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ORIGINAL PAPER

ANALYSIS OF PLASMA BIOMARKERS AS AN INDICATOR FOR EARLY DIAGNOSIS OF ACUTE MESENTERIC ISCHEMIA IN RAT

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Abstract

Objectives. The aim of the present study was to investigate the correlation between routine plasma markers after acute mesenteric ischemia, in order to determine an algorithm which could provide early diagnosis of the disease.

Methods. Fourteen male Sprague Dawley rats were allocated into two groups: Group I (n=7) – Partial ischemia group; Group II (n=7) – Diffuse ischemia group. Blood samples were taken three times for each animal: preoperatively, as well as at 24 hours and 48 hours after the ischemia. Changes within each test, as well as the correlations between them, were investigated.

Results. There were almost no changes in the level of BUN and CRP values after ischemia. Similarly, ALT and C3 levels were only minimally increased ($p>0.05$). However, AST, LDH and fibrinogen levels significantly increased in the first 24 hours after the mesenteric ischemia, then reduced minimally 48 hours later ($p<0.05$).

Conclusion. Partial and diffuse acute mesenteric ischemia caused a decrease in blood glucose, increases in AST, LDH and fibrinogen and no significant changes in ALT, CRP, BUN and C3 levels in the rats.

Résumé

Analyse des biomarqueurs plasmatiques en tant qu'indicateur d'un diagnostic precoce de l'ischemie mesenterique chez le rat

But. L'objectif de l'étude présente est d'analyser la corrélation entre les marqueurs plasmatiques de routine après l'ischémie mésentérique aigue afin d'établir un algorithme capable de fournir un diagnostic précoce de la maladie.

Méthodes. Quatorze rats mâles Sprague Dawley ont été répartis en deux groupes: I-er Groupe (n=7) groupe d'ischémie partielle; IIe Groupe (n=7) groupe d'ischémie diffuse. Des échantillons de sang étaient prélevés trois fois pour chaque animal; avant l'opération ainsi qu'après 24 et 48 heures suite à l'ischémie. Les changements dans le cadre du test, ainsi que les corrélations entre eux sont aussi investigués.

Résultats. Il n'y a presque aucun changement dans les niveaux de valeurs d'urée et protéine C-réactive (CRP) après l'ischémie. Similairement, les niveaux de ALT et C3 ont faiblement augmenté ($p>0,05$). Bien que les niveaux de AST, LDH et du fibrinogène aient augmenté de manière significative dans les 24 premières heures suite à l'ischémie, ils étaient réduits au minimum 48 heures après ($p<0,05$).

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Keywords: mesenteric ischemia, plasma marker, biochemical.

Conclusion. L'ischémie mésentérique aigue partielle et diffuse provoque une baisse du glucose dans le sang, une augmentation de AST, LDH et du fibrinogène et pas de changements significatifs dans les niveaux de ALT, CRP, urée et C3 chez les rats.

Mots-clés: ischémie mésentérique, marqueur plasmatique, biochimique.

Introduction

Acute mesenteric ischemia (AMI) is an uncommon medical condition in young and healthy individuals. It only occurs in patients who have underlying vascular or hematologic disease, such as arthritis, cardiac embolism, hypercoagulability from protein C and S deficiency, factor V Leiden mutation, pregnancy and oral contraceptive use in this age group[1-3]. However, it is more common in elderly patients, particularly after cardiac operations, and patients with cardiac rhythm disorders. The signs and symptoms of AMI are generally non-specific and can vary within a wide range, from quiet presentation to severe acute abdomen presentation. Therefore, physical examination is rarely effective in diagnosis[4]. Laboratory tests are also not beneficial to make diagnosis as, unfortunately, there is no plasma marker at this time that is an accurate indicator of AMI[5]. Thus, advanced radiological investigations, including CT scans and angiography, remain the only options for the diagnosis. Nonetheless, even radiological imaging techniques for early diagnosis of the disease can be challenging. Early diagnosis and timely treatment, however, are key factors to decrease the mortality and morbidity in AMI[6].

