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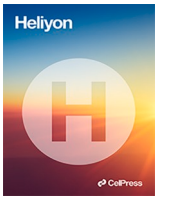

Decreased muscle strength in adjuvant-induced rheumatoid arthritis animal model: A relationship to behavioural assessments

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disorder with unknown aetiology. Patients suffering from RA face persistent pain due to joint inflammation, and tissue destruction. Behavioural phenotyping is an approach to target the role of different behavioural traits associated with disease progression. The study aimed to assess behavioural patterns associated with decreased muscle strength in the adjuvant-induced rheumatoid arthritis animal model. The study was conducted on male Albino Wister rats (n = 30) [Control, Vehicle, and Disease groups]. After taking ethical approvals RA was induced by complete Freund's adjuvant (CFA) intradermally base of tail. The weight of animals, macroscopic analysis of inflammatory signs, and arthritic scores were measured weekly. Grip strength, ganglia-based movement, cataleptic activity, and motor-coordination-related behaviours were assessed among the groups. Radiographs and spleen index assay were performed followed by data analysis using one-way and two-way ANOVA (Analysis of Variance). A significant decrease in weight and an increase in arthritic scores among the diseased group was observed. Behavioural analyses confirmed that diseased animals had significantly decreased grip strength and increased cataleptic activity with less motor coordination. Radiographic images and spleen index assay confirmed the pattern of RA. Therefore, it can be suggested that the development of the disease animal model is an effective approach to identifying the disease progression and associated behavioural changes. Moreover, this prepared laboratory animal model may be utilised for pathway analyses to understand the key role of immune regulators and genetic insight into molecular pathways associated with acute and chronic phases of RA.

1. Introduction

RA is developed due to an abnormal immune system where the immune system loses self-tolerance and damages the body's cellular and tissue components. Inflammation plays a key role in swelling and redness which causes pain followed by bone destruction in the later stage of the disease [1]. RA is a progressive autoimmune disorder that initially attacks small joints starting from the hand, wrist, and knee followed by the destruction of multiple joints [2]. In the development of RA cell-mediated, and humoral immunity both are

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ABBREVIATIONS

RA	Rheumatoid Arthritis
CFA	Complete Freund's Adjuvant
ANOVA	Analysis of Variance
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
DMARDs	Disease-Modifying Antirheumatic Drugs
CVD	Cardiovascular Diseases
GIT	Gastrointestinal Tract
APC	Antigen-Presenting Cells
IACUC	Institutional Animal Care and Use Committee
E	Effect Value
PBS	Phosphate Buffer Saline
FIA	Freund's Incomplete Adjuvant
HSD	Honestly significant difference
SPSS	Statistical Package of Social Sciences

involved. In cell-mediated immunity, the antigen-presenting cells (APC) attack the synovial membrane to cause synovitis followed by the infiltration of immune cells including, lymphocytes and polymorphonuclear leukocytes [3]. At this stage, the disease is asymptomatic, whereas autoreactive B-cells are involved in the release of autoantibodies which leads to the chronic stage of the disease [4]. The presence of antigenic determinants on *Mycobacterium tuberculosis* for various proteins (glycolipids and lipoprotein). These proteins can be recognized by the APCs and mimic the production of inflammatory mediators that contribute to the progression of RA [5,6].

Currently, limited treatment options are available to manage the symptoms and complications associated with the progression of the disease. Opioid analgesics [7] and nonsteroidal anti-inflammatory drugs (NSAIDs) are choices of drugs prescribed as pain relievers for the first-line management of RA, but the antagonistic effects surpass the benefits [8]Disease-modifying antirheumatic drugs (DMARDs) and anti-inflammatory injections are the choice of medicine as the second line of management against RA [8,9]. These drugs increase the susceptibility of other comorbidities related to cardiovascular diseases (CVD) and gastrointestinal tract (GIT) diseases among RA patients [10]. Globally, it affects 1 % of the population [11], whereas in Pakistan it affects 0.14 %–0.55 % population [12].

The development of a diseased animal model is an efficient approach to studying the disease progression, pathways analysis, drug therapy, behavioural traits, and pain related to the disease [13]. Various studies reported the development of RA-induced animal models which are limited to identifying novel therapeutic strategies and pathophysiology [14,15]. However, there is limited data available regarding the role of behaviours in the pathogenesis of rheumatoid arthritis. According to the American Psychological Association [16], behaviours contribute to developing and controlling the disease [16,17]. The studies reported the presence of neurological symptoms including depression, continuous pain, fatigue, and avolition, among 13–42% of progressive arthritis patients [18,19]. Another study revealed the relationship between neurologic and muscle disorders through the continuous use of medications by RA patients could lead to occasional neurological effects [20].

