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Changes of biochemical parameters in rat intestinal mucosa induced by methotrexate and effects of enteral administration of glutamine

Katica BAJIN-KATIĆ
Karmen STANKOV
Zoran KOVAČEVIĆ

DEPARTMENT OF BIOCHEMISTRY, MEDICAL FACULTY NOVI SAD, NOVI SAD, SERBIA AND MONTENEGRO

BACKGROUND: *Rapidly proliferating crypt cells of the intestinal epithelium, the precursors of the mature enterocytes, are extremely sensitive to the effects of cytostatic agents. We investigated the effects of the methotrexate on rat intestinal mucosa in order to get the information on biochemical indicators of intestinal damage.*

METHODS: *Biochemical parameters were investigated in isolated intestinal mucosa of Sprague-Dawley rats, previously treated with methotrexate by intraperitoneal administration. Glutamine was dissolved in water and administered orally.*

RESULTS: *The activity of glutaminase and alkaline phosphatase showed the enzymatic response to different doses of methotrexate. The activity of both enzymes was significantly lower in the mucosa of treated animals, compared to control group.*

CONCLUSION: *Minimal mucosal damage and regeneration time is dose dependent and influenced by the dosage schedule of antitumor therapy.*

KEY WORDS: *Methotrexate; Glutamine; Intestinal Mucosa; Rats; Drug Toxicity*

INTRODUCTION

Methotrexate (MTX) is an antineoplastic drug frequently administered to patients having either cancer or autoimmune diseases. Despite the fact that the dose of MTX administered to malnourished patients is generally small, the toxic side effects are numerous and often the limiting factor for its use. Gastrointestinal side effects are the most common complications of MTX administration (1-5).

The mechanisms by which the MTX causes the gastrointestinal toxicity are not well known. Methotrexate undergoes polyglutamylation and inhibits the enzymes of purine and pyrimidine biosynthesis. The administration of MTX induces focal vacuolization and ultrastructural damage to the immature intestinal crypt cells. As the mature cells are sloughed off, the injury to the villous tip progenitors exposes the mucosa to the erosive pancreatic and biliary secretions, and autodigestion of the intestinal epithelium follows. These morphological changes are associated with the reduction of the intestinal mucosal mass, mucosal protein and DNA content, body weight loss, a significant increase in intestinal transit time and increased intestinal permeability (1,6).

In vivo animal studies have shown that glutamine is the principal respiratory substrate for enterocytes, and that glutamine-fortified parenteral and enteral diets significantly improve the intestinal morphology and function (1). Glutamine itself is absent from all currently available commercial parenteral nutrient solutions and it is present in only few enteral diets.

The glutamine is less stable in aqueous solution, compared to other amino acids, because its amide nitrogen group is easily hydrolyzed. The glutamine is spontaneously degraded and forms toxic products (pyroglutamic acid and ammonia). The recent studies show that utilization of glutamine-containing dipeptides and sterilization by ultrafiltration instead of high temperature allows prolonged stability of sterile glutamine solutions (7).

The effectiveness and safety of glutamine administration in catabolic patients have been shown in many studies (8,9). Protective effects of glutamine administration are shown in clinical and experimental studies where its enteral administration has prevented the development of enterocolitis induced by chemo- or radiotherapy (10). Damage of the intestinal mucosa occurs when the level of circulating glutamine decreases and when the utilization of glutamine is prevented. Following the administration of glutaminase inhibitors in several species, a rapid onset of vomiting and profuse diarrhea develops in association with intestinal mucosal ulceration and necrosis (1).

Address correspondence to:
Katica Bajin-Katić, MD, Assist. Prof., Department of Biochemistry, Medical Faculty Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Serbia and Montenegro

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It is still unclear whether the glutamine has its impact on the intestine as a metabolic fuel, as a precursor of metabolites that are essential for cellular replication, or as a regulator of enterotropic hormones. We aimed in our study to setup the experimental conditions by testing some biochemical parameters (enzymes), which enable follow-up of controlled intestinal mucosa damage induced by MTX, and to test whether glutamine decreases or prevents this damage. Therefore, we presented the preliminary results of this investigation.

MATERIALS AND METHODS

In our study we used Sprague-Dawley rats, body weight of 250 g approximately. The experimental animals constituted the control group (50 animals), and the experimental group, where either methotrexate (MTX) alone or MTX and glutamine were administered (450 animals in total). All experimental animals were further stratified according to the experimental model. The animals received standard nutritive formula for rats. The MTX was administered by intraperitoneal application. Water solution of 1% glutamine was prepared daily and administered orally.

For the analysis of the dose-dependent effects, we decided to follow-up the enzyme activity on day 7 after the administration of 0, 20, 30, 40, and 60 mg of MTX per kg of body weight. Each subgroup, including control group, comprised 30 animals, 150 in total.

The time course of the MTX intestinal effects was analyzed in isolated intestinal mucosa of animals treated by MTX (20 mg /kg BW) on day 0, 5, 7, 10, 20, and 60 after the initial treatment. The number of animals in different subgroups was 30 per each subgroup, including control group.

For the analysis of the possible protective role of glutamine, the animals were stratified in three groups. The first group consisted of 20 control animals, the second group included the animals treated with 20 mg MTX/kg and killed after 20 days (30 animals), while in the third group the MTX treatment was carried out in the same manner as in the second group, but after the day 20 the animals were drinking the 1% fresh glutamine for 7 days and then they were killed as well (30 animals).

The isolation of enterocytes was performed according to the method of Kralovanszky et al. (11). The enterocytes were obtained by "scrapping" method from the 90 cm long intestine, starting from the end of duodenum. The mucosa was homogenized in Potter-Elveheim homogenizer in isotonic KCl with 10 mmol/l mercaptoethanol at 0°C. After the centrifugation on 15000 rpm in Eppendorf centrifuge for the analysis of studied parameters, the supernatant was used, as well as for the total protein content determination. The "scrapping" method provides homogenous experimental material and therefore, all the parameters are calculated according to the exactly measured length of the intestines and the protein content. The isolated cells were collected in one combined cell fraction. The experimental animals were continually observed and all behavioral changes, toxic gastrointestinal damages, and the survival periods for each animal were recorded.

In cell homogenate the protein content was determined using the Biuret method. The activities of enzymes glutaminase and alkaline phosphatase were determined by kinetic method (12).

All experiments were done in duplicate. The results are presented as mean value. Statistical analysis for comparison of all the measured parameters in intestinal mucosa homogenate of treated animals versus non-treated animals was performed by the Student's *t* test (with $p<0.05$ as the level of significance).

RESULTS

Considering the main goal of our study, the glutamine protection of the intestinal mucosa from the damage induced by methotrexate, two main experimental approaches were postulated: 1. Determination of biochemical parameters that may serve as markers of mucosal damage; therefore, we measured the activity of glutaminase, one of the key enzymes in metabolic transformation of glutamine, and the activity of alkaline phosphatase. 2. Follow-up of MTX effects in function of concentration and duration of MTX treatment, in order to test the conditions, which enable the significant but not irreversible intestinal damage.

The effects of different MTX doses on enterocytic glutaminase and alkaline phosphatase are shown in table 1. In all cases observed, the animals were killed on day 7 after the administration of MTX. As it is shown in Table 1, the increase of MTX dose induces decreased activity of glutaminase and alkaline phosphatase, while the animals could not survive the dose of 60 mg/kg.

Table 1. The activity of glutaminase and alkaline phosphatase in rat intestinal mucosa 7 days after the methotrexate administration

MTX (mg/kg)	Glutaminase (nmol/min/mg proteins)	ALP (nmol/min/mg proteins)
0	68.96 (30)*	296.79 (30)
20	44.92 (30)	101.00 (30)
30	36.50 (30)	76.12 (30)
40	22.74 (30)	49.51 (30)
60	animals did not survive	animals did not survive

* number in parentheses indicates the number of treated animals

We detected the effects of MTX following the changes in behavior of the animals. On days 1 and after the application of the MTX, we did not detect any significant changes in behavior of the animals. However, on day 3, the rats were lethargic, and those receiving higher doses suffered from hemorrhagic diarrhea.

