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Research Article
Stem Cell Mobilization with G-CSF and Cyclophosphamide Ameliorated Collagen-Induced Arthritis in Wistar Rats

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Abstract

Background and Objective: Rheumatoid arthritis is an inflammatory autoimmune disorder which affects multiple joints and causes cartilage erosion. It's treatment depends on the systemic use of anti-inflammatory and immunosuppressive drugs. The aim of this study was to test effect of combination of Granulocyte-Colony Stimulating Factor (G-CSF), cyclophosphamide (CTX) and Bone Marrow (BM) cells on Collagen Induced Arthritis (CIA) in Wistar rats. **Materials and Methods:** Collagen Induced Arthritis (CIA) was induced in Wistar rats by subcutaneous (s.c.) injection of 200 and 100 µL/rat of collagen II at tail base in on days 0 and 12, respectively. Then, rats were treated with either intravenous injection of fresh Bone Marrow (BM) cells (5×10^6) or s.c. injection of G-CSF (5 µg for s.c. for 5 consecutive days) in presence or absence of a single intraperitoneal injection of cyclophosphamide (4 µg/rat). Inflamed paws, x-ray and histopathological examination were assessed during the experiment. Serum levels of IL-4, IL-8 and TNF-α were also measured. **Results:** All treatments decreased inflammation in the paws as reflected by decreased paw thickness in fore and hind limbs and confirmed by x-ray and histological examination. Of note, the anti-CIA effects after treatment with CTX plus G-CSF were higher than of CTX plus BM cells and other treatment modalities (CTX or BM alone). Interestingly, all treatment modalities induced decreases in levels of IL-4, IL-8 and TNF-α. Among them, CTX/G-CSF showed the highest inhibitory effect on TNF-α. **Conclusion:** Treatment of CIA rats with the combination of G-CSF and CTX induces potential anti-arthritic effect. This anti-arthritic effect is through down regulating the elevated levels of IL-4, IL-8 and TNF-α cytokines.

Key words: Bone marrow, collagen, cyclophosphamide, Granulocyte-colony stimulating factor (G-CSF), rheumatoid arthritis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease that affects multiple joints and is characterized by progressive erosions, cartilage destruction, stiffness and synovitis[1]. It is associated with an aberrant joint fibroblast activation that contributes to joint destruction. Current conventional treatments, such as steroids, non-steroidal anti-inflammatory drugs (NSAIDs), glucosamine, hyaluronic acid and steroids for RA focuses on relief of symptoms, rather than improvement of the biochemical micro environment of joints or their regeneration[2]. With this context, regenerative medicine could be a potential means to induce regeneration of injured cartilages[3]. Different approaches of stem cell-based transplantation of Hematopoietic and Mesenchymal Stem Cells (HSCs, MSCs) were used based on the administration of exogenous stem cells[4] rather than focusing on endogenous stem cells.

Bone Marrow (BM) is an important microenvironment involved in migration, selective retainment and function of innate and adaptive immune cells[5]. The BM has anti-inflammatory, angiogenic and immunomodulatory properties that can potentially enhance cartilage repair or improve the disease process, as it is a rich source of HSCs and MSCs, as well as several chemokines and cytokines[6]. Pre-clinical studies demonstrated that bone marrow, adipose, synovial and umbilical cord-derived MSC all suppress the functions of different immune cells raising the possibility of new therapies for autoimmune diseases including RA[4]. As such, finding modalities that depends on HSCs to treat RA is of paramount significance. However, due to the limited number of stem cells in BM, growth factors such as Granulocyte-Colony Stimulating Factor (G-CSF) are used alone or in combination with chemotherapeutic drugs such as; Cyclophosphamide (CTX) to mobilize stem cells in bone marrow[7].

G-CSF induces mobilization of hematopoietic stem/progenitor cells from BM into peripheral blood by causing marked alterations in the hematopoietic stroma in the marrow[8]. Such mobilizing effects of G-CSF are more pronounced when it's administered after treatment with chemotherapeutic drugs in particular cyclophosphamide (CTX)[9]. CTX is an alkylating agent that is one of the most potent immunosuppressive therapies available and has been used extensively to treat severe manifestations of a variety of autoimmune and inflammatory diseases[10]. Similar to some disease modifying anti-rheumatic drugs, CTX appears to have a clinically and statistically significant benefit on the disease activity of patients with RA[11].

Besides its mobilizing effects on stem cells, G-CSF has been reported to express anti-inflammatory effects[12] may be by inducing the expansion of the type 2 plasmacytoid dendritic cells[13] which are known to induce tolerance[14]. For example, in a Mycobacterium tuberculosis-induced joint inflammation model, G-CSF has a protective role in disease severity due to reduced antigen-presenting capacity and subsequent reduction in lymphocyte proliferation and IFN-γ secretion[15].

The aim of this study was to compare the anti-arthritic effects of G-CSF, which induces increases in the numbers of endogenous hematopoietic stem cells and administration of exogenous bone marrow cells rich in hematopoietic stem cells. Given that Collagen-Induced Arthritis (CIA) shares many similarities with human RA[16], this study used this model in Wistar rats to address the aim.

MATERIALS AND METHODS

This study area was conduct at Center of Excellence in Cancer Research (CECR), Tanta University, Tanta, Egypt during the period from September-November, 2019.

Experimental animals: This study followed the ethical criteria approved by the Ethical Committee of the National Research Center of Egypt. The Animal Ethics Committee of the Faculty of Science, Tanta University, Tanta, Egypt, provided an approval to the protocol of this study.

