Original Article / Özgün Araştırma

Folinic Acid (Leucovorin) Treatment in Lipopolysaccharide-Induced Systemic Inflammation in Rats

Sıçanlarda Lipopolisakkarid ile İndüklenen Sistemik İnflamasyon Modelinde Folinik Asit (Lökovorin) Tedavisi

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Abstract

Objective: The antioxidant and anti-inflammatory effects of Folinic acid [(FA); Leucovorin] in a septic shock model have been investigated.

Materials and Methods: FA (100 mg/kg) given thirty minutes before lipopolysaccharide (LPS; 5 mg/kg). The blood and tissue samples of control, FA, LPS and FA + LPS groups were obtained sixteen hours later of LPS intervention.

Results: Sepsis resulted in a remarkable increase in alanine aminotransferase, aspartate aminotransferase, urea, creatinine, lactate, nitric oxide, Malondialdehyde (MDA), procalcitonin, tumour necrosis factor-alpha, Interleukin 1-beta levels and consumed antioxidants catalase (CAT) and glutathione (GSH) levels of the tissues. MDA, nitric oxide, GSH and CAT levels were ameliorated by FA treatment.

Conclusion: FA can be used as a safe way of restoring the (anti)oxidant status of kidney and liver tissue in sepsis clinic. It can be effective in controlling inflammatory mediators, decreasing procalcitonin and regulating the metabolic process of sepsis.

Keywords
Endotoxemia, folic acid, LPS, rat, sepsis, vitamin B9

Öz

Amaç: Folinik asit [(FA); Lökovorin]'in septik şok modelinde antioksidan ve antiinflamatuvar etkisi araştırıldı.

Gereç ve Yöntemler: FA (100 mg/kg), lipopolisakkarid (LPS; 5 mg/kg)'ten 30 dakika önce uygulandı. Kontrol, FA, LPS ve FA + LPS gruplarının kan ve doku örnekleri, LPS uygulamasından 16 saat sonra alındı.

Bulgular: Sepsis, alanin aminotransferaz, aspartat aminotransferaz, üre, kreatinin, laktat, nitrik oksit, malondialdehitin (MDA), prokalsitonin, tümör nekroz faktörü, interlökin-1b düzeylerinde belirgin artışa ve dokularda antioksidanların [katalaz (CAT) ve glutatyon (GSH) düzeyleri] tüketilmesine yol açtı. FA tedavisi ile MDA, nitrik oksit, GSH ve CAT değerlerinde iyileşme sağlandı.

Sonuç: FA, sepsiste böbrek ve karaciğer dokularında (anti)oksidan durumu onarmak için kullanılacak güvenli bir yoldur. İnflamatuvar mediyatörleri kontrolde, prokalsitonini azaltmada ve sepsisin metabolik sürecini düzenlemede etkili olabilir.

Anahtar Kelimeler
Endotoksemi, folik asit, LPS, sıçan, sepsis, vitamin B9

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Introduction

A life-threatening organ dysfunction, sepsis, is accompanied by metabolic disturbances (1). Massive cytokine release, oxidant stress and mitochondrial dysfunction can be counted as some of the underlying mechanisms of overwhelming inflammatory response in sepsis (2,3). Fluid administration strategies, metabolic and bioenergetic reformation routes, inflammatory pathway modulations and antibiotics might be used as some of the therapeutic approaches of the disease control but still, further investigations for the treatment are needed (4). Considering that, patients are highly vulnerable to iatrogenic complications (4); the right medication and co-treatments should be chosen in the safest doses in intensive care units.

Folic acid [(FA); Vitamin B9] cannot synthesize in mammalian cells, it must be obtained entirely from dietary sources (5). It is given to pregnant women to prevent neural tube defects (6), for the treatment of megaloblastic anemia (7) and alcohol-associated vitamin deficiencies (8). During the medical treatment of methotrexate, aminopterin, pyrmethamine and trimethoprim, it can be given as folinic acid supplementation to restore the folate metabolism (5). The importance of folate in metabolic syndrome has been shown that its deficiency results in hyperhomocysteinemia, increased blood pressure and insulin resistance in spontaneously hypertensive rats (9). A recent study has also suggested that FA might contribute to the control of inflammation by controlling monocytes' recruitment to the inflamed tissues (10).

In the intensive care unit, many nutritious solutions are given altogether to patients, they include many vitamins in different amounts to support the metabolisms' requirement; but so far, the treated power of only one of the vitamins has not been revealed properly. This point has drawn our attention and previously, we have already published that dexpanthenol (Vitamin B5) is highly effective on lipopolysaccharide (LPS)-induced endotoxic shock model (11). Therefore, we have continued our research with another vitamin-drug, FA to evaluate its safety and efficacy on the hepatic and kidney tissue injuries of endotoxemia.

Materials and Methods

Animals and Experimental Procedures

Male Wistar rats, 12-15 weeks old, were gathered from Aydın Adnan Menderes University Laboratory of Experimental Animals, and the experiments were applied after the approval of Animal Local Ethics Committee of Adnan Menderes University (decision number: 33, date: 25.02.2016). All animal care and experimental procedures were in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Thirty-two rats were randomly assigned into four groups of eight animals each. A single intraperitoneal (i.p.) injection of saline solution was given to the rats as a control group. A single i.p. injection of LPS (5 mg kg^{-1} in 1 mL of saline; *Escherichia coli* 055: B5, Sigma-Aldrich L-2880, Interlab, İstanbul, Turkey) was administered to the rats to produce the animal model of sepsis (LPS group). To see the safety of FA (100 mg/kg; folinic acid, Calcium Folinate DBL, Hospira UK Limited, Warwickshire, UK), it has been given to the healthy rats as a third group. Thirty minutes before LPS (5 mg kg^{-1} in 1 mL of saline) injection of the left side, 100 mg per kg FA was administered via i.p. injection to the abdomen at the right region (3,11) as a treatment group (FA + LPS group).

Sixteen hours after all interventions, blood samples of the four groups were obtained by a cardiac puncture under the anaesthesia of Ketamine (50 mg/kg) and Xylazine (5 mg/kg). Blood was centrifuged (1000 X g for 10 minutes) and serum were separated and stored with the tissues at -80 °C.

Blood Analyses

A spectrophotometric auto-analyzer (Architect C 8000, Abbott, IL, USA) was used for the assessment of serum urea, creatine, lactate, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Lipid peroxidation was specified by malondialdehyde (MDA) production (12). Nitric oxide (NO; nitrite + nitrate) was assessed using a method of cadmium-reduction (13). By the Beutler method, tissue total glutathione (GSH) level was determined (14). The Aebi method was used to measure the activity of catalase (CAT) (15). The reduction rate of peroxide (H_2O_2) was accounted for 30 seconds at 240 nm wavelength.

Tumour necrosis factor-alpha (TNF-alpha; Fine Test Rat TNF-alpha ELISA kit ER1094); procalcitonin (PCT;

Fine Test Rat PCT ELISA kit ER1235); Interleukin 1-beta (IL-1b; Fine Test Rat IL-1 beta ELISA kit ER1094) were purchased from Fine Wuhan Biological Technology, China. Test results of serum were accounted via ELISA plate reader (BioTek Inc., ELX800, USA) using standard curve at 450 nm. The boundary of detections used for either PCT or TNF-alpha was 9.37 pg/mL, and for IL1-b was 18.75 pg/mL. The intra-assay and inter-assay variation coefficients were 8% and 10%, respectively. 15.6-1000 pg/mL assay range was used for both PCT and TNF-alpha. Additionally, 31.25-2000 pg/mL for IL-1b was used. Process was carried through the producer instructions.

Tissue Analyses

After weighting the liver and kidney samples, they were homogenized and centrifuged at 20,000 g for 15 minutes, and supernatant was collected and stored at -80 °C for detection of NO, MDA, GSH, and CAT activity levels (in 7.4 pH, 50 mM phosphate buffer saline).

