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NEPHROTOXICITY OF NSAIDS AND MTX

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Abstract

Rheumatoid arthritis (RA) is a chronic disease requring potencial nephrotoxic therapy with nonsteroidal antinflamatory drugs (NSAIDs) and disease modifying antireumatic drugs (DMARDs). The aim of this study is to identify early renal changes by means of specific biomarkers, as the most sensitive parameter.

Methodology: 100 patients (80-RA^{sero+}, 20-RA^{sero-}) with chronic rheumatic pain were treated with NSAIDs and 8 to 16 weeks by metotrexat (MTX) in comparison with the control group. The follow up was 3 times during the treatment. Besides conventional markers of renal function (serum/urine creatinin determined by Jaffe methods, enzymaticassay for urea in serum and GFR calculated by Cockcroft Gaunt formulas) we used imunoturbodimetric assay for urine α1 Microgloglobulin (α1M) and microalbuminuria, to monitor glomerular and tubular functioning. Any history of kidney disease was exclusion criteria to enter the study.

Results: Following 16 weeks' treatment with combined therapy with NSAIDs and MTX, no changes were found in the serum creatinine and serum urea, compared with the specific biomarker α1 Microgloglobulin (α1M) and microalbuminuria in all patients (RA $^{sero+}$ and RA $^{sero-)}$ with 99% interval of confidence (CI), and probability of $p<0.01$ compared with the control group of healthy patients.

Conclusion: We found changes on the glomerular and tubular level, despite the normal values of all the assayed conventional markers for renal function, and we confirmed their sensitivity. We can't confirm the nephrotoxicity, but, if we follow the elevation of the level of the specific biomarkers, we can use them as early signals for nephrotoxicity.

Keywords:Biomarkers, Nephrotoxicity, Nonsteroidal antiinflamatory drugs, Rheumatoid arthritis**.**

Introduction

The first drugs of the anti-inflammatory analgesic group (NSAID) is acetylsalicylic acid, which was synthesized by Feliks Hofman in 1897. Indomethacin was synthesized in 1960. In 1971, cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX -2), leading to the detection of the NSAID mechanism, and later COX 3 (COX-3) was discovered, which contributed to the detection of new types of NSAIDs. Today, these drugs are among the most used drugs in the world. About 30 million people consume daily, about 70 million are in regular therapy and are estimated to be about 500 million times a year (Seager et al., 2001). The use of analgesic, antipyretic and anti-inflammatory NSAIDs in the treatment of various rheumatic diseases and other diagnoses is a major achievement in medicine.

About 1% of the general population in the world is attacked by rheumatoid arthritis (RA) and is treated with many NSAIDs and other medicines as disease-modifying antirheumatic drugs (DMARDs) (Gavdel et al., 2013). Any NSAID treatment aims to reduce the duration of the pain as well as preventing complications of the disease (Griffin et al., 2000; Gokcen et al., 1983). Despite many positive effects, however, NSAIDs do not meet the expected results. In recent years, many studies have confirmed that the use of NSAIDs for the treatment of rheumatic diseases has some side effects, the most common being gastrointestinal, renal, hematopoietic, cardiovascular and others. Negative gastrointestinal effects are more investigated and quantitative, but much less known for the nephrotoxic effect of NSAIDs (Huerto et al., 2005).

The NSAID action is based on blocking cyclooxygenase, an important enzyme for the synthesis of prostaglandins. Cycloxigenase 1 (COX-1) synthesizes prostaglandins necessary for normal physiological functions of the organism, and cyclooxygenase 2 (COX-2), which is normally not present in the organism and is only present in the inflammatory processes and participates in the synthesis of pathological prostaglandins mediating inflammation. It is understood that blocking COX-2, can stop

the inflammatory process. The paradox is that most NSAIDs block and COX-1, not just COX -. 2, which means that these drugs block the synthesis of all prostaglandins, as well as normal physiological functions (Parezela et al, 2001). The non-specific blockage of the enzyme leads to a continuous vasoconstrictive activity of leukotrience, angiotensin II, vasopressin, endothelin and catecholamines. In persons with balanced status of electrolytes and fluids, there is no reduction in the glomerular filtration rate (GFR), but in conditions of kidney hypoperfusion it can result in: - Acute kidney injury (AKI). According to many authors, damages are completely reversible after 24 hours of termination of therapy (Adams, et al, 1986; Blackshear, 2012; Koppert et al., 2006; Penders et al 2004).
The problem gains larger dimensions in patients with high predisposition for nephrotoxicity described in "at-risk" patients, including methotrexate-treated patients, in the continued use of NSAIDs (Unswort et al, 1987; Welton, 1999).

