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Impact of 6-mercaptopurine and 6-mercaptopurine riboside on pro-inflammatory cytokines and oxidative stress in blood plasma of rats with complete Freund's adjuvant (CFA) arthritis

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ABSTRACT

Inflammatory arthritis is a chronic autoimmune disorder characterized by joint inflammation and pain, often requiring effective immunosuppressive treatment. In a rat model of inflammatory arthritis, the anti-immunosuppressive effects of 6-mercaptopurine (6-MP) and its derivatives were evaluated. Paw edema was induced via subplantar injection of CFA, followed by oral administration of 6-MP and 6-MP riboside (3, 6, or 10 mg/kg). Footpad thickness was measured to assess edema, and blood plasma samples were analyzed for pathogenic mediators (PGE_2), oxidative stress biomarkers (H_2O_2), and pro-inflammatory cytokines (TNF-α, IL-1, IL-6) using ELISA. Results showed that 6-MP reduced PGE_2 and pro-inflammatory cytokines in a dose-dependent manner, significantly decreasing edema. Repeated 6-MP doses (6 and 10 mg/kg) dramatically lowered pro-inflammatory cytokine production, similar to the NSAID diclofenac. Plasma PGE_2 levels for 6 and 10 mg/kg 6-MP were 2357±624.7 pg/mL and 1670±150.2 pg/mL, respectively, compared to control. For 6-MP riboside, levels were 1931±305.2 pg/mL and 1710±249.2 pg/mL. TNF-α concentrations decreased significantly in plasma treated with 6 mg/kg 6-MP (36.18±4.71 pg/mL) and 10 mg/kg 6-MP riboside (42.02±3.46 pg/mL). IL-6 levels also showed significant reductions, while IL-1 remained unchanged. Additionally, peroxide and glutathione levels improved. Notably, the 10 mg/kg dose of both compounds exhibited superior immunosuppressive potency compared to diclofenac. These findings suggest that 6-MP and its riboside suppress pro-inflammatory mediators while enhancing antioxidant defense. Thus, 6-MP possesses anti-inflammatory and immunosuppressive properties beneficial for early-stage inflammatory arthritis and may serve as a promising disease-modifying anti-rheumatic drug (DMARD).

Keywords: *6-mercaptopurine; 6-mercaptopurine riboside; pro-inflammatory cytokines; PGE_2; and glutathione*

INTRODUCTION

A complex biological reaction to vascularized tissue and cell injury, inflammation can be categorized as either acute or chronic depending on when it initially manifests. At commencement, activated macrophages release several inflammatory mediators, including prostaglandin E_2, nitric oxide (NO), interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (Kneusels et al., 2021). These cytokines play a major role in the initiation and progression of inflammatory conditions. TNF-α, IL-6, and interleukin-1 beta (IL-1β) are among the cytokines that are primarily responsible for the systemic inflammatory response syndrome. According to Mettelman et al. (2022), excessive production and prolonged circulation in the body system might also result in the production of pathogenic cytokines. Rheumatoid arthritis (RA) and other forms of inflammatory arthritis are chronic autoimmune diseases marked by severe joint abnormalities brought on by tendon destruction and bone erosions. A complex network of cytokines mediates the chronic inflammatory process of arthritis. Additionally, oxidative stress species, NO, cyclooxygenase (COX), PGE_2, and other pro-pathogenic inflammatory mediators are crucial to the disease's early pathophysiological processes. However, the formation of reactive oxygen species is also linked to the inflammatory process, particularly in rheumatoid arthritis, and the depletion of synovial fibroblast antioxidant defense, such as glutathione, is an early hallmark of acute early arthritis (Hassan et al., 2011).

Its pathophysiology may be mostly attributed to pro-inflammatory cytokines like as TNF-α and IL-1β, which have been demonstrated to be increased in both synovial fluid and blood. Regardless of treatment, all patients had high levels of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, granulocytes-macrophage colony stimulating factor (GM-CSF), and chemokines like IL-8, according to an analysis of cytokine mRNA and protein in rheumatoid arthritis tissue. The increased synthesis of cytokine inhibitors like soluble tumor necrosis factor-receptor (TNF-R) and interleukin-1 receptor antagonist (IL-1ra) as well as anti-inflammatory cytokines like IL-10 and TGF partially offsets this. However, this upregulation in homeostatic regulatory mechanisms is not sufficient as these are unable to neutralize all the major pro-inflammatory cytokines includings TNF-α, IL-1 and IL-6 had produced (Zheng et al., 2013). The specificity of cellular responses to oxidative stress is determined by the up-regulation of redox-sensitive transcription factors, such as the hypoxia-inducible factor 1 (HIF-1), which is activated by cytokines through a crucial network interaction in reactive oxygen species (ROS)-dependent pathways (Taylor & Scholz, 2022).

According to Hassan et al. (2011), oxidative stress and compromised antioxidant systems play a significant role in the etiology of RA, the pathophysiology of joint tissue injury, and persistent inflammation can result in connective tissue deterioration and joint and periarticular abnormalities. The physiologically active species known as ROS, which include hydroxyl radical (OH.), hydrogen peroxide (H_2O_2), superoxide (O_2-), and reactive nitrogen species like nitric oxide (NO) and peroxynitrite (NO_3-), are increasingly understood to play important roles in vascular biology through redox signaling. To shield biological systems from ROS and oxidative damage, a number of defense mechanisms have developed. The main intracellular enzymes those involved in defense mechanisms such as superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT). In inflammatory arthritis such as rheumatoid arthritis infiltration of large numbers of polymorphonuclear leukocytes and macrophages into the joint space occurs (Kneusels et al., 2021). These phagocytic cells are known to experience a respiratory burst upon activation, which is followed by the production of extremely reactive oxygen species, including hydrogen peroxide (H_20_2) and superoxide anion (0_2^-). One of the main reactive oxygen species produced under oxidative stress conditions is peroxide, such as hydrogen peroxide (H_2O_2). Pathological illnesses like aging, diabetes, atherosclerosis, cataracts, inflammatory arthritis, and neurological diseases have all been connected to elevated levels of peroxide production (Rajkapoor et al., 2007). Based on in vitro research, glutathione (GSH) is the most prevalent and is thought to play a significant part in reactive oxygen species (ROS) defenses. Glutathione also is involved in detoxification of hydrogen peroxide through glutathione oxidase and glutathione peroxidase, the primary mitochondrial enzymes defense from hydrogen peroxide, is upregulated by p53. Deficits of important enzymes involved in glutathione metabolism, such as glutathione synthase, glutathione reductase, and glucose-6-phosphate dehydrogenase, are associated with low levels of antioxidant defenses. Furthermore, the prostaglandin cascade and the autoxidation of small molecules like hydroquinones, flavoproteins, pyrogallols, and others can also produce free radicals in inflammatory joints.

