ANNOTATION

dissertation work for the degree of Doctor of Philosophy

Topic: Effect of mesenteric blood flow revascularization on intestinal microflora translocation (experimental study)

Specialty: 6D110100 Medicine

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Relevance of the research topic

The relevance of the experimental study of acute mesenteric ischemia (AMI) is due to the fact that, despite the low percentage of overall morbidity (from 0.09 to 0.2% of all emergency calls to the emergency department), and the fact that AMI is a rare cause of abdominal pain syndrome , mortality in this pathology remains quite high (from 50 to 80%) [1,2]. OMI also acts as a leading syndrome of a number of diseases such as systemic thrombosis, embolism, is pathognomonic in the infringement of various hernias of the anterior abdominal wall, strangulation type of intestinal obstruction, during autotransplantation, systemic lesions of the main vessels, acute pancreatitis, etc. [3, 4,5] According to the literature of various periods, the lack of a consensus on the damaging systemic effects of short-term ischemia does not allow in clinical practice to unambiguously determine the time interval during which the exclusion of the small intestine from the blood circulation does not increase the risk of developing multiorgan complications. The study of the conditions for the development of bacterial translocation in acute disorders of the main blood flow in the small intestine and its role in the development of systemic inflammatory response syndrome is the subject of many studies, but this issue is still debatable [6]. The proportion of fatal complications in mesenteric ischemia of occlusive genesis, even after surgical reperfusion, is more than 70%. First of all, the development of complications in this pathology is associated with ischemic disorders of the morphostructure of the intestinal epithelium and reperfusion syndrome [7]. The severity of ischemic and reperfusion damage to the morphological structures of the small intestine wall is determined by the level of ischemia, the degree of development of collateral circulation, and the initial state of the macroorganism [8], but the role of reperfusion in the development of extremely severe complications of AMI cannot be denied. Also, in the issue of studying AMI, there is not enough information about the optimal timing of simulated ischemia, which would give a visualization of the process of microbial translocation with the development of systemic inflammatory response syndrome (SIRS), without causing the death of the animal and irreversible pathmorphological changes in the structure of the intestinal tissue. The possibility of using various radioactive methods as markers of bacterial translocation has not found wide application; they are expensive and time-consuming. Therefore, the search for new opportunities for experimental complex detection of bacterial translocation is undoubtedly relevant. Also, the experimental model of mesenteric ischemia is an ideal substrate for studying this phenomenon. More than 2000 studies were found published in the Pubmed database for the query “acute mesenteric ischemia AND reperfusion” and about 500 in the Web of Science Core collection database over the past 20 years, which certainly indicates the relevance of this issue in the scientific community. At the same time, only 1 publication was found, made by Kazakh authors, which indicates insufficient attention paid to the problem of mesenteric ischemia in our region, despite its undeniable relevance. The study of the phenomenon of microbial or bacterial translocation in the context of mesenteric circulation disorders has only 32 publications by Russian-speaking authors, including Kazakh ones, over the past 30 years, while no publications were found before 1990. In the PubMed database, the trend in the number of publications is constantly growing, which illustrates the increased interest of researchers in this problem.

Purpose of the study

To study the level of translocation of the intestinal microflora in acute disorders of the mesenteric circulation in the absence of mesenteric blood flow and after its restoration

Research objectives

1)To develop and implement an experimental model of mesenteric ischemia with the possibility of revascularization and a method for introducing fluorescent microorganisms into the gastrointestinal tract with an assessment of the effectiveness of their use in detecting the translocation of intestinal microflora;;

2) Assess the nature of morphological changes in internal organs during the development of experimental acute mesenteric ischemia and during the restoration of blood flow;3) To study the features of E.Coli GFPP translocation into internal organs using the MI model before and after revascularization;

4) To study the features of E.Coli GFPP translocation into internal organs using the MI model before and after revascularization;

5)On the model of mesenteric ischemia, to study the significance of biomarkers of intestinal translocation, ischemia and SIRS in the pre- and post-reperfusion period;

6) Determine the relationship between translocation biomarkers, SIRS, microbiological outcomes, under conditions of ischemia and reperfusion;

7) To develop a scheme reflecting the role of bacterial translocation in the pathogenesis of the development of experimental acute mesenteric ischemia and restoration of blood flow with methods for verifying this phenomenon in the body.

