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Research Article

Effects of CDP-Choline and Choline on COX Pathway in LPS-Induced Inflammatory Response in Rats

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Abstract

Background and Objective: Cytidine-5-diphosphate-choline (CDP-choline) and choline activate the cholinergic anti-inflammatory pathway in case of inflammation. This study investigated the role of CDP-choline and choline along with the contribution of the cyclooxygenase (COX) pathway on the lipopolysaccharide (LPS)-induced endotoxemia model in rats. **Materials and Methods:** Endotoxemia model was induced by LPS administration. CDP-choline or choline 5 min before and 6 hrs after LPS injection. The sepsis severity, body weight changes, survival rate were evaluated. Serum prostaglandins, Tumour Necrosis Factor (TNF)-α, total choline levels were measured. COX-2 mRNA expression and protein levels were analyzed. Spleen tissues were evaluated histomorphological. One-way analysis of variance analysis (ANOVA) or Kruskal Wallis tests was used for statistical analysis. **Results:** COX-2 expressions in liver and brain tissues, serum prostaglandin E_2, 6-keto prostaglandin $F_{1\alpha}$, Thromboxane A_2 and TNFα levels were increased 24 hrs after LPS administration. Administrations of CDP-choline or choline were decreased COX-2 expression in the liver. Serum prostaglandin levels were decreased in the CDP-choline-treated group, whereas, only prostaglandin E_2 level was decreased in the choline-treated group. Total choline levels in serum and brain were increased after CDP-choline or choline administration. Accordingly, serum TNFα levels and TNFα expression in the liver were decreased in CDP-choline and choline-treated groups. TNFα expression in the brain was decreased in the choline-treated group, whereas, increased in the CDP-choline-treated group. **Conclusion:** CDP-choline and choline decreased LPS-induced COX-2 enzyme expression and prostaglandin levels in the periphery by increasing serum and brain total choline levels in the LPS-induced endotoxemia model in rat.

Key words: CDP-choline, choline, lipopolysaccharide, cyclooxygenase 2, cholinergic anti-inflammatory pathway, endotoxemia, prostaglandin levels

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sepsis, a systemic inflammatory response to infection, triggered by some antigenic components of microorganisms, is characterized by widespread inflammation leading to multiple organ failure[1]. The rat endotoxemia model is commonly used as it resembles the sepsis-associated acute inflammatory response. Lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria, is a well-known inducer of pathogen-associated molecular pattern (PAMP) recognition receptors (toll-like receptors, TLRs) is used in endotoxemia models[2,3]. Exposure to LPS leads to endotoxemia which activates mononuclear phagocytic cells (MPCs) through TLRs to initiate systemic inflammation[2]. Activated MPCs release various pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα), interleukin 1β (IL-1β) and IL-6, causing hemodynamic changes that eventually lead to multiple organ failure[4]. Furthermore, direct action of endotoxin and stimulation of cytokines simultaneously causes the release of prostaglandins produced via an inducible form of cyclooxygenase (COX-2), an enzyme that contributes to further release of inflammatory mediators involved in cardinal signs of inflammation[5-8].

The cholinergic anti-inflammatory pathway (CAP) is a part of the endogenous response to systemic inflammation throughout the body and defined as a neural mechanism that inhibits pro-inflammatory cytokine release resulted from the activation of the vagus nerve via cholinergic receptors[9]. Alpha7 nicotinic acetylcholine receptors (α7nAChR), expressed in numerous kinds of MPCs are required for the interaction between cholinergic nerves and the immune system. Activation of α7nAChRs on macrophages by acetylcholine (ACh) inhibits pro-inflammatory cytokine release including TNFα and interleukins in different experimental inflammation models[10]. Therefore, α7nAChR-activating agents appear to be valuable therapeutics for the modulation of systemic inflammatory response.

Cytidine-5-diphosphate-choline (CDP-choline) is rapidly cleaved into choline and cytidine by phosphodiesterases (PDEs) in the cell membrane which in turn increases choline levels in blood circulation and brain[11]. CDP-choline is an important component of phosphatidylcholine (PC) synthesis, the main phospholipid in the brain, by the kennedy pathway. Additionally, PC is the source of bioactive lipids including arachidonic acids[12]. PC can be hydrolysed by phospholipase A_2 (PLA_2) which contributes to the production of arachidonic acid from membrane phospholipids, prostaglandins and leukotrienes[13] and it has been demonstrated that PLA_2 activity significantly increased in systemic inflammatory conditions[14]. Moreover, central purinergic signalling has been shown to contribute to the immune response and exacerbation of inflammatory state in sepsis[15]. Owing to its cytidine moiety, CDP-choline might also exert some anti-inflammatory effectiveness through purinergic receptor activation[16].

Choline, a precursor of neurotransmitter ACh, interacts directly with α7nAChRs and induces cholinergic neurotransmission[17]. Several studies performed on animal models of sepsis/endotoxemia have demonstrated that stimulation of the CAP via CDP-choline and choline enhancing ACh release, moderate pro-inflammatory cytokine levels and improve survival rates by preventing multiple organ failure due to inflammation[4,17-21].

Therefore, this study investigated the effects of CDP-choline and choline as well as the role of the COX-2 pathway in the LPS-induced endotoxemia model in rats.

MATERIAL AND METHODS

Study area: This research project was carried out at the Department of Pharmacology, Dokuz Eylul University, Izmir, Turkey from June, 2018-February, 2019.

Materials: Lipopolysaccharide (LPS) from *Escherichia coli* (O55:B5), Choline chloride, CDP-choline (Cytidine 5-diphosphocholine sodium salt dihydrate) were obtained from Sigma-Aldrich (St. Louis, Mo., USA). All drugs were dissolved in saline (0.9% sodium chloride).

Animals: The experimental protocol of this study was approved by the Dokuz Eylul University, Turkey Multidisciplinary Laboratory Animal Studies Local Ethics Committee (No: 51/2017). Thirty-eight male rats (Wistar albino, 180-200 g) used in the study were individually housed during the experiment under standard controlled conditions (12 hrs dark/light cycle, at $22\pm2°C$). Rats freely accessed food and water during housing. All procedures were performed according to the 'Principles of Laboratory Animal Care' by the 'National Institute of Health' publication.

