Establishment of Chemically Induced Arthritis and Diabetes Combinedly in Wistar Rats: An Experimental Study

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Abstract - The purpose of the present study was to establish combined animal model of chemically induced rheumatoid arthritis and diabetes and study various parameters. The study was conducted on adult male Wistar rats. Arthritis was induced by injecting CFA (Freund's complete adjuvant) followed by administration of Alloxan monohydrate intra-peritoneally. The levels of TNF-α, IL-1β, IL-6, and IL-10 in sera were measured using ELISA. Histopathological changes in joint tissues, kidney and pancreas were examined using haematoxylin and eosin (H&E). Swelling and inflammation occurred within 24 hr with a significant increase in paw volume which showed no resolution till the 30th day. There was significant increase in blood glucose levels. Bodyweight, GSH and IL-10 levels were decreased in serum of diseased group animals. Spleen & thymus index, nitric oxide, MDA, MPO, GSH, and serum triglyceride levels were elevated in diseased animals. Also, pro-inflammatory cytokine levels were extremely high in diseased animals as compared to control group. The joint histology showed synovial hyperplasia and inflammatory cell infiltration in joint tissues. The pancreas histology results showed disruption and reduction in the size of Islet of Langerhans. Radiological analysis showed deformation in bones. All these parameters confirmed the successful establishment of combined animal model for diabetes and arthritis.

Keywords: Rheumatoid Arthritis, Diabetes, Freund's complete adjuvant, Alloxan Monohydrate

I. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that leads to distortion of the joints and physical disability. It affects 0.5% to 1% inhabitants all over the world with perversiveness of 0.75% in India [1]. It leads to unalterable joint damage with systemic complications which lead to substantial morbidity and increased mortality. Scientists have reported some link between rheumatoid arthritis and diabetes mellitus. The possibility of getting diabetes is about 50% higher in patients suffering from autoimmune forms of arthritis, such as rheumatoid arthritis and psoriatic arthritis [2]. Since the past few decades researchers are trying to find the alliance between these two major autoimmune diseases. Recently, Centre for disease control and prevention have reported that over 52% of the patients having arthritis are likely to develop diabetes also [3]. Diabetes mellitus is linked with insulin resistance; rheumatoid arthritis, on the other hand, is the excruciating inflammatory condition of joints. These diseases are although utterly dissimilar, but there can be a correlation among these two. The precise mechanism of how arthritis can pilot to diabetes or vice a versa is not known. But experts advocate that this can be due to inflammation involved in RA, genetically or due to physical immobility [4]. Recently Dong and co-workers have reported the correlation between RA & diabetes mellitus. They reported that women with RA are more prone to develop diabetes type 2 than men with RA conditions. Also, women with psoriatic arthritis are highly susceptible to diabetes mellitus [5]. Development of type 2 diabetes in women are predicted by elevated levels of C reactive protein (CRP) and interleukin 6, both well-known markers of systemic inflammation [6]. Some other researchers have found that markers of inflammation such as CRP, amplified WBC cell count, and stumpy serum albumin levels are associated with the advancement of diabetes over a prolonged period [7, 8]. Tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) appear to obstruct the function of insulin at the receptor level; as well, C-reactive protein and plasminogen activator inhibitor–1 negatively affect insulin sensitivity. Although in literature, the correlation between these two diseases is mentioned theoretically but to best of
our knowledge, experimentally these two diseases were not studied in combined animal model, which is very essential to facilitate the mechanism, correlation and symptoms of rheumatoid arthritis and diabetes together in animals. Therefore murine model is essential for understanding of the mechanism of disease and development of new therapy.

Keeping all these points in mind, the combined arthritis and diabetes model was established chemically by injecting complete Freund’s adjuvant (CFA) and Alloxan monohydrate in wistar rats followed by their physical examination, histopathological studies, radiological observation and immunological analysis of various inflammatory markers.

