Research Article

Differential Effects of Atorvastatin and Prednisolone on Inflammation, Oxidative Stress and Hematological Biomarkers on Freund's Adjuvant Induced-Arthritis in Rats

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ABSTRACT

Rheumatoid arthritis (RA) is chronic systemic, immune-mediated inflammatory disorder that attacks flexible joints due to persistent overproduction of pro-inflammatory and oxidative biomarkers. Hematological disturbances have been recognized as major complications upon rheumatoid arthritis progression. This study aimed to investigate the anti-inflammatory and anti-oxidant effects of Atorvastatin along with their modifying role on hematology in comparison with Prednisolone in adjuvant –induced arthritis in rats. Forty female rats were divided into 4 equal groups: first group as normal control group, the other three groups received 0.4 ml of Complete Freund's adjuvant (CFA) as single dose every 4 days for 12 days, one served as positive control group and the other two groups received Atorvastatin (10 mg/kg/day) and Prednisolone (10 mg/kg/day) respectively for 15 consecutive days. At the end of experiment, blood samples were collected for hematological examination and serum samples were used for detection of inflammatory and oxidative biomarkers. Results revealed that Prednisolone and Atorvastatin monotherapies significantly reduced all parameters of inflammation and arthritis. This evidenced by significant decrease in serum Tumor Necrosis Factor alpha (TNF-α), Interleukin -6 (IL-6), Malonaldehyde (MDA) and reduced Glutathione (GSH). Along with amelioration in hematological parameters that were impaired by CFA where remarkable increase in Red Blood Cells (RBCs), Hemoglobin (Hb), Platelet and Hematocrit (Hct) values concomitant with inhibition of White Blood Cells (WBCs) count were recorded. These results further confirmed by histopathological studies. The present work demonstrated that Atorvastatin exert potent anti-inflammatory and anti-oxidant effects with favorable role on deterioration of hematological parameters.

Keywords: Rheumatic Arthritis; Atorvastatin; Prednisolone; Interleukin -6; Tumor Necrosis Factor alpha, Complete Freund's adjuvant.

INTRODUCTION

Rheumatoid arthritis is a common, chronic, inflammatory, autoimmune disease of unknown etiology affecting approximately 1% of the world population[1]. It is characterized by joint swelling, joint tenderness and destruction of synovial joints associated with progressive disability, systemic complications, early death, and socioeconomic costs[2]. Therefore, critical issues concerning the effect of therapy were to control symptoms and signs of the disease for prolonged periods and retard the damaging effect of inflammation on articular cartilage and bone[3].

Prednisolone is a fast acting glucocorticoid, potent anti-inflammatory drug where Short-term use of it can reduce synovitis and its long-term decrease joint damage[4].

Glucocorticoids incur substantial adverse risks, such as infections and osteoporosis, and their overall risk/benefit ratio was deemed unfavorable[5]. In addition, some studies have demonstrated that glucocorticoids could increase the risk of cardiovascular disease in patients with rheumatoid arthritis due to their potential deleterious effects on lipid, glucose tolerance, and hemoglobin, abnormalities as well as development of hypertension and/or obesity[4].

In recent days, researchers were directed towards traditional system of medicine for the discovery of drugs that were long acting anti-inflammatory with minimum side effects.

Atorvastatin belonging to statins, one of these classes of drugs that represent a well-established class for effectively lowering serum cholesterol level by competitive inhibition of 3-hydroxy-3-methyl glut aryl coenzyme A (HMG-CoA) reductase and were widely prescribed for treatment of hypercholesterolemia[4]. Some of the clinical benefits of statin therapy may be independent of their cholesterol lowering effects.

These so called pleiotropic effects were believed to include anti-inflammatory actions and immunosuppressive properties suggesting a new face of statin therapy which makes them important not only in the treatment of dyslipidemias but also in chronic systemic inflammatory disease like R.A[6]. The present study aimed to shed light on promising influences of Atorvastatin on inflammatory markers along with oxidative stress and hematology in the treatment of rheumatoid arthritis.

MATERIALS AND METHODS

Animals

Adult female Wister Albino rats, weighing 170–200 g obtained from the Modern Veterinary Office for Laboratory Animals, Cairo, Egypt. They were housed with

free access to commercial diet and tap water. After one week of acclimatization, rats were randomly divided into four equal groups of 10 rats each.

Drugs and Chemicals

Complete Freund's adjuvant (Difco Laboratories Co-USA)

1. Prednisolone acetate powder (Epico, Egypt)

2. Atorvastatin calcium crystalline powder(Sigma Pharmaceutical Industries, Egypt)

All drugs were used as freshly-prepared solutions (dissolved in 1% v/v tween 80).

