Comparative Study of the Effect of Allopurinol and Nabumetone either Alone or Combined on Freund’s Adjuvant-Induced Arthritis in Rats

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Abstract

Background: Non steroidal anti-inflammatory drugs (NSAIDs) partially alleviate symptomatic manifestation of rheumatoid arthritis (RA) but do not provide long term protection from articular damage. Nabumetone possesses a powerful anti-inflammatory profile as conventional NSAIDs, with little gastric and renal side effects due to selective blocking of Cyclooxygenase-2 (COX-2), thus prevent formation of prostaglandins (PGs) responsible for the cardinal signs of inflammation. Over expression of XO enzyme, overproduction of free radicals plus release of inflammatory cytokines has been shown to be involved in the inflammation-induced tissue damage.

Xanthine oxidase (XO) inhibitors as allopurinol may retard disease progression most probably due to an antioxidant effect. Hence, XO inhibitors/NSAIDs combinations may help to achieve both long term prevention of disease progression as well as rapid onset of symptomatic control.

Aim of the Work: This study was designed to compare the biochemical and pharmacological effects of allopurinol, nabumetone and their combination in RA.

Material and Methods: Forty male rats were used, divided into five groups (8 rats each): Control group, Complete Freund's Adjuvant (CFA) induced RA group-not treated-, arthritic group treated with allopurinol, arthritic group treated with nabumetone and arthritic group treated with allopurinol plus nabumetone. After 6 weeks of treatment, the anti-inflammatory and antioxidant effects of the tested drugs on the severity of arthritis was evaluated by ELISA assay of serum TNF-α, a critical inflammatory mediator in this condition, calorimetric assay of serum uric acid due to the evidence of increased XO activity in serum and tissue in patients with RA and by calorimetric assay of the oxidative stress parameters, lipid peroxides (LPO) and superoxide dismutase (SOD), due to overproduction of reactive oxygen species (ROS) during inflammatory process.

Results: In the arthritic non-treated group, the serum levels of TNF-α, uric acid and LPO were significantly higher while the activity of SOD was significantly lower than in the control group; also there was a significant difference in right hind paws thickness between the two groups. In the allopurinol treated group, the activity of SOD was significantly increased while the serum levels of TNF-α, LPO, uric acid and right hind paws thickness were significantly decreased in comparison with the arthritic non-treated group. Nabumetone induced a significant decrease in the levels of TNF-α and LPO and a significant increase in SOD activity accompanied by a significant decrease in right hind paws thickness in comparison with the arthritic non-treated group. Combination of allopurinol and nabumetone induced also a significant improvement of all serum bio-indices and right hind paws thickness compared to other treated group, this improvement was still significantly different from the non-arthritic control group.

Conclusion: Both allopurinol and nabumetone showed anti-inflammatory and antioxidant effects in CFA-induced arthritis in rats. Addition of allopurinol to nabumetone induced a synergistic effect evidenced by reducing paw edema, free radical generation and improves antioxidant status.

Key Words: Rheumatoid arthritis – Allopurinol – Nabumetone – Antioxidants.

Introduction

RHEUMATOID arthritis is polyarticular autoimmune disease affecting 1-2% of the population; it is characterized by joint swelling, hyperplasia of synoviocytes, mainly of synovial fibroblasts resulting in progressive destruction and disability [1].

Immunization of rats with Complete Freund's Adjuvant (CFA) leads to development of adjuvant-induced arthritis as a model of chronic inflammation that bears resemblance to RA. Freund’s Adjuvant is an antigen solution emulsified in mineral oil that is effective in stimulating cell-mediated immunity and may lead to the potentiation of the production of certain immunoglobulins [2].

The pro-inflammatory cytokines TNF-α, IFγ, IL-1, 2, 6, 8 PGs are shown to play an important role in the pathophysiology of arthritis development in animal models and in humans [3]. Cyclooxygenase (COX) enzyme is the key enzyme in the bio-
Comparative Study of the Effect of Allopurinol

Cyclooxygenase-2 (COX-2) has been shown to be induced in vivo under inflammatory conditions. Selective inhibitors of COX-2 are potential therapeutic agents expected to have anti-inflammatory effects similar to those of conventional NSAIDs as they decrease formation of PGs mainly PGE2; the key prostaglandin mediating the cardinal signs of inflammation, but with improved side effects profile on the gastric and renal systems [5].

Nabumetone-a selective COX II inhibitor-is converted in liver to an active metabolite. Its half life is about 24 hours, so it is suitable for once daily dosing. Also, it is not renally excreted; this gives it an advantage in many rheumatic diseases that are associated with renal pathology [6].

Overproduction of free radicals has been shown to be involved in the inflammation-induced tissue damage [7]. The triggering mechanisms for the production of free radicals are intracellular enzymes like xanthine-oxidase (XO), microsomal NADPH-dependent cytochrome P-450 reductase and mitochondrial membrane bound dehydrogenases. All these enzymes can initiate the production of oxygen radicals which are mainly responsible for further initiation of lipid peroxidation [7].

A variety of well known endogenous enzymes such as SOD, catalase and glutathione peroxidase act as scavengers for free radicals, in contrast other endogenous enzymes such as XO can produce free radicals [8]. Allopurinol, a well known XO inhibitor, has been shown to prevent ischemia-reperfusion injury-induced tissue damage [9]. It has been shown that allopurinol has a preventive effect on rejection in organ transplantation, in chronic gastric ulceration, in chronic inflammation and in focal cerebral ischemic injury [10].

There is an evidence for increased circulating levels of XO in human plasma samples from patients with RA. Allopurinol ameliorates the symptoms of arthritis in animal models of RA; at least, some of these effects are likely related to the antioxidant actions of the compound [8,11].

Allopurinol may be a novel strategy in the treatment of various cellular autoimmune disorders due to antioxidant effect [10].

The aim of the present study was to determine the possible antioxidant and anti-inflammatory effects of allopurinol and to compare these effects with those of nabumetone. The effect of their combination was also studied.

Material and Methods

Animals:
Forty adult male albino rats, weighing 150-170 g each, were used in this study. Rats were purchased from the National Research Center (NRC), Cairo, Egypt and kept in the Department of Pharmacology, Faculty of Medicine, Suez Canal University where the experiment was conducted.

Each animal was left alone in a polyethylene cage and allowed for acclimatization before the start of the study for 7 days. They were kept on a standard rodent chow and water was freely provided. The transportation, care and use of animals were harmless and directed by well trained and experienced person, taking in consideration the avoidance or minimization of discomfort, distress, and pain. The treatment protocol was approved by the National Research Center Animal Rights Committee.

Drugs and chemicals:
Allopurinol:
It was supplied as a white powder from Sigma pharmaceuticals.

Nabumetone:
It was supplied as white crystalline powder from Smithkline Beecham pharmaceuticals.

Complete Freund’s Adjuvant (CFA):
It was provided from Sigma biosciences, Egypt as 10ml amber viscous emulsion. Each milliliter contains 1mg heat killed and dried mycobacterium tuberculosis emulsified in 0.85ml mineral oil and 0.15ml mannide monooleate, dissolved in 85 per- centage saline. It was stored at 2-8 °C.

Kits:
TNF-α (using cytoscreen immunoassay Kit for rat TNF-α Catalog No. KRC3012, bioscience International, Inc., Cmarillo, USA). Uric acid, LPO and SOD kits were obtained from bio-diagnostic company (Cairo, Egypt).

Induction of arthritis:
Arthritis was induced by a single subcutaneous- ly injection of 0.1ml CFA corresponding to 100 microgram mycobacterium T.B. for each animal in the right hind paw [12].

Study design and animal groups:
Forty animals were used, divided into the following 5 groups (8 animals each); treatment was
given once daily orally (P.O.) by oral gavage for 6 weeks starting with the day of CFA injection to investigate the effect of tested drugs on induction of arthritis.

Control group: Non-arthritic that received no medication.

Arthritic control group: Injected once by 0.1ml CFA and received 0.5ml water P.O.

Allopurinol group: Arthritic group received allopurinol alone P.O. suspended in 0.5ml water in a dose of 50mg/Kg. The dose of allopurinol was selected as the optimal dose that does not cause nephrotoxicity [11,13].

Nabumetone group: Arthritic group received nabumetone alone P.O. suspended in 0.5ml water in a dose of 300mg/Kg. The dose represented the average human therapeutic dose [14].

Combination group: Arthritic group received allopurinol + nabumetone P.O. in the same above mentioned doses.

All rats were sacrificed by decapitation after 6 weeks from the onset of the experiment. The degree of severity of the induced arthritis and the effects of the tested drugs were evaluated by two parameters; blood tests for some biomarkers and the thickness in the hind paws.

Blood tests:
Five ml blood was collected from retro-orbital plexus of each rat in a clean sterile tube inserted at the inner canthus of the eye. The blood was left to be clotted, blood samples were centrifuged and serum separated to be stored at –20°C until analyzed.

Serum level of the inflammatory mediator (TNF-α): These kits depend on solid-phase sandwich Enzyme-linked immunosorbent assay (ELISA) [15].

Serum levels of uric acid: Using uric acid Kits, colorimetric method [16].

Serum levels of lipid peroxides (LPO): By thiobarbituric acid (TBA) test. The sample under test is treated by TBA and a pink chromogen is measured. In TBA reaction, one molecule of malondialdehyde (MDA) reacts with 2 molecules of TBA with the production of pink pigment detected by spectrophotometer with an absorption maximum of 532nm [17].

Superoxide dismutase (sod) activity: This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate (PMS)-mediated reduction of nitroblue tetrazolium dye by spectrophotometer with an absorption maximum of 560nm [18].

Evaluation of the rat paw edema:
The thickness of right hind paw of each rat was measured in m.m. by using hand screw micrometer [14,19]. The reading was taken when the metal facets of the micrometer made firm contact with the skin without compressing the soft tissues.

Statistical analysis:
The data was coded and entered using the statistical package SPSS version 15. The results were expressed as mean ± S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post hoc multiple comparison; bonferroni test, to test the significance among group means. p value ≤0.05 was considered statistically significant at confidence interval 95% [20].

Results

Serological studies:
As seen in Table (1 and Fig. (1, panel A), induction of arthritis by CFA in rats showed a significant increase in the serum level of TNF-α (129.9±3.1 pg/ml) compared to non-arthritic control rats (33.1±2.6). Administration of allopurinol, nabumetone or their combination resulted in a significant decrease in serum level of TNF-α (84.4±2.9, 67.3±2.8 and 43.8±1.7 respectively) (p<0.05) compared to arthritic untreated group. Combination of the two drugs resulted in a significant decrease in TNF-α serum level compared to the other treated groups and the control group.

Also, serum level of uric acid was significantly increased in the arthritic untreated rats (2.7±0.8 mg/dl) compared to non-arthritic control rats (1.3±0.8) as shown in Table (1 and Fig. (1, panel B). Oral administration of allopurinol either alone or combined with nabumetone resulted in a significant decrease in uric acid serum level (1.9±0.4, 1.7±0.9 respectively) compared to arthritic untreated group. Combination of allopurinol and nabumetone resulted in a significant decrease in uric acid serum level compared to nabumetone alone treated group and the non-arthritic control group.

Regarding LPO, as shown in Table (1 and Fig. (1, panel C), there was a significant increase in its serum level in the arthritic untreated rats (1.7±0.04 nmol/ml) compared to non-arthritic control rats (0.62±0.06). Oral administration of allopurinol, nabumetone or their combination induced a signif-
significant decrease in LPO serum level (0.76 ± 0.04, 0.8 ± 0.04 and 0.59 ± 0.02 respectively) compared to arthritic untreated group. Combination of the two drugs resulted in a significant decrease in LPO serum level compared to the other treated groups and the non-arthritic control group.