6-MP is an immunosuppressive medication linked to the suppression of cell proliferation, including intestinal epithelial cells, smooth muscle cells, T-lymphocytes, and endothelial cells (Kurakula et al., 2015). The literature contains research on the protective effects of 6-mercaptopurine (6-MP) against intramyocardial injections of streptococci, protein antigens, experimental allergic encephalomyelitis, and primary and secondary immune responses in skin and renal transplants (Laufer et al., 1963; Schwartz & Dameshekthe, 1960). Various strategies have been developed to reduce and block the expression of pro-inflammatory cytokines and pathogenic mediators with additional aim which is to sustain oxidative regulation system in the affected patients. These therapeutic approaches include the use of new drug dosages to enhance disease management, treatment with specific inhibitor or antagonizing effect of antibodies. Interestingly, there is a possibility of 6-MP to be used in the treatment of arthritis patients. However, there is poor evidence related to mechanism and pathway exerted by 6-MP to inhibit inflammation in arthritis. Therefore, as 6-MP has the capability to act as anti-inflammatory and immune modulatory agent, this study was conducted to investigate the effects of 6-mercaptopurine and 6-mercaptopurine riboside on plasma circulation cytokines, oxidative stress biomarkers related to NF-κB pathway

through prostaglandin E_2 production on CFA-induce inflammation arthritis in rat model.

METHODOLOGY

Measurement of cytokines in rat blood plasma

Male adult Sprague dawley rats (200–300 g) were purchased and kept in cages with controlled temperature (23 ± 2 °C) on a 12-h light–dark cycle. All animals were fed ad libitum and allowed to acclimatize to their new surroundings for 7 days before any experimental manipulation was undertaken. Animal experiments were conducted at animal house of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The experiments were conducted in accordance with the care and ethical guidelines (Zimmermann, 1983), approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (Ref: UPM/IACUC/AUP-R104). In compliance with the Institutional Animal Ethics Committee's (IAEC) requirements, the least number of animals (n=8/group) and the least amount of toxic chemicals were utilized to show the effects of the drug or drugs being treated. On day 28, the groups of treated and control animals were put to sleep and killed in order to collect plasma samples. The obtained blood samples were placed in tubes containing heparin for biochemical analysis and ethylene-diamine-tetra-acetic acid (EDTA), an anticoagulant, for hematological analysis. Centrifugation at 3000 g for 10 minutes was used to separate the heparinized plasma for biochemical analysis, which was then refrigerated at -80 °C until it was tested using commercial kits using the Hitachi 902 automated analyzer (Japan). The levels of PGE_2, TNF-α, IL-1β and IL-6 in the plasma exudates were measured by using specific commercially available ELISA kits (Thermo Scientific Rockford, IL USA) cytokines assay following manufacturer's instructions recommendation.

Enzyme-linked immunosorbent assay (ELISA) of plasma cytokines

ELISA was performed in accordance with the manufacturer's instructions. The EDTA tube containing the whole blood of the sacrificed rats was centrifuged at 3000 g for 10 minutes at 4 °C in order to extract the plasma. Following the manufacturer's instructions, the ELISA kits (Thermo Scientific, Rockford, IL USA) were used to measure the amounts of TNF-α, IL-1β, and IL-6 in the rats' blood plasma.

Determination of oxidative stress level

The heparinized blood samples were centrifuged. Plasma were collected and used for determination of oxidative stress parameters. Peroxide and glutathione (GSH) activity, two biochemical indicators of oxidative stress, were measured in plasma samples from the normal, arthritic, and therapy groups. On day 28, the animals were killed, and the whole blood was extracted by heart puncture and given out in tubes that contained heparin (Zimmermann, 1983). Samples of heparinized blood were centrifuged. Oxidative stress parameters were determined by collecting plasma.Single Reflection ATR sampling accessory (PIKE Technologies, USA). Data were recorded as absorbance between 4000 and 400 cm^{-1} using a LabSolutions IR analytical data system (Shimadzu, Japan) with 45 scans accumulated per sample at a resolution of 8.0, which is in the recommended range for balancing spectral detail and potential peak broadening in solid or liquid samples (Resolution and Aperture, n.d.). The wavelength range (4000 to 400 cm^{-1}) was chosen for its effectiveness in analysing lip cosmetic functional groups. Pure samples were placed directly on the crystal plate with a spatula without a substrate. Spectral readings were taken in ten replicates before trace samples were prepared. The ATR crystal plate was cleaned with acetone (Bendosen) to prevent contamination and erroneous readings. Basic pre-treatment steps such as background scans of the clean crystal and blank substrates were conducted.

Determination of peroxide and gluthatione/reduced gluthatione

The peroxide and gluthatione/reduced gluthatione concentration were determined using assay kit Quantichrome™ Peroxide assay kit Diox-250 and Quantichrome™ Glutathione assay kit, Bioassay Systems, Hayward, CA, U.S.A.), respectively. The supernatants (plasma samples) were instantly measured using the ELISA kits. All procedures of the assay were strictly followed in accordance to procedures recommended by manufacturer.

Statistical analysis

For each template experiment, the data were presented in triplicate sets as mean ± standard error of mean (± S.E.M). Tukey's multiple comparison test across groups and Student's t-test for comparison between means of two groups were performed after the ELISA cytokine expression and oxidative stress indicators data were submitted to analysis of variance (ANOVA). Variations were deemed significant when p values were less than 0.05 ($p<0.05$). Microsoft Office Excel 2013 (Microsoft, USA) and GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) were used for all the analyses.