II. MATERIALS AND METHOD

Animals: Adult Wistar rats, 7-8 weeks old, weighing 160-180g were obtained from Central Animal House, Panjab University, Chandigarh. All the animals were given an antibiotic-free diet and water ad labium. Ethical clearance was sought from the Institutional Animal Ethics Committee before the start of the study. The rats were housed five per cage prior to initialization of experiments and were acclimatized for 2 days. All the experiments were carried out in triplicate. The error bars in graphs are representative of the standard deviation in each experiment.

Drug and Reagents: Freund’s complete adjuvant (CFA), Alloxan monohydrate, Anthrone reagent, Ellman reagent and Serum triglyceride determination kit were purchased from Sigma Aldrich Chemical Co. USA. Rat ELISA IL-6, TNF-α, IL-1β and IL-10 kits were purchased from Peprotech US. All the other chemicals used in this study were of analytical grade.

Experimental protocol: The experiments were conducted for 5 weeks. The animals were randomly allocated to two experimental groups of 10 rats each and the experimental arthritis was induced in rats by Freund’s complete adjuvant according to the method of Vincenzi et al. [9]. Briefly, all the rats were challenged with 0.1 ml of Freund's complete adjuvant (CFA) into the sub-plantar region of right hind paw under mild ether anesthesia. To augment the severity of arthritis, a booster injection with 0.1 ml of CFA was administered in the same manner on the 7th day. Likewise, the establishment of diabetes was done by intraperitoneal administration of alloxan monohydrate dissolved in sterile normal saline at a dose of 150 mg/kg body weight in arthritic rats by the method as described by Srinivasan & Ramarao, [10].

Measurement of Paw volume: The thickness of the right hind paw was deliberated; left hind paw serves as a control. During the experimental period, the severity of arthritis was assessed 2 times a week by measuring the paw thickness by vernier caliper till the 30th day.

Body weight Measurement: Body weight was measured twice a week till day 30 after CFA and Alloxan monohydrate administration for experimental and control group animals.

Thymus and spleen index assay: On day 23rd rats were euthanized via anesthesia. The spleen and thymus were removed from the aseptic environment and weighed instantly after removal. The thymus index = wet weight of thymus (mg)/ rat body weight (g). The spleen index = wet weight of spleen (mg)/ rat body weight (g).

Radiological Imaging: Radiographic severity of adjuvant-induced arthritis was assessed blindly on the 24th day. High-resolution digital X-ray imaging of hind limbs was performed on all animals. Radiological visual scoring was performed by two different observers. The limb regions from the digitized images were manually cropped and were stored for further analysis.

Estimation of Glutathione reductase (GSH) activity: To evaluate GSH level in samples, 15 μL of hemolysate was mixed with 260 μL of assay buffer (0.1 M sodium phosphate and 1 mM EDTA, pH: 8) and 5 μL Ellman reagent. Samples were incubated for 15 min at room temperature and the TNB formation was estimated in a spectrophotometer at 412 nm. Absorbance values were compared with a standard curve generated from known GSH [11].

MPO activity: Rats were sacrificed and their blood serum was collected and further used for estimation of MPO according to the method given by Chhibber et al [12]. A Bio-Rad microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to log the optical density at 490nm. MPO was calculated as the change in optical density (O.D) x dilution factor.

MDA assay: Malondialdehyde (MDA), a metabolite resulting from lipid peroxidation, which was detected by the method described by Saini et al [13]. Briefly, joint tissue was isolated, homogenized, and 0.1 ml of the homogenate was mixed with 0.2 ml of the 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.8% thiobarbituric acid (TBA). The mixture was heated at 100°C for 1 h, and then 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1; v/v) was added. After centrifugation at 10000 rpm for 10 min, the absorbance of the upper organic layer was measured at 532 nm. MDA concentration was expressed as nanomole/mg protein.