Experimental protocol: An experimental endotoxemia model was performed with a sublethal dose of LPS administration (10 mg kg^{-1})[22]. Animals with no significant change in their basal body weights were randomly separated into four experimental groups, 1. Control (n = 6), 2. LPS+saline

(n = 12), 3. LPS+375 mg kg^{-1} CDP-choline (n = 10) and 4. LPS+90 mg kg^{-1} choline (n = 10). Saline or LPS were administered 5 min after the drug injections. Saline, CDP-choline and choline injections were repeated at the 6th hrs of the experimental protocol. All injections (in 1 mL) were performed intraperitoneally (i.p.). The CDP-choline dose was determined as the equimolar dose of choline as reported earlier[21].

The severity of endotoxemia was evaluated at the beginning of the experiment and the 6th and 24th hrs during the protocol by using a sepsis scoring system with the highest total score of 28[23]. Sepsis scoring performed blindly by 3 researchers was averaged and a sum of all individual parameters presented as total sepsis score. Rats were weighed at the beginning and 24th hrs. Survival rates during the experiments were monitored at the 6th and 24th hrs following the LPS exposure. Rats were sacrificed by decapitation at the 24th hrs under mild ether anaesthesia and their trunk blood was collected for prostaglandin and choline measurements. Total RNA and protein samples isolated from liver and brain tissues were used to determine COX-2 mRNA expression and protein levels via real-time reverse transcription-polymerase chain reaction (rtRT-PCR) and Western Blot analyses, respectively. Brain samples were excised with a razor blade by cutting from the edges of hypothalamic tissue[22,24]. Spleen samples were dissected for histomorphological evaluations. All specimens were transferred into cryotubes and stored at -80°C until the analysis.

Histomorphological analysis: For histomorphological examination, spleen tissue samples were fixed with 10% formalin then embedded in paraffin blocks. Sections of 5 µm thickness were stained with hematoxylin-eosin (H-E) and examined for hemorrhagic and inflammatory changes. A grading system was used to score the tissue injury semi-quantitatively as (0) absent, (1) slight, (2) moderate and (3) severe. Each section was evaluated blindly and the average score determined[25].

Image analysis methods: All sections were digitally photographed and analysed by using a computer-assisted image analyser system coupled to a microscope (Olympus BX-51, Japan) with a high-resolution video camera (Olympus DP-1, Japan). For morphometric evaluation, a computerized image analysis system (UTHSC Image Tool software version 3.0, University of Texas Health Science Center, San Antonio, TX) was used.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis: Liver and brain tissues were rapidly removed and homogenized following the sacrification. Total RNA was isolated by an extraction kit (GeneJET RNA Purification Kit, ThermoScientific) according to the protocol provided by the manufacturer. The quality of RNA was calculated by OD_{260}/OD_{280} nm absorption ratio. RNA from each sample was converted to complementary DNA using a cDNA synthesis kit (OneScript Plus G236, Applied Biological Materials, Richmond, BC, Canada) according to the manufacturer's instructions. The cDNA samples were amplified with RT-PCR (Biorad CFXconnect) by using forward and reverse primers of COX-2 and *TNFα* gene. Cycling conditions were 95°C for 10 min for polymerase activation/denaturation and 40 cycles (95°C for 15 sec and 55°C for 60 sec for COX- 2/56°C for 60 sec for TNFα) for amplification followed by a dissociation stage (65°C for 5 sec then 5 sec each at 0.5°C for increments between 65-95°C). House-keeping gene β-actin was used as an internal positive control for normalization (Table 1). ΔΔCt method was employed for the relative quantification of mRNA expression[22].

Western blot analysis: To evaluate the effects of CDP-choline and choline on the COX-2 pathway, liver and brain tissue homogenates were prepared with a lysis buffer and run on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins separated in the gel were transferred onto polyvinylidene fluoride (PVDF) membranes and immunoblotted with primary antibodies specific to COX-2 and β-actin followed by incubation with goat anti-rabbit IgG antibody-HRP conjugate as a secondary antibody. After blotting, protein bands were visualised by using Enhanced Chemiluminescence (ECL) reagent (ThermoScientific 32132)[26].

Measurement of serum prostaglandin, TNFα and choline levels: Serum levels of the inactive non-enzymatic hydrolysis product of PGI_2, 6-keto-PGF1α, (Abnova, KA0295), PGE_2 (Cusabio, CSB-E07967r), TxA_2 (Abnova, KA0295), TNFα

Table 1: Primer sequences used in real-time quantitative RT-PCR

Target gene	Forward sequence	Reverse sequence
COX-2	TGTACAAGCAGTGGCAAAGG	TAGCATCTGGACGAGGCTTT
TNF α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
β-actin	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA

(Invitrogen, BMS622) and total choline level in serum and brain (Biovision, K615-100) were measured via ELISA according to the manufacturer's instructions.

Statistical analysis: One-way analysis of variance analysis (ANOVA) with post hoc Tukey-Kramer multiple comparisons for parametric results, Kruskal Wallis for non-parametric results and Student's t-test or Mann-Whitney U tests for comparison between two groups were employed where appropriate. The survival rate was analyzed by log-rank (Mantel-Cox) test. Statistical analysis were performed by using GraphPad 5(La Jolla, USA). Data were shown as Mean±Standard Error of the mean (SEM) and p<0.05 was accepted as significant.

RESULTS

Survival percentage: LPS+Saline group (n = 12) has 41.6 and 33.3% survival at the 6th and 24th hrs, respectively. The survival ratio decreased in the LPS+Saline group compared to that of the control group (n = 6) at the 6th and 24th hrs (p<0.05 and p<0.01). The percentage survival in LPS+CDP-choline and LPS+choline groups (n = 10) compared to LPS+Saline group was improved to 80.0% (p = 0.07) at the 6th hrs, whereas that of LPS+CDP-choline group and LPS+choline group at 24th hrs was 50.0 (p = 0.22) and 60.0% (p = 0.44), respectively (Table 2).

