

Research Article

Lysoenzymuria as a Hypotetical Tracer for Evaluation of Nephrotoxicity in Patients with Rheumathoid Arthritis Treated with Most Used Slow Acting Antirheumatic Drugs-Saards

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Summary

The aim of this study is to determine the effect of initial therapy with Methotrexate and Ketoprofen on glomerular and tubular integrity in patients with Rheumatoid arthritis (RA), to quantify nephrotoxicity of Methotrexate and Ketoprofen with measurement of enzyme excretion which correlates with the degree of damage of tubular epithelium. To determine whether there is a change in clinical and laboratory indicators of renal function (creatinin in serum and urine, serum urea, glomerular filtration rate (GFR) in course of the follow up of treatment and whether that change correlates with the dynamics of the quantity of enzymes excreted in urine and reactants of the acute phase: Rheumatoid factor (RF), C-reactive protein (CRP), Erythrocyte sedimentation rate (ESR) and Index of intensity of disease (Disease Active Score DAS28). To determine whether changes in tubular enzymes excreted in urine depend on the disease evolution. Microalbuminuria is used as a marker for glomerular damage, and urine excretion of N-Acetyl- β -D-glucosaminidase (NAG) as indicator of proximal tubular damage. Using colorimetric method for determination of NAG (ROCHE), as well as immunoturbidimetric method for detection of microalbuminuria (RANDOX Laboratories Limited), samples of 70 participants are examined (35 RA patients treated with Ketoprofen only, 35 RA patients treated with combined use of Methotrexate and Ketoprofen). The follow up is in 5 time-intervals in the course of 24 weeks. It is determined that there is weak correlation between NAG and microalbuminuria ($r=0,34$) in the group of patients treated with Ketoprofen only, while poor correlation ($r=0,21$) in group of patients with combined use of Methotrexate and Ketoprofen. NAG enzymuria in size, number of patients registered, and time of appearance, is greater and appears earlier in the group with the combined use of Methotrexate and Ketoprofen compared with the mono-therapy with Ketoprofen. Mean urinary NAG induction is increasing with the concomitant use of Methotrexate and Ketoprofen. Methotrexate is more potent NAG inductor of Ketoprofen and provokes greater tubular enzymuria than Ketoprofen.

Keywords: N-acetyl- β -D-glucosaminidase; Microalbumin; Rheumathoid Arthritis; Ketoprofen; Methotexate

Introduction

Traditional treatment of RA includes non-steroid anti-inflammatory drugs (NSAIDs), drugs that modify the disease (DM-RADs), steroids and immunosuppressive cytotoxic drugs.

Methotrexate in low dose regime is the most subscribed drug of the DMRADs, while in the group of NSAIDs is Ketoprofen (Niflam®, Ketonal®). Lysosomal system of the tubules is dynamic system, and low level of lysosomal enzymes found in normal urine is result of normal exocytotic and pinocytotic activity of the tubular endothelial cells (1). Enzymuria depends on the place and intensity of damage. Increased enzyme activity is a reflection of disease activity and residual functional capacity of kidney (2). Perturbation in renal tubules initially will affect lysosomal/plasma membrane system in cells, resulting in increased loss of enzyme in the urine in early stadium. Latter increase of enzyme excretion is connected with structural damage of cells resulting with cell necrosis. With the absence of the toxic stimulus, decrease of the urinary enzyme activity will be followed by regeneration of tubular cell function.

Enzymuria is connected with several possible processes:

1. Change in cell epithelisation.
2. Increased re-absorption of lysosomotropic substances.
3. Enzyme induction
4. Membrane induction
5. Necrosis of tubular cells due to exogenous chemical substances (drugs, toxins, pesticides).

Criteria that make urinary enzymes suitable for determination of certain abnormalities in renal function are:

1. Low level of present enzymes in physiologic conditions.
2. Sensitive methods for determination of enzyme activities in urine.
3. High concentration of examined enzymes in renal tissue.