Inflammatory mediators: The release of nitric oxide (NO) in the synovial tissue homogenate was evaluated through the measurement of its oxidized nitrite and nitrate metabolites, according to the method of Saini et al. [13]. Since inflammatory cytokines are considered to be good markers of the severity of infections and inflammatory diseases. We calculated the levels of IL-1β, TNF-α, IL-6 and IL-10 in the
blood serum (control) and diseased (CFA + Alloxan monohydrate immunized) groups. Levels of total TNF-α, IL-1β, IL-6, and IL-10 in the serum of FCA induced arthritic rats were investigated by commercially available ELISA kits (Peprotech, US). The cytokines were measured according to the manufacturer’s instructions provided along the kit.

**Blood Glucose estimation:** One week after the alloxan monohydrate injection, fasting blood glucose levels were recorded. The blood sample was collected by tail clipping method of Kapoor and Sarwate [14] and checked by the glucose oxidase method using a pre-calibrated Glucometer (Accu-check active™).

**Serum triglyceride level estimation:** The total serum Triglycerides (TG) levels were estimated by Serum triglyceride determination kit sigma from Sigma Aldrich Chemical Co. USA following the procedure given by Buccolo and David [15]. Levels of serum triglyceride were measured according to the manufacturer’s instructions provided along the kit. Absorbance readings were taken on 540 nm and total triglyceride concentration of the sample was calculated as follows.

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\frac{(A_{\text{SAMPLE}} - A_{\text{BLANK}})}{(A_{\text{STANDARD}} - A_{\text{BLANK}})} \times \text{Concentration of Standard c}
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**Histopathology:** The animals were sacrificed on day 23rd by cervical dislocation. Hind paw ankle joints, pancreas, and kidneys were separated aseptically weighed and immersed in 10% buffered formalin for 24 hrs followed by decalcification in 5% formic acid and processed for paraffin embedding sections. The sections were stained with hematoxylin and eosin (H&E) and examined under the light microscope [16].

**Statistical analysis:** Data are expressed as means ± standard deviation (SD) of mean and statistical analysis was performed with Graph Pad Instat Software (Version 5.00, Graph Pad Software, San Diego, California, USA) using Student’s t-test for calculations of mean and standard deviation while one-way ANOVA. Variation with \( P < 0.05 \) was considered statistically significant.

### III. RESULTS

**Measurement of Paw thickness:** Signs of RA became prominent after 24hrs of adjuvant treatment, as the mean right paw circumference of the experimental rats increased compared to controls (Fig.1). A significant increase \( (P < 0.001) \) in rat paw volume was observed in adjuvant treated rats when compared to those in the normal non-immunized group (Fig. 2).

**Body weight:** During the experiment, normal body weight growth pattern significantly reduced in the diseased group (arthritis + diabetes) as compared to the control group. A significant decrease in body weights of diseased animals can be derived from inflammation, increased immobility, pain and febrile condition of rats (Fig. 3).

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Fig. 1: Increase in paw thickness in FCA injected animals. Values are expressed as mean ±SD (n = 6) in each group, **P<0.001.**

Fig. 2: Morphological representations of rat paw before and after subplantar administration of complete Freund’s adjuvant (CFA) indicated production of inflammation (A) Control rat with normal foot pad (B) CFA treated rat showing severe edema.
Fig. 3: Body weight growth pattern post-CFA and Alloxan monohydrate injection till day 28. Body weight is expressed in grams, body weight growth become significant after day 8th. n=6, ns $P > 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control group.

**Thymus and spleen index study:** As shown in Fig. 4 A and B, the thymus indexes and spleen indexes were significantly increased after FCA and Alloxan immunization compared with control group.

**Radiological Imaging:** Severity of adjuvant-induced arthritis was assessed radiographically on day 42nd. High-resolution digital radiography of hind limbs was performed on animals and it has been revealed that there was severe swelling in soft tissue of FCA treated group animal hind limbs. Also, finger bones were deformed and there was a reduction in the gap between the tibia and femur bone of the diseased animal. Control animals were observed with normal bone structure without any swelling of soft tissue (Fig. 5).

