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EXPERIMENTAL STUDY

Choline and citicoline ameliorate oxidative stress in acute kidney injury in rats

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ABSTRACT
OBJECTIVES: The purpose of this study is to investigate the effects of cholinergic anti-inflammatory pathway (CAP)-activating drugs, choline and citicoline (Cytidinediphosphate-choline, CDP-choline), on lipopolysaccharide (LPS)-induced acute kidney injury (AKI) parameters and the contribution of NADPH Oxidase4 (NOX4) p22phox.
BACKGROUND: Endotoxemia induces a systemic inflammatory response characterized by the production of pro-inflammatory mediators and reactive oxygen species (ROS), which eventually develops acute kidney injury (AKI). NADPH Oxidase4 (NOX4) p22phox pathway contributes to the development of endotoxemia-induced AKI. Inflammatory response can be controlled by CAP.
METHODS: Expressions levels of KIM-1, TNF-α, NOX4, p22phox and NFκB in the kidney tissues of rats were analyzed via RT-PCR in experimental groups; 1. Control, 2. LPS (10 mg/kg) + saline, 3. LPS + CDP-choline (375 mg/kg) and 4. LPS + choline (90 mg/kg). Choline and ROS levels in kidney tissues were also measured by a spectrofluorometric assay.
RESULTS: LPS-induced elevations of ROS levels were decreased by CDP-choline or choline administration ($p < 0.001$). LPS-elevated KIM-1, TNFα, NOX4, p22 phox, and NFκB expressions were significantly decreased by choline or CDP-choline treatments ($p < 0.001$).
CONCLUSION: Decreased ROS production in kidney tissues in treatment groups suggests that choline or CDP-choline may have therapeutic potential in endotoxemia-associated AKI via downregulating NOX4 and p22phox expressions *(Tab. 1, Fig. 5, Ref. 45)*. Text in PDF *www.elis.sk*
KEY WORDS: endotoxemia, choline, cytidine diphosphate choline, acute kidney injury, reactive oxygen species.

Introduction

Acute kidney injury (AKI) is a very common clinical problem in intensive care units with the mortality rates ranging between 26–88 % and may require renal replacement therapy (1). Inflammation and oxidative stress are among the main mechanisms in AKI development. Inflammation can be triggered by some antigenic molecules as well as by lipopolysaccharide (LPS), a gram-negative bacterial endotoxin, which activates inflammatory cells by interacting with toll-like receptors (TLR) (2).

Systemic inflammatory response causes multiple organ dysfunctions due to uncontrolled production of proinflammatory cytokines and reactive oxygen species (ROS) (3). Inflammation and oxidative stress are closely associated with many pathologies including cancer, cardiovascular, pulmonary and Alzheimer's diseases. Studies showed that expressions of Tumour Necrosis Factor (TNF)α, Interleukin (IL)1β, Cyclooxygenase (COX)2, inducible Nitric Oxide Synthase (iNOS), Nicotinamide adenine dinucleotide phosphate oxidase (NOX)2, NOX4, and xanthine oxidase increased in heart and kidney tissues of endotoxemic animals (4–6). It has been also shown that LPS-mediated inflammation and oxidative stress contributes to AKI development in endotoxemic animals (7, 8).

NOX is an enzyme which catalyzes superoxide radical formation and ROS production. NOX4 is the most profound isoform of NADPH oxidases in kidney tissues and its stabilization and activation require p22phox, a membrane-bound protein. Studies proposed that NOX4 is regulated at the transcriptional level (9, 10). Previous data showed that, NOX4 expression is induced upon LPS activation in sepsis-associated AKI (11, 12). Malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and cytokine levels increased, whereas catalase and glutathione levels decreased in the brain tissues of endotoxemic mice.

Cholinergic anti-inflammatory pathway (CAP) is a neural mechanism that controls inflammatory response by inhibiting the

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Tab. 1. Primers used in real-time quantitative RT-PCR.