Scientific novelty

A new minimally invasive, apodactylally reproducible experimental model of acute mesenteric ischemia with the possibility of revascularization has been developed to study various pathogenetic mechanisms in violation of mesenteric blood flow and during its recovery (certificate IS No. 2573 dated August 7, 2018, Turgunov E.M., Amanova D.E., Ivachev P.A., Korobeinikov T.S. "Method of modeling mesenteric ischemia in rats (work of science)";

A new method of introducing fluorescent microorganisms has been developed (Certificate on the object of copyright No. 1857 dated February 20, 2019 (work of science) National Institute of Intellectual Property of the Republic of Kazakhstan; 2018 "Method of introducing a suspension of microorganisms into the gastrointestinal tract of rats");

For the first time, fluorescent strains of GFP E.Coli were used to detect translocation of intestinal microflora on the original model of mesenteric ischemia and reperfusion (certificate IS No. 3833 dated 04.06.19 on the object of copyright "Method for detecting translocation of intestinal microflora into internal organs in experimental intestinal obstruction" (work of science));

For the first time on an experimental model of mesenteric ischemia with reperfusion, a comprehensive morphological study was performed, an assessment of the dynamics of the levels of biomarkers of intestinal microflora translocation, systemic inflammatory response of the body and ischemia in their relationship (certificate No. organs in experimental intestinal obstruction" (work of science));

For the first time, a scheme has been developed that reflects the role of intestinal microflora translocation in the pathogenesis of the development of complications of experimental acute mesenteric ischemia and restoration of blood flow and methods for its verification (Copyright certificate No. 21732 dated November 16, 2021. reperfusion).

The main provisions for defense:

The model of mesenteric ischemia in rats makes it possible to study the features of translocation under conditions of disturbance and restoration of mesenteric blood flow, it is minimally invasive and easily reproducible. The most optimal model for studying TCM at various times of reperfusion is the duration of ischemia of 30 minutes;

The developed route of introduction of fluorescent microorganisms ensures the integrity of the gastrointestinal tract with the precise localization of the suspension of bacteria in the intestine, maintaining their viability;

Based on a morphological study, it was found that when modeling mesenteric ischemia and reperfusion, acute inflammatory changes occur in the liver, kidneys, spleen and necrotic changes in the intestine;

Microbiological studies using fluorescent strains of E.Coli showed that the main route of translocation of this strain is hematogenous, through the portal vein system, while the main target organs are the liver, spleen and kidneys;

In ischemia, LBP and PCT act as markers of translocation of the intestinal microflora. With the restoration of perfusion, the level of TCM markers decreases in the early stages;

The level of CRP correlates with the level of L-lactate and ferritin using the average feed-forward;

Between the LBP level of L-lactate there is a direct average strength significant relationship.

Practical significance

The universality of the mechanisms makes it possible to use the developed model of mesenteric ischemia and revascularization as a basis for studying the phenomenon of bacterial translocation in the future, extrapolating to other pathological conditions.

The developed microbiological method using fluorescent microorganisms can be used for other scientific areas in the biomedical industry, and the strains of fluorescent microorganisms are further cultivated, which creates prospects for their wide use for various scientific purposes.

The revealed significance of biomarkers can be used in clinical practice to detect the translocation of intestinal microflora in various pathological conditions.

Author's personal contribution

The dissertation student independently completed the experimental part of the work on laboratory animals (modeling mesenteric ischemia, introducing a suspension of fluorescent microorganisms, collecting and preparing material for microbiological, histological and immunological studies, removing animals from the experiment). Together with the head of the LKP SIC Akhmaltdinova L.L. a laboratory blood test was performed. Under the guidance of Doctor of Medical Sciences, Professor of the Department of Pathology Tusupbekova M.M. a histological study of the internal organs of experimental animals was carried out on the basis of the pathomorphological laboratory of the department of NAO "MUK". All material is systematized, documented and presented in the form of a dissertation personally by the author.