Sepsis severity: Total sepsis score, defined as zero in all groups at the beginning of the experiment was increased by LPS+Saline treatment at 6th and 24th hrs (p<0.01), decreased by CDP-choline or choline treatments at 24th hrs (p<0.05) (Table 3). The activity was improved significantly in the LPS+CDP-choline group compared to that of LPS+Saline at the 6th hrs (1.0±0.3 vs. 2.0±0, p<0.05). The levels of consciousness, active respiration rate (0.2±0.2 vs. 2.3±0.7 and 0.5±0.2 vs. 1.5±0.3 respectively, p<0.05) and response to stimulus (0.0±0.0 vs. 1.7±0.5, p<0.01) scores in the 24th hrs were improved significantly by LPS+choline treatment compared to that of LPS+Saline. The appearance, respiration rate and quality were improved by LPS+CDP-choline treatment compared to that of LPS+Saline at the 24th hrs (1.0±0 vs. 2.7±0.7, 0.0±0.0 vs. 1.5±0.3 and 0.0±0.0 vs. 0.7±0.2 respectively, p<0.05).

Body weight loss: Decrease in body weights at the end of the experimental protocol were -27.4±2.1, -21.7±5.2 and -13.6±6.7g in LPS+Saline, LPS+CDP-choline and LPS+choline groups, respectively. In LPS+Saline and LPS+CDP-choline groups, body weight loss was significantly higher than that of the control (p<0.0001 and p<0.005). However, there was no significant difference between the LPS+choline and control groups (p = 0.11) as well as LPS+CDP-choline and LPS+choline groups compared to the LPS+Saline group (p = 0.38 and p = 0.14, respectively) (Fig. 1a).

Histomorphological evaluation in spleen tissue: Histomorphological injury score was significantly increased in spleen tissue in the LPS+Saline group (2.2±0.2) compared to the control group (0.4±0.1) (p<0.001). In the LPS+CDP-choline (1.2±0.0) and LPS+choline (1.5±0.0) groups the

Table 2: Percentage survival in the experimental groups

	Percentage survival in experimental groups			
Time (hrs)	Control (n = 6)	LPS+Saline (n = 12)	LPS+CDP-choline (n = 10)	LPS+Choline (n = 10)
6th	100.0%	41.6%†	80.0%	80.0%
24th	100.0%	33.3%††	50.0%	60.0%

Survival percentage was analyzed by log-rank (Mantel-Cox) test, (n = 6-12 per group), (†), (††); p<0.05, p<0.01 vs. control group

Table 3: Evaluation of the sepsis severity in experimental groups

	Score (6th hrs)			Score (24th hrs)		
Variables	LPS+Saline	LPS+Choline	LPS+CDP-choline	LPS+Saline	LPS+Choline	LPS+CDP-choline
Appearance	2.0±0.0**	2.0±0.0**	1.6±0.2	2.7±0.7**	1.3±0.3	1.0±0.0†
Level of consciousness	2.5±0.3**	1.2±0.5	2.2±0.6	2.3±0.7*	0.2±0.2†	0.8±0.4
Activity	2.0±0.0**	0.8±0.4	1.0±0.3†	1.7±0.2**	0.3±0.2†	0.8±0.4
Response to stimulus	2.3±0.5**	0.6±0.4	1.6±0.2	1.7±0.5††	0.0±0.0††	0.4±0.4
Eyes	2.0±0.4**	1.4±0.6	1.0±0.6	2.0±0.8*	0.3±0.3	0.4±0.2
Respiration rate	1.5±0.5*	1.2±0.4	1.2±0.2	1.5±0.3**	0.5±0.2†	0.0±0.0†
Respiration quality	0.7±0.2*	0.5± 0.2	0.8±0.4	0.7±0.2*	0.3±0.2	0.0±0.0†
Total score	14.0±1.1**	8.3±2.5	8.6±2.4	14.0±2.2**	3.0±0.8†	3.0±1.1†

Total sepsis score was 0 at the 6th and 24th hrs in the control group. Kruskal-Wallis and Mann-Whitney U tests were used for statistical analysis. Data were shown as Mean±SEM (n = 4-6 per group). (*), (**); p<0.05, p<0.01, p<0.001 vs. control group, (†), (††); p<0.05, p<0.01 vs. LPS+Saline group

