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Metabolites of Tryptophane and Phenylalanine as Markers of Small Bowel Ischemia-Reperfusion Injury

"The role of aromatic amino acids in intestinal ischemia and reperfusion"

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Abstract: Ischemic-reperfusion injury of the small intestine is an acute clinical condition with high mortality rate. This study describes the changes in levels of phenylalanine and tryptophan metabolites in intestinal homogenates and urine samples of Wistar male rats after 60 minutes of mesenteric ischemia and different reperfusion periods (1, 24, 30 hours) in comparison with a control group without the ischemia. The ischemic-reperfusion injury was quantified by the histopathological injury index. The levels of serotonin, epinephrine, and norepinephrine were determined in the intestinal homogenate and epinephrine, vanillylmandelic acid, and the 5-hydroxyindoleacetic acid was analyzed in urine using the HPLC method. The statistically significant increased level of serotonin, epinephrine and norepinephrine were detected in the intestinal samples after 24 hours of reperfusion ($p<0.01$); even the most elevated serotonin level was observed after one hour of reperfusion ($p<0.001$). A statistically significant decreased level of vanillylmandelic acid was observed after one hour of reperfusion, but it significantly increased after 24 hours ($p<0.05$) in urine. The elevated level of the 5-hydroxyindoleacetic acid after one hour and 24 hours after reperfusion ($p<0.05$) were determined in the urine. The most significant elevated epinephrine level was observed after 24 hours of reperfusion ($p<0.001$) in urine. Results showed a potential role of serotonin as an early biomarker (after one hour of reperfusion) of small intestinal damaged homogenate, while the best predictor of intestinal injury in urine was epinephrine, which was elevated after 24 hours.

Keywords: ischemic-reperfusion injury of small intestine; serotonin; catecholamines.

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1 Introduction

Intestinal ischemia and following reperfusion injury (IIRI) is a frequent complication of many medical conditions such as shock (hypovolemic, septic etc.) and surgical procedures. Patomechanism of IIRI is based on the deficiency of blood flow, which is manifested in the acute phase as significant changes in the motility and functional status [1].

The highest degree of the injury is not associated with the ischemia itself but with the following reperfusion period. This period of reperfusion is associated with the damage of the villi, an alteration of the intestine-blood barrier, bacterial and toxins translocation, bacteraemia and even multiple organ failure and death. A similar pathomechanism of the barrier function alteration and endotoxin, bacteria and metabolites translocations also plays an important role in other diseases such as inflammatory bowel diseases or small bowel transplantation [2].

Small bowel transplantation is still a great challenge in organ transplantation. It leads to multiple clinical complications with the graft function. The sufficient functional status of transplanted intestines is necessary for the success of the entire process. In the early stages after the transplantation, the functional status and degree of damage is the most important predictor of complications such as early rejection. Nonspecific signs and symptoms of the acute IIRI and higher frequency of surgical procedures involving the small intestine create the need to develop proper diagnostic methods [3,4].

Serotonin (5-hydroxytryptamine) is a biologically active substance that plays an important role in various aspects of cognition, behavior and physiology, including mood, sleep, but also energy balance, tissue regeneration, platelet coagulation, gastrointestinal functions, as well as immunity [5].

Serotonin is synthesized and stored in the enterochromaffin cells of the intestine. In the intestine, there are two sources of serotonin: neural and mucosal. Enteric neuronal serotonin is clearly required for gastrointestinal motility, while that from the mucose layer plays only a minor role. Under normal physiological conditions, most of the circulating serotonin is stored in platelets, and only a small fraction circulates in the free form [6].

Abnormalities of a serotonergic function contribute to the pathomechanism of functional, inflammatory and infectious diseases of the intestine. Intestine serotonin has been recently reported as a possible marker of ischemia - reperfusion injury in animal models [7,8].

Catecholamines (epinephrine, norepinephrine) are the major metabolites of phenylalanine. Catecholamines act as neurotransmitters when released into the synaptic space and as hormones when released into the bloodstream. The epinephrine increases catabolism of glycogen to glucose in the liver, thereby elevating the blood sugar level. At the same time, epinephrine begins the breakdown of lipids in fat cells and has a suppressive effect on the immune system. Catecholamines can affect virtually every cell within the gastrointestinal tract. The intestine also produces catecholamines, which modulate blood flow and thereby intestinal perfusion, proliferation, differentiation and apoptosis of various cells [9].

This work studies serotonin, epinephrine, norepinephrine in intestinal homogenate and epinephrine, vanillylmandelic acid and 5-hydroxyindoleacetic acid in urine as potential biomarkers of small intestinal injury after intestinal ischemia and following reperfusion injury during different time periods (one hour, 24hours, 30days).