Gene	Forward Sequence	Reverse Sequence
NOX4	GTGAACGCCCTGAACTTCTC	TTCTGGGATCCTCATTCTGG
p22 phox	TGTTGCAGGAGTGCTCATCTGTCT	AGGACAGCCCGGACGTAGTAATTT
β-actin	TGCAGAAGGAGATTACTGCC	CGCAGCTCAGTAACAGTCC
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
KIM-1	ACTCCTGCAGACTGGAATGG	CAAAGCTCAGAGAGCCCATC
NF-κB	CAAAGCTCAGAGAGCCCATC	CCAGGTCATAGAGAGGCTCAA

release of inflammatory cytokines via activating vagus nerve and cholinergic receptors. It has been shown that alpha 7 nicotinic acetylcholine receptors (α7nAChR), members of cholinergic receptors, are required for communication between cholinergic system and immune system cells (13). Alpha7nAChR agonists inhibit the release of cytokines (i.e. TNF-α) and interleukins as well as increasing survival in *in vivo* and *in vitro* inflammation models. Therefore, potential therapeutic role of α7nAChR agonists in inflammatory disorders has been suggested (13). The role of choline and CDP-choline, a choline donor, is extensively studied *in vivo* models of endotoxemia due, in part, to their agonistic effect on CAP via α7nAChR interaction (14, 15). Studies demonstrated that CDP-choline and choline increase survival rates in endotoxemic animals and produce positive effects on endotoxin-induced tissue damage and multiple-organ failure (16–18) by CAP-mediated decrease in release of inflammatory mediators from mononuclear cells (19).

Regarding the oxidative stress and CAP interaction, citicoline (Cytidine Diphosphate Choline or CDP-choline), administration decreased MDA levels in the brain tissues of animals with head trauma (20). Administration of carbachol, a cholinomimetic agent, decreases xanthine oxidase and MDA levels in heart tissues of animals with sepsis (21). MDA levels decreased whereas SOD activity increased after citicoline administration in rats with myocardial damage (22). Citicoline reduced ischemia-reperfusion-induced oxidative stress and apoptosis in neonatal rats (23). Phosphorylcholine exerted antioxidant and renoprotective effects by decreasing MDA levels and increasing SOD activity on kidney tissues in rats with contrast induced AKI (24).

We previously showed that choline and CDP-choline administration downregulate LPS-induced Cyclooxygenase (COX)-2 pathway along with prostaglandin production while producing positive effects on sepsis severity and survival of animals with endotoxemia (25). COX pathway and prostaglandin production contribute to oxidative damage in animals with various disorders including inflammation (5, 26). There is insufficient evidence about the effects of choline and CDP-choline in LPS-induced AKI and oxidative stress mediated mechanisms. Therefore, this study investigates effects of CDP-choline and choline on the LPS-induced ROS production via NOX4 pathway in the kidney tissues of the endotoxemic rats.

Materials and methods

Archival kidney samples of male Wistar rats were used in this study. These samples were obtained from our previous study approved by Dokuz Eylul University Multidisciplinary Laboratory Animal Studies Ethics Committee (51/2017). Animal Ethics Committee approval for this study about using collected kidney tissues of rats was also obtained (59/2021).

Four experimental groups archival kidney tissues stored in cryovials at –80 °C for molecular analysis were used in this study: 1. Control (n = 6), 2. LPS+saline (sublethal dose, 10 mg/kg, n = 12), 3. LPS+ CDP-choline (375 mg/kg, n = 10) and 4. LPS+ choline (90 mg/kg, n = 10). Saline or LPS were administered 5 min after the drug injections. Saline, choline, and CDP-choline injections were repeated at the 6th hour. The CDP-choline dose was determined as equieffective choline dose as reported earlier (25,27). Rats were sacrificed by decapitation at the 24th hour under mild ether anesthesia at the end of the experimental protocol and kidneys were removed and cryopreserved immediately for further analyses.

Chemicals

Lipopolysaccharide (LPS; from *E. coli*), Choline chloride, CDP-choline (Cytidine 5- diphosphocholine sodium salt dihydrate) obtained from Sigma-Aldrich (St. Louis, Mo., USA). All drugs were dissolved in saline (0.9 % sodium chloride) and administered intraperitoneally.

Measurement of reactive oxygen species

Reactive oxygen species (ROS) levels in kidney tissue analyzed with the fluorometric ROS kit (Elabscience, E-BC-K138-F, USA) according to the instructions in the kit (28).

RT-qPCR analysis on KIM-1, TNF-α, NOX4, p22phox and NFκB expression

Total RNA extraction in kidney homogenates was performed according to the manufacturer instructions (GeneJET RNA Purification Kit, ThermoScientific, USA). cDNA of each sample was synthesized by a commercial kit (ABM 6236, USA) and amplified in an RT-PCR device (Bio-Rad CFXconnect) by using Master Mix (ABM G013, USA). Beta-actin was used as a positive internal control for normalization of the gene transcription. The oligonucleotide primer sequences used in the study are shown in Table 1. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels (29).