As illustrated in Table (1 and Fig. (1, Panel D), SOD activity was significantly decreased in arthritic untreated rats (116.1 ± 5.3%) when compared to control non-arthritic rats (341.4 ± 7). This activity was significantly increased again after oral administration of allopurinol, nabumetone or their combination for 6 weeks (238.6 ± 6.7, 211.1 ± 7.1 and 277.5 ± 9.1 respectively) when compared to arthritic untreated rats. The highest increase was observed in the combination group that was significantly different from the other treated groups and the non-arthritic control group.

**Evaluation of the rat paw edema:**

As presented in (Table 2), induction of arthritis induced a significant increase in the right hind paw thickness by about 9 folds compared to non-arthritic control group. Administration of allopurinol, nabumetone or their combination resulted in a significant decrease in the hind paw thickness by about 37.5%, 46% and 56% respectively comparing to arthritic untreated group.

Combination of allopurinol and nabumetone resulted in a significant decrease in right hind paws thickness compared to the other treated groups, but this decrease still significantly different from the control group.

![Fig. (1): The effect of the tested drugs on serum biomarkers at the end of week 6.](image_url)

* = Significantly different from control group at $p \leq 0.05$,
# = Significantly different from arthritic group at $p \leq 0.05$,
$ = Significantly different from allopurinol group,
@ = Significantly different from nabumetone group.

CON = Normal control.
ART = Arthritic control.
ALLO = Allopurinol.
NABU = Nabumetone.
COMB = Combination.
Table (1): The effect of allopurinol, nabumetone or their combination on serum biomarkers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Arthritic control</th>
<th>Allopurinol</th>
<th>Nabumetone</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>33.1±2.6</td>
<td>129.9±3.1*</td>
<td>84.4±2.9*#</td>
<td>67.3±2.8 *$</td>
<td>43.8±1.7*#$@</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>1.3±0.8</td>
<td>2.7±0.8*</td>
<td>1.9±0.4*#</td>
<td>2.5±0.14*#</td>
<td>1.7±0.9*#@</td>
</tr>
<tr>
<td>LPO (nmol/ml)</td>
<td>0.62±0.06</td>
<td>1.70±0.04*</td>
<td>0.76±0.04*#</td>
<td>0.80±0.04*#</td>
<td>0.59±0.02*#$@</td>
</tr>
<tr>
<td>SOD%</td>
<td>341.4±7</td>
<td>116.1±5.3*</td>
<td>238.6±6.7*#</td>
<td>211.1±7.1*#$</td>
<td>277.5±9.1 *#$@</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E. n=8.
* : Significant difference from control group.
# : Significant difference from arthritic control group.
$ : Significant difference from allopurinol group.
@: Significant difference from nabumetone group.
Data are analyzed by using one way ANOVA followed by bonferroni test at p<0.05.

Table (2): The effect of allopurinol, nabumetone or their combination on the right hind paws thickness (in mm).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Arthritic control</th>
<th>Allopurinol</th>
<th>Nabumetone</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right hind paw thickness</td>
<td>0.9±0.05</td>
<td>8.3±0.13*</td>
<td>6.1±0.06*#</td>
<td>4.5±0.12*#$</td>
<td>3.7±0.24*#$@</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E. n=8.
* : Significant difference from control group.
# : Significant difference from arthritic control group.
$ : Significant difference from allopurinol group.
@: Significant difference from nabumetone group.
Data are analyzed by using one way ANOVA followed by bonferroni test at p<0.05.

Discussion

The present study was designed to find out the possible role of allopurinol on induction of arthritis. An animal model of RA was induced by CFA which was proved as a useful model of RA [2,21]. Also the study aimed to compare the effect of allopurinol with nabumetone and to illustrate the effects of their combination.

