Electronic Journal of Biotechnology 76 (2025) 67–78

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage: www.elsevier.com/locate/ejbt

Research article

# The biological agent rituximab ameliorates DNA damage and repair efficiency in the somatic cells of arthritic mice ☆

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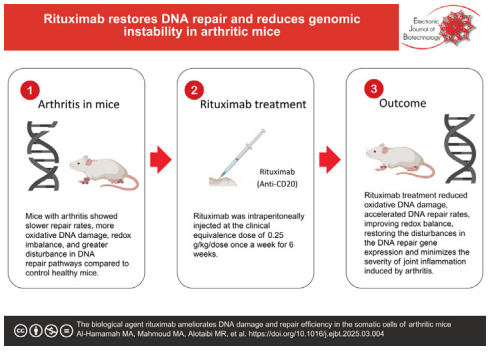
## GRAPHICAL ABSTRACT

The biological agent rituximab ameliorates DNA damage and repair efficiency in the somatic cells of arthritic mice




## ARTICLE INFO

*Article history:*
Received 5 October 2024
Accepted 11 March 2025
Available online 27 May 2025

*Keywords:*
Anti-CD20 monoclonal antibody
Arthritic mice
Autoimmune disease
DNA damage
DNA repair
Genomic instability
Inflammatory stress
Malignancy
Rheumatoid arthritis
Rituximab

## ABSTRACT

*Background:* Rheumatoid arthritis (RA) is an autoimmune disorder that deteriorates joints and can affect various physiological systems if not properly managed. The persistent joint inflammation and oxidative damage caused by RA can lead to genomic instability, a primary feature of most cancer cells. In RA, the prevalence of malignancy is comparatively higher than in the general population. However, it is unclear if the disease itself or its treatments induce susceptibility to neoplastic disorders. Our goal was to study genomic instability in the arthritic mouse model, namely DNA damage and repair and determine if the long-term use of the biological agent rituximab can impact these changes.
*Results:* Our results show that rituximab did not disrupt genomic stability at the tested regimen. Arthritic mice had more spontaneous DNA damage and a slower repair rate than control mice. Redox imbalance, oxidative DNA damage, and disturbance in the DNA repair pathways are also increased in arthritic mice. Meanwhile, rituximab treatment reduced oxidative DNA damage and accelerated DNA repair rate, improving redox balance and restoring the disturbances in the DNA repair gene expression at both mRNA as determined by $RT^2$ Profiler PCR array and protein levels as determined by Western blotting analysis in arthritic animals. Additionally, the ameliorative effect of rituximab has also been shown by

☆ Audio abstract available in Supplementary material.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso

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https://doi.org/10.1016/j.ejbt.2025.03.004

Somatic cells

its ability to reduce the severity of joint inflammation and histopathological alterations induced by arthritis.
*Conclusions:* The findings indicate that rituximab is a non-genotoxic, safe, effective drug for the treatment of RA and its complications.
**How to cite:** Al-Hamamah MA, Mahmoud MA, Alotaibi MR, et al. The biological agent rituximab ameliorates DNA damage and repair efficiency in the somatic cells of arthritic mice. Electron J Biotechnol 2025;76. https://doi.org/10.1016/j.ejbt.2025.03.004.


## 1. Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder characterized by the deterioration of articular cartilage and erosion of the bone. It manifests in 1–2% of the global population and can adversely impact several physiological systems, such as the eyes, skin, lungs, blood vessels, and heart, in some individuals [1]. While RA can develop at any stage of life, its occurrence rises as individuals' age. Its cause is uncertain; however, various investigations have shown a mix of genetic predisposition and environmental influences. These factors contribute to impairments in immunoregulation and a range of inflammatory processes [2]. Although there is no known cure for RA, clinical research suggests that early initiation of treatment with disease-modifying antirheumatic medicines (DMARDs) greatly increases the chances of symptom remission. The objective of achieving efficient treatment strategies for individuals with RA has resulted in the discovery of biological drugs that have enhanced the patient's capacity to manage the manifestations and symptoms of RA and its complications [3].

Recent progress in B cell investigations has shown that B cells are crucial in the pathology of RA and its related manifestations [4]. The FDA has approved rituximab (RTX) for the treatment of RA and is currently recommended to manage disease symptoms and delay the progression of structural damage in adults with moderately to severely active RA who have not shown sufficient response to one or more treatments, including TNFα blockers [5]. The hybrid mouse/human monoclonal antibody RTX targets the CD20 antigen on pre-B and mature B lymphocytes. This antibody causes a reduction in CD20-positive B cells. By binding to CD20, RTX induces B cell lysis and depletion of both circulating and tissue B cells for a prolonged duration, lasting up to 6 to 8 months. However, anti-CD20 antibody-induced B cell depletion dramatically accelerates the development and propagation of tumors, especially lung cancer and lymphoma [6,7]. It is crucial, therefore, to continuously monitor the long-term safety of using B cell-depleting therapies.

Numerous clinical studies have proven the safety of RTX [8]. However, the certainty of the safety of recurrent peripheral B-cell depletions, especially considering the possible risk of malignancy, remains incomplete. Several studies have shown that RA is linked to a higher likelihood of severe infection episodes, lymphomas, and increased mortality rates caused by cardiovascular disorders [9,10]. Although numerous clinical trials have evaluated the longer-term safety of RTX and examined the possible consequences of repeated therapy over an extended period [8], the impact of RTX on DNA damage and repair efficiency has not been investigated.

Patients with RA have a 1.5–3-fold increased risk of lymphoma compared to the general population. This risk is further heightened by symptoms that indicate disease activity and severity, therefore providing evidence for the concept of a line between lymphomagenesis and autoimmunity in RA [9]. Yet, the question arises as to whether the susceptibility to neoplastic diseases is caused by the disease itself, namely by exposure to chronic inflammation and persistent oxidative stress, or by the medications used to treat RA patients. It is postulated that the heightened vulnerability of RA patients to cancer may be linked to elevated levels of DNA damage, ineffective DNA repair mechanisms, and susceptibility to substances that damage DNA [11,12].

Numerous studies have documented an aberrant DNA damage repair system in RA [13,14]. In recent years, there has been a growing recognition of the interaction between the innate immune response and the DNA damage repair pathway. Numerous studies have shown that a change in the balance of the DNA damage repair system, caused by either exposure to genotoxicants or disruption of DNA repair processes, leads to the development of genetic damage that can function as powerful immunostimulators [15]. The association between DNA damage/repair and immune response is proposed by the observation that loss of immunological homeostasis and prolonged inflammatory response from many sources can result in DNA damage and disrupt the DNA repair system [16].

Given that the currently licensed DMARDs for the management of RA are associated with serious adverse effects, it is crucial to search for safe DMARDs. Therefore, the anti-CD20 antibody RTX has great potential as a safe treatment drug in combating RA. Our prior studies observed repeated RTX injection to naïve control DBA/1J mice did not suggest any notable harmful consequences [17,18,19,20]. In addition, the repeated administration of RTX to arthritic DBA/1J mice significantly reduced the disturbances in male germ cells [17,19] and reduced somatic cell clastogenicity/aneugenicity [20] as well as reduced the arthritis-induced immune disturbances [18]. To gain more insights into the DNA damage and repair changes that occur in RA and to determine whether RTX can ameliorate these changes, we aimed to investigate the genomic instability, in terms of DNA strand breaks and deficient DNA repair, in collagen-induced arthritis in DBA/1J mouse model. Furthermore, elucidating the mechanism(s) by which RTX can counteract DNA repair failure in the DBA/IJ mouse model would enable the discovery of safe therapeutic strategies for treating RA.

## 2. Materials and methods

### 2.1. Animals

The study utilized DBA/IJ male 8–10 weeks old mice obtained from Jackson Laboratories (Bar Harbor, ME, US). These mice had an increased risk of arthritis following collagen immunization [21]. In a specialized pathogen-free environment, the mice were maintained at around 22°C and subjected to a 12-h light–dark cycle. They were provided with standard rodent chow and available water without limitations. The Institutional Animal Care and Use Committee at King Saud University (KSUSE-2164) granted final approval for every experiment. Animals were housed in plastic cages in a well-ventilated room for acclimatization during the experiment.

