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Reduction of Some Extra-Articular Complications Associated with Arthritis Development in Rats by Low Dose γ-Irradiation

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ABSTRACT

Rheumatoid arthritis (RA) is considered a systemic inflammatory disease that affects not only the joints, but also other organs in the body causing many extra-articular complications such as kidney and liver impairments as well as hematological disturbances. Low-dose radiation (LDR) modulates a variety of immune responses that have exhibited the properties of immune hormesis. LDR has been used clinically for the treatment of autoimmune diseases. Several molecular mechanisms and cellular components contribute to the clinical efficacy of low dose radiotherapy (LD-RT). The present study aims at evaluating the possible capability of low dose γ-irradiation to reduce some of these extra-articular complications in adjuvant-induced arthritis model in rats. The current results revealed a significant increase in liver parameters in serum including ALT, AST activities as well as creatinine level in adjuvant induced arthritic rats that indicate liver and kidney impairments respectively. The results also revealed a sharp increase in total leukocytic count (TLC). All of these biochemical and hematological disturbances were found to be significantly improved after exposure to low dose γ-irradiation.

Keywords: Rheumatoid arthritis, extra-articular complications, adjuvant induced arthritis, low dose γ-irradiation

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects the physical and psychosocial wellbeing of patients and is a major cause of work disability [1]. It affects principally the joints and is usually accompanied by one or more of extra-articular manifestations [2]. Rheumatoid arthritis has several potentially life-threatening extra-articular complications. It is often associated with inflammation in other organs and tissues [3, 4]. Comorbid diseases are the medical conditions associated with RA. The mechanisms of this association may be due to the pathogenesis of RA itself, the effects of medications used for treating RA, or only a coincidence. The continuous systemic inflammation and immune dysfunction characteristic for RA plays a critical role in the development and acceleration of comorbidities [5, 6]. Low-dose radiation (LDR) modulates a variety of immune responses that have exhibited the properties of immune hormesis. LDR has been

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used clinically for the treatment of autoimmune diseases [7]. Several molecular mechanisms and cellular components contribute to the clinical efficacy of low dose radiotherapy (LD-RT). A large body of evidence indicated that low-dose (single dose ≤1.0Gy) irradiation predominantly induced anti-inflammatory activation of macrophages [8, 9]. Despite the known efficacy of low dose radiotherapy in RA treament, irradiation regimens are still not well established. Also, the role of ionizing radiation in treatment of comorbid diseases associated with RA is still unknown. Therefore, further research efforts using a new irradiation protocol are needed to elucidate this role. Rat adjuvant arthritis is an experimental model of polyarthritis which has been widely used for preclinical testing of numerous anti-arthritic agents which are either under preclinical or clinical investigation or are currently used as therapeutics in this disease [10].

2. Materials and Methods

2.1. Experimental animals

Forty female Wister albino rats with an initial weight of 180±20 g were used for the present study. Animals were obtained from the animal breeding unit of the National Center for Radiation Research and Technology (NCRRT), The Atomic Energy Authority, Cairo, Egypt. They were maintained under appropriate conditions of a good ventilation, atmospheric temperature (22 ± 4˚C), average humidity (50 ± 4%) and a 12/12h light/dark cycles along the experimental period. The animals were allowed free access to water and were fed a standard rodent pellet diet, containing all necessary nutritive elements. Rats were kept for about one week before the start of the experiment to acclimatize laboratory conditions. All animal procedures adopted in this study were in accordance with the recommendations for the proper care and use of laboratory animals approved by the Ethics Committee of the NCRRT, Cairo, Egypt.

2.2. Adjuvant induced arthritis (AIA) model

According to the method described by Holoshitz et al. [11], rats were injected subcutaneously with a single dose of 0.1ml of complete Freund's adjuvant (CFA) (supplied through Sigma-Aldrich, USA) containing 100 µg dry heat-killed Mycobacterium tuberculosis (H37Ra) suspended in mineral oil (Paraffin) into the dorsal root at the base of the tail. The day of adjuvant injection was referred to as day zero.

