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ORIGINAL ARTICLE

Investigation of Protective Effect of L-Carnitine on L-Asparaginase-Induced Acute Pancreatic Injury in Male Balb/c Mice

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Abstract
Introduction The present analysis deals with the biochemical and histopathological effects of L-carnitine in mice with L-asparaginase (ASNase)-induced experimental acute pancreatic injury (API).
Methods A total of 32 male Balb/c mice were divided into four groups as follows. Group I (control) was injected with single saline via the intraperitoneal route. Group II received 500 mg/kg of L-carnitine daily with the injected volume of 62.5–75 µl for 25–30 g mice using a Hamilton microinjector applied for 5 days. Group III received a single 10,000 IU *Escherichia coli* ASNase/kg body weight dose of ASNase at a dose of 500 mg/kg. Group IV received 500 mg/kg of L-carnitine daily and a single dose of 500 mg/kg of ASNase and were decapitated on the fifth day following the injection. Blood and pancreatic tissue samples were obtained for evaluation of histopathological structure and levels of malondialdehyde (MDA), reduced glutathione (GSH), total sialic acid (TSA), glucose, amylase and triglyceride.
Results In group III, compared to group IV and group I it was determined that levels of GSH and amylase were significantly lower while levels of MDA, TSA, glucose and triglyceride were higher. Levels of GSH, MDA, TSA, glucose, triglyceride and amylase, especially in group IV, approached that of group I. As a result, L-carnitine for ASNase-induced API mice may be protective against pancreatic tissue degeneration and oxidative stress or lipid peroxidation.

Keywords Mice · Pancreas · L-Carnitine · L-Asparaginase · Sialic acid · Lipid peroxidation

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Introduction

Asparaginase is used in treatment of lymphoid malignancies such as acute lymphocytic leukemia and T cell lymphomas. L-asparaginase is an integral component of induction chemotherapy for adult and pediatric patients with acute lymphoblastic leukemia (ALL). The levels of L-asparagine, which is an essential amino acid for protein synthesis, are decreased converting to L-aspartic acid by this drug. In this way, development of leukemic cells is prevented and also hepatic production of various plasma proteins related to hemostasis is reduced [1, 2].

ASNase has side effects such as central nervous system dysfunction, hypersensitivity reactions, coagulation abnormalities, hyperglycemia, liver dysfunction, and pancreatitis

[3]. ASNase-induced pancreatic injury needs detailed explanations in terms of mechanism. Pathologically, the pancreas is hemorrhagic, suggesting the role of coagulation abnormalities. Nakamura et al. [4] reported simultaneous occurrence of pancreatitis in patients during treatment with ASNase. The effect of ASNase on lipid metabolism during chemotherapy induction of childhood lymphoblastic leukemia was studied and changes in serum lipid were found [5]. Pancreatitis can be lethal, and chemotherapy may need to be terminated in some cases. Previous reports claim that ASNase is the most likely cause of such pancreatitis at a rate about 2–16 % of clinical cases [6, 7]. During ASNase toxicity, symptoms such as anorexia, vomiting, nausea, abdominal and/or back pain are observed. Some patients develop symptoms of diabetes owing to degeneration in islet cells, and following reduction in reactions of insulin synthesis, which is important to levels of blood glucose. It is reported that severity of hyperglycemia can be decreased if ASNase is received after prednisone [8].

L-Carnitine is an essential cofactor in mitochondrial respiration and plays an important role in the transfer of long-chain fatty acids from cytosol to mitochondria. By combination with carnitine to form acylcarnitine, acyl groups could be transferred from cytosolic coenzyme A on the outer surface of the mitochondrion membrane, then to the inner surface by exchanging with free carnitine using an antiport mechanism. The acyl groups are then transferred from carnitine to coenzyme A within the mitochondrion [9]. Carnitine is also associated with buffering of excess acyl-Co A, which is potentially toxic to the cells, and it was reported that L-carnitine has a protective effect on lipid peroxidation by reducing the formation of hydrogen peroxide [10, 11]. L-carnitine was also able to improve antioxidant status in rats and showed free radical scavenging activity as well [11, 12].

