Vitamin D Improves the Anemic Condition, Reduces Inflammation, Oxidative Stress and Suppress Immunity in Rheumatoid Arthritis Induced by Complete Freund's Adjuvant In Rats

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ABSTRACT

Rheumatoid arthritis (RA) is the most common chronic systemic, immune-mediated inflammatory disorder that attacks flexible joints and also may affect many tissues and organs. The present study was designed to assess the effect of vitamin D and to compare it with that of Prednisolone in adjuvant induced arthritis in female Wistar albino rats. Fourty rats were divided into (4) groups, each of 10 rats. Group I was kept as control. The other three groups received 0.4 ml of Complete Freund's Adjuvant dose every 4 days for 12 days, one group served as positive control group. Group III and IV were treated with prednisolone and vitamin D, respectively. Blood samples were collected after four weeks of the last dose of treatment for detection of inflammatory markers, oxidative stress markers and hematological markers. A significant increase in serum tumor necrosis factor α (TNF α), interleukine-6 (IL-6), MDA, and WBCs were observed in arthritis rats accompanied by a significant decrease in GSH, RBCs, Hb, platelet count and Hct value. Treatment with vitamin D significantly decreased serum tumor necrosis factor α (TNF α), interleukine-6 (IL-6), MDA and WBCs and significantly increased GSH, RBCs, Hb, platelet count and Hct value. In conclusion, vitamin D showed beneficial protective effects against adjuvant – induced arthritis.

Keywords: Rheumatoid arthritis; Complete Freund’s Adjuvant; prednisolone; vitamin D.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory auto immune disease with unknown etiology characterized by joint swelling, joint tenderness, and destruction of synovial joints leading to severe disability and premature mortality1 and 2. The inflammation associated with the RA disease process results in elevated circulating levels of inflammatory cytokines, including multiple interleukins and tumor necrosis factor-alpha3.

Although there are reasonably good drugs used in the symptomatic relief of arthritis, such as non-steroidal anti-inflammatory drugs, current treatment is still not satisfactory to modify fundamental pathologic processes responsible for the chronic inflammation.4

Glucocorticoids (GCs) such as prednisolone are excellent anti-inflammatory medications. They inhibit several components of the inflammatory process including cytokines, inflammatory enzymes, adhesion molecules, and permeability factors but Long-term GC therapy in chronic inflammatory disease remains controversial due to the widely accepted list of adverse effects associated with GC use5.

The activated form of vitamin D, 1,25-dihydroxyvitamin D3, not only plays a central role in bone and calcium metabolism, but also has important general effects on cell proliferation and differentiation.

Moreover, it behaves as a paracrine factor in the immune system as it can be produced by monocytes and has potent actions on all the cellular components of the immune system6.

The present study aimed to investigate the possible beneficial influences of Vitamin D on inflammatory markers along with immunological, oxidative stress and hematological markers in the treatment of rheumatoid arthritis.

MATERIALS AND METHODS

Animals

All the experimental procedures were conducted using Wistar adult female albino rats, weighing 180 ± 20 g, provided by the Modern Veterinary Office for Laboratory Animals, Cairo, Egypt. the National Cancer Institute, and left to accommodate in the animal facility of the Faculty of Pharmacy, Nahda University, for 1 week before being subjected to experimentation. All animals were maintained under a 12-h light–dark cycle, with controlled humidity (60–80%) and constant temperature (22 ± 1°C). Throughout the study, food and water were supplied ad libitum.

All experimental procedures were controlled and approved by the Ethics Committee of Faculty of Pharmacy, BeniSuef University.

Induction of adjuvant arthritis

Rats were injected intra-peritoneally by complete Freund’s adjuvant as a single dose of 0.4 ml every 4 days for 12 days in the planter surface of the right hind paw7, and then treatments were started on day 12 for 2 weeks.
Drugs and chemicals

Complete Freund’s adjuvant (Difco Laboratories Co-USA) which contains mycobacterium tuberculosis and prepared from non metabolizable oils (paraffin oil and mannidemonooelate).

