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Original article

The effects of enteral supplementation of glutamine and arginine in lipopolysaccharide (LPS) induced sepsis

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Summary. Sepsis is the leading cause for death in critically ill patients. While the exact mechanisms are not clear, multiple organ dysfunction syndrome induced by sepsis might cause mitochondrial dysfunction. Spesific nutrients may help regulating immunological and inflammatory responses. Glutamine and arginine amino acids have both important roles in growth, tissue repair, cell renewal and collagen synthesis. There are different results of studies with immunmodulatory nutrients in critically ill patients such, they have no effect on mortality or decrease sepsis incidance. The aim of this study is to evaluate the effects of enteral supplementation of glutamine, arginine and glutamine and arginine combination in lipopolysaccharide (LPS) induced sepsis. Twenty eight male Sprague Dawley rats were randomly divided into four groups: Glutamine, Arginine, Glutamine + Arginine and Control. Glutamine group received 500 mg/kg/day glutamine, Arginine group recieved 500 mg/kg/day arginine, Glutamine + Arginine group received 250 mg/kg/day glutamine and 250 mg/kg/day arginine containing suspension. Rats were fed for 10 days and 3 mg/kg LPS was implemented. 24 hours later, all rats were sacrified. C-Reactive Protein (CRP), Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor-α(TNF-α), Aspartat aminotransferase (AST) and Alanine aminotransferase(ALT) levels were studied, livers were examined histopathologically. Serum TNF-α levels were significantly lower in Glutamine + Arginine group in comparison with Control group. Liver histopathology analysis showed that apsis, hepatocyte damage, kupffer cell proliferation and portal inflammation were more frequent and severe in control group than all groups. These results revealed that enteral supply of glutamine, arginine and their combination have positive effects on liver damage and inflammation.

Key words: arginine, glutamine, sepsis, endotoxemia, immunonutrition

Introduction

Glutamine and arginine amino acids have both roles on growth, tissue repair, cell renewal and collagene synthesis (1). Glutamine is the most abundant amino acid but its storage is immediately depleted from muscles in catabolic stages such as trauma, sepsis and burn. For this reason glutamine is defined as conditionally essantial.

Arginine has also roles in nitrogene metabolism, creatine and polyamine synthesis and is the major substrate for nitric oxide synthesis. Thus it has an important role on immune response. Arginine is not an essential amino acid for healthy subjects, but in stress phases such as sepsis, it might be essential (2). Low plasma arginine levels and increased arginine requirement which could not be met by endogenous synthesis in septic patients are related with worsening prognosis (3, 4).

Prevalance of hospital deaths caused by sepsis has been incresing as 90% since last 20 years. Sepsis and inflammation cause multiple organ dysfunction syndrome (MODS) which is usually the cause for death in intensive care units. Decreasing or inhibiting stress response with substrats like glutamine may help preventing from MODS thus mortality in critically ill patients (5).

The results of animal models of arginine supplementation in sepsis are unsteady. The results are almost the equal mix of benefit, harm and no effect (6). Heyland et al. (7) have published a meta-analysis indicating that arginine supplemented diets have no benefit even might be harmful and have potential side effects in 2001, while in elective surgery patients arginine supplementation is found to be benefical by decreasing infection risk (8).

Moreover, many studies showed that glutamine and arginine supplementation have positive effects on preventing from sepsis, but more studies are needed for the use of combination of glutamine and arginine in enteral feeding (9). In this study the effects of enteral supplementation of glutamine, arginine and glutamine and arginine combination in lipopolysaccharide induced sepsis in rats was investigated aiming to contribute to present literature.

Material and Methods

Study Design

Study has been approved by Yeditepe University, Animal Research Ethics Committee. 24 Sprague Dawlay male rats weighted 300,2 g (270-330 g) were randomly diveded four groups as Glutamine (n=6), Arginine (n=6), Glutamine + Arginine (n=6) and Control (n=6). Additially 4 rats were kept for testing the dose of LPS *(Sigma-Aldrich* O55:B5) to prove the dose has an inflammating effect, but low enough for 24h survival. At the second try, 3 mg/kg/day LPS dose is decided to be used and 2 rats remaining were added to Glutamine and Arginine groups.