4. Visible increase of enzyme activity in urine in renal damage.

Increase of urine enzyme activity could show the place of the primary renal tubular damage because of their localization in brush border region (microsomal Alanin Aminopeptidase (AAP), E.C.3.4.11.2) and tubular lysosims (NAG E.C.3.2.1.30). They can be useful in early diagnose of acute renal damage as nephrotoxicity caused by immunosuppressive drugs, contrast substances, antibiotics and cadmium exposition (3-10). Urinary enzyme activity normally is very low in urine and is increased in renal tubular cell damage (11). Urinary enzymes, especially NAG, AAP and Alkaline Phosphatase (AF) are very sensitive indicators of renal parenchimal damage in comparison with functional measurements as GFR, creatinin and inulin clearance. Relatively low sensitivity of GFR could be explained with great functional reserve of the kidney and its great capability of compensation. The use of urinary enzymes is relatively simple, cheap, quick, non-invasive method in detection of very early stadium of disease and follow up of renal failure. In last 30 years a great attention is paid in evaluation of urinary enzymes as non-invasive markers for determination of tubular damage. Urinary activity of NAG is one of the most evaluated urinary enzymes and is very sensitive marker for renal tubular damage (12-28).

Patient and Methods

A subspecialist in this field did the clinical evaluation. All patients took part in this study voluntarily, so the ethical criterion was not breached during our work.

In the patients examined for this study, the diagnosis of the disease was established on the basis of revised diagnostic criteria for the classification of RA, suggested in 1987 by the American Association for Rheumatism (ARA) (29). In order for a patient to be diagnosed with rheumatoid arthritis, he or she must fulfill at least four out of seven criteria. Criteria from one to four are present for at least six month. In this study 70 patients are examined, divided in 2 groups. In the group treated with Ketoprofen only are included 35 patients with RA. Of them 27 (77%) are women, 8 (23%) are men. Mean age of patients in this group is 53.7 ± 10.4 years (min=28, max=65 years). The disease

lasted mean 72.2 ± 52.7 months (min=1, max=360 months). In the group with combined use of Methotrexate and Ketoprofen are included 35 patients with RA. Of them 29 (83%) are women, and 6 (17%) are men. Mean age of patients in this group is 53.9 ± 9.7 years. The disease lasted mean 121.3 ± 109.3 months (min=3, max=456 months).

None of the patients included in this study has medical record for past or present renal disease. 6 patients are previously treated with oral steroids in the group of patients treated with combined use of Methotrexate and Ketoprofen before the beginning of follow up. The rest negate use of previous NSAIDs, golden salts and other drugs, especially antibiotics before use of Ketoprofen and Methotrexate. Samples are collected in the period of 2 years.

Criteria for inclusion: The study includes patients suffering from RA, age 18-65 years, till now not treated with NSAIDs and DMARDs.

Criteria for exception from the research: From this research were accepted all the patients with a disease or condition which could directly or indirectly influence a change in results:

1. Patients with previous medical record for diseases of the spleen, thyroid gland, hepatal damage, renal, hematologic, cardiovascular, neurotic and lung damage, autoimmune disease, AIDS, aged <18 years.
2. Patients with diabetes mellitus, acute infections, malignant neoplasm, febrile conditions.
3. Patients treated with antibiotics and salicylate in the period of six months prior to the beginning of the study.
4. Patients with hypertension arterialis, uric arthritis, uric infections, SLE, Sjögren, mixed conjunction texture disease, vasculitis.
5. Patients treated with antihypertension, antidiabetic and cardiac therapy.
6. Patients with anamnesis for transfusion of blood and overweight.
7. Hypersensitive to some of the medicines or their compo-

nents.

8. Excepted patients who together with these medicines take medicines from basic line.

9. Excepted patients whose results show that in 0 spot there is a glycemia, or increased level of degraded

products: creatinine in serum and urine, urea in serum and disorder of the hematologic and enzymatic status.

All patients took part in this study voluntarily, so the ethical criterion was not breached during our work.