### 2.2. Induction of arthritis and drug therapy

0.2 mg of immunization-grade calf type II collagen (CII) formulated in the same volume of Freund's complete adjuvant, including Mycobacterium tuberculosis (2 mg/ml), were administered intradermally to animals at the base of the tail on day 0. On day 21 of the experiment, the mice were administered another dose of 0.2 mg CII formulated with the same volume of incomplete Freund's adjuvant. Two times a week, mice were scored blindly, starting from the fourth week of immunization until they were euthanized to evaluate the severity of arthritis. As previously described, the arthritic scores were computed on a scale ranging from 0 to 4 [22]. For each mouse, the total score was calculated by adding the scores of its four paws. The highest possible total score per mouse was 16. Two groups of arthritic animals were assigned to receive intraperitoneal treatment with either RTX or an equivalent volume of saline once a week from week 4 to week 10 of the study, following the start of arthritis.

RTX was given at the clinical equivalence dose of 0.25 g/kg/dose throughout weeks 4 to 10 following collagen administration [23,24]. In addition to the experimental arthritic groups, two other groups of naïve DBA/1J animals were intraperitoneally injected with RTX or saline and included in the experiment. As a positive control genotoxicant, a group of naïve DBA/IJ animals were intraperitoneally treated with a single dose of 40 mg/kg cyclophosphamide. Following drug administration, the animals were provided with unrestricted access to food and drinking water until they were euthanized. The animals were lethally euthanized using isoflurane inhalation anesthesia, and subsequently, the femur and knee tissues were extracted 24 h following the final administration of RTX. Femoral bone marrow cells were transferred into aseptic glass tubes by flushing them with saline solution. The knee tissues were immediately used for histopathological investigations or frozen in liquid nitrogen at −80°C until needed for molecular analysis. In preparation for use, the knee tissues were positioned on filter sheets to remove moisture, then weighed and crushed before homogenization.

### 2.3. Estimation of endogenous DNA strand breaks

Spontaneous DNA strand breaks were assessed in bone marrow cells using the standard comet assay according to the recommendations of the OECD, as previously described [25]. The cells were suspended in a low melting point agarose (0.75%) solution and applied onto slides previously coated with agarose with a standard melting point (1.5%). Upon gel solidification, the coded slides were kept overnight in a refrigerator with the lysing solution. The slides were immersed in a cold electrophoresis buffer for 20 min. The electrophoresis, neutralization, and ethidium bromide staining procedures were carried out according to the prior description [26]. The procedure involved preparing two slides from each mouse and analyzing at least 150 stained bone marrow cells on each slide using the Comet Assay IV software (Fig. 1). The level of DNA damage was determined by tail intensity and tail moment markers. Tail intensity represents the proportion of DNA in the comet tail compared to the overall DNA in the bone marrow cell. The tail moment calculates the number of broken/relaxed pieces (expressed by the intensity of DNA in the tail) and the lowest observable size of migrating DNA (shown by the comet's tail length).

### 2.4. Estimation of endogenous oxidative DNA strand breaks

A DNA repair endonucleases-based modified comet test was conducted on slides to identify the presence of oxidized purine and pyrimidine bases [27]. After the lysis process, the slides were washed with enzyme buffers and then incubated with 50 μL of formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III) enzymes, which precisely detect DNA breaks at modified purine and pyrimidine bases, respectively or endonuclease buffers at 37°C for 40–45 min. Following incubation with endonucleases, the slides were immersed in an electrophoresis solution and subjected to electrophoresis, neutralization, staining, and analysis, as previously explained. The presence of oxidative DNA strand breaks was assessed by measuring the difference in tail intensity between incubations with endonuclease and buffers.

### 2.5. Estimation of DNA repair capacity

The DNA repair capacity was investigated by conducting the standard comet test multiple times with bone marrow cells following radiation exposure to induce DNA lesions. The cells were obtained from each animal and cultivated in RPMI 1640 media supplemented with 2% phytohemagglutinin at 37°C. A total dose of 4 Gy of radiation was exposed to the cells using a GammaCell-220 device ($^{60}$Co gamma-ray source) around 30 min after culture initiation [28]. Following radiation exposure, the cells were directly examined for DNA strand breakage or subjected to different incubation times at 37°C before the standard comet test was conducted. Following radiation, the cell viability exceeded 95%, as shown by the trypan blue dye exclusion test. Cells exposed to sham radiation were used as controls. Aliquots of the cell suspensions were collected at 0 min (no incubation), 15, 30, 60 min, and 120 min following radiation. The DNA repair kinetics were

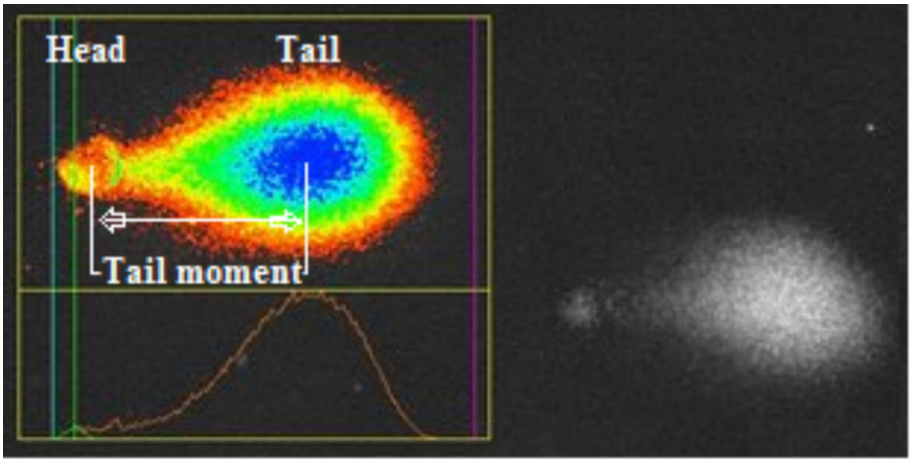


**Fig. 1.** An example image of the comet analysis by the Comet Assay IV software.

assessed using the standard comet test procedure, as described above.

### 2.6. Estimation of oxidant/antioxidant balance

The potential effects of RTX on the imbalance between oxidants and antioxidants in arthritic animals were investigated by evaluating reduced glutathione (GSH) levels and reactive oxygen species (ROS) production in bone marrow cells. An assessment of GSH was conducted using 5,5′-dithiobis (2-nitrobenzoic acid) according to the Ellman procedure [29]. The spectrophotometric measurement of the absorbance of the resulting color was conducted at a wavelength of 412 nm, compared to a blank. The data are presented in units of μmol/g protein. Protein concentration was measured using the Lowry method, which used calf serum albumin as the reference standard [30]. The production of ROS was evaluated using spectrofluorimetry. This method relied on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate to generate 2′,7′-dichlorofluorescein (DCF) fluorescein, as previously explained [31]. The fluorescent level was quantified using a spectrofluorimetric reader at 485/520 nm. The results are shown as fold differences compared to the untreated control naïve DBA/1J animals.

### 2.7. Gene expression analysis

To clarify the molecular processes that generate DNA strand breaks, we analyzed the mRNA expressions of genes related to DNA damage and repair in knee tissues using an $RT^2$ Profiler PCR array. Knee tissues were subjected to RNA extraction using the RNeasy Kit following the manufacturer's instructions (Qiagen). The $RT^2$ PCR Array first strand kit was used to generate first-strand complementary DNA in a 20-μL reaction mixture containing 2.0 μg of the extracted RNA. The synthesized complementary DNA was subsequently mixed with RT master mix and pipetting into the wells of a 96-well $RT^2$ PCR Array plate (PAMM029Z); then, the array reactions were performed using the 7500 RT-PCR System [31,32]. Data were normalized to *B2m, Actb, Gapdh, Gusb*, and *Hsp90ab1* housekeeping genes involved in the plates. A fold-change was computed for each evaluated gene using the $2^{-\Delta HC}T$ technique. Genes showing a substantial difference and a fold-change of more than 1.5 compared to those of the control DBA/1J animals were included for analysis.

