours. *Conclusion*: The levels of the inflammatory biomarkers were increased in LPS-induced acute systemic inflammation up to 24 hrs; also, there was an increase in the sensitivity and specificity for the assessment of acute systemic inflammation.

Keywords: Inflammation, hsCRP, TNF-α, Lipopolysaccharides, COX-2, Caspase-3.

1. INTRODUCTION

Inflammation is a complex pathophysiological response to infection and its pathogenesis is associated with excessive cytokines production like IL-6 and TNF-α; the later magnifies the inflammatory cascades through activating macrophages/monocytes to secrete other pro-inflammatory cytokines, besides

its role in the pathogenesis of the early stage of shock (Bohannon et al., 2012). Consequently, these cytokines produce tissue damage and initiation of apoptosis (Lowes et al., 2013).

In hepatic tissues, there is an increase in apoptosis that has been recognized as a critical step in the induction of acute hepatic failure, probably associated with further complication of systemic bacterial sepsis (Marshall, 2001). At low tissue concentration, TNF-α initiates the defense response to local injury and might have beneficial effects against infections through augmentation of the host defense mechanisms (Feldmannand Steinman, 2005); meanwhile, it can lead to exaggerated inflammatory response and tissue damage at high cellular concentrations. Furthermore, it mediates a variety of direct pathogenic effects and enhances the production of other inflammatory mediators along with tissue destruction through the activation of multiple inflammatory cascades (Tracey et al., 2008; McClain et al., 2004). Moreover, evaluation of hsCRP became a standard sensitive approach to assess many inflammatory disorders (Ridker, 2003; Ridker et al., 2007; Song et al., 2017). In this regard, the hepatoprotective role of IL-6 in alcoholic liver disease is complicated and not well recognized; this could be attributed to the apoptotic effects that facilitate mitochondrial DNA repair after alcoholic liver injury (Hong et al., 2002).

In contrast, IL-17 production may be enhanced by IL-6 expression during alcohol-induced liver inflammation (Gao, 2005). Meanwhile, IL-10, the anti-inflammatory cytokine can regulate TNF-α production during endotoxemia (Mathurin et al., 2000) and reduces other pro-inflammatory cytokine production (Louis et al., 2003). Myeloperoxidase (MPO) is released by the neutrophils and is involved in the generation of ROS (Klebanoff, 2005; Brown et al., 2001), so, it can be employed as an inflammatory marker during infection-induced inflammation (Haegens et al., 2009). Caspases-3 plays a major role in the apoptotic machinery (Pop & Salvesen, 2009) and its suppression is associated with the reduction of pro-inflammatory signaling and hepatocyte damage (Thapaliya et al., 2014; Mohamed & Magdy, 2017).

The cyclooxygenases (COX) utilize arachidonic acid for the production of prostaglandins, prostacyclins, and thromboxanes (Cha & Du Bois, 2007), and the COX-2 isoenzyme expression is enhanced due to the effects of some inflammatory cytokines in the presence of intracellular and extracellular stimuli (Emanuela & Garret, 2011; Martín-Sanz et al., 2017). The rate of COX-2 expression is increased in Kupffer cells and damaged hepatocytes due to the increase in macrophages and other inflammatory cells, especially during an antigen-induced inflammation (Wójcik et al., 2012; Andersen et al., 2013; Zhong et al., 2006). No enough previous data were available to compare these mediators in different time intervals depending on the ROC analysis as an effective measure of the accuracy of these inflammatory mediators for the assessment of acute systemic inflammation.

Therefore, the aim of present study was to evaluate the relationship between various inflammatory markers at different time intervals (6 and 24 hrs) and compare their sensitivity and specificity in the assessment of acute inflammation of the liver induced by intra-peritoneal injection of LPS in rats.

2. MATERIALS AND METHODS

Study design

The study has been carried out on 270 age-matched albino rats, with their weights ranging from 200-250 grams. The animals were subdivided into 150 rats as a control group and 120 rats as a test group. Animals were fed a standard diet and housed in the animal house of the Faculty of Medicine, Mu'tah University. The albino rats were allowed to have a 12:12 hour light/dark cycle with a simple access to water and food during the whole time of the experiment.

The experiment was done as follows: group I, the control group was subdivided into two groups, each of 75 rats; they were given a single injection of normal saline intra-peritoneally. Then, the rats were sacrificed after 6 hours, while the remaining 75 rats were sacrificed after 24 hours of inflammation induction in the test group. Group II, the test group was given a single intra-peritoneal injection of 100 μg/kg body weight of LPS derived from Salmonella abortusequi (L5886, Sigma, Poole, UK). LPS was suspended in sterile saline according to the method adopted by Teeling et al., (2010).