Clinical evaluation of disease activity: A subspecialist in this field did the clinical evaluation. The activity of the disease was evaluated using DAS 28 Index (30-33). The index is a mathematical formula that allows us to get a uniquely composed quantitative score, which constitutes from palpation painful sensitive joints (max number 28), swollen joints (max number 28), Westergren ESR, and the patient's global assessment of the activity of disease (0-100 mm Visual Analogous Scale, (VAS)) and the morning rigid (minutes). DAS 28 index is ranked from 0 to 10 and a score under 3.2 ranks the disease as low-active. The assessment of GFR was calculated using the Cocroft&Gault equation

Laboratory Assessment

For clinical estimation of the disease it was necessary to take in consideration following laboratory variables: blood count, differential, reactants of the acute phase:CRP, RF and ESR, Anti CCP 2 antibodies, alkaline phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactate dehydrogenase (LDH), urea and creatinine in serum. Examples of urine were taken not only for routine urine analysis, but also for detection of NAG, creatinine in urine and microalbuminuria. For processing are used samples from second urine, not frozen, immediately processed, preserving the rules for good laboratory practice.

Colorimetric Assay for the Determination of N-Acetyl- α -D Glucosaminidase (Nag) in Urine (Roche)

Principle of the assay: 3-Cresolsulfonphthaleinyl-N-Ace-

tyl- β -D-glucosaminide, sodium salt, is hydrolysed by N-Acetyl-b-D-glucosaminidase (NAG) with the release of 3-cresol-sulfonphthalein, sodium salt (3-cresol purple), which is measured photometrically at 580nm using **ROCHE MANCHEIM** tests. Turbid urines should be centrifuged and the supernatant decant.

Reference value: NAG urine 0.27-1.18 U/mmol creatinine

Immunoturbidimetric Assay for the Determination of Urinary Albumin (Randox Laboratories Limited)

Principle of the assay: An undiluted sample is added to a buffer containing the antibody specific for human serum albumin. The absorbance (340 nm) of the resulting turbid solution is proportional to the concentration of albumin in the sample urine. By constructing a standard curve from the absorbance of standards, the albumin concentration of the sample can be determined. The assay can be carried out manually (at room temperature) or with an automated analyser using DAKO tests (34,35).

Sample collection and storage: For random urinary albumin measurement, use an early morning mid-stream specimen. Centrifuge cloudy samples before use and analyse clear supernatant in the assay.

Reference value: 2.0-20.0 mg/L.

Urea in serum was detected with the method of » Kassirer «. Reference values are 3–7.8 mmol/L.

Creatinine in serum and urine was detected with the »Jaffe« method. Reference values for creatinine in serum are 45-109 μ mol/L and for creatinine in urine 7–17 μ mol/dU.

Rheumatic factor (RF) was determined using the test of agglutination (Latex RF test) (BioSystems S.A. Reagents&Instruments Costa Brava 30, Barcelona, Spain). Reference values are under 8 mg/L RF in serum

C-reactive protein (CRP) was found using the test of agglutination (Latex CRP test), (BioSystems S.A. Reagents&Instruments Costa Brava 30, Barcelona, Spain). Reference values are under 6 mg/L CRP in serum.

For the specification of **red cell sedimentation (ESR)**, we

used the method after Westergren, and

normal values were: for males 7–8 mm, for females 11–16 mm.

Statistical Analysis

Student's t-test was used for testing the difference between two arithmetic means comparing two groups, and Wilcoxon-matched test was used for independent examples. The association between various group data was calculated with the Pearson or Spearman rank correlation tests for parametric and non-parametric data respectively. Analysis of relations between attributive statistical series is made with Pearson's χ^2 test. Test of the significance of the differences among three or more arithmetical means is made with Analysis of variance (ANOVA); as well as Freedman's analysis of variance (Fr χ^2). Statistical series are shown with tables and figures according to the defined variables of interest. Statistical software Statistica, release 7.0 for data processing was used. Data are expressed as Mean+SD and range when appropriate. P values less than 0.05 were considered significant.

Results

Analysing the distribution of patients according to the values of NAG in the five probes, in the group of patients treated only with Ketoprofen one could conclude that evaluated values of NAG are registered in 27 (77%) of patients in 16th week (4th probe), when mean urinary NAG induction is the highest (1.65 \pm 0.74).

But, analysing the group of patients treated with combined use of Methotrexate and Ketoprofen, in relation with the distribution of patients according to the values of NAG in the five probes, one could conclude that elevated values of NAG are registered in 31 (89%) of patients in the 8th week (3rd probe), when the degree of mean urinary NAG induction is highest (1.99 \pm 1.00).

