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Biochemical, molecular, and histopathological changes of rheumatoid arthritis induced experimentally in rats

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ABSTRACT

Complete Freund's adjuvant (CFA) is a mixture of desiccated mycobacterium in mannide monooleate and paraffin oil that causes tissue death, lesions, and inflammation. Immunologists have been inducing inflammatory conditions and autoimmune diseases in laboratory animals using CFA for more than 50 years. Rheumatoid arthritis (RA) is a disease marked by ongoing inflammation that affects more than just joints. Twenty male rats were divided into two groups (10 each), Normal control group: Rats were given no medication. CFA-induced arthritis group: Rats were injected with Complete Freund's adjuvant (0.1 ml, once). After 60 days blood samples and Stifle joints were collected for biochemical assay and genetic study of some signaling transducers including IFNγ, Interleukin-4, STAT3 and Interleukin-12. The results revealed that CFA induced rheumatoid arthritis in rats causing an increase in serum liver enzymes and serum concentration of creatinine, urea, FBS, RF, Anti-CCP and CRP, while decreasing in serum total antioxidant capacity (TAC) concentration. Moreover, there was up regulation of IL-12, IFNγ and STAT3 gene, and down regulation of IL-4 gene in CFA-induced arthritis group contrasted with the control group. On conclusion, CFA injection into leg footpad of rats induced arthritic animal model associated with similar inflammatory changes recorded in RA patients

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, debilitating inflammatory autoimmune illness characterized by synovial inflammation, joint lesions, and bone damage (Bullock *et al*., 2018). It is a clinical condition that encompasses a number of disease subgroups and frequently entails a number of inflammatory cascades, eventually, this causes persistent synovial inflammation and damage to the articular cartilage and underlying bone (Bae *et al*., 2022). Although the exact etiology of RA is unclear, some variables, such as obesity, infectious germs and viruses, hormone changes, and external factors, may raise the chance of disease development. Therefore, to prepare for future clinical and public health requirements, reducing the prevalence and effects of arthritis is essential (Zheng *et al*., 2022).

An adjuvant is a component added to some immunizations to help recipients' immune systems respond more powerfully (Seya *et al*., 2022). A variety of adjuvants, including those for Freund's complete adjuvant, Freund's incomplete adjuvant, *Mycobacterium* TB, *Bordetella pertussis*, bacterial lipopolysaccharide (LPS), synthetic polynucleotides (poly IC/poly AU) and other adjuvants, are currently available (Karch and Burkhard, 2016). Freund's Adjuvant is one of the most prevalent adjuvants used in studies today.

It is utilized in rodent studies to elicit an inflammatory humoral antibody response for the generation of high titer antibodies and to assess the efficacy of specific anti-arthritic medications (Ain' Sabreena *et al.*, 2021). CFA is a type of immune substance that heightens the antigenic reaction containing *Mycobacteria* that have been destroyed by heat (typically *Mycobacterium tuberculosis*) (Cooper *et al.*, 2020). CFA is useful in promoting cell-mediated immunity and potentiating T helper cells, which in turn promotes the production of specific immunoglobulins and effector T cells and creating arthritic or inflammatory conditions in animals. (Grötsch *et al*., 2019)

In mice and rats, CFA can be administered subcutaneously in the leg or intraperitoneally. The skin irritation caused by the injection in the paw starts to become reddish and distended as early as two hours after the procedure and culminates between six and eight hours later. This severe irritation lasts for three to four days (Cinzia *et al.*, 2019) which begins to show a rise in blood neutrophil and leukocyte numbers on the 4th day following CFA injection, as well as erythrocyte sedimentation rate (ESR) values. At 1-2 weeks after the CFA injection, formation of oedema and hyperalgesia in the foot (Rodrigues *et al.*, 2020).

Aim of work: It was aimed to assess the effect of complete Freund's adjuvant for induction of rheumatoid arthritis and its effect on some biochemical parameter and some signaling transducers for genetic study.