All of the animals had free access to standard laboratory rat chow and tap water in a room at constant temperature (24°C) and under a 12-h light–dark cycle, 3 or 4 rats were set into each cages.

Experimental groups recieved;

Glutamine (n=7): 500 mg/kg/day glutamine,

Arginine (n=7): 500 mg/kg/day arginine,

Glutamine+Arginine (n=6): 250 mg/kg/day glutamine and 250 mg/kg/day arginine containing suspension by orogastric route (9) additionally to standart food and water access. 5 grams of amino acids containing 2 ml water suspension was given according to the weights of animals consecutive 10 days at 4 pm everyday. Control group (n=6) recived water by orogastric route according to their weight for generating the same stress with experimental groups. At 5. day of the experiment one rat died after enteral implementation. No reason was found and it has been decided to be a heart attackt. At 10. day of the study after enteral implementation (at 5 pm) all of the rats were infected with 3 mg/kg LPS by interperional way. Next day till 9 am food access was inhibited and 24 hours after LPS administration all of the rats were sacrified, blood samples were collected in the meantime of decapitation. Their livers were processed in 0.9% NaCl solution and send to histopthologic analysis in 10 % formaldehyde.

Blood and tissue analysis

CRP, TNF-α, IL-1β, IL-6 levels are studied as sepsis and inflammation parameters. Latex agglunitation test is used for CRP analysis. It is a qualitatively analysis method and used for inflammatory indication but has a mild specifity. TNF-α, IL-1β, IL-6 are analysed with ELISA (Beckman Coulter Elisa Plate Reader) method (Boster Immunoleader Kits) AST, ALT parameters were studied (Cobas Integra 400 Clinical Chemistry Autoanalyzer) as organ damage and liver histopathology is evaluated.

Statisticall analysis

MINITAB 17 and SPSS 17 programmes are used for statisticall analysis $p<0.05$ is accepted as statistically significance.

Results

Plasma ALT, AST, IL-6 and TNF-α Levels

CRP levels are found between normal levels in all groups. IL-1β values are found 250 pg/ml in all groups

which is the maximum level that could be counted in studied concentrations. Thus, CRP and IL-1β levels were not able to be compared between groups.

Plasma ALT (U/L), AST (U/L), IL-6 (pg/ml) and TNF-α (pg/ml) levels are shown in Table 1. Plasma TNF-α levels are found 7.29 pg/ml in Glutamine + Arginine group which is significantlu lower than control group ($p<0.05$).

Histopathological analysis

All livers from all groups are analysed histopathologically. Abscesses, hepatocyte damage, kupffer cell proliferation and portal enflmammation are evaluated.

Presence of abscesses are statistically significant between groups ($p<0.05$). In Arginine group, no abscesses are found while in control group all of the liver samples had abscesses, 3 rats had grade I, 2 rats had grade II and 1 had grade III. In Glutamine and Glutamine + Arginine groups, none of the rats had grade I abscesses. In glutamine + Arginine group only 1 rat had grade III abscesses. The resting 5 rats in this group did not have any abscesses. The images of control and Arginine group are shown in Figure 1 and 2.

According to hepatocyte damage, difference between groups is statistically significant ($p<0.05$). In control group, all of the rats presented grade II hapatocyte damage while all of rats in Arginine group presented grade I. 5 rats in Glutamine group and 4 rats Glutamine + Arginine group presented no damage In both groups 1 rat presented grade II hepatoscyte damage.