### 2.8. Western blotting analysis

Using RIPA lysis buffer, the total protein was extracted from knee tissues, and its concentration was measured using the Lowry method [30]. A total of 25 μg of the extracted protein was separated using SDS-PAGE gels (10%) and then transferred onto PVDF membranes. Following incubation in the blocking buffer, the membranes were incubated with primary antibodies against Ogg1, Parp1, Gadd45a, P53, Rad50, and Xrcc1 at 25°C room temperature for 120 min. After that, the membranes were incubated with a secondary antibody that was linked to HRP at a 1:1,000 dilutions for 60 min at room temperature. Following washing, the bands were detected by exposing them to a Luminata Western-HRP Chemiluminescence Substrate and recorded using a C-DiGit. Measurement of intensity was conducted using the ImageJ program. Each band's intensity was normalized to the intensity of the comparable loading control β-actin bands.

### 2.9. Histopathological investigations

After immersing knee tissues in a 10% formalin solution for at least two days, they were prepared for additional processing for histopathological investigations. Subsequently, the specimens were cut into slices, and after undergoing standard automated processing, the knee specimens on the histopathology slides were stained with hematoxylin and eosin stains. An experienced histopathologist examined the stained slides with and without indications of arthritis.

### 2.10. Statistical analysis

Data are displayed as the mean ± SD. Initial assessments of the results' homogeneity and normality were conducted using the Bartlett's and Kolmogorov-Smirnov tests, respectively. A comparison was made between datasets using either the parametric Student's *t*-test and ANOVA with a Tukey-Kramer posthoc test or the non-parametric Mann-Whitney *U* test and Kruskal-Wallis with Dunn's posthoc test. The analyses were performed using GraphPad Prism. All findings were deemed significant if the *p* value was less than 0.05.

## 3. Results

### 3.1. Influence of RTX on arthritis development and joint histopathology

To explore the possible relevance of RTX in CIA, we initially assessed the clinical features of arthritis in animals. Following the second CII administration, animals exhibited arthritis around 21 d later, characterized by a gradual increase in paw edema/swel-

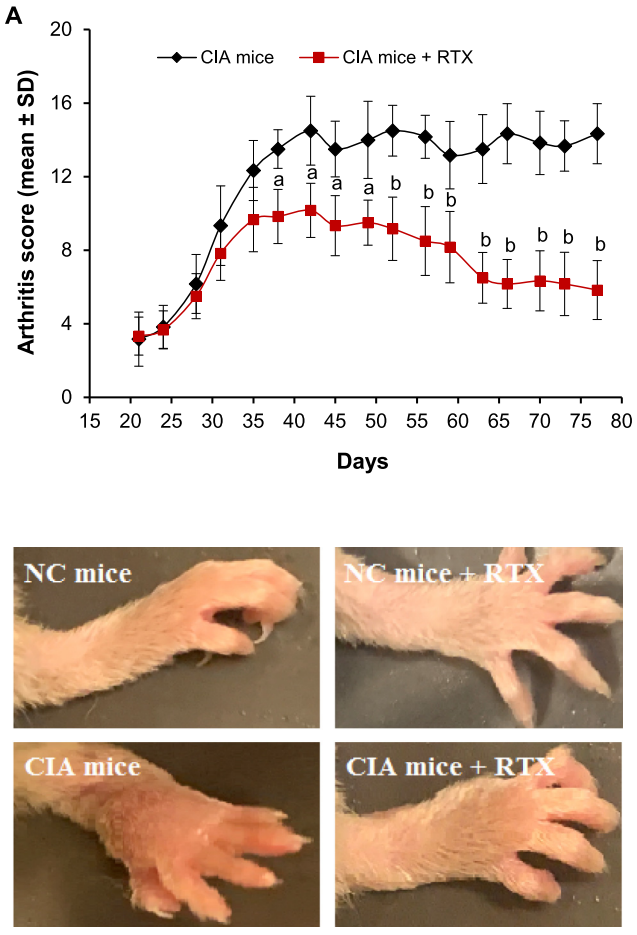


**Fig. 2.** Treatment with rituximab (RTX) decreased inflammatory edema of joints in a mouse model of collagen-induced arthritis (CIA). (A) Scores indicating the severity of arthritis (n = 6, mean ± SD). (B) Swelling of the paws in the various groups. a: $p < 0.05$ and b: $p < 0.01$ *versus* untreated CIA mice (Mann-Whitney *U* test).

ling. Following the initiation of CIA, animals administered RTX showed reduced arthritis severity scores compared to the naïve DBA/IJ control animals (Fig. 2A). An evident decrease in paw edema was noted following the treatment of RTX compared to the untreated CIA animals (Fig. 2B). In the histological analysis (Fig. 3A), the knee joint sections of the naïve DBA/IJ control animals showed normal, clear, and healthy joint space and tissues. Untreated CIA animals exhibited an apparent deterioration of joint spaces accompanied by inflammatory responses and bone degradation caused by forming a pannus and activated fibrous tissues. In contrast, CIA animals treated with RTX had a less severe inflammatory process without noteworthy damage to the joint structures. As depicted in Fig. 3B, the elevated histopathological scores in the joints of CIA animals were markedly reduced following the injection of RTX ($P < 0.01$). Administration of RTX alone did not result in substantial alterations in joint histology compared to the naïve DBA/IJ control animals. However, it effectively prevented the induced histological alterations.

### 3.2. RTX suppresses the increased levels of DNA strand breaks in CIA animals

The data presented in Fig. 4 indicate that the frequency of DNA strand breaks as evaluated by tail intensity (Fig. 4A) and tail moment (Fig. 4B) in naïve DBA/IJ animals treated with the positive control cyclophosphamide was markedly greater than in naïve DBA/IJ control mice ($p < 0.01$). These results validate the sensitivity of the comet assay in precisely evaluating DNA strand damage. No significant difference in the percentage of DNA strand breaks was observed between the control naïve DBA/IJ animals and the RTX-treated naïve DBA/IJ animals. Furthermore, there was a notable increase in endogenous DNA strand breakage in CIA mice compared to the naïve DBA/IJ control mice ($p < 0.01$). Notably, CIA mice that were administered RTX showed a significantly reduced rate of DNA strand breakage compared to untreated CIA mice that were not ($p < 0.01$).

### 3.3. RTX suppresses the increased levels of oxidative DNA strand breakage in CIA animals

Fig. 5 demonstrates that adding DNA glycosylases to the control bone marrow cells on comet sides significantly enhanced the extent of DNA strand breaking compared to the slides that were only treated with enzyme buffers, as observed by the modified comet test. This pertains to identifying oxidized DNA backgrounds and the related DNA strand breaks specific to each enzyme on these backgrounds. After subjecting the bone marrow cells from CIA animals to incubation with Fpg (Fig. 5A and Fig. 5B) or Endo