Measurement of total choline levels

Total choline levels in kidney tissue were analyzed via a spectrophotometric total choline/acetylcholine kit (Biovision, K615, USA) according to the manufacturer instructions (25).

Statistical analysis

One-way analysis of variance analysis (ANOVA) with post hoc Tukey–Kramer multiple comparisons for parametric results were employed for group comparisons. Statistical analyses 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.

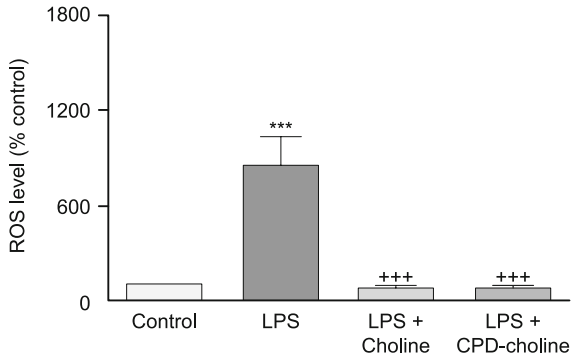

Fig. 1. Relative tissue reactive oxygen levels in kidney tissues of experimental groups. One-way analysis of variance (ANOVA) with post hoc Turkey-Kramer multiple comparison was used for statistical analyses. Data are shown as mean and S.E.M (n = 4–6 in each group). ***, p < 0.001 vs control group; †††, p < 0.001 vs LPS group.

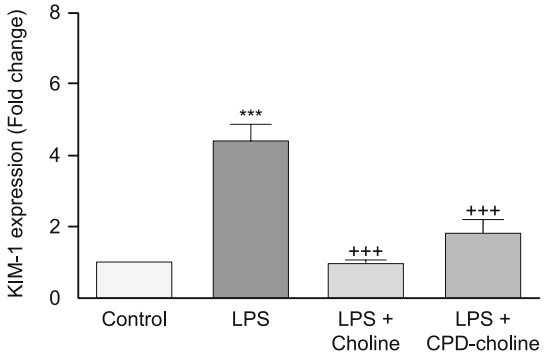

Fig. 2. KIM-1 mRNA expression (fold change) in kidney tissues of experimental groups. $2^{-\Delta\Delta Ct}$ method was employed for the relative quantification of mRNA expression. One-way analysis of variance (ANOVA) with post hoc Turkey-Kramer multiple comparison was used for statistical analyses. Data were shown as mean and S.E.M (n = 4–6 per group). ***, p < 0.001 vs control group; †††, p < 0.001 vs LPS-group.

Results

ROS levels in kidney tissues

Tissue ROS levels significantly increased in LPS+Saline treated group compared to controls ($p < 0.001$) while significantly decreased in LPS+Choline and LPS+CDP-choline treated groups compared to LPS+Saline group ($p < 0.001$) (Fig. 1).

KIM-1 mRNA expression in kidney tissues of endotoxemic animals

Kidney Injury Molecule (KIM)-1, marker of AKI, significantly increased in LPS+Saline treated group compared to control ($p < 0.001$) while significantly decreased in LPS+Choline and LPS+CDP-choline treated groups compared to LPS+Saline group ($p < 0.001$) (Fig. 2).

NOX 4 and p22 phox mRNA expressions in kidney tissues

NOX 4 mRNA and p22phox mRNA expressions significantly increased in LPS+Saline treated group compared to controls ($p < 0.001$), while significantly decreased in LPS+Choline and LPS+CDP-choline treated groups compared to LPS+Saline group ($p < 0.001$) (Fig. 3).

TNFα and NF-κB mRNA expressions in kidney tissues

TNFα and NF-κB expressions, significantly increased in LPS+Saline treated group compared to controls ($p < 0.001$) while decreased significantly in LPS+Choline and LPS+CDP-choline treated groups comparable to LPS+Saline group ($p < 0.001$) (Fig. 4).

Total choline levels in kidney tissues

Total Ch/ACh levels significantly increased in LPS+Choline and LPS+CDP-choline treated groups comparable to control group ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 5).