Prednisolone was provided from Egyptian Int. Pharmaceutical Industries Co. E.I.P.I. Co) and was dissolved in sterile saline at a dose of 10 mg/kg/day.9 Vitamin D was purchased from Sigma –Aldrich, USA and was dissolved in sterile saline at a dose of 0.050 µg/kg/day orally10.

All other chemicals were of the highest grade commercially available.

Both prednisolone and vitamin D were dissolved in sterile saline shortly before administration to animals. The concentrations of the drugs were adjusted so that each 100g animal’s body received orally 1ml of either suspension containing the required dose.

Experimental protocol and procedure

After acclimatization period of one week rats were randomly allocated into 4 groups (n= 10 rats per group).

Group 1: Non-arthritic healthy control rats. This group served as normal control group.

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 day followed by daily dose of sterile saline orally for two consecutive weeks. This group served as positive control group.

Group3: 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 daily dose of prednisolone (10mg/kg/day) for two weeks.

Group4: 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 daily dose of vitamin D (0.05µg/kg/day) for two weeks.

Assessment of arthritis progression

At the end of the study, on day 27, blood samples were collected from the medial epicantus of the animal’s eyes by non-heparinized capillary tube 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.

For histopathological examination, the knee joints were removed for microscopic examination to determine the extent of joint inflammation. Rats were sacrificed using ether anesthesia, and the arthritic knee joints from different rat groups were collected for histopathological analysis. Formaldehyde-fixed knee joints were decalcified with a solution containing 10% ethylene di amine tetra acetic acid.

Assessment of immunological and inflammatory parameters

Tumor necrosis factor (TNF- α) expressed as pg/ml was determined using an enzyme-linked immunosorbent assay according to the principle of11 and interleukine 6 (IL-6) expressed as pg/ml was measured as according to12 using test reagent kits.

Oxidative stress parameters

Malondialdehyde (MDA) was expressed as n mol/ml and was assayed according to the method described by13 and Glutathione (GSH) expressed as Mmol/L and was measured as according to the method of14.

Hematological parameters

Hemoglobin (Hb) expressed as g/dl, platelets expressed as 10⁹/L, Red blood cells (RBCs) expressed as 10⁹/mcl, White blood cells (WBCs) expressed as 10⁹/Ml and hematocrite (Hct) expressed as % were estimated by adopting standard procedures6.

Histopathological examination

After decalcification, knee joint tissues were then sectioned, embedded in paraffin, and sliced for hematoxylin and eosin (H&E) staining.

Statistical Analysis

All data were expressed as mean ± standard error of mean (S.E.) of 10 rats per experimental group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student-Newman-keuls multiple comparisons test by the aid of Graph bad prism and Graph pad instant computer software, San Diego, USA. The P values smaller than 0.05 were selected to indicate statistical significance between groups.

RESULTS

Effect of prednisolone or vitamin D on immunological and inflammatory parameters

Complete Adjuvant arthritis produced a significant increase in serum levels of TNF- α and IL-6 as compared to normal control group. Administration of prednisolone or vitamin D on arthritic rats for two weeks produced a significant decrease on serum levels of TNF- α, IL-6. (Table 1)

Effect of prednisolone or vitamin D on oxidative stress parameters

As shown in (table 2) Complete Adjuvant arthritis significantly increased MDA level and significantly decreases serum GSH level as compared to normal control group Administration of prednisolone or vitamin...
D significantly reduced serum MDA level and significantly increased serum GSH level as compared to arthritic non-treated rats.

**Effect of prednisolone or vitamin D on hematological parameters**

As shown in table 3 adjuvant induced arthritis resulted in a significant increase in WBCs count and a significant decrease in RBCS count, Hb concentration, platelet count and Hct % as compared to untreated group.

Administration of Prednisolone or vitamin D on arthritic rats for two weeks produced a significant decrease in WBCs count and a significant increase in RBCs count, Hb concentration, platelets count and Hct% group as compared to arthritic non-treated rat group.

