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Can heat shock protein 32 be used for the early diagnosis of acute mesenteric ischemia?

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ABSTRACT

Objective: Acute mesenteric ischemia is a challenging and fatal disease. The aim of this study was to detect the heat shock protein 32 (HSP32) response in intestinal tissue and systemic blood to intestinal ischemia and ischemia/reperfusion to define a tool for the early diagnosis of acute mesenteric ischemia.

Material and Methods: Thirty female Wistar albino rats were equally divided into 3 groups. Group 1 rats underwent simple laparotomy and closure (control). In Group 2 rats, 1-hour intestinal ischemia followed by 5-hour reperfusion was performed, and Group 3 rats were subjected to 6-hour intestinal ischemia. The experiment was repeated with a 24-hour waiting period. At the end of the waiting period, blood was withdrawn from the tail veins of the rats and the rats were sacrificed via cardiac puncture. Re-laparotomy was subsequently performed and intestinal tissue and luminal samples were obtained for biochemical and pathological investigations. The HSP32 levels of intestinal tissues, luminal contents and blood levels were compared among the groups.

Results: At the end of the 24-hour waiting period, the median tissue HSP32 levels were 0.43 (0-6.6) ng/mL for Group 1, 9.51 (2.5-49.9) ng/mL for Group 2 and 43.13 (6.3-121.3) ng/mL for Group 3 (p=0.001). The median blood HSP32 levels were 0.11 (0.1-1.4) ng/mL for Group 1, 0.42 (0.1-0.7) ng/mL for Group 2, and 0.25 (0.1-1.2) ng/mL for Group 3 (p=0.047). The HSP levels in the luminal contents were undetectable.

Conclusion: Both ischemia and ischemia/reperfusion significantly raised intestinal tissue HSP32 levels in comparison with the control group. However, this change was not reflected in the circulating blood or luminal contents.

Keywords: Acute, heat shock protein 32, ischemia/reperfusion, mesenteric ischemia

INTRODUCTION

Acute mesenteric ischemia is a serious, fatal disease that occurs because of tissue damage in the superior mesenteric artery which is the major blood supply of the small intestines and the right colon. The disease is usually observed in the elderly and has mortality rates as high as 56-93% (1). The main reason for this high mortality is late diagnosis (2). Therefore, diagnosis of this condition at earlier stages is very important. The most important diagnostic tool for a timely diagnosis is mesenteric angiography, which is a complex and expensive procedure that requires specialized equipment and educated technicians. In addition, performing the procedure is not feasible for all suspicious cases. Therefore, there is a great necessity for a non-invasive, simple diagnostic method.

As the tissue perfusion in the intestines diminishes, the mucosal layer cells are the first to be affected. When this situation arises, the levels of heat shock proteins (HSPs) rise significantly (3). Heat shock proteins are small proteins that weigh less than 100 kDa and are produced in response to injury due to heat or toxic substances (3-5). The basic roles of HSPs in cells include cytoprotection, neurodegenerative disorders, signal transduction and cancer immunology (6). The levels of these proteins can be measured in the systemic circulation (3, 4). It has also been reported that reperfusion injury causes a significant increase in cellular HSP levels (7).

Mueller et al. (8) were able to detect HSPs in the urine samples of subjects suffering from renal ischemia. This report led us to investigate the detection of HSPs in the feces of patients suspected to be suffering from mesenteric ischemia, which might represent an inexpensive and rapid method for the early diagnosis of acute mesenteric ischemia. HSP32 (aka heme oxygenase-1), which is an antioxidant enzyme complex (4, 7, 9-11), appeared to be a good candidate for this purpose. However, although it has been clearly demonstrated that HSP32 levels rise rapidly following ischemia (7), there is a paucity of data regarding how HSP32 responds to reperfusion injury. Thus, we performed an experimental study to identify the HSP32 response to ischemic and reperfusion injury of the intestinal mucosa. However, we did not obtain the expected results from that study and because we concluded that the unsatisfying results were mainly due to the short waiting period (6 hours), we conducted a second study with a similar methodology in which we lengthened the waiting period from 6 hours to 24 hours. In this paper, the findings of these two experimental studies both of which were planned and performed to identify an inexpensive and practical method for the early diagnosis of acute mesenteric ischemia are summarized.