Thirty five female Wistar rats of 7-8 weeks old (n = 7 in each group) and weighing 140-180 g were purchased from Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt. Animals were handled and kept at the animal facility, Faculty of Science, Tanta University, Egypt. Rats were housed in a room with a controlled ambient temperature (22-25°C) and a 12 h light/dark cycle and received sterilized food and water and acclimated to the housing conditions and handled for 7 days before experiments.

Reagents and antibodies: Cyclophosphamide (CTX: Sigma-Aldrich Co., China) was dissolved in Phosphate Buffered Saline (PBS) and frozen at -80°C until use. Recombinant

human (rh) G-CSF (Neupogen)® was purchased from (United Pharma Co., Cairo, Egypt). Lyophilized collagen II was purchased from Chondrex (Redmond, WA 98052, USA), acetic acid and Incomplete Freund's Adjuvant (IFA) was purchased from Sigma Aldrich (Cairo, Egypt). Ammonium Chloride Potassium (ACK) lysis buffer was purchased from Lonza, Bio Whittaker, USA. Rat IL-4 ELISA kit was purchased from Abcam (CA, USA) while Rat IL-8 and TNF-α ELISA kits were purchased from Biotech Co., Ltd. (Shanghai Blue Gene, Shanghai, China). The antibodies including: APC-conjugated anti-rat CD34 (Santa Cruz-Biotechnology, USA) and PE-conjugated anti-rat CD90 (Biolegend, USA) were used to identify stem cells in bone marrow.

Arthritis induction and assessment: Collagen Induced Arthritis (CIA) was made by prime-boost immunization of Wistar rats by collagen II in Incomplete Freund's Adjuvant (IFA) as described previously[16]. Collagen was dissolved at 2 mg mL^{-1} in 0.05 M acetic acid by gently stirring overnight at 4°C. The IFA was added to an equal volume of collagen solution drop-wise with mixing at low speed until a stiff emulsion was produced. The CIA was induced in rats by a single subcutaneous (s.c.) injection of 0.2 mL of CII-IFA emulsion at the tail base. To ensure a high incidence and severity of arthritis, a booster injection (0.1 mL of the emulsion) was given s.c. on day 12 after immunization at the tail base. Arthritis development was monitored in all four limbs by determining paw inflammation using a digital caliber equation[17]:

$$V = \frac{W(2)\times L}{2\ mm^2}$$

where, V is tumor volume, W is tumor width and L is tumor length.

Treatment of CIA rats: Three days after immunization with the booster dose of collagen II/IFA, the rats were treated with one of the following modalities: (1) s.c. injection of 200 µL PBS, (2) Single intraperitoneal injection of 200 µL CTX (4 mg/rat) as previously described[18,19], (3) Single intravenous injection of BM cell (5×0^6/rat), (4) s.c. injection of PBS containing G-CSF (5 µg/rat) as previously described by Rubinstein *et al.*[7] and Salem *et al.*[20], (6) CTX+G-CSF and (7) CTX+BM. One group of naïve rats were used as a negative control for the disease.

Preparation of BM cells: Single cell suspension of Bone Marrow (BM) cells was prepared as previously described[21]. The BM cells were obtained from the tibia and femur of adult (>8 weeks) Wistar rats. Whole femurs and tibias were obtained and kept in PBS until isolation of BM. Bone ends were cut off and bones were flushed with PBS using a syringe with a 23-G needle. Bone Marrow (BM) cells were collected in a 70 mm mesh sieve placed on a 50 mL tube and gently pushed through with the plunger of a syringe. The cells were rinsed with PBS to collect all cells in the tube then centrifuged for 8 min at 200 g. The cell pellet was re-suspended in lysis buffer to remove red blood cells then centrifuged for 8 min at 200 g and the pellet was re-suspended in PBS. The viable cells were counted using trybane blue dye exclusion assay. Cells were re-suspended at a concentration of 10^7 cells mL^{-1}.

Flow cytometry for stem cells: Fresh single-cell suspensions and 1×10^6 cells were treated with anti-CD16/CD32 for 5 min on ice. Cells were then stained with the indicated conjugated mAb, including APC anti-CD34 and PE anti-CD90 and incubated for 30 min on ice. The cells were washed twice and re-suspended in 0.3 mL of 0.5% BSA and 0.02% sodium azide solution. Cells were analyzed by flow cytometry (BD FACS Canto, CA, USA) using the Cell Quest software package (Becton Dickinson, San Jose, CA).

Evaluation of CIA by radiological imaging: After 20 days of treatments, rats were anesthetized and X-ray image was taken in the posterior table by Dental Occlusal X-ray film and scanned using a digitizer (Faculty of Dentistry, Tanta University, Egypt).

Evaluation of CIA by histopathology: The CIA rats were sacrificed on day 20 after treatments and their fore paws and hind paws were examined. The ankle joints were separated and kept in 10% neutral buffered formalin for 24 h prior to placement in surgipath decalcifier I for approximately 1 week. Paws were de-calcified with a solution containing HCl and 0.1 MEDTA for 21-30 days. After completion of the de-calcification process, the ankle joints were transacted into two equal halves in the longitudinal plane and were embedded in paraffin, sectioned and stained with hematoxylin-eosin.

Detection of plasma levels of cytokines using ELISA: One week after treatment, rats were bled by heparinized capillary tubes via orbital plexus and samples of peripheral blood were collected in 3.8% sodium citrate and plasma was stored at -80°C. Levels of TNF-α, IL-4 and IL-8 in plasma were measured by Enzyme-Linked Immunosorbent Assay (ELISA) according to protocols of the Rat Interleukin 4 ELISA kit (Abeam, USA) and Rat IL-8 and TNF-α ELISA kits (Shanghai Blue Gene Biotech Co., Ltd., Shanghai, China).