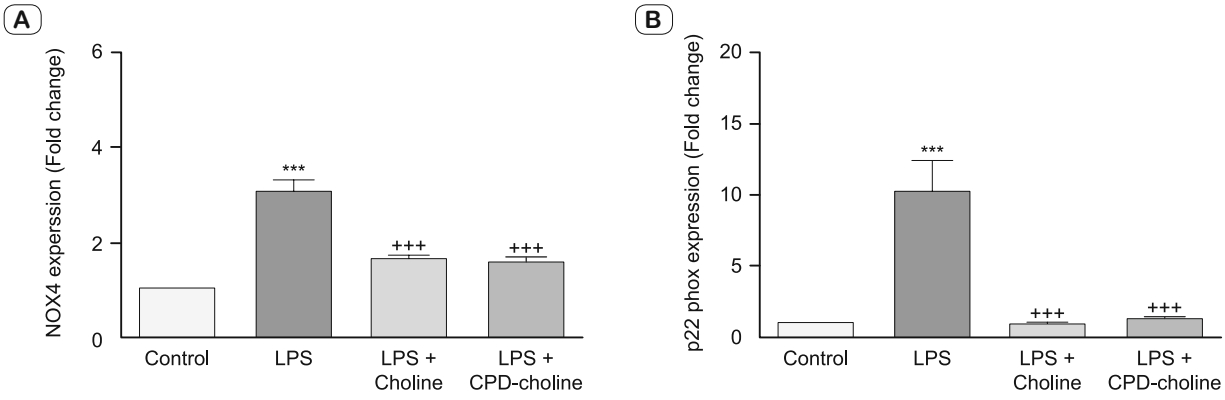

Fig. 3. NOX4 and p22 phox mRNA expression (fold change) in kidney tissues of experimental groups. $2^{-\Delta\Delta Ct}$ method was employed for the relative quantification of mRNA expression. One-way analysis of variance (ANOVA) with post hoc Turkey-Kramer multiple comparison were used for statistical analyses. Data are shown as mean and S.E.M (n = 4–6 per group). ***, p < 0.001 vs control group; †††, p < 0.001 vs LPS-group

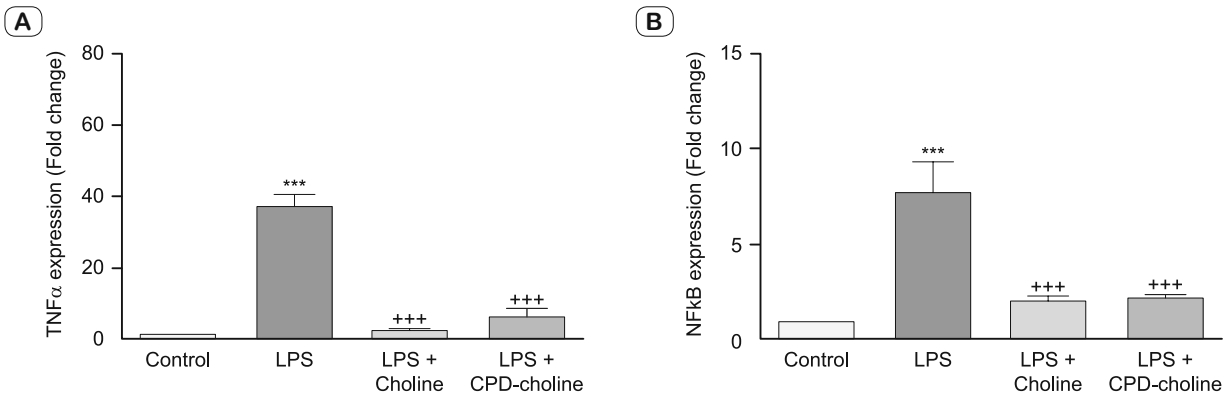

Fig. 4. TNF-α and NF-κB mRNA expression (fold change) in kidney tissues of experimental groups. $2^{-\Delta\Delta Ct}$ method was employed for the relative quantification of mRNA expression. One-way analysis of variance (ANOVA) with post hoc Turkey-Kramer multiple comparison were used for statistical analyses. Data are shown as mean and S.E.M (n = 4–6 per group). ***, p < 0.001 vs control group: †††, p < 0.001 vs LPS-group.