Routine laboratory tests are not beneficial for early diagnosis since the results are mostly non-specific[7]. Moreover, investigations into laboratory testing for AMI are predominantly focused on new and plasma markers that are not routinely used, such as D-lactate and α-Glutathione S-transferase[8-9]. However, none of these new experimental biomarkers is currently employed in clinical practice. We believe that changes in the routine plasma biomarkers and correlations between them have not been sufficiently studied. Therefore, we hypothesize that changes in blood chemistry must happen when acute mesenteric ischemia occurs and the reflection of these changes should be detected through routine laboratory analysis.

This study aims to investigate the correlations between the routine plasma biomarkers during the early and late phases of acute mesenteric ischemia, and to attempt to determine a reliable pattern to indicate the diagnosis. This study was approved by Baskent University Ethical Committee for Experimental Research on Animals (project no: DA13/02).

Materials and methods

Fourteen male Sprague Dawley rats, weighing between 200-250 g, were purchased from Baskent University experimental animals breeding center, in Ankara, Turkey. The animals were housed in the Baskent University Experimental Research Center (Ankara, Turkey) in accordance with the guidelines established by the Turkish Government. Food and water were not restricted from the animals. The rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). The abdomen was cleansed with povidone iodine and a laparotomy was performed via a midline incision. The two study groups were designed as follows:

Group I (n=7): Partial ischemia group; draw 1 ml blood, laparotomy, only colonic ischemia is achieved by ligating left colon and transverse colon vessels, draw 1 ml blood first and second postoperative days, sacrification.

Group II (n=7): Diffuse ischemia group; draw 1 ml blood, laparotomy, diffuse ischemia is achieved by ligating colonic and 1/3 distal jejunal segment vessels, draw 1 ml blood first and second postoperative days, sacrification.

Surgical Procedure

The abdomen was opened via a midline incision. An operating microscope (Carl Zeiss OPMI 9-FC, Germany) was used to visualize the bowel vessels. Direct manipulation of the bowel was minimized, to avoid mechanical injury to the bowel. A wet cotton swab was used when necessary to handle the bowel. Partial ischemia was achieved by ligating the middle colic and sigmoid arteries of the colon (Figure 1).

In this way, the transverse colon and the descending colon became ischemic (Group I). Diffuse intestinal ischemia was achieved by ligating to 1/3 distal jejunal segment's arteries in addition to partial

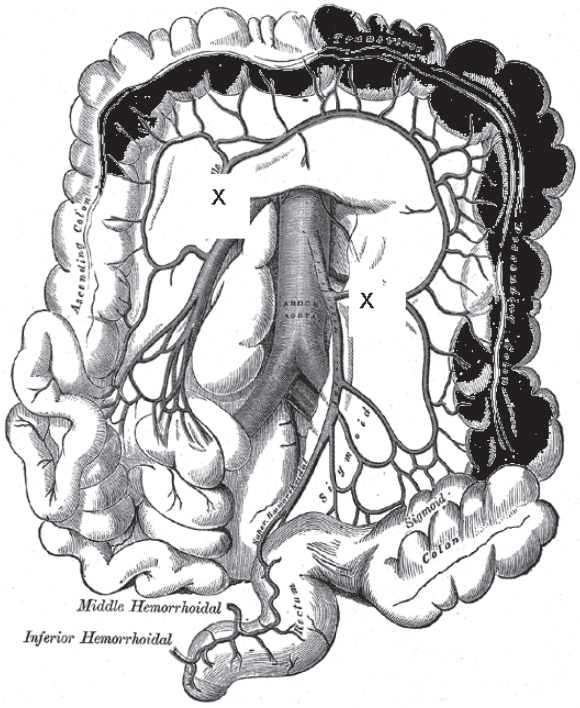


Figure 1. Partial mesenteric ischemia model.