NSAIDs and Methotrexate (MTX)

Recently, methotrexate (MTX) has been successfully used in combination with NSAIDs for treating RA. According to Gawdel (Gawdel et al., 2013), 15% of RA patients were using only MTX, but in many patients they were used in combination with NSAIDs. According to Vucinic (Vucinic, 2000), renal excretion of MTX is performed through glomerular filtration and active tubular excretion and during the use of drugs that reduce renal blood circulation, such as NSAID, may prolong the elimination of MTX. Numerous clinical studies show that the combination of NSAIDs with MTX can cause a reduction in MTX elimination, even at relatively low doses, as well as the risk of increased nephrotoxicity, as well as the higher MTX concentration level in serum appears as an additional risk (Perin et al., 1990; Grönroos, 2008). The use of ketoprofen, indomethacin or diclofenac in patients receiving MTX leads to a different level of toxicity, including nephrotoxicity. Azza et al. has shown a different level of nephrotoxicity in patients treated with combination therapy with MTX and NSAID (Azza et al., 2006). Also, according to Pathan et al in the chronic administration of NSAID and MTX combination therapy in RA patients, an asymptomatic increase in serum creatinine levels was observed (Pathan et al., 2003).

However, according to Svendsen and Spasovski, in the treatment of RA patients with other anti-rheumatic drugs (DMARDS), with normal urine creatinine did not detect renal impairment during tracking enzim and albumin in urine (Svendsen et al, 2005; Spasovski et al., 2007; Spasovski et al, 2008a). However recent studies suggest that kidney damage associated with NSAIDs and MTX can be more extensive than previously thought (Unswort et al., 1987).

Most chronic rheumatoid arthritis (RA) patients treated with various non-steroidal anti-inflammatory drugs (NSAIDs) show reversible microalbuminuria that rarely develops until the last stage of renal insufficiency. Subclinical renal impairment can not be identified with routine tests (serum / urine creatinine, serum urea and glomerular GFR) which, besides being non-sensitive and non-specific, do not allow early identification of renal impairment (Liangos et al , 2007), and serum creatinine changes when there is damage to renal function more than 50% (Prasad et al, 2005). Microalbuminuria is a sensitive indicator during kidney dysfunction and microalbuminuria monitoring can be used as a useful tool for managing RA patients without clinical nephropathy as indicators of glomerular function (Penders et al., 2004; Spasovski et al., 2007; Spasovski et al, 2008a). Previously, microalbuminuria was detected during short or prolonged therapy in nephrotoxic agents, such as Cisplatin, Ifosfamide and MTX, as well as antibiotics, such as Gentamicin (Vaidya et al., 2008). The effects of NSAID on glomerular and tubular function may be of clinical significance and should be followed by more sensitive methods

Urinary excretion of albumin and protein with molecular mass greater than (> 80000) is indicative of glomerular changes , while small molecule mass proteins such as α1 - Microgloglobulin (α1M) indicate changes in the tubules.
(Davis et al, 1994).According to Belleit and associates during the NSAID misuse, the massive expression of α1M protein in the urine is detected (Bellei et al, 2012). Compared with β2M, urinary α1M is more stable at PH intervals in clinical routine practice, making it the most preferred biomarker for tubular proteinuria in humans. It has been found to be a specific biomarker for proximal tubular dysfunction, even in the early stages of injury, before histologic lesions are identified (Kern, et al., 2000).
There are still outstanding dilemmas associated with nephrotoxicity in the combination of NSAIDs with other drugs such as (MTX). (Orfeas et al., 2009). As a challenge to address some of the aforementioned issues, we have set the purpose of the work. to identify early changes in renal function in patients treated with combination therapy with MTX and NSAID following urinary levels of microalbuminuria and α1M.

Materials and Methods

For the determination of certain purposes, we used urine and venous blood samples from 100 patients, of whom 80 meet the criteria for RA $^{sero+}$ and 20 for $^{sero-}$ RA status (based on the immunoturbodymmetricdetermination of Reuma Factor-RF) suffering from chronic rheumatic pains treated with combined NSAID and MTX therapies for the prevention of disease complications, with a minimum dose of 7.5 mg to 10 mg once per dose. Patients were followed before therapy with MTX and after 8 and 16 weeks. The results are compared with reference intervals and control group of 80 healthy individuals. Patients were informed about the mode of application and the purpose of the research before giving the written statement. The search was conducted according to

the protocol designed according to the ethical principles of the Helsinki Declaration for Medical Research on People (WMA, 2000), and all the analyzes were processed according to the laws of good laboratory practice.