RESULTS

Pro-inflammatory cytokines production

Tumor necrosis factor alpha (TNF-α), interleukin (IL)-1 and IL-6 are pleiotropic cytokines that have been implicated as key pro-inflammatory cytokines in inflammatory response in biological system. The effects of 6-mercaptopurine and 6-mercatopurine riboside on cytokine production in plasma induced CFA rat were assessed by ELISA method and the results were described as below.

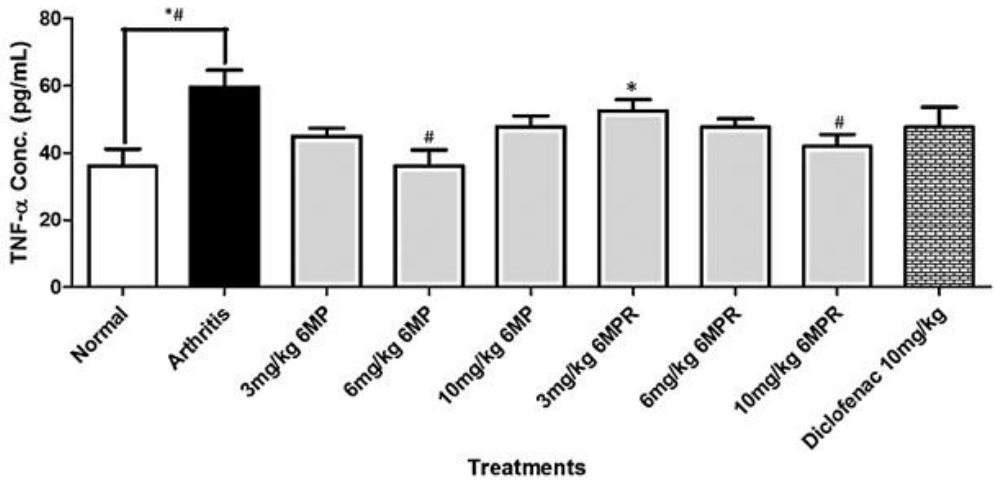
Tumor Necrosis Factor-Alpha (TNF-α)

Figure 1 was showed the effect of various treatments of 6-mercatopurine and 6-mercaptopurine riboside (3, 6 and 10 mg/kg) and 10 mg/kg diclofenac on plasma TNF-α production in CFA induced rats. The control (Arthritic) group showed a strong significant increase ($p<0.05$) in TNF-α level in RA induced rats as compared with the naive (Normal) group. 6-mercaptopurine riboside compound was demonstrated a dose-dependent inhibitory effect upon plasma TNF-α production with significant suppressive at $p<0.05$ but not on 6-mercatopurine.

The middle dose of 6-mercaptopurine, 6 mg/kg (36.18 ± 4.71 pg/mL) and highest dose of 6-mercaptopurine riboside, 10 mg/kg (42.02 ± 3.46 pg/mL) showed a significant suppressive ($p<0.05$) effect as compared with control (Arthritic) group (59.54 ± 5.067 pg/mL). Interestingly, data showed that treatment with both 6-mercaptopurine and 6-mercaptopurine riboside exhibited a significant suppression activity of plasma TNF-α as compared to 10 mg/kg of diclofenac as a control (positive). This study found that that middle dose of 6-mercaptopurine and the highest dose of 6-mercaptopurine riboside are the optimum doses in reducing the systemic TNF-α in plasma CFA-induced rat model. The decreasing of TNF-α level demonstrated by three dosages of 6-mercaptopurine riboside showed almost same potency strength when compared with 10 mg/kg dosage of 6-mercaptopurine and diclofenac.

Figure 1

6-Mercaptopurine and 6-Mercaptopurine riboside's impact on plasma TNF-α levels in rats with CFA-induced RA



*Notes: *Significantly different at p<0.05 compared to control (Normal). #Significantly different at p<0.05 compared to control (Arthritic). (p<0.05 as compared with control; n=8, mean ±S.E.M)*

Interleukin-1 Beta (IL-1β)

Figure 2 showed the effect of various treatments on IL-1β excretion in CFA induced rats. There are no significant different observed between normal and arthritic groups on IL-1β excretion. Only 3 mg/kg 6-mercaptopurine riboside and 10 mg/kg diclofenac were showed significant different when compared to arthritic group at $p<0.05$. Other treatment groups showed a small changes reduction of IL-1β concentrations as compared to control arthritic with no significant different. Interestingly, the two highest dosages of both compounds were demonstrated a significant difference when compared to drug control, 10 mg/kg diclofenac.

Interleukin-6 (IL-6)

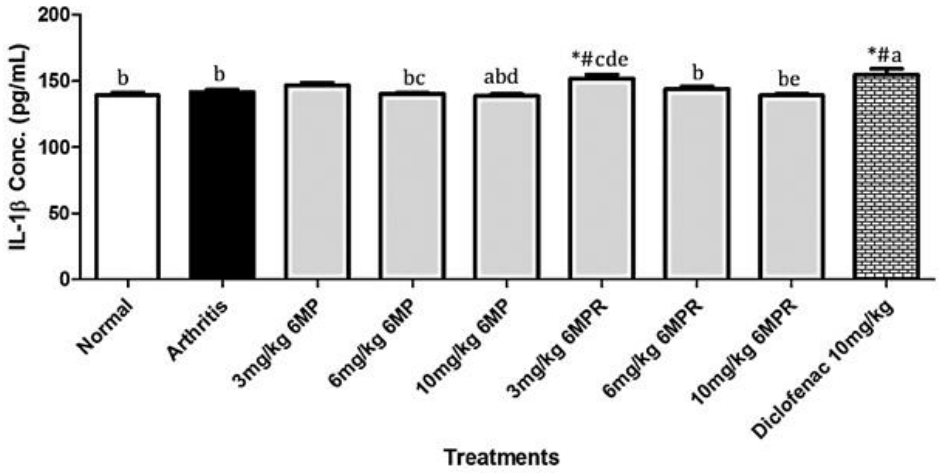
In the present study, it was found that arthritic control group demonstrated a significant increased ($p<0.05$) of IL-6 level production in CFA induced rats as compared with the normal control group (Figure 3). However, a

significant decreased of plasma IL-6 level in CFA induced RA rats were found following treatment of all dosages of 6-mercaptopurine and 6-mercaptopurine riboside compounds as well as 10 mg/mg diclofenac, as compared with the arthritic control group (p<0.05) with the reduction dose-dependent inhibitory effect from lowest to highest dosages in 6-mercaptopurine upon IL-6 production. The 10 mg/kg (59.18±17.56) which was the highest dosage of 6-mercaptopurine and the middle 6 mg/kg (33.66±18.09) dosage of 6-mercaptopurine riboside showed a very good potency capability than positive control 10 mg/kg diclofenac (74.79±11.73). Generally, 6-mercaptopurine and 6-mercaptopurine riboside demonstrated suppressive activity toward IL-6 in all treatment doses compared to control 10 mg/kg diclofenac.

The results suggested that 6-mercaptopurine and 6-mercatopurine riboside also suppressed IL-6 in a similar trend to that demonstrated for TNF-α. Both drugs, 6-mercaptopurine and 6-mercaptopurine riboside showed the strongest suppressive properties on IL-6 production at 10 mg/kg dose as compared to 10 mg/kg diclofenac.

Figure 2

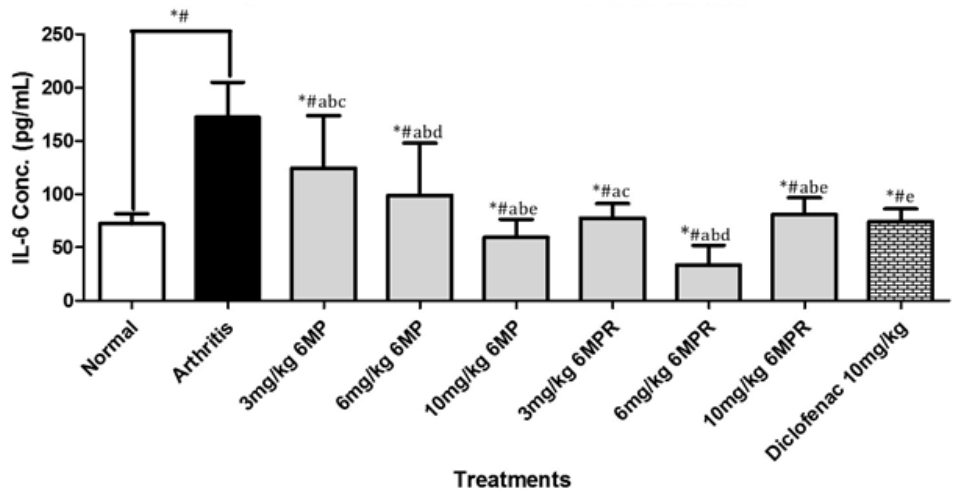
6-Mercaptopurine and 6-Mercaptopurine riboside's impact on plasma IL-1β levels in rats with CFA-induced RA



*Notes: *Significantly different at p<0.05 compared to control (Normal). #Significantly different at p<0.05 compared to control (Arthritic). [a]Significantly different at p<0.05 compared to group with equivalent dosage. [b]Significantly different at p<0.05 compared to 10 mg/kg Diclofenac. Values with same lower case letters (c,d,e) of different groups are significantly different at p<0.05. (p<0.05 as compared with control; n=8, mean ±S.E.M)*

Figure 3

6-Mercaptopurine and 6-Mercaptopurine Riboside's impact on plasma IL-6 levels in rats with CFA-induced RA



*Notes: *Significantly different at p<0.05 compared to control (Normal). #Significantly different at p<0.05 compared to control (Arthritic). [a]Significantly different at p<0.05 compared to group with equivalent dosage. [b]Significantly different at p<0.05 compared to 10 mg/kg Diclofenac. Values with same lower case letters (c,d,e) of different groups are significantly different at p<0.05. (p<0.05 as compared with control; n=8, mean ±S.E.M).*

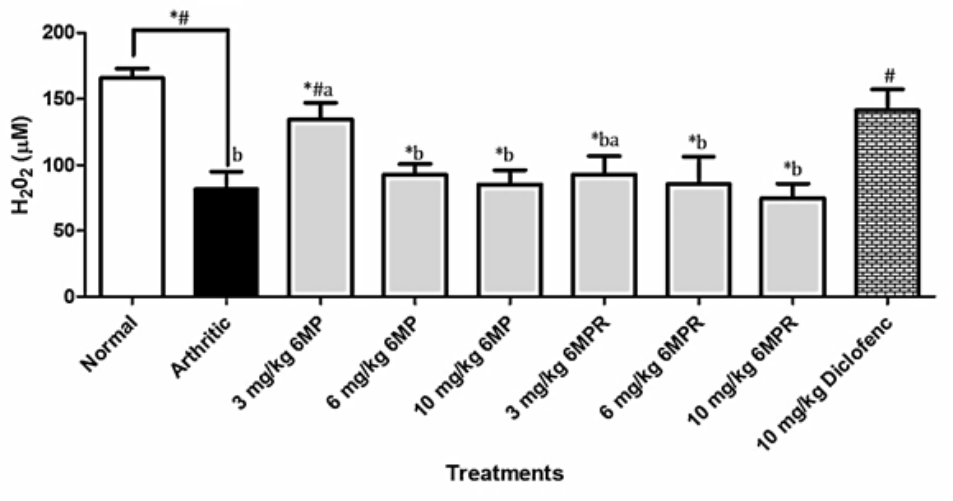
Plasma oxidative markers circulation in CFA-induced rat

Hydrogen peroxide

A significant reduction ($p<0.05$) in the hydrogen peroxide (H_2O_2) level in RA-induced rats in arthritic control groups as compared with the normal control group (Figure 4). 6-mercaptopurine and 6-mercaptopurine riboside were demonstrated a reduced dose-dependent peroxide effects upon CFA-induced in rat as low as 3 mg/kg of both compounds. All dosages except 3 mg/kg of 6-MP demonstrated a significant decreased ($p<0.05$) in the plasma level peroxide in CFA-induced RA rats as compared with the control normal. But only 10 mg/kg diclofenac was showed a significant different when compared to arthritic control group. Interestingly, all the high dosage of both compounds were demonstrated a very strong suppression activity especially 6-mercaptopurine riboside when compared to 10 mg/kg diclofenac.