It was proved that increase oxidative stress is one of the major hypotheses purposed to explain the development and progression of arthritis. Once formed, reactive oxygen species (ROS) induce lipid peroxidation and deplete antioxidant defenses such as SOD, which converts superoxide radicals to hydrogen peroxide, rendering the affected cells and tissues more susceptible to oxidative damage [22].

In the present study, treatment with allopurinol (50mg/kg/d P.O.) and nabumetone (300mg/kg/d P.O.) either alone or combined led to significant reduction in serum level of TNF-α and LPO and significant increase in SOD activity compared to arthritic control group. The combined treatment was superior in the beneficial effect and induced a significant benefit comparing to other treated groups. These results were in agreement with Bae, et al. [23] who proved that oxidative injury and inflammatory reactions in RA were associated with increased levels of inflammatory mediators like nitric oxide (NO), PGs, TNF-α, IL-6 and blocking of these mediators formation can improve the clinical condition. Also, Inglis, et al. [24] proved that treatment with TNF-α antagonists is of benefit in RA patients; in addition to exerting an anti-inflammatory effect and slowing the progression of RA, anti-TNF-α therapy produces a profound and rapid analgesia.

Decreasing lipid oxidation is an important line of treatment of inflammatory conditions because oxidized lipids enhance endothelial cell superoxide anion production and stimulate the expression of several genes and their dependent cytokines and adhesion molecules in the synovial membrane. In addition oxidized lipids are chemotactant for monocytes and lymphocytes and enhance macrophages to produce toxic ROS, cytokines, proteases and growth factors [25]. Similarly, Kuzkaya and Stone [26] found that SOD activity was significantly lower in arthritis as inflammatory cytokines reported to have a suppressant effect on the gene expression and activity of SOD and blockage of xanthine oxidase (XO) enzyme significantly elevated this level [27]. On the other hand, the SOD activity in the current study was in contrast with Edmonds, et al.
[28] and Schimdt et al. [29] who showed that anti-oxidant therapies had no effect on RA disease activity or indices of inflammation, but only improve pain, probably due to central analgesic mechanism.

The effect of allopurinol was explained by Zhang, et al. [30] who suggested that arthritis is associated with increased articular formation of nitrotyrosine, which may contribute to injury. Nitrotyrosine is formed by nitration of tyrosine by reactive nitrogen species, the formation of which may be enhanced by XO that can generate NO from nitrite/nitrate, and O free radicals during xanthine metabolism. So XO inhibitors may have anti-oxidant and anti-inflammatory properties by decreasing nitrotyrosine formation.

Meanwhile, oral administration of allopurinol alone or combined with nabumetone induced significant decrease in uric acid serum level. These results were supported by the findings of Nemeth, et al. [31] who described that uric acid is considered one of the non-enzymatic antioxidants, but increased production of uric acid means increased free radical production due to the activation of the XO enzyme system.

Nabumetone alone induced a non-significant decrease in uric acid serum level compared to arthritic untreated group. This can be explained as its mechanism of action is not through XO enzyme [8]. The underlying mechanism by which allopurinol exerts a protective effect on free radical-mediated pathological conditions as RA has been studied. It is a scavenger of the highly reactive hydroxyl radicals [32]. The drug also prevents the formation of free radicals through XO inhibition [1]. As an intracellular enzyme, two forms of XO, a dehydrogenase (type D) and an oxidase (type O) have been described. In normal conditions, type D of the enzyme converts the xanthine to uric acid; during inflammation, this form of enzyme transforms into type O which is responsible for the production of free radicals [33,34].