The presented results represent the average value of the three measurements, under identical conditions. As a sample for analysis, it was used by 5 ml of blood collected in special tubes without anticoagulants, all the material for analysis was transported to the Clinic Hospital-Tetovo and Clinical Biochemistry in Skopje, in a special refrigerator at 20-80C. The serum obtained after centrifugation at 3000 rotations / min for 10 to 15 minutes was used to determine parameters .

For the determination of creatinine and specific proteins (α1M and microalbuminuria), the first morning urine was used. After proper processing the pure supernatate is used for further processing. (D.Yzeiri Havziu et al, 2016).

Method of work

For creatine serum / urine, is used the Jaffe method - during the reaction of the creatinine with the basic reagents (Flex reagent cartridge) a complex of red color is formed which is followed by measuring the change of absorbance at a time interval of 510 nm (Dimension Rxl).

Urea serum, the enzymatic-urea hydrolysis under the influence of the urease enzyme, the formed ammonia (NH3) reacts with the catalytic effect of the GLDH (Flex Reagent Cartridge), α-KG (Flex Reagent Cartridge) and NADH (Flex Reagent Cartridge). As a result of the reaction, glutanamic acid and NAD are formed. The decrease in absorbance due to the reduced NADH oxidation is proportional to the release of the urea NH3. Measured at a value of 340, 383nm (Dimension Rxl).

GFR with the Cockcroft Gaunt formula.

For the determination of urinary albumin, microalbuminuria and α1-M, the immunoturbodymmetric method (Cobas Mira Plus) (D.Yzeiri Havziu et al, 2016).

Statistical data processing

Statistical data processing is done in Microsoft Excel, calculating the average value (M) and the standard deviation (standard deviation, SD). Differences between variance of patients during the course of therapy are recorded by variance analysis (ANOVA) and Student T-test.

Results and discussion

100 patients from whom 80 meet the criteria for RA $^{sero+}$ and 20 for RA $^{sero-}$ (based on immunoturbodymmetric determination of -RF), were treated with NSAID and (MTX) therapy during continuous monitoring of renal impairment in the post-treatment period before MTX treatment and after 8 and 16 weeks in comparison to the control group of healthy individuals according to the protocol, which includes the parameters that are presented in Table 1

Table 1 It is noted thatvalues of urea serum, creatinine of serum/ urine and GFR in patients$RA^{sero(-)}$ and patients with RA $^{sero(+)}$ status compared to the control group of individuals prior to therapy, as well as after 8 and 16 weeks with MTX therapy, no significant statistical difference was found in any parameter

Biochemical parameters	RA sero(-) n=20 M±SD	P value	RA sero(+) n=80 M±SD	P value	Control group n=80 M±SD	Reference values
Urea (serum) mmol/L *M±SD AT* *M ± SD for 8 weeks M ± SD for 16 weeks M±SD*	 5.8±1.1 6.8±1.8 5.8±1.4	p=0.0518	 5.45±1.3 5.9 ±2.1 5.3±1.7	p=0.076	5.65±1.3	2,0 – 8,3
Creatinin (serum) µmol/L *M±SD AT* *M±SD for 8 weeks* *M±SD for 16 weeks*	 82.6±15.2 79.5±12.6 82.3±15.2	P=0.755	 89.1±12.7 83.1±16.5 84.6±18.9	p=0.054	82.9±14.3	45 – 115
Creatinin (urina) mmol/du *M±SD AT* *M±SD for 8 weeks* *M±SD for 16 weeks*	 7.0±3.9 8.2±3.6 9.8±3.9	P=0.074	 7.5±2.1 7.8± 3.7 8.9±5.1	p=0.0539	7.5± 4.2	Men5,3-22,1 Females5.1-3.3
GFR ml/min *M±SD AT* *M±SD for 8 weeks* *M±SD for 16 weeks*	94.6±23.5 92.0±22.4 98.2±20.4	P=0.675	97.2±19.6 90.2± 24.2 96.3±19.5	P=0.08	110.3± 27.3	125 ±15

p <0.01 ** represents a very high statistical difference at values with a 99% confidence interval
p <0.05 * statistically significant difference in values with a safety interval of 95 %.