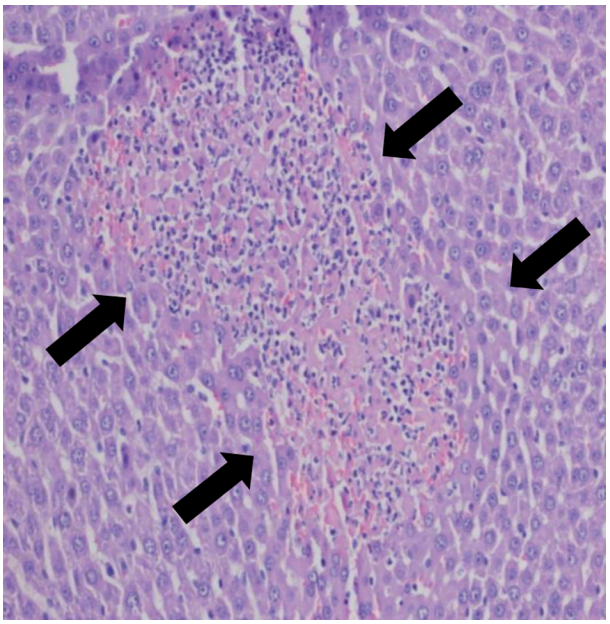

Figure 1. Liver abscesses in control group H-E x 200

Table 1. Plasma ALT, AST, IL-6 and TNF-α Levels

Parameter	Groups	Minimum	Median	Maximum	IQR	p
ALT (U/L)	Arginine	46	63	826	272	
	Glutamine	47	118	7000	3483	0.224
	Glutamine+Arginine	38	45	931	231	
	Control	46	139	848	412	
AST (U/L)	Arginine	121	181	1025	353	
	Glutamine	126	245	7000	4498	0.895
	Glutamine+Arginine	131	162	1079	258	
	Control	106	271	1047	482	
IL-6 (pg/ml)	Arginine	10.89	18.89	30,61	9,66	
	Glutamine	14.24	19.41	30.66	7.49	0.889
	Glutamine+Arginine	13.02	18.43	24.71	8.54	
	Control	10.28	17.23	24.61	9.37	
TNF(pg/ml)	Arginine	6.76	10.15	19.92	9.87	
	Glutamine	4.80	16.57	25.38	12.53	0.034*
	Glutamine+Arginine	4.80	7.29	13.84	4.82	
	Control	11.03	15.15	18.71	3.88	

IQR; Inter Quartile Range.
*Kruskal-Wallis Test *p<0.05*

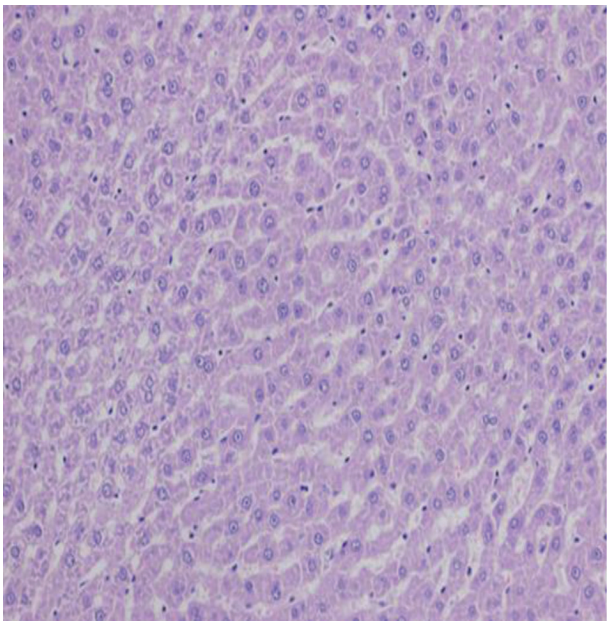

Figure 2. No abscesses are found in Arginine group H-E x 200

Kupffer cell proliferation is significantly different between groups ($p<0.05$). All of the rats developed grade II kupffer cell proliferation in control group. All of the rats in Glutamine, Arginine and Glutamine + Arginine groups developed grade I proliferation.

Portal inflammation is also significantly different between groups ($p<0.05$). In control group all of tha rats had grade I portal inflammation while in Arginine group none of the rats had portal inflammation. 1 rat in Glutamine group and 2 rats in Glutamine + Arginine group had portal inflammation.

Discussion

It is known that morbirty and mortality rates due to sepsis are still unacceptibly high (10). While the effects of glutamine and arginine amino acis on prevention from sepsis are known, together use of these amino acids still need further research (9).

In a meta-analysis evaluating four randomised controlled trials with glutamine has shown that high prevalance of infectous complications are caused by intestinal permeability, colonic apoptosis and decreased secretuar immunglobulin A function, abnormal lymphosyte and macrophage functions and glutamine supplementation might reverese all these effects (11).