Most interestingly, the anti-inflammatory and antioxidant effects of allopurinol may be not related to ROS generation [38]. In addition to blocking uric acid production, inhibition of XO causes an increase in hypoxanthine and xanthine, which are converted to adenosine and guanosine monophosphates [35]. Adenosine is an intrinsic anti-inflammatory mediator involved in several immunoregulatory cascades [36]. Other researchers attributed the immunomodulatory effect of allopurinol to its affinity to bind adenosine receptors; particularly A2 receptors which are found on the leucocytic immune effector cells [37]. Allopurinol is reported to interfere with adenosine metabolism [37]; yet, allopurinol might share the adenosine release/adenosine receptor activation hypothesis on immune-effector leucocytes.

Allopurinol pretreatment prevented the increased synthesis of leukotriene B4 (LTB4) in hind limb ischemia-reperfusion in rabbits and LTB4 has been shown to be increased in synovial fluid during arthritis [38]. So there is a possibility that allopurinol may provide an additional beneficial effect by inhibiting the synthesis of LTB4 in adjuvant arthritis.

The effect of nabumetone can be explained by the fact that lipid peroxidation and ROS can be formed as by-products of COX pathway, during PGG2 to PGH2 conversion, which generated hydrogen peroxide [39]. So there is increasing evidences suggest that COX-II inhibitors may exert a beneficial effects in oxidative stress.

In the present study, the bio-indices results were confirmed by the direct physical evidence. Injection of CFA led to more that 89% increase in hind paw thickness compared to the control. These results are in agreement with Miletić et al. [40] who showed that immunization with CFA induced an increased expression of heat shock protein (HSP) 47, a molecular chaperone involved in the synthesis and assembly of collagen molecules in adjuvant arthritis, in joints of rats, which exhibited severe clinical signs of arthritis at the time of disease peak.

Oral administration of allopurinol, nabumetone or their combination induced as significant decrease in the right hind paws thickness by about 37.5%, 46% and 56% respectively compared to arthritic control rats. The combined regimen resulted in a significant decrease compared to other treated groups.

The anti-inflammatory effect of allopurinol can be explained also as it suppresses the production of TNF-α and down-regulates the expression of Intracellular Adhesion Molecule-1 (ICAM-1) and P2X7 receptors on monocyte/macrophages. ICAM-1 serves as a ligand for Leukocyte Functional Antigen-1 (LFA-1) on T lymphocytes, allowing proper antigen presentation. P2X7 receptors are thought to be involved in IL-1 β release, mitogenic stimulation of T lymphocytes and the probable cytoplasmic communication between macrophages and lymphocytes at inflammation sites [41].

These results are in agreement with Pacher, et al. [37] who approved that allopurinol may be useful
as additive therapy in RA due to its antioxidant properties. The effect of nabumetone of hind paws thickness was supported by the results of Somia, et al. [4] and Chang, et al. [42] who demonstrated that nabumetone induced significant reduction in the mean arthritic score of hind paws of rats. This can be explained due to inhibition of COX-2, the inducible enzyme that can be expressed in inflamed tissues and in synovial tissues from patients with RA and experimental animals respectively. The COX-2 plays a pathophysiological role by production of prostanoids involved in inflammation, pain and pyrexia [43]. Inhibition of COX-2 also decreased production of ROS formed during prostaglandin synthesis [39]. In a short-term double blind comparative study, nabumetone was proved to be equally effective to indomethacin in improvement of arthritis but with improved side effect profile on ulcers [44]. Again, Cardin, et al. [45] demonstrated that nabumetone was more effective (70%) than indomethacin (55%) in reducing paw edema. Other selective COX-2 inhibitors also improved the arthritic index in mice with collagen-induced arthritis [46].

Conclusion: Both allopurinol and nabumetone showed anti-inflammatory and antioxidant effects in CFA-induced arthritis in rats. Addition of allopurinol to nabumetone reduced free radical generation and improved antioxidant status. The combine regimen resulted in a significant improvement of arthritis compared to each drug individually. Combination of allopurinol and nabumetone may effectively enhance the impaired oxidant/antioxidant system and may be useful in delaying the complication of RA by different mechanisms.

References