**Estimation of Glutathione reductase (GSH) activity:** Oxidative stress is associated with many diseases including arthritis and diabetes. Here oxidative stress was evaluated by measuring the level of GSH in the blood serum of diseased animal. FCA induced arthritis and Alloxan monohydrate induced diabetes decreased the tissue GSH, as compared to control healthy rats (Fig. 6).

Fig. 4: A) Spleen index was calculated. Spleen index = spleen weight (g)/body weight (g) × 100%. Then the thymus index was calculated. Thymus index = thymus weight (g)/body weight (g) × 100%. Both the index of thymus and spleen index in the diseased group were markedly increased when compared with the normal group.

Fig. 5: Radiographic images showing (A & B) normal control animals and (C & D) CFA induced experimental arthritis in Wistar rats.

Fig. 6: Effect of CFA and Alloxan monohydrate on GSH levels in blood serum of animal in comparison to healthy animals.
Nitric oxide (NO) estimation: Nitric oxide levels evaluated in blood plasma of arthritis and diabetes-induced animals were significantly higher in diseased animals at 7th, 14th and 21st days after the induction of disease as shown in Fig. 7.

**Fig. 7:** It is clearly demonstrated that there is a statistically significant ($P<0.001$) increase in NO expression in arthritis and diabetes-induced group. Data are expressed as mean ±SD.

MPO activity: Myeloperoxidase (MPO) activity, which is accepted as an indicator of neutrophil infiltration was determined in different animal groups. The effect of CFA and Alloxan monohydrate on MPO activity in serum is represented in Fig. 8. This graph clearly shows that Serum MPO activity significantly has increased in arthritis and diabetes-induced group compared to the healthy animal group.

**Fig. 8:** Comparison between the different studied groups according to MPO. Values are represented as mean ±SD, **$P<0.05$, ***$P<0.001$.**

MDA assay: The marked increase in serum MDA was observed in arthritic rats which can be due to overproduction of ROS in adjuvant arthritis model leads to a considerable oxidative stress (Fig. 9).

**Fig. 9:** Graph shows MDA levels in animal blood serum. Values are expressed as ± SD. **$P<0.05$, ***$P<0.001$.**

Cytokine estimation: The levels of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and anti-inflammatory cytokines (IL-10) in serum samples were measured in serum at day 7, day 14 and day 21 post-CFA and Alloxan monohydrate immunization. A significant increase in the level of IL-1β, TNF-α, IL-6, and IL-10 in serum was detected ($P<0.05$, $P<0.001$) in disease induced rats, as compared by to healthy rats as shown in Fig. 10.

**Fig. 10:** A
Fig. 10: Cytokine levels in serum following CFA and Alloxan administration in rats. The levels of IL-1β, TNF-α, IL-6 and IL-10 in serum (A, B, C, D). Values are represented as mean ±SD. *P<0.05, **P<0.01, ***P<0.001, significantly different from control group levels.

**Blood glucose estimation:** Administration of a single dose of Alloxan monohydrate (150 mg/kg, i.p.) resulted into a rise in fasting blood glucose levels, which was maintained over a period of 3 weeks. Whereas healthy control group rats showed normal blood glucose levels as shown in Fig. 11.

**Serum triglyceride levels:** Administration of the alloxan led to a significant rise (P < 0.001) in the level of triglycerides, in the blood serum of diseased animals (Arthritis + diabetes). In contrast, triglyceride levels remain normal in the blood serum of control group rats (Fig. 12).

**Histopathology of ankle joints:** control group rats showed intact cartilage, synovium, and bone. Also, there was no inflammation in the surrounding tissues (Fig. 13 A&C). Whereas in the test group there was heavy neutrophil infiltration and arthritic rats showed synovial hyperplasia, cartilage erosion and mononuclear cell infiltration in the surrounding tissue (Fig. 13 B&D).