Approbation of work

The main provisions of the study were reported at the following scientific events at the 30th Congress of the World Society of Surgeons, Gastroenterologists, Oncologists IASGO Moscow, Russia, 2018; Congresses of the European Society for Surgical Research ESSR-(2018,2019,2020 Madrid, Geneva, Innsbruck); Congresses of the European Society for Pathological Research ECP 2019, Nice, France; 2020 Glasgow, Scotland)

Publications on the topic of dissertation.

14 scientific papers were published on the topic of the dissertation, including: 4 articles in international scientific journals included in the Scopus information database (of which with a percentile of 25 or more at the time of publication - 2), 3 publications in scientific journals of Kazakhstan recommended by the Control Committee in the field of education and science of the Ministry of Education and Science of the Republic of Kazakhstan; 1 monograph, 4 certificates of state registration of rights to the subject of copyright; 2 publications in the proceedings of international conferences.

Design and research methods

The dissertation research is a fragment of the research topic of grant funding of the Ministry of Education and Science of the Republic of Kazakhstan IRN No. AP05134304 "Comprehensive assessment of the phenomenon of bacterial translocation into tissues, internal organs and systemic blood flow on models of intestinal obstruction", as well as a fragment of the research topic with funding from the internal grant of NJSC "MUK": "Application of fluorescent microorganisms for the detection of translocation of intestinal microflora on the model of mesenteric ischemia "(experimental study)", approved by the decision of the Academic Council dated 11.12.2017. Protocol No. 6

Experimental work was carried out on the basis of a vivarium and a shared laboratory of the Scientific Research Center of the Nenets Autonomous Okrug Medical University of Karaganda. Histological examination was performed at the Department of Pathological Anatomy under the guidance of Prof., MD. Tusupbekova M.M. All experimental

manipulations were carried out in accordance with the principles of the European Convention for the Protection of Vertebrate Animals; euthanasia of animals was carried out in accordance with the principles of AVMA [10]. This study was approved by the Bioethics Committee protocol No. 4 dated September 25, 2017. To carry out the practical part of the study, standards of operating procedures (SOPs) were developed, approved by the Bioethics Committee of the NAO MUK. The experimental procedures were performed according to the algorithms described in the SOP. All invasive manipulations and euthanasia of animals were performed under general anesthesia by intramuscular injection of Sol.Ketamini 0.5 mg/kg of animal body weight.

The material of the study was the homogenizates of internal organs, histological preparations of internal organs and blood serum.

The study used 255 white male rats of comparable weight and age.

Study design

1. Characteristics of the study design of the experimental model

Table 1. The design of creating experimental model

Episode (I), min	**Group (R)**	**Quantity of rats**
30	60 min	8
	120 min	8
	24h	8
60	60 min	8
	120 min	8
	24h	8
120	60 min	8
	120 min	8
	24h	8
Control group(intact)		8
All		**80 rats**

The animals were divided into 3 series of experiments, 24 rats each, according to the time of ischemia modeling (I) (30,60,120 min, respectively). Each series was divided into 3 groups according to the reperfusion time (R) (60 min, 120 min and 24 h) - 8 animals in each subgroup.

All animals of these groups also underwent a histological examination of the following samples of organs and tissues of the abdominal cavity:

- Kidney;

- Liver;

- Intestines;

- Mesentery with lymph nodes;

- Spleen;

- A total of 400 samples were studied.

2. Design for the development of a technique for introducing a suspension of fluorescent E.Coli strains. and microbiological research

Used 107 white male rats, comparable in sex, age and weight.

1) Group 1 - intact rats (n=5) - no surgery was performed, rats were used to control sterility

2) The sham control group (n=51) - rats that underwent laparotomy without MIR modeling and the introduction of a suspension were divided into subgroups according to the distribution of experimental groups according to the observation period:

- 30 min - 15 rats

- 90 min - 15 rats

- 150 min - 45 rats

3) Group 3 - rats with the MIR model (n=51) were divided into subgroups, according to the parameters of the experimental model:

- subgroup with ischemia/reperfusion time: 30 min/60 min (I30/R60)–17 rats;

- subgroup with ischemia/reperfusion time: 30 min/120 min (I30/R120) – 17 rats.