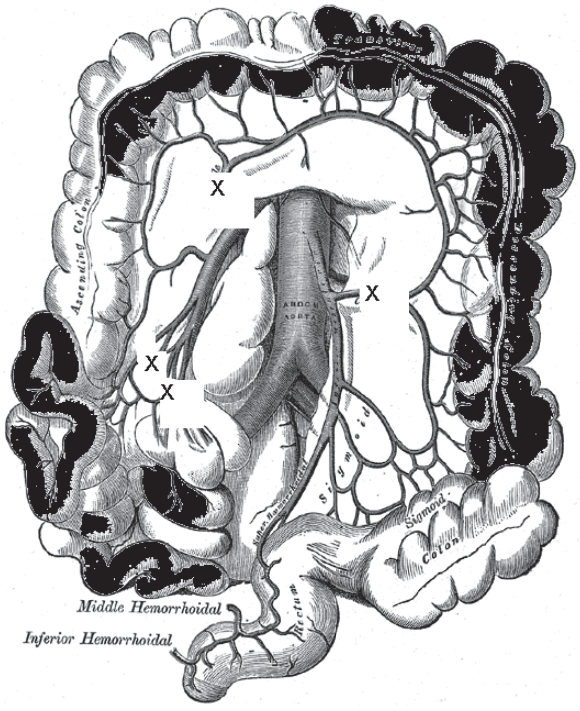


Figure 2. Diffuse mesenteric ischemia model.

group vessels (Figure 2). In this way, the transverse colon, the descending colon and the distal jejunum became ischemic.

Postoperative care: After closing the abdomen, 10 ml of saline was given subcutaneously for fluid replacement and this was repeated daily. An analgesic was given intramuscularly for pain treatment. Analgesic treatment was repeated once a day until the end of the experiment. Postoperatively, the rats were kept on a warm blanket and under a heat lamp for the first night. They usually recovered from anesthesia within 1 hour of the operation. The rats were given regular water and food *ad libitum*.

Taking blood samples and the study parameters

According to our study design, 1 ml blood samples were taken immediately before the operation and then once a day, postoperatively, until the animal's general condition deteriorated. Blood samples were obtained by cardiac puncture under light general anesthesia. The drawn blood was immediately poured into an empty biochemistry tube; then, 0.1 ml citrate was added and the tube was lightly shaken. After this, blood samples were centrifuged at 3,500 revolutions per minute (RPM) for 10 minutes, and the resulting plasma was removed and stored in a –70° C temperature-controlled device. The frozen prepared plasma samples were subsequently thawed and tested on a Siemens ADVİA 1800 Clinical Chemistry System. The results from each of the groups were then totaled and averaged to calculate a mean value of the parameters. The study parameters included, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C reactive protein (CRP), complement 3 (C3), fibrinogen, blood urea nitrogen (BUN), and glucose.

Statistical analysis

The results are expressed as mean ±SD. The differences between the groups were analyzed by the Kruskal-Wallis test, followed by the Dunn test. Probability values of $p<0.05$ were considered significant. The SPSS 17.0 (SPSS Ver. 17.0, Chicago IL, USA) program was used for analysis.

Results

All animals recovered from anesthesia and reached the first postoperative day. On the first postoperative day, the general conditions of the animals began to deteriorate; however, with fluid replacement and analgesic treatment animals, they recovered and reached the second postoperative day. Animals had an unwell appearance and the general conditions in both groups deteriorated further on the second postoperative day. On physical examination, their abdomens were distended. Therefore, it was decided to terminate the study on the second postoperative day. All animals were sacrificed immediately after blood was drawn by cardiac puncture and the study was terminated.

In this study, the animals' first blood samples were used as the control value for each test and they are represented by "0 hour" on the graphics. Each of the parameters was tested for all animals prior to the ischemia; thus, we obtained 7 normal values for each group, which meant 14 in total. These values were similar to each other and comparable with the reference values[10]. Therefore, it was not necessary to add another control group to the experiment. Each parameter's mean, standard deviation, as well as the minimum and maximum values of the preoperative blood samples are illustrated in Table I.

The results of partial mesenteric ischemia group (Group I)

First postoperative day: LDH, AST and fibrinogen levels were dramatically elevated to 2163±978 U/L, 336±106U/L and 413±57 mg/dl, respectively. The differences between the control values were statistically significant ($p<0.05$). ALT and BUN levels were slightly elevated to 74±17 U/L and 33±22mg/dL, respectively. However, the differences between the control values did not reach a level of statistical difference ($p>0.05$). The glucose level was reduced significantly to 141±23mg/dL ($p<0.05$). CRP and C3 levels were not changed at all. The results are summarized in Table II.

Second postoperative day: LDH level reduced to 1207±1078 U/L and AST levels reduced to 254±107 U/L; however, these levels were still significantly elevated when compared with the control values ($p<0.05$). Fibrinogen levels did not change and were the same as in the first postoperative day (412±216 mg/dL). ALT and BUN levels returned to normal values, glucose levels were similar to the first postoperative day level (145±70 mg/dL). There were no changes in the CRP and C3 levels.

