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CONTRIBUTION OF TOLL-LIKE RECEPTORS AS A THERAPEUTIC TARGET IN ADJUVANT INDUCED ARTHRITIC RAT MODEL

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ABSTRACT:

Background: Rheumatoid arthritis (RA) is an inflammatory joint disease characterized by a variety of abnormal cellular processes. Toll-like receptors (TLRs) have been linked to immunological abnormalities in RA patients.

Objectives: The purpose of this study was to look at the role of TLR2 and TLR4 in progression of arthritis and their modulation by N-(2-hydroxyphenyl) acetamide.

Material and Methods: Arthritis was induced in rats by Heat-killed Mycobacterium tuberculosis (MT37Ra). Progression and impact of disease was observed macroscopically and at molecular level. Reactive oxygen species (ROS) and antioxidants production was estimated in serum to assess the immune response.

Results: We observed over expression of TLRs on bone marrow cells parallel to the severity of arthritis progression in arthritic control group. Over-expression of TLRs triggered ROS expression followed by a decrease in antioxidants resulting in destruction of bones and cartilage in arthritic control group. Treatment with N-(2-hydroxyphenyl) acetamide (NA-2) reduced severity of arthritis by reducing the expression of toll like receptors TLR2 ($^{*}P < 0.043$) and TLR4 ($^{**}P < 0.001$) on BMCs in arthritic rats. Production of Reactive oxygen species, a marked increase in the GSH and SOD.

Conclusions:

Our findings suggest that TLRs may play a role in the etiology of arthritis by causing direct activation of BMCs. Our data further suggest that NA-2 inhibits TLR-mediated joint inflammation and other arthritis-related symptoms, implying that it could be used to treat arthritis.

Keywords: Pro-inflammatory cytokines, reactive oxygen species, antioxidants. N-(2-hydroxyphenyl) acetamide

1. INTRODUCTION

Rheumatoid arthritis is a chronic inflammatory, systemic autoimmune disease which results in progressive joint damage and lifelong disability (1). RA is characterized by inflation of inflammatory cells in synovium along with synovial hyperplasia, pannus formation, angiogenesis and cartilage damage which may lead to bone degradation (2). Unusual activation and phenotypic changes in circulating neutrophil in RA has already been observed in previous studies. Delayed apoptosis and increased reactive oxygen species (ROS) and cytokines production leads to destruction of cartilage and bones of joints (3-6).

Toll-like (TLRs) receptors are special receptors that are expressed on innate immune cells and can recognize special patterns. They are responsible for maintaining the inflammatory loop in chronic inflammation and have a significant role in progression of RA (7). These receptors are expressed both extra cellularly and intracellularly. When they are expressed extracellularly, they can recognize lipopolysaccharide (LPS), flagellin, and dsRNA which are bacterial products and are referred to as pathogen associated molecular pattern (PAMPs) (8). Some toll like receptors like Toll-like receptors (TLRs)- 3, 7, 8 and 9 are expressed intracellularly and can be activated by self and foreign nucleic acid structures. TLR-2 can recognize lipoprotein of gram-positive bacteria whereas lipopolysaccharide of gram negative bacteria can be recognized by TLR-4 (9,10). It is supported by various studies that TLRs have significant impact in the progression of various chronic inflammatory diseases like rheumatoid arthritis. TLRs can be expressed on various immune cells including B cells, T cells, macrophages, and dendritic cells. When TLRs are stimulated it results in increased production of pro-inflammatory cytokine, and up regulation of co-stimulatory molecules due to which they form a bridge between innate and adaptive immunity. In addition to the exogenous ligands derived from bacteria and viruses, TLRs can also be activated by endogenous proteins and oligosaccharides. The endogenous ligands are released from damaged tissue and are known as damage associated molecular patterns (11,12). Other endogenous ligands include a group of proteins called acute phase proteins, for example amyloid A protein. TLRs signaling involves two inflammatory cascades which are MyD88 dependent and MyD88-independent or TRIF pathway. Both of these pathways result in stimulation of nuclear factor kappa-B (NFkB) and production of pro-inflammatory cytokines (13).

Reactive oxygen species (ROS) are unstable molecules which contains oxygen and peroxides and can easily react with other molecules. They are formed as by products during the metabolism of oxygen in electron transport chain in mitochondria (14,15). In inflammatory disorders they also act as signaling molecules leading to progression of disease. During inflammation, oxidative stress produced by polymorph nuclear leukocytes (PMNs) causes an opening at inter-endothelial junction allowing the migration of inflammatory cells across the endothelial barrier. The function of migrated cells is to clear the pathogens but in RA these migrated immune cells interact with resident cells in synovial tissue resulting in qualitative alteration of cell phenotype and increased inflammation

. Migrated leukocytes to the joints develop both innate and adaptive responses causing a powerful burst of oxidative stress under the influence of inflammatory factors (16,17). During this period, a large amount of oxygen is consumed and converted into a variety of free radicals forming a local environment of highly reactive oxygen species, which plays a role in the occurrence and persistence of RA (18). Reactive oxygen species (ROS) are molecules with at least one oxygen atom and one or more unpaired electrons and can exist independently (19). This group includes oxygen and nitrogen radicals as well. Under physiological conditions, they are formed in small amounts in cells and participate in oxygen respiration. In addition, ROS are primarily signaling molecules and can induce cell differentiation and apoptosis. There is a balance between the formation and clearance of free radicals in the body (20). When ROS are overproduced, antioxidant system of the body gets damaged, leading to tissue damage, which is called oxidative stress Currently, the evidence strongly suggests that oxidative stress plays a role in the pathogenesis of RA (21). According to one study, there is a