Figure 4

The effect of 6-mercaptopurine and 6-mercaptopurine riboside on plasma peroxide level in CFA-induced RA rats



*Notes: *Significantly different at p<0.05 compared to control (Normal). #Significantly diferent at p<0.05 compared to control (Arthritic). [a]Significantly different at p<0.05 compared to group with equivalent dosage. [b]Significantly different at p<0.05 compared to 10 mg/kg Diclofenac. (p<0.05 as compared with control; n=8, mean ±S.E.M)*

Gluthatione/reduced gluthatione

Figure 5 showed the effect of the various treatments on plasma glutathione circulation in CFA-induced rat model. A high significant increased ($p<0.05$) in the plasma gluthathione level in RA-induced rats in arthritic control groups as compared with the normal control group. All dosages of both mercaptopurine as well as 10 mg/kg diclofenac (15.23 ± 3.89 µM) caused significant reduction ($p<0.05$) in the glutathione level in CFA-induced RA rats as compared with the arthritic group. The concentration level of glutathione in plasma CFA-induced rat after treatment with 6-mercaptopurine were 9.85 ± 0.43, 19.29 ± 5.81 and 24.24 ± 8.41 µM respectively. Meanwhile, the concentration level of glutathione in plasma CFA-induce rat after treatment 6-mercaptopurine riboside were 9.73 ± 0.49, 10.07 ± 0.48 and 19.64 ± 3.89 µM, respectively. All the concentration values of gluthathione after treatment with 6-mercaptopurine and 6-mercaptopurine riboside compounds were restored back to normal level.

Interestingly, both oxidative stress biomarkers peroxide and glutathione were revealed a decreased and an increased pattern, respectively against increasing dosages. After treatment with the compounds the level of both biomarkers almost restored back to the normal condition as compared to normal control group. Peroxide and glutathione metabolisms were played critical roles in drug detoxification and working as dual-balanced functions (García-González et. al., 2015; Veselinovic et. al., 2014).

Pathogenic Mediators in Systemic RA

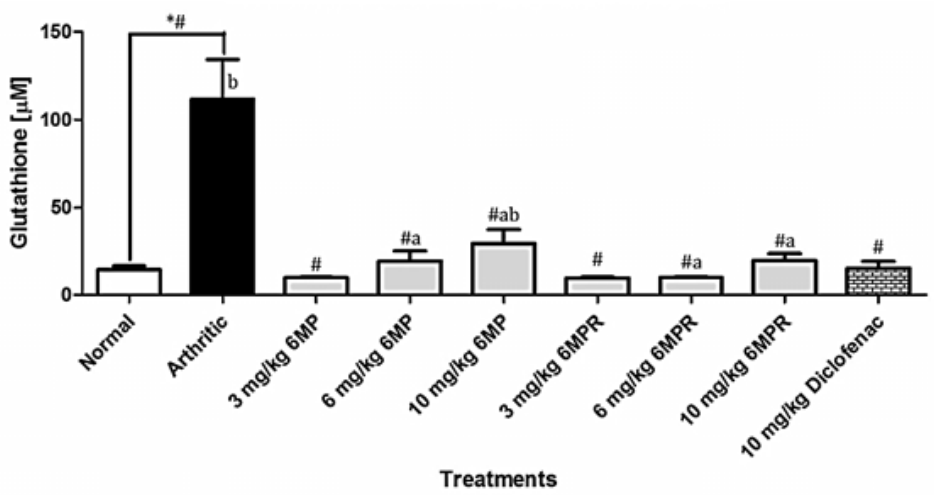
Prostaglandin E_2 (PGE_2)

In this study, the arthritic control group was found to show a high significant increase ($p<0.05$) in PGE_2 level in RA induced rats as compared with the normal control group (Figure 6). All dosages of 6-mercaptopurine, 6-mercaptopurine riboside and 10 mg/kg diclofenac showed a significant difference ($p<0.05$) as compared with the controls group (normal and arthritic). Interestingly, a decreased dose dependent of PGE_2 level were demonstrated

by three selected dosages in both compounds. Surprisingly, the treatment of 6-mercaptopurine and 6-mercaptopurine ribosides to the arthritic rats cause a dose dependent inhibition of PGE_2 production pattern started at lower dosage, but no significant differences between dosages. At low dosage, suppression of PGE_2 production was about 11-12 folds lower than arthritic values. Generally, all treatments were demonstrated same potency in reducing PGE_2 concentration back to normal level as same as Normal control group. The highest dosage of 6-mercaptopurine (10 mg/kg) and 6-mercaptopurine riboside (10 mg/kg) exhibited similar potency capability with standard drug, 10 mg/kg diclofenac.

Figure 5

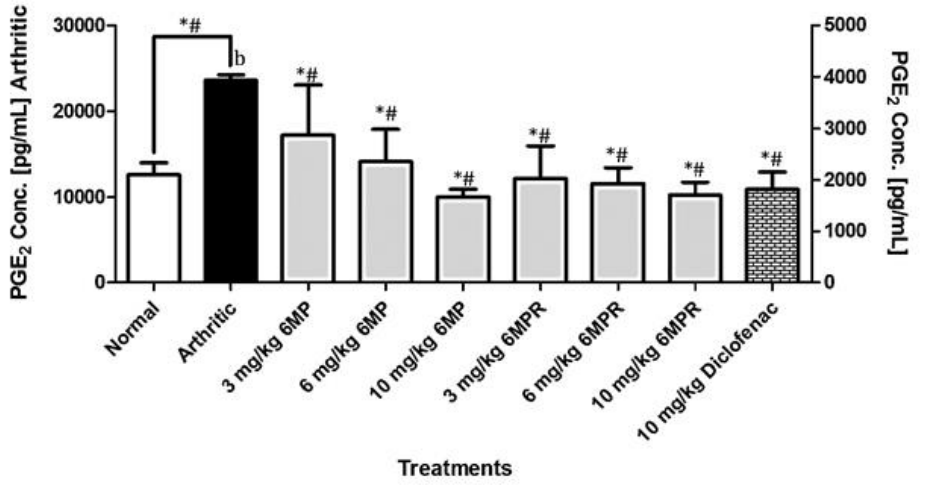
The effect of 6-mercaptopurine and 6-mercaptopurine riboside on plasma glutathione level in CFA-induced RA rats