2.3. Irradiation

Whole body gamma irradiation of experimental rats was performed at theNCRRT ,Cairo, Egypt, using the Canadian gamma cell-40 (caesium-137 irradiation unit). Rats were exposed to γ-radiation with a total dose 1Gy which was delivered as fractionated doses 0.25Gy per week for 4 weeks. The dose rate was 0.44Gy/min calculated according to the dosimeter department in the NCRRT at the time of the experiment.

2.4. Experimental design

Forty female wistar albino rats (180±20 g) were equally divided into four main groups of 10 rats each as follows:

A- Adjuvant free groups:

- Normal Control group (NC): Rats daily received 1ml of distilled water via oral tube till the end of the experiment.
- Irradiated group (IRR): Rats were whole body exposed to a fractionated dose of γ-radiation at a dose level of 0.25Gy, four times at the 15th, 23th, 30th and 37th days of the experiment up to a total dose of 1Gy and received 1ml of distilled water via oral gavage for 30 consecutive days starting from the 15th day of the experiment

B- Adjuvant induced groups:

- Arthritic group (AR): Rats were injected subcutaneously with a single dose of 0.1ml of complete Freund's adjuvant (CFA) containing 100 µg dry Mycobacterium tuberculosis into the dorsal root of the tail. Also, they orally received 1ml of distilled water for 30 consecutive days starting from the 15th day of the experiment. The day of adjuvant injection is referred to as day zero.
- Arthritic irradiated group (AR+IRR): Rats were inoculated by adjuvant inducer (CFA) as described in group IV. Starting from the 15th day, each arthritic rat orally received 1ml of distilled water for 30 consecutive days and was exposed to a whole body gamma radiation as in group III on the 15th, 23th, 30th and 37th days following CFA inoculation.

At the end of the experiment, after an overnight fast, all experimental rats were anesthetized with diethyl ether and immediately whole blood samples were withdrawn from each rat by heart

puncture. Each blood sample was divided into two parts; the first part was allowed to clot at 37oC for 15 min in a plain tube. The clotted blood was then centrifuged at 4,000 rpm for 10 min. The separated serum was used for the assessment of alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and creatinine levels while the other part of blood sample was taken on a tube contains di-sodium ethylenediaminetetraacetic acid (EDTA) for total leukocytic count (TLC).

2.5. Biochemical measurements

- Assessment of liver enzymes

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed in serum by the kinetic method using a commercial kit (Linear, Spain) according to the method described by Winn-Deen et al. [12].

- Kidney function test

The concentration of creatinine was determined by the kinetic colorimetric method using available commercial kit (Linear, Spain) according to the method described by Bartels and Böhmer [13].

- Assessment of inflammatory responses

Total leukocytic count (TLC) was performed using the hemocytometer according to the method described by Turgeon [14].

2.6. Statistical analysis

Statistical analysis of results including the mean, standard error (SE) and p values was performed using GraphPad prism (version 5.01) for windows. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test for comparison means of different groups. The data were expressed as mean ± standard error. Differences were considered statistically significant at $P \leq 0.05$, highly significant at $P \leq 0.01$ and very highly significant at $P \leq 0.001$.

3. RESULTS

3.1 Liver function tests

Alanine aminotransferase (ALT) and Aspartate Aminotransferase (AST)

The results are illustrated in Figures (1&2). The experimental data obtained from the control group revealed that the mean value of serum ALT and AST activities were 25.43 ± 0.96 U/L and 44.18 ± 2.69 U/L, respectively. The results obtained in the present study revealed that subcutaneous injection of CFA into the dorsal root at the base of tail of the experimental rats produced a significant increase ($P \leq 0.001$) in the ALT & AST activities by 68.62% and 63.81%, respectively in comparison with the control group. On the other hand, the whole body γ-irradiation of the experimental arthritic rats with 0.25 Gy/Week for four weeks revealed a significant decrease ($P \leq 0.001$) in serum ALT & AST activities by 31.44% and 34.66%, respectively in comparison with arthritic untreated group.