strong link between oxidative stress levels and synovial fluid oxidative damage markers (22). ROS has strong positive relationships with clinical and biochemical indicators present in the blood of RA patients (23). ROS could be useful indicators for tracking disease development. large amount of ROS produced by phagocytes, recruited immune cells and proliferating synovial stromal cells in the RA synovitis microenvironment, but the complex interactions between ROS and these cells remain unclear. Therefore, the primary aim of present study is to evaluate the role and molecular mechanisms of ROS in various cells in the RA synovial microenvironment and summarize the therapeutic intervention of ROS to restore the redox balance in RA patients, providing a new research direction for treatment. In RA patients, reactive oxygen intermediates (ROI) are abundant in the synovial fluid (SF) and peripheral blood, while glutathione (GSH) antioxidant protection may be impaired (24-25). This could be due to inflammatory processes or dietary insufficiency of stimulation of TLR-2 and TLR-4 on synovial tissue resulting in increased production of pro- inflammatory cytokines which could further flare-up the inflammation associated with arthritis. Blocking TLR-2 or TLR-4 or down-regulating their expression can decrease the spontaneous release of pro- inflammatory cytokines from synovial explants (27-29). Therefore, TLR inhibitors can be used as potential therapeutic agent for the treatment of rheumatoid arthritis. Keeping in view the role of TLRs, the present study was carried out with the purpose of exploring the effect of novel anti-arthritic compound N-(2-hydroxyphenyl) acetamide (NA-2) on the expression levels of TLRs, downstream ROS and antioxidants in AIA model of rat. NA-2 is a derivative of salicylic acid and has shown a very promising anti-inflammatory and anti-arthritic activity in our earlier studies (30). Therefore, we aimed to explore its therapeutic effects on the above d mentioned marker involved in the pathogenesis of RA.

2. Materials and Methods

2.1. Animals

Female Sprague Dawley rats (SD) weighing 160-240 g were used for the experiments. Five to six animals were housed in a cage in a controlled humidity and temperature environment with a 12/12- h dark-light cycle. Animals were given normal diet which includes standard laboratory rat food pellets and water. Ethical guidelines of the International Association for the Study of Pain in conscious animals (31) and the Scientific Advisory Committee on Animal Care, Use, and Standards, International Center for Chemical and Biological Sciences (ICCBS) were followed to care for the experimental animals.

2.2 Adjuvant/Drugs:

Disease was induced in the animals by Heat-killed Mycobacterium tuberculosis (MTH37Ra) purchased from DIFCO Laboratories (Detroit, MI, USA). It was used as adjuvant and injected at the tail base of each animal. Indomethacin was used as reference drug and N-(2-hydroxyphenylg) acetamide (derivative of salicylic acid) was used as test drug and were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.3. Preparation of adjuvant:

A uniform suspension of heat killed mycobacterium (MTH37Ra) in mineral oil (MP Biomedicals, Germany) with final concentration of 10 mg/ml was prepared at the beginning of each experiment. It acts as complete Freund's adjuvant (CFA).

2.4. Induction of Arthritis & Treatment:

Arthritis was induced by injecting 0.1ml of freshly prepared adjuvant intradermally at the tail base of each animal under anesthesia (32). After the induction of disease, animals were carefully monitored until the arthritic score 4 was developed in the arthritic control group, receiving no other treatment. Treatment with reference and test drug was started on the day of induction of arthritis. Grouping of animals and treatment given to the animals are shown in table 1. Treatment doses were set by our preliminary dose finding studies and literature review.

Table 1: Treatment regime followed for the administration of N-(2-hydroxyphenyl) acetamide or indomethacin (n = 12/ group).

Group	Treatment Given	Doses	Route of Administration
GI *(Arthritic Control)*	-	-	-
GII *(Normal Control)*	-	-	-
GIII *(Drug Control)*	Arthritic animals receiving Indomethacin	5 mg/kg/day	Intraperitoneal (i.p.)
GIV *(Test Group)*	Arthritic animals receiving N-(2-hydroxyphenyl) acetamide	5 mg/kg/day	Intraperitoneal (i.p.)
GV *(Test Group)*	Normal animals receiving N-(2-hydroxyphenyl) acetamide	5 mg/kg/day	Intraperitoneal (i.p.)

2.5. Assessment of Disease Progression:

Progression of disease was measured by observing rise in paw volume, decrease in body weight, and increase in pain sensation on alternate days after induction of disease (33).

2.6. Assessment of Reactive Oxygen Species& Endogenous antioxidants:

After establishment and validation of model Reactive oxygen species (nitric oxide and peroxide) and endogenous antioxidant (glutathione and superoxide dismutase) levels were measured calorimetrically in the serum samples of animals using kit method (Bio Assay system, CA, USA).

2.7. Isolation of Bone Marrow Cells (BMCs):

Cells were obtained under sterilized conditions from the rats' femur bones at the end of each experiment and used for BMC isolation and culture. Cells were cultured in DMEM cell culture media (supplemented with 5% FBS. 1% penicillin/ streptomycin 11% amphotericin B, 1% glutamine, and 1% pyruvate). Confluent cells were used for reverse transcriptase polymerase chain reaction (RT-PCR) for mRNA expression and protein of TLR2 and TLR4.

2.8. Determination of mRNA Expression of TLRs by RT-PCR:

2.8.1 RNA Isolation:

The primary cultured BMCs were trypsinized and centrifuged (800-1000 rpm) for 20 seconds. The supernatants were discarded and the cells pellets were used to isolate RNA using SV total RNA isolation system (Promega).

2.8.2 c DNA synthesis and RT-PCR:

The isolated RNA samples were reverse-transcribed into cDNA using oligo deoxy thymidine (dT) primers and first strand cDNA synthesis kit (USB, Corporation). Manufacturer protocol was followed for cDNA synthesis. The transcribed cDNA was amplified by Reverse Transcriptase-Polymerase Chain Reaction, using Omni script RT kit (QiagenInc Valencia, CA, USA) and oligonucleotide primers of respective genes.

2.8.3. Gel Electrophoresis:

After amplification DNA sequence was resolved by 1% agarose gel electrophoresis. The bands were visualized in Gel-Dock System (Fluor Chem, Alpha INNOTECH) under UV- lamp. Density of each band was quantified. For mRNA analyses, housekeeping gene, GAPDH density was used to normalize the density of amplified gene product. The integrated density value (IDV) was obtained through the corresponding pixel intensity using Spot density Tools of the Gel-Doc system for comparison purposes.