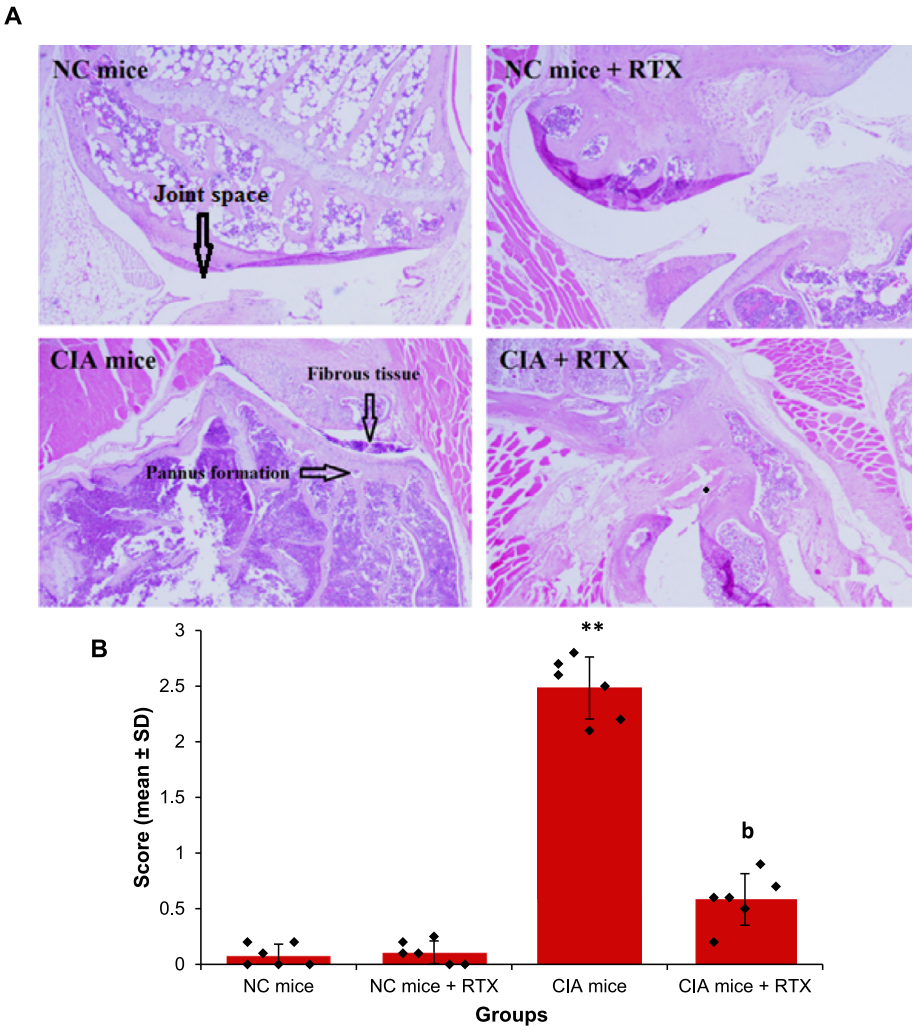


**Fig. 3.** Treatment effects of rituximab (RTX) on the histological alterations in mice with collagen-induced arthritis (CIA) model. Under a light microscope (100x), histological examinations (A) were conducted on knee joints stained with hematoxylin and eosin (50 μm scale bar). Statistical pathology scores were computed for each group and reported as the mean ± SD (B). **: $p < 0.01$ *versus* NC mice and b: $p < 0.01$ *versus* untreated CIA mice (Kruskal-Wallis Test with Dunn's posthoc test). NC mice: naïve control DBA/1J mice.

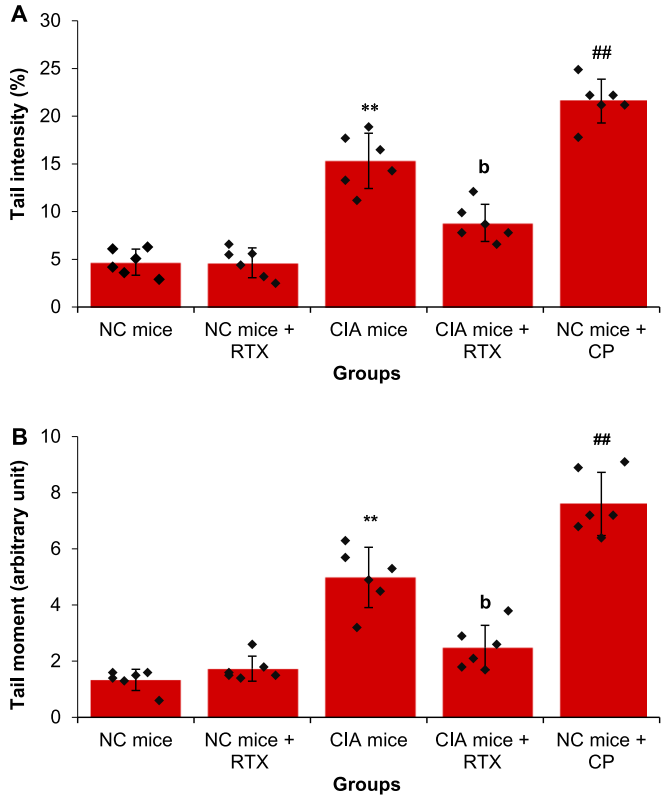


**Fig. 4. Administration of rituximab (RTX) reduced the increased levels of endogenous DNA strand breaks**; tail intensity (A) and tail moment (B) seen in the bone marrow cells of the collagen-induced arthritis (CIA) mice model (n = 6; mean ± SD). **: $p < 0.01$ *versus* NC control mice; b: $p < 0.01$ *versus* untreated CIA mice (Kruskal-Wallis Test). ##: $p < 0.01$ *versus* NC control mice (Mann-Whitney *U*-Test); NC mice: naïve control DBA/1J mice; CP: the positive control cyclophosphamide.

III (Fig. 5C and Fig. 5D) endonucleases, we observed a higher percentage of DNA strand breaks (tail intensity and tail moment). These levels were much greater than those observed in naïve DBA/IJ control animals ($p < 0.01$). This observation implies that the DNA strand breaks seen in arthritic animals are caused by oxidative damage in the DNA's pyrimidine and purine bases. Comparing CIA mice that received RTX with those not treated revealed a markedly reduced incidence of oxidative DNA strand breaks in the treated CIA mice. No statistically significant difference was found in the frequency of DNA strand breaks between naïve DBA/IJ mice treated with RTX alone and untreated naïve control DBA/IJ mice.

### *3.4. RTX decreases susceptibility to radiation and enhances the effectiveness of DNA repair systems*

Fig. 6 illustrates the extent of DNA strand breakage, tail intensity (Fig. 6A), and tail moment (Fig. 6B) in the bone marrow cells obtained after 0, 15, 30, 60, and 120 min post-radiation exposure, along with the sham controls. The occurrence of DNA strand breaks at 0 min (initial DNA damage formation) indicates the initial vulnerability to radiation, while its variation with time quantifies the effectiveness of the DNA repair system. No statistically significant difference in DNA strand breaks was found in the unirradiated bone marrow cells from naïve control DBA/1J animals compared to the earlier comet assay data. This indicates that the preparation techniques alone did not cause substantial DNA damage. Unirradiated bone marrow cells from CIA animals had a notably greater strand breakage than naïve control DBA/1J mice. This finding further confirms that spontaneous DNA strand breaks are more common in bone marrow due to arthritis.

Following radiation, cells from the naïve DBA/1J control and CIA mice models showed an initial rise in DNA strand break at 0, 15, and 30 min, followed by a gradual decrease at 60 and 120 min. This phenomenon indicates the activation of the spontaneous DNA repair processes. CIA bone marrow cells exhibited the highest degree of DNA strand breakage 15 min after radiation. The damage levels detected at all sampling times were significantly greater in CIA mice than those of irradiated naïve DBA/IJ control bone marrow cells. This indicates that arthritis increases the vulnerability of cells to radiation damage. While the DNA strand breakage decreased significantly from 15 to 120 min after irradiation in both CIA and naïve DBA/IJ control animals, the reduction rate was slower in CIA mice bone marrow cells compared to naïve DBA/IJ control cells. This indicates that CIA mice bone marrow cells have defective DNA repair function.

Following a 120-min repair incubation period, the cells derived from CIA mice successfully repaired about 46% of the DNA strand breaks induced by the radiations, compared to the peak observed at 15 min. In contrast, the irradiated bone marrow cells from naïve DBA/IJ control animals exhibited a rapid repair rate. These cells successfully eradicated about 55% of the DNA strand breaks compared to the peak level observed at 15 min. Remarkably, the administration of RTX significantly enhanced the rate of repairing the damage caused by irradiation in CIA animals as compared to