- subgroup with simulation of 30-minute mesenteric ischemia without formation of reperfusion –I30/R0- 17 rats

Abdominal organs (liver, spleen, intestinal area with mesentery, kidneys) were taken for microbiological examination. To standardize the results of microbiological studies, all animals were injected with strictly 2 ml of a suspension of fluorescent microorganisms.

3. Design of the study of biomarkers of ischemia and translocation of microorganisms

The study used 68 white male rats. The number of animals for this type of study was also calculated using formula (1). At E=15, the number of animals in the group should be 17. We decided to take 5 rats in each control subgroup, taking into account the cost of the study,

and 16 rats in each subgroup of the experimental group. Also, 5 intact rats were used as a reference of normal concentration. All animals were divided into 3 main groups (Table 2).

Table 2. Design of creating research of biomarkers

Group	**Subgroup (observation period, reperfusion(R)), min**	**Quantity**	**All**
1-intact		5	5
2- sham	30 min	5	15
	90 min	5	
	120 min	5	
3- ischemia 30 min (I30)	R0	16	48
	R-60	16	
	R-120	16	
All			**68 rats**

Research methods

All procedures were carried out in accordance with the developed standards of operating procedures, certificates of intellectual property were obtained for the author's methods.

Morphological study

A histological analysis of preparations of organs and tissues was carried out in order to diagnose morphological changes in the body that occur during bacterial translocation and impaired mesenteric blood flow with microscopy of finished preparations using a computerized microscope from LeicaDM 1000 with further digital color microphotography and interpretation and recording of the results.

Fluoroscopy and microbiological examination of internal organs.

The material for microbiological research is homogenizates of organs and tissues. To determine the qualitative and quantitative composition of the microflora, inoculation is carried out from the homogenizate on Luria-Bertani with 0.2% L-arabinose (Sigma-Aldrich Inc., St. Louis, Missouri, USA) with 100 mg/l of ampicillin (Sigma-Aldrich Inc.) with the identification of GFP-producing E. Coli.

Immunological study

The level of biomarkers in blood serum was determined by solid-phase chromogenic ELISA using commercial kits for the determination of C-reactive protein (CRP), procalcitonin (PCT), lipopolysaccharide-binding protein (LBP), ferritin (Fer) and lactate (Lac). The registration of the results was carried out at different wavelengths, according to the instructions for the kits.

Statistical processing of results.

Statistical processing of the results and graphic design of the study were carried out using IBM SPSS Statistics 22.0 and the Excel spreadsheet editor from the Microsoft Office 2010 office software package. For each indicator, the average value (M), standard deviation

(SD), median (Me), and interquartile interval were calculated (IQR). Statistical hypotheses were tested for reliability using non-parametric methods: to evaluate quantitative data, the Mann-Whitney test was used for 2 independent groups (experiment-control comparison) and the Kruskal-Wallis test for k independent samples to determine the significance of the results in subgroups at different reperfusion times; to assess qualitative variables - the □2 test (Pearson's chi-square) or Fisher's exact test. A significant level of differences in the groups was considered at $p<0.05$.

Correlation analysis to determine the relationship between various translocation criteria (translocation biomarkers and SIRS, microbiological analysis data) was carried out using the calculation of the Spearman correlation coefficient (rs). In the calculations it was considered that at rs=1 there is a direct connection, and at rs= -1 there is a feedback. If the correlation coefficient is equal to zero, then the relationship between the quantities is practically absent. Due to the fact that the coefficient is a method of non-parametric analysis, a check for the normality of the distribution is not required.