**RESULTS**

**Table 1:** Effects of Prednisolone or Vitamin D on inflammatory markers in adjuvant-induced arthritis rats.

<table>
<thead>
<tr>
<th>Groups Parameters</th>
<th>Non-arthritic healthy control rats</th>
<th>Untreated adjuvant arthritis rats</th>
<th>Prednisolone</th>
<th>Vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>32.776 ± 1.249</td>
<td>117.55 ± 1.749*</td>
<td>51.538 ± 3.305* @</td>
<td>51.950 ± 3.465* @</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>34.764 ± 1.223</td>
<td>130.95 ± 4.838*</td>
<td>58.888 ± 4.323* @</td>
<td>69.963 ± 6.760* @</td>
</tr>
</tbody>
</table>

N= 10 rats per group.; Data were expressed as mean ± SEM; Statistical analysis is carried out using one way ANOVA followed by Newman-kuels multiple comparisons test; *: Significantly different from normal control at p < 0.05; @: Significantly different from RA control at p < 0.05

**Table 2:** Effects of Prednisolone or Vitamin D on oxidative stress markers in adjuvant-induced arthritis rats.

<table>
<thead>
<tr>
<th>Groups Parameters</th>
<th>Non-arthritic healthy control rats</th>
<th>Untreated adjuvant arthritis rats</th>
<th>Prednisolone</th>
<th>Vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA(nmol/ml)</td>
<td>1.109 ± 0.0678</td>
<td>12.238 ± 0.4810*</td>
<td>2.933 ± 0.2982* @</td>
<td>3.384 ± 0.3269* @</td>
</tr>
<tr>
<td>GSH(Mmol/L)</td>
<td>55.375 ± 0.918</td>
<td>17.054 ± 0.568*</td>
<td>43.075 ± 1.921* @</td>
<td>42.813 ± 3.468* @</td>
</tr>
</tbody>
</table>

N= 10 rats per group.; Data were expressed as mean ± SEM; Statistical analysis is carried out using one way ANOVA followed by Newman-kuels multiple comparisons test; *: Significantly different from normal control at p < 0.05; @: Significantly different from RA control at p < 0.05

**Table 3:** Effects of Prednisolone or Vitamin D on hematological parameters in adjuvant-induced arthritis rats.

<table>
<thead>
<tr>
<th>Groups Parameters</th>
<th>Non-arthritic healthy control rats</th>
<th>Untreated adjuvant arthritis rats</th>
<th>Prednisolone</th>
<th>Vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs(10⁹/mL)</td>
<td>8.550 ± 0.6112</td>
<td>21.738 ± 1.143*</td>
<td>11.775 ± 0.3849* @</td>
<td>12.013 ± 1.004* @</td>
</tr>
<tr>
<td>RBCs(10⁵/mL)</td>
<td>3.175 ± 0.1830</td>
<td>1.633 ± 0.1374*</td>
<td>2.950 ± 0.0866@</td>
<td>3.068 ± 0.0796@</td>
</tr>
<tr>
<td>Hb(g/dl)</td>
<td>13.925 ± 0.1567</td>
<td>8.210 ± 0.2749*</td>
<td>12.225 ± 0.3211* @</td>
<td>12.313 ± 0.5423* @</td>
</tr>
<tr>
<td>Platelets(10⁹/L)</td>
<td>351.25 ± 9.946</td>
<td>271.50 ± 9.668*</td>
<td>315.88 ± 2.587* @</td>
<td>310.95 ± 6.269* @</td>
</tr>
<tr>
<td>Hct(%)</td>
<td>53.275 ± 1.221</td>
<td>38.900 ± 2.227*</td>
<td>52.375 ± 0.9753@</td>
<td>47.375 ± 2.340@</td>
</tr>
</tbody>
</table>

N= 10 rats per group.; Data were expressed as mean ± SEM; Statistical analysis is carried out using one way ANOVA followed by Tukey-kramer multiple comparisons test; *: Significantly different from normal control at p < 0.05; @: Significantly different from RA control at p < 0.05

**Histopathological examination**

Photomicrograph of knee joint sections of Non-arthritic healthy control rats stained with Hematoxylin and Eosin (H & E x200) stain showed normal knee joint as regard A smooth articular surface (black arrow) and a regular tide mark (white arrow) separating the articular cartilage (C) from the underlying subchondral bone (B) and no inflammatory cells noticed. Fig (1)

Photomicrograph of knee joint sections of Non-treated arthritic rat group stained with Hematoxylin and Eosin (H & E x200) stain showed Articular surface of an osteoarthritic joint with a disrupted articular surface (black arrow) Fig (2)

Photomicrograph of knee joint sections stained with Hematoxylin and Eosin (H & E x200) of rats treated with prednisolone or Vitamin D showed similar results of A smooth articular surface (black arrow). Thickened articular cartilage(C) and subchondral bone (B) can be observed. Fig (3), (4).