The concentrations of the drugs were adjusted so that each 100g animal's body received orally 1ml of either suspension containing the required dose.

Induction of Adjuvant Arthritis

To develop a rat model of adjuvant arthritis, rats were injected with 0.4 ml Complete Freund's Adjuvant subcutaneously in the planter surface of the right hind paw Divided in three doses [one dose every four Days] for 12 days[6].

Experimental Groups

After acclimatization period for one week rats were divided into four groups as follows:

Group 1: Served as normal control group .This group received regular diet and water ad libitum and didn't receive any medication.

Group 2: Rats were injected subcutaneously by complete Freund's adjuvant as a single dose of 0.4 ml in the planter surface of the right hind paw divided in three doses [one dose every four Days] for 12 days. This group served as positive control group.

Group 3: Rats were injected subcutaneously by complete Freund's adjuvant as a single dose of 0.4 ml in the planter surface of the right hind paw divided in three doses [one dose every four Days] for 12 days followed by 15 days administration of Prednisolone (PO , 10 mg/kg/day)[4].

Group 4: Rats were injected subcutaneously by complete Freund's adjuvant as a single dose of 0.4 ml in the planter surface of the right hind paw divided in three doses [one dose every four Days] for 12 day followed by 15 days treatment with Atorvastatin (PO , 10 mg/kg/day)[4].

By the end of the treatment period, blood samples were collected from orbital venous plexus under light anesthesia. Each sample was divided into two portions; the first was collected in clean dry Eppendorf tubes containing EDTA as anticoagulant to be used for hemogram studies. The second part was collected into non heparinized tubes and centrifuged at 3000 rpm for 10 minutes for separation of the serum. The collected sera were stored at -20°C for biochemical estimation.

For detection of histopathological changes, right/left ankle joints were collected into 10% neutral buffered formalin and then processed for complete decalcification in EDTA.

Parameters Measured

Assessment of immunological and inflammatory parameters

Serum Tumor Necrosis Factor α [TNF α] and Serum Interleukin 6 [IL-6] were determined by *in vitro* Enzyme Linked Immunosorbent Assay [ELISA] kit, using colorimetric reaction method as instructed in the kit manualrespectively[7-8].

Assessment of oxidative stress parameters

1. Reduced Glutathione level was measured in blood serum as described by[9].

2. Malonadialdehyde (MDA) was measured as an indicator for lipid peroxidation. Using method described by[10].

Assessment of hematological parameters

Blood smears that containing EDTA were prepared as soon as possible after blood collection on a glass slide, quickly dried, and stained with Giemsa and May-Grunwald stain[6]. Determination of total number of leukocytes [WBCs], Total number of erythrocytes [RBCs], Hemoglobin [Hb]concentration, Platelet count and Hematocrit value [Hct%] were estimated by adopting standard procedures[11].

Histopathological Examination:

After decalcification paws were embedded in paraffin, sectioned longitudinally at 5µm and stained with hematoxylin and eosin. Sections were examined for arthritic changes in the control as well as in the drug-treated rats[12].

Statistical Analysis

The values of the measured parameters were expressed as mean ± S.E.M. Comparison between the mean values of different groups was carried out by using one way analysis of variance (ANOVA), (F value) has been performed to show inter-group significance followed by Student-Newman-keulsfor multiple comparisons. The P values smaller than 0.05 were selected to indicate statistical significance between groups using graph bad instat programme.

RESULTS

Effect of Prednisolone and Atorvastatin on Immunological and Inflammatory Parameters

Complete Freund's adjuvant significantly increased proinflammatory cytokines TNF-α (117.550 ± 1.75 pg/ml), IL-6 (130.950 ± 4.84 pg/ml).

Oral treatment of rats with Prednisolone or Atorvastatin was associated with significant decrease in serum level of

TNF-α and IL-6, as compared to that of arthritic group (Table 1).

Effect of Prednisolone and Atorvastatin on Oxidative Stress Parameters

As shown in figure 2 Complete Freund's adjuvant significantly increased MDA level (12.238 ± 0.48 nmol/ml) and significantly decreased GSH level (17.054 ± 0.57 µmol/L) when compared with non-arthritic control rats.

Treatment with Prednisolone or Atorvastatin resulted in significant lower level of serum MDA and higher levels of serum GSH when compared to that of untreated group.

It was to be noted that Atorvastatin showed no significant difference in serum MDA level or GSH level when compared to Prednisolone.