3.2. Creatinine level

The results are graphically illustrated in Figure (3). The experimental data obtained from the control group revealed that the mean value of serum creatinine concentration was 0.44± 0.03 mg/dl. The results for healthy rats exposed to whole body γ-radiation (0.25 Gy/Week for four weeks) revealed no significant changes in serum creatinine concentration compared to the control group value. Data revealed that subcutaneous injection of CFA into the dorsal root of tail of the experimental rats induced renal toxicity as evidenced by the significant sharp increase ($P \leq 0.001$) in serum creatinine level by 72.73%, in comparison with control values. Treatment of the experimental arthritic rats with low dose γ-irradiation revealed a significant decrease at ($P \leq 0.01$) in the elevated serum creatinine concentration by 26.32% in comparison with arthritic untreated group.

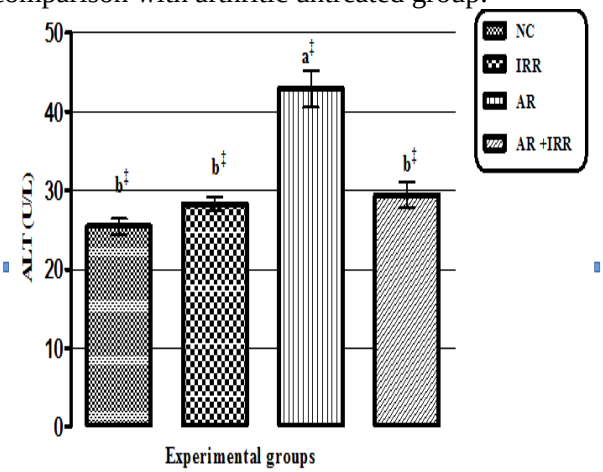


Fig. (1): ALT activities in serum of different animal groups. a‡, b‡ denote significant change at ($P \leq 0.001$) versus control and arthritic groups, respectively

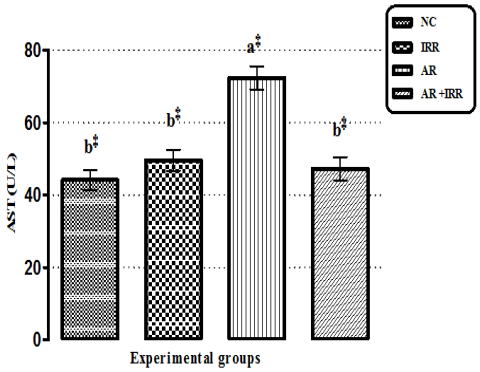


Fig. (2): AST activities in serum of different animal groups. a‡, b‡ denote significant change at (P ≤ 0.001) versus control and arthritic groups, respectively

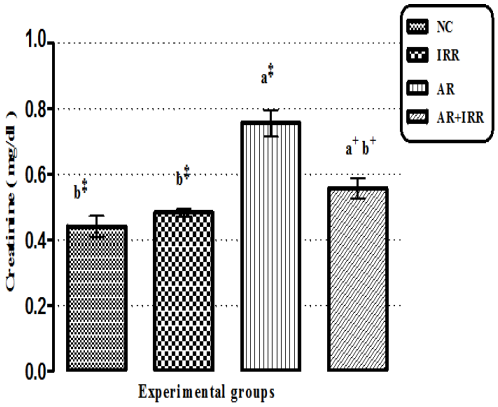
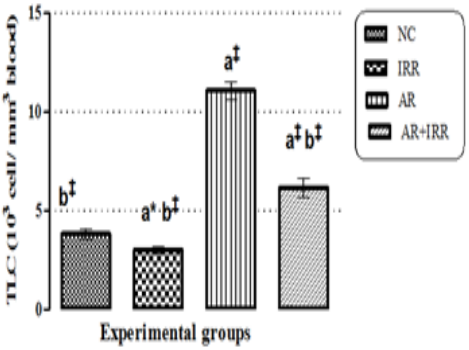