Parenteral glutamine administration for four weeks to rats with protein energy malnutrition decreased inflammation and prevented from organ damage due to sepsis (12). In another study on rats evaluating the effects of glutamine on intestinal functions has shown that decreasing amino acid concentration due to endotoxemia is related with enterocyte dysfunction, thus oral or enteral glutamine supplementation could not be as effective as parenteral glutamine administration due to impaired intestinal capasity (13).

Differently, a study investigating intra-abdominal sepsis model in rats has shown that enteral glutamine supplementation decreases bacterial translocation and increases mucosal thickness and antioxidant levels (14). Another study in rats also determined that enteral glutamine does not prevent from bacterial translocation but significatntly decreases bacterial spread (15).

A study investigating the effects of glutamine on cytokine formation has shown that lipopolysaccharide injection to blood samples of healthy subjects and glutamine administration to endotoxemic blood samples might have positive effects on mortality and morbidity (16).

Differently, a randomised controlled double blinded study conducted on 40 centers on 1443 critically ill patients and showed that glutamine supplementation did nor improve clinical outcomes and increased MODS caused mortality (17). Similarly, in our study plasma IL-6 and TNF-α levels are not significanly different between the groups but the highest levels are found in glutamine supplemented group.

In a study investigating arginine safety on critically ill and healthy volunteers with 13 septic patients 5 with septic shock and 7 healthy volunteers it has been found that all of the participants had similar total body arginine production (3).

Arginine supplementation to rats with severe inflammation is found to be able to regulate IL-6, TNF-α and nitric oxide prouction (18) and oral arginine supplementation at a dose of 2 % of total energy for 7 days significantly decreases bacterial translocation due to intestinal obstruction at blood, mesenteric lymph node, liver, spleen and lungs (19).

A study investigating short term (8 hours) response to arginine supplementation in septic shock patients has shown that at the beginning there is an increased protein breakdown and arginine supplementation both decreased protein breakdown and protein synthesis. As a result, in sepsis arginine supplementation at a dose that might increase plasma arginine levels four fold might regulate arginine and nitric oxide levels and might decrease whole body protein breakdown without any adverse effect (20). The effects of glutamine and arginine supplementation on inflmammatory cytokines and intestinal mucosa in lipopolysaccharide induced endotoxemia in rats are found that glutamine did not increase plasma glutanmine and arginine levels while arginine supplementation increased plasma arginine levels. Combination of glutammine and arginine could not increase plasma arginine levels as much as arginine supplementation alone. Oral supllementation of glutamine, arginine or their combination all increased villus lenght in jejenum and ileum and they had sinergetic effects on intestinal integrity and benefical on inflammatory cytokines (9). Similarly we have found that the group supplemented with combination of glutamine and arginine amino acids had significant lower levels of TNF-α in comparison with control group. But there is a conflict that if the decrease in TNF-α levels was due to the use of combination of these amino acids or due to the dose we have used for these amino acids. In combination group, rats recieved 250 mg/kg/day per amino acid with a total 500 mg/kg/day while glutamine and arginine groups recieved 500 mg/kg/day glutamine or arginine. Present literatüre emphasis that a dose of 500 mg/kg/day glutamine supplementation is benefical and a range between 142-428 mg/kg/day is apropriate according toy he pathology while maximum tolerated dose is 501 mg/kg/day when there is not any complication. As a result of supply of 250 mg/kg/day glutamine to rats in comparison with 500 mg/kg/day, it is found that 500 mg/kg/day is more effective for decreasing the spread of E.coli (15, 21, 22). 2000 mg/kg/day supplementation of glutamine with the aim of determining potential harm or toxicity has shown that even that dose did not cause any side effects (23). These results support that the reason of highest IL-6 and TNF-α levels in glutamine group was not caused by glutamine toxicity. It is also known that arginine is well tolorated by adult rats at 214-570 mg/kg/day doses (24). In a study investigating the effects of arginine doses on inflammation it has been shown that a dose of 5000 mg/kg/day arginine supplementation has a significant antiinflammatory effect on peritoneal macrophages without any side effects (25).