Effect of Prednisolone and Atorvastatin on Hematological Parameters

Hematological analysis in table [3] showed significant increase in WBCs count (172.586 ± 8.46 10^3/µL), significant decrease in RBCs count (1.633 ± 0.141$0^6$/mcl), Hb concentration (8.210 ± 0.27 g/dl), and platelet count (277.625 ± 9.93 billion/l) and Hct (38.900 ± 2.23 %) in Complete Freund's adjuvant treated rats as compared to normal control group. Treatment with Prednisolone or Atorvastatin produced significant decrease in WBCs count with significant increase in RBCS count, HB concentration and Hct % as compared to untreated group. On the other hand drug treatment with Prednisolone or Atorvastatin resulted in slight increase in platelet count when comparing to arthritic group as shown in table [3].

Table 1: Effect of two weeks daily dose administration of atorvastatin and prednisolone on immunological and inflammatory biomarkers of complete freund's adjuvant induced rheumatoid arthritis in rats

Experimental groups	TNF-α (pg/ml)	IL-6 (pg/ml)
Control	32.776 ± 1.25	1.22 ± 34.764
Untreated	117.550 ± 1.75 *	130.950 ± 4.84 *
Prednisolone	57.738 ± 2.95 * @	58.888 ± 4.32 * @
Atorvastatin	64.300 ± 2.76 * @	62.638 ± 5.87 * @

N= 8-10 rats per group.; Data were expressed as mean ± SEM; Statistical analysis is carried out using one way ANOVA followed by Student-Newman-keuls multiple comparisons test; * Significantly different from control value at p < 0.05; @ Significantly different from Rheumatoid arthritis control value at P < 0.05

Table 2: Effect of two weeks daily dose administration of atorvastatin and prednisolone on oxidative stress biomarkersof complete freund's adjuvant induced rheumatoid arthritis in rats

Experimental groups	GSH (µmol/L)	MDA (nmol/ml)
Control	55.375 ± 0.92	1.109 ± 0.07
Untreated	17.054 ± 0.57 *	12.238 ± 0.48 *
Prednisolone	41.936 ± 2.10 * @	3.880 ± 0.41 * @
Atorvastatin	38.663 ± 2.74 * @	4.941 ± 0.55 * @

* N= 8-10 rats per group.; Data were expressed as mean ± SEM; Statistical analysis is carried out using one way ANOVA followed by Student-Newman-keuls multiple comparisons test; * Significantly different from control value at p < 0.05; @ Significantly different from Rheumatoid arthritis control value at P < 0.05

Table 3: Effect of two weeks daily dose administration of atorvastatin and prednisolone on hematological biomarkersof complete freund's adjuvant induced rheumatoid arthritis in rats

Experimental groups	WBCs (10^3/µL)	RBCs (10^6/mcl)	Hb (g/dl)	Platelets (billion/l)	Hct (%)
Control	8.550 ± 0.61	3.175 ± 0.18	13.925 ± 0.16	351.250 ± 9.95	53.275 ± 1.22
Untreated	21.738 ± 1.14 *	1.633 ± 0.14 *	8.210 ± 0.27 *	277.625 ± 9.93 *	38.900 ± 2.23 *
Prednisolone	14.013 ± 0.38 * @	2.950 ± 0.09 @	10.988 ± 0.30 * @	309.625 ± 2.85 * @	46.813 ± 1.33 * @
Atorvastatin	16.188 ± 0.91 * @	2.801 ± 0.18 @	12.063 ± 0.50 * @	314.125 ± 0.06 * @	47.825 ± 1.21 * @

N= 8-10 rats per group.; Data were expressed as mean ± SEM; Statistical analysis is carried out using one way ANOVA followed by Student-Newman-keuls multiple comparisons test; * Significantly different from control value at p < 0.05; @ Significantly different from Rheumatoid arthritis control value at P < 0.05

Histopathological Examination

Histopathological examination of joint sections of sham control rats stained with Hematoxylin and Eosin **(H &E x 200)** stain showed a smooth articular surface **(black arrow)** and a regular tide mark **(white arrow)** separating the articularcartilage **(C)** from the underlying subchondral bone Figure [1A]. Arthritis produced by FCA was associated with histopathologic changes in the joint tissue

as revealed by a disrupted articular surface **(black arrow)** Figure [1B].

Rats treated with Prednisolone showed smooth articular surface **(black arrow)** with thickened articular cartilage **(C)** and subchondral bone **(B)** when stained by **(H &E x 200)** Figure [1C].