Two groups of rats each of 60 were sacrificed 6 hours and 24 hours, respectively, after the induction of inflammation. A sample of venous blood was collected from each rat under ether anesthesia. Later, the rats were sacrificed to obtain the liver for further analysis of the tissue inflammatory markers. Tissues were prepared for biochemical analyses according to a standard procedure (Aksoy et al., 2014).

Biochemical analyses

All the targeted inflammatory mediators were analyzed using the ELISA method (Engvall et al., 1971). The kits for MPO, COX-2, ALT, Caspase-3, IL-10, TNFα, IL-6, and hsCRP were purchased from Cusabio/USA, Abcam/UK, and MyBiosource/USA/Canada. ELISA was done according to the manufacturer's protocol instructions, where 100 mg of tissue was rinsed with 1X PBS to estimate

the activity of caspase-3, COX-2, ALT, and MPO in the liver tissue homogenates, which were homogenized in 1 ml of 1X PBS and stored at -20°C overnight. The homogenates were centrifuged at 5000 x g for 5 min at 4°C, after performing two freeze-thaw cycles for breaking the membranes of the cells. The supernatant was removed, subdivided into aliquots, and stored at -80°C till the time of assays. The MPO activity in the liver tissue homogenates was assessed according to the method proposed by Kuebler and colleagues (1996). Meanwhile, IL-10, TNFα, IL-6, and hsCRP were estimated in the serum.

Statistical analyses

Data was processed in IBM SPSS Software version 20 and expressed as mean±SD. Groups were compared using t-test and ROC Curve to evaluate sensitivity and specificity for the studied mediators. The correlation between the numerical data was also performed using Spearman's correlation coefficient (r). P-value <0.05 was considered statistically significant.

3. RESULTS

In table 1, the levels of the studied inflammatory mediators were decreased after 24 hours of the induction, except for the levels of MPO, which were elevated after 24 hours compared with its levels after 6 hours post-induction of inflammation. A statistically significant difference ($p<0.05$) in the mean values of MPO, hsCRP, COX-2, Caspase-3, and TNF-α was reported at 24 hours intervals compared with the 6 hours intervals. Meanwhile, there was no statistically significant difference between the mean values of IL-6, IL-10, and ALT markers.

Table 1 Effect of a challenge with LPS on the inflammatory markers after 6 and 24 hours of inflammation of induction in albino rats

Parameters	6 hr post-induction		24 hr post-induction		*P*-value
	Control (no= 75)	Test (no= 60)	Control (no= 75)	Test (no= 60)	
MPO (ng/ml)	3.27±0.82	6.26±1.45*a	3.77±1.3	7.24±1.63*b	0.006
hsCRP (ng/ml)	3.99±1.39	8.17±1.72*a	2.84±0.79	6.09± 1.64*a	0.000
COX-2 (ng/ml)	27.1±10.5	48.04±13.05*a	24.8±10.3	41.6±10.2*a	0.025
Caspase-3 (ng/ml)	2.8±1.2	6.0±1.9*a	1.8±1.0	4.0±1.3*b	0.000
ALT (IU/L)	34.4±11.3	64.8± 13.2*a	32.0±12.1	60.2± 12.9*a	0.110
IL-6 (pg/ml)	84.4 5±2 9.5	351.6±166.5*a	169.7±93.8	351.5±103.2*a	0.280
IL-10 (pg/ml)	164.0 ± 87.0	661.2±253.1*a	141.0±109.2	527.5±207.4*a	0.150
TNF-α (pg/ml)	30.8 ±2 0.8	78.8±10.6*a	19.4± 10.6	53.4±16.0*b	0.000