Statistical analysis: All results were represented as mean±SD. Statistical comparisons among prospective groups were analyzed using a one-way analysis of variance (ANOVA) Statistical significance was determined by a *post hoc* test followed by Dunnett's multiple comparison tests to compare treatment means vs. respective controls. All p-values considered significant at $p<0.05$.

RESULTS

Effect of G-CSF based treatment on stem cell mobilization in CIA rats: By comparing the effects of different treatments on the numbers of hematopoietic stem cells in peripheral blood in CIA rats through 3 consecutive weeks post injections, increases in the numbers of these cells in CIA rats were observed regardless the type of treatment modality. Rats treated with CTX/G-CSF, G-CSF, BM or CTX showed a significant increase in the percentage of hematopoietic stem cells (CD90-CD34$^+$) in the peripheral blood (1.35, 1.37, 1.52 and 1.29%), respectively. While treatment with CTX/BM resulted in decreases in the percentage (0.63%) as compared to CIA rats (Table 1).

Treatments with G-CSF and BM cells ameliorated the clinical signs of CIA: The data of the clinical signs of CIA are presented in Fig. 1a-b. Approximately 15 days after boosting the CIA rats, periarticular erythema and edema appeared in the hind paws of all rats. By day 35 of the 1st immunization, swelling, redness, bone deformity and ankylosis appeared in the fore and hind paws as well as in ankle joint in treated groups as compared to the naive group which showed normal for and hind paws (Fig. 1a-b). Rats treated with either CTX or CTX/BM cells showed moderate amelioration in the inflamed fore and hind paws (Fig. 1a). While rats treated with CTX/G-CSF showed a marked reduction in the inflammation of fore and hind paws as compared to untreated CIA rats. Treatment with G-CSF alone or BM cells alone showed a slight reduction in inflammation of fore and hind paws.

Treatments with G-CSF and BM cells reduced paw thickness in CIA rats: With regard to the hind paws, the thickness of the right and left hind paws in CIA rats markedly increased until week five, which reached 7.8 and 8.0 mm^3, respectively, as compared to those of non-CIA rats, which measured 4.2 mm^3 (Fig. 2a-b). Interestingly, treatment of CIA rats with CTX/G-CSF resulted in significant amelioration of the inflammation of the right and left hind paws, where their thickness measured 4.5 mm^3, which indicates to the amelioration of inflamed paw. However, treatment with CTX/BM cells, G-CSF, BM cells or CTX induced slight amelioration of the inflammation, where the right hind paw measured 5.9, 6.6 and 6.4 mm^3, respectively and the left hind paw measured 6.2, 6.0 and 6.3 mm^3, respectively. Of note, treatment with CTX alone did not induce any appreciated effects, where the right and left hind paw measured 7.3 and 7.7 mm^3, respectively (Fig. 2a-b).

With regard to the fore paws, the untreated CIA rats showed severe inflammation till week five, which measured 6.2 and 6.3 mm^3, respectively, as compared to the right and left fore paws in naive rats, which showed

Table 1: Numbers of hematopoietic stem cells (CD90-CD34$^+$) in the peripheral blood of CIA rats

Week	Number	Naive	CIA/PBS	CIA/CTX	CIA/CTX+BM	CIA/CTX+G-CSF	CIA/G-CSF	CIA/BM
1st	%	3.8	5.4	26.3	20.3	7.9	2.3	37
	Abs	0.60±0.42	0.91±0.35	1.29±0.39	0.63±0.52	1.35±0.49	1.37±0.56	1.52*±0.7
2nd	%	2.4	2.3	29.2	103.6	4.8	6.6	30.9
	Abs	0.39±0.45	0.39±0.47	1.43*±0.48	1.14±0.46	0.81±0.58	0.40±0.43	1.27±0.61
3rd	%	2.8	8.4	18.9	39.4	4.9	1.9	34.1
	Abs	0.45±0.34	1.40±0.371	0.93±0.24	1.22±0.33	0.84±0.34	1.14±0.341	1.40*±0.36

Each reading represents mean±SD (n = 7 rats), *Significance of difference after treatment was tested by one way ANOVA analysis, Percentage shown are the relative number of stem cells (CD90-CD34$^+$) analyzed by Flow cytometry, the absolute cell numbers of stem cells were calculated as: [total WBCs count (cells/μL)×stem cells %]/100%, Abs: Absolute number, G-CSF: Granulocyte-colony stimulating factor, CTX: Cyclophosphamide, BM: Bone marrow, CIA: Collagen-induced arthritis, PBS: Phosphate buffer solution