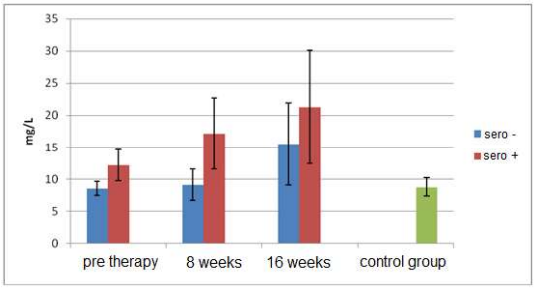
From the results obtained we note that no changes in the values of conventional markers have been observed. According to Liangos, they are termed as traditional biochemical parameters and at the same time thought to be non-specific and non-sensitive for early discovery of glomerular and tubular damage, as well as for adequate detection and differentiation of different stadiums acute renal impairment (Adams et al, 1986, Liangos et al, 2007). The findings correlate with results of Spasovski and associates who during the 24-month follow-up of the group of patients treated with ketoprofen and the group of patients treated with combination therapy ketoprofen and MTX with parameters (serum urea, creatinine serum and urine) no changes were confirmed..

However, Perini and Seidman have proven the opposite, in combination therapy with NSAID and MTX, a reversible increase in creatinine and urea levels (Perrin et al., 2006), and decline in GFR (Seideman et al., 1993) where renal dysfunction corresponds to MTX dose (Perrin et al, 2006). Also, according to Pathan et al. (Pathan et al., 2003) in the chronic administration of NSAID and MTX combination therapy in RA patients, an asymptomatic increase in serum creatinine levels was observed.

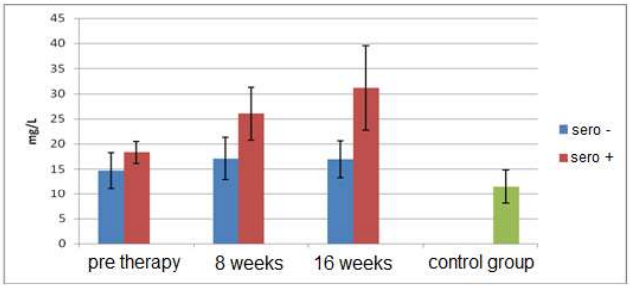
Given the fact that serum creatinine and some of the biochemical parameters according to standard nephrology protocol differ when 50% of renal function is reduced, the most sensitive biomarkers are monitored for early identification of renal impairment in RA patients. For this reason further studies with more specific urine biomarkers for renal dysfunction in the glomerular and tubular levels are needed (Figure 1).

Monitoring of microalbuminuria and α1M for 80 patients with RA$^{sero\ (+)}$ and 20 patients with RA $^{sero\ (-)}$ status in the pre-MTX therapy period and during MTX treatment of 8 and 16 weeks with MTX are graphically described in Figure 1 .

a)



b)



From Figures1 during a)microalbuminuria and b) α1M monitoring in patients with RA $^{sero\ (+)}$ and R $^{sero\ (-)}$ status, compared to the pre-treatment group and after 8 and 16 weeks of combined MTX therapy significant statistically significant change in safety intervals of 99% and p <0.01, which again confirm the changes observed at glomerular and proximal tubule levels (Figure1and 2).

If the values are compared only from the 16th month, with the control group of individuals, results are obtained that suggest us for apparent changes in epithelial cells in proximal tubes (Table 2).

Specific biomarkers	**RA $^{sero(-)}$ n=20 M±SD**	**P value**	**RA $^{sero(+)}$ n=80 M±SD**	**P value**	**Group control**	**Reference values**
Mikroalbuminurija mg/L *M±SD* for 16 weeks	16.93±3.70	p‹ 0.01 **	31.16±8.45	p‹ 0.01**	11.43±3.3	2-20
α 1 M mg/L *M±SD* for 16 weeks	15.52±6.45	p‹ 0.01 **	21.28±8.88	p‹ 0.01**	8.7±1.4	Up to 12

p <0.01 ** represents a very high statistical difference at values with a 99% confidence interval

p <0.05 * statistically significant difference in values with a safety interval of 95 %.