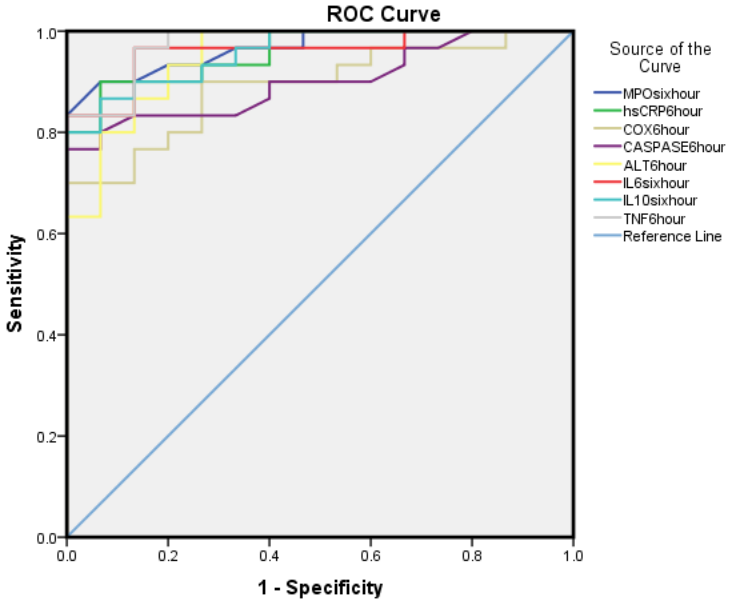
Values expressed as mean±SD; * significantly different compared with corresponding control within the same time (P<0.05);
Values of test group at different periods (6 and 24 hrs) with different superscripts (a, b) are significantly different (P<0.05).

The ROC curve analysis has demonstrated an area under the curve (AUC) for all the studied inflammatory mediators after 6 and 24 hours of the induction of inflammation (Table 2 and Figure 1). The results showed that the lowest AUC was reported for COX-2 (0.891 and 0.854) after 6 and 24 hours, respectively; while the highest value was reported for TNF-α after 6 hours and for hsCRP after 24 hours (0.976 and 0.969, respectively). Moreover, the only mediator that showed an increase in AUC after 24 hours of the induction of inflammation was the hsCRP compared with its value after 6 hours. Pearson's correlations analysis was performed for all the studied mediators at a 6-hours interval. The (r^2) values were positive among all parameters ranging from 0.334-0.712 (Table 3). Regarding the 24 hrs intervals, all parameters showed positive (r^2) values ranging from 0.208 -0.726 (Table 4).

Table 2 ROC analysis and the area under the curve (AUC) at both 6 hours and 24-hrs intervals

Test variables (6 hrs post-induction)	AUC	Test variables (24 hrs post-induction)	AUC
MPO	0.967	MPO	0.946
hsCRP	0.958	hsCRP	0.969
COX-2	0.891	COX-2	0.854
Caspase-3	0.900	Caspase-3	0.883
ALT	0.949	ALT	0.934
IL-6	0.960	IL-6	0.882

IL-10	0.958	IL-10	0.900
TNF-α	0.976	TNF-α	0.951



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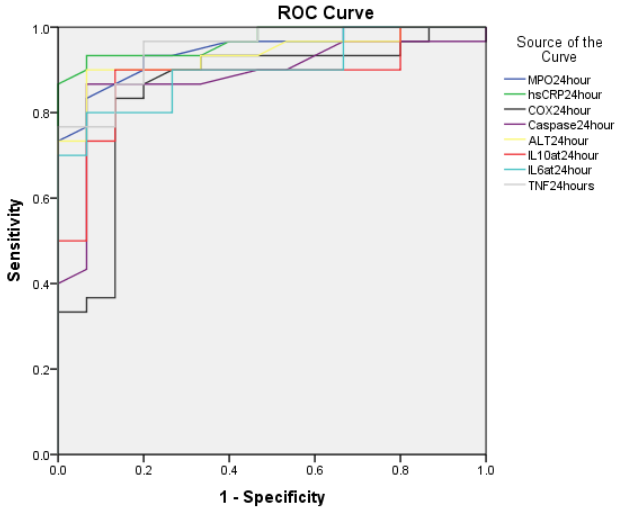

B

Figure 1 ROC Curve analysis of the studied inflammatory mediators (A) after 6 hours (B) after 24 hrs

Table 3 the correlations between the studied mediators after 6 hours of the induction of systemic inflammation

	MPO	hsCRP	COX	Caspase	ALT	IL-10	IL-6	TNF
MPO Pearson Correlation (r^2) Sig. (2-tailed)	1	0.587** 0.000	0.665** 0.000	0.712** 0.000	0.455** 0.000	0.550** 0.000	0.524** 0.000	0.587** 0.000
hsCRP Pearson Correlation (r^2) Sig. (2-tailed)	0.587** 0.000	1	0.558** 0.000	0.395** 0.007	0.686** 0.000	0.534** 0.000	0.643** 0.000	0.680** 0.000