Changes in histopathology after Atorvastatin treatment showed narrow area of disrupted articular surface **(black arrow)**. Thickened articular cartilage **(C)** and subchondral bone **(B) (H &E x 200)** Figure [1D].
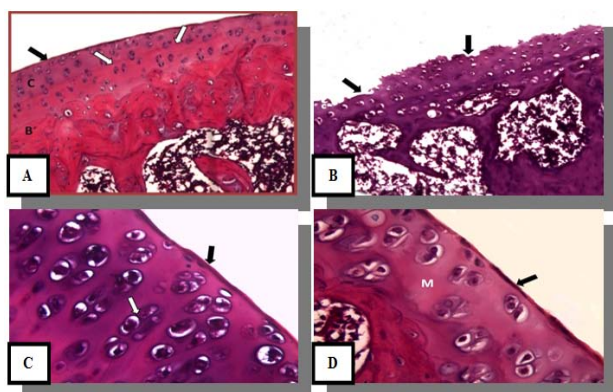
Figure 1: Effect of two weeks daily dose administration of atorvastatin and prednisolone on Histopathological evaluation of knee joints of complete freunds adjuvant induced rheumatoid arthritis in rats. (A) Section of a joint of normal control rats (H &E x 200). (B) Section of a joint of an untreated arthritic rat (H &E x 200). (C) Section of a joint of Prednisolone treated rats (H &E x 200). (D) Section of a joint of Atorvastatin treated rats (H &E x 200).

DISCUSSION

In the present study we explored the possibility of subcutaneous injection of FCA containing MB to the rats induced inflammation and arthritic lesions during two weeks in the animals. Rat adjuvant arthritis was believed to be the result of a sequence of immune pathologic events involving sensitization to antigen, proliferation of immune competent cells, cellular hypersensitivity and mediator release[13]. When compared to the normal control group, the untreated arthritic group elucidated an elevation in several pro-inflammatory cytokines such as TNF-α and IL-6 due to the stimulation of cell mediated immunity that leads to potentiation of the production of certain immune-globulins by FCA causing RA[14-15].

Inflammation and tissue injury related oxidative stress have been implicated in the pathogenesis of rheumatoid arthritis. Free radicals were enormously produced at the site of inflammation and tissue injuries[16].

Lipid peroxides that were generated at the site of inflammation of tissue injury diffuse into blood and can be estimated in serum, which in turn reflect the severity of the tissue damage. Thus, the elevated lipid peroxidation observed in the present study in Freund's adjuvant (FA) induced arthritis can be related to excessive generation and diffusion of lipid peroxides from the inflamed or injured joints of rheumatoid arthritis. In addition, the present study has observed a change of non-enzymatic antioxidants as compared to normal control rats[16].

Reduced glutathione was a well-known antioxidants which play an important role in protecting the lipids of lipoproteins and other bio membranes against peroxidative damage by intercepting oxidants before they can attack the tissues[17]. An inverse relationship between lipid peroxidation and non-enzymatic antioxidants has been well documented[18]. Hence, the decrease in level of non-enzymatic antioxidants can be correlated to impairment in the antioxidant defense mechanism, due to excess utilization by the inflamed tissues to scavenge the excessive lipid peroxides that were generated at inflammatory sites, or to scavenge accumulated lipid peroxides.

Circulating red blood cells possess the ability to scavenge ROS generated extra cellular by activated neutrophils[16]. Thus, RBC may be important in regulating oxidant reactions in the surrounding medium thereby preventing free radical-mediated cytotoxicity[19]. Hence, the RBC with decreased antioxidant levels were easily destroyed[20]. The significantly decreased values of RBC, Hb, Hct and platelet in the blood of RA group observed in our study were supported by other workers who reported that increased ROS production was indicative of RBC destruction in RA. Also, the hematological results showed that induction of rheumatoid arthritis caused a significant leukocytosis accompanied with neutrophilia due to stimulation of the immune response to help the body to fight infection by producing antibodies that circulate widely in the blood stream, recognizing the foreign particles and triggering inflammation[21].

Glucocorticoids such as Prednisolone have been used to treat rheumatoid arthritis for the last half century and recently, there has been renewed interest in these medications[22]. Short-term glucocorticoids reduce synovitis and their long-term decrease joint damage[23].