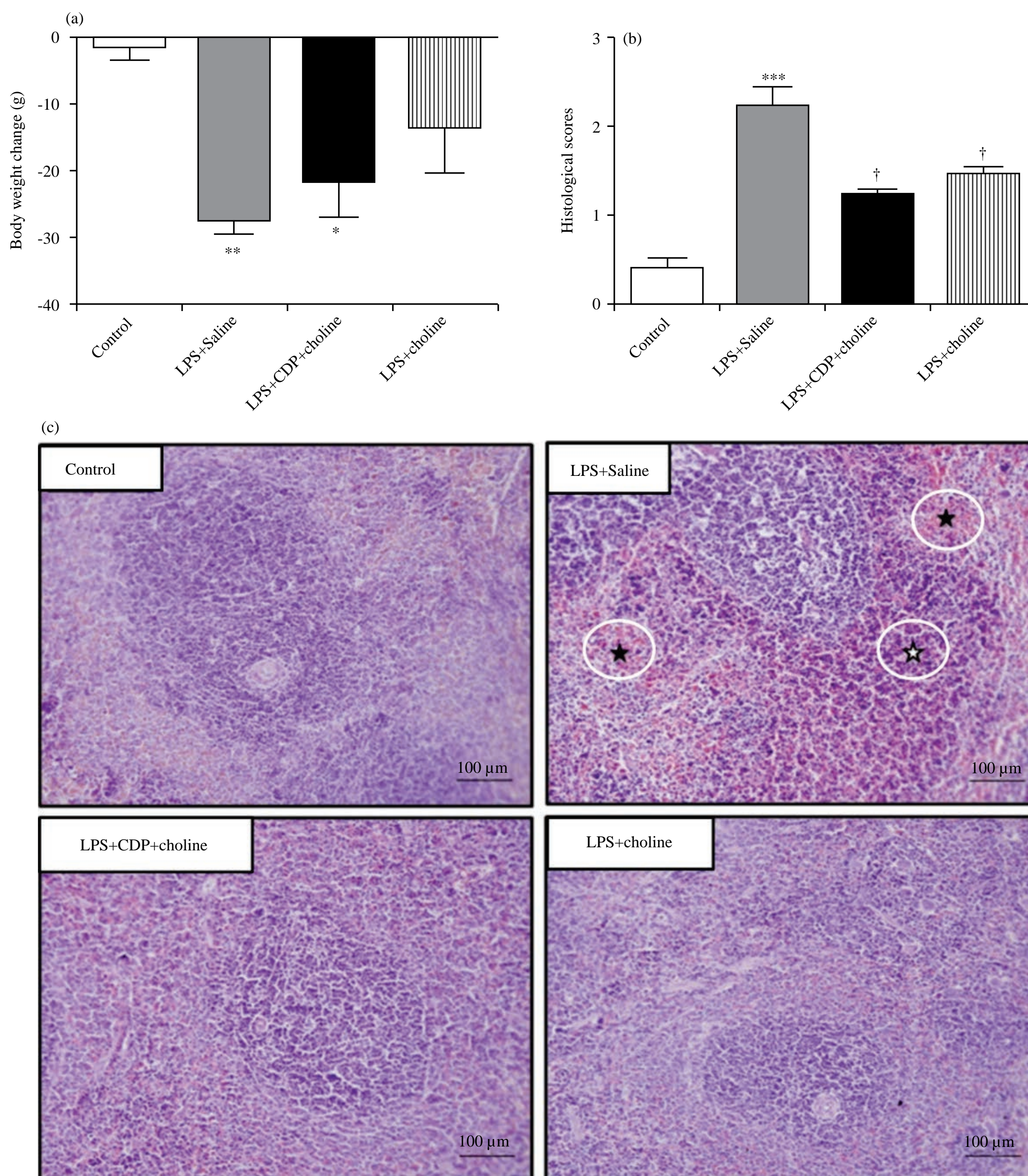


Fig. 1(a-c): Effects of CDP-choline (375 mg kg^{-1}) and choline (90 mg kg^{-1}) on sepsis severity

Shown are body weight loss (A), histomorphological scores (B) and Representative light-microscopic images of H-E staining in spleen tissue (C) of experimental groups; (☆) indicates mononuclear cell infiltration and (★) indicates haemorrhage in spleen tissue. One-way analysis of variance analysis (ANOVA) with post hoc Tukey-Kramer multiple comparisons, Kruskal Wallis with Mann Whitney U and Student's t-tests were used for statistical analysis. Data were shown as Mean±S.E.M. (n = 4-6 per group). (*), (**), (***); p<0.05, <0.01, p<0.001 vs. control group. (†), p<0.05 vs LPS+Saline group

histomorphological damage score decreased compared to LPS+Saline group (p<0.05) (Fig. 1b). H-E staining revealed a normal histological structure of spleen tissue in the control group. Spleen tissues showed diffuse and severe haemorrhage and mild inflammation with Mononuclear Phagocytic Cell (MPC) infiltration in LPS+Saline group, reduced spleen injury with little haemorrhage and inflammation in LPS+CDP-choline and LPS+choline groups (Fig. 1c).

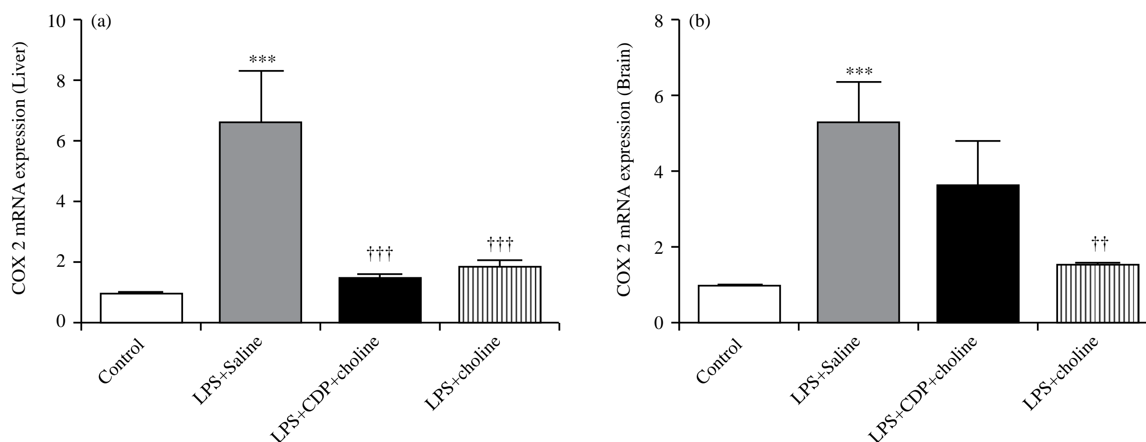


Fig. 2(a-b): COX-2 enzyme mRNA expressions in the liver (A) and brain (B) tissues in experimental groups

ΔΔCt method was employed for the relative quantification of mRNA expression. Kruskal Wallis and Mann Whitney U tests were used for statistical analysis. Data were normalized to beta-actin and shown as Mean±S.E.M. (n = 4-6 per group). (**), (***); p<0.01, p<0.001 vs. control group. (††),(†††); p<0.01,p<0.001 vs. LPS+Saline group

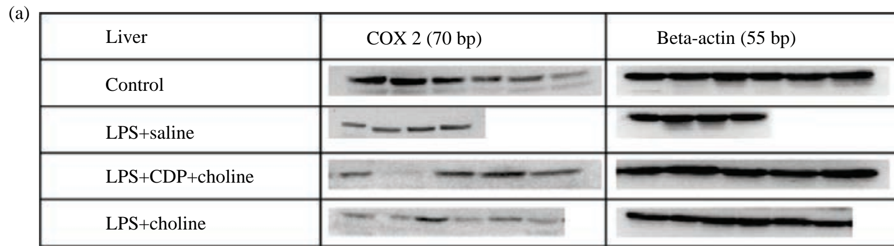
COX-2 mRNA and protein expression: COX-2 mRNA expression increased significantly in liver and brain tissues by LPS+Saline treatment compared to that of control (p<0.001, Fig. 2a, b). COX-2 mRNA expressions in liver samples from CDP-choline- and choline-treated groups decreased significantly compared to the LPS+Saline group (p<0.001, Fig. 2a). Although not statistically significant, COX-2 mRNA expression in brain samples increased in CDP-choline-treated group compared to that of the control. However, COX-2 mRNA expression in the brain significantly decreased in a choline-treated group compared to the LPS+Saline group (p<0.01). In the LPS+saline group, COX-2 protein expressions in liver and brain samples increased compared to the control group (p<0.001 and p<0.05, respectively). COX-2 protein expressions in liver samples from CDP-choline- and choline-treated groups decreased significantly compared to the LPS+Saline group (p<0.01 and p<0.05 respectively, Fig. 3a) without any significant change in the brain (Fig. 3b).