COX Pearson Correlation (r^2) Sig. (2-tailed)	0.665* 0.000	0.558** 0.000	1	0.548** 0.000	0.334* 0.025	0.373* 0.012	0.538** 0.000	0.534** 0.000
Caspase Pearson Correlation (r^2) Sig. (2-tailed)	0.712** 0.000	0.395** 0.007	0.548** 0.000	1	0.351* 0.018	0.519** 0.000	0.551** 0.000	0.560** 0.000
ALT Pearson Correlation (r^2) Sig. (2-tailed)	0.445** 0.002	0.686** 0.000	0.334* 0.025	0.351* 0.012	1	0.537** 0.000	0.507** 0.000	0.689** 0.000
IL-10 Pearson Correlation (r^2) Sig. (2-tailed)	0.550** 0.000	0.534** 0.000	0.373* 0.012	0.519** 0.000	0.537** 0.000	1	0.658** 0.000	0.628** 0.000
IL-6 Pearson Correlation (r^2) Sig. (2-tailed)	0.524** 0.000	0.643** 0.000	0.538** 0.000	0.551** 0.000	0.507** 0.000	0.658** 0.000	1	0.537** 0.000
TNF Pearson Correlation (r^2) Sig. (2-tailed)	0.587** 0.000	0.680** 0.000	0.534** 0.000	0.560** 0.000	0.689** 0.000	0.628** 0.000	0.537** 0.000	1

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

Table 4 the correlations between the studied mediators after 24 hours of the induction of systemic inflammation

	MPO	hsCRP	COX	Caspase	ALT	IL-10	IL-6	TNF
MPO Pearson Correlation (r^2) Sig. (2-tailed)	1	0.592** 0.000	0.439** 0.000	0.403** 0.006	0.434** 0.003	0.429** 0.003	0.463** 0.001	0.626** 0.000
hsCRP Pearson Correlation (r^2) Sig. (2-tailed)	0.592** 0.000	1	0.726** 0.000	0.562** 0.000	0.546** 0.000	0.417** 0.004	0.482** 0.001	0.433** 0.003
COX Pearson Correlation (r^2) Sig. (2-tailed)	0.439** 0.003	0.726** 0.000	1	0.616** 0.000	0.528** 0.000	0.411** 0.005	0.404** 0.006	0.387** 0.009
Caspase Pearson Correlation (r^2) Sig. (2-tailed)	0.403** 0.006	0.562** 0.000	0.616** 0.000	1	0.565** 0.000	0.469** 0.001	0.282 0.060	0.482** 0.001
ALT Pearson Correlation (r^2) Sig. (2-tailed)	0.434** 0.003	0.546** 0.000	0.528** 0.000	0.565** 0.000	1	0.619** 0.000	0.322* 0.031	0.564** 0.000
IL-10 Pearson Correlation (r^2) Sig. (2-tailed)	0.429** 0.003	0.417** 0.004	0.411** 0.005	0.469** 0.001	0.619** 0.000	1	0.208 0.170	0.441** 0.002
IL-6 Pearson Correlation (r^2) Sig. (2-tailed)	0.463** 0.001	0.482** 0.001	0.404** 0.006	0.282 0.060	0.322* 0.031	0.208 0.170	1	0.540** 0.000
TNF Pearson Correlation (r^2) Sig. (2-tailed)	0.626** 0.000	0.433** 0.003	0.387** 0.009	0.482** 0.001	0.564** 0.000	0.441** 0.002	0.540** 0.000	1

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

4. DISCUSSION

Systemic inflammation is a major source of inflammatory mediators, based on the induction of systemic inflammation using LPS endotoxins extracted from different sources including *E. coli, Salmonella, or Haemophilus species*. Regarding the study of the effect of LPS on various mediators with different time intervals, it has been claimed that the induction of inflammation with LPS resulted in an acute elevation of plasma TNF-α level (Culley et al., 2014). However, the elevation in plasma TNF-α was resolved within 24-48 hours. This finding was consistent with the results of the present study within 6 hours. However, such an outcome of the present study could be attributed to the high dose of injected LPS. The endotoxin produces injury of liver tissue through TNF-α, which plays a major role in stimulating the apoptosis of the hepatocytes (Shimizu et al., 2005). Another study showed the association between liver injury and increased TNF-α production by Kupffer cells, which is consistent with the results obtained in the present study (Konno et al., 2002).