**Fig. 11:** Effect of CFA and Alloxan monohydrate on blood glucose (mg/dl) levels in Wistar rats. Blood glucose levels were significantly high in diseased group animals as compared to control, ***P<0.001.

**Fig. 12:** Graph represents a rise in triglyceride levels after CFA and Alloxan monohydrate treatment in Wistar rats. Results are expressed in mean ± SD. n=6 ***p<0.001.
Fig. 13: Histopathological changes in the ankle joints of FCA treated animal and control animal. A) Control: The normal joint surface cartilage and synovium. B) FCA treated. C) Control: normal no inflammation. D) FCA treated: Dense inflammation

**Histopathology of Kidney**: Fig. 14 B) shows normal control animal kidney tissue showing normal cellular architecture. Fig. 14 A) diseased animal kidney tissue induced with 150mg/kg of Alloxan showed a thickening in the basement membrane and the edematous proximal convoluted tubule. Necrosis in the glomerulus is well observed in test animal kidney tissue.

Fig. 14: The necrotic area of the glomerulus in the diabetic kidney is shown with arrows. The thickening in the basement membrane and the edematous proximal convoluted tubule could be well observed. Image B shows the control group animal glomerulus image which is intact in shape.

**Histopathology of Pancreas**: Based on HE-stained tissue sections of pancreas Alloxan administration along with CFA has caused noticeable changes like the severe injury of pancreas and disruption of the structure of islet of
Langerhans. The islets were shrunken in the diabetic rat when compared with normal rat (Fig. 15 A & B).

Fig. 15: Histopathological studies of pancreas B) post Alloxan monohydrate injection (150mg/kg). A) Control group animal pancreas histopathology image.

IV. DISCUSSION

Understanding the etiology and pathogenesis of a disease is crucial to develop different strategies to eradicate or improve diseased conditions. To understand this better, there is a need for simulation of a real system which mimics the human disease conditions. Rheumatoid arthritis and diabetes both are life-threatening autoimmune diseases. Scientific literature suggests that there is some link between these two diseases but there is no proper in-vivo validation of this theory. In the current investigation, the combined effects of RA and diabetes were investigated on Wistar rats. Results revealed that when healthy male wistar rats were challenged with 0.1 ml of CFA in the subplantar region and 150 mg/kg body weight alloxan monohydrate was administered intra-peritoneally, all the animals developed arthritis and diabetes-like symptoms [9, 10]. Paw swelling being the earliest visible symptom was studied and found that there was a marked increase in paw volume of diseased animals. In addition, there was a significant weight loss in CFA and Alloxan monohydrate immunized animal group whereas the control group showed normal weight gain pattern [17]. This documented downfall in weight growth pattern can be due to insufficient nutrient absorption by the intestine and muscle loss due to inflammatory conditions [18, 19]. In our study, we used blood sugar levels, triglyceride levels, and immune organ indexes to access the disease progression. Results revealed that there was a significant increase in blood sugar levels and triglyceride levels in diseased group animals also immune organs were enlarged which indicates inflammation [20, 21].

Various inflammatory cytokines (pro-inflammatory cytokines IL-1β, TNF-α and anti-inflammatory cytokine IL-10) were assessed in both, the control and the test animals. The levels of inflammatory cytokines, IL-1β, and TNF-α produced during inflammation in the serum of normal healthy rats were much lower than in the serum of diseased rat [22, 23]. Anti-inflammatory cytokines IL-10 levels were lower in diseased rats which indicated inflammation. TNF-α and IL-1β advance cartilage and bone damage, whereas IL-10, provide anti-inflammatory and immunoregulatory actions [24]. TNF-α is cytotoxic to glomerular, mesangial and epithelial cells and may induce significant renal damage [7].