Sialic acids are a family of nine-carbon neuraminic acid derivatives that are found in terminal residues of oligosaccharide chains of mucins, glycoproteins and glycolipids of cell membranes [13, 14]. High levels of reactive oxygen species, which indicates the oxidative stress, are known to induce lipid peroxidation that can be further shown by increased MDA levels [15, 16]. The levels of TSA may be used as an indicator of the disruption of cell membranes due to lipid peroxidation along with increased MDA concentration [13, 15, 16]. Reduced GSH including the thiol group prevents oxidative damage to red blood cells in organisms. In addition, GSH and MDA concentrations in the blood have been used as markers of oxidative stress [17].

The protective effects of L-carnitine against pancreatic cell degeneration are not well documented in biochemical and histopathological terms. Therefore, this study was designed to evaluate the effect of L-carnitine in mice with experimental API, in biochemical and pathological terms.

Materials and Methods

Animals

The ethical approval of the study was confirmed by Kafkas University Animal Care and Use Committee (Registration Number: KAU HADYEK 2012/82). All procedures were conducted in accordance with the "Guide for Care and Use of Laboratory Animals", published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. Thirty-two male Balb/c mice (12–14-weeks old, 25–30 g bw) were obtained from Kafkas University, Animal Research Farm. The mice were acclimatized one week prior to the planned experiments and fed with standard pelleted diet ad libitum. The mice were housed in stainless-steel cages (26 × 15 × 50 cm in size) in a controlled environment with temperature at 22 ± 2 °C, humidity 50 ± 5 % and a 12-h light/dark cycle.

Experimental Design

The mice were divided into four groups each consisting of eight animals as follows. Group I (control) was injected with single saline (0.02 ml/g body weight, 0.9 % NaCl; Baxter, Mediflex, Eczacibasi, Istanbul, Turkey) via the intraperitoneal (i.p.) route. Group II received 500 mg/kg of L-carnitine daily (Carnitene®, injectable solution in 5 ml sterile ampoule containing 200 mg/ml L-carnitine, 0.014 ml 10 % hydrochloric acid and injectable water, Santa Farma Ilac Sanayii A.S., Istanbul, Turkey) with the injected volume of 62.5–75 µl for 25–30 g mice using a Hamilton microinjector applied for 5 days. Mice in group III received a single i.p. 10,000 IU/kg *Escherichia coli* ASNase (lyophilized powder, Sigma Aldrich Chemical Company). Mice in group IV received 500 mg/kg of L-carnitine daily (starting 5 days before ASNase injection via the i.p. route) and a single dose of ASNase and were decapitated on the fifth day following injection, respectively.

Biochemical Analysis

Blood samples were collected from heart via cardiac puncture under light ether anesthesia for GSH, MDA, TSA, amylase, glucose and triglyceride measurements before putting the animals to sleep at determined time intervals. Level of GSH in blood was measured from whole blood. For MDA, TSA, amylase, glucose and triglyceride measurements, all tubes were centrifuged (at 1,200×*g* at 4 °C) for 10 min to obtain the serum. The serum samples were kept at −25 °C until they were analyzed. Serum amylase, glucose and triglyceride concentrations were determined using a commercially available kit and using an auto analyzer (Olympus Chemistry Analyzer AU 640, Type:

Table 1 Levels of GSH, MDA, TSA, glucose, triglyceride and amylase in ASNase and L-carnitine treated mice