In our study, the levels of hsCRP were markedly increased in the test group 6 hrs post-induction of inflammation compared with the controls in the same time interval; then it was decreased after 24 hours in the test group but was still higher than the control group. The use of different endotoxins such as Freund's adjuvant or colony-forming units of *Staphylococcus aureus* may give results that are similar to some of the results deduced in the present study. The use of different endotoxins and the wide interval time followed during the assay could explain the difference in the obtained results (Abbak & Ulutaş, 2017). In this regard, Song and colleagues (2017) showed a significant elevation in the level of hsCRP among the rats maintained on a high-fat diet compared with those fed a normal diet as a control group. Moreover, hsCRP level was increased among rats with fatty liver disease of the nonalcoholic etiology, especially nonalcoholic steatohepatitis, which is consistent with the results of the present study regarding the elevated level of hsCRP in the inflammatory status of the hepatic tissue. Meanwhile, increased levels of IL-6 were detected after 4 hours of acute systemic LPS- induce inflammation, and the findings of the present study seem to be in tune with these data (Nezić et al., 2009).

In this regard, it has been found that serum IL-6 levels were significantly increased after 2 hrs and dropped back to the baseline levels after 6 hrs (Teeling et al., 2010). However, these results do not coincide with the findings of the present study as it indicated a relatively high serum level of IL-6 after 24 hrs, it may be due the small sample size and type of animals used in the study. Regarding the influence on IL-10 levels, the obtained results in this study was in agreement with those reported by Biesmans and colleagues (2016) where the serum level peaks 1-hr after LPS injection and normalized within the first 24 hrs. Moreover, another study reported a marked increase in the serum IL-10 level after 6 hrs of LPS injection and remained significantly high up to 48 hrs, which might be explained by the use of a higher dose of LPS isolated from *E. coli* (Li et al., 2012). Regarding the influence of LPS on the tissue MPO activity, high MPO levels were detected in the tissues of liver and lung, respectively, compared with the controls (Li et al., 2016; Islam et al., 2017).

The present study has also observed an increased MPO activity that sustained up to 24 hrs and can be interpreted according to the suggestion of Simsek et al., (2018), who stated that the increase in MPO level is considered as a counter-regulatory mechanism to reduce the inflammatory burdens. The influence of the inflammatory process on COX-2 expression was previously evaluated in an experimental animal model of alcoholic liver injury (de AraújoJúnior et al., 2016). Moreover, another study showed a significant rise in the level of COX-2 after 6 hours of LPS challenge, which confirms that COX-2 can be induced by various factors including cytokines and endotoxins (Ganey et al., 2001). Caspase-3, the effector subtype of caspases, was recruited especially when extrinsic signals triggered apoptosis during LPS-induced (Deaciuc et al., 2004), and its activity was significantly elevated after 6 hrs post-induction of inflammation. This might be different from the results obtained by Zhong et al., (2006) and Emam and Abo El Gheit, (2015) who reported a significant elevation in the activity of caspase-3 after 8, 6, and 24 hrs of LPS injection, respectively.

The exposure to LPS exposure leads to hepatic injury and impaired liver function elicited by elevated serum ALT levels (Hagar, 2009; Ajuwon et al., 2014; Chung et al., 2015); our results were in tune with these findings. Based on the ROC results, the present study has shown a reduction in the AUC after 24 hrs of the induction of inflammation in all studied mediators, except for hsCRP; we recommend its use as a unique mediator for the assessment of acute inflammation within 24 hrs.

5. CONCLUSION

According to the results of the inflammatory markers were influenced by LPS-induced acute systemic inflammation up to 24 hours, especially hsCRP. However, further evaluation is recommended regarding different types and doses of the LPS to demonstrate the systemic effect and the time needed until the restoration of normal or near-normal levels of the involved inflammatory mediators.

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Authors Contribution

Eman Mohammad Albataineh - Concepts or Ideas, Design, Literature search, Experimental studies, Data acquisition, Manuscript preparation, Manuscript editing, Manuscript review. Saad Abdulrahman Hussain - Literature search, Manuscript editing, Manuscript review; Sinan Subhi Farhan - Literature search, Manuscript editing, Manuscript review; Heba M Abd El Kareem - Literature search, Manuscript editing, Manuscript review; Samir Saad Mahgoub - Concepts or Ideas, Design, Literature search, Experimental studies, Data acquisition, Manuscript preparation, Manuscript editing, Manuscript review. All the authors approved the final version of the manuscript.

Ethical considerations

The study was approved by the Ethics Committee, Faculty of Medicine, Mu'tah University, Jordan (reference no. 201415).

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Conflict of Interest

The authors declare that there are no conflicts of interests.

Data and materials availability

All data associated with this study are presented in the paper.

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