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2. MATERIAL AND METHODS

2.1. Animals:

Twenty healthy Albino rats, 4-5 weeks old and average body weight 170-200 g were provided with a standard diet and water was given *ad-libitum*. Rats were housed in metal cages at controlled room temperature of 29 ± 2 ºC. Before the trial began. Rats were allowed for a period of two weeks to acclimatize. Experiment was conducted according to the guide for Institutional Animals Care and Use Committee approved by Research Ethics Board, Faculty of Veterinary Medicine, Benha University. (Approval no. BUFVTM 01-11-22).

2.2. Reagents:

Complete Freund's adjuvant (CFA) at concentration of 10 mg/ml was purchased from Sigma Chemical Company (USA)

2.3. Experimental design:

Rats were divided into two groups (n=10) as following:

- Normal control group: Rats received no drugs.
- CFA-induced arthritis group: were injected S.C with single dose of 0.1 ml of CFA/rat, into right hand leg footpad of rats for induction of rheumatoid arthritis (Nasuti *et al.,* 2019).

2.4. Sampling after 60 days of CFA injection rats:

2.4.1. Blood samples and biochemical analysis

Blood samples were collected in dry, clean tubes and incubated for 30 min. at room temperature. Serum was collected in Eppendorf tubes using automated micropipettes and stored at -20 °C until it was used for estimating biochemical parameters:

AST by colorimetric Assay as (Reitman and Frankel, 1957). ALT by colorimetric assay as (Winn-Deen *et al.,* 1988). FBS Enzymatic determination as (Trinder, 1969). Urea by Enzymatic determination as (Patton and Crouch, 1977). Creatinine by Enzymatic determination as (Jaffe, 1986). RF by ELISA as (Swedler *et al.,*1997). Anti-CCP by ELISA as (Senshu *et al.,*1992). CRP by ELISA as (Hanson and Wadsworth, 1979). Total Antioxidant Capacity (TAC) (Koracevic *et al.,* 2001).

2.4.2. Stifle joints samples:

Stifle joints were removed, cleaned by rinsing in cold saline, placed in Eppendorf tubes, and promptly held in liquid nitrogen and preserved at -80°C until RNA extraction for molecular analysis: IL-12, IL-4, STAT 3 and IFNγ by using HERA^{Plus} SYBR® Green qPCR KIT (WF1030800X), Maxime RT PreMix Kit for 20 µl rxn Oligo $(dT)_{15}$ primer cat. No. 25081 Random Primer cat. no.25082 (table 1).

Table 1: Forward and reverse primers sequence for real time PCR Gene

Gene	Forward primer	Reverse primer
IL-4	ATCATCGGCATTTTGAACG AGGTC	ACCTTGGAAGCCCTACA GACGA
IFNγ	CAGCAACAGCAAGGCGAA AAAGG	TTTCCGCTTCCTGAGGCT GGAT
STA T-3	AGGAGTCTAACAACGGCA GCCT	GTGGTACACCTCAGTCT CGAAG
IL-12	ACGAGAGTTGCCTGGCTAC TAG	CCTCATAGATGCTACCA AGGCAC

2.4.3. Preparation of Stifle joint for Histopathological study:

Stifle joint was separated from the hand paw, immediately fixed in a 10% neutral buffered formalin. After proper fixation, tissue paraffin section was routinely prepared and stained with hematoxylin and eosin according to Bancroft and Gamble (2008).

2.5. Statistical Analysis:

Results were expressed as mean ± SE using SPSS (18.0 software, 2011). Using one-way ANOVA followed by Duncan's test, data were analyzed. Values were statistically significant at $p < 0.05$.

3. RESULTS

3.1. Determination of the biochemical parameters:

The data in table (2) demonstrated that, when compared to a normal control group, CFA-induced arthritis rats had highly significant serum levels of urea, creatinine, FBS concentration, ALT, and AST activities.