In Vitamin D-treated group the chondrocytes (white arrow) showing hypercellularity and aggregation Fig (4).

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**Figure 1:** Normal control
Rheumatoid arthritis is an autoimmune disorder resulting in an immune mediated inflammatory attack on synovial joints. The inflammation associated with the RA disease process results in elevated circulating levels of inflammatory cytokines, including multiple interleukins and tumor necrosis factor-alpha and interleukin 6. Recent data showed that approximately 35% of RA sufferers in the US claim work disability within ten years of disease onset.

Many experimental models for RA have been developed to get the exact events that illustrate the disease. Adjuvant arthritis model is the most rheumatoid arthritis models that exhibit similar clinical and pathological features to human rheumatoid arthritis.

In the present study, intraperitoneal administration of CFA to rats caused severe poly arthritis evidenced by significant increase in serum levels of many inflammatory and oxidative stress predictors such as TNF-α, IL-6, MDA, and WBCs count. Likewise significant decrease in serum levels of GSH, RBCs count, Hb %, platelet count and Hct% as compared to normal saline treated rat group. Histopathological examination further supports RA-induced by CFA.

Similarly, previous investigations showed comparable results concerning RA induced by CFA in rats where reported similar increase in TNF-α and IL-6 in adjuvant induced arthritis model. In addition, similar increase in serum WBCs count induced by intra peritoneal administration of CFA content was reported by and.

On the other hand, the decreases in serum level of GSH, RBCs count, Hb%, Platlets count and Hct % was in harmony with the results reported by who observed similar decrease in GSH, RBCs, Hb, Platlets and Hct activity in adjuvant induced arthritis model.

Although the pathogenic mechanisms of rheumatoid arthritis (RA) remain elusive, advances in both molecular biology and clinical research have identified a unique orchestration of immune system cell subsets, cell surface markers, and soluble cell products that have a role in the process of inflammation associated with RA.

Inflammation and subsequent degradation of the synovial tissue are initiated by the influx of lymphocytes (B cells, CD4+, and CD8+ T cells) into the synovial tissue. In the simplest model, CD4+ T lymphocytes are activated by antigens in the joint and stimulate plasma cells, mast cells, macrophages, and synovial fibroblasts to produce inflammatory mediators.

It is well established that TNF-α and interleukins play an important role in the pathology of RA, as it can induce collagenase production that may contribute directly to cartilage destruction and bone resorption found in RA.

The end product of lipid peroxidation MDA is also harmful, and may be responsible for some of the overall effect, leading to release of cell contents and cell death, causing tissue and organ damage.

Circulating human red blood cells possess the ability to scavenge ROS generated extracellularly by activated neutrophils. Thus, RBC may be important in regulating oxidant reactions in the surrounding medium thereby preventing free radical-mediated cytotoxicity. Hence, the RBC with decreased antioxidant levels are easily destroyed. The significantly decreased values of RBC and haemoglobin in the blood of RA patients observed in our study are supported by other workers who reported that increased ROS production is indicative of RBC destruction in patients with RA.

Glucocorticoids (GCs) are excellent anti-inflammatory medications. They inhibit several components of the inflammatory process including cytokines, inflammatory enzymes, adhesion molecules, and permeability factors. GCs have been found to provide rapid and dramatic improvement in functioning and reduce joint damage in
patients with RA but long-term GC therapy in chronic inflammatory disease remains controversial due to the widely accepted list of adverse effects associated with GC use. The proposed adverse effects of GC therapy on cardiovascular functioning include hypertension, elevation of blood glucose, accelerated atherosclerosis and lipid disturbances.