Behavioural analysis is an important aspect in various research purposes, that provides facts about behavioural manifestations of molecular changes that occur during the disease progression [21–24]. Behavioural phenotyping is the systematic approach that provides a better understanding of underlying mechanisms, behavioural patterns, and physiological changes involved in the disease progression. The current study is based on a translational research approach to assess behavioural patterns associated with the progression of decreased muscle strength in adjuvant-induced rheumatoid arthritis animal models.

2. Material and Methods

2.1. Reagents and chemicals

Animal feed, Animal bedding (sawdust), Vernier calliper, FCA [(Imject™ Freund's Complete Adjuvant), (Catalog number: 77140), Thermo Scientific™], FIA [Imject™ Freund's Incomplete Adjuvant), (Catalog number: 77145), Thermo Scientific™], PBS [(Phosphate buffer saline), (Catalog number: 10010023), Thermo Scientific™], Formalin Solution, 10 % (Histological), Fisher Chemical™, Sodium thiopental (40mgkg^{-1}).

2.2. Animals

Locally bred male Albino Wister rats weighing 150–200 g (5–6 months old) were purchased from the DUHS (Dow University of Health Sciences), OJHA campus, Karachi, Pakistan. Animals were kept for 1 week of acclimation period to reduce any physiological effect, making them familiar with the new environment and reducing the handling stress. All the animals were singly housed in propylene cages to restrict social interaction with the levelled-cage illumination and *ad libitum* access to food and water. The standard rodent diet was provided containing fat (25 %), carbohydrates (50 %) and protein (25 %) which makes calories up to 4.47 kcalg^{-1}

[25], whereas tap water was provided. The housing conditions include 12:12 light/dark conditions within controlled temperature (20 °C) and humidity (55 %). All the behavioural analyses were monitored and carried out using a balanced design model. The study was performed after taking ethical approval from KIBGE (Karachi Institute of Biotechnology and Genetic Engineering) (Ref No: KIBGE/ICE/077/June 25, 2021) and IACUC (Institutional Animal Care and Use Committee) of BUHS (Bahria University of Health and Sciences) (Ref No: ERC 30/2022) under the mentioned reference IDs. All the protocols were conducted as per the guidelines of the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised1985) and Scientific Procedures Act 198 (UK Animals) and approved by the BUHS (Bahria University of Health and Sciences) Animal House facility.

2.3. Experimental protocol

The sample size was calculated by the crude method which is also referred to as the resource equation method [(E = Total number of animals x Total number of groups) − Total number of groups). The E (effect value) range between 10 and 20 is considered an adequate sample size to conduct the study [26]. Thirty rats were divided into three groups [Control group (n = 10), Vehicle group (n = 10), and Disease group (n = 10)]. No treatment was provided to the control group, solvent (PBS) was provided with the same procedure to the vehicle group, and the drug was induced in the disease group. The objective of this study was to identify the animal grip strength and severity of pain with the disease progression. The animal grip strength and severity of pain were estimated by different parameters including, body weight, paw morphology, paw measurements, rheumatoid arthritis index calculation, behavioural models, and radiographic images.

2.4. CFA (Complete Freund's adjuvant) induced arthritis

In the diseased group, 0.2 mL of FCA [(Imject™ Freund's Complete Adjuvant, Catalog number: 77140), Thermo Scientific™] was injected intradermally from the base of the tail followed by the booster dose (0.1 mL) after 48 h and two subsequent booster doses (0.2 mL) of FIA [Imject™ Freund's Incomplete Adjuvant), Catalog number: 77145, Thermo Scientific™]. The control group was provided with similar environmental conditions without inducing any drug, whereas in the vehicle group, the solvent PBS (phosphate buffer saline, pH: 7.4) was injected. Adjuvants were diluted in the PBS in a 1:1 ratio as mentioned in the product description. Animals were kept under observation to record body weight and the swelling in paws was measured (in mm) by vernier calliper and rheumatoid arthritis index calculation (Table 1). The experiment was carried out eight weeks after the confirmation of the disease (Fig. 1).

2.5. Behavioural analyses

2.5.1. Kondziela inverted screen test

The Kondziela inverted screen test was performed to assess the grip strength of the rat's hindlimbs and forelimbs. The test was executed according to the reported protocol [27]. The size of the screen was 43 cm square comprised of 12 mm wire mesh with a diameter of wire 1 mm. The mesh-wired screen is beaded with 4 cm deep wood to prevent the climbing of rats to the other side of the screen. The rat was placed at the centre of the screen and inverted to check the grip of the rat's limbs. The time (sec) of the fall was noted, where the cut-off time was 120 s.

2.5.2. Pole test

The ganglia-based movement is the major feature of depression therefore, the pole test experiment was performed. In this test, the rat was placed over the 100 cm height and 2.5 cm wide wooden rod and supported by the wooden frame. The animal was kept over the top of the rod and the time of fall of the animal was noted as per the reported protocol [28].

