Antiinflammatory and antioxidant effects of gemcitabine in collagen-induced arthritis model

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Background/aim: Gemcitabine (GEM) has antiproliferative effects on lymphocytes, which are potent pathogenic actors of rheumatoid arthritis (RA). The aim of the study was to investigate the therapeutic potential of GEM on collagen-induced arthritis (CIA).

Materials and methods: Arthritis was induced by the intradermal injection of chicken type II collagen with incomplete Freund’s adjuvant into albino Wistar rats. Doses of 5 and 20 mg/kg GEM were administered twice a week after the 14th day, which marked the onset the arthritis. Serum IL-17, TNF-α, malondialdehyde, catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels and tissue heme oxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf2) levels were analyzed.

Results: Histopathologically prevalent inflammation and cartilage/bone destruction were observed in the arthritis group. Moreover, in the arthritis group serum IL-17, TNF-α, and malondialdehyde levels were significantly increased while catalase, SOD, GPx, HO-1, and Nrf2 levels were significantly decreased. However, in the GEM-treated groups, decreased TNF-α, IL-17, and malondialdehyde levels; increased SOD, catalase, GPx, Nrf2, and HO-1 levels; and ameliorated perisynovial inflammation and cartilage/bone destruction were observed.

Conclusion: GEM suppresses cytokine levels and enhances antioxidant activity. It also prevents cartilage/bone destruction in the CIA model. GEM may be a viable candidate for research into the treatment of RA.

Key words: Rheumatoid arthritis, collagen-induced arthritis, gemcitabine, western blotting

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory illness characterized by persistent immune activation (1). B and T cells play crucial roles in the pathogenesis of RA (2,3). There are several mechanisms that trigger immune activation in RA. Leukocyte migration, mediated by chemokines and chemokine receptors, is recognized to have a crucial role in the progression of inflammation in rheumatoid synovium (4). Leukocytes including T cells infiltrate the synovium and produce cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-17. T cell-mediated immune response is considered a critical contributor in the initiation and progression of RA pathogenