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ACTIVITIES OF PROXIMAL TUBULE ENZYMES AND ALBUMIN CONCENTRATION IN URINE OF CHILDREN TREATED WITH METHOTREXATE

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Abstract: In order to study methotrexate nephrotoxicity, the activities of proximal tubule epithelial cell membrane enzymes: alanine aminopeptidase (AAP) and gamma-glutamyltransferase (GGT), as well as of lysosomal N-acetyl-beta-D-glucosaminidase (NAG) and urinary albumin concentrations were determined in 12-h-urine samples of 30 patients with lymphoblastomous leukemia. The patients were *i.v.* receiving 4 individual methotrexate doses of 2000 mg/m^2 every 15 days followed by leucovorin as a protector. Control and methotrexate-treated group, each consisting of 30 examinees, included 4–10 years old children of both sexes.

Statistically significant increase of AAP and GGT activities, expressed as U/mmol creatinine was observed after the first two ($p < 0.05$), as well as after the remaining two therapies ($p < 0.01$) in relation to the control. Enzymatic activities of these two enzymes decreased to control value before the second and the third methotrexate application, but they increased again after the third application and remained elevated up to the end of experiments. Significant increase of NAG activity expressed as U/mmol creatinine ($p < 0.01$), as well as urinary albumin levels (mg/mmol creatinine; $p < 0.01$) were registered after the third methotrexate therapy and this elevation of the same statistical significance of the differences remained stable till the end of the therapy. Based on these results it can be concluded that during the time period of two first applications nephrotoxic methotrexate action is reversible and at the level of proximal tubule epithelial cell membranes. During the last two applications impairment is irreversible and at the level of cell organelles and glomerular filtration.

Keywords: nephrotoxicity, alanine aminopeptidase (AAP), gamma-glutamyl transferase (GGT), N-acetyl-beta-D-glucosaminidase (NAG), albumin, urine, methotrexate.

1. INTRODUCTION

Methotrexate, a known folate synthetase inhibitor represents one of the first and most frequently used drugs in chemotherapy of malignancies. It has been applied as chemotherapeutic for almost five decades. It was shown to be efficient against numerous malignancies such as leukemia, lymphoma, choriocarcinoma, osteosarcoma and breast, head and lung tumors [1−5]. Methotrexate is usually applied by *i.v.* route in high, moderate and low doses [6]. High doses of 500 mg/m^2 or even higher are used in the cases of leukemia, lymphoma, leptomeningeal metastases and osteosarcoma. Medium doses of 50 to 500 mg methotrexate *per* m^2 are given in cases of gestational trophoblastic diseases, while low doses (50 mg/m^2 or lower) are applied during anti-

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inflammatory therapy of rheumatoid arthritis and psoriasis [7,8].

Intravenous methotrexate doses over 1.0 g/m^2, frequently result in a series of systemic toxicities. Besides the skin, mucous membranes, liver and brain, they also affect the kidneys [9]. Methotrexate can exert toxic effects on glomerular and tubular functioning, but some clinical studies showed that it is not a general phenomenon [10]. Even low and moderate intravenously applied methotrexate doses aggravate the existing glomerular and tubular cell necrosis. The risk of nephrotoxic methotrexate action can be elevated by genetic polymorphism included in folate metabolism [11].

In the studies published so far, determination of methotrexate nephrotoxicity was performed during the application of low doses of the drug in the cases of anti-inflammatory treatment of rheumatoid arthritis and psoriasis. In earlier reports, glomerular function was checked by measuring clearance of inulin, creatinine and ethylenediaminetetraacetate (EDTA), as well as by increased albumin concentration in urine [12]. Tubular function was estimated by determining the blood concentration of electrolytes, content of alfa-1- or beta-2-microglobulins in urine and by tubular enzymuria [13,14].

The present study aimed at determining possible nephrotoxic methotrexate effects in the representative number of patients suffering from acute lymphoblastomous leukemia treated with high doses of the drug. Glomerular toxicity was estimated *via* the determination of albuminuria, and tubular toxicity by determining enzyme activities of AAP (EC 3. 4. 11. 2), GGT (EC 2. 3. 2. 2), and NAG (EC 3. 2. 1. 52), known as very sensitive markers of epithelial proximal tubule cell damages.

2. EXPERIMENTAL SECTION

Sixty children aged between 4 and 10 years, all of them patients of the Pediatric Hospital of the University Clinical Center, Banjaluka were included in the present study. The experimental group consisted of 30 patients with diagnosed lymphoblastomous leukemia receiving *i.v.* four intramittent methotrexate doses of 2000 mg/m^2 every 15 days, each of them followed by protective leucovorin doses of 15 mg/m^2, 42, 48 and 54 h after methotrexate. The control group included 30 examinees without either lymphoblastomous leukemia or some other kidney- or urinary tract-connected disorders and without methotrexate application.