2 Materials And Methods

2.1 Materials

Serotonin, examined catecholamines (epinephrine and norepinephrine), 5-hydroxyindoleacetic acid (5-HIAA), vanillylmandelic acid (VMA), formic acid, methanol and millipore filter (0.45 μm), saline solution, p-formaldehyde, 0.1 M perchloric acid were obtained from Sigma-Aldrich (St.Louis, MO, USA). Allure™ PFP Propyl (5μm particle diameter, 60Å pore size) was purchased from Alltech Associates (Deerfield,IL).

2.2 Animal Model of Ischemia and Experimental Design

Adult male Wistar rats (mean body weight 320 g, total n = 66) were housed in a cages under standard conditions with a temperature of 22 ± 2°C, and periodical variations of day light in 12 hour intervals. Small intestine ischemia was induced after the midline laparotomy of the abdominal cavity by the occlusion of *artheria mesenterica cranialis* for 60 minutes by an atraumatic clamp under general anaesthesia induced by intraperitoneal application of Ketamine 10mg/100g (Narketan inj. ad us. vet.) and Xylasine 1.5mg/100g (Xylariem inj. ad us.vet.). Normothermic conditions (37°C) during the surgical procedure were monitored by the microthermistor placed in the ear. The temperature was maintained using a homeothermic blanket. After the 60-minute ischemia period, targeted animals were subjected to periods of reperfusion for one hour, 24 hours and 30 days (groups IR1, IR24, IR30, each group n = 16). The control groups of animals (groups K1, K24, K30, each N=6) were prepared according to the same protocol without the occlusion of *a. mesenterica*.

This experiment was approved by the Committee for Ethics on Animal Experiments at the Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovakia, and the experimental protocol was approved by the State Veterinary and Food Administration of the Slovak Republic No. 2843/08-221a.

2.3 Sampling

2.3.1 Preparation of urine samples

After prescribed reperfusion periods, animals were euthanized by exsanguination under the general anesthesia. Urine samples were collected by bladder puncture after prescribed reperfusion periods. Samples of the urine were stored immediately after the collection in eppendorf tubes at -80°C.

2.3.2 Preparation of intestinal homogenate samples

Samples of small intestines (approximately 6 cm long taken from the ligament of Treitz) were immediately rinsed in the cold saline solution and small piece of small intestine (2 cm) was fixed in 4% p-formaldehyde for next histological examinations. Other 2 pieces (2 cm) of the small intestine were stored immediately in eppendorf tubes at -80°C.

2.3.3 Preparation of urine samples before HPLC analysis

The samples of urine were centrifuged 10 min at 4000 rpm and purified by using Millipore filter (0.45 μm). The supernate (200 μl) was added to the mobile phase (800 μl) before HPLC analysis and was injected into the HPLC system to detect epinephrine, 5-hydroxyindoleacetic acid and vanillylmandelic acid.

2.3.4 Preparation of intestinal homogenate samples before HPLC analysis

The piece of small intestine (2 cm) was homogenized in 500 ml of 0.1 M perchloric acid. Homogenates were centrifuged for 30 min at 10 000 rpm. The supernatant was filtered through a millipore filter (0.45μm). Filtrate (40 μl) was injected into the HPLC system to detect serotonin, epinephrine, and norepinephrine.

2.4 Methods

2.4.1 Histological examination

Histological slides were stained with Hematoxylin-Eosin (HE) for the visualization and quantification of histopathological damage by using an Olympus BX50 microscope equipped with the camera (Olympus, Tokyo, Japan) in blinded manner. The semi-quantitative Park/Chiu grading system was used for the quantification of histopathological damage. In this scoring system zero grade indicated no damage of the intestine wall and an eight grade represented massive damage with the ulceration of the intestine [10].

2.4.2 HPLC analysis

The liquid chromatograph Model SLC-10A VP (Shimadzu Co., Kyoto, Japan) with a solvent delivery system, a low-pressure gradient flow control valve, UV-VIS detector (220 and 280nm) and fluorescence detector were used for the analysis of metabolites of tryptophane (serotonin, and 5-hydroxyindoleacetic acid) and phenylalanine (epinephrine, norephinephrine, vanillylmandelic acid). The mobile phase composed of ammonium formate (20 mM, pH = 3) and methanol in ratio 90:20 was prepared prior to each quantification. The flow rate of the mobile phase was 1ml/min. The injection standard solution (2.5μg/mL) of epinephrine, and norepinephrine serotonin, 5-hydroxyindoleacetic acid and vanillylmandelic acid was injected every four samples to evaluate drift in retention time.