3. RESULTS:

After induction for arthritis, severity and progress of disease was closely monitored by measuring paw volume, body weight and nociception. Clinical feature of disease was further validated by measuring level of reactive oxygen species and antioxidant in the serum.

3.1. Measurement of Reactive Oxygen Species Levels in Serum:

A significant increase in the serum concentration of nitric oxide (NO) (*P< 0.001) and peroxide (PO) (*P < 0.004) was observed in arthritic rats as compared to normal control animals (Table 2). We observed a decrease in NO and PO serum concentration of arthritic rats after treatment with indomethacin and NA-2 (Table 2).

Table 2: Effect of NA-2 (5 mg/kg dose) on serum level of reactive oxygen species and endogenous antioxidants. *p < 0.005 is taken as significant value.

Groups of Animals	Reactive oxygen species (mg/dl)		Endogenous antioxidant (mg/dl)	
	Nitric oxide	Peroxide	glutathione	Superoxide dismutase
Normal	119.6	43.53	56.4*	94.21*
Arthritis	261.4*	92.21*	13.2*	44.16*
Arthritis+Indo(5mg/kg)	147.2	52.19	41.8*	63.78*
Arthritis +NA-2(5mg/kg)	185.7	53.56	29.0*	63.50*

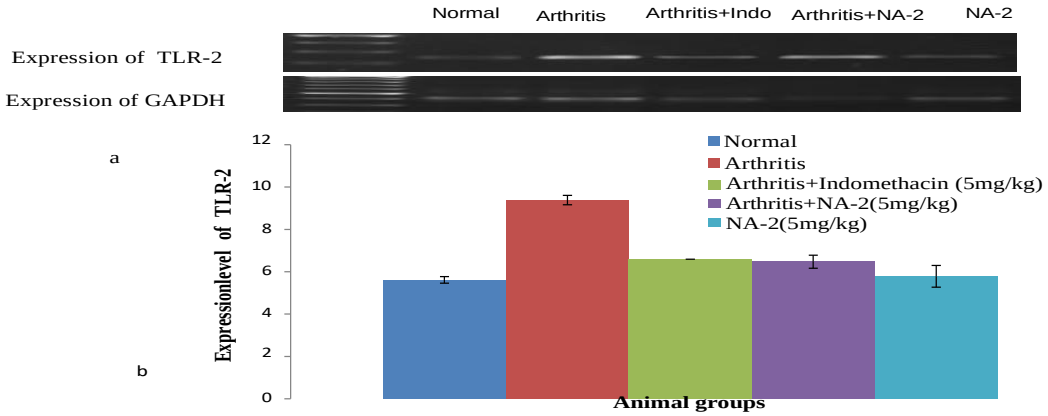
3.2. Measurement of Serum Endogenous Antioxidants:

The serum levels of glutathione (GSH) and superoxide dismutase (SOD) were estimated in normal control, arthritic control and arthritic animals treated with NA-2 and indomethacin (Table 2). In comparison to the normal control significantly decreased levels of GSH (P < 0.001) and SOD (P < 0.007) were observed in the nontreated arthritic rats. When non-treated arthritic group was compared with indomethacin and NA-2 treatment group, a marked increase in the GSH (P< 0.034 and (P< 0.01) and SOD (P< 19.04 and P <0.03) was noted respectively (Table 2).

3.4. Measurement of mRNA Expression by PCR:

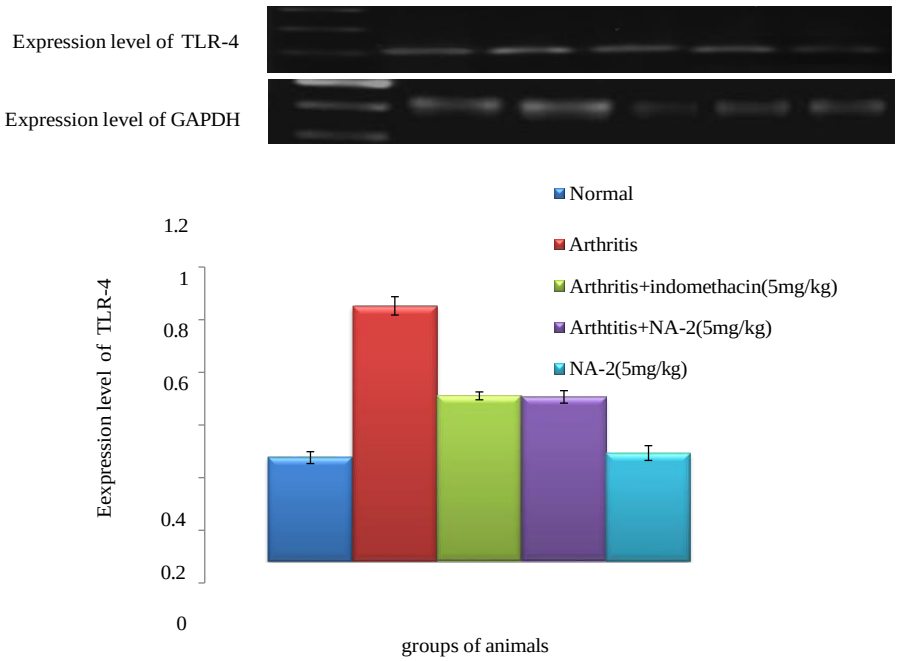
Figures 1&2 show bands indicating comparisons of TLR-2 and TLR-4 mRNA levels in cultured BMCs from different experimental groups included in this work, as well as bar graphs for TLR-2 and TLR-4 integrated densities after normalization with housekeeping gene GAPDH. BMCs from the normal control group had the lowest TLR-2 and TLR-4 mRNA expression, hence they were utilized as a baseline to compare the treated and untreated arthritic groups. A significant increase in the expression of TLR-2 (P < 0.043) and TLR-4 (P< 0.001) was observed in arthritic control group as compared to normal animals. A significant decrease in expression level of TLR-2 and TLR-4 mRNA was observed in indomethacin and NA-2 treated arthritic rats as compared to arthritic control groups (P<0.001). Values for the significant difference between different groups are given in the rows under the figures.