*Notes: *Significantly different at p<0.05 compared to control (Normal). #Significantly diferent at p<0.05 compared to control (Arthritic). [a]Significantly different at p<0.05 compared to group with equivalent dosage. [b]Significantly different at p<0.05 compared to 10 mg/kg Diclofenac. (p<0.05 as compared with control; n=8, mean ±S.E.M)*

Figure 6

The effect of 6-mercaptopurine and 6-mercaptopurine riboside on plasma PGE_2 level in CFA-induced RA rats



*Notes: The left scale is represented for arthritic control group only. *Significantly different at p<0.05 compared to control (Normal). #Significantly diferent at p<0.05 compared to control (Arthritic). [a]Significantly different at p<0.05 compared to group with equivalent dosage. [b]Significantly different at p<0.05 compared to 10 mg/kg Diclofenac. (p<0.05 as compared with control; n=8, mean ±S.E.M)*

DISCUSSION

Pro-inflammatory cytokines like TNF-α, IL-1β, IL-2, IL-6, and PGE_2 mediate inflammation, which is the immune system's initial reaction to infection or irritation. The creation of effective medication dosages that suppress the synthesis and secretion of pro-inflammatory cytokines and regulate and block the main pathogenic molecules in the afflicted area is a promising strategy to lessen arthritic inflammation. 6-MP is an immunosuppressive medication that has been shown to have several biological effects, including immunomodulatory and anti-inflammatory properties (Kurakula et al., 2015). As an immunosuppressive drug, 6-MP has been shown to inhibit proliferation of different cells such as lymphocytes, smooth muscle cells, endothelial cells and intestinal epithelial cells at the highest dosages (Marinkovic et al., 2014). While the compound's interference with humoral and cell-mediated immune responses may be related to the alkylation of thiol groups in the lymphocyte cell membrane, its anti-metabolic effects are achieved by inhibiting DNA synthesis by 6-mercaptopurine and its metabolites (Sahasranaman et al., 2008). Severe cases of inflammation, including Crohn's disease (Dubinsky, 2004), an inflammatory reaction linked to elevated prostaglandin levels, allergic encephalomyelitis, acute lymphoblastic leukemia and cancer, and inflammatory bowel disease (Dubinsky, 2004), have improved with the use of 6-MP in both human and experimental animals.

In this present study, a significantly higher plasma concentrations level of TNF-α, IL-6, PGE_2, and glutathione were recorded in control pathologic group with inflammation arthritis but in contrast, lower plasma concentration level of IL-1β and peroxide were demonstrated. A great variations were seen in the excreted patterns of pro-inflammatory cytokines and antioxidant biomarkers concentration between different treatment groups, although, interestingly, the findings revealed that not all cytokines were elevated in the same samples. This variation among different cytokines in the same sample reflects the intricate cytokine network and its regulatory functions. Although the major pathology in RA occurs in the synovial; synovial fluid is more readily accessible, and as such the production of cytokines like IL-1 was first documented but the synovial fluid of rats in this study is very limited (Proundman et al., 1999).

This study showed that the plasma level of TNF-α was considerably higher in the arthritic group compared to the control (normal) group in animals with early RA (Spraque dawley rats). Following 6-mercaptopurine and 6-mercaptopurine riboside treatment, plasma TNF-α levels gradually decreased in comparison to the arthritic control. Most of the animals examined in this study exhibited mild to moderate disease activity and were in the early stages of RA. Only 6 mg/kg of 6-mercaptopurine and 10 mg/kg of 6-mercaptopurine riboside demonstrated a substantial (p<0.05) decrease in the plasma TNF-α when compared to the arthritic control, despite the fact that all medication dosages utilized in this investigation indicated a trend of inhibition. Surprisingly, both doses of the drugs were showed higher reduction potency strength as compared to 10 mg/kg diclofenac, a commonly used drug for treating arthritis. Thus, this result indicated that the respective dosages might be optimum in reducing systemic TNF-α. Based on the Figure 1, 6-mercaptopurine and 6-mercaptopurine riboside seem to be a dose-dependent to reduce circulating TNF-α might be due to the high level of IL-6 in the rats were shown in Figure 3. TNF-α is an autocrine stimulator as well as a potent paracrine inducer of other inflammatory cytokines, including IL-1, IL-6, IL-8, and granulocyte-monocyte colony-stimulating factor (Joosten et al., 2003). Moreover, in this study, the level of TNF-α and IL-6 CFA-induced rats treated with 6-mercaptopurine and 6-mercaptopurine riboside were significantly decreased compared with the arthritic group with decreasing pattern propational to increase dosages. Our findings are in line with previous studies which reported high concentrations of TNF-α and IL-6 in inflammation diseases (Isomäki & Punnonen, 1997).

Although the overall pre-clinical response to the 6-mercaptopurine was excellent when compared to 10 mg/kg diclofenac, in this regard, the plasma TNF-α concentration after treatments were showed the strongest association with physical assessment appearances of the disease progression. However, 6 mg/kg 6-mercaptopurine and 10 mg/kg 6-mercaptopurine riboside, did not totally restored to its normal condition which may suggest that TNF-α alone, might not be the only main pro-inflammatory cytokine in causing paw inflammation in CFA-induced RA rat model. Other pathological inflammatory pathways associated with RA may also exist that were inhibited by 6-mercaptopurine and 6-mercaptopurine riboside. A studied reported that the high concentration of 6-mercaptopurine not azathioprine (Aza, parent drug of 6-mercaptopurine) fully inhibit the production of pro-inflammatory cytokines especially common pro-inflammatory cytokines likes TNF-α, IL-1β and IL-6 on blood serum in inflammatory bowel disease (IBD) patients (Louis et al., 2000). This suggests that the effect on pro-inflammatory cytokines observed with high dose of 6-MP may be due to the high nitro-imidazole metabolite produced during synthesis of 6-MP from Aza (Loius et al., 2000). These revelation findings, the measurement of plasma TNF-α might be able to provide additional clinical information that may consider the treatment strategy in the future in treating inflammation arthritis disease.