According to data in table (3), serum concentrations of RF, CRP, and anti-CCP were highly significant in the CFA-induced arthritis when compared to a normal control group, while serum TAC concentrations were significantly lower.

The results shown in table (4) the CFA-induced arthritis group showed significantly up-regulated STAT3, IL12, and IFN gene expression while IL4 gene expression showed significantly down-regulated.

Table 2 Effect of CFA in serum urea, creatinine and FBS concentration and ALT and AST activities in rheumatoid arthritis induced experimentally in rats.

	Urea (mg/dl)	Creatinine (mg/dl)	AST (U/L)	ALT (U/L)	FBS (mg/dl)
Normal control	23.92±1.91	0.43±0.08	61.84±2.35	21.60±1.74	64.18±4.69
CFA-induced arthritis	51.67±3.05**	2.13±0.11***	122.27±2.55***	53.08±4.10**	114.51±4.91**

The data is given as (Mean S.E.). S.E. stands for standard error. ***,** **, and *** indicated statistically significant at P < 0.05., P < 0.01 and P < 0.001, respectively.

Table3 Effect of CFA in serum RF, CRP, Anti-CCP and TAC concentration in rheumatoid arthritis induced experimentally in rats.

	RF (mIU/ml)	CRP (mg/L)	Anti-CCP (U/mL)	TAC (mM/L)
Normal control	6.19±0.08	1.93±0.25	2.30±0.34	13.58±0.70
CFA-induced arthritis	7.42±0.11**	11.37±0.99**	20.29±0.65***	1.06±0.06***

The data is given as (Mean S.E.). S.E. stands for standard error. ***,** **, and *** indicated statistically significant at P < 0.05., P < 0.01 and P < 0.001, respectively.

Table4 Effect of CFA on IL-4, STAT3, IL12 and IFNγ gene expression in synovial membrane on rheumatoid arthritis induced experimentally in rats.

	IL4	STAT3	IL12	IF
Normal control	1.06±0.03	0.98±0.01	1.03±0.02	0.94±0.03
CFA-induced arthritis	0.06±0.01***	2.62±0.56*	2.90±0.50*	8.64±1.60**

The data is given as (Mean S.E.). S.E. stands for standard error. ***,** **, and *** indicated statistically significant at P < 0.05., P < 0.01 and P < 0.001, respectively.

3.2. Histopathological findings

The microscopic examination of the knee joint of rats in control group showed thin synovial lining cell layer of the intema which formed from macrophage-like synoviocytes and fibroblast-like synoviocytes. The underlying stroma was formed from fibro-adipose connective tissue contained

few blood capillaries (Fig. 1A). In contrast, the examined joints of rats in CFA-induced arthritis group revealed severe histopathological changes in the synovia represented by focal disruption of the cells lining the intima and increases in the collagen fibers between the adipocytes in the stroma in some examined joints (Fig. 1B). While the other examined joints showed focal synovial hyperplasia with increases in the number of macrophages like synoviocytes. Moreover, synovitis represented by massive mononuclear cellular aggregation, fibrin deposition and congestion of blood vessels in the sub-lining stroma at the injected hind paw tissue was prevalent (Fig. 1C). Hypertrophy of the muscular layers of blood vessels with perivascular fibrosis was also noticed (Fig. 1D).