Twelve-h-urine samples in triplicates, collected in the morning 24 h before and 24 h after each methotrexate treatment were kept at −25 ^{o}C till the analysis. The data on age, sex, body height and mass, and the health status of the patients were introduced into a questionnaire during the first urine collection.

Before urine analysis, the enzymes were separated by gel filtration [15]. Enzymatic activities of AAP [16], GGT [17] and NAG [18,19] expressed as U/mmol creatinine [20], and concentrations of urinary albumin [21,22] expressed as mg/mmol creatinine, were determined by spectrophotometry.

The results analyzed by standard statistical methods are presented graphically and in tables as mean values ± standard deviation. Thesignificance of differences between the experimental group and the control was estimated by Student ***t***-test.

3. RESULTS

Enzymatic AAP, GGT and NAG activities, as well as urinary concentrations of albumin presented as mean values ± S.D. are described in Table 1-4. As seen in Table 1, a statistically significant increase of AAP activity in urine samples of patients treated with methotrexate in relation to the control was recorded after each of four therapies.

Table 1. Average values (± SD) of the activities AAP in urine of children treated with four therapeutic doses of methotrexate in relation to the corresponding controls

methotrexate therapies	control group, values in units	experimental group, values in units	p
I	0.37 ± 0.12	0.47 ± 0.17	$p < 0.05$
II	0.38 ± 0.11	0.54 ± 0.19	$p < 0.05$
III	0.35 ± 0.10	0.60 ± 0.20	$p < 0.01$
IV	0.37 ± 0.12	0.69 ± 0.23	$p < 0.01$

Similarly to AAT, the mean values of enzymatic GGT activity were significantly increased after the first, after the second, after the third and after the fourth therapy in experimental group in relation to the control (Table 2)

Twenty-four hours before the onset of the second and third methotrexate treatment the activities of AAP and GGT were decreased to control value. However, the activities of these enzymes after the third, as well as before the fourth methotrexate treatment remained significantly elevated up to the last experimental point, i. e. 24 h after the last application of the drug.

Table 2. Average values (± SD) of the activities GGT in urine of children treated with four therapeutic doses of methotrexate in relation to the corresponding controls

methotrexate therapies	control group, values in units	experimental group, values in units	p
I	3.84 ± 1.12	4.85 ± 1.61	$p < 0.05$
II	3.62 ± 1.20	5.45 ± 1.84	$p < 0.05$
III	3.45 ± 1.15	5.72 ± 1.92	$p < 0.01$
IV	3.51 ± 1.17	5.57 + 1.85	$p < 0.01$

Changes in NAG activity during the experimental period are described in Table 3. As shown, a statistically significant increase of this enzyme activity in methotrexate-treated group of patients in comparison with the control was observed after the third treatment to remain significantly elevated up to the end of the therapy.

An increased albumin level in the form of microalbuminuria was recorded after the third treatment with methotrexate and the same level of statistical significance of the differences was recorded till the end of the therapy (Table 4)

Table 3. Average values (± SD) of the activities NAG in urine of children treated with four therapeutic doses of methotrexate in relation to the corresponding controls

methotrexate therapies	control group, value in units	experimental group, value in units	p
I	0.27 ± 0.09	0.29 ± 0.08	$p > 0.05$
II	0.31 ± 0.11	0.32 ± 0.10	$p > 0.05$
III	0.29 ± 0.09	0.46 ± 0.15	$p < 0.01$
IV	0.28 ± 0.07	0.59 ± 0.19	$p < 0.01$

Table 4. Average values (± SD) of the concentrations albumine in urine of children treated with four therapeutic doses of methotrexate in relation to the corresponding controls

methotrexate therapies	control group mg/mmol creatinine	experimental group mg/mmol creatinine	p
I	1.99 ± 0.66	2.20 ± 0.71	$p > 0.05$
II	1.98 ± 0.68	1.96 + 0.68	$p > 0.05$
III	2.33 ± 0.77	5.30 ± 1.81	$p < 0.01$
IV	2.28 ± 1.0	7.11 ± 2.83	$p < 0.01$

4. DISCUSSION

In the present work, nephrotoxic effects of *i.v.* methotrexate therapy were studied in the group of children suffering from lymphoblastomous leukemia, aged between 4 and 10 years of both sexes. The increased enzymatic activities of AAP and GGT (enzymes of the proximal tubule epithelial cell membranes) in urine pointed to methotrexate-induced injuries of this kidney tissue. At the same time, harmful effects of methotrexate application were seen as a significantly increased activity of lysosomal enzyme NAG. In addition, significantly elevated albumin levels in urine of the patients demonstrated disturbances in glomerular filtration.