Fig.1



Comparison Groups	*P*-values BMSCs
Normal and Arthritic Control	(0.001)
Arthritic Control and Indomethacin	(0.001)
Arthritic Control + NA-2	(0.001)

Figure 1. (a) Representative bands of TLR-2 in bone marrow cells and housekeeping Gene GAPDH in normal, arthritic and treated groups (b) Bar diagrams showing expression level of TLR- 2 in BMCs after normalization with house-keeping gene showing a significant increase in expression of TLR-2 in arthritic animal compared with normal group. Treatment of indomethacin and NA-2 reduce the expression of TLR-2 significantly compared with arthritic animals.



Comparison Groups	*P*-values BMSCs
Normal + Arthritic Control	(0.001)
Arthritic Control + Indomethacin	(0.001)
Arthritic Control + NA-2	(0.001)

Figure 2. (a) Representative bands of TLR-4 in bone marrow cells and housekeeping Gene GAPDH in normal, arthritic and treated groups (b) Bar diagrams showing expression level of TLR- 4 in BMCs after normalization with housekeeping gene showing a significant increase in expression of TLR-4 in arthritic animal compared with normal group. Treatment of indomethacin and NA-2 reduce the expression of TLR-4 significantly compared with arthritic animals.

3.5 Immunocytochemistry for TLR-2 and TLR-4:

Figure 3a & 4a shows the expression of TLR-2 and TLR-4 proteins on the bone marrow cell surface of different experiment groups. Image J software was used to find out the intensities for the expression of TLR-2 & TLR-4 are represented in figures 3b & 4b by bar graph. The expressions of TLR-2 & TLR-4 in normal samples were considered as baseline and the arthritic control groups and arthritic treated group were compared with it. A significant up-regulation of TLR-2 & TLR-4 (**P< 0.001) in BMCs of arthritic control group was observed compared to normal control. A significant down-regulation of TLR-2 & TLR-4 expression (**P < 0.001) in NA-2 and indomethacin treated groups was observed as compare to arthritic animals.

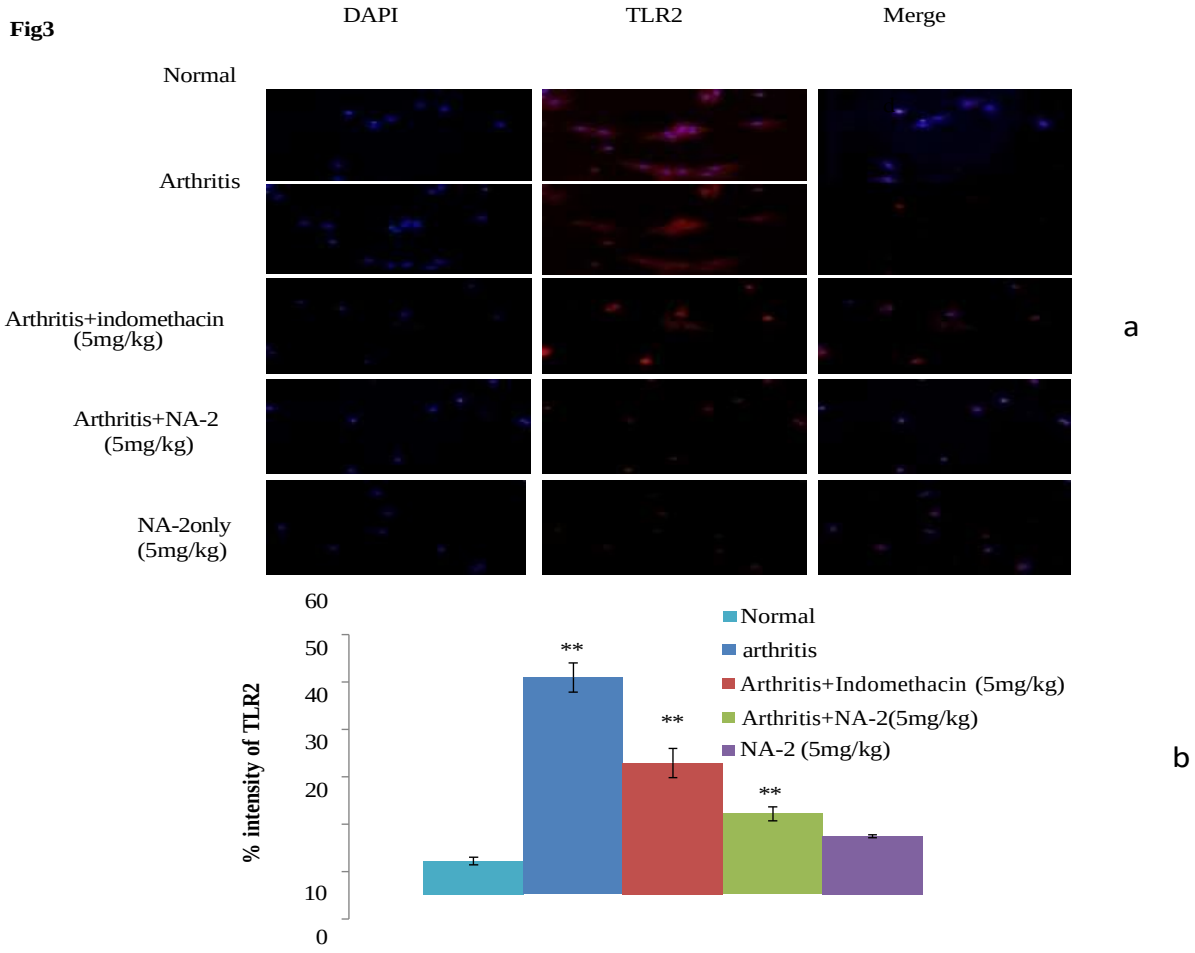


Figure 3. (a) Immunocytochemistry of active TLR-2 expression in bone marrow cells obtained from normal, arthritic control, indomethacin and NA-2 treated arthritic rats (b) graphical illustrations of immunostaining performed to analyze intensity of TLR-2 protein expression. Images showing a marked down regulation of TLR-2 expression in NA-2 and indomethacin treated treated groups as compare to arthritic animals. Histograms demonstrate the intensity of expression of TLR-2 in %. Each bar represents mean ± S.E.M of three independent experiments. Significant difference in expression between the arthritic group and indomethacin treated group was ** p< 0.001.