Cytokines such as tumor necrosis factor alpha (TNF-a) and interleukin-6 (IL-6) have been shown to display potent pro-inflammatory actions that were thought to contribute to the pathogenesis of RA[24]. TNF-a and IL-6 were involved in inflammation, differentiation and proliferation of T and B cells and bone resorption[25-26]. Glucocorticoids such as Prednisolone, were known to down-regulate proinflammatory cytokine production, such as IL-6 and TNF-a, normally produced by macrophages and monocytes through diffusion into the cell and bind with a cytoplasmic glucocorticoid receptor, which moves to the nucleus where it induces the transcription of IkBa[26]. This action inactivates NF-kB, decreasing the proinflammatory cytokine production[26], this in turn lead to increasing GSH level an decreasing MDA level by the anti-inflammatory effect of glucocorticoids[4].

This study also showed that Prednisolone can modulate hematological parameters such as increasing RBCs, Hb, and Hct and platelet values. This action including direct regulation of hematopoietic cell-specific transcription factors c-Myb and GATA-1[27-28]. It also stimulates erythropoiesis indirectly by increasing Epo production in the kidney[29]. On the other hand one of the remarkable effects of GCs was their ability to decrease WBCs count and to inhibit the infiltration of inflammatory leucocytes to specific tissue sites of inflammation[30].

Statins were class of drugs that were known by preventing cardiovascular diseases through their lipid-lowering activity, these drugs have also been shown to possess anti-inflammatory and immune modulatory effects[31-32].

In the present study, Atorvastatin exhibited anti-inflammatory and antioxidant effects in addition to its basic lipid-lowering effects when used for treatment of induced rheumatoid arthritis in rats, the anti-inflammatory effects of statins were induced via peroxisome proliferator-activated receptors (PPARs) signaling-pathway through suppression of NF-κB mediated-target gene activation especially TNF-α, IL-6, adhesion molecules[33].

Another possible mechanism for the action of Atorvastatin was the inhibition of neutrophil migration with a subsequent decrease in local production of pro-inflammatory cytokines[34-35]. This came in consistent with the results of the study that showed that Atorvastatin, specifically attenuate cytokine-induced endothelial iNOS expression by interfering with activation of both NF-κB and signal transducer and activator of transcription (STAT-1). This effect appeared to be independent of a blockade of HMG-CoA reductase[36]. In addition, statins improve endothelial function before significant reduction in serum cholesterol levels occurs. This, in part was mediated by up regulation of eNOS[4].

Statins affect eNOS expression and activity mainly through three mechanisms. First, statin increase eNOS expression by prolonging eNOS mRNA half-life rather than by inducing eNOS gene transcription. Second, statin reduce caveolin-1 abundance. Caveolin-1 was an integral membrane protein, binds to eNOS, thereby inhibiting NO production directly[37]. Third, statin can activate phosphatidylinositol 3 kinase/protein kinase Akt pathway that phosphorylates and activates eNOS[38]. Nagila et al. observed that Atorvastatin treatment caused a significant reduction in oxidative stress by decreasing levels of all lipid oxidation markers including MDA and increased total antioxidant substance like GSH due to its ability to reduce the production of ROS[39] (GGPP) through the mevalonate pathway[40].

In the present study treatment with Atorvastatin resulted in restoring of the hematological changes that accompanied the inflammation. Atorvastatin was able to restore RBCs, platelets, Hb and Hct values. These results were in harmony with the study that supposed that statins can treat anemia of chronic diseases as rheumatoid arthritis through interference with the IL-6 signaling pathway[41-42] and reduce hepcidin levels which regulate iron deficiency caused by immune mediated changes in iron homeostasis where their levels expression was increased during inflammation[43-44].

Since cellular immune activation and oxidative stress play a major role in the pathogenesis of RA, the anti-inflammatory capacity of statins could contribute to reduce WBCs count[45]. This happen through preventing leukocyte recruitment and adhesion to the vascular endothelium this was done through[46-47]. Moreover, the anti-inflammatory effects of Atorvastatin have been proposed to be due to inhibition of the production of isoprenoids, which share a biosynthetic pathway with cholesterol[48]. Since these isoprenoids have effects on G-protein, adhesion molecules, and cell proliferation, blocking their production could have a profound effect on these inflammation-related functional systems such as WBCs[49-50]. The protective effect of Prednisolone or Atorvastatin was further confirmed histologically through improvement of joint structure.

CONCLUSION

From the current study, we can conclude that Prednisolone and Atorvastatin showed a powerful anti-rheumatoid with anti-oxidant effects against Freund's Complete Adjuvant induced rheumatoid arthritis in rats with a significant ameliorative effects against hematological changes associated with RA progression.

The use of Prednisolone or Atorvastatin was recommended for better management of rheumatoid arthritis with promising control role on hematological deteriorations events induced by rheumatoid arthritis.

However, further investigations to support this idea were still needed.

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