Our body has a proficient mechanism to prevent or neutralize the free radical induced damage. For this a set of endogenous antioxidant enzymes, such as NO, MPO, MDA, GSH etc. are involved. When the balance between ROS (reactive oxygen species) production and antioxidant protection is misbalanced, ‘oxidative stress’ results, which deregulate the cellular function through a series of events, leading to serious pathological conditions [25]. So biomarkers of oxidative stress were determined in blood serum of diseased group animals and compared to the healthy control rats. A marked increase in serum MDA was observed in diseased animals leading to oxidative stress and it also destroys beta cells [20]. Increased levels of NO in blood serum support the report by Adela et al. [26] which states hyperglycemia enhances nitric oxide production in diabetes. Overproduction of NO promotes tissue injury which is commonly seen in arthritis patients [27]. An additionally high level of MPO in diseased rats demonstrates granulocyte infiltration and inflammation [9]. A study conducted by Odobasic et al. [28] has reported that MPO contributes to the progress of arthritis despite suppressing adaptive immunity in secondary lymphoid organs. Tachi and colleagues have shown in their study that high glucose levels reduce the intracellular glutathione (GSH) content and the pace of uptake of cystine, which maintains the GSH level [29]. Another study conducted by Ueno et al. [30] has reported that dietary glutathione protects rats from diabetic nephropathy and neuropathy also GSH plays an important role in the protection of cells and tissue structure in arthritic conditions. These studies corroborate...
our result where GSH levels have been gone down in diseased rats as compared to healthy control rats.

Other very important information related to the pathology of arthritis that has been obtained during this study was a radiographic examination of the paws. Tissue swelling and inflammation are the earlier signs, as the disease progresses more severe changes like joint space narrowing and subchondral erosions can be observed [31]. X-ray images of our experimental animals revealed soft tissues swelling around the joints, the gap between bones and deformities in finger bones which indicates that arthritis has been induced successfully.

The observed histopathological changes in diseased animal hind joints, on day 23rd, revealed prominent abnormalities from the ordinary healthy joint. Changes observed were edema, degeneration, and erosion of the cartilage and extensive infiltration of inflammatory exudates in the articular surface. Kidney tissues of healthy control animals were found to be normal while diseased (diabetes+ arthritis) group showed a high level of cellular abnormalities like glomerular damages, edema in convoluted tubules, tubular necrosis, and thickening in the basement membrane. It is also reported that Alloxan monohydrate administration to experimental rats causes pancreatic β-cell membrane disruption and cytotoxicity [32]. Further observations of histopathology of pancreas revealed that 150mg/kg dose administration of Alloxan monohydrate had impaired the normal histology of pancreas and there a severe reduction of β cells.

Findings of this study have revealed that CFA and Alloxan monohydrate can induce arthritis and diabetes in Wistar rats respectively. This combined model of two diseases can be used to explore different treatment measures. Also, the effect of some drug or combination of drugs can be checked on both the diseases together and develop the treatment strategy.

V. CONCLUSION

Rheumatoid arthritis and diabetes both are crucial autoimmune diseases which are entirely dissimilar but somehow interlinked. The aim of present study was to develop a chemically induced combined arthritis and diabetes model in Wistar rats. Increase in blood sugar and triglyceride levels along with destruction of β-cells in animal confirmed the progression of diabetes in diseased group. It was found that there was narrowing of joint spaces and subchondral erosion which are indicative of arthritis. Radiographic examination revealed the presence of gaps between bones and deformities in finger bones. In conclusion, all the parameters confirmed the successful establishment of the experimental combined animal model of diabetes and arthritis. The combined model of diabetes and arthritis can be used further for research on treatment methods to cure both the diseases together.

Conflict of Interest
No conflict of interests exist.

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Ethics approval
All animal experimental procedures were approved by the Institutional Animals Ethical Committee (IAEC), Panjab University, Chandigarh.

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