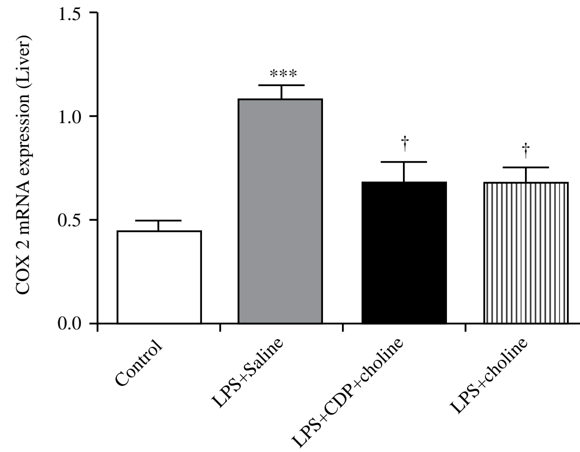
Prostaglandin levels: Serum levels of PGE_2, 6-keto-$PGF_{1\alpha}$ and TXA_2 increased (in pg mL^{-1}: 13.3±3.1-39.0±6.0, 1387.0±296.3-2975.0±869.9 and 539.1±74.4-1227.0±121.2, p<0.05, p = 0.06 and p<0.05, respectively) in the LPS+Saline group compared to that of control, whereas decreased significantly in LPS+CDP-choline group compared to that of LPS+Saline (in pg mL^{-1}: 13.7±4.1, 779.7±143.5, 520.0±105.6, p<0.05). Only serum PGE_2 levels were decreased significantly (15.6±5.4 pg mL^{-1}, p<0.05) by LPS+choline comparable to that of LPS+Saline (Fig. 4a). Although the serum 6-keto-$PGF_{1\alpha}$ and TXA_2 levels decreased in the LPS+choline group, the data did not reach any statistical significance (Fig. 4b-c).

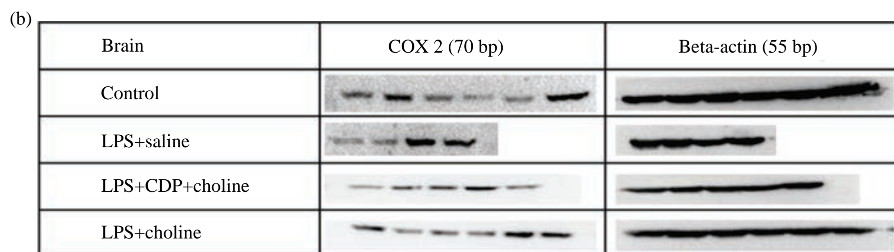
Serum and brain total choline levels: Total choline levels in serum and brain were not altered significantly in the LPS+Saline group compared to that of the control group (in μM: 301.7±15.3 vs. 298.8±6.0 p = 0.84 and -181.4±30.9 vs. 124.7±3.8 p = 0.053, respectively). Serum and brain total choline levels increased significantly in LPS+CDP-choline (in μM: 396.0±19.44 and 155.5±14.19, p<0.05, p<0.05) and LPS+choline (in μM: 387.0±31.6 and 185.8±7. 6, p<0.05, p<0.05) groups comparable to control (Fig. 5).

TNFα mRNA expression and protein level: TNFα mRNA expression was increased significantly in liver and brain tissues by LPS+Saline treatment compared to that of control (p<0.001, Fig. 6a-b). TNFα mRNA expressions in liver samples from CDP-choline- and choline-treated groups decreased significantly compared to the LPS+Saline group (p<0.001). TNFα mRNA expressions in brain samples significantly increased in CDP-choline- treated group (p<0.001) while significantly decreased in the choline-treated group (p<0.01) compared to the LPS+Saline group (Fig. 6a-b).

Serum TNFα levels increased (in pg mL^{-1}: 48.5±3.9, p<0.01) in the LPS+Saline group compared to that of control (in pg mL^{-1}: 18.4±3.4), whereas decreased significantly in LPS+CDP-choline group (in pg mL^{-1}: 16.1±5.2, p<0.01) and LPS+Choline group (in pg mL^{-1}: 15.1±4.9, p<0.01) compared to that of LPS+Saline (Fig. 6c).







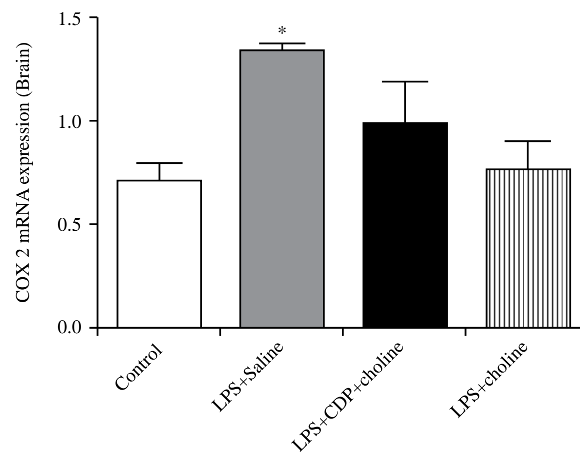


Fig. 3(a-b): COX-2 protein expressions in the liver (A) and brain (B) tissues from experimental groups