Parameters	Groups			
	Control	L-carnitine	ASNase	ASNase plus L-carnitine
GSH (mg/L)	29.62 ± 2.06[b]	32.11 ± 2.14[a]	17.20 ± 1.93[d]	25.50 ± 2.79[c]
MDA (μmol/L)	5.50 ± 0.53[c]	4.37 ± 0.51[d]	10.25 ± 1.16[a]	6.62 ± 0.74[b]
TSA (mg/L)	48.50 ± 3.74[c]	39.44 ± 3.12[d]	70.66 ± 3.64[a]	61.66 ± 3.48[b]
Glucose (mg/dl)	74.43 ± 4.43[b]	68.43 ± 4.12[a]	94.33 ± 3.08[a]	76.00 ± 3.65[b]
Triglyceride (mg/dl)	109.89 ± 17.51[b]	88.57 ± 5.38[c]	140.50 ± 26.73[a]	117.00 ± 5.89[b]
Amylase (U/L)	1,314.9 ± 36.9[a]	1,333.0 ± 105.3[a]	1,108.1 ± 104.7[c]	1,205.9 ± 110.5[b]

Data are presented as mean ± standard deviation. Values with different letters within the same row indicates significant differences ($P < 0.001$)

640-03, Japan). TSA was measured calorimetrically using a spectrophotometer (UV-1201, Shimadzu, Japan) by the method of Sydow [18] in that all bound sialic acid was separated by acid per-chloride in plasma and tissue homogenates, and then the supernatants were boiled by Erlich reagent, and finally the product was read at 525 nm. GSH concentration was assayed by the method of Beutler et al. [19] based on the spectrophotometric measurement of sulphydril (-SH) groups forming complexes with 5, 5'- (2-dithiobis nitrobenzoic acid) which give rise to colored products that were read at 412 nm. Measurement of MDA concentrations was carried out by the method of Yoshoiko et al. [20] based on the reaction between thiobarbituric acid and MDA produced as an end product of lipid peroxidation. The end products were read at 535 nm.

Histopathological Examinations

For histopathological examinations, pancreas samples excised from mice were fixed in 10 % neutral buffered formalin, and were dehydrated and embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin-eosin (H&E). The histological sections were examined with an Olympus BX51 optical microscope (Olympus Optical Co., Osaka, Japan) and photographed.

Pancreas injury scores were evaluated semi-quantitatively. The stained sections were graded as follows: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked; 5 = severe. The severity of the changes was determined by the degeneration, necrosis and degree of vacuolization per cell and the amount of tissue involved.

Statistical Analysis

For the statistical analysis, differences between the groups were tested by analysis of variance (ANOVA) and Tukey test using SPSS for Windows version 20.0. Data were presented as mean ± standard deviation (X ± SD), and P values less than 0.001 were considered significant.

Results

Biochemical Findings

Levels of GSH, MDA, TSA, glucose, triglyceride and amylase in control, L-carnitine, ASNase-induced API and ASNase-induced API plus L-carnitine groups are shown in Table 1. According to these data, in the ASNase-induced API group compared to ASNase-induced API plus L-carnitine and control groups, it was determined that levels of GSH and amylase were significantly decreased ($P < 0.001$) while levels of MDA, TSA, glucose and triglyceride were significantly increased ($P < 0.001$). It was determined that levels of GSH, MDA, TSA, glucose, triglyceride and amylase, especially in the ASNase-induced API plus L-carnitine treated group, approached that of the control group.

Histopathological Findings

The pancreas samples that were excised from the control group and only L-carnitine applied group were either normal (Fig. 1a, b) or showed minimal degenerative changes in the cytoplasm of some acinar cells. Histopathological changes were, however, found in the pancreas of mice receiving daily doses of ASNase.

Table 2 compares histopathological grading scores for the pancreas of mice receiving only L-carnitine or ASNase and L-carnitine plus ASNase. In general, pathological changes were significant in the pancreas from the ASNase group. The toxic effects of the ASNase were mainly seen in the exocrine pancreas. Vacuolar degeneration of acinar cells was detected in only the ASNase applied group (Fig. 1c). Cytoplasmic vacuolization consisted of small, clear, discrete vacuoles within the pancreatic acinar cells. Occasionally a single large vacuole was noted. The vacuoles exhibited a perinuclear preponderance. The islets of Langerhans were morphologically normal (Fig. 1d), dispersed throughout the affected acinar tissue and without reduction in their number. In some cases coagulative necrosis of the pancreatic acinar cells either interspersed through parenchyma or diffuse necrosis