Fig. 4

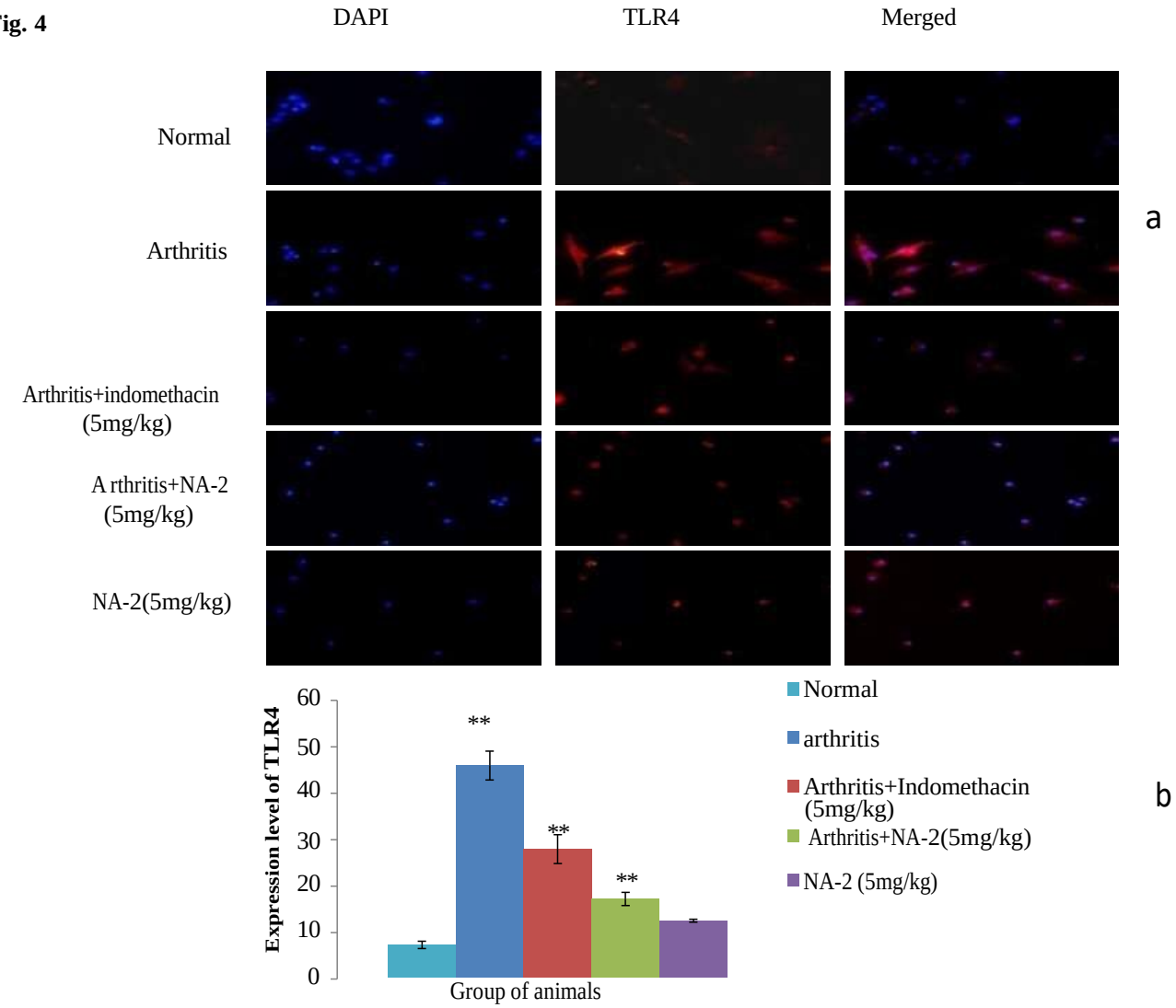


Figure 4. (a) Immunocytochemistry of active TLR-4 expression in bone marrow cells obtained from normal, arthritic control, indomethacin and NA-2 treated arthritic rats (b) graphical illustrations of immunostaining performed to analyze intensity of TLR-4 protein expression. Images showing a marked down regulation of TLR-2 expression in NA-2 and indomethacin treated groups as compare to arthritic animals. Histograms demonstrate the intensity of expression of TLR-4 in %.Each bar represents mean ± S.E.M of three independent experiments .Significant difference in expression between the arthritic group and indomethacin treated group was **p< 0.001.

4. Discussion:

Our findings indicate N-(2-hydroxyphenyl) acetamide's potentially essential features, such as its powerful anti-oxidative stress effects along with downregulation of TLRs (TLR2 & TLR4) on AIA-induced arthritic rats. The evidence implies its complicated multi-targeted action on reactive oxygen species, antioxidants and toll-like receptors (TLRs), which are major participants in the pathophysiology of rheumatoid arthritis and critical regulators of inflammation in general.

Adjuvant-induced arthritis (AIA) rodent models are commonly used to test and develop anti-arthritic medicines (34). We used an SD rat rodent model to assess the effect of NA-2, a salicylic acid derivative, on oxidative stress markers linked to RA, with a focus on TLRs (TLR-2 and TLR-4) expression. Heat killed (MTH37Ra) suspension in mineral oil was employed as an adjuvant in this investigation [34]. Multiple features of the disease can be control by TLRs. Therefore, to find out the therapeutics of the disease TLRs or their signaling cascades inhibitors can be used to target inflammation and other arthritis related markers.