From a clinico-biochemical point of view, this suggests that combined MTX therapy significantly affects changes in proximal tubular epithelial cells, which is directly related to increased urinary secretion of α1M low molecular weight proteins (early biomarkers for tubular dysfunction) . The prospect of α1M compared to other biomarkers is its stability within the pH values of routine analyzes. Based on the microalbuminuria follow-up (as a marker for early identification of renal impairment at the glomerular level), glomerular changes have been detected, which may be presented as complications of the disease itself or under the influence of combined MTX therapy.

The obtained results correspond to the data of Azza et al. Who demonstrated different levels of nephrotoxicity in patients treated with MTX and NSAIDs (Azza et al., 2006). But Mandel and colleagues confirmed that MTX toxicity has increased in patients with renal insufficiency due to prolonged drug excretion. which is performed mainly by the glomerular filtration kidneys and tubular secretion (Mandel et al., 1976). Similar results have been demonstrated by numerous clinical studies with relatively low doses of Ketoprofen, Indomethacin and Diclofenac with MTX, causing different levels of toxicity. (Thyss et al., 1986. Stewart et al., 1991; Cassano et al., 1989; Singh et al., 1986).

Conclusion

- There are changes in the glomerular and tubular levels, although other biochemical parameters according to the nephrological protocol did not show any change;
- Sensitivity of the most sensible bio-indicators has been verified to detect nephrotoxicity at an early stage:
- We cannot confirm nephrotoxicity with this method. We realized that we need long-term research based on histopathological data for these, but we concluded that if we follow the set up of biomarkers, we can use them as early signals for nephrotoxicity due to the changes at this stage which are reversible and after cessation of therapy, the situation may be normalized.

Bibliography:

1. Adams, D.H., Homer, A.J., Michael, J., Mc Konkey, B., Bacon, P. 1986 *Adu.Nonsteroidal anti-inflammatory drugs and renal failure,*Lancet.;1:57-59.
2. Azza.A.K El Sheikh. 2006. *Interaction of NSAID with MRP 2 / ABCC2 and MRP4/ABCC4 –Mediated Metotrexat Transport*, JPET #110379 27
3. Blackshear, J.L., Napier, J.S., Davidman, M., Stillman, M.T.1985. *Renal complications of nonsteroidal anti-inflammatory drugs: Identification and monitoring of those at risk Semin Arthritis.* Rheum.14:165-75.
4. Bellei, E., Cuoghi, A., Monari, E., Bergamini, S., et al. 2012. *Proteomic analysis of urine in medication – overuse headache patients: possible relation with renal damages*. J Headache pain.;13:45-52.

5. Cassano,W.F. 1989. *Serious methotrexate toxicity caused by interaction with ibuprofen.* Am J Pediatr Hematol Oncol 11**:** 481**–**482.
6. Davis, M.E., Berndt, W. 1994. *Renal methods for tocicology. Principles and methods of toxicology. Third edition,* Raven Press Ltd, New Yor.;871-894.
7. .D.Yzeiri HavziuM.Hiljadnikova-Bajro,T. Kadifkova PanovskaN.Bexheti.2016.*Renal function of patients with rheumatoid arthritistreated withpiroxicam;*Acta Medica Balkanica/International Journal of Medical Sciences;VOL1NO2PP.1-13