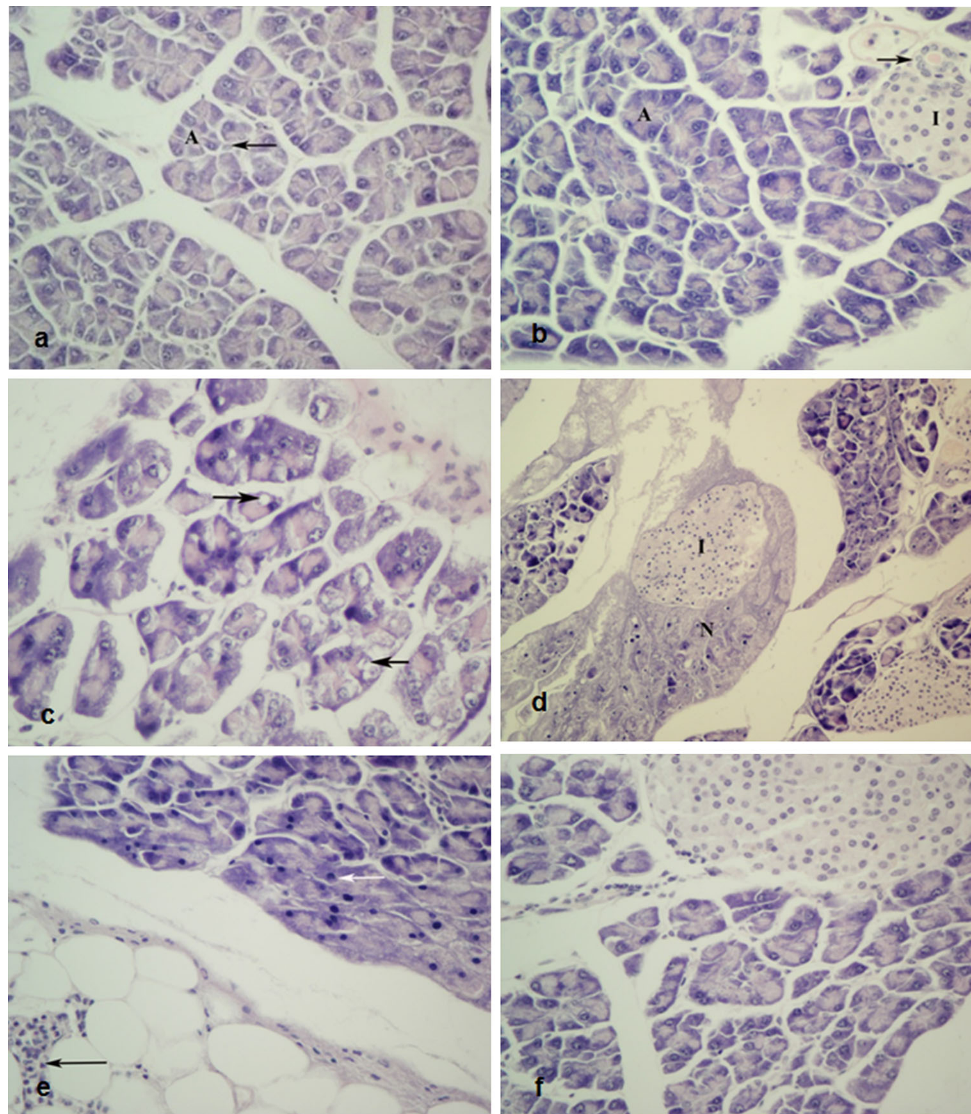

Fig. 1 Light micrographs of pancreatic tissue from mice. **a** In the control group, normal findings are observed. Pancreatic acini (*A*); centroacinar cell (*arrow*) (*HE,* ×400). **b** In the L-carnitine group no pathological changes are seen. Pancreatic acini (*A*); islet of Langerhans (*I*), intercalated duct (*arrow*) (*HE*, ×400). **c** In the ASNase group, small vacuoles are seen in the cytoplasm of acinar cells (*arrows*) (HE, ×400). **d** Diffuse necrosis of the pancreatic acinar cells (N). Note islets of Langerhans (*I*) with relatively preserved architecture. ASNase group (*HE*, ×200). **e** The nuclei of some acinar cells that are undergoing necrosis showing pyknosis (*white arrow*). Undamaged peripancreatic adipose tissue is infiltrated by mixed inflammatory cells (*black arrow*). ASNase group (*HE*, ×400). **f** In the ASNase plus L-carnitine group, normal findings are seen (HE, ×400)

Table 2 Histopathological grading scores for pancreas injury of mice receiving asparaginase

Groups	Pancreas
Control	0.125 ± 0.03
L-carnitine	0.25 ± 0.05
ASNase	1.02 ± 0.16
ASNase plus L-carnitine	0.25 ± 0.07

Data are presented as mean ± standard deviation. Values with different letters within the same row indicates significant differences ($P > 0.001$)

were present (Fig. 1d). The nuclei of some acinar cells that were undergoing degeneration were pyknotic (Fig. 1e) or karyorrhectic. Peripancreatic adipose tissue was infiltrated by mixed inflammatory cells (Fig. 1e). In the L-carnitine plus ASNase group, no vacuolization in the acinar cells was detected and the histology appeared to be normal (Fig. 1f).

Discussion

Despite of the great efforts in studies for development of new agents that counteract the toxic or metabolic changes