One-way analysis of variance analysis (ANOVA) with post hoc Tukey-Kramer multiple comparison tests were used for statistical analyses. Data were normalized to beta-actin and shown as mean±S.E.M. (n = 4-6 per group). (*), (***); p<0.05, p<0.001 vs. control group. (†); p<0.05 vs. LPS+Saline group

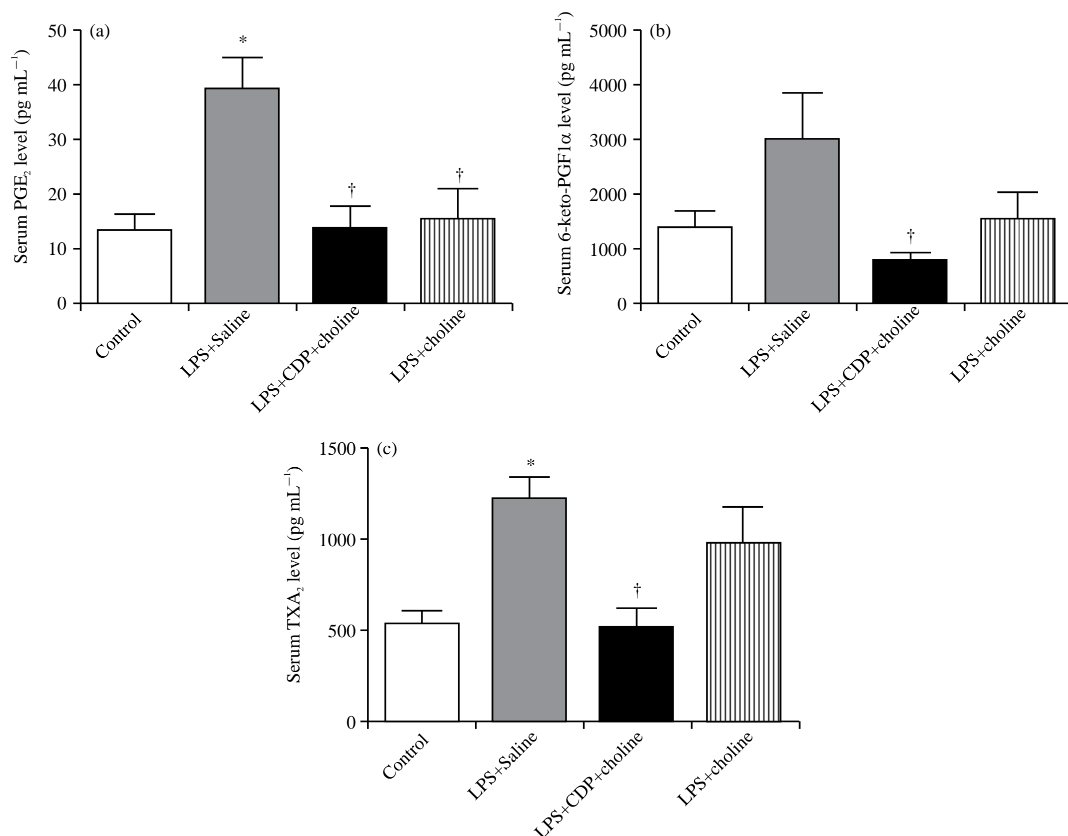


Fig. 4(a-c): Serum prostaglandin levels in experimental groups

Shown are PGE2 (A); 6-keto-PGF1a (B), TxA2 (C) (PGE2: Prostaglandin E2, 6-keto-PGF1a: 6-keto Prostaglandin F1a, TxA2: Thromboxane A2. One-way analysis of variance analysis (ANOVA) with post hoc Tukey-Kramer multiple comparison tests were used for statistical analyses. Data were shown as mean±S.E.M. (n = 4-6 per group). (*); p<0.05 vs. control group. (†); p<0.05 vs. LPS+Saline group

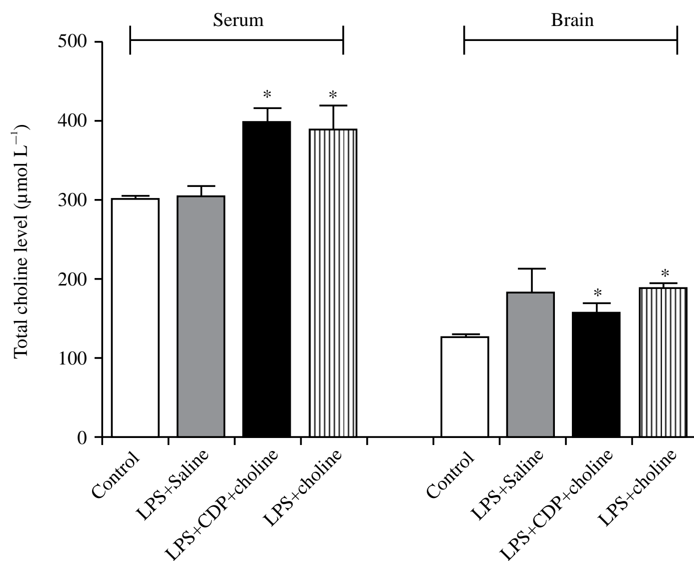


Fig. 5: Serum and brain total choline levels in experimental groups

One-way analysis of variance analysis (ANOVA) with post hoc Tukey-Kramer multiple comparison tests and Student's t-tests were used for statistical analyses. Data were shown as mean±S.E.M. (n = 4-6 per group). (*), p<0.05 vs control group