The result of diffuse mesenteric ischemia group (Group II)

First postoperative day: Similarly to the partial ischemia group, LDH, AST and fibrinogen levels were dramatically elevated to 1678±800U/L, 287±103 U/L and 399±42mg/dL, respectively. The differences between the control values were also statistically significant ($p<0.05$). ALT and BUN levels, were again slightly elevated to 70±22 U/L and 32±19mg/dL, respectively. The differences between the control values did not reach a statistical difference ($p>0.05$). In the same way as the other group, glucose levels were reduced to 147±36mg/dL ($p<0.05$). There were no

Table I. Mean, standard deviations, minimum and maximum values of the parameters before the ischemia as a control in all animals

n=14	Mean	SD	Minimum	Maximum	Reference
Glucose (mg/dl)	251	27	209	302	106-208
LDH (U/L)	521	212	206	947	272-1965
ALT (U/L)	51	10	34	76	35-80
AST (U/L)	95	25	74	173	28-140
BUN (mg/dl)	22	2	17	28	10-22
Fibrinogen (mg/dl)	180	23	158	231	109-212
C3 (g/L)	0.38	0.03	0.3	0.4	0-05
CRP (mg/L)	0.2	0	0.2	0.2	0-1

Table II. The results of the partial mesenteric ischemia group (Group I).

n=7	Control	24 Hours	48 Hours	P
Glucose (mg/dl)	251±27	141±23	145±70	<0.05
LDH (U/L)	521±212	2163±978	1207±1078	<0.05
ALT (U/L)	51±10	79±11	56±32	NS
AST (U/L)	95±25	386±90	254±107	<0.05
BUN (mg/dl)	22±2	33±22	22±12	NS
Fibrinogen (mg/dl)	180±23	413±57	412±216	<0.05
C3 (g/L)	0.38±0.03	0.3±0	0.4±0	NS
CRP (mg/L)	0.2±0	0.01±0	0.2±0	NS

Table III. The results of the diffuse mesenteric ischemia group (Group II).

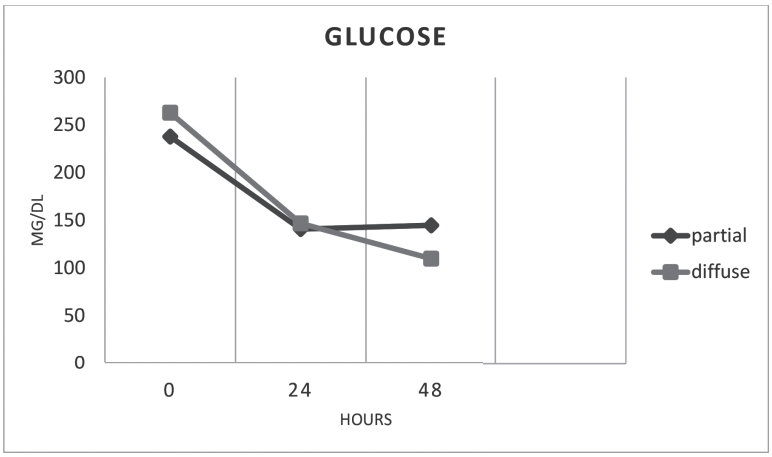
n=7	Control	24 Hours	48 Hours	P
Glucose (mg/dl)	251±27	147±36	122±58	<0.05
LDH (U/L)	521±212	1678±800	1293±797	<0.05
ALT (U/L)	51±10	70±22	48±17	NS
AST (U/L)	95±25	287±103	211±80	<0.05
BUN (mg/dl)	22±2	32±19	20±8	NS
Fibrinogen (mg/dl)	180±23	399±42	405±195	<0.05
C3 (g/L)	0.38±0.03	0.3±0	0.4±0	NS
CRP (mg/L)	0.2±0	0.2±0	0.2±0	NS

changes to the levels of CRP and C3 in this group. The results are summarized in Table III.