Fig. (3): Creatinine levels in serum of different animal groups. a+, b+ denote significant change at (P≤ 0.01) versus control and arthritic groups, respectively. a‡, b‡ denote significant change at (P≤0.001) versus control and arthritic groups, respectively

3.2.Inflammatory responses

Total leukocytic count (TLC) is illustrated in Figure (4) . The experimental data obtained from the normal control (NC) group showed that the mean value of TLC was 3.81± 0.25 (10^3 cell/mm^3 blood). The whole body γ-irradiation of healthy rats with 0.25 Gy/Week for four weeks produced a slightly significant decrease (P≤0.05) in TLC by **20.73** % in comparison with the normal control values. The results obtained in the present study revealed that subcutaneous injection of CFA into the dorsal root at the base of tail of experimental rats produced a significantly sharp increase (P≤0.001) in TLC by 190.81% in comparison with the normal control. On the other hand, the whole body γ-irradiation of the experimental arthritic rats with 0.25 Gy/Week for four weeks staring from day 15 after CFA inoculation revealed a highly significant reduction (P≤0.001) of elevated TLC by **44.40**% compared to the arthritic group.



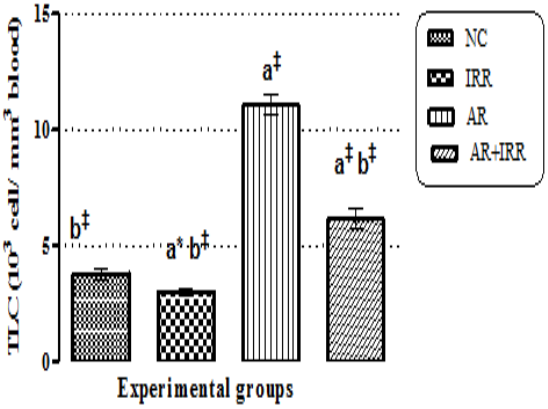


Fig. (4): Total leukocytic count in different animal groups. **a*:** significant against normal control at (P ≤ 0.05), **a‡:** very highly significant against normal control at (P ≤ 0.001), **b‡:** very highly significant against arthritic group at (P ≤ 0.001)

4. DISCUSSION

Since RA is an autoimmune disease, it can affect any part of the body and can contribute to the development of a whole plethora of life threating conditions **[15].** Adjuvant-induced arthritis (AIA) in rats is considered a model of chronic inflammation that is similar to RA in human. This model mirrors much of the pathology of RA; including hyperplasia of the synovial tissues, inflammatory infiltration of the joints, and destruction of bone and cartilage in the synovial joint [16, 17].

To study the systemic effects of RA on liver, the activity of liver enzymes in serum was assayed. In the current study, subcutaneous injection of CFA into rats induced a marked rise in liver marker enzymes, a finding that is in harmony with previous studies of Kshirsagar *et al.* [18] and Mbiantcha *et al.* [19] that reported, a significant rise in the serum activities of AST and ALT in the arthritic control group after injection of CFA into rats compared to normal control rats. Also, Banji *et al.* [20**]** demonstrated that liver impairment is a typical feature in adjuvant arthritis when they assessed liver injury in adjuvant induced arthritis by ascertaining the serum activities of ALT and AST. The marked elevation in the serum activities of ALT and AST in arthritic rats might be due to injury of the architecture of hepatic cells resulting in leaching of these enzymes from the damaged cells of the liver into the circulation [20]. This injury of hepatic cells might be attributed to free radical-mediated lipid peroxidation of liver cell membrane. Free radicals were found to be produced in large quantities at the site of inflammation [21]. Similarly, CFA injection into rats causes a significant release of these reactive oxgen species [22]. On the other hand, the present results results revealed that exposure of arthritic rats to a low dose of γ-radiation reduced the elevated activities of ALT and AST and resulting in a significant improvement as compared to arthritic untreated rats. This data is in accordance with a pervious study of Rashed *et al.* [23] that reported a significant reduction in the elevated serum activities of ALT and AST after being exposed to a low dose of γ-irradiation. The current data also agree with Moustafa *et al.* [24] who reported that a low dose of radiation (LDR) possess hepatoprotective activity and restored tissue vitality in liver induced damage model in rats. Moreover, a low-dose of γ-irradiation was found to induce an endogenous antioxidant defense in the liver which could be beneficial in protecting the liver cells from oxidative stress [25, 23].