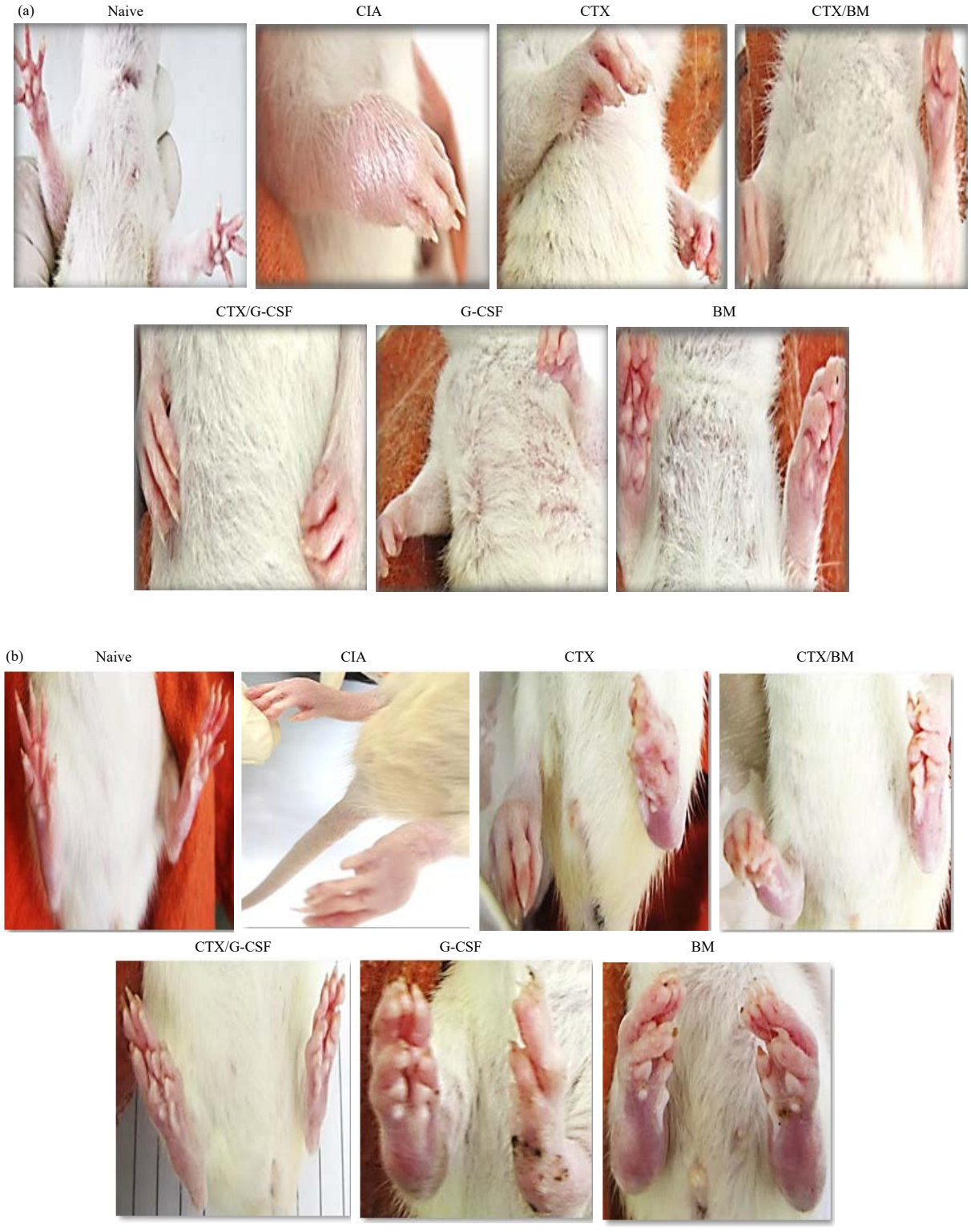

Fig. 1(a-b): Effects of treatment with BM, CTX, G-CSF or their combination on clinical signs of pad swelling of (a) Fore and (b) Hind limbs

Native: Normal rat, CIA: Collagen-induced arthritis, CTX: Cyclophosphamide, CTX/BM: Cyclophosphamide/bone marrow, CTX/G-CSF: Cyclophosphamide/granulocyte-colony stimulating factor, G-CSF: Granulocyte-colony stimulating factor, BM: Bone marrow

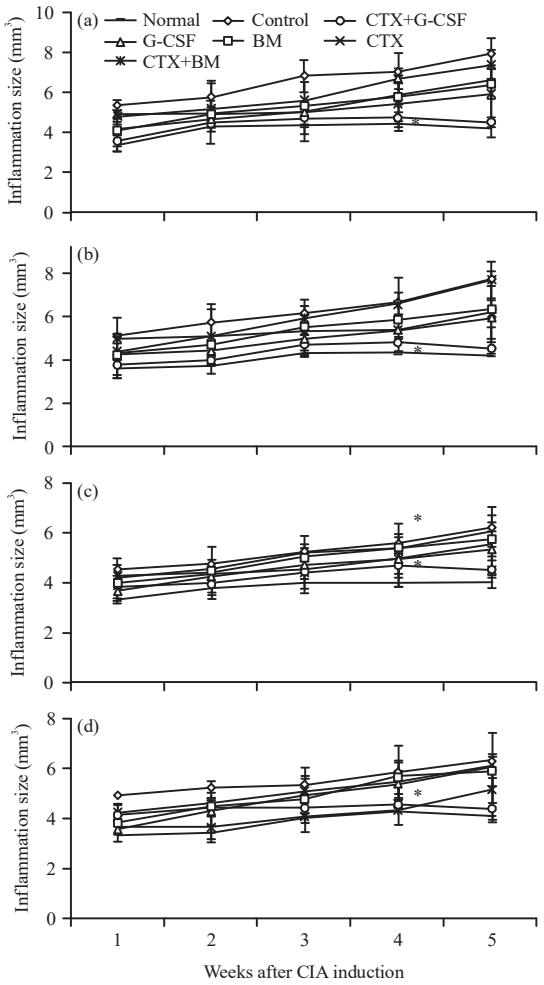

Fig. 2(a-d): Effects of treatment with BM, CTX, G-CSF or their combination on pad swelling of fore and hind limbs. The data show difference in the paw measurements of (a) Right hind inflamed paw, (b) Left hind inflamed paw, (c) Right fore inflamed and (d) Left fore inflamed paw through five weeks of the experiment

Wistar rats were treated with CTX, CTX/BM, CTX/G-CSF, G-CSF alone and BM alone. Both length and width of the inflamed paws were determined using ellipsoidal formula, V = (Width (A)×2 length (B))/2 mm^2. Data are presented as mean±SD animals, group, From n = 7 animals/group, ANOVA analysis of variance was done, *p≤0.05 as compared with untreated rats

normal thickness, scored 4 mm^3. Treatment of CIA rats with CTX/G-CSF induced significant amelioration of the thickness of both the right and left fore paws which measured 4.5 and 4.3 mm^3, respectively (Fig. 2c-d). Treatments of CIA rats with CTX/BM cells, G-CSF, BM cells and CTX also induced significant amelioration in the thickness of the right fore paws, but with lesser effects scoring 5.5, 5.3, 5.7 and 6.02 mm^3, respectively. The left fore paws in these CIA treated rats measured 5.2, 6.1, 5.9 and 6.1 mm^3, respectively. Taken together, these data concluded that G-CSF treatment combined with CTX showed the highest anti-inflammatory effect.