After validation of RA model, role of TLR-2 and TLR-4 in the progression of disease was examined. We used our test drug NA-2 against the TLRs. They are targeted for therapy of the disease. Both the TLRs (TLR-2 & TLR-4) which are considered in the study were found to be up-regulated on the bone marrow cells of arthritic control animals as compare to normal animals. TLRs (TLR-2 & TLR-4) are overexpressed as a result of both exogenous and endogenous ligands. Peptidoglycan and lipopolysaccharide operate as external ligands present in the cell wall of Mycobacterium tuberculosis (used as an adjuvant), while damage-associated molecular pattern acts as an endogenous ligand (35,36). They are produced in condition of cell stress and/or structural damage and are responsible for primary immune response that result in initiation of autoimmunity (37-39). It is already found that different pathways of stress signaling pathways are activated in the synovial membrane and synovial fluid of RA patients (40). Literature also cites that up-regulation of TLR-2/TLR-4 in the presence of endogenous ligands results in increased production of IL-12/IL-18, and MMPs leading to inflammatory changes that result in irreversible joint destruction (41).

It is already observed that migrated leukocytes to the joints stimulates both innate and adaptive immune responses (42). In the joints they stimulate a powerful oxidative burst under influence of inflammatory factors. Under this condition a large amount of oxygen is consumed and converted into a variety of free radicals and develop local environment of highly reactive oxygen which plays a very important role in the development and persistence of RA (43).

In the present study an increase in paw oedema of arthritic control rat in comparison to the normal animals is observed after an increase in expression of toll like receptor in arthritic animals as compare to normal animals. It indicates that inflammatory reactions take place in the arthritic control rats and paw oedema occurs due to the infiltration of immune. Oedema is a result of infiltration of immune cells and inflammatory mediators such as cytokines, prostaglandins, and reactive oxygen (ROS) species and antioxidant is observed in inflamed tissue which are mainly responsible for cartilage destruction. We observed a decrease in disease progression with concomitant reduction in the TLR- 2 and TLR-4 expression in bone marrow cells in NA-2 treated arthritic animals compared to arthritic control animals. Bone marrow cells play a critical role in the pathogenesis of RA. The primitive bone marrow mesenchymal cells can transverse cortical bone through pores and reside in the synovium, where they produce mediators that enhance synovitis before the onset of clinical disease. The bone marrow also contributes cells of lineage that travel to the synovium and aggravates the arthritis features. The NA-2 treatment was able to reduce proinflammatory mediators such as ROS improves antioxidant and significantly reverse the increased expression of TLRs.

Previous studies show that increased expression of TLRs results in increase production of proinflammatory cytokines. It is already proved in previous studies that the increase in inflammatory cytokines production is responsible for progression of disease as they are responsible for destruction of joints and pain (44-51). Salicylic acid, a parent compound of NA-2 has been studied that it inhibits the activation of the transcription factor NF-κB in human monocytes through a cyclooxygenase independent mechanism This further inhibits the secretion of cytokines and other inflammatory mediators. The repressive action of NSAIDs provides us with the evidence that NA-2 may inhibit cytokines secretion by blocking NF-kB which can be activated by TLRs (52-54).

Oxidative stress or reactive oxygen species (ROS) and nitrogen species serves as mediators in pathogenesis of cartilage destruction (55). Increased oxidative enzyme activity, along with decreased antioxidant levels (SOD and GSH) in sera and synovial fluids of arthritic patient and experimental animals have been observed by several groups (56). Results of present study are in accord with the previous studies showing increased level of peroxide and nitric oxide in the serum of control arthritic rats.

In the present study indomethacin is used as reference drug established an inhibitory effect on the level of peroxide and nitric oxide with a parallel increase in GSH and SOD level treated arthritic rats. It is already studied that indomethacin has inhibitory action on ROS production by inhibiting COX and prostaglandin E2 (57,58) which support our present study data. The inhibitory effect of indomethacin might be responsible to increase the level of SOD. Increase in generation of superoxide ions due to the inhibitory action of indomethacin results in availability of increase levels of SOD

show activity. These studies validate our choice of using indomethacin as a reference drug. It is also observed in the present study that treatment of NA-2 significantly reduces the levels of ROS with concurrent increase in the GSH and SOD activities. Salicylic acid the parent compound of NA-2 has also been studied previously and it is found out that it has suppressive effects on IL-1β-induced NF-κB production which regulate the expression of ROS (iNO) and antioxidants (59-60). In the light of these studies, it can be concluded that the antioxidant activity of NA-2 might be due to its intervention in NF-kB pathway.

5. Conclusion

In the present study we have established that NA-2 compounds possess both anti-inflammatory and anti-rheumatic activities in arthritic animal model. It effectively reduces the TLRs (TLR-2, and TLR-4) expression on BMCs and reduced oxidative stress by modulating reactive oxygen species and antioxidant. Thus, our study establishes that NA-2 may serve as a probable candidate to be further developed as an anti-arthritic agent.

Conflict of Interest

Authers involves in the manuscript report no conflicts of interest. The author alone is responsible for the content and writing of the paper.

Ethics approval

Ethical approval has been taken from the scientific Advisory Comity on Animal Care and use, International Center for Chemical and Biological Sciences, University of Karachi (Protocol No:1209004)

References:

1. Josef S S, Daniel A, Iain B MC Rheumatoid arthritis.Lancet.2016;388:2023– 2038.doi: 10.1016/S0140 - 6736 (16)30173-8. [PubMed] [CrossRef] [Google Scholar]
2. McInnes I.B., Schett G. The Pathogenesis of Rheumatoid Arthritis. N. Engl. J. Med. 2011; 365:2205–2219.doi: 10.1056/NEJMra1004965. [PubMed]
3. Wright H.L., Lyon M., Chapman E.A., Moots R.J., Edwards S.W. Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines, Reactive Oxygen Species, and Neutrophil Extracellular Traps. Front.Immunol. 2021;11:584116.doi: 10.3389/fimmu.2020.584116. [PMC free article] [PubMed] [CrossRef] [Google Scholar]
1. 4.Trofimenko A.S., Mozgovaya E.E., Bedina S.A., Spasov A.A. Ambiguities in Neutrophil Extracellular Traps. Ongoing Concepts and Potential Biomarkers for Rheumatoid Arthritis: A Narrative Review. Curr.Rheumatol.Rev. 2021;17:283293.doi:
2. 10.2174/1573397116666201221113100. [PubMed] [CrossRef] [Google Scholar]
4. Lee K.H., Kronbichler A., Park D.D.-Y., Park Y., Moon H., Kim H., Choi J.H., Choi Y., Shim S., Lyu I.S., et al. Neutrophil extracellular traps (NETs) in autoimmune diseases:A comprehensive review. Autoimmun. Rev. 2017;16:1160–1173. doi: 10.1016/j.autrev.2017.09.012. [PubMed] [CrossRef] [Google Scholar]
5. Ryan T, Giselle C , Sandra S. Emerging role of endosomal toll-like receptors in rheumatoid arthritis. Front. Immunol 2014; 5: 1.
6. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. J Clin Invest 2008; 118: 205-216.
7. Akira S, Takeda K. Toll-like receptor signaling. Nat Rev Immunol 2004; 4(7): 499–511.
8. Page TH, Midwood KS.Targeting DAMP activation of toll-like receptors: Novel pathways to treat rheumatoid arthritis?, Rheumatoid Arthritis - Treatment, Dr. Andrew Lemmey (Ed.) 2012; DOI: 10.5772/28110

3. 10.Asea A, Rehli M, Kabingu E, Boch JA, Bare O, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 2002; 277:15028-15034.
4. 11. Park JS, Svetkauskaite D, He Q, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box1 protein. J Biol Chem 2004; 279(9): 7370-7377
5. 12. Bobacz K, Sunk IG, Hofstaetter JG, et al. Toll-like receptors and chondrocytes: the lipopolysaccharide-induced decrease in cartilage matrix synthesis is dependent on the presence of toll-like receptor 4 and antagonized by bone morphogenetic protein 7. Arthritis Rheum 2007; 56: 1880-1893
6. 13.Hiratsuka S ، Watanabe A, Sakurai Y, et al. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. Nat Cell Biol 2008; 10:1349-1355.
7. 14.Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39(1):44-84
8. 15. Henrotin YE, Bruckner P, Pujol JP. The role of reactive oxygen species in homeostasis and degradation of cartilage. Osteoarthr . Cartil. 2003; (10):747-55.
9. 16.Maurice MM, Nakamura H, van der Voort EA , et al. Evidence for the role of an altered redox state in hyporesponsiveness of synovial T cells in rheumatoid arthritis. JImmunol 1997; 158: 1458-1465.
10. 17. Jakubczyk, K.; Dec, K.; Kałdu ´nska, J.; Kawczuga, D.; Kochman, J.; Janda, K. Reactive oxygen species—Sources, functions, oxidative damage. Pol. Merkur. Lek. 2020, 48, 124–127.
11. 18. Dröge, W. Free radicals in the physiological control of cell function. Physiol. Rev. 2002, 82, 47–95. [CrossRef]
12. 19. Sies, H. Oxidative stress: A concept in redox biology and medicine. Redox Biol. 2015, 4, 180–183. [CrossRef]
13. 20. da Fonseca, L.J.S.; Nunes-Souza, V.; Goulart, M.O.F.; Rabelo, L.A. Oxidative Stress in Rheumatoid Arthritis: What the Future Might Hold regarding Novel Biomarkers and Add-On Therapies. Oxid. Med. Cell. Longev. 2019, 2019, 7536805. [CrossRef]
14. 21. Phull, A.-R.; Nasir, B.; Haq, I.U.; Kim, S.J. Oxidative stress, consequences and ROS mediated cellular signaling in rheumatoid arthritis. Chem. Biol. Interact. 2018, 281, 121–136. [CrossRef] [PubMed]
15. 22. Datta, S.; Kundu, S.; Ghosh, P.; De, S.; Ghosh, A.; Chatterjee, M. Correlation of oxidant status with oxidative tissue damage in patients with rheumatoid arthritis. Clin. Rheumatol. 2014, 33, 1557–1564. [CrossRef] [PubMed]
16. 23. Mateen, S.; Moin, S.; Khan, A.Q.; Zafar, A.; Fatima, N. Increased Reactive Oxygen Species Formation and Oxidative Stress in Rheumatoid Arthritis. PLoS ONE 2016, 11, e0152925. [CrossRef] [PubMed]
17. 24.Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39(1):44-84 25.Maurice MM, Nakamura H, van der Voort EA , et al. Evidence for the role of an altered redox state in hyporesponsiveness of synovial T cells in rheumatoid arthritis. JImmunol 1997; 158: 1458-1465.
18. 26. Pattison DJ, Silman AJ, Goodson NJ, et al. Vitamin C and the risk of developing inflammatory polyarthritis: prospective nested case-control study. Ann RheumDis 2004; 63: 843-847.
19. 27.Asea A, Rehli M, Kabingu E, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 2002; 277:15028-15034.
20. 28.Bobacz K, Sunk IG, Hofstaetter JG, et al. Toll-like receptors and chondrocytes: the lipopolysaccharide-induced decrease in cartilage matrix synthesis is dependent on the presence of toll-like receptor 4 and antagonized by bone morphogenetic protein 7. Arthritis Rheum 2007; 56: 1880-1893
21. 29. Pedras - Vasconcelos J, Puig M, Verthelyi D. TLRs as therapeutic targets in CNS inflammation and infection. FrontBiosci (Elite Ed) 2009; 1: 476-487