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MATERIAL AND METHODS

Both studies were performed after receiving the written approval of the Uludağ University Ethics Committee for Animal Studies. The first step of the study was completed using 21 out of 30 pathogen-free male Wistar-albino rats and the second step was completed using 27 out of 30 pathogen free male Wistar-albino rats weighing 200-210 grams. All rats were kept in the laboratory for at least 24 hours prior to the experiment to allow acclimatization and exclude physiological stress.

In both sets of experiments, 3 groups were created; a sham group (SG), an ischemia group (IG) and a reperfusion group (RG). The animals were equally divided among the groups.

Mesenteric ischemia was simulated using a superior mesenteric artery (SMA) occlusion model.

Surgical Procedure

Following laparotomy, the superior mesenteric artery (SMA) and vein (SMV) of the rats were identified. Then a 00-silk suture was passed through the origin of the superior mesenteric bundle (Figure 1).

In the *sham group*; the laparotomy was closed.

In the *ischemia group*; the suture was knotted and the laparotomy was closed.

In the *reperfusion group*; a temporary slip knot was added and the free ends of the suture were taken out through both sides of the abdominal walls. At this point, care was taken to insert the suture properly to prevent later tying. The abdomen was closed using a running 00 silk suture and the skin was stapled. The free suture ends were cut to leave two centimeters outside the abdominal walls. Adhesive tape was wrapped around the rat's torso to prevent it from eating the silk sutures. After the *planned waiting period*, the adhesive tape was cut using scissors and the free suture ends were pulled in opposite directions until the unknotting of the slip-knot was felt. The suture was pulled out of the body by pulling in only one direction (Figure 2).

The *planned waiting period* was 60 minutes in the first-step experiments and 24 hours in the second-step experiments.

A 5-milliliter saline solution was administered to the posterior neck of all the rats to restore fluid loss over the past six hours. After the operation, all animals were allowed to spontaneously recover from anesthesia. The animals were not fed until the next procedure. Additional saline solution was administered every 6 hours in the second-step experiments.

All of the animals were once again anesthetized after 60 minutes in the first-step experiments and after 24 hours in the second-step experiments. Following laparotomy, the superior mesenteric bundle was examined for pulsation, and a non-pulsating SMA was accepted as an exclusion criteria for the reperfusion group. Then, the small intestines were excised. Intraventricular blood samples were obtained and the animals were sacrificed using cardiac puncture.

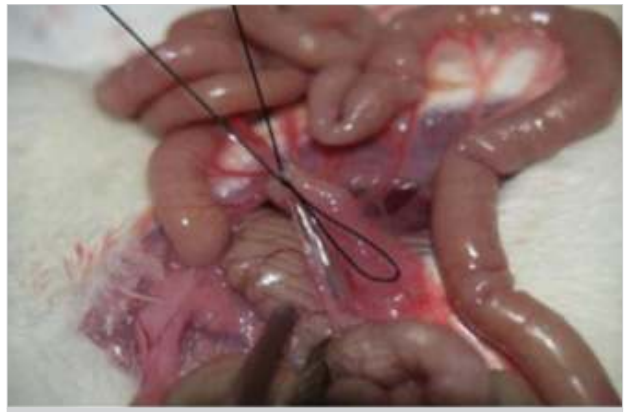

Figure 1. Superior mesenteric artery and vein of the rats were identified. Then a 00-silk suture was passed through the origin of the superior mesenteric bundle

Figure 2. The free suture ends were pulled in opposite direction until the unknotting of the slip knot was felt

The excised intestinal lumens were opened and stool samples were obtained. Intestinal tissues were equally divided into 5 pieces and the first and fourth pieces were submitted to pathological investigation whereas the other pieces were submitted to biochemical analysis for HSP.

Tissue, Stool and Blood Samples

The tissue and blood samples were prepared and the HSP measurements were performed by the same physician.

Tissue samples of intestine and luminal stools were flash frozen with liquid nitrogen and stored at -70°C until examined. Frozen materials were covered with liquid nitrogen in a mortar. After evaporation, the liquid nitrogen-frozen tissues were ground with a pestle until a fine powder was obtained. One tablet of protease inhibitor cocktail (PIC) (SigmaFAST™ Protease Inhibitor Tablet, Sigma-Aldrich, Inc., Saint Louis, Missouri, USA) was dissolved in 10 mL of extraction reagent, which is available in the kit, and 1 mL was used for each 0.5 cm^3 tissue sample. Tissue and stool suspensions homogenized with a pestle were cooled to 4°C and centrifuged at 21.000 g for 10 min. Subsequently, the supernatants were collected in labeled polypropylene tube and stored at -80°C until the analysis was performed.