2.5.3. Inclined plane test

Depression in medical conditions is associated with the cataleptic effect. Therefore, the inclined plane test was performed by the reported method [29]. The test was conducted by placing the animal in an unusual position (inclined plane) and the time to change in position was recorded as latency time (seconds). The cataleptic activity was calculated by [(latency time/cut-off time) x 100], where the cut-off time is 180 s [30].

2.5.4. Beam walking test

The beam walking test is associated with motor coordination [31]. In this test, beams of different diameters (3 cm, 2 cm, 1 cm) hang

Table 1
Arthritic index score calculation.

Score	Evaluations
0	No evidence of redness/swelling
1	Redness with slight swelling (one digit or full paw/tarsal joints or ankle)
2	Redness with mild swelling (only one or two joints/extending from the ankle to the tarsal joint)
3	Redness with moderate swelling (swelling in three or more joints/extending from the ankle to the metatarsal joint)
4	Severe swelling (swelling in all digits or entire paw affected/the ankle, foot, and digits or ankylosis of the limb are affected)

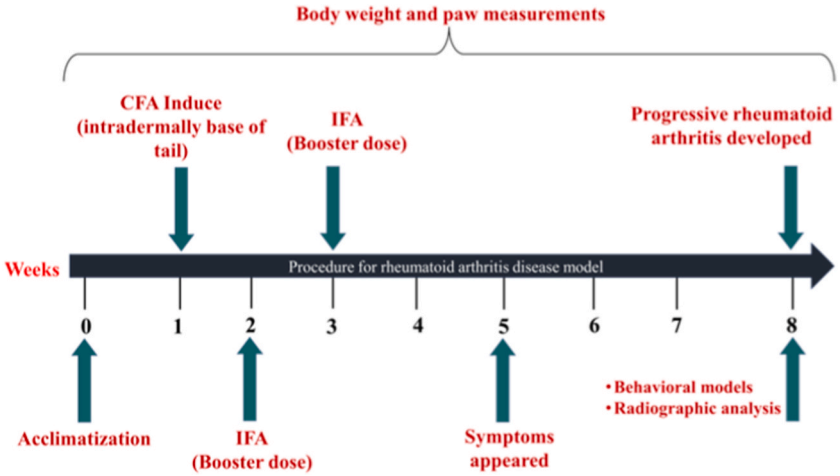

Fig. 1. The experimental timeline of rheumatoid arthritis induced animal model, where CFA is complete Freund's adjuvant and IFA is incomplete Freund's adjuvant.

in such a way that it connects the start point with the cage. The rods were supported by two pillars at 50 cm in height. The experimental animals were initially trained to walk over beams of different sizes from widest to narrowest [32]. The latency time (time to cross each beam) and the number of slips of each animal were recorded.

2.6. Evaluation of arthritic index score

The progression of arthritis was recorded by macroscopic analysis of inflammatory signs including redness and swelling. The disease severity score is characterized by an arthritic index score from 0 to 4, where 0 = No evidence of redness/swelling, 1 Redness with slight swelling (one digit or full paw/tarsal joints or ankle), 2 Redness with mild swelling (only one or two joints/extending from the ankle to tarsal joint), 3 = Redness with moderate swelling (swelling in three or more joints/extending from the ankle to metatarsal joint) and 4 = Severe swelling (swelling in all digits or entire paw effected/the ankle, foot, and digits or ankylosis of the limb are affected) [33]. Additionally, the paw swelling, and thickness were measured by using a Vernier calliper (mm) as per the reported double-blind protocol to avert any error [34].

2.7. Radiographic (X-ray) analysis

The disease progression was assessed by radiological images (X-ray) to observe bone erosion, joint deformity, and narrowing of joints. The experimental animals were sedated by the intraperitoneal injection of sodium thiopental (40 $mgkg^{-1}$). The paws were observed under the traditional radiography system, where animals were placed over the X-ray plate and focused from 25 cm apart.

2.8. Spleen index assay

Animals were anaesthetized (as mentioned above) followed by decapitation to ensure complete death. Animals were dissected for further experiments and spleens were weighed and divided by the total body weight to analyse the spleen index assay. The weight of the spleens from all groups was measured.

2.9. Statistical analysis

All the data were presented as Mean ± SD (Standard deviation) evaluated by one-way and two-way ANOVA (Analysis of Variance) followed by post hoc Tukey's HSD (honestly significant difference) test to measure the difference among the intergroups. Significance level $p < 0.05$ is considered throughout the study, where the statistical analyses were performed by the Statistical Package of Social Sciences (SPSS) program version 23 and GraphPad Prism 8.0.1.

3. Results

3.1. Effect of disease progression on the body weight

It was observed that among three groups, the weight of animals was increased in the control (Mean = 148g), vehicle (Mean = 148g), and disease group (Mean = 154.8g), and results were not statistically significant ($p > 0.05$) till the fourth week. On the other hand, after the induction of two booster doses of IFA (Incomplete Freund's Adjuvant), it was observed that the weight of animals from

the diseased group was significantly decreased (Mean = 112g, $p < 0.001$) in comparison to the other two groups i.e., control group: mean = 190.1g and vehicle group = 181.9g, $p > 0.05$ (Fig. 2).

3.2. Limbs swelling and arthritic score index

The paws measurements revealed a significant increase ($p < 0.001$) in the diameter of forelimbs among the disease group after the fourth week from 10 mm to 16.1 mm, whereas the diameter of forelimbs was found to be non-significant (n.s) among control and vehicle group (Fig. 3A). Additionally, the diameter of hindlimbs was also significantly ($p < 0.001$) increased after the booster dose (second week) from 13.4 mm to 18.4 mm, while on the contrary the diameter of hindlimbs was found to be statistically nonsignificant (Fig. 3B). The rheumatoid arthritis index showed that the score was significantly increased ($p < 0.001$) among the disease group in comparison to the control and vehicle group, whereas among the vehicle group arthritic score was found to be non-significant ($p > 0.05$) after the first booster dose (Fig. 3C). Based on the mentioned scoring system swelling was recorded (Fig. 3D)

3.4. Association of behavioural models with disease progression

The Kondziela inverted screen test showed that the grip of rheumatoid arthritis-induced rats was weakened and unable to use limbs hence, a significant ($p < 0.001$) decrease in fall time was recorded among the disease group in comparison to the control and vehicle group. On the other hand, control and vehicle groups maintain the grip till the cut-off time of 120 s (Fig. 4A; Fig. S1). The Pole test behavioural model showed the pattern of fall among the experimental groups, where the time of fall was significantly ($p < 0.001$) decreased among the disease group, whereas animals from control groups significantly met the cut-off time (Fig. 4B; Fig. S2). An inclined plane test showed that cataleptic activity was significantly higher in the disease group ($p < 0.001$) than in the control and vehicle groups (Fig. 4C; Fig. S3). The beam walking test revealed that the time of fall and slips were found to be significantly higher among animals from the diseased group as they were unable to walk over the rod of 3 cm, 2 cm, and 1 cm (Fig. 4D; Fig. S4). However, no significant difference ($p > 0.05$) was observed between the time of fall and slips of animals from the control and vehicle groups (Fig. 4E).

3.5. Bone destruction by radiographic (X-ray) analysis

The radiographic images revealed bone destruction among the diseased group in comparison to the vehicle and control group. The radiographic images of the control and vehicle group seemed to be normal in comparison to the diseased model (Fig. 5A and B). However, the radiographic images of the diseased group showed the major rheumatoid arthritis features including bone erosion, loss of joint space, hyperostosis, and ulnar deviation or digit deformation (Fig. 5C).

3.6. Spleen index assay

Splenomegaly is the key feature of rheumatoid arthritis disease. It was observed that the weight of the spleen significantly increased ($p < 0.001$) in comparison to the control group and vehicle group (Fig. 6A). The spleen index was obtained to be significant ($p < 0.001$) among the disease group in comparison to the control group and vehicle group (Fig. 6B). Moreover, it was observed that the size of the spleen is larger than the control and vehicle groups (Fig. 6C).

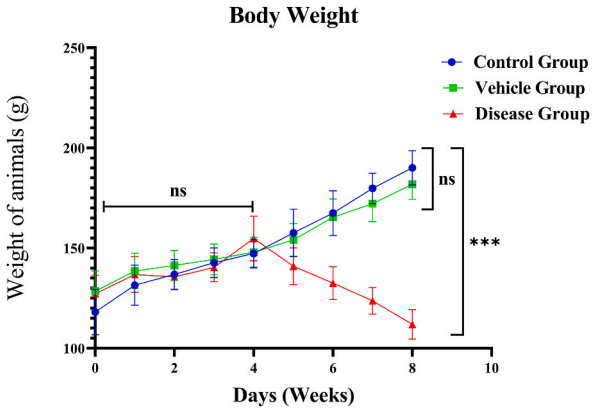

Fig. 2. Representation of the comparative change in body weight of rats among three targeted groups, where n.s. represented non-significant and *** represented statistically significant probability score ($p < 0.001$) taken from one-way ANOVA followed by post hoc analysis (Tukey's test).

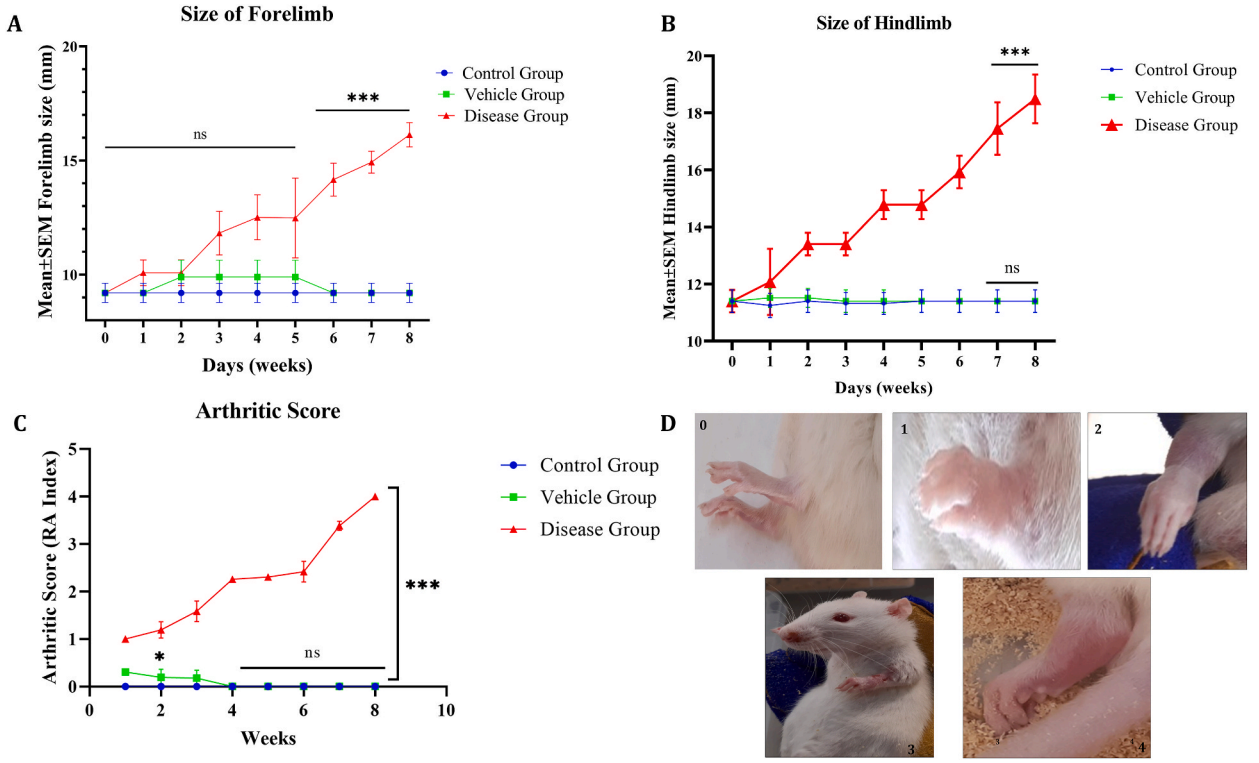

Fig. 3. (A) Diameter of forelimbs (mm) among the targeted groups (B) Change in hindlimb diameter among the targeted groups throughout the eight weeks. (C) Representation of arthritic index among the experimental groups. (D) The images of swelling in paws with the score 0 to 4. Results are represented in the form of repetitive means (n = 10) analysed by the one-way analysis of variance (ANOVA) followed by Tukey's test, where *** indicates $p < 0.001$, * indicates $p < 0.05$ and n.s is non-significant.

4. Discussion

Disease animal models play an important role in identifying key mechanisms involved in the acute and chronic phases of the disease. The current study demonstrates the development of a rheumatoid arthritis (RA) animal model to assess the impact of behavioural traits in the progression of the disease. Behaviours majorly associated with the ability of animals [10] to recover from disease is the major feature that contributes to the disappearance of the symptoms [35]. It was observed that after the induction of the first dose of CFA (Complete Freund's Adjuvant) animals were found to be lethargic for 24 h and an increase in weight was observed till the fourth week followed by the booster dose of IFA (Incomplete Freund's Adjuvant). The weight of diseased animals was reduced up to 8.9 % of total body weight from the start of the experiment, where the results were consonant with the previous studies [34,36]. The first dose of CFA slowly dispersed, and it was observed that diseased rats were unable to maintain their grip on the hind limbs and started falling within the cage (Fig. S5; Video S1). Moreover, the animals from the diseased group showed abnormality in gait behaviour, and changes in the walking pattern (Fig. S6; Videos S2 and S3), where the symptoms disappeared after 72 h and gradually reappeared in the fifth week after the first booster dose of IFA which is the key feature of adjuvant-based arthritis model [37]. The initiation of RA was observed by inflammatory cardinal signs including rubor (redness), calor (heat), tumour (swelling), and dolour (pain). In diseased animals, the small blood vessels of paws become dilated and cause redness. In this study a significant increase in the size of forelimbs and hindlimbs as the disease progressed [34]. Furthermore, animals after eight weeks start crawling with a significant increase in the arthritis index score in comparison to the control group and vehicle group due to continuous swelling of paws [34,38].

Studies reported that patients suffering from rheumatoid arthritis face severe illness including, joint disabilities which result in continuous pain and compromise the quality of life [39–41]. Analysis of behavioural patterns provides a better understanding of underlying mechanisms, behavioural traits, and physiological changes involved in the disease progression. Thus, behavioural models were applied for the confirmation of disease and to assess the impact of animal behaviours in the pathophysiology of the rheumatoid arthritis rat model. The grip strength of animals was analysed by KIST (Kondziela inverted screen test) which showed that animals from the diseased group were unable to hold the wire meshed screen and unable to use hindlimbs which resulted in rapid falling [42]. A study reported that there is a direct link between RA and the CNS (Central Nervous System) [43], ganglia are responsible for various activities including, movement, learning, and behaviours, therefore, a pole test was performed which revealed that animals suffering from RA are unable to hold the rod and unable to retain position till the cut-off time (60 s) [44]. The disease-related behavioural patterns include fear of moving around and falling, which leads to sedentary behaviour, depression, and loss of appetite. Studies reported that depression is correlated to cataleptic activity which results in freezing action against unusual stimuli [45]. A significant

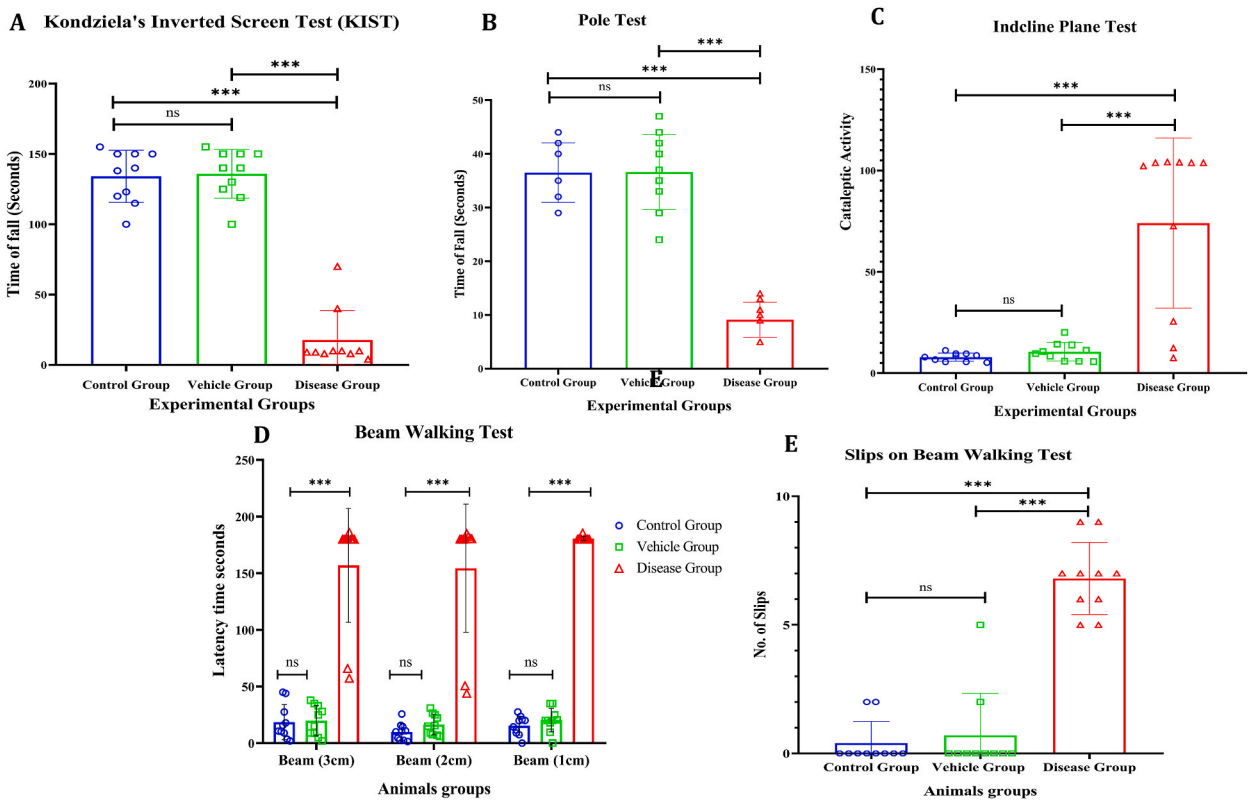

Fig. 4. (A) The representation of the Kondziela inverted screen test (KIST) showing the time of fall in seconds for grip strength of limbs (B) The representation of the Pole test showing a time of fall in seconds, where the cutoff time is 60 s (C) Representation of Inclined plane test (IPT) where cataleptic activity was noted (D) The representation of beam walking test (BWT) showing latency time in seconds and (E) number of slips in each group. All the Results are represented in the form of repetitive means (n = 10) analysed by the one-way and two-way analysis of variance (ANOVA) followed by Tukey's test, where *** indicates $p < 0.001$ and n.s is non-significant.

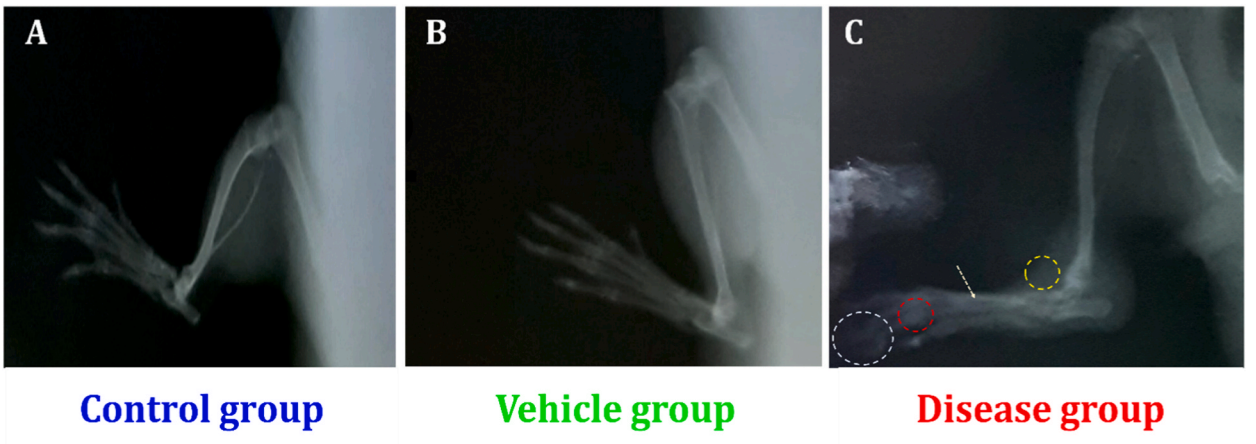

Fig. 5. Representation of rat limbs from the control group (A) and vehicle group (B) showing no destruction in bone. (C) Representation of rat limbs from the control group, where the yellow circle indicated the presence of oedema, the arrow indicates loss of joint space or narrowing of the joint, the red circle showed the presence of hyperostosis, and the white circle pointed to ulnar deviation or digits deformation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

increase in cataleptic activity among diseased animals was observed when kept in the unusual position (inclined plane) which confirmed the presence of disease [29]. The gait behaviour and motor coordination were analysed by beam walking test which revealed that the animals from the disease group were unable to walk over the rods (2 cm and 1 cm) with the increased number of slips

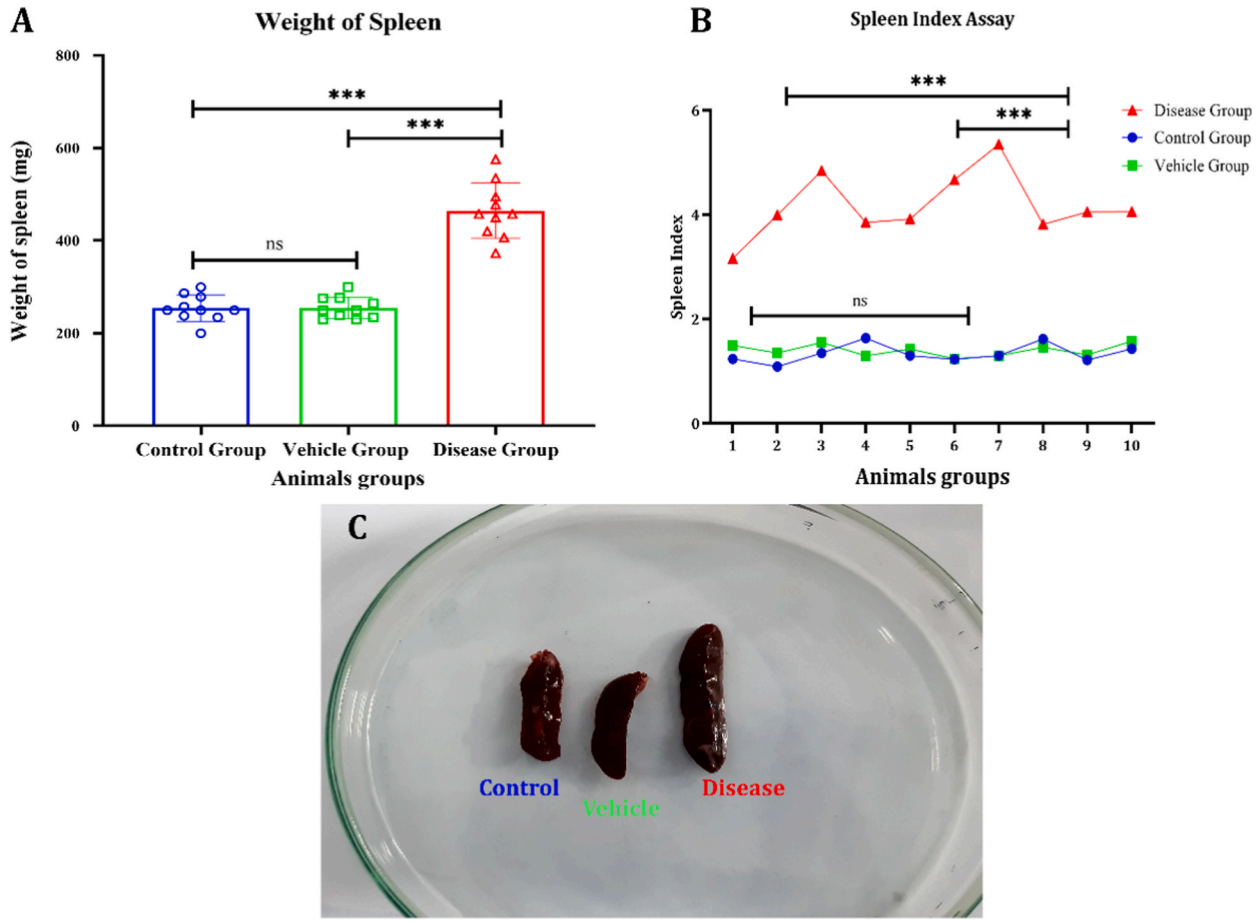

Fig. 6. Representation of weight of spleen (mg) (A) and spleen index assay (B) among the targeted groups including blue = control group, green = vehicle group, and red = disease group. Results were analysed by the analysis of variance (ANOVA) followed by Tukey's test, where *** indicates $p < 0.001$ and n.s is non-significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in comparison with the control and vehicle group. It also confirmed that animals from the control and vehicle groups had better learning capabilities in comparison to the disease group [46,47]. Other behaviours such as escape behaviour and bulging of eyes were also noticed among the diseased group which is associated with depression and stress (Figs. S6 and S7).

Bone erosion, narrowing of joint space, and ankylosis with swan-neck deformity are the major features of RA, and this feature was found to be predominant among the diseased group rats [34,48]. However, RA features were not present among the control and vehicle groups. Previously, polymorphism-based studies were conducted on human RA patients which revealed that joint deformity is the major feature of the chronic stage of rheumatoid arthritis where symptoms appear [8,39,40]. Splenomegaly is the key feature of rheumatoid arthritis disease [49] which confirmed the successful development of CFA induced rat animal model. The adjuvant-induced arthritis rat model may be beneficial in the identification of behavioural phenotypes and susceptibility patterns of disease. Due to the inherent complexity of human behaviour may not be replicated by the animal models that are used in behavioural research, thereby restricting the direct application of results to human circumstances. Therefore, macroscopic analyses were performed which mimic the symptoms of RA and were further confirmed by the radiographic analysis. The prepared rheumatoid arthritis model will be utilized to study the involvement of key immune regulators in the progression of the disease and to get genetic insights into molecular pathways associated with acute and chronic phases of the disease (RA).

5. Conclusions

Studies confirmed that negative emotions directly impact the immune system and play a critical role in the pathophysiology of different diseases like rheumatoid arthritis (RA), cardiovascular disease, and osteoporosis. The current study reported the successful development of a CFA-induced RA animal model that mimics the progressive joint disorder with exact symptoms occurring in both acute and chronic phases. Reduction in weight along with increased paw volume was observed in diseased rats. Since behaviours play an essential role in disease development, the study emphasized that motor-related behavioural models are better criteria for the

assessment of disease progression. Radiographic images and spleen index assay confirmed that the laboratory animal model is successfully developed and can be utilized to target key immune regulators that have been involved in the disease progression. The study also confirmed that behavioural models are an important source in identifying the progressiveness of disease before sacrificing the animal.

Funding statement

This research did not receive any funding for financial assistance.

Ethical statement

All the behavioural analyses were monitored and carried out using a balanced design model. The study was performed after taking ethical approval from KIBGE (Karachi Institute of Biotechnology and Genetic Engineering) (Ref No: KIBGE/ICE/077/June 25, 2021) and IACUC (Institutional Animal Care and Use Committee) of BUHS (Bahria University of Health and Sciences) (Ref No: ERC 30/2022) under the mentioned reference IDs. All the protocols were conducted as per the guidelines of the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised1985) and Scientific Procedures Act 198 (UK Animals) and approved by the BUHS (Bahria University of Health and Sciences) Animal House facility.

Data availability

All the data analysed and generated in this study is available in the manuscript and supplementary files.

Response to data availability

Has data associated with your study been deposited into a publicly available repository? No.

CRediT authorship contribution statement

Maham Ghouri: Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Mehreen Lateef:** Supervision, Resources, Project administration. **Laraib Liaquat:** Methodology. **Ahsan Zulfquar:** Resources. **Saima Saleem:** Writing – review & editing. **Sitwat Zehra:** Writing – review & editing, Supervision, Resources, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23264.

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