Enzymatic AAP and GGT activities were significantly increased already after the first methotrexate application, but a 15-day-period between the first and the second therapy was sufficient for recuperation of kidney cells, seen as decreased activities of these two enzymes to the control levels. A similar trend was also observed after the second methotrexate treatment, i. e. after an initial increase in AAP and GGT activities registered 24 h upon the therapy, the activities of these two enzymes declined to control values before the third methotrexate application. These results are in accordance with our previous findings demonstrating that AAP and GGT represent early, reliable and very sensitive markers of reversible changes in epithelial cell membranes of proximal

tubules [23]. The activities of AAP and GGT also significantly increased upon the third methotrexate treatment to remain elevated till the end of the experimental period. These results ainindubitably pointed to severe and irreversible methotrexate-induced injuries of proximal tubule epithelial cells. After the first two methotrexate treatments of the patients, no significant changes in NAG activity were observed. However, the activity of this enzyme was significantly increased after both the third and the fourth methotrexate application, clearly demonstrating serious and irreversible damages in proximal tubule epithelial cells at the level of cell organelles. These results are in agreement with the report of Wilanda *et al.* [24], although they do not support the findings of Hempel *et al.* [9].

After the third methotrexate therapy, albumin concentration in urine was significantly increased pointing to methotrexate-induced damages of glomerules. These changes at the level of microalbuminuria persisting up to the end of the experiment are partially in accordance with earlier findings of Vujić *et al* [25] and Ferrari *et al*. [26] and Spasovski *et al.* [27].

5. CONCLUSION

Based on the results obtained throughout the present study, it can be concluded that high methotrexate doses given to children with acute lymphoblastomous leukemia exert severe nephrotoxic effects. After the first two methotrexate therapies these effects are expressed as reversible changes in epithelial proximal tubule cells. However, the next two therapies lead to irreversible changes accompanied by an impairment at the level of kidney cell organelles and significant reduction of glomerular filtration.

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АКТИВНОСТИ ЕНЗИМА ПРОКСИМАЛНИХ ТУБУЛА И КОНЦЕНТРАЦИЈА АЛБУМИНА У УРИНУ ДЈЕЦЕ ТРЕТИРАНЕ МЕТОТРЕКСАТОМ

Сажетак: Ради утврђивања нефротоксичности метотрексата, одређивани су ензимска активност ензима мембрана ћелија епитела проксималних тубула, аланинаминопептидазе (ААП) и гама-глутамилтрансферазе (ГГТ), лизозомалног ензима Н-ацетил-бета-Д-глукозаминидазе (НАГ) и концентрација албумина у урину. Одређивања су вршена у узорцима 12-часовог урина код 30 испитаника који су били обољели од лимфобластне леукемије. Њима је метотрексат аплициран интравенски у четири појединачне дозе од 2000 mg/m^2 са размаком од 15 дана и уз заштиту са леуковорином. Иста одређивања су вршена и у 12-часовном урину 30 испитаника контролне групе. Обје групе су се састојале од испитаника оба пола, старости од 4 до 10 година.

Статистички значајно повећање активности ААП и ГГТ, изражених у U/mmol кретинина, експерименталне у односу на контролну групу је регистровано након прве двије терапије ($p < 0,05$) и након друге двије терапије ($p < 0,01$). Активности ова два ензима експерименталне групе нормализовале су се до почетка друге, односно треће апликације метотрексата, али су се опет повећеле након треће апликације и остале увећане све до краја експеримента. Сигнификантни порасти активности НАГ у U/mmol креатинина ($p < 0,01$) и концентрације албумина у mg/mmol креатинина ($p < 0,01$) су регистровани након треће терапије и повећања са истом статистичком значајношћу су задржана све до краја терапије. На основу добијених резултата може се закључити да је, за вријеме двије прве примјене лијека, нефротоксично дјеловање метотрексата

реверзибилно и на нивоу ћелија мембрана епитела проксималних тубула. За вријеме двије посљедње примјене лијека оштећења су иреверзибилна и на нивоу ћелијских органела и гломеруларне филтрације.

Кључне ријечи: аланинаминопептидаза (ААП), гама-глутамилтрансфераза (ГГТ), Н-ацетил-бета-Д-глукозаминидаза (НАГ), албумин, урин, метотрексат.