To determine the systemic effects of RA as well as the possible effects of the proposed treatment agent on the kidney, serum creatinine level was determined in the present study since it is the most commonly used parameter for renal excretory function [26]. The present results showed that subcutaneous injection of CFA into rats induced renal toxicity as evidenced by a significant sharp increase in serum creatinine level as compared to normal control values. These results agree with Ekambaram *et al.* [27] who found an increase in serum creatinine levels in adjuvant induced arthritic rats when compared with the normal control group which indicates the kidney dysfunction in arthritic rats. Similarly, Ramadan and EL-menshawy [28] also reported that serum creatinine levels were significantly increased in the adjuvant induced arthritis group compared to the control group. Addionally, Filho *et al.* [29**]** also reported an alteration and impairment in kidney function in arthritic rats. Under normal condition, oxidative stress is just kept at low level and unharmful to the kidney **[30].** CFA injection into rat causes a significant release of ROS as mentioned by Cascão *et al.* [22]. Accordingly, the increase in creatinine level in arthritic rats might be attributed to an increase of renal oxidative stress due to up-regulation of ROS production and down-regulation of the anti-oxidants expressions [31].

On the other hand, the present results showed that treatment of arthritic rats with a low dose of γ-irradiation revealed a significant decrease in the the elevated serum creatinine concentration in comparison with arthritic untreated group. However, the exact mechanism, by which a low dose of γ-irradiation causes this reduction in the elevated creatinine concentration in adjuvant induced arthritic rat model, is still unknown, it has been reported by Aunapuu *et al.* [32] that **a** low dose of irradiation causes a reduction in the elevated serum creatinine levels in renal injury model in rats. Furthermore, it was found that LDR significantly prevents renal damage and dysfunction in diabetic rats through attenuation of inflammation and oxidative stress by activation of the Akt signaling pathway and up-regulation of multiple renal antioxidant levels that mediated by nuclear factor E2-related factor-2 (Nrf-2) expression in the kidney [33, 34, 30]. The latter is a key transcription factor that regulates intracellular redox balance and is a sensor of oxidative stress and it positively regulates the expression of several downstream genes (SOD-1, NQO-1 and HO-1) playing an important role in the prevention of oxidative stress and damage [35, 30]. These genes are up-regulated through the antioxidant response regulatory element in response to oxidative stress [36].