Testing the significance in differences in both groups in 0 spot, in the group of patients treated only with Ketoprofen the mean value of the urinary NAG induction ranges 0.93 \pm 0.48, in the group of patients treated with combined use of Methotrexate and Ketoprofen 1.13 \pm 0.54. It shows that Methotrexate is more potent NAG inductor compared with Ketoprofen, but during

their combined use mean urinary NAG induction is increased considering size and time of appearance. Table 1, Figure 1 and 2.

Table 1. NAG, microalbumin and other laboratory variables in the group of patients treated with Ketoprofen only, and in the group with combined use of Ketoprofen and Methotrexate.

| | KETOPROFEN GROUP No 35 | | | KETOPROFEN+METOTREXAT GROUP No 35 | | |
|-----------|---------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|----------------|---------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-----------|
| | NAG +>1.18 (U/mmol/crea) value (M ± SD) normal range (%) / elevated values (%) | MICROALBUMINURIA +>20 (mg/L) value (M ± SD) normal range (%) / elevated values (%) | GFR | NAG +>1.18 (U/mmol/crea) value (M ± SD) normal range (%) / elevated values (%) | MICROALBUMINURIA +>20 (mg/L) value (M ± SD) normal range (%) / elevated values (%) | GFR |
| 0 spot | 1.13±0.54 22 (63%) / 13 (37%) | 17.91±11.17 30 (86%) / 5 (14%) | 99.5±22.2 5 | 0.93±0.48 25 (71%) / 10 (29%) | 16.35±7.41 30 (86%) / 5 (14%) | 95.9±32.6 |
| 4th week | 1.27±0.48 17 (49%) / 18 (51%) | 19.55±11.46 28 (80%) / 7 (20%) | 99.9±24.1 | 1.27±0.47 18 (51%) / 17 (49%) | 18.21±7.63 26 (74%) / 9 (26%) | 97.1±39.4 |
| 8th week | 1.49±0.67 12 (34%) / 23 (66%) | 18.91±13.03 30 (86%) / 5 (14%) | 102.9±3.01 | 1.99±1.00 4 (11%) / 31 (89%) | 20.50±9.69 25 (71%) / 10 (29%) | 98.8±36.1 |
| 16th week | 1.65±0.74 8 (23%) / 27 (77%) | 18.08±11.68 32 (91%) / 3 (9%) | 101.2±2.55 | 1.94±1.02 10 (29%) / 25 (71%) | 17.02±5.64 29 (83%) / 6 (17%) | 95.9±32.6 |
| 24th week | 1.36±0.57 15 (43%) / 20 (57%) | 18.14±12.65 32 (91%) / 3 (9%) | 98.8±22.3 | 1.36±0.83 21 (60%) / 14 (40%) | 14.74±8.56 32 (91%) / 3 (9%) | 98.9±44.3 |

Figure 1. Mean values of N-acetyl- D-glucosaminidase (NAG) in the group of patients with the combined use of Methotrexate and Ketoprofen in the five probes

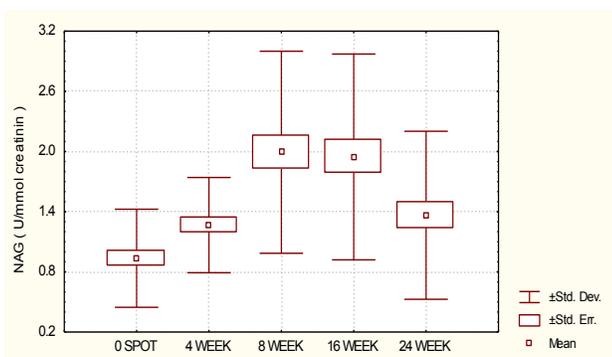
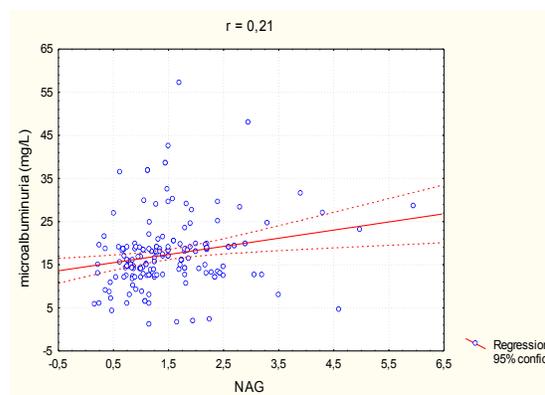