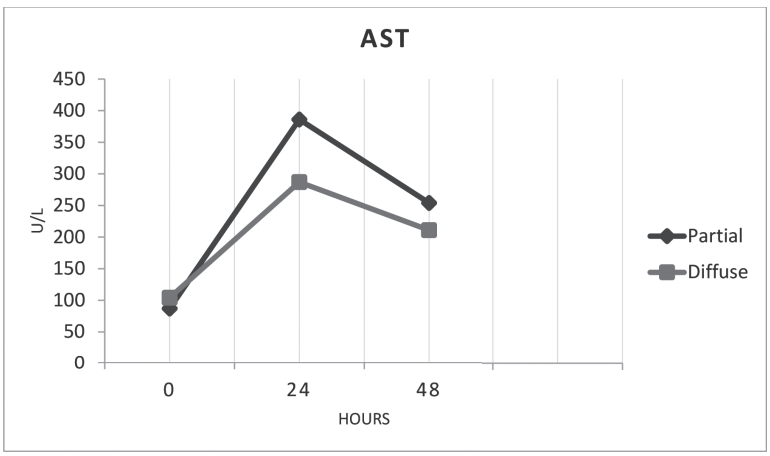
Second postoperative day: LDH and AST levels were slightly reduced to 1293±797 U/L and 211±80 U/L, respectively. However, these levels were again significantly high when compared with the control values ($p<0.05$). Fibrinogen levels continued to increase up to 405±195 mg/dl. In the same way as Group I, ALT and BUN levels returned to normal values. The glucose levels continued to reduce down to 122±58mg/dL. There were no noted changes in the CRP and C3 levels.

The results of the partial and diffuse mesenteric ischemia groups were comparable to each other. There were no statistical differences between the two groups in any of the parameters studied ($p>0.05$). This parallelism is shown in Graphics I, II, III, IV, V and VI for glucose, AST, LDH, BUN, fibrinogen and ALT, respectively.

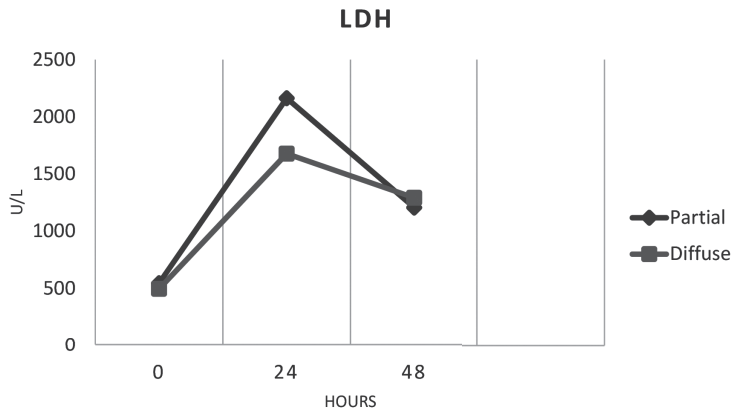
GRAPHIC 1. Alterations in plasma glucose over time ($p<0.05$)



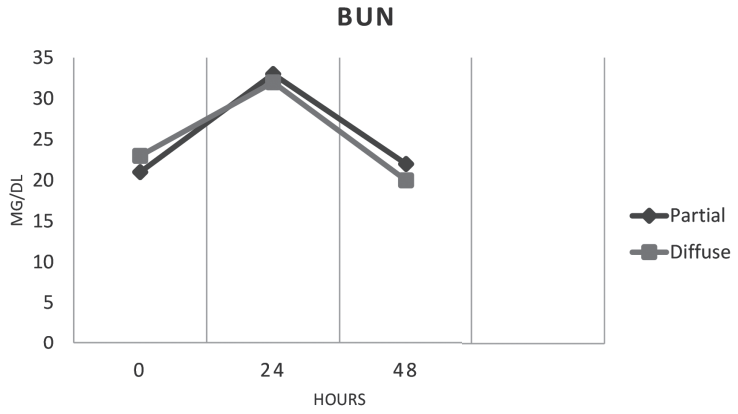
GRAPHIC 2. Alterations in plasma AST over time ($p<0.05$)