Regarding inflammatory responses, leukocytes (white blood cells) are the major cellular elements of the immune system that comprise different lineages of lymphocytes (B and T cells) as members of an antigen-specific effector response, as well as polymorphonuclear (PMN) and mononuclear cells (MC) as components of the innate immune system [37]. The present results showed that subcutaneous injection of CFA into rats produced a significant sharp increase in total leukocytic count (TLC) as compared to normal control. This finding is in accordance with pervious observations reported by Ekambaram *et al.* [27] who found changes in many hematological parameters in CFA-induced arthritic rats such as sharp increase in white blood cells (WBC) count when compared with the control rats. Additionally, Glenn *et al.* [38] reported leukocytosis (increase in the number of leukocytes) on day 21 post-induction of arthritis. More to the point, Franch *et al.* [39**]** found leukocytosis with a high percentage of neutrophils (neutrophilia) and a high number of lymphocytes. The authors also observed a marked increase in the number of both CD4+ and CD8+ T-cells in arthritic rats, but the rise in CD8+ T-cells was more pronounced than the increase in CD4+ T-cells. They suggested that these fluctuations in white blood cells are probably secondary to the inflammatory process present in adjuvant arthritis. The significant increase in TLC in adjuvant-induced arthritic rats in the present investigation might be attributed to an increase in CD8+ (T-cells) subset that carry specific receptors which recognise mycobacterial determinants present in CFA preparation as suggested by Raulet [40] and Born *et al.* [41]. Also, the increase in CD4+ (T-cells) subset may be another factor for the elevated TLC since the number of CD4+ T-cells has been shown to increase in human RA [42]. Moreover, it has been dmonestrated that the rise in TLC in arthritic condition may be due to release of IL-1β inflammatory response which increases the production of both granulocyte and macrophages colony stimulating factor [43].

On the other hand, exposure of arthritic rats to a low dose of γ-radiation caused a highly significant reduction in the elevated TLC as compared to arthritic untreated group. This finding agrees with Bogdándi *et al.* [44**]** who reported that a low dose of irradiation caused quantitative alterations in the numbers of leukocytes. Additionally, Tsukimoto *et al.* [45**]** found a reduction of CD8+ (T-cells) subset after repeated irradiation with 0.5Gy/week for 4 weeks in an animal model of autoimmune disease. In rheumatoid arthritis, the inflammatory infiltrate in the synovia consists of activated fibroblasts, monocytes and lymphocytes, which show histochemical signs of dysregulation of apoptosis [46]. Induction of apoptosis or necrosis of these activated inflammatory cells by ionizing irradiation might theoretically be a successful way to control the activity of this destructive disease. In adjuvant induced arthritis, such efficacy has been suggested by Trott *et al.* [47] and Hildebrandt *et al.* [48]. Beside its central role in cellular homeostasis, apoptosis significantly impacts on immune regulation and radiation response. In line with that, Voll *et al.* [49] reported that a low dose radiotherapy produced its anti-infllammatory effect by sending an immunosuppressive signal, which is received by the thrombospondin receptor (CD36) on activated leukocytes (mononuclear cells) resulting in down-regulation of pro-inflammatory cytokines (TNF-α and IL-1) and up-regulation of the anti-inflammatory cytokine IL-10. Kern *et al.* [50] also reported a dose-dependent induction of apoptosis in leukocytes (mononuclear cells) after a low dose radiotherapy at a single dose rate of 0.3 and 0.7Gy and suggested that this response may be a contributing factor to the overall anti-inflammatory effect induced by low doses of radiation. This mechanism has gained much support in many other studies [51, 52, 53, 54]. The current results revealed a slight decrease in TLC after whole body γ-irradiation of healthy rats as compared to the normal control values despite its role in improving other inflammatory and arthritic parameter and improving the disease state in arthritic rats. This observation is in accordance with Park and Lee [55] who reported that using low radiation doses for treatment of inflammatory disorders had minimal side effects on most normal tissues, thus the risks of radiation exposure always have to be weighed against the therapeutic benefits. The authors also mentioned that low dose radiotherapy for these disorders may be safely and effectively used, especially in older patients who are reluctant to use other treatment options. Therefore, this reduction in TLC by irradiation cannot be described as diseased state comparing with its benefits.

5. CONCLUSION

The current investigation demonstrates that a low dose of γ-irradiation at the specified dose level of 0.25Gyx4 has a positive effect upon already-established arthritis in rats. It normalized the haematological and biochemical abnormalities occurred after induction of arthritis and reduce the extra-articular complications occurred in the liver and kidney. Further histopathological, radiological and molecular studies are needed to elucidate in detail precise mechanisms for these outcomes.

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