Figure 2. Pearson's coefficient of correlation (r) between the values of N-acetyl- D-glucosaminidase (NAG) and microalbuminuria in the group of patients with the combined use of Methotrexate and Ketoprofen



In the group of patients treated with Ketoprofen, the distribution of patients according to the values of microalbuminuria in the five probes, one could conclude that elevated values of microalbuminuria are registered in 7 (20%) of patients in the 4th week (2nd probe) when the level of microalbuminuria is highest (19.55±11.46).

Analysing the distribution of patients according to the values of microalbuminuria in the five probes, in the group of patients treated with the combined use of Methotrexate and Ketoprofen, we can conclude that increased values of microalbuminuria above normal range are maximally registered in 10 (29%) of patients in the 8th week (3rd probe) with size of induction 20.50±9.69.

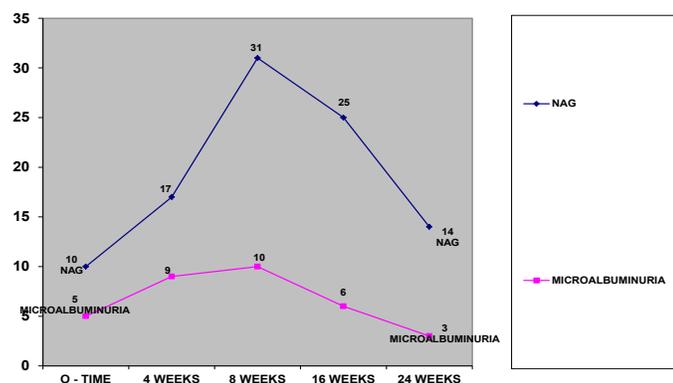
Testing the significance of difference in both groups in 0 spot, in the group of patients treated with Ketoprofen only mean value of microalbuminuria is 17.91±11.17, while in the group treated with combined use of Methotrexate and Ketoprofen is 16.35±7.41. This explains why Methotrexate gives identical appearance of microalbuminuria compared with Ketoprofen, but with their combined use microalbuminuria is increased both in size and time of appearance.

Analysis with Pearson's χ^2 test showed that there is moderate correlation between NAG and microalbuminuria in the five probes in the period of 24 weeks in the group of patients treated only with Ketoprofen.

Analysis with Pearson's χ^2 test showed that there is statisti-

cally significant correlation ($r=0.21$) between the elevation of values of NAG and microalbuminuria in the five probes in the period of 24 weeks in the group with combined use of Methotrexate and Ketoprofen. Figure 3.

Figure 3. Distribution of patients in the group with the combined use of Methotrexate and Ketoprofen according to the elevated values of N-acetyl- D-glucosaminidase (NAG), microalbuminuria and other laboratory variables in the five probes



Nag, Microalbuminuria and Calculated Creatinin Clearance

$Fr\chi^2$ showed that there are not statistically significant differences between mean values of calculated creatinin clearance (CCC) in the five probes in the group of patients treated only with Ketoprofen ($Fr\chi^2=0.1521$; $p=0.9618$), as well in the group with combined use Methotrexate and Ketoprofen ($Fr\chi^2=0.0551$; $p=0.9943$).

Analysing the mean values of CCC in the two groups in 0 probe, in patients treated only with Ketoprofen and in the group of patients treated with combined use Methotrexate and Ketoprofen, ANOVA showed that there are not statistically significant differences between the mean values of the CCC in patients of the two groups: $F=0.130$, $p=0.9419$. There is not statistically significant correlation between the elevation of the mean values of NAG, microalbuminuria and CCC in the two groups. Table 1.

Discussion

In untreated RA, primary is affected tubular apparatus on proximal renal tubules. (36). Glomerular integrity in the group of patients with RA, with mono or combined use of Methotrexate and Ketoprofen stays intact, which is not the case with the

tubular integrity.