Results and its discussion

1) The proposed model of mesenteric ischemia allows to study the pathomorphological substrate of the process of microbial translocation with visualization of the stages and degree of ischemia, as well as pathogenetic and histological changes that occur after the restoration of blood circulation. The apodactile technique of model formation makes it possible to comply with the conditions of sterility and atraumaticity, increases the survival rate of the rat and postoperative complications of modeling. With ischemia of the intestinal wall, there is a histological destruction of the epithelial barrier, the development of microthrombosis of the vessels of the mesentery, necrotic changes in the structures of the intestinal wall, which are the morphological basis for the development of the phenomenon of translocation of the intestinal microflora. Effective visualization of the process of microflora translocation from the intestinal lumen in the experiment occurs at 30 minutes of ischemia and reperfusion time not exceeding 120 minutes ($p=0.01$);

2) Pathological changes in the abdominal organs correspond to the picture of alternative inflammation, with the transition to the exudative phase, more characteristic of microbial etiology, as the duration of ischemia and reperfusion prolongs. Inflammatory morphological changes in the liver, spleen, and kidneys during late MI modeling indicate the role of intestinal microflora migration through the portal vein system into the bloodstream;

3) To visualize the process of migration of intestinal microflora from the intestinal lumen to the systemic circulation and internal organs, the fluorescent strains of GFPP E.Coli used as a marker statistically significantly showed that this strain does not spread in the animal body beyond the intestinal wall ($p = 0.001$), in under conditions of preserved passage and normal blood supply to the intestine, as evidenced by the absence of growth of fluorescent E. Coli both in the intact group and in the group with the sham operation.

The main route of translocation of fluorescent E. Coli in mesenteric ischemia is hematogenous, through the portal vein system. The main target organs are the liver (translocation frequency - 35.3%) and spleen (translocation frequency - 32.8%). Also,

changes in the morphological structures of the liver, kidneys and spleen indicate the involvement of the translocation mechanism in the development of these changes;

The developed method of introducing a suspension ensures the integrity of the intestine with clear visualization and control of the installation of the probe, avoids aspiration of intestinal contents into the respiratory tract, and accurately measures the amount of introduced suspension.

4)The data obtained from bacteriological studies allow us to reliably state that the introduced fluorescent E. coli into the intestinal lumen does not spread in the animal's body beyond the intestinal wall, under conditions of a preserved passage and normal blood supply to the intestine, as evidenced by the absence of growth of fluorescent E. coli in the group with surgery sham. For the fluorescent strains of Escherichia coli used in this work, the intact mucosa is an impenetrable barrier. Bacteriological examination of the abdominal organs confirms the hematogenous pathway of translocation of the intestinal microflora, mainly through the portal vein system of the liver, as evidenced by the greatest growth of fluorescent E. Coli in the liver and spleen.

5)The most significant marker of ischemic disorders and associated generalized complications is L-lactate, the concentration of which increases by 32% with an increase in reperfusion time after ischemia from 30 to 60 minutes ($p=0.017$). Ferritin is of lesser diagnostic value for the syndrome of mesenteric circulation disorders. its level responds equally to both the presence of ischemia and its absence ($p\geq0.05$ in comparison with the control group).

The most specific of the studied markers of the systemic inflammatory response syndrome associated with the phenomenon of microbial translocation is LBP, the concentration of which increases by 2.0 times in the group with ischemia for 30 minutes compared to the control group. With reperfusion from 60 min to 120 min, the LBP level decreases within 60 min to the values in the group of intact rats, and after 2 hours it increases by 22%. Procalcitonin, CRP, and IL-6, based on the results of this study, are less specific markers of SIRS under investigation that occurs against the background of impaired mesenteric blood flow.

6)Between lactate and SIRS markers there is a direct medium-strength correlation between LBP ($rsp=0.434$, $p=0.0001$) and CRP ($rsp=0.220$, $p=0.0001$). Ferritin also significantly correlates with CRP levels ($rsp=0.220$; $p=0.044$) showing a direct mean correlation.

The level of SIRS markers is not significantly associated with positive results of organ cultures ($p\geq0.05$).

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Приложение 1.

Схема роли БТ в патогенезе и методов верификации БТ при экспериментальной мезентериальной ишемии и реперфузии