In addition, the levels of excreted TNF-α in RA synovia tissue is directly proportional with inflammation and bone erosion. Furthermore, a series of potential therapeutic compounds trials as TNF-α blocking agent has been conducted. Recently, TNF-α blockade agent has been reported to prevent cartilage erosion and bone destruction in RA patients (Fox, 2000). Prolonged inflammation can occur when the blood's soluble versions of TNF receptors (sTNF-R) are overloaded by excessive TNF synthesis. The elevated levels of TNF inhibitors in RA are noteworthy because they rule out the idea that the inability to create inhibitory factors normally plays a significant role in the pathophysiology of RA (Grell et al., 1995).

IL-1β is also a main target pro-inflammatory as important as TNF-α in current RA study. IL-1β activates proliferation of synoviocytes and induces the secretion of many other mediators and chemokines in RA (Firestein, 2003). IL-1β is mainly secreted via macrophages activation in the physiopathogenesis of RA and act as messenger molecules in paracrine system which induces the initiation of biological responses to other immune cells (Kay and Calabrase, 2004). From the results, all dosages of 6-mercaptopurine and 6-mercaptopurine riboside failed to reduce circulating IL-1β significantly. Based on the Figure 2, the probably reasoned was the selected dosage failed to reduce circulating IL-1β might be due to the different mechanism of IL-1β response and production in the 6-mercaptopurine and 6-mercaptopurine pathway and metabolism. It seem using difference pathway with common finding stated that the TNF-α production plays roles in inducing the production of IL-1β and IL-6 thus might be the 6-mercaptopurine and its derivatives not directly suppressed the level of IL-1β via TNF-α regulation but probably synergistic with IL-6 thus effecting the expression of TNF-α as result were demonstrated.

Beside the TNF-α activation via NF-κB pathway, it is also well established that a distinct pro-inflammatory cytokines cascades involved during inflammatory processes can contribute to the development of inflammatory pain and hyperalgesia. The increased production of IL-1β during inflammation stimulates and enhances the expression of other pro-inflammatory mediators such as IL-6 and granulocytes-macrophage colony-stimulating factors thus facilitates inflammatory cell infiltration and promotes induction of COX enzymes via prostaglandin E_2 pathway (Guo et al., 2008). Both TNF-α and IL-1β are contribute to a hyperalgesia condition induced by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) as well as N-methyl-D-aspartate (NMDA), which mainly mediate excitatory synaptic transmission (Kawasaki et al., 2008) through activation and sensitization of peripheral nociceptors thus lowering the threshold sensation of nociceptor during the inflammation (Sibilia et al., 2006).

In RA joints, pro-inflammatory cytokines like PGE2, TNF-α, and other ILs stimulate fibroblast-like synoviocytes (FLS) to produce IL-6 (Firestein et al., 2003). It is an essential cytokine in RA that triggers the production of inflammatory cells like B lymphocytes in the inflammatory process (Bugatti et al., 2014). By promoting osteoclast maturation and pannus development brought on by vascular endothelial growth factor (VEGF), IL-6 can encourage joint degeneration (Srirangan & Choy, 2010). In this study, IL-6 production was found significantly to be inhibited in a dose-dependent manner in all treatment groups reducing the systemic IL-6 as showed in Figure 3. IL-6 has been believed to play a synergistic role with TNF-α and also functioned as pro-inflammatory cytokine with multiple biological activities and exhibiting a pivotal pathogenic role in RA but somehow overlapped with other cytokines especially TNF-α itself via different mechanism of actions (Fox, 2000). Interestingly, in this present study, the production of IL-6 was significantly induced probably mainly by TNF-α but not IL-1β protein. This present study suggested that 6-mercaptopurine might have a strong supressive property toward IL-6 and TNF-α than others pro-inflammatory cytokines such as IL-1β thus preventing the pannus formation via blocking the stimulating vascular endothelial growth factor (VEGF), further distort the mechanism of joint destruction (Yang et al., 2016).

Generally, 6-mercatopurine and 6-mercaptopurine riboside were shown to have inhibitory activities towards systemic pro-inflammatory cytokines involved, such as TNF-α, IL-1β and IL-6 in RA CFA-induced rat model. From the findings and discussions above, a proposed mechanism those probably blockage the inflammation of main cytokines, TNF-α, IL-1 and IL-6 pathways involved in rheumatoid arthritis by 6-MP and 6-MPR.

Apart from pro-inflammatory cytokine pathways discussed above, it is also well established that a distinct antioxidative systemic biomarkers pathway also contributed to inflammation, working synergistic with cytokines and chemokines in development of inflammatory pain and recovery period (Shah et al., 2011). The effects of oxidative antioxidant plasma biomarkers conducted in this present study were discussed in detailed below.

The responses induced by exposure of cells to oxidative stress vary from necrotic or apoptotic cell death to inflammation depending on the cell type and oxidant exposure. The role of peroxide (H_2O_2) and glutathione in rheumatoid arthritis (RA) is still not clear (Quiñonez-Flores et al., 2016). The importance of selenium and selenoproteins compound such as GSH-Px to human health has been well-documented (Brown & Arthur, 2001). In this present study, after 27 days rat received CFA immunization via injection into the palmar surface of the left hind paw the level of plasma peroxide on arthritic group (control) was significantly reduced almost half compared to normal group (Figure 4), meanwhile level of plasma reduced glutathione was highly increased almost 6 times when compared to normal (Figure 5). Moreover, the level of both biomarkers were significantly reduced and increased respectively upon 6-mercaptopurine and 6-mercaptopurine riboside treatments and restored back to normal level after about two weeks supplementation.

Production of H_2O_2 is related to insufficient supply of O_2 demands in inflammation condition and much related to xanthine oxidase (XO) enzyme catalyzed during purine degradation. XO is one of the key enzyme involved in thiopurine metabolism. 6-MP is metabolized by xanthine oxidase (XO) leads to thiouric acid, an inactive metabolite excreted in urine (Roblin et al., 2016). In addition, exposure to H_2O_2 *in vitro* activated NF-κB pathway, however the response was not universal and depends on the cell type and transformation state (Jaspers et al., 2001). An increasing level of reactive oxygen species (ROS), either by direct addition of chemical such as H_2O_2 or by adding stimulant agents will also increase intracellular ROS levels, has been demonstrated to activate NF-κB in some cell lines (Manna et al., 1998).