Combination of CTX and G-CSF ameliorated the joints injury: The X-ray data are shown in Fig. 3a-b. To confirm the effects of the anti-arthritic effects of CTX/G-CSF showed, the present study monitored the deformity in the bone of ankles and in hind and fore limbs by X-ray scan. As compared to naive rats which showed intact bony outlines and normal joint space in both hind and fore limbs, the untreated CIA rats showed severe destructive abnormalities of all the metatarsal bones showing definite bone erosion as well as edema of soft tissues and mild osteoporosis. Consistent with the paws thickness above, treatment with CTX/G-CSF induced significant inhibition of damage in joints, which apparently was similar to those in normal rats. Joints from rats treated with CTX or CTX/BM cells exhibited a moderate reduction of damage in both hind and fore limbs. Treatment with G-CSF alone or BM cells showed a slight reduction of bones deformity.

Combination of CTX and G-CSF ameliorated the histopathological changes in joints injury: To further confirm the anti-arthritic effects of CTX/G-CSF, histopathological examination on the joints were performed (Fig. 4a-g). As compared with normal joints, untreated CIA rats showed severe destructions, febrile formation, erosion and irregular thickness of the articular cartilage. The wide joint space showed inflammatory debris and highly proliferating edematous synovial membrane extending into the joint cavity and eroding the surface cartilage and bone by the formation of pannus as well as cartilage islets. By examining the synovial pannus sections of joints examined at later stages of inflammation, the current study found eroded cartilage and subchondral bone of CIA rats. Interestingly, CIA rats treated with CTX/G-CSF showed normal joint space and the normal regular articular surface of the cartilage and its underlying subchondral bone with prominent tide mark separating the cartilage from the bone. CTX treated CIA rats showed decreased joint space and its filling with fibrinoid material. However, they showed subchondral bone thinning and

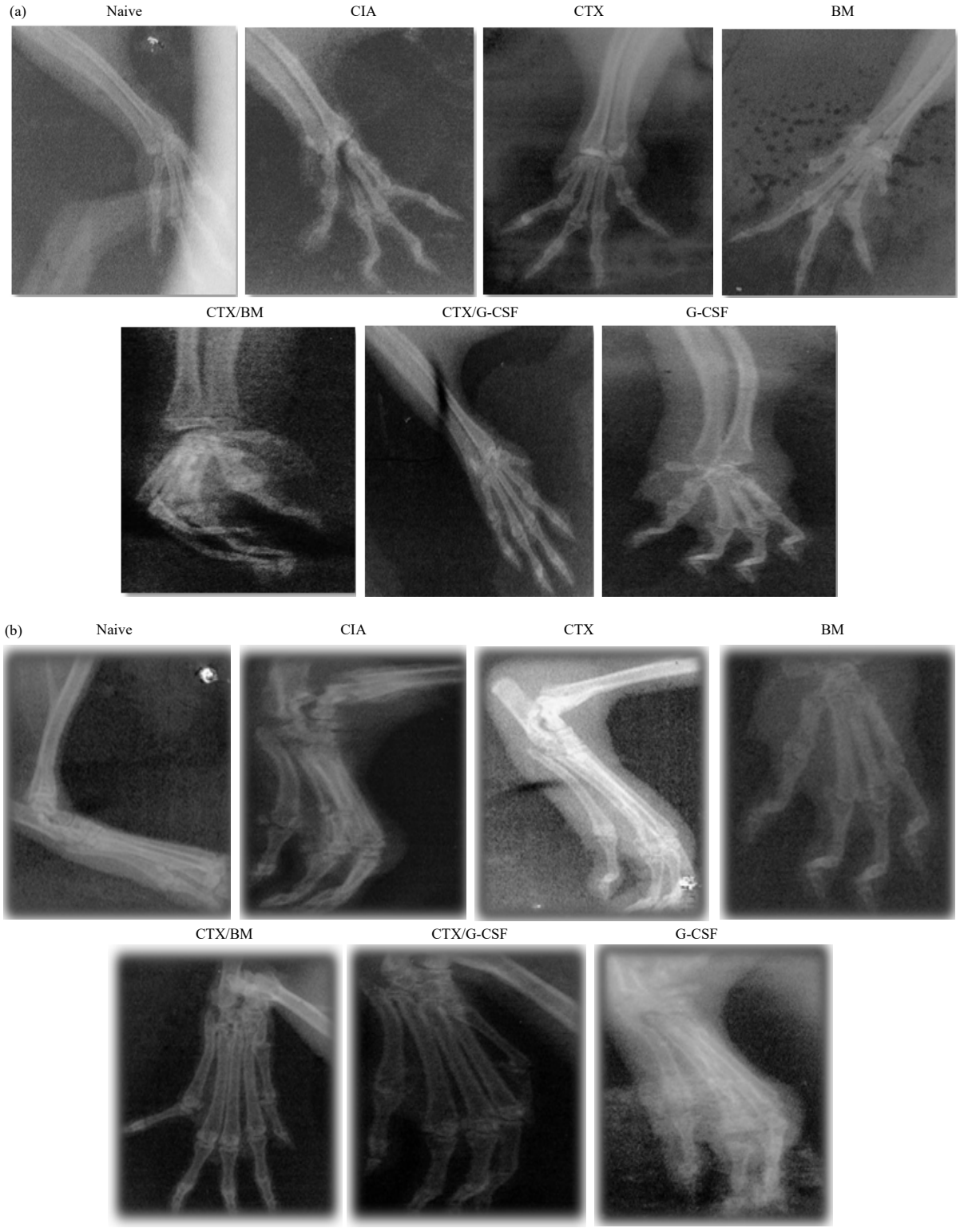

Fig. 3(a-b): Roentgen graph of (a) Fore and (b) Hind limbs in control CIA rats and after treatment with BM, CTX, G-CSF or their combination

Native: Normal rat, CIA: Collagen-induced arthritis, CTX: Cyclophosphamide, BM: Bone marrow, CTX/BM: Cyclophosphamide/bone marrow, CTX/G-CSF: Cyclophosphamide/granulocyte-colony stimulating factor, G-CSF: Granulocyte-colony stimulating factor

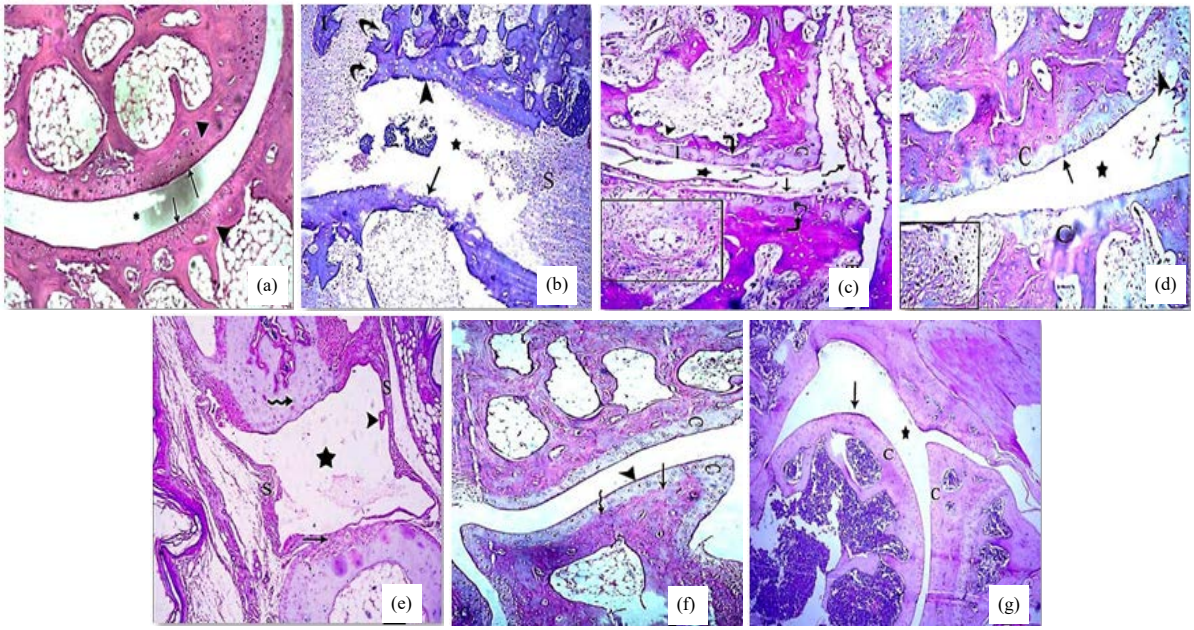

Fig. 4(a-g): Effects of treatment with BM, CTX, G-CSF or their combination on the histopathological changes in CIA rats, (a) Naive: normal articular cartilage with normal thickness and regular surface and absence of infiltrate in the synovium (HE stain×200), (b) CIA group: severely distorted joint displaying fibrillation and irregular thickness of the articular cartilage, marked infiltration of inflammatory cells and pannus formation (HE stain×200), (c) CTX-treated group: replacement of the cartilage and bone with granulation tissue (pannus), minor infiltration of inflammatory cells (HE stain×400), (d) BM-treated group: normal joint space with regular articular surface and reparative cartilage tissue (HE stain×400), (e) CTX/BM-treated group: wide joint space, regular articular cartilage at one side and fibrous pannus on the other side and synovial membrane depicts small nodules (HE×100), (f) CTX/G-CSF-treated group: normal joint space with regular articular cartilage and prominent tide mark separating the cartilage from the bone (HE×200) and (g) G-CSF-treated group: normal joint space with regular articular cartilage (HE×200)

*Joint space, (wavy arrow): Articular cartilage, →: Fibrous pannus, S: Synovial membrane, ▸: Small nodules, Square: Granulation tissue (pannus), C: Cartilage

erosions. Treatment with BM cells also showed normal joint space and a few fibrinoid materials. The synovial membrane was seen at one side eroding the articular cartilage and its subchondral bone (pannus). In contrast, treatment with CTX/BM cells showed increased joint space. Treatment with G-CSF alone did not show any obvious tide mark.

Effects of treatments on IL-4, IL-8 and TNF-α: Compared with normal rats (785 pg mL^{-1}), CIA rats showed significant high serum levels of TNF-α which reached about 1000 pg mL^{-1}. CIA rats treated with CTX/BM cells or BM cells showed 951.5 and 878.3 pg mL^{-1}, respectively (Fig. 5a). The levels of TNF-α in CIA rats treated with CTX/G-CSF showed marked decrease reaching 732 pg mL^{-1}. The levels of TNF-α in CIA rats treated with CTX alone and G-CSF alone showed 854.7 and 545.7 pg mL^{-1}, respectively.

The CIA rats showed a significant ($p<0.001$) increase in the serum levels of IL-4 (Fig. 5b) reaching about 800 pg mL^{-1}, which overall significantly decreased after all treatments. Specifically, treatment with CTX/G-CSF decreased it to 96.2 pg mL^{-1}. While treatment with G-CSF alone, CTX/BM cells, BM cells alone and CTX alone decreased its levels to 353.1, 440, 568.7 and 700.3 pg mL^{-1}, respectively. As compared to normal rats which showed 101 pg mL^{-1}, CIA rats showed 416 pg mL^{-1}. Overall, treatments of CIA rats resulted in significant decreases in the serum levels of IL-8 (Fig. 5c). Specifically, treatments of CIA rats with BM, G-CSF, CTX/BM cells, CTX and CTX/G-CSF showed 56.8, 68.9, 51.0, 52.3 and 104 pg mL^{-1}, respectively.

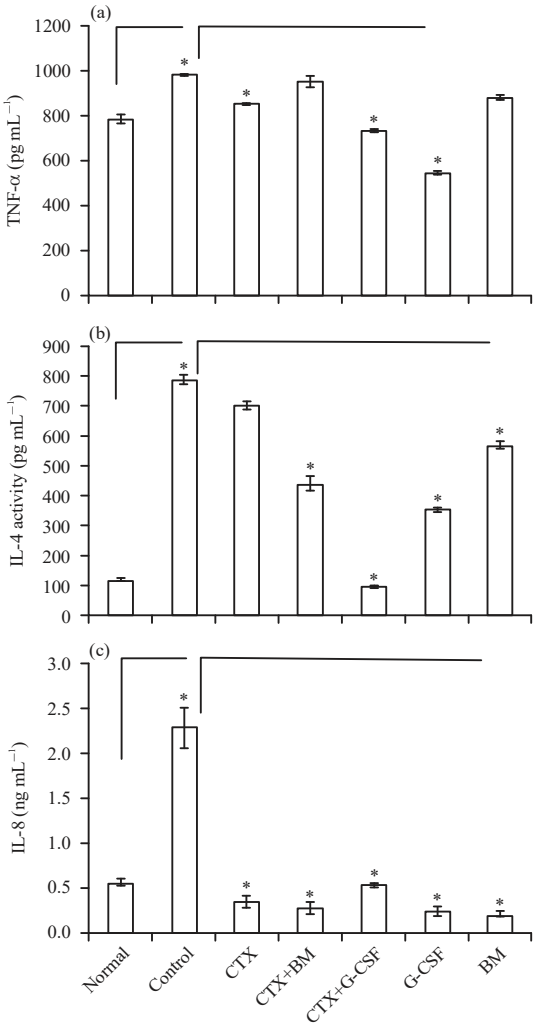

Fig. 5(a-c): Effects of treatment with BM, CTX, G-CSF or their combination on the plasma levels of inflammatory and anti-inflammatory cytokines in CIA rats. Plasma levels of (a) TNF-α, (b) IL-4 and (c) IL-8 CII-induced arthritis in Wistar rats

Wistar rats were injected with CIA, CTX, CTX/BM, CTX/G-CSF, G-CSF or BM, The concentrations of TNF-α and IL-4 in plasma were determined with ELISA kits, data represent the mean±SD (n = 7), ANOVA analysis of variance was done, *$p \leq 0.05$ as compared with untreated rats

DISCUSSION

Stem cell therapy in rheumatoid arthritis is based on transplantation of hematopoietic or administration of mesenchymal stem cells from different sources including umbilical cord blood, synovial fluid[21], adipose tissues[22] rather than mobilization of endogenous stem cells[23]. The main goal of this study was to test the effect of the combination of G-CSF and BM cells with CTX as a potential remedy for arthritis using an experimental CIA model. This study used flow cytometry to confirm the mobilization of hematopoietic stem ($CD90\text{-}CD34^+$) cells from BM into peripheral blood of CIA rats. Consistent with recent data in naive Wistar rats[24], this study found comparable increases in the numbers of $CD90\text{-}CD34^+$ cells after treatment with BM, G-CSF and CTX/G-CSF. The thickness of the inflamed paws, X-ray and histopathological examination were assessed as well as the serum levels of IL-4, IL-8 and TNF-α. The present study found that although treatment with BM in combination with CTX or individually induced decreases in paw thickness in fore and hind limbs which was confirmed by x-ray and histological examination, treatment with both CTX and G-CSF induced the most ameliorating effect against CIA. These data indicated that mobilization of endogenous stem cells by CTX and G-CSF may play a significant role in the treatment of experimental CIA providing insights into a new therapeutic approach to rheumatoid arthritis.

Radiography has traditionally been considered the most objective measurement technique for assessing the severity and progression of experimental arthritis[25]. Consistent with previous reports showing destructive bones in CIA rats measured by X-ray[26], this study found that X-ray of hind joints taken on the 35th day after primary immunization in CIA model showed that joints swollen joints coincided with erosion in cartilage and bone along with severe destruction in the metatarsal bones where at least one of inner metatarsal joints has been completely eroded leaving some bony joint outlines partly preserved. Results of the present study for X rays images presented a clear difference between normal, untreated and treated CIA rats. Joints from rats treated with CTX combined with G-CSF exhibited significant inhibition of damage in joints, which resembled the joints from the normal rats. However, joints from rats treated with CTX alone, G-CSF alone or CTX combined with BM exhibited a moderate reduction in the damage induced in both hind limbs and forelimbs.