to be concluded with pancreatitis, investigation of effective treatments that would suppress pancreatitis is important in terms of minimization of drug side effects. In the present study, administration of L-carnitine was tested in mice with API. The results show that toxic alterations detected by biochemical and histopathological means were ameliorated by i.p. injection of L-carnitine. In biochemical analysis, in the ASNase-treated mice compared to the control group, it was determined that the levels of TSA, MDA, glucose and triglyceride significantly increased, and levels of GSH with amylase also decreased. L-carnitine administration was determined to normalize these values.

The ASNase has been applied to cure ALL since the 1960s [21, 22]. It was reported that use of ASNase with vincristine and prednisone importantly improved rate of remission in induction therapy of childhood [8]. However, it is possible that pancreatitis is caused by ASNase as related to the source of the enzyme and its half-life ($t_{1/2}$) or administrated dose [22–24]. In a study, it was stated that pancreatitis post *E. coli* sourced ASNase occurred at a mean of 12 d after ASNase administration in humans [7]. Clinical signs related to pancreatitis in the dog in this case were expressed as about 2 d. It has been recorded that shortened time frame could be explained by a difference in plasma half-lives and median $t_{1/2}$ since *E. coli* ASNase is 14 h in the dog and 26 h in humans [24, 25]. In the clinical treatment of ALL, *E. coli* ASNase was used by Suzuki et al. [22] in the first induction therapy at a dose of 200 IU/kg or 6,000 IU/m^2 given in a series of six or nine intramuscular or intravenous injections in order to deplete the circulating asparagine. The half life of ASNase is reported to be shorter in rats than humans (5–6 vs 26 h) [22, 26]. In terms of reduced side effects in ASNase, treatment research on the protective precautions is needed. In the present study, L-carnitine injection was used as a different method when administrated 10,000 IU/kg dose of ASNase with a single i.p. injection in order to induce API by the agency of ASNase in mice.