2.4.2.1 Quantification and validation

To obtain a good resolution and symmetric peak shapes of the analytes, the chromatographic conditions, such as the composition of mobile phase and total analysis were optimized through three trials. All compounds were determined by HPLC with fluorescence detection according to the Magera method et.al 2003 [11] with modifications. The retention times of the analytes were approximately 7.59 min for epinephrine, 4.3 min for norepinephrine, 30.23 min for serotonin, 27.33 min for 5-hydroxyindoleacetic acid, 7.33 min for vanillylmandelic acid respectively. The epinephrine, norepinephrine, serotonin, vanillylmandelic acid and 5-hydroxyindoleacetic acid amounts were calculated using the calibration curve by evaluating the peak intensity. The precision was expressed as the relative standard deviation of each calculated concentration. The intra-day precision was tested with three samples at three concentration levels. The inter-day assay precision was determined for the same sample on five different days. Intra- and inter-assay coefficients of the variation for epinephrine, norephinephrine were less than 3% and 4%, respectively, 1.6% and 3.2% for serotonin and less than 7% for vanillylmandelic acid and 5-hydroxyindoleacetic

acid. Detection limits for urinary epinephrine, and vanillylmandelic acid were 0.03 μg/mL and for epinephrine in the intestinal homogenate it was 0.09 μg/mL; and for the urinary 5-hydroxyindoleacetic acid and norephinephrine and serotonin in intestinal homogenates it was 0.06 μg/mL.

2.5 Statistic

Statistical analysis was performed using SPSS version 20.0 for Windows (IBM Corp. 2011. Published SPSS, version 20.0 ARMONK, NY: IBM Corp.). The values of each parameter were expressed as Mean ± SD. The quantitative results (results of HPLC methods) were compared between experimental and analog control groups and analyzed by a Student t-test. Semi-quantitative results (HII) were analyzed by a non-parametric Kruskal-Wallis test. Values of $p < 0.05$ were considered significant.

3 Result

3.1 Histopathological changes

In all adequate control groups, the detected jejunal mucosa was almost intact (C1 = 0.8±0.21; C24 = 0.4±0.12; C30 = 0.1±0.05, Figure 1). Statistically significant damage was observed after one hour of reperfusion compared to the equal control group (R1=5.2± 1.5; p<0.001 C1 *vs.* R1; Figure 1). After this time period, an improved jejunal histological architecture was observed in the remaining groups with only a slight but significant damage compared to the control after 24 hours (R24=1.4 ± 0.4; p<0.001 C24 *vs.* R24), as well as after 30 days (R30=0.54 ± 0.14; p<0.001 C30 *vs.* R30).

3.2 Changes of serotonin concentration in intestinal homogenates

The highest serotonin levels were detected in intestinal homogenates (Table 1) of subjects from the R1 group (0.63±0.05 μg/mL), which were approximately three times higher and statistically different than the concentration in C1 (0.17±0.01 μg/mL; *p<0.001*). Additionally, a significant (*p<0.01*) difference was observed between serotonin concentrations in R24 (0.38±0.023 μg/mL) and C24 (0.16±0.01 μg/mL). In samples from group R30, we observed higher serotonin concentrations (0.25±0.02 μg/mL) than in C30 (0.18±0.023 μg/mL) but the difference was nonsignificant.

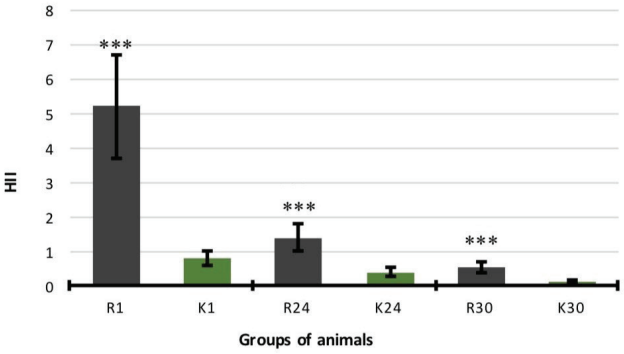

Figure 1: Histopathological injury index in different experimental groups *(IR1 vs. C1; IR24 vs. C24; IR30 vs. C30 p<0.001)*.

Table 1: Changes of serotonin, epinephrine, norepinephrine in intestine homogenates of experimental groups affected by different degree of intestinal injury *(** p<0.01; *** p<0.001)*.