By Ohkowa method, lipid peroxidation (for MDA production) has been assessed in tissues (12). With thiobarbituric acid generation, MDA forms a compound which may be detected at 532 nm absorbance measurement. The levels of absorbance were accounted by a spectrophotometer. As a standard, 1.1'.3.32' tetraethoxy propane was utilized and the results were stated as micromol/mg protein.

For the assessment of NO a modification of the cadmium-reduction method of Navarro-Gonzalves et al. (13) was used. In this method, nitrite production was assessed via sulfanilamide diazotization and coupling with naphthyl ethylenediamine. The specimens were examined via spectrophotometric microplate device (ELX800, BioTek Instruments, Vermont, USA) and automatically measured according to KNO_3 normal curve and the outcomes were stated as micromol/mg protein.

The reduction rate of H_20_2 was followed at 240 nm for 30 seconds at room temperature for CAT activity in tissue by the method of Stankova et al. (15). Beutler method was used for GSH assesment (14). At 412 nm the absorbance levels were surveyed via a spectrophotometer model of Shimadzu UV-160. By standard aqueous solutions GSH levels were measured.

Statistical Analysis

The results of the disease group (LPS) were compared with the control. Also, to make sure of FA safety, we compared this group with control. Then, we evaluated the treatment power of FA in the disease group. A non-parametric Mann-Whitney U test was used to assess biochemical parameters and a percent rate for the clinical approach. Results were expressed as mean ± SEM and $p<0.05$ was statistically significant.

Results

Blood Results

Blood markers of control animals were not affected by alone FA treatment ($p>0.05$; Table 1); but, they have been elevated 3 to 4 fold in LPS group, except CAT level which has a 63.95% declined ($p<0.001$; Table 1).

FA treatment has significantly restored the liver function (AST and ALT) and kidney function tests (creatinine, urea), and declined the lactate levels. Additionally, PCT and nitrite/nitrate levels increased in LPS group. Oxidant MDA levels were reduced and antioxidant CAT levels were partially renovated by FA treatment (Table 1).

Each TNF-alpha and IL-1b levels were increased about three times with the LPS administration ($p<0.001$; Table 1). TNF-alpha level of FA + LPS treatment group was only increased 32% and IL-1b increment was only 16% in this group.

Tissue Oxidant/Antioxidant Levels

By LPS intervention MDA and NO levels were increased in tissues of liver and GSH and CAT results were diminished. Meanwhile, NO and MDA levels were improved statistically significant. Furthermore, CAT and GSH levels were increased by FA therapy ($p<0.001$; Table 2).

In the kidney tissue, MDA and NO levels were increased after LPS intervention while the CAT and GSH levels highly reduced. With the help of FA treatment, MDA level was completely restored; NO, GSH, CAT tissue levels were significantly restored ($p<0.001$; Table 3).

Discussion

The possible benefit of FA supplementation might reduce the disease severity. Liver and kidney functions, (anti)oxidant and cytokine pathways, lactate and PCT were evaluated with regard to this hypothesis in LPS-induced endotoxemia rat model.

Table 1. Blood parameters of all experimental groups, Mean ± SEM

Group	ALT (U/L)	AST (U/L)	Urea (mg/dL)	Creatinine (mg/dL)	Lactate (mg/dL)	Nitrite + nitrate (umol/L)	MDA (µmol/L)	Catalase (nmol/min/mg)	Procalcitonin (pg/mL)	TNF-a (pg/mL)	IL-1b (pg/mL)
Control	63.38±2.95	77.13 ±2.92	38.50±0.80	0.42±0.01	22.65±1.09	21.63±0.78	11.81±0.53	147.75±10.46	35.25±2.94	36.53±1.00	49.59±3.10
LPS	183.50±23.25[#]	241.25 ±19.27[#]	105.50±7.83[#]	1.51±0.02[#]	90.39±2.83[#]	94.84±2.52[#]	47.28±1.65[#]	53.25±2.93[#]	107.63±4.34[#]	116.86±7.20[#]	140.19±11.15[#]
Folinic acid	64.87±3.28	77.25 ±2.78	37.38±1.25	0.42±0.01	25.13±1.44	10.96±1.07	13.01±0.66	160.00±8.17	32.13±1.71	35.90±1.27	44.20±2.43
Folinic acid + LPS	89.14±6.62[†]	101.00 ±5.24[‡]	44.86±4.18[‡]	0.57 ±0.05[‡]	30.27±1.40[#†]	22.48±1.76[‡]	29.13±1.90[#‡]	97.43±4.08[#‡]	51.14±3.69[‡]	48.32±5.26[#‡]	57.71±9.02[#†]