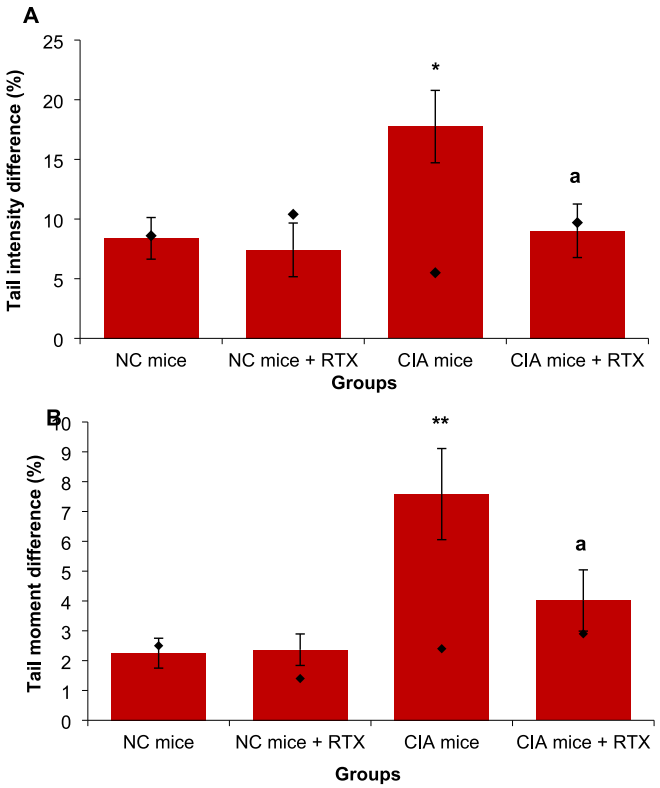


**Fig. 5.** The administration of rituximab (RTX) reduced the elevated levels of oxidative DNA strand breaks (tail intensity differences and tail moment differences) evident in the bone marrow cells of the collagen-induced arthritis (CIA) mice model (n = 6; mean ± SD). The bone marrow cells were smeared on comet gel and exposed to either the Fpg enzyme for 30 min (A and B) or the Endo III enzyme for 45 min (C and D). The difference is the difference between the strand breaks observed in the presence of the repair enzyme (oxidized bases + endogenous DNA strand breaks) and the strand breaks observed in the absence of the repair enzyme (endogenous DNA strand breaks only). *: $p < 0.05$; **: $p < 0.01$ *versus* NC control mice; a: $p < 0.05$; b: $p < 0.01$ *versus* untreated CIA mice (Kruskal-Wallis Test); ##: $p < 0.01$ *versus* NC control mice (Mann-Whitney *U*-Test); NC mice: naïve control DBA/1J mice.

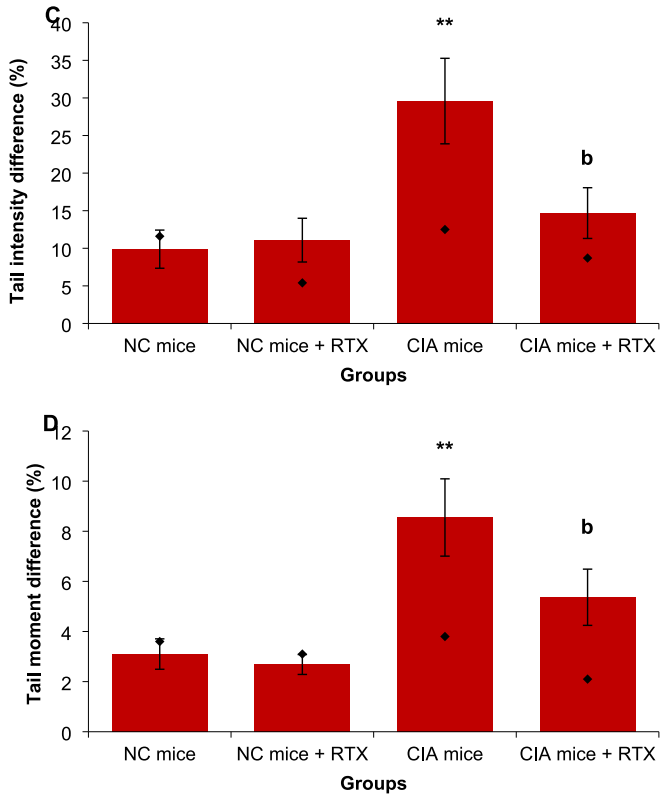


**Fig. 5** (*continued*)

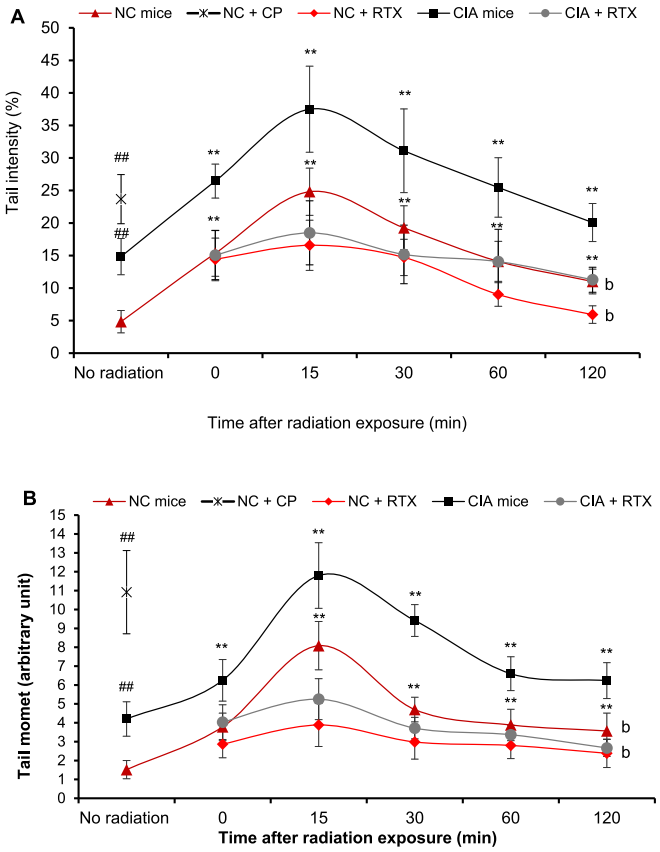


**Fig. 6.** Collagen-induced arthritis (CIA) increased the impaired DNA repair capacity, and the rituximab (RTX) treatment recovered it; tail intensity (A) and tail moment (B) (mean ± SD; n = 6). **: $p < 0.01$ *versus* NC control mice; b: $p < 0.01$ *versus* untreated CIA mice (Kruskal-Wallis Test); ##: $p < 0.01$ *versus* NC control mice (Mann-Whitney *U*-Test); NC mice: naïve control DBA/1J mice.

untreated CIA animals. Also seen in irradiated naïve control DBA/IJ bone marrow cells was a comparable impact, indicating that RTX improves the repair of DNA damaged by radiation in both cases.

### 3.5. RTX reduces redox imbalance in mouse models of CIA

The quantification of cellular oxidants and antioxidants balance was achieved by measuring ROS generation and GSH levels in bone marrow cells. Fig. 7A demonstrates that the production of ROS in the DBA/IJ naïve animals did not exhibit any notable disparity following treatment with RTX, compared to the naïve DBA/IJ control mice that did not receive any treatment. The CIA mice showed a 3.5-fold rise in ROS generation compared to the naïve DBA/IJ control mice ($p < 0.01$). In contrast, the administration of RTX substantially reduced the production of ROS induced by arthritis, resulting in levels much lower than those seen in CIA animals that did not get therapy ($p < 0.05$). No significant difference was seen in the GSH level between the naïve DBA/IJ mice treated with RTX and the naïve DBA/IJ control animals (Fig. 7B). In contrast, the GSH level was lower in the CIA animals compared to the naïve DBA/IJ control mice ($p < 0.01$). However, when RTX was administered to CIA mice, there was a statistically significant rise in GSH concentration to the control level. Furthermore, CIA mice treated with RTX showed a substantial increase in GSH levels compared to untreated CIA animals ($p < 0.01$).

### 3.6. RTX reduces disturbances of DNA damage and repair gene expression in CIA animals

Knee tissues were analyzed using a PCR Array to evaluate changes in the relative gene expression of 84 genes involved in DNA damage and repair. Table 1 provides a summary of the notable alterations in gene expression compared to the untreated naïve control DBA/IJ animals. The hierarchical clustering approach presents heat maps illustrating the fold-change patterns among groupings (Fig. 8). CIA animals exhibited differential expression of thirteen genes, with a *P* value less than 0.05, compared to untreated naïve control DBA/IJ animals. Among the genes examined, seven (*Bax, Cdc25a, Msh2, Msh3, Ogg1, Parp1*, and *Parp2*) showed a substantial increase in expression, whereas six (*Gadd45a, Mre11a, Rad17, Rad50, Trp53,* and *Xrcc1*) showed a significant decrease in expression in CIA animals compared to their expression in untreated naïve control DBA/IJ animals. Administration of RTX in CIA animals effectively corrected the observed alterations in expression, therefore restoring them to their normal level, as compared to untreated CIA animals. Notably, *Rad1* was shown to be upregulated in CIA animals treated with RTX.