Groups	Serotonin in homogenates (μg/mL)	Epinephrine in homogenates (μg/mL)	Norepinephrine in homogenates (μg/mL)
R1	0.63±0.05 ***	0.18±0.02	0.158±0.012
C1	0.17±0.01	0.19±0.018	0.16±0.01
R24	0.38±0.023 **	0.37±0.021 **	0.37±0.021 **
C24	0.16±0.01	0.19±0.016	0.19±0.013
R30	0.25±0.02	0.23±0.013	0.22±0.012
C30	0.18±0.01	0.2±0.02	0.18±0.015

3.3 Changes of epinephrine concentration in intestinal homogenates

Nonsignificant changes (*p=0.29*) of epinephrine concentration between groups R1 (0.18±0.02 μg/mL) and C1 (0.19±0.018 μg/mL) were observed in intestinal homogenates (Table 1). However, when comparing R24 (0.37±0.021 μg/mL) and C24 (0.19±0.016 μg/mL), a significant increase was observed (*p<0.01*). A non-significant increase was observed in the R30 group (0.23±0.013 μg/mL) compared to the control C30 (0.2±0.02 μg/mL).

Table 2: Changes of epinephrine, 5-hydroxyacetic acid and vanillylmandelic acid in urine of experimental groups affected by different degree of intestinal injury *(*p<0.05; *** p<0.001)*.

Groups	Epinephrine in urine (μg/mL)	VMA in urine (μg/mL)	5-HIAA in urine (μg/mL)
R1	0.69±0.02	0.27±0.01 *	0.19±0.01 *
C1	0.62±0.025	0.38±0.02	0.105±0.01
R24	0.85±0.021 ***	0.21±0.012 *	0.27±0.021 *
C24	0.27±0.01	0.12±0.08	0.12±0.009
R30	0.36±0.01	0.1±0.01	0.12±0.01
C30	0.31±0.01	0.11±0.011	0.11±0.012

3.4 Changes of norepinephrine concentration in intestinal homogenates

The concentration of norepinephrine in intestinal homogenates (Table 1) in both the R1 (0.158±0.012 μg/mL) and C1 (0.16±0.01 μg/mL) groups was almost equal. A significant increase was detected in R24 (0.37±0.021μg/mL) compared to C24 (0.19±0.013μg/mL) (*p<0.01)*. A non-statistically increase was observed in R30 (0.22±0.012 μg/mL) compared to C30 (0.18±0.015 μg/mL).

3.5 Changes of epinephrine concentration in urine

A non-significant increase in the epinephrine concentration of urine (Table 2) was observed in R1 (0.69±0.02 μg/mL) compared to C1 (0.62±0.025 μg/mL), as well as R30 (0.36±0.009 μg/mL) compared to C30 (0.31±0.01μg/mL). In addition, a significant increase (*p<0.001)* was detected between R24 (0.85±0.021μg/mL) and C24 (0.27±0.01 μg/mL).

3.6 Changes of vanillylmandelic acid concentration in urine

A significantly (*p<0.05*) lower concentration of urine vanillylmandelic acid (Table 2) was detected in R1 (0.27±0.01 μg/mL) compared to C1 (0.38±0.02 μg/mL). Conversely, the vanillylmandelic acid concentration was significantly (*p<0.05*) higher in R24 (0.21±0.012 g/mL) compared to C24 (0.12±0.008 μg/mL). Values of vanillylmandelic acid concentration in R30 (0.10±0.01 μg/mL) and C30 (0.11±0.011 μg/mL) were almost equal and did not differ statistically (*p=0.06)*.

3.7 Changes of 5-hydroxyindole acetic acid concentration in urine

A significant ($p<0.05$) increase of 5-hydroxyindole acetic acid concentration in R1 (0.19±0.01 μg/mL) compared to C1 (0.105±0.01μg/mL) was detected in urine (Table 2). A significant increase of 5-hydroxyindole acetic acid ($p<0.05$) was observed in the R24 group (0.27±0.021 μg/mL) compared to C24 (0.12±0.009 μg/mL). After comparing the experimental R30 to the C30 control, no significant difference was observed as values were almost the same.

4 Discussion

The functional state and eventually the damage of the small intestine should be evaluated during different surgical procedures or emergency conditions [12].

Ischemia of the small intestine develops due to the reduction or complete halt of oxygen and nutrient supplies, hence the first step in the restoration of reperfusion is in addition to the oxygen supply and the replenishment of high-energy matter, "reenergetisation." Reperfusion opens up the possibility for energy generation by oxidizing the reduced coenzymes ($NADH+H^+$ and $FADH_2$) in the mitochondria.