[§]p<0.01, [#]p<0.001 according to control; [†]p<0.01, [‡]p<0.001 according to LPS group. LPS: Lipopolysaccharide, ALT: Alanine transaminase, AST: Aspartate aminotransferose, SEM: Standard ernor of the mean

Sixteen hours later of LPS intervention, serum AST, ALT, creatinine and urea levels have markedly elevated as indicative of hepatorenal damage; the animal model was successfully established. Given alone FA (100 mg/kg) in one of the groups did not show any tissue damage findings. Before exploring the beneficial effect the FA, it has been shown that this dose of FA is safe.

It has been published that folate deficiency was associated with alterations in antioxidant enzyme activities in the liver, kidney and heart (9). One of the previous study was used a forced swimming model to mimic the clinical situation of fatigue and produced oxidative stress in mice; supplementation of FA and its modified form have been found highly effective antioxidant with theirs' scavenging activity (16). As it is well known, sepsis produces many inflammatory oxidants (2,11), scavenging activity of FA molecule might result in a better serum profile of (anti)oxidant systems as we have determined both in serum and tissues.

FA has a crucial role in the generation of Methyl-tetrahydrofolate (5,17), which joins in methionine-homocysteine metabolism (17). Dysregulated methionine metabolism has been implicated in sepsis (1). Produced S-adenosylmethionine is highly important to the synthesis of GSH (1). Indeed, in our study, both GSH levels of liver and kidney tissues have been consumed as a marker of oxidative stress and significantly restored by FA treatment. Also, FA treatment significantly reduced both serum and tissue MDA levels in sepsis.

The imbalance between produced pro-inflammatory cytokines (IL-1, TNF-alpha and IL-6) and anti-inflammatory mediators of septic shock may result in organ failure (18,19). Their detection may be useful to understand the pathophysiology, severity and diagnosis of sepsis (18). The severity of sepsis is associated with NO production, in other words mitochondrial dysfunction (2,19,20). Bhattacharjee et al. (21) have been shown produced reactive oxygen species, NO and TNF-alpha increment in nicotine-induced pancreatic islet cell damage model. Given FA supplementation was able to restore oxidant mechanisms, decrease blood glucose level, increase insulin secretion, decrease pro-inflammatory mediator (NO, TNF-alpha and IL-6) levels, blunted

Table 2. Liver tissue levels of (anti)oxidants in all experimental groups, Mean ± SEM

Group	MDA (umol/mg protein)	NO (uM/mg protein)	GSH (uM/mg protein)	Catalase (nmol/min/mg protein)
Control	0.71±0.03	25.04±2.81	27.05±1.16	28.45±1.56
LPS	2.28±0.14$^{\#}$	85.51±4.08$^{\#}$	10.84±0.73$^{\#}$	12.69±0.81$^{\#}$
Folinic acid	0.84±0.06	26.09±3.07	28.64±1.85	27.83±1.70
Folinic acid + LPS	1.14±0.16*‡	32.83±5.44$^{\#}$	25.99±1.38‡	29.14±3.61‡

*p<0.05, §p<0.01, $^{\#}$p<0.001 according to control; †p<0.01, ‡p<0.001 according to LPS group. LPS: Lipopolysaccharide GSH: Glutathione, MDA: Malondialdehyde, NO: Nitric oxide, SEM: Standard ernor of the mean

Table 3. Kidney tissue levels of (anti)oxidants in all experimental groups, Mean ± SEM