### 3.7. RTX reduces disturbances of DNA damage and repair proteins in CIA animals

In order to evaluate the relative protein levels of specific genes identified by the $RT^2$ Profiler PCR Array analysis as being expressed at statistically significant levels in knee tissues of treated and untreated mice, Western blotting analysis was performed. Fig. 9A shows illustrative electrophoretic bands for the protein ladder (marker), protein under investigation and the corresponding β-actin electrophoretic bands. The densitometric analysis results in Fig. 9B demonstrate no notable differences in the levels of the proteins under investigation between the mice treated with RTX and the untreated naïve control DBA/IJ group. Significantly greater levels of Ogg1 and Parp1 expressions were seen in CIA animals compared to naïve control DBA/IJ mice ($p < 0.01$). The data also

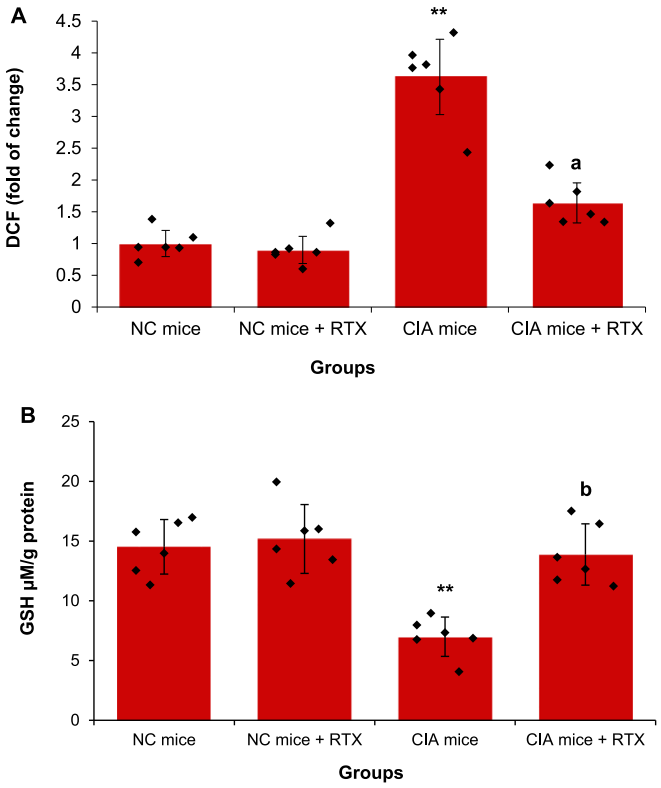


**Fig. 7.** Investigation of the impact of rituximab (RTX) on the generation of reactive oxygen species (ROS, A) and the reduced glutathione (GSH, B) levels in an animal model of collagen-induced arthritis (CIA). ROS production was evaluated by quantifying the fluorescence intensity of 2′,7′-dichlorofluorescein (DCF) (mean ± SD; n = 6). **: $p < 0.01$ *versus* NC control mice; a: $p < 0.05$; b: $p < 0.01$ *versus* untreated CIA mice (one-way ANOVA); NC mice: naïve control DBA/1J mice.

revealed a notable reduction in the protein concentrations of Gadd45a, P53, Rad50, and Xrcc1 in the CIA animals compared to the control DBA/IJ mice ($p < 0.01$). In mice treated with CIA, RTX efficiently reversed inappropriate expressions of these genes.

## 4. Discussion

In RA, the prevalence of cancer is comparatively higher than those in the general population [6,7,9]. The etiology of this vulnerability to cancer remains uncertain, whether it is attributed to the RA itself or therapy with DMARDs. DNA damage is a characteristic feature of human cancer and is responsible for causing instability in the genome. To further understand the DNA damage and repair alterations that occur in RA and determine if the biological DMARD RTX can impact these changes, we aimed to examine the genomic instability in the arthritic mouse model, explicitly focusing on DNA damage and repair. The current investigation revealed a markedly increased level of spontaneous DNA damage in arthritic animals compared to the control naïve DBA/1J mice. A distinct disparity was seen between naïve control DBA/1J and arthritic mice following radiation exposure. The arthritic animals exhibited higher levels of DNA strand breaks and a slower repair rate than the naïve control DBA/1J mice. Moreover, there was an apparent increase in redox imbalance, oxidative DNA strand breakage, and disruption of DNA repair pathways.

It was shown that RTX did not cause disturbances in genomic stability. Meanwhile, RTX decreased the incidence of spontaneous DNA strand breaks and expedited the process of DNA repair, enhancing redox balancing in animals with arthritis. Furthermore, RTX therapy effectively restored the impaired DNA repair mechanisms in mice with arthritis. These data indicate that RTX is a non-genotoxic agent and an effective treatment for RA by reducing inflammation severity, decreasing oxidative DNA damage, and restoring gene expression in the DNA system. Additionally, the protective effect of RTX has also been shown by its ability to reduce the histological alterations caused by arthritis, indicating that RTX is a safe therapeutic agent for the treatment of RA and its complications.

The spontaneous DNA strand breaks in RTX-treated and untreated CIA animals were assessed using the standard comet assay. Naïve DBA/IJ mice exposed to the positive control DNA-damaging compound cyclophosphamide exhibited a higher DNA strand breakage frequency than untreated naïve DBA/IJ controls. These data are in line with the cytogenetic studies conducted on mouse bone marrow [33], which have confirmed the sensitivity of the comet assay. Moreover, the observed spontaneous DNA strand breaks in CIA mice were statistically greater than those in naïve DBA/IJ control animals. Conversely, the treatment with RTX substantially decreased the extent of DNA strand breaks in CIA animals compared to those that were not treated. This observation is consistent with the previous research that reported higher levels of spontaneous DNA damage in samples from patients with RA. Specifically, peripheral blood mononuclear cells obtained from patients with RA exhibited increased spontaneous DNA damage compared to healthy subjects [11,12].

**Table 1**
Molecular expression patterns of genes associated with DNA damage and repair in tissues of the knee joint.

| Gene symbol and description | | Gene Bank | Fold change[a] | | |
|---|---|---|---|---|---|
| | | | NC mice + RTX | CIA mice | CIA mice + RTX |
| *Bax* | Bcl2-associated X protein | PPM02917E | NS | +5.78 | +2.36 |
| *Cdc25c* | Cell division cycle 25 homolog C (*S. pombe*) | PPM04967F | NS | +11.2 | NS |
| *Gadd45a* | Growth arrest and DNA-damage-inducible 45 alpha | PPM02927C | NS | –0.40 | NS |
| *Mre11a* | Meiotic recombination 11 homolog A (*S. cerevisiae*) | PPM03445C | NS | –0.41 | NS |
| *Msh2* | MutS homolog 2 (*E. coli*) | PPM04993F | NS | +4.84 | NS |
| *Msh3* | MutS homolog 3 (*E. coli*) | PPM04961A | NS | +6.27 | NS |
| *Ogg1* | 8-oxoguanine DNA-glycosylase 1 | PPM05282A | NS | +10.52 | NS |
| *Parp1* | Poly (ADP-ribose) polymerase family, 1 | PPM05150C | NS | +13.56 | NS |
| *Parp2* | Poly (ADP-ribose) polymerase family, 2 | PPM05429B | NS | +7.54 | NS |
| *Rad1* | RAD1 homolog (*S. pombe*) | PPM03136A | NS | NS | +12.5 |
| *Rad17* | RAD17 homolog (*S. pombe*) | PPM03276F | NS | –0.13 | NS |
| *Rad50* | RAD50 homolog (*S. cerevisiae*) | PPM03278C | NS | –0.37 | NS |
| *Trp53* | Transformation-related protein 53 | PPM02931C | NS | –0.22 | NS |
| *Xrcc1* | X-ray repair complementing defective repair in Chinese hamster cells 1 | PPM04396A | NS | –0.23 | NS |

[a] The values of fold change were obtained for each evaluated gene using the method of $2^{-\Delta HC}T$ method. The expression levels of these genes were markedly decreased (–) or increased (+) by more than 1.5 times relative to the NC mice. All genes included in the table exhibited statistically significant variations (Student's *t*-test, $p < 0.05$) *versus* untreated NC mice; RTX: rituximab; NS: not significant; NC mice: naïve control DBA/1J animals; CIA mice: collagen-induced arthritis animals.

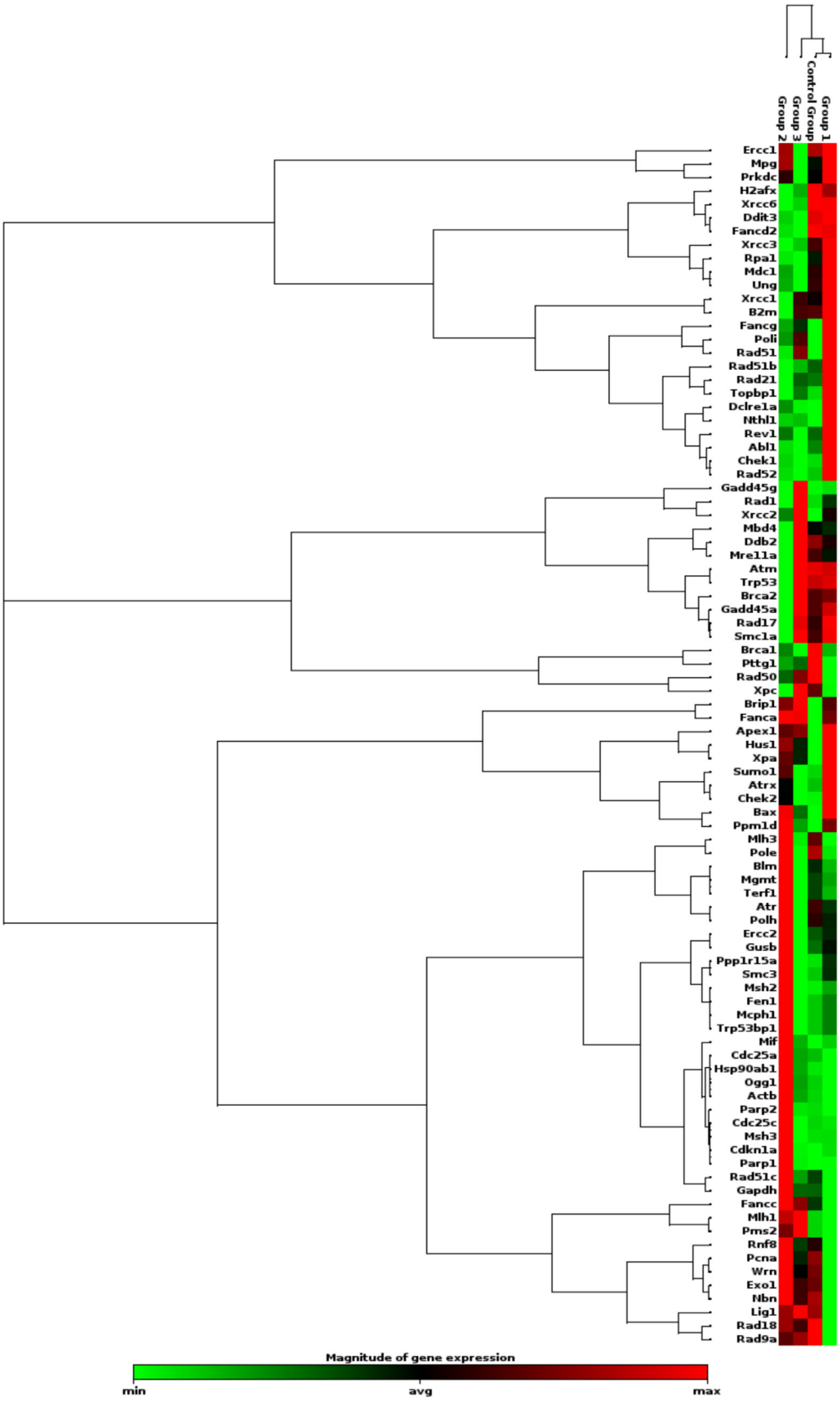


**Fig. 8.** Hierarchical clustering of DNA damage and repair gene expression as recognized by $RT^2$ Profiler PCR Array in the knee joints. A dendrogram quantifies the degree of overlap in gene expression within each group. Group 1: NC mice received RTX; Group 2: collagen-induced arthritis (CIA) mice; Group 3: CIA animals received RTX, together with control NC mice. RTX: rituximab; NC mice: naïve control DBA/1J animals; CIA mice: collagen-induced arthritis animals.

Insufficient DNA repair capability and heightened vulnerability to damaging agents may be reflected in these DNA strand breaks. Numerous investigations have examined the responsiveness of cells from patients with RA to DNA-damaging agents, such as radiation, to provide predictive insights to individuals. In patients with RA, blood cells are more vulnerable to DNA damage induced by radiation [11,34]. In order to assess the DNA repair capacity in CIA animals, bone marrow cells were cultured in RPMI media and exposed to radiation. The rates of DNA damage and repair were then assessed by conducting sequential comet experiments at different times after radiation. Evidence indicated that the bone marrow cells from CIA animals exhibited heightened sensitivity to radiation damage and a notably slower rate of repair compared to cells from naïve DBA/IJ control animals. This indicates that CIA animals are more susceptible to radiation damage and lack efficient DNA repair mechanisms. The heightened vulnerability observed aligns with the proposed impacts of particular genetic variations on DNA repair and the aberrant control of DNA repair genes characterized by RA [35,36].

Notably, the administration of RTX improved the rate at which radiation-induced DNA strand breaks were repaired in the CIA model and naïve control animals. This indicates that RTX increases the repair of fragmented DNA caused by radiation and that cell status does not influence this effect. Overcoming oxidative stress-induced oxidative damage in mammals involves utilizing at least two defensive pathways: mechanisms for DNA repair and antioxidant defenses. Previous investigations demonstrated that administering biologic therapies that target TNF-α decreases the elevated oxidative stress in patients with RA [37,38]. Furthermore, our earlier investigation demonstrated that RTX has antigenotoxic properties against micronuclei induction and chromosomal damage caused by arthritis. These effects are linked to elevated levels of antioxidants in treated arthritic mice [20].

The higher rates of DNA strand breakage observed in CIA animals are almost certainly caused by the imbalance between oxidants and antioxidants that arises from chronic inflammation. Pathophysiological events of inflammation and oxidative stress are intricately interconnected, with one being readily triggered by the other and both being associated with cancer development. Many researchers have proposed that oxidative stress plays a role in the development of RA lesions. Highly increased formation of ROS and reduced levels of scavenging free radical molecules were observed in arthritic patients compared to healthy people [39,40]. An elevated redox imbalance has also been observed in animal models of arthritis [41]. The present work revealed elevated levels of spontaneous oxidative DNA strand breaks in CIA animals using a modified comet assay utilizing the Endo III and Fpg enzymes. This indicates a higher production of oxidants or a reduced antioxidant ability, consistent with previous research findings in arthritis [39,40,41]. Furthermore, RTX reduced the levels of oxidative DNA strand breaks compared to untreated CIA animals. This effect may be ascribed to RTX's ability to decrease inflammatory stress on DNA molecules. Thus, RTX may beneficially affect the defense against DNA strand breaks caused by arthritis by lowering inflammatory stress and recovering oxidant-antioxidant balance. The results presented here align with the published findings on the development of oxidative stress in individuals with arthritis, which is diminished after the administration of biological DMARDs [37,38]. Consistent with our previous research on germ cells, the current finding indicates that RTX defends the DNA molecules in arthritic mice by augmenting its existing antioxidant system and decreasing ROS generation.

Genomic repair and immunological response are intricately linked processes. Their collaboration is to address several adverse stresses and carry out certain physiological functions that necessitate intentional DNA damage and reorganization [42]. The preservation of genomic stability after DNA damage relies on the DNA repair mechanisms, which contribute to the maintenance of ordinary immune system functioning. Variations in the activity of DNA repair genes have been linked to the development of several autoimmune and inflammatory disorders, including RA [2]. Furthermore, mutations in these genes are believed to contribute to tumorigenesis by increasing the incidence of DNA strand breaks, as documented in various investigations [43]. The observed modifications could be associated with variations in the functionality of the encoded protein molecules, resulting in a disordered repair mechanism after DNA damage caused by either internal or external factors.