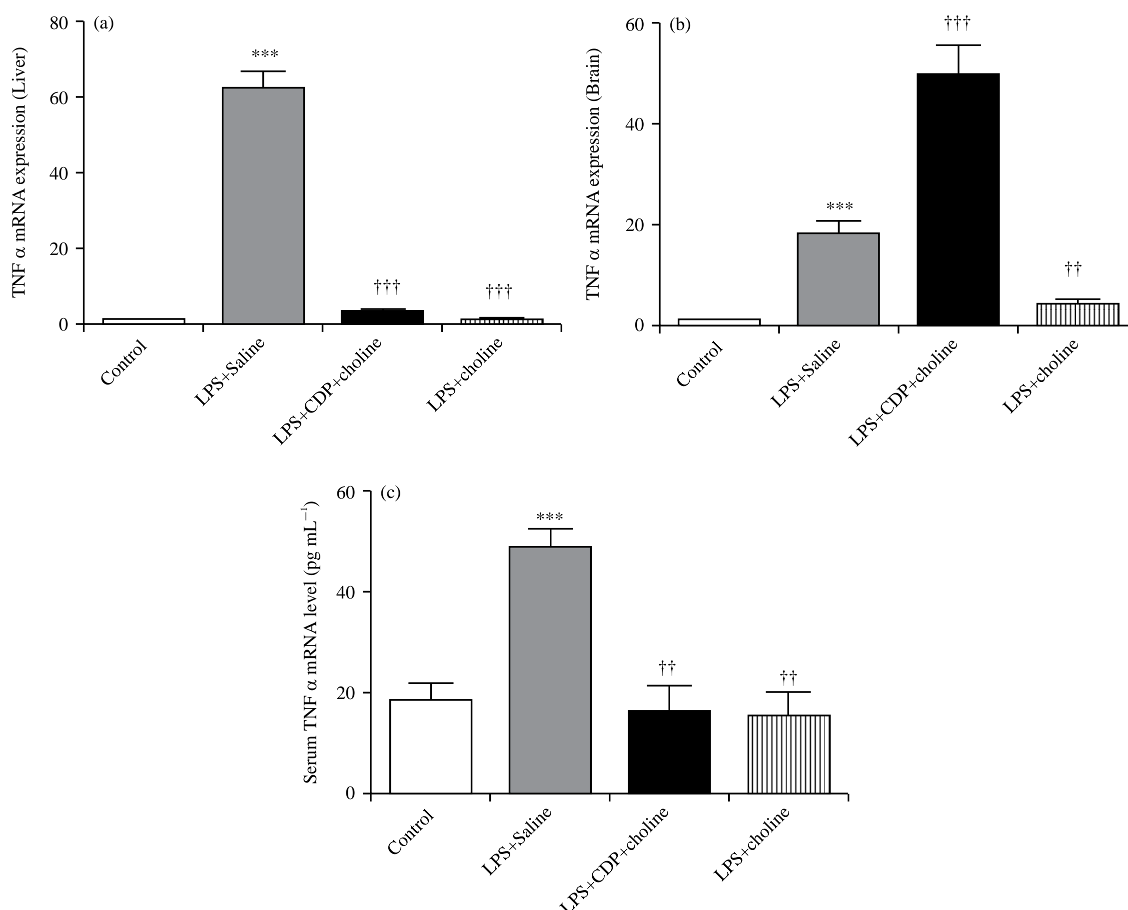


Fig. 6(a-c): TNF a mRNA expressions in the liver (A) and brain (B) tissues and serum TNF a levels (C) in experimental groups.
ΔΔCt method was employed for the relative quantification of mRNA expression. One-way analysis of variance analysis (ANOVA) with post hoc Tukey-Kramer multiple comparison tests and Student's t-tests were used for statistical analyses. Data were normalized to beta-actin and shown as Mean±SEM, (n = 4-6 per group). (**), (***); p<0.01, p<0.001 vs. control group. (††),(†††); p<0.01, p<0.001 vs. LPS+Saline group

DISCUSSION

This study investigated the role of cytidine-5-diphosphate-choline (CDP-choline) and choline as well as the contribution of the COX-2 pathway on the inflammatory response induced by lipopolysaccharide (LPS) in endotoxemic rats.

In this study, endotoxemia was induced by 10 mg kg^{-1} LPS[22] and the success of the model was determined by evaluating the rats' survival rates, sepsis scoring, body weight changes and histomorphological changes in the spleen. The dose was in the upper limit of the related reported studies and the rate of survival, increase in sepsis scoring and body weight decrease were compatible with the previous studies in LPS induced endotoxemia. Although not statistically significant, CDP-choline and choline have been detected beneficial effects on improving survival rates in early phases of endotoxin challenge. The survival rate (20% in 24 hrs) in the present study was similar to that published earlier. Shrum *et al.*[23] demonstrated that survival rates diminish to 20.0% in a murine animal model with total sepsis scores over 15. Previous observations reported that CDP-choline and choline increased survival rates by diminishing LPS induced organ injury, preventing disseminated intravascular coagulation, changing serum lipid responses and decreasing systemic inflammatory parameters in endotoxemic animals[21,27]. Along with previous reports, CDP-choline and choline treatments appear to improve the survival rate via exerting protective effects in multiple organ systems in endotoxemic animals.

Sepsis is an acute inflammatory response and endotoxemia is a model of acute inflammation, therefore we used a novel scoring system composed of different variables demonstrated to evaluate sepsis severity in animal models which provides reliable and consistent results[23,28]. The

evaluated parameters in sepsis scoring in rats were appearance, level of consciousness, activity, response to stimulus, eyes, respiratory rate and quality. The data showed that total sepsis scores as well as individual parameters, were significantly poor in the LPS+Saline group compared to the control group. Whereas CDP-choline and choline treatments significantly decreased total sepsis scores and individual parameters at late stages of sepsis challenge.

Response to stimulus in sepsis scoring was improved in the LPS+choline group compared to the LPS+Saline group. These novel findings might be related to the beneficial effects of choline on motor coordination. It was reported that the impaired motor coordination in endotoxemic mice[29]. Choline deficient diet is correlated with increased motor dysfunction in rats[30,31]. Respiratory parameters were improved in both treatment groups compared to the LPS+Saline group. CDP-choline and choline decreased the respiratory inflammation in the lung by decreasing inflammatory mediators released from mononuclear phagocytic cells (MPCs) were reported earlier[32,33]. Consistent with our findings, improvement in respiratory parameters might be related to the decreased pulmonary inflammation which should be confirmed by further studies.