When we examined pancreatic tissue with a light microscope, there were no obvious pronounced histopathological changes in the control and L-carnitine alone applied groups; however, we found some pathological changes in the pancreas of mice receiving daily doses of ASNase. Under microscopy, vacuolar degeneration and necrosis of acinar cells were detected in only the ASNase applied group. The formation of vacuolar degeneration during experimental API was also reported in other studies [22, 27]. The levels of serum amylase and lipase have been used as markers of pancreatitis because their raised levels in patients are highly suggestive of development of pancreatitis. Our results showed that L-carnitine administration significantly increased serum amylase compared to the ASNase applied group. Decreasing serum amylase and increasing glucose concentrations in mice receiving ASNase may reflect the secretions of carbohydrate digestive enzymes by pancreatic cells. During exocytosis of pancreatic digestive enzymes from zymogen granules in the ASNase applied group, blockage is possible. In experimental models of API, blockage of secretion has been suggested as the initiating event, leading to the accumulation of zymogen granules within acinar cells [22]. This pathway is continued by excess of digestive enzymes and lysosomal hydrolases within vacuoles and, eventually, over enzyme activity within pancreatic cells and acute intracellular damage [28, 29].

The levels of GSH, which is known to be an important mediator in detoxifying lipid hydroperoxides and MDA levels for lipid peroxidation, may be an indicator in terms of oxidative stress [12, 16]. This study revealed that the role of oxygen-derived free radicals in the pathogenesis of ASNase-induced API in mice was possible. Our findings related to MDA of mice with API are consistent with high level of MDA findings belonging to an applied study on rats with experimental API [30]. L-carnitine itself has a protective effect as an antioxidant agent due to its direct antioxidant effect. The antioxidant effect of L-carnitine was impressively utilized to prevent or reduce the toxic effect of several chemicals. For example, cisplatin-induced nephrotoxicity, where oxidative stress and lipid peroxidation are thought to play a major role in the pathophysiology of nephrotoxicity, administration of L-carnitine in Sprague–Dawley rats normalized kidney function. In addition, L-carnitine attenuated the increased MDA and reduced GSH levels [31]. Methamphetamine neurotoxicity due to peroxynitrite radicals was recorded to be prevented by L-carnitine administration [32]. L-carnitine was also reported to reduce ethanol-induced lesions of gastric mucosa and protect against lipid peroxidation as well as standardize GSH levels in gastric mucosa of rats [33]. In this study, in mice with ASNase-induced API, decrease in blood GSH level and increase MDA level were determined. By L-carnitine injection, these levels were concluded to be able to be normalized as the control group.

The levels of SA have been declared that may be used as a marker to specify tissue degeneration in several disease conditions such as rheumatoid arthritis, cancer, bacterial infections and liver dysfunctions [13, 34]. The exact mechanisms of increases of SA levels in metabolic disorders are unclear. A conceivable explanation might be that tissue sialic acid originates from the terminal oligosaccharide chain of some acute phase proteins such as ceruloplasmin, fibrinogen and haptoglobin, which are known to increase in these conditions [35]. It has been suggested that SA levels can be useful in the diagnosis and evaluation of the tumor location in patients with primary pancreatic cancers because the location of tumors in the pancreas

affects SA levels [36]. Sialylation of acute phase proteins has been claimed to reflect the intensity of inflammatory response during API and could be used as a prognostic parameter for disease severity [37]. In addition, it has been declared that serum level of TSA in the alcoholics depends on the concentration of the most sialylated glycoproteins [38]. In an earlier study, the level of liver α_1-acid glycoprotein which SA contains was reported as higher in poisoned rats administering lead compared to the control [39]. Based on our findings, levels of high serum TSA in mice with ASNase-induced API is possible to be explained by the production of highly sialylated acute phase proteins related to inflammation. The levels of lower serum TSA in L-carnitine treated compared to ASNase applied mice may be explained with the protective effect of L-carnitine on inflammation. Moreover, changing serum TSA levels in all groups are thought to depend on the concentration of the most sialylated glycoproteins.

As a result, administration of ASNase by itself has been found to have the ability to directly constitute histological damage in the pancreas and L-carnitine to have antioxidative and preventive effects on tissue degeneration in ASNase-induced API. Our findings regarding the protective effects of L-carnitine administration are based on experiments on mice. Additionally, L-carnitine may be important to patients with pancreatitis and for quality increase in treatment of ALL.

Conflict of interest None.

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