Blood samples were collected in dry tubes and allowed to clot for 30 minutes. Subsequently the samples were centrifuged at 2700 g for 10 min and serum samples were transferred to labeled polypropylene tubes. Serum aliquots were stored at -80 °C until analysis was performed.

Heme oxygenase (HO) - 1 (aka HSP32) concentrations were quantified with commercially available ELISA kits specific for rat HO-1 (Assay designs, Stressgen, MI, USA) according to the manufacturer's protocol.

All measurements were performed in duplicate and the results were averaged. The physician performing the tests was blinded for groups during processing of all samples. The absorbance was measured in a microplate reader at 450 nm. By plotting a standard curve from known concentrations versus measured absorbance, the amount of HO-1 in the sample was estimated. The concentration of the HO-1 was expressed as ng per milliliter (ng/mL), according to the manufacturer's protocol. The minimum detectable concentration of HO-1 was 0.036 ng/mL.

Pathological Evaluation

The small intestines were examined by a blinded pathologist who examined the coded tissues without knowing which tissue belonged to which animal. The pathologist macroscopically classified the small intestines as normal, ischemic or with ischemia-reperfusion injury. The microscopic evaluation of intestinal mucosal injury was performed using hematoxylin-eosin staining and the scale developed by Chiu et al. (12) (Table 1).

Table 1. Microscopic criteria for grading intestinal mucosal injury (12)

Grade	Description
0	Normal mucosal villi
1	Slight elevation of epithelium from lamina propria at the apex of villi
2	Moderate elevation of epithelial layer from lamina propria
3	Massive epithelial lifting down the sides of villi
4	Denuded villi with lamina propria exposed and dilated capillaries
5	Disintegration of lamina propria; hemorrhage and ulceration

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences [SPSS® for Windows ver. 17.0 (SPSS, Chicago, Illinois, USA)]. Mann-Whitney U test was used to compare two groups and Wilcoxon test was used to compare more than two groups. A p value smaller than 0.05 was regarded as significant.

RESULTS

Nine rats were excluded during the first phase of the experiment; 2 rats in control, 3 rats in ischemia and 1 rat in reperfusion groups died due to hemorrhage, 1 rat in control group could not be awaken from anesthesia and 2 rats in reperfusion group were excluded due to non-pulsating SMA. The first phase of the experiment was completed with 7 rats in each group.

Three rats were excluded during the second phase of the experiment; 1 rat in control and 1 rat in ischemia groups died due to hemorrhage, and 1 rat in reperfusion group was excluded due to non-pulsating SMA. The second phase of the experiment was completed with 9 rats in each group.

The results of the first-step experiments are summarized in Table 2. The median weights of the 3 groups of rats were similar (p=0.68). Microscopic evaluation revealed that although there was no significant difference between the IG and RG in the means of the Chiu score (p=0.10), the median Chiu score of the SG was significantly lower than those of the other groups (p=0.0004).

The median tissue HSP32 levels were comparable between the IG and RG (p=0.48) whereas the SG tissue median HSP32 level was significantly lower (p=0.0017). However, HSP32 could not be detected in either blood or stool samples.

The results of the second-step experiments are summarized in Table 3. The median weights of the 3 groups of rats were similar (p=0.66). Microscopic evaluation revealed that while there was no significant difference between the IG and RG for the Chiu score means (p=0.12), the median Chiu score of the SG was significantly lower than the other groups (p=0.0003). The

Table 2. The results of the first-step experiments

	Sham Group (n=7)	Ischemia Group (n=7)	Reperfusion Group (n=7)	p
Macroscopic	Normal	Ischemic injury	Ischemia/reperfusion injury	–
Weight (gr)	200 (200–210)	200 (190–210)	200 (200–210)	0.68
Tissue HSP32 (ng/mL)	3.4 (0.5–4)	114.6 (55.2–125.9)	90.1 (52–116.3)	0.0011*
Blood HSP32 (ng/mL)	0.7 (0–3.6)	0.8 (0.4–1)	0.7 (0.1–2.6)	1

*Kruskal Wallis test. HSP: heat shock protein

Table 3. The results of the second-step experiments

	Sham Group (n=9)	Ischemia Group (n=9)	Reperfusion Group (n=9)	p
Macroscopic	Normal	Ischemic injury	Ischemia/reperfusion injury	–
Weight (gr)	270 (260-290)	260 (250-280)	280 (260-300)	0.66
Tissue HSP32 (ng/mL)	0.43 (0-6.6)	43.1 (6.3-121.3)	9.5 (2.5-49.9)	0.001*
Blood HSP32 (ng/mL)	0.11 (0.1-1.4)	0.25 (0.1-1.2)	0.42 (0.1-0.7)	0.064

*Kruskal Wallis test. HSP: heat shock protein

median tissue HSP32 levels were comparable between the IG and RG (p=0.093) whereas the SG tissue median HSP32 level was significantly lower (p=0.003).