The IIRI model shows the highest injury localized during the first hour of reperfusion while gradually decreasing with longer time period. Our previous studies have shown that the residual damage can be detected even after 30 days of reperfusion [13]. The recent study demonstrated similar results. A high degree of injury was detected during the first hour of reperfusion with a rapid drop after 24 hours and 30 days of reperfusion. However, the histopathological damage was still slightly higher compared to the control group after 30 days of reperfusion. The acute ischemic-reperfusion model on the rodents allowed us to determine the acute changes in the intestine and its impact on various biochemical parameters and to monitor different acute conditions including the shock state, possible bowel transplantation in a few hours after performing the surgery. The longer period of 30 days of reperfusion with the remaining histopathological changes allowed us to monitor biochemical parameters for a longer time period after the acute condition. Results have shown the highest formation of both metabolites of phenylalanine (epinephrine and norepinephrine) in the intestinal homogenates in experimental samples after 24 hours of reperfusion. However, in contrast to norepinephrine, the amount of epinephrine was increased after 24 hours of reperfusion almost twice the amount, and after 30

days of reperfusion returned to a level comparable to the concentration of epinephrine in the control group. The concentration of norepinephrine significantly increased (but less than the level of epinephrine) after 24 hours of reperfusion while after 30 days of reperfusion there was only a slight increase.

The inflammation damaged tissue and initiated the defense mechanism in all tissues which led to the repair processes. These processes were influenced by the sympathetic adrenergic system, namely epinephrine released from the adrenal medulla. High physiological levels of adrenaline interfered with the uptake of glucose from the extracellular space. We assumed that there was a higher epinephrine excretion in urine after 24 hours of reperfusion. Any pathophysiological conditions associated with hypovolemia, resulted in a strong activation of the sympathetic nervous system and a release of norepinephrine. Under these conditions, the vasoregulatory role of epinephrine in the gastrointestinal tract was believed to be negligible. The work of [15] describes that both catecholamines significantly increased systemic oxygen delivery (epinephrine by 140% and norepinephrine by 60%) while epinephrine induced only minor changes in gastric hemoglobin saturation [15]. Catecholamines (metabolites of phenylalanine) play a very important role in the restoration of reperfusion, that stimulate glycogenolysis in the liver and skeletal muscle. Catecholamines increase in plasma free fatty acid mobilization and lactate formation and generally stimulate the metabolism [14].

The ultimate degradation product of catecholamines (in 35%) is vanillylmandelic acid, norepinephrine and epinephrine which is are excreted into the urine only in small quantities. Surprisingly, A non-significant decrease of vanillylmandelic acid concentration in urine was observed after one hour in which could be explained by the control group having shown a sharp elevation in vanillylmandelic acid. In urine samples, the levels of epinephrine were assessed. The elevation of epinephrine levels was observed (probably as a result of stress etc.) after the first hour of reperfusion, while after 24 hours of reperfusion, epinephrine was sharply increased, which correlated well with the results determined in the small intestinal homogenate.

Many studies have highlighted the correlation of enterochromaffin cells count and serotonin concentration from the intestine samples with the histopathological findings [13,14]. Large population of serotonin producing cells are presented in most segments of the gastrointestinal tract which are the main source of serotonin in human body [15]. In addition to a well documented physiological role, increasing evidence supports the concept of serotonin being directly involved in pathological mechanisms, as well as in the modulation of immune/inflammatory responses within the gut [17-19].

Serotonin captured by enterocytes is rapidly metabolized to various end products, of which 5-hydroxyindoleacetic acid is the most abundant and excreted in urine [5]. Results of the highest amount of serotonin degradation product was observed in urine after one hour of reperfusion which was significantly higher compared to the control group. However, the level of 5-hydroxyindoleacetic acid concentrations in urine after 24 hours of reperfusion was increased even more significantly. This result correlates with a higher level of serotonin in the experimental samples of intestinal homogenate after one and 24 hours of reperfusion. These data indicate the possibility of considering serotonin in small bowel mucosa as a parameter of small bowel grafts ischemic injury. Studies in diverse species have demonstrated a serotonin release from the gut after experimental intestinal ischemia and reperfusion. Serotonin was suggested as a good marker of ischemia reperfusion injury after allotransplantation of the small bowel in rats [19]. In their experience, the release of serotonin in the lumen bowel after reperfusion inversely correlated with the number and quality of serotonin cells [20].

5 Conclusion

Results suggest that the determination of serotonin levels, as well as epinephrine and norepinephrine in the homogenate of the small intestine (as type of non-invasive technique) may be a helpful marker to detect reperfusion injury following ischemia of the small intestine during the transplantation period or acute/chronic intestine changes during various diseases. The best predictor of intestinal injury in urine according to results was epinephrine. However larger randomized humans studies are needed.

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