A study investigating the effects of oral supplementation of glutamine and arginine on inflammatory cytokines and intestinal mucosa has similar results with our study. 300 mg/kg/day glutamine and arginine supplementation and a combination of these two amino acids 150 mg/kg/day per amino acid with a total of 300 mg/kg/day amino acid supplementation are compared and it has been decided that the combination of these amino acids are more benefical on intestinal mucosa and inflammatory cytokines (9).

Using half doses of glutamine and arginine in combination group might be misleading as both of the amino acids have been discussed to have potential harm in the present literature. In order to clarify, the study should be planned as all off the groups are suppleented with the same dosed active ingredient.

CRP is an acute phase protein discovered in 1930. It adheres to Gram-positive and Gram-negative bacteria and by this way it helps leukocytes phagocyte them. CRP is produced in liver and peaks in 24-38 hours after inflammation. While it is used in the diagnosis of infectious and non-infectious inflammation, it is not an optimal indicator for sepsis diagnosis (26). In our study we found CRP levels in all groups between normal levels (negative according to qualitative method). This might be due to that 24 hours period is not long enough for the expected increase. Additionally, latex agglutination text is a qualitative method which is an easy and low cost method but mildly sensitive and spesific (27). Thus, the test method also might be the reason for unexpected CRP levels.

We could not compare IL-1β levels between groups because we have found 250 pg/ml in all gropus which was the maximum level that could be analysed in those concentrations. In order to determine significant results, the samples should have been analysed at more diluted concentrations.

Multiple organ dysfunction sydrome (MODS) which mostly occurs with liver and kidney damage is

the leading cause for mortality and morbidity in sepsis. Liver dysfunction has a primary and progressive effect on MODS because liver is not only the source of inflammatory mediators but also the target organ effected by inflammatory mediators (28).

There was not a significant difference between AST and ALT levels in all groups while both parameters were at highest levels in contol group and the lowest in Glutamine+Arginine group. ALT is one of the most important liver enzymes and mostly more spesific to liver damage and more active in comparison with AST. ALT levels might increase due to liver damage but also it might increase because of hemolysis (29).

Histopathological analysis of liver has shown that apsis, hepatocyte damage, kuppfer cell proliferation and portal inflammation were significantly different between supplemented groups and control group. Similarly, 7 days of 500 mg/kg/day paranteral glutamine supplementation has found to decrease liver damage in rats with ischemia perfusion (30). In another model ischemia perfusion, one dose of 750 mg/kg intraperitoneal glutamine administration, it is found that glutamine protects liver from tissue damage (31) and enteral gutamine supplemetation for 7 days at 1000 mg/kg/day dose is found to be preventive from oxidative damage of liver (32). On a study evaluating the effects of arginine on iscehmia perfusion has found that oral arginine supplementation for 5 days with a dose of 500 mg/kg/day has preventive effcets on liver tissue (33).

According to the results of liver pathology, use of glutamine and arginine even alone or together has significant preventive effects on liver damage in comparison with control group. Glutamine, arginine and glutamine and arginine use in critically ill patients need guidelines for disease and pathology specified doses and supplementation route.

Conclusion

Studies with glutamine and arginine supplementation in sepsis have paradoxical results both in human and animal experiments. We could not find significant difference in groups according to their AST, ALT and IL-6 levels while serum TNF-α levels were significantly higher in control group in comparison with Glutamine + Arginine group ($p<0.05$). Histopathological analysis of liver has shown that abscesses, hepatocyte damage, kupffer cell proliferation and portal enflmammation are significantly higher in control group than Glutamine, Arginine and Glutamine + Arginine groups ($p<0.05$).

Limitations

Firstly, we have found CRP levels between normal levels in all samples. This might be due to the quantitative analysis method we have used.

Secondly, in Glutamine + Arginine group, both amino acids are used at a dose of 250 mg/kg/day with a total 500 mg/kg/day amino acid supplementation, while Glutamine and Arginine groups recieved 500 mg/kg/day Glutamine or Arginine alone. This might cause a conflict about the significant TNF-α decrease in Glutamine + Arginine group that if the effect was due to the dose or usage of combination.

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