Since we induced significant effects on glutaminase activity with 20 mg/kg of MTX without influence on animal survival, we select-

ed this dose for testing the MTX effects in function of time. Table 2 shows the levels of glutaminase and alkaline phosphatase activity in function of time. The effects of unique dose of MTX were observed for 60 days. The results show that the significant inhibition of these enzymes occur on day 10 and that the effect of altered dose is reversible, thus on day 60 the activity is close to initial values.

Table 2. The activity of glutaminase and alkaline phosphatase in rat intestinal mucosa in function of time. The animals were treated by unique methotrexate dose of 20 mg/kg

Days after the treatment	Glutaminase (nmol/min/mg proteins)	ALP (nmol/min/mg proteins)
0	68.96 (30)*	296.79 (30)
5	51.00 (30)	169.04 (30)
7	44.92 (30)	101.00 (30)
10	46.99 (30)	89.45 (30)
20	47.22 (30)	119.73 (30)
60	53.40 (30)	202.58 (30)

* number in parentheses indicates the number of treated animals

The observed changes in function of MTX dose and time after the initial treatment were useful basis for testing the effects of glutamine on protection and regeneration of intestinal mucosa, after the damage induced by this cytostatic. Our results (Table 3) show significantly higher activity of alkaline phosphatase in the group of animals that were drinking the glutamine than in the MTX group ($p<0.01$).

Table 3. The mean values of effects of 1% glutamine on glutaminase and ALP activities in rat intestinal mucosa treated with 20 mg MTX/kg

Experimental conditions	Glutaminase (nmol/min/mg proteins)	ALP (nmol/min/mg proteins)
Control	68.32 (20)*	289.13 (20)
MTX	47.52 (30)	119.73 (30)
MTX + glutamine	51.06 (30)	164.36 (30)**

* number in parentheses indicates the number of treated animals
** the activity of ALP in MTX+glutamine group in comparison with MTX group is significantly higher ($p<0.01$)

DISCUSSION

Our investigation suggests that glutamine supplementation via oral route can reduce MTX-induced toxicity of epithelial cells by lining the small intestine (enterocytes). Our analysis of alkaline phosphatase activity may indirectly support the hypothesis that the glutamine protects the intestinal mucosa from the damage induced by MTX. The measurement of glutaminase activity did not show such significant effect, thus indicating that this biochemical parameter is less useful marker for detection of intestinal mucosa damage. Our results show that the changes of alkaline phosphatase activity are better markers of MTX intestinal mucosa damage than the changes in glutaminase activity.

Glutamine is metabolized in similar fashion whether it enters the mucosal cells from the lumen or from the blood, and its utilization is dependent on the circulating level of glutamine. The enzyme glutaminase catalyzes the initial step in glutamine degradation in the enterocytes and the alterations in the activity of enzyme may increase the rate of intestinal glutamine utilization. Enterocytic glutaminase activity decreases with prolonged fasting, and therefore the improvement of nutritional state, either by parenteral or enteral feeding, is very important for intestinal mucosa integrity (13,14).

The protective role of glutamine is also well supported by experimental findings of other researchers (15-17). Numerous clinical studies have been conducted utilizing glutamine in conjunction with radiation and chemotherapy with promising results. In studies on patients undergoing radiation and chemotherapy (cis-platinum and 5-fluorouracil) for esophageal and colorectal cancer, the glutamine supplementation prevented an increase in gut permeability (15,18,19). The mechanism is unclear, but may involve methotrexate conjugation by glutamine, or possibly increased glutathione synthesis and subsequent cellular protection promoted by glutamine supplementation. Glutamine is a precursor for the synthesis of glutathione, which is an important intracellular antioxidant and hepatic detoxifier (20). Cao et al. (21) showed that doxorubicin dose-intensive therapy for breast cancer is limited by a cardiomyopathy that often results in congestive heart failure. It was reported that dietary glutamine supplementation might diminish doxorubicin-induced oxidative damage and thus cardiotoxicity through the increase of cardiac glutathione metabolism (21).

All these findings suggest glutamine supplementation for the patients undergoing radiation and chemotherapy in order to prevent cytotoxicity of different tissues, especially of the gastrointestinal mucosa.

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