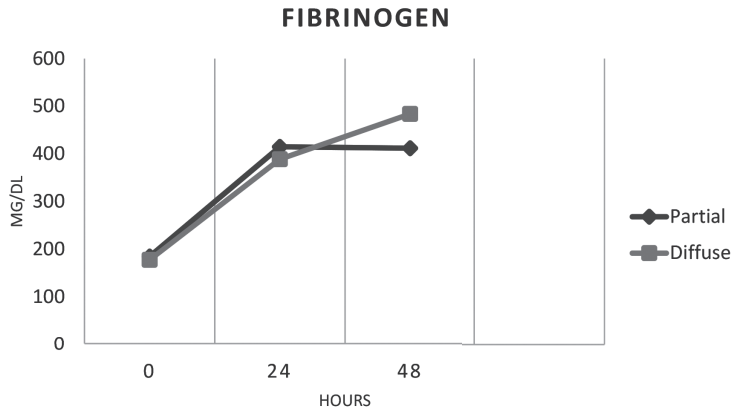
GRAPHIC 3. Alterations in plasma LDH over time ($p<0.05$)



GRAPHIC 4. Alterations in plasma BUN over time ($p>0.05$)



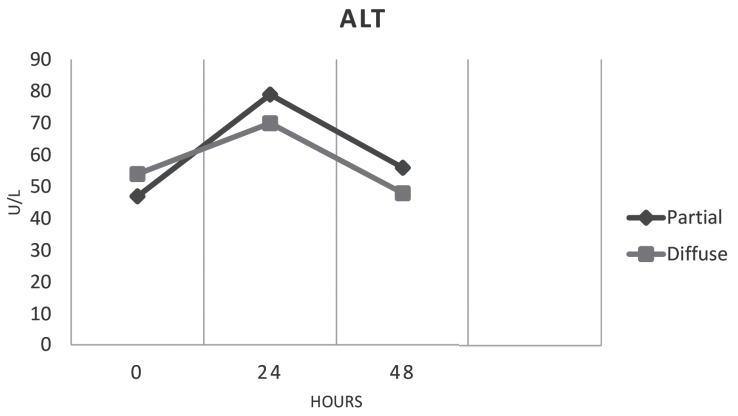
GRAPHIC 5. Alterations in plasma fibrinogen over time ($p<0.05$)



Discussion

Various candidate plasma markers have been studied such as D-dimer, ischemia-modified albumin, urinary and plasma fatty-acid proteins (FABPs), D-lactate, α-Glutathione S-transferase and L-lactate; however, there is currently no single plasma marker that could be regarded as sufficiently reliable to diagnose acute mesenteric ischemia[8-9, 11-14]. Therefore, none of these experimental plasma markers for acute

GRAPHIC 6. Alterations in plasma ALT over time (p>0.05)



mesenteric ischemia has yet entered in routine clinical practice[15,16].

In this study, we principally focused on the correlation of the routine plasma markers to determine a diagnostic pattern that could indicate the early diagnosis of acute mesenteric ischemia. For this purpose, we chose a limited number of plasma markers, since very limited blood samples can be obtained from rats. Moreover, only 1 ml of blood can be drawn each day from the rats and this amount is not sufficient to work with a wide range of plasma markers. Part of our selection criteria was the routinely used markers as well as the sensitivity of the test to the ischemic injury. The test should also be generally available and the results should be delivered within minutes, reaching the clinician shortly thereafter.

Acute mesenteric ischemia may alter the liver and kidney functions, and therefore ALT and AST were chosen as liver function tests and BUN as a kidney function test. Glucose is an important plasma marker during stress and inflammation. LDH are the enzymes which are involved in lactate metabolism and lactate is the end product of anaerobic glycolysis. Fibrinogen, complement 3 and CRP are routinely used acute phase reactants and therefore, they were chosen for the study.

Acute mesenteric ischemia caused a significant blood glucose decrease in the study. During stress and inflammation, hyperglycemia can be expected since glycogenolysis and gluconeogenesis are triggered by complex neuroendocrine reactions[17]. Both partial and diffuse acute mesenteric ischemia, however, reduced blood glucose levels in rats when compared with the control animals. The nutrition of the animals diminished remarkably, whereby the animals ceased eating foods and drinking water after acute mesenteric ischemia; this could be the reason for the blood glucose reduction in the rats.

Plasma lactate dehydrogenase levels of the animals dramatically increased in the first 24 hours after the acute ischemic insult. It is well known that lactate is a product of anaerobic glycolysis and is converted from pyruvate by LDH[11]. Thus, it could be assumed that LDH will increase when lactate levels increase. Ischemic gut during acute mesenteric ischemia is an anaerobic metabolism, which would result in increased lactate release from the gut into the portal system. LDH appeared to be an effective marker to indicate gut ischemia in our model. Even the animals with partial acute mesenteric ischemia demonstrated the same LDH increase as the diffuse ischemia group of animals.