22. 30. Perveen K, Hanif F, Jawed H, et al . Protective efficacy of N-(2-hydroxyphenyl) acetamide against Adjuvant-Induced Arthritis in Rats. Bio Med res intl 2013; 2013:635143.
23. 31.Zimmermann M. Ethical consideration in relation to pain in animal experimentation. Acta Physiol Scand 1986 ; 128(suppl.554): 221-223.
24. 32. Perveen K, Hanif F, Jawed H, et al. N-(2-hydroxy phenyl) acetamide: a novel suppressor of Toll-like receptors (TLR-2 and TLR-4) in adjuvant-induced arthritic rats. Mol Cell Biochem 2014; 394 (1-2): 67-75.
25. 33. Bendele AM. Animal Models of Rheumatoid Arthritis. J Musculoskel Interact 2001; 4: 377–385.
26. 34. Jawed H, Shah UA, Jamall S, Simjee SU. N-(2-hydroxy phenyl) acetamide inhibits inflammation-related cytokines and ROS in adjuvant-induced arthritic (AIA) rats. IntImmunopharmacol 2010; 10(8): 900-905.
27. 35. Min-Fu. Review: Pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors. J Endotoxin Res 2007; 13(1):6-14.
28. 36.Guo-Yun C, Nicholas KB, Wei W, et al., Broad and direct interaction between TLR and Siglec families of pattern recognition receptors and its regulation by Neu1.Elife3 2001; e04066.
29. 37. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat Med 1999; 5(11): 1249-1255.
30. 38 Johnson G L, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298(5600): 1911-1912.
31. 39. Matzinger P. The danger model: a renewed sense of self. Science 2002; 296(5566): 301-305.
32. 40. Schett G, Tohidast-Akrad M, Smolen JS, et al., Activation, differential localization and regulation of the stress-activated protein kinases, extracellular signal–regulated kinase, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis.Arthritis Rheum 2000; 43(11): 2501-2512.
33. 41. Van Lent PL, Van de Loo FA, Holthuysen AE, et al . Major role for interleukin-1 but not tumor necrosis factor in early cartilage damage in immune complex arthritis in mice.J Rheumatol 1995; 22:2250–2258
34. 42.O'Neil, L.J.; Kaplan, M.J. Neutrophils in Rheumatoid Arthritis: Breaking Immune Tolerance and Fueling Disease. Trends Mol. Med. 2019, 25, 215–227. [CrossRef]
35. 43. Mateen, S.; Moin, S.; Khan, A.Q.; Zafar, A.; Fatima, N. Increased Reactive Oxygen Species Formation and Oxidative Stress in Rheumatoid Arthritis. PLoS ONE 2016, 11, e0152925. [CrossRef] [PubMed] .
36. 44. Johnson GB, Brunn GJ, Kodaira Y, et al . Receptor mediated monitoring of tissue well-being via detection of soluble heparin sulfate by Toll-like receptor 4. J Immunol 2002; 68: 5233–5239.
37. 45.Keffer J, Probert L, Cazlaris H, al., Transgenic mice expressing human tumor necrosis factor: a predictive genetic model of arthritis. EMBO J 1991; 10(13): 4025-4031. 36. Schaible HG, Grubb BD. Afferent and spinal mechanisms of joint pain. Pain 1993; 55 (1): 5-54.
38. 46. Kinne RW, Brauer R,Stuhlmuller B, et al .Macrophages in rheumatoid arthritis. .Arth research 2000; 2 (3): 189-202. 47.Bingham CO. The pathogenesis of rheumatoid arthritis: pivotal cytokines involved in bone degradation and inflammation. J Rheumatol 2002; 65: 3-9.
39. 47. Rommel C, Camps M, Ji H. PI3Kδ and PI3Kγ: partners in crime in inflammation in rheumatoid arthritis and beyond? Nat Rev Immunol 2007; (3): 191-201
40. 48. Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 1994; 265(5174): 956-959.
41. 49. Farivar RS, Chobanian AV, Brecher P. Salicylate or aspirin inhibits the induction of the inducible nitric oxide synthase in rat cardiac fibroblasts. Circ Res1996; 78(5):759- 768.
42. 50.Aizman E, Blacher E, Ben-Moshe O, et al .Therapeutic effect of farnesylthiosalicylic acid on adjuvant-induced arthritis through suppressed release of inflammatory cytokines.Clini & Exp Immunology 2014; 175(3) : 458-467.

43. 51. Joosten LA, Helsen MM, van de Loo FA, et al. Anti-cytokine treatment of established type II collagen induced arthritis in DBA/1 mice: a comparative study using anti-TNF-a anti-IL-1a/ß, and IL-1ra. Arthritis Rheum 1996; 39:797-809.
44. 52 Hoffmann, M.H.; Griffiths, H.R. The dual role of Reactive Oxygen Species in autoimmune and inflammatory diseases: Evidence from preclinical models. Free Radic. Biol. Med. 2018, 125, 62–71. [CrossRef]
45. 53. Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 1994; 265(5174): 956-959.
46. 54. Farivar RS, Chobanian AV, Brecher P. Salicylate or aspirin inhibits the induction of the inducible nitric oxide synthase in rat cardiac fibroblasts. Circ Res1996; 78(5):759- 768.
47. 55. Khojah, H.M.; Ahmed, S.; Abdel-Rahman, M.S.; Hamza, A.-B. Reactive oxygen and nitrogen species in patients with rheumatoid arthritis as potential biomarkers for disease activity and the role of antioxidants. Free Radic. Biol. Med. 2016, 97, 285–291. [CrossRef]
48. 56.Mahajan A, TandonV R. Antioxidants and rheumatoid arthritis.J Indian Rheumatol Assoc 2004; 12: 139-142.
49. 57. Hrab´ak A, Vercruysse V, Kah´an IL, et al. Indomethacin prevents the induction of inducible nitric oxide synthase in murine peritoneal macrophages and decreases their nitric oxide production. Life Sci 2001; 68(16): 1923–1930.
50. 58. Friman C, Johnston C, Chew C, et al . Effect of diclofenac sodium, tolfenamic acid and indomethacin on the production of superoxide induced by N-formyl methionyl leucyl-phenylalanine in normal human polymorph nuclear leukocytes
51. 59. Pourcyrous M, Leffler CW, Bada HS, et al. Speroxide anion generation in asphyxiatedpiglets and the effect of indomethacin at therapeutic dose. Pediatr Res 1993; 45: 366–369.
52. 60. Aizman E, Blacher E, Ben-Moshe O, et al. Therapeutic effect of farnesyl thiosalicylic acid on adjuvant-induced arthritis through suppressed release of inflammatory cytokines.Clini & Exp Immunology 2014; 175(3) : 458-467.