Different cell types or pathological cell conditions were demonstrated different activation of NF-κB after stimulated with H_2O_2. For examples, a subclone of Jurkat T and HeLa cells have been shown to activate NF-κB

after stimulation with H_2O_2, however a number of other cell types, including monocytic cells, astrocytoma cells and KB epidermal cells (Brennan & O'Neill, 1995), appear to be insensitive to stimulation with H_2O_2. In addition, stimulation of a transformed human dermal microvessel endothelial cell line (HMEC-1) with H_2O_2 increased NF-κB activity, whereas it had no effect on NF-κB activity in another study (True et al., 2000). Furthermore, although there are numerous studies demonstrating activation of NF-κB in vascular endothelial cell culture models stimulated with H_2O_2, some studies demonstrated the opposite effect. For example, high concentrations of H_2O_2 (1 mM) increased NF-κB DNA binding in human umbilical vein endothelial cells (HUVECs), whereas lower concentrations (50 to 200 μM) were unable to increase NF-κB activity in the HUVEC culture model. Modulation of H2O2 production may represent a novel strategy for controlling neutrophilic inflammation in the joints. This present study showed an evidence whereas, 6 mg/kg and 10 mg/kg of 6-MP and 6-MP riboside dosage were able to stimulate and provide a suitable environment for H_2O_2 production to inhibit NF-κB signaling pathway thus reduced the inflammation on the affected area instantly within 2 weeks.

Our study suggested that the presence of high glutathione levels was likely to be caused by inflammation arthritis just at an earlier stage. This circumstance probably different if the patients had been suffering with RA for long periods. The studied evidences regarding to these had been documented. Previous in vivo studies on solid tumors reported that elevated glutathione levels are associated with drug resistant as well (O'Dwyer et al., 1995). Treatment of 6-MP and 6-MP riboside on CFA-induced rat arthritis in this study provide a new insight whereas plasma glutathione was elevated, the level of plasma peroxide is reduced. This is an ideal balancing of homeostasis in regard to antioxidant system in blood circulation. Interestingly, following treatment with 6-MP and 6-MP riboside, the level of glutathione was restored back to normal as demonstrated in Figure 5 however it was elevated at high dosage treatments.

PGE_2 plays an important role in inflammation diseases and associated with increase production of prostaglandin E_2 including RA and it can be detected in the blood of RA patients (Gheorghe et al., 2011). This present study was demonstrated that concentration of PGE_2 was very high in arthritic group compared to normal group. Upon treatment with 6-MP and 6-MP riboside, the level of concentration of PGE_2 in circulation was significantly reduced in a dose-dependent manner (Figure 6). This study demonstrated that prostaglandin E_2 production was suppressed by 6-mercaptopurine and 6-mercaptopurine riboside were suggested that these inhibitory effects may underlie the drugs anti-inflammatory actions. These present findings also suggested that PGE_2 might be one of the main pathogenic pro-inflammatory cytokines to cause RA inflammation although some others pathological pathways of the disease might be existing (Hussein et al., 2012) that were inhibited by 6-mercaptopurine and its derivatives. 6-MP has been shown to have a very strong anti-inflammatory effects by inhibiting prostaglandin synthesis and neutrophil trafficking into inflammatory asthmatic tissue (Dean et al., 2004) and Rac1 inhibition in T cells and gut epithelial cells.

These present study demonstrated that 6-mercaptopurine and 6-mercaptopurine riboside were inhibited the excessive productions of pro-inflammatory cytokines and pathogenic mediators by its anti-inflammatory properties in CFA-induced rat arthritis model by elevating GSH concentration and retarded the production of hydrogen peroxide, a very harmful reactive oxygen species (ROS). This study may open a new approach in understanding the relationship between adjuvant arthritis in rats and rheumatoid arthritis in human since both having interrelated or overlapped cytokines involved. This study may help to explain why alkylating agents have a favorable, suppressive effect on the inflammatory reactions of connective tissue disease. Altogether, these findings indicate that 6-MP and 6-MP riboside have an anti-inflammatory effects and inhibitory properties in suppressing cytokines and accelerates the level of antioxidant status on arthritis condition thus improve the remission until fully recovery.

CONCLUSION

In conclusion, this study found that drug 6-mercaptopurine and 6-mercaptopurine riboside were inhibited inflammatory response induced by CFA-induce arthritis in-vivo using rat model. 6-mercaptopurine and 6-mercaptopurine riboside were significantly inhibits the development of adjuvant-induced inflammation arthritis and accelerate the endogenous antioxidant level in the rat model. It can alleviate early arthritis inflammation and have a further therapeutic effect for rheumatoid arthritis. Its mechanisms may be related to reduce the expression of pro-inflammatory cytokines, pathogenic mediators and enhance glutathione in blood circulation thus alleviate inflammatory response. In addition, 6-MP attenuates TNF-α and IL-6 production, but not IL-1β in blood circulation through inhibition of PGE_2 activation probably via NF-κB pathway. The level of gluthatione and peroxide, the major oxidative biomarkers in blood circulation also were improved upon treatments. Data presented in the current study disclosed a previously unknown role of 6-MP in early inflammation arthritis as a new DMARD for treating arthritis. As these experiments were performed using animal, future studies should be conducted and focus on testing of 6-MP in synoviocytes models of synovial cells to determine the possible mechanism and molecular pathway.

AUTHOR CONTRIBUTIONS

The following study was undertaken by Che Ku Dahlan-Daud as a part of his PhD project under the supervision of Muhammad Nazrul Hakim. Zetty Nadia Zain and Tham Chau Ling were the co-supervisors contributed to the *in vitro* studies and laboratory analysis of data. Yong Yoke Keong also co-supervised and contributed to the *in vivo* studies. The manuscript was written by Che Ku Dahlan-Daud and Muhammad Nazrul Hakim edited and reviewed the content.

ETHICS APPROVAL

Animal experiments were conducted in accordance with the care and ethical guidelines, approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (Ref: UPM/IACUC/AUP-R104).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest in this work.

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