According to this study, prednisolone showed a protective effect in treatment of RA where it significantly reduced many inflammatory parameters such as TNF-α, IL-6 and WBCs count. These findings confirm the work of and who reported reduction of serum level of the previous parameters. The anti-inflammatory and immune modulatory effect of prednisolone could be attributed to its ability of decreasing the migration of neutrophils, and inhibiting neutrophil aggregation.

Beside the anti-inflammatory action of prednisolone, it has a potent anti-oxidant activity and this action is evidenced in this study by significant decrease in serum level of MDA and significant increase serum level of GSH. These findings are in agreement with who reported similar decrease in serum level of MDA, and also in harmony with the results showed similar increase in serum GSH level reported by induced by prednisolone.

The anti-oxidant activity of prednisolone is due to its ability to keep the balance between oxidative stress and the internal anti-oxidant defense mechanisms. Prednisolone reduces generation of ROS due to inhibition of neutrophil aggregation as it was illustrated before which plays an important role in the production of ROS and so it protects the synovial joints from peroxidation.

In addition, prednisolone in this study showed improvement in the anemic condition induced by CFA as compared to RA untreated group evidenced by significant increase in RBCs count, Hb%, Platelets count and Hct % and these results was in harmony with the results reported by and.

The anti anemic effect produced by prednisolone may be due to suppression of inflammatory and oxidative stress biomarkers so it protects the RBCs from peroxidation.

However due to the side effects of prednisolone, there is an orientation to get safe and effective drug used in treatment of RA.

Vitamin D is a steroid vitamin, a group of fat-soluble prohormones, which encourages the absorption and metabolism of calcium and phosphorous.

In the liver vitamin D is converted to calcidiol, which is also known as Calcifediol (INN), 25-hydroxycholecalciferol, or 25-hydroxyvitamin D—abbreviated 25(OH)D.

Part of the calcidiol is converted by the kidneys to calcitriol, the biologically active form of vitamin D [1,25(OH)2D][1,25 dihydroxycholecalciferol].

In this study, vitamin D showed potent anti-inflammatory and immune-modulatory effect through decreasing joint inflammation macroscopically, microscopically and biochemically. Vitamin D showed significant decreasing in serum levels of TNF-α, IL-6 and WBCs and These results are in harmony with results of and who reported the same results in RA models induced in rats.

It is thought that The anti-inflammatory effect of vitamin D is related to inhibition of cytokine production such as TNF-α and IL-6 which play a key role in the inflammation process. In addition, vitamin D may reduce expression of Prostaglandins ES (the enzyme finally responsible for biosynthesis of Prostaglandins E2) and increase expression of Prostaglandins DS by antigen presenting cells called dendritic cells from synovium rich tissue.

Vitamin D also has immune-modulatory properties, acting on the immune system both in an endocrine and in a paracrine manner. It appears to regulate the immune response by a variety of mechanisms, such as decreasing antigen presentation, inhibiting the pro-inflammatory T helper type 1 profile and TNFα and inducing regulatory T cells.

In this study vitamin D also showed anti-oxidant effect where it significantly decreased MDA and significantly increased GSH and these results are in agreement with the results of and.

In addition in the present study complete blood picture showed a significant decrease in number of WBCs compared with RA group indicating the anti inflammatory and immune modulatory effect of Vit. D and a significant increase in RBCs count, Hb, Hct and platlets indicating the effect of vitamin D in treatment of anemia induced by CFA. These results are in agreement with results of and who reported that circulating human red blood cells possess the ability to scavenge ROS generated extracellularly by activated neutrophils. Thus, RBC may be important in regulating oxidant reactions in the surrounding medium thereby preventing free radical-mediated cytotoxicity. Hence, the RBC with decreased antioxidant levels are easily destroyed and. The significantly decreased values of RBC and haemoglobin in the blood of RA observed in our study are supported by other workers who reported that increased ROS production is indicative of RBC destruction in patients with RA.

CONCLUSION

It is concluded that Vitamin D Improves the anemic condition, reduces inflammation, oxidative stress and suppress immunity in Rheumatoid Arthritis induced by Complete Freund’s Adjuvant in Rats with less side effects than prednisolone.

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