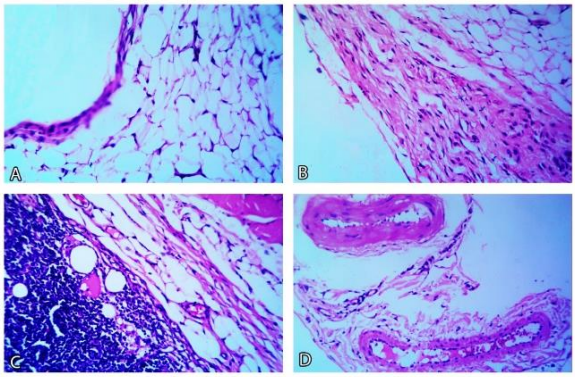

Fig. 1 Photomicrograph of control group, showing macrophage-like synoviocytes and fibroblast-like synoviocytes in the intema with fibro-adipose connective stroma of synovial membrane (A). CFA-induced arthritis group showing focal disruption of the cells lining the intima and increase in the collagen fibers of the stroma (B), synovitis represented by extensive mononuclear cellular infiltration and congestion of blood capillary (C) and hypertrophy of the muscular layers of blood vessels with perivascular fibrosis (D). H&E stain × 200

4. DISCUSSION

In comparison to the control group, the adjuvant exhibited elevated serum levels of the liver and kidney markers, urea and creatinine concentration. This finding is in agreement with Aloke *et al.* (2021), who found that elevated creatinine indicates a variation in glomerular filtration rate while increasing urea level may be connected to changes in tubular function.

In addition to increasing in AST and ALT activities in CFA-induced arthritis group this is similar to Kumar *et al.* (2016), who reported that although they are also considered to be properties of adjuvant arthritis, in the inflammatory process, AST and ALT amounts are important in the creation of active chemical molecules such as bradykinins.

The existing results showed significant increasing in serum FBS level in CFA-induced arthritis group, this is agreed with Casey *et al*. (2010) results that reported CFA significantly increased pancreas beta cell apoptosis and the onset of hyperglycemia.

CFA-induced arthritis group showed increasing the diagnostic RA markers: RF and anti-CCP, this is identical to the findings of Mahdi *et al.* (2018), who reported that CFA work by extending the half-life of the autoantigen that is injected, promoting its efficient transport to the immune system, and delivering a complicated collection of signals to the innate immune compartment. This alters leukocyte proliferation and differentiation, which causes a loss of tolerance to citrulline-containing proteins and results in the production of autoantibodies like anti-CCP and RF.

CFA-induced arthritis group showed increasing in serum CRP concentration comparing with normal group this result is similar to Geyer *et al.* (2020), who observe that CRP levels increase in reaction to inflammation, and the autoimmune illness also produces significant amounts of inflammation in addition to other signs like swollen and aching joints. Zhang *et al.* (2021) approved that CFA injection induce chronic inflammation and pain.

Decreasing of TAC serum level indicated in CFA-induced arthritis group this result confirmed by the results of ArezooMoradi *et al.* (2022), who discover that oxidative stress, a state in which the pool of reactive oxygen species, rises over time either by their increased production or because TAC is the main protection against oxidative stress, there is an inverse relationship between TAC and the illness.

CFA induced autoantigen-specific responses (Luo *et al.*, 2020). Including accelerated phagocytosis, increased cytokine production by mononuclear phagocytes, temporary stimulation, and proliferation of CD4+ lymphocytes, as well as fast uptake of adjuvant components by dendritic cells and phagocytosis (Kumar *et al.*,2016).

The results of our research showing up-regulating in pro inflammatory cytokines (IFNγ, IL-12 and STAT3) and down-regulating of anti-inflammatory cytokine (IL-4) in CFA-induced arthritis group comparing with control group and this is similar to Zhang *et al.* (2017), who reported that activation of lymphocytes such as CD4 T cell and natural killer (NK) cells leading to secretion of interferon-gamma which activated the JAk-STAT pathway ,through this pathway, pro-inflammatory signaling is elevated while anti-inflammatory signaling is reduced.

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5. CONCLUSION

CFA injection S.C with single dose of 0.1 ml into right hand leg footpad of rats created the arthritis complications shown in human RA patients. It effects on different investigations, including the presence of rheumatoid factor, C-reactive protein, Anti-CCP in the blood, inflammatory markers and signaling transducers including IFNγ, STAT3 and IL-12. Inversely showed decreasing of anti-inflammatory marker including IL-4. Also, the results showed an undesirable effect on liver and kidney enzymes.

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