LPS-induced endotoxemia/sepsis is known to be associated with sickness behaviour including appetite loss and body weight decreases caused by the central effect of cytokines[34]. LPS-induced body weight loss was partially recovered in treatment groups compared to the LPS+Saline group in this study. Although not statistically significant, CDP-choline and choline treatment decreased body weight loss. Accordingly, previously demonstrated that choline-supplemented diet recovered maternal, fetal and placental weight loss in LPS induced endotoxemia model of rats through the action on α7nAChRs and cholinergic anti-inflammatory pathway[35]. We consider that CAP activation by CDP-choline and choline might account for its protective effects on LPS induced body weight loss in endotoxemic rats. Taken together, the protective effects of CDP-choline and choline on LPS-induced body weight loss via α7nAChRs needs to be studied further.

The spleen is a major organ that contributes to cholinergic control of systemic inflammatory response[36]. Histomorphological examination of lung, liver and kidney tissues from rats with CLP-induced sepsis showed that inflammation, vascular degeneration, haemorrhage and necrosis parameters were improved by CDP-choline treatment without affecting the spleen[37]. Recent studies have been implicated that CDP-choline exerts tissue-protective effects by reducing oxidative stress in ischemic and inflammatory conditions[37,38]. COX-2 inhibitor celecoxib ameliorated tissue damage in spleen, liver and lungs in rats with CLP model of sepsis via decreasing COX-2 mediated oxidative damage[39]. We observed that severe haemorrhage and mild inflammation with MPC infiltration decreased in the spleen tissues from LPS-induced endotoxemic rats. Based on our observation, decreased circulating prostaglandin levels and improved sepsis severity suggest that inflammatory response might be suppressed with CDP-choline and choline treatments in the spleen. However, the exact role of CDP-choline or choline on LPS-induced tissue damage together with the involvement of COX-2 downregulation and antioxidant effect of the compounds needs to be clarified.

COX-2 expression and its products increased in the LPS+Saline group compared to that of control confirming the impact of COX-2 upregulation in LPS-induced endotoxemia[40,41]. COX-2 upregulation is also known to contribute to inflammation by releasing prostaglandins[42]. Furthermore, COX-2 upregulation together with increased prostaglandin levels under the inflammatory conditions is associated with increased vascular permeability, the unrestrained release of inflammatory mediators and reactive radicals, hemodynamic changes eventually yielding multiple organ failure and death[5,6,7]. Co-administration of choline and acetylsalicylic acid in LPS-induced endotoxemia in mice showed a synergistic anti-inflammatory interaction between the compounds via affecting the arachidonic acid pathway[41]. Accordingly, our data showed that COX-2 expressions in the liver decreased in LPS+CDP-choline and LPS+choline groups. Accordingly, PGE_2, a stable PGI_2 metabolite 6-keto-PGF1α and TXA_2 levels were decreased in LPS+CDP-choline group compared to that of LPS+Saline. Consistent with our observations, several studies showed activation of the cholinergic anti-inflammatory pathway by CDP-choline and choline attenuates pro-inflammatory cytokine levels in animal sepsis models [4,11,20].

There are some conflicting results about the action of CDP-choline and choline in brain tissues. In study investigating blood pressure and heart rate in healthy rats revealed that intracerebroventricular CDP-choline administration increased COX-2 reactivity in the hypothalamus[43]. Nicotine administration causes an increase in COX-2 expression and PGE_2 level in microglial cells by activating the cholinergic anti-inflammatory pathway[44]. In this study, CDP-choline decreased the LPS induced COX-2 expression in the liver without affecting brain levels compared to the LPS+Saline group.

It is well known that endotoxemia leads to inflammation and an increase in TNFα levels in the brain[45]. Elevation in IL1β and TNFα levels increases PC hydrolysis and PLA_2 activity.

Evidence suggest that CDP-choline attenuated the loss of phospholipids and increase arachidonic acid in case of cerebral ischemia[46]. The study showed that i.p. CDP-choline significantly increased LPS induced TNFα expression without affecting COX-2 expression under the hypothesis suggest that beneficial effects of CDP-choline in the brain are opposed to TNFα via counteracting TNFα mediated events[47]. In our study downregulated COX-2 activity was also confirmed by decreased serum prostaglandin and TNFα levels.

Several studies demonstrated that exogenously administered CDP-choline and choline elevates total choline levels in the brain and blood circulation[11,20,48]. As shown earlier, the LPS challenge increases serum total choline concentrations by activating endogenous cholinergic anti-inflammatory pathway which is further enhanced by administration of choline and CDP-choline activating cholinergic receptors expressed on MPCs[9,18]. According to our findings, total choline levels did not increase in serum in response to LPS administration whereas it slightly increased in brain tissues although statistically not significant. However, i.p. administration of CDP-choline or choline elevated total choline concentrations in serum and brain tissues of endotoxemic animals. Briefly, besides peripheral anti-inflammatory effects, increasing total choline levels in the brain and central activation of the cholinergic anti-inflammatory pathway may account for protective effects of CDP-choline or choline in endotoxemic rats.

The underlying mechanism of CDP-choline and choline on the COX pathway in LPS-induced inflammatory response and its severity were not examined in the scope of the present experimental protocol. Moreover, it was not possible to monitor changes in COX-2 expression levels for a longer period due to poor survival rates in the LPS+Saline group. The sensitivity of the ELISA kit was not high enough to determine tissue prostaglandin levels. Although appears to be relevant at the pre-clinical level, the protective effects of CDP-choline and choline through the COX pathway and cholinergic receptor involvement in LPS-induced endotoxemia/sepsis needs to be confirmed unequivocally by further studies.

CONCLUSION

This study demonstrated that the anti-inflammatory effects of CDP-choline or choline appear to be associated with downregulation of COX-2 expression and proportional decreases in prostaglandin and TNFα levels in the periphery, however, the central action of CDP-choline counteracted this effect possibly via TNFα mediated mechanisms. CDP-choline or choline improved survival rate and decreased sepsis severity in endotoxemic rats.

SIGNIFICANCE STATEMENT

This study discovers the effects of CDP-choline and choline on cyclooxygenase (COX)-2 activity and prostaglandins in the central and periphery, which are known to contribute cardinal signs of inflammation. The results are providing supportive evidence on the anti-inflammatory role of CDP-choline and choline along with other pre-clinical studies allowing the development of candidate cholinomimetics in the treatment of sepsis and other inflammatory diseases.

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