The median blood HSP32 levels were different between groups (p=0.047). However, neither of the differences in two-group comparisons reached statistical significance. Therefore, a one-way ANOVA test with Bonferroni correction was performed that indicated a p value of 0.064. Unfortunately, HSP32 could not be detected in the stool samples.

DISCUSSION

In these two steps of experiments that tested the availability of HSP32 as a diagnostic tool in the early detection of acute mesenteric ischemia; it was observed that although the level of HSP32 significantly increases in both ischemic and reperfusion injury of the small intestine, stool HSP32 measurement is not a diagnostic tool candidate, nor can systemic blood HSP32 levels be used for this purpose.

The high levels of HSPs in both the IG and RG even after the 24-hour waiting period might indicate equivalent damage caused by ischemia and perfusion. Although the regeneration rate of the intestinal epithelium is fairly rapid (13), 24 hours might be insufficient.

Heat shock proteins are stored in small amounts in cells and produced when the cell is under stress (14). Protein synthesis is similar for many types of proteins and lasts approximately 6-7 hours (15). The high amounts of HSPs in the intestinal cells of study groups compared with the control group even after 24 hours reflect the facts that the hazardous effects of reperfusion remain even after 24 hours and that 24 hours is not enough for the cells to repair themselves. In human subjects, collateral vessels can develop in the event of chronic occlusion of the superior mesenteric artery (16). However, 24 hours is not enough for development of collateral blood vessels. We did not investigate whether collateral vessels occurred at the end of the 24-hour period, which is a limitation of our study.

In our study, by the end of the 24-hour waiting period, the HSPs were comparable in the IG and RG. These findings might indicate that the effects of ischemia and reperfusion on cells are similar, or that even 1-hour ischemia results in irreversible damage to intestinal cells. Sukhotnik et al. (17), performed 30-minute ischemia followed by 24-hour reperfusion and demonstrated that mucosal damage was significantly higher in the study group compared with the control group. However, their study did not have an ischemia group. We did not assess mucosal damage in our study but HSP levels reflect the magnitude of tissue damage because HSPs are bound to molecules that cause tissue damage or occur as a result of tissue damage. If the organism could control tissue damage, the HSP levels would not have been higher compared with the control group. As the HSP32 levels were similar in the IG and RG, we can conclude that the main problem was the reperfusion hazard rather than the 1-hour ischemia time. However, in our experimental model, it was impossible to determine if HSP32 levels were high because of ongoing hazard as a consequence of reperfusion, or if 24 hours was insufficient for tissue repair.

There was no significant rise in systemic blood HSP32 levels. Since HSPs are intracellular molecules, this observation was most likely related to the preservation of cell integrity even after a 24-hour ischemia period. Otherwise, we should have been able to detect HSP32 in the blood and stool samples of the study group rats. Another potential issue with the stool samples might be the use of inappropriate buffering tools. Intraluminal HSPs might be degraded by intestinal luminal content that could include proteinases. We were aware of such a risk; however, our investigations for an appropriate buffer for stool analyses did not reach a feasible method. In addition, we did have the chance to measure stool proteinase levels. Our model should be repeated with an appropriate buffer for stool to ensure that stool HSP32 measurement is not a feasible method for the early diagnosis of acute mesenteric ischemia.

CONCLUSION

Our study demonstrated that tissue HSP32 levels rise significantly 24 hours after the onset of either ischemia or ischemia/reperfusion in a similar manner; however, this rise was not reflected in the systemic blood or intestinal luminal content. Therefore, our attempts to determine a method for the early diagnosis of acute mesenteric ischemia via the measurement of stool HSP32 levels did not lead to introduction of a useful method. In addition, even the differences between the blood HSP32 levels of the IG and RG in comparison with the control group were only of borderline significance. Nevertheless, further studies should be conducted that compensate for the weaknesses in our study, particularly involving the use of the correct buffers for the stool sample analyses. Early detection of acute mesenteric ischemia is of vital importance as such detection currently appears to be the only way to reduce mortality rates associated with this devastating disease.

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