8. Gawdel, S.R., Shetty, Y.C., Merchant, S., Kulkarni1, U.J.et al..2013.*Drug Utilization Pattern and Cost Analysis in Rheumatoid Arthritis Patients – A Cross– Sectional Study in Tertiary Care Hospital, Mumbai.*British Journal of Pharmaceutical Research 3(1): 37-45.
9. Griffin, M.R., Yared, A., Ray, W.A..2002. *Nonsteroidal anti-inflammatory drugs and acute renal failure in elderly persons.* Am J Epidemiol;151:488-496.
10. Grönroos M. 2008. *Treatment-related renal side-effects in pediatric cancer patients.* Sarja - Ser. D OSA - tom. ;824 Medica – Odontologica.
11. Huerto, C., Castellsague, J. et al. 2005.*Nonsteroidal antinflamatori drugs and risk of ARF.* Am J kidney; Dis ;45;531-539.
12. Kern, W., Braess, J., Kaufmann, C.C., Wilde, S., Schleyer, E., Hiddemann,W. 2000.*Microalbuminuria during cisplatin therapy: relation with pharmacokinetics and implications for nephroprotection .*
13. Koppert, W., Frotsch, K. et al.. 2006.*The Effects of Paracetamol and Parecoxib on Kidney Function in elderly Patients Undergoing Orthopedic Surgery.* Anesth Analg;103:1170-6.
14. .Liangos, O., Perianayagam, M.C., Vaidya, V.S., Han,W.K., Wald, R. et al. 2007 *Urinary N-acetyl-beta-(D)- glucosaminidase activity and kidney injury molecule-1 level are associated ëith adverse outcomes in acute renal failure.* J. Am. Soc. Nephrol; .18:904**–**12.
15. Mandel MA.1976.*The synergistic effect of salicylates onmethotrexate in man.* Plast Reconstruct Surg, 57:733**–**7.
16. Orfeas L. , Hocine Tighiouart M.S. et al. 2009. *Comparative Analysis of Urnary Biomarkers for Early Detection of Acute Kidney Injury Following Cardiopulmonary Bypass.* NIH-Public Access;14(60):423-431
17. Penders, J., Delanghe, J.R. .2004 *Alpha 1-microglobulin: clinical laboratory aspects and applications.* Clin. Chim. Acta;346:107**–**18.
18. Pathan, E., Shilpa, G., Rajadhyaksha, S., Sule Gayatri Mittal, A., Joshi, VR. 2003. *A Longitudinal Study of Serum Creatinine Levels in Patients of Rheumatoid Arthritis on on Long Term NSAID Therapy.* JAPI • NOVEMBER VOL. 51; 1045:1049.
19. .Parezela, M.A., Tras, K.. 2011.*Selective cyclooxygenase-2 inhibitors: a pattern of nefrotoxicity similar to traditional nonsteroidal anti-inflammatory drugs.* Am J Med;111:64-67.
20. Prasad, D., Jonthan, M..2005. *Barasch Method for early detection of renal injuryc.* New Jork,NY (US) ;116/096,113.
21. Perrin, A. et al. 2006. *Biohemical and pharmacological consecuences of the intereaction between methotrexat and ketoprofenin the rabit.* 36 voie Romaine 2006;,06054 Nice France.
22. Seideman, P. et al. 1993. *Renal effects of Aspirin and Low dose Methotrexate in rheumatoid Arthritis.* AN. Rheum. Dis.;52:613-615 doi
23. Singh, R.R., Malaviya, A.N., Pandey, J.N. and Guleria, J.S. . *Fatal interaction between methotrexate and naproxen.*Lancet, 1;1986 **:** 1390.
24. Seager, J.M., Hawkey C.J. 2001. *Indigestion and Nonsteroidal antinflamatori drugs.* BMJ;3231236-1236-1239.
25. Spasovski D. Gruev T. et al.b. 2007. *Vrednost N-acetil-ß-D-glukozaminidaze i mikrolabuminurije kod reumatoidnog artritisa.*Journal fo Medical Biochemistry;Vol. 26,Br.4, str.300-308.
26. Spasovski D., Gruev T., Marina N., Calovski J., Rajcevska L., et al.,a. 2008.a*Alanine Aminopeptidase, g-Glutamil Transferase and b2-microglobulin as diagnostic markers in patients with rheumatoid arthritis*.JMB ;27: 59**–**63.
27. Stewart, C.F., Fleming, R.A., Germain, B.F., Seleznick, M.J. and Evans, W.E. 1991. *Aspirin alters methotrexate disposition in rheumatoid arthritis patients.* Arthritis Rheum;34**:**1514**–**1520.
28. Thyss, A., Milano, G., Kurbar, J., Namer, M., Schneder, M. 1986. *Clinical and Pharmacokinetic evidence of a life-thretaening interaction between methtrexate and ketoprofen.* Lancet;I:256-258.
29. Unsworth, S.Struman, J.Lunec and Blake. 1987. *Renal imparment associted with non-steroidal anti-iflamatori drugs.* Annals of the Reumatic Disease ;46,233-236
30. Vishal S. Vaidya, Michael A.Ferguson and Joseph V. 2008. *Bonventre Biomarkers of Acute Kidney Injury.* Annu Rev Pharmacol Toxicol,:48:463-493:doi:10.1146/annurev .pharmtox.48113006.094615.
31. Vucinic V.M . 2000. *What is the future of methotrexate in sarcoidosis ?* A study and revive Current in Pulmonary medicine:8:470-476.
32. Whelton, A. 1999.. *Nephrotocicity of nonsteroidal antiinflammatory drugs:Physiologic foundations and clinical implications.* Am J Med;106:13s-24s.
33. WMA-World Medical Association Declaration of Helsinki Ethical principles for medical research involving human subjects.2000. JAMA ;284:3043-3045.