Acute phase reactant levels of the animals changed diversely in our study. Fibrinogen levels of the animals increased significantly, although CRP and C3 levels were only minimally changed. CRP is a substance in the serum of the patients with acute inflammation that reacts with the C-polysaccharide in the liver. CRP increases when an inflammation occurs via microorganisms which contain phosphorylcholine in their membranes. This structure complex also stimulates the activation of complement 3 as well. One of the main biological activities of CRP is complement activation and opsonization[18-19]. Thus, CRP and C3 activities are closely related. Acute mesenteric ischemia injures the bowel and causes anaerobic metabolism. However, it does not cause bacterial overgrowth, at least in the early phase of the injury. This situation may explain our results in terms of CRP and C3.

Fibrinogen is one of the acute phase proteins that originate from the liver. It plays an important role in the acute phase response after tissue damage and inflammation. It has a long half-life and, therefore, it is normally not expected to increase in the early acute phase response in humans[19]. However, fibrinogen increased significantly and initially after

tissues were damaged in this study. This may be due to the fact that fibrinogen levels are more sensitive to bowel damage in rats. Fibrinogen levels of the diffuse mesenteric ischemia group in our study increased dramatically in the first 24 hours after ischemic insult, and continued to increase after 48 hours.

Transaminases are a group of enzymes that catalyze the interconversion of amino acids and oxoacids by transfer of amino groups. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are the two transaminases of greatest clinical significance. However, since the concentration of ALT is significantly less than AST in all cells except hepatic cytosol, ALT serum elevations are less common in non-hepatic disorders. Following myocardial infarction and some inflammations, AST activity is consistently increased. Similarly, AST levels were much higher than ALT levels in this study[20]. Ischemic injury of the gut caused significant AST elevation in the rats when compared with ALT.

BUN is a valuable screening test for the evaluation of renal disease. Acute mesenteric ischemia has systemic effects on the body and may cause renal dysfunction. Therefore, BUN levels were evaluated in this study to determine how and when the level of BUN changes after acute ischemic insult of the gut. Our results revealed that ischemic injury of the gut has no effect on BUN levels either in the early or late period of the insult. More than 99% of urea synthesis occurs in the liver and its primary source is dietary protein. It is decreased by low-protein diets, malnutrition or starvation. Animals were unable to eat food properly after ischemic insult in this study. Although subcutaneous fluid replacement was performed, starvation was inevitable. This could be the explanation for the BUN changes in our study.

One of the main purposes of the study was the investigation of plasma biomarkers' changes simultaneously to examine whether a reliable correlation or pattern may occur after ischemic insult to the gut, which may be an indicator for early diagnosis of the disease. When time is considered as a constant variable, a positive correlation between time and plasma levels of ALT, LDH and fibrinogen was found, while a negative correlation between time and plasma levels of glucose was also discovered. These positive correlations are shown in Graphics II, III, and V and the negative correlation is shown in Graphics I. ALT and LDH increased in the first 24 hours and reduced in 48 hours, revealed in very similar graphics (Graphics II and III). Glucose and fibrinogen results, however, were similar but in opposite directions, as illustrated in Graphics I and V.

According to our laboratory results, a plasma markers changing pattern was found after the initial period of the insult, which may assist in making earlier diagnosis. This pattern was reflected in: 1) plasma fibrinogen, AST and LDH levels increasing; 2) glucose levels reducing; and 3) BUN, ALT, C3, CRP levels unchanged or minimally changed in the first 24 hours after ischemic insult. This picture was very clearly defined in our study on rats. However, the question of whether the same laboratory pattern can be observed in humans remains unanswered. If this can be established, this could help clinicians to make mesenteric ischemia diagnoses much earlier.

In summary, several routine plasma marker alterations were studied together to determine a reliable pattern profile which could be an indicator for disease in this study. It was found that fibrinogen, AST and LDH all significantly increased, glucose significantly reduced and CRP, BUN, C3 and ALT were either unchanged or showed minimal changes in the initial period of the ischemic insult to the gut. These findings could be helpful for clinicians for earlier diagnosis when clinical symptoms of the disease exist.

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