Group	MDA (umol/mg protein)	NO (uM/mg protein)	GSH (uM/mg protein)	Catalase (nmol/min/mg protein)
Control	1.24±0.15	24.80±1.07	15.58±1.40	20.32±1.29
LPS	2.84±0.11$^{\#}$	69.93±3.74$^{\#}$	4.86±0.63$^{\#}$	7.78±0.70$^{\#}$
Folinic acid	1.08±0.08	28.63±1.56	15.23±0.77	18.58±1.25
Folinic acid + LPS	1.14±0.05‡	35.01±2.55‡	12.64±0.94‡	12.96±1.22‡

$^{\#}$p<0.001 according to control; ‡p<0.001 according to LPS group. LPS: Lipopolysaccharide, SEM: Standard ernor of the mean

the loss of mitochondrial membrane potential and limit the excessive apoptosis (21). Additionally, it has been demonstrated that TNF-alpha, IL-1b and NO production were inhibited by FA in BV-2 microglia cells against LPS activation (22). Although we have been used another inflammation model, blood IL-1b, both tissue and serum TNF-alpha and NO levels can be restored by FA similar to these literatures. One of the recent study by Samblas et al. (10) supports our findings that they found FA is highly effective for preventing of IL-1b and TNF-alpha expression (10).

Metabolic alterations such as mitochondrial dysfunction, energy metabolism, insulin resistance and high lactate level have been found responsible for inflammation and damaging of the organs (23). FA treatment has been shown significantly lowered blood urea nitrogen and blood lactate level, increased the storage of hepatic glycogen and improved energy metabolism in fatigue syndrome (16). This paper supports our study that FA treatment lowered blood urea and lactate level in septic rats.

Mitochondrial respiratory dysfunction, swelling, and in part uncoupling of oxidative phosphorylation have been produced by LPS intervention (24) and this mitochondrial dysfunction and cellular ATP depletion may lead to organ failures and death (23). Mitochondrial functions like ATP production levels and complex I inhibition rates via NO generation could not be evaluated in this study, but the lactate level of serum has been defined as an inflammatory metabolic marker. The follow-up of lactate levels are suggested for evolving the sepsis diagnosis (18). In sepsis, the reduced ATP generation may be compensated by the increase in the synthesis of glycolytic ATP which is accompanied by the increase in lactate generation (23). In the present study, lactate levels were nearly tripled (299% increment) and it was only stayed 33% increased with FA treatment. Actually, lactate generation is induced by TNF-alpha (23) and controlling of its increase may be helpful in reducing lactate levels. FA treatment brings additional benefits on metabolic inflammation of sepsis (23). PCT level measurement is also recommended to follow-up of sepsis clinic for determining the improvement (18). PCT has increased 205.33% in our study and shown that it is also a suitable marker for drug research in this accepted and widely used animal model (3). PCT elevation of FA treated group was dramatically prevented and it was only 45%.

Intensive care patients take many vitamins for a supplement via the intravenous route. Previously, we have published that dexpanthenol is effective to alleviate the inflammation of endotoxemia/sepsis patients (11). Consequently, we continued our hypothesis with FA to search the treatment power of vitamins on critically ill patients. FA has already

been approved for several indications and is ready to use pharmaceutical formulation (ampoule) in many clinics. For just the dose adjustment, the new indication can be attributed to FA.

Conclusion

FA treatment restores kidney and liver functions which are increased by sepsis-induced organ injury at some point. It has an antioxidant power on regulation of CAT, GSH and MDA levels. Additionally, the anti-inflammatory activity is revealed by level of PCT, IL-1, TNF-alpha and NO. It is also capable of ameliorating hyperlactatemia which is the important biomarker of metabolic cell stress. FA treatment has not any side effect on studied markers, hence it should be taken into account as an economical, safe and efficient adjuvant therapy in endotoksemia.

Ethics

Ethics Committee Approval: The experiments were applied after the approval of Animal Local Ethics Committee of Adnan Menderes University (decision number: 33, date: 25.02.2016).

Informed Consent: Experimental study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Animal Care: B.D., Concept: B.D., H.B.U., Design: B.D., H.B.U., Data Collection or Processing: H.B.U., M.Y., Analysis or Interpretation: B.D., H.B.U., M.Y., Literature Search: B.D., H.B.U., M.Y., Writing: B.D.

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