Changes in activity of NAG, but also of microalbuminuria is characterised with frequent monophasic excretion. The microalbuminuria follows the parabola of NAG, visually more in the group with combined use of Methotrexate and Ketoprofen, as well with monotherapy with Ketoprofen.

Analysis with Pearson's χ^2 test showed that there is statistically significant correlation ($r=0.21$) between the elevation of NAG and microalbuminuria in the five probes in the follow up period of 24 weeks in the group with combined use of Methotrexate and Ketoprofen, while there is moderate correlation between NAG and microalbuminuria ($r=0.34$) in the five probes in the follow up period of 24 weeks in the group of patients treated with Ketoprofen only.

Initial increase of the activity is a result of the changes in cell synthesis and not always enzymuria can be result of the lytic or necrotic processes. However, with the appearance of the second peak one can be certain of the necrotic process provoked by these drugs. Significant elevation of the activity of NAG is 2-3 times higher than the values gained in the probe with maximal induction (8th week) in combined use of Methotrexate and Ketoprofen (0.93 ± 0.48 vs 1.99 ± 1.00) in correlation with the isolated Ketoprofen (1.13 ± 0.54 vs 1.65 ± 0.74) in the probe with maximal induction (16th week), without bi-phase appearance.

Very important finding in the study, besides high frequency of abnormal urinary excretion of NAG is decrease of the frequency of high NAG values in the course of the combined use of Methotrexate and Ketoprofen. Significant decrease of the NAG excretion is noticed in the 24 week as a result of the reduction of the disease itself by the favourable beneficial effect of Methotrexate on inflammation, correlates with ESR, which is in concordance with the findings of other authors (21-23,26). The kidney is highly plastic organ and decrease of NAG excretion is a result of the adaptive characteristics of the kidney in the presence of external matter.

Lysosomal enzymes show expressible tendency towards normalisation. This probably is result of the regeneration processes which is proved on the level of the tubular epithelium, also noticed by other authors (37). However, that is not the case with the membrane AAP, g-glutamyl transferase, (3 -GT), AF,

which after the treatment stays on high significant levels in terms of decreased activity. Regenerated tubular epithelium shows high resistance towards influences of drugs.

Low dose Methotrexate regime does not cause significant damage of the renal proximal tubules in the most of the followed patients. Nephrotoxicity in the monotherapy with Methotrexate is bigger than with Ketoprofen. Methotrexate is more potent NAG inductor in comparison with Ketoprofen. The size of the NAG enzymuria and microalbuminuria is bigger in monotherapy with Methotrexate in comparison with the isolated use of Ketoprofen, but especially mean urinary NAG induction is increased with the concomitant use of Methotrexate and Ketoprofen. NAG induction, in size is bigger and appears earlier in combined use of Methotrexate and Ketoprofen in comparison with the monotherapy with Ketoprofen. Early detection of high NAG enzymuria or microalbuminuria before the treatment with Methotrexate can be of use considering the anticipation for possible Methotrexate toxicity probably connected with impaired renal clearance of Methotrexate. Parameters as age of patients, duration of disease in months, previous or current treatment, do not allow us to anticipate the decrease of NAG enzymuria.

There is not change of the clinical parameters of the renal function regarding degradation products of the nitric metabolism (kreatinin in serum and urine, GFR) in the course of follow up. The least sensitive markers for early nephrotoxicity caused by Methotrexate and Ketoprofen are concentration of creatinin in serum and urine and urea in serum, as well as the level of CCC. These tests point at the changed, decreased glomerular filtration, but not at the changes in renal tubular function. We think that the use of these parameters can find application in the clinical practice in cases when there is much longer therapy with Methotrexate and Ketoprofen, combined with antibiotics, when they can indicate impairment of the glomerular filtration.

Conclusion

Determination of the urinary NAG together with the urinary creatinin excretion could serve as a more sensitive test for renal lesions in patients suffering from RA, as an additional diagnostic tool, and the information for the status of the disease.

Obtained results in this study confirmed the safety and efficacy of Methotrexate and Ketoprofen in treatment of RA. Following the renal function with enzyme activity in urine for evaluation of the effective metabolic exfoliative turnover of tubular cells, avoidance of the frequent use of drugs, as well as individual adaptation of the doses are measurements for avoidance of the nephrotoxicity of these drugs.

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