Consistent with the established histological evaluation of the paws in CIA rats[27], this study also revealed signs of severe arthritis; severe destruction and sloughing of the covering cartilaginous cap, irregular bony margins and dense inflammatory cellular infiltration and hyperplasic synovial membrane. CTX or BM alone induced decreases in the joint space and its filling with fibrinoid material and the subchondral bone displays thinning and erosions. Of note, the effects of BM were more efficacious than CTX. The synovial membrane is seen at one side eroding the articular cartilage and its subchondral bone. In contrast, treatment with CTX combined with BM showed an increase in joint space, which might be due to the granulocytic lineage in the exogenous BM

and its further proliferation and maturation upon CTX treatment since neutrophils play a critical role in rheumatoid arthritis[28]. With this regard, it has been found that infiltration of mononuclear cells and neutrophils to the sub-synovial region induces vigorous proliferation of synovial cells, resulting in pannus formation and destruction of the cartilage and bone[29].

Although treatment with G-CSF alone did not show any significant effects on CIA rats, CIA rats treated with G-CSF combined with CTX, however, showed almost normal joint space, the normal regular articular surface of the cartilage and its underlying subchondral bone with prominent tide mark separating the cartilage from the bone. Indeed, besides its mobilizing effects, prophylactic treatment with CTX was found to inhibit collagen-induced arthritis in mice by acting as an act at the induction phase of arthritogenic lymphocytes[30]. This effect of CTX might be further enhanced when combined with the effects of G-CSF which has been found to express direct anti-inflammatory effects on activation and functional properties of CD4+ and CD8+ T cells, which express G-CSF receptors, resulting in decreases in IFN-ɣ and increases in IL-4 production *in vitro* and *in vivo* suggesting that G-CSF could play a significant role both in preventing the development of excessive and potentially damaging inflammatory reactivity and in constraining the expansion of potentially cytodestructive by skewing the T helper cells response from Th1 to Th2 type cells.

G-CSF has been found to mobilize not only hematopoietic stem cells from BM but also mesenchymal stem cells[31]. For instance, treatment of mice with G-CSF induced precise kinetics of stem cell mobilization, where at early time point it mobilized hematopoietic stem cells in BM while at later time point it mobilized mesenchymal stem cells in the peripheral blood[32]. In another study, G-CSF was able to mobilize mesenchymal stem cells into peripheral blood (fourfold increase), where these cells expressed the typical BM mesenchymal stem cell markers including CD73, CD44, CD90, CD106, CD31 and CD45. These cells were able to integrate into injured cerebral tissue[33]. This effects of G-CSF may explain why treatment of adjuvant arthritis in Lewis rats, induced by s.c. injection of *Mycobacterium tuberculosis* in mineral oil, with G-CSF, induced a decrease in the disease severity and joint destruction[15]. It seems that the beneficial effects of G-CSF is mainly due to its mobilizing effects of stem cells since the endogenous levels of G-CSF were found to increase both in the serum and in inflamed paws of arthritic mice and its blockade reduced numbers of neutrophils in blood and resulted in a significant reduction in arthritis severity[34].

Although the anti-arthritic effects of CTX/G-CSF could be attributed to several mechanisms, current study evaluates that this treatment on the balance between the pro-inflammatory cytokines namely TNF-α and IL-8 versus the anti-inflammatory cytokines namely IL-4. IL-8 as a neutrophil chemoattractant and a mediator of angiogenesis is responsible for the increased number of neutrophils in RA joints[35] and, therefore, for the clinical manifestation of joint swelling and pain[36]. *In vivo*, it has been shown that inoculation of only one intra-articular injection of IL-8 induces synovial hyperplasia similar to the human RA[37]. Further, the concentration of IL-8 in serum and synovial fluid is a significant marker of disease activity and progression of synovial inflammation in RA joints[38]. In the present study, there was an elevation in the levels of IL-8 in untreated CIA rats as compared to naive rats. Treatment of these rats with the tested modalities, in particular, those containing G-CSF associated with decreased serum levels of IL-8.

TNF-α is a central component in the cytokine cascade and is responsible for stimulating the production and recruitment of other inflammatory mediators into the rheumatoid joint[39], resulting in bone and cartilage degradation[40]. Compared with normal rats, the present study found that CIA rats show elevated levels of TNF-α, which was slightly decreased after treatment with CTX alone or in combination with G-CSF. However, a substantial decrease was found after treatment with G-CSF alone or combined with and CTX. Compared with normal rats, CIA rats showed high serum levels of IL-4, which was slightly diminished after treatment with G-CSF alone, BM alone or CTX combined with BM. Treatment with CTX/G-CSF diminished the increased levels of IL-4. Given that IL-4 as a Th2 cytokine that mediates the development and progression of arthritis through Th17-induced inflammation[40], it could be suggested that the anti-arthritic effects of treatment with G-CSF/CTX are mediated by inhibition of the Th2 type cells, in particular, its production of IL-4 which have pathogenic effects during the early phase of arthritis[41].

Taken data together, it can be suggested that mobilizing the endogenous stem cells by CTX and G-CSF results in anti-arthritic effects than the use of exogenous BM cells even in the presence of administration of CTX. This anti-arthritic effect are mediated by decreases in the pro-inflammatory cytokine TNF-α and increases in the anti-inflammatory cytokine IL-4.

CONCLUSION

Treatment of CIA rats with G-CSF and CTX showed potential anti-arthritic effect through modulating the associated inflammatory response resulting in amelioration of bone destruction.

SIGNIFICANCE STATEMENT

This study discover that treatment of CIA rats with the combination of G-CSF and CTX induces potential anti-arthritic effect. It can be suggested that mobilizing effects of the endogenous stem cells by CTX and G-CSF results in anti-arthritic effects than the use of exogenous BM cells even in the presence of administration of CTX. Thus a new theory on anti-arthritic effect through down regulating the elevated levels of IL-4, IL-8, TNF-α cytokines.

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