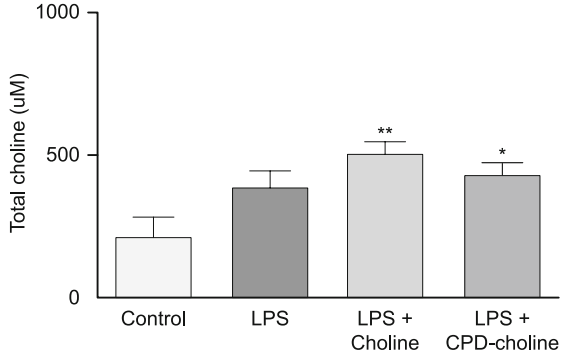

Fig. 5. Total choline levels in kidney tissues of experimental groups. One-way analysis of variance (ANOVA) with post hoc Turkey-Kramer multiple comparison were used for statistical analyses. Data are shown as mean and S.E.M (n = 4–6 in each group). *, p < 0.05; **, p < 0.01 vs control group.

Discussion

This study shows the inhibitory effects of CDP-choline and choline on the LPS-induced ROS production via NOX4 pathway in the cryopreserved kidney tissue samples isolated from the endotoxemic rats.

Kidney Injury Molecule-1 (KIM-1) is a transmembrane protein expressed in the proximal tubules which increases with acute kidney injury (AKI) and accepted as an early biomarker (30, 31) induced in LPS-induced AKI (32, 33). Studies showed that elevated KIM-1 expressions are correlated with acute tubular damage and acute tubular necrosis (34). KIM-1 was demonstrated as an early biomarker in the diagnosis of septic AKI and correlated with poor prognosis in clinical practice (35). There is a lack of evidence regarding the interaction between KIM-1 and choline-containing compounds. Earlier study showed an increase in choline radioactivity in regenerating kidney tissues in rats with nephropathy induced by mercury, suggesting an association between renal regeneration and choline-containing phospholipids (36). In this study, KIM-1 levels significantly increased in LPS group, comparable to control. Briefly, significant decrease in KIM-1 levels by CDP-choline and choline suggests that these agents may have a clinical impact on prophylaxis of endotoxemia-induced AKI.

The pathophysiology of sepsis-induced AKI includes ischemic or toxic injury to the renal tubular epithelium leading to necrosis, apoptosis and production of reactive oxygen species (ROS), which is characterized clinically as tubular damage (37, 38). NADPH oxidases (NOX) are important sources of ROS production, and NOX4 isoform is widely expressed in the kidney. Stabilization and activation of NOX4 requires p22 phox protein interaction (39). Elevations of NOX4 and p22 phox expressions were demonstrated in different kidney injury models *in vivo* (29, 40, 41). Previous data showed that NOX4 expression induced upon LPS activation in sepsis was associated with AKI (11, 12). Choline downregulates angiotensin II-mediated NOX expression and mitochondrial ROS via muscarinic ACh receptor activation in vascular smooth muscle cells *in vitro* (42). In summary, significant decreases in LPS-induced NOX4 and p22phox expressions may account for the possible therapeutic effectiveness of choline and CDP-choline in endotoxemia.

Inflammation and oxidative stress are closely related with sepsis-induced AKI. Studies showed that expressions of TNFα, IL1β, COX2, iNOS, NOX2, NOX4, and xanthine oxidase increased in heart and kidney tissues of endotoxemic animals (4, 6, 43). Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), cytokine levels increased, and catalase and glutathione levels decreased in brain tissues of endotoxemic mice while α7nAChR protein, ACh levels and choline acetyl transferase activities decreased (44). These observations were supported by the previous histomorphological evidence that endotoxemia-induced kidney damage along with hemodynamic parameters were significantly improved by CDP-choline administration (45). In addition to previous findings, the present study further suggests a therapeutic potential of choline and CDP-choline prevention of LPS-elevated

ROS, TNFα and NF-κB levels possibly due to the inhibition of NOX4 and p22phox expressions in rat kidney.

Conclusion

Taken together, this study showed that CDP-choline and choline administrations significantly decreased LPS-induced NOX4 and p22phox expressions by increasing total choline levels resulting in decrease in the ROS levels in rat kidney tissues. Together with previous data, our results suggest that CDP-choline and choline may have a therapeutic value in LPS-induced AKI and that further studies can be conducted to evaluate the clinical implications of these findings.

Learning points

Citicoline (CDP-choline) and choline treatments decreased KIM-1 levels that indicate acute kidney injury (AKI) in rats with endotoxemia.

Citicoline (CDP-choline) and choline decreased AKI via downregulating LPS induced NOX4 and p22phox expressions along with ROS.

Citicoline (CDP-choline) and choline decreased ROS levels via increasing total choline levels in kidney tissues.

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