Gene expression research has shown that various genes associated with DNA repair are significantly altered in arthritic patients compared to healthy persons [2,35,36,44], indicating that oxidative stress and impaired DNA repair mechanisms are involved in the pathomechanisms of RA. Using PCR Array, we conducted gene expression profiling of knee tissues in CIA mice. This analysis showed a significant disruption in the expressions of many genes associated with DNA damage and repair. However, RTX treatment

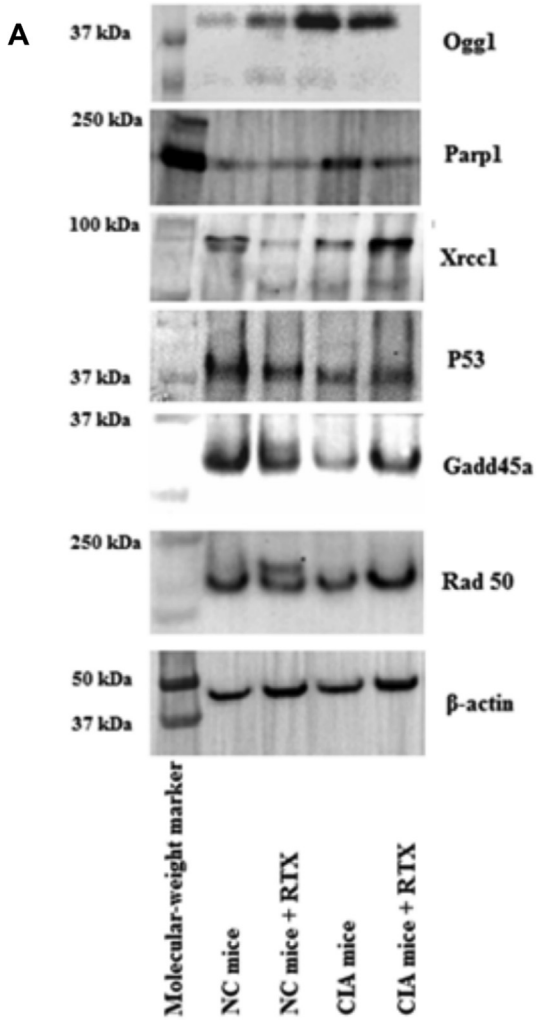


**Fig. 9. Investigating DNA damage and repair-related proteins in knee tissues using Western blot analysis**. (A) Representative spectra of the bands for the protein ladder (marker), each protein under investigation, and the control group with β-actin loading. (B) Differences in expression levels of the chosen proteins associated with DNA damage and repair (mean ± SD; n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* NC control mice and a: $p < 0.05$, b: $p < 0.01$ *versus* untreated CIA mice (one-way ANOVA). NC mice: naïve control DBA/1J mice.); RTX: rituximab; NC mice: naïve control DBA/1J animals; CIA mice: collagen-induced arthritis animals.

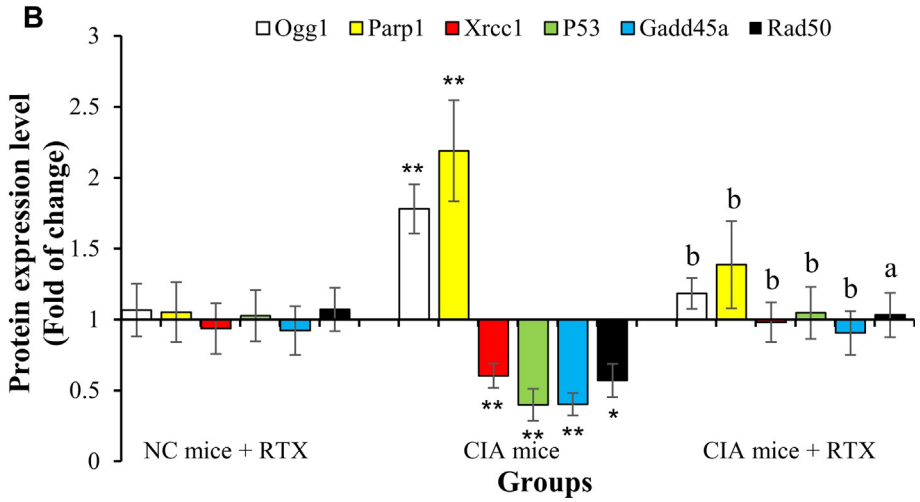


**Fig. 9** (*continued*)

restored these expressions. The genes *Bax, Cdc25a, Msh2, Msh3, Ogg1, Parp1*, and *Parp2* showed considerable up-regulation in CIA mice compared to the corresponding levels in naïve control DBA/IJ animals. Conversely, in CIA mice, *Gadd45a, Mre11a, Rad17, Rad50, Trp53*, and *Xrcc1* were considerably downregulated.

The aberrant expression of these DNA repair genes provides more proof that an impaired DNA repair function is accountable for the accumulation of DNA damage detected in CIA animals, which aligns with previous research demonstrating altered expression of the repair mechanism in RA [2,35,36]. In contrast, the administration of RTX effectively restored the altered DNA repair mechanism in the CIA animals by accurately recovering the mRNA expression of *Bax, Cdc25a, Msh2, Msh3, Ogg1, Parp1, Parp2, Gadd45a, Mre11a, Rad17, Rad50, Trp53,* and *Xrcc1* to their normal levels. Thus, RTX could protect DNA by recovering the expression of altered genes implicated in DNA repair. A Western blot analysis was conducted to evaluate the degree of correlation between the mRNA transcription and protein translation of certain changed genes. CIA mice exhibited markedly increased expressions of Ogg1 and Parp1 compared to naïve control DBA/IJ mice. Additionally, the CIA mice showed considerable downregulation of Gadd45a, P53, Rad50, and Xrcc1 compared to the naïve control DBA/IJ animals. These results validate the previously stated dysregulations in the DNA repair pathway [2,35,36]. Nevertheless, the administration of RTX effectively restored these alterations, as evidenced by the $RT^2$ PCR Array results. Consequently, the altered genes were transformed into their respective proteins, and RTX protected DNA strand breakage by recovering the impaired DNA repair mechanism.

## 5. Conclusions

Administering RTX with arthritis development markedly decreased the clinical symptoms and histological alterations in mice with arthritis. Treatment with RTX reduced the occurrence of oxidative DNA strand breaks and accelerated the rate at which they were repaired in arthritic animals, maybe by maintaining the redox balance and recovery of disrupted DNA repair processes. The results emphasize that RTX is a non-genotoxic biological agent and a safe treatment for patients with RA. The current work also addresses the role of genomic instability in the pathology of RA and offers intriguing insights for the use of effective and safe treatments to reduce the increased risks of genomic instability in RA patients.

## CRediT authorship contribution statement

**Mohammed A. Al-Hamamah:** Writing – review & editing, Writing – original draft, Resources, Methodology, Data curation, Conceptualization. **Mohamed A. Mahmoud:** Visualization, Validation, Methodology. **Moureq R. Alotaibi:** Supervision, Funding acquisition, Formal analysis, Data curation. **Ahmed Nadeem:** Validation, Software, Resources, Data curation. **Mushtaq A. Ansari:** Validation, Resources, Methodology. **Sheikh F. Ahmad:** Validation, Resources, Investigation, Data curation. **Saleh A. Bakheet:** Visualization, Supervision, Data curation. **Gamaleldin I. Harisa:** Resources, Project administration, Formal analysis, Data curation. **Sabry M. Attia:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

## Financial support

This work was supported by the Ongoing Research Funding Program (ORF-2025-748), King Saud University, Riyadh, Saudi Arabia.

## Declaration of competing interest

None.

## Acknowledgments

The authors acknowledge the language editing assistance to the Investigator Support Unit at the Prince Naif Health Research Center, Saudi Arabia.

## Supplementary material

https://doi.org/10.1016/j.ejbt.2025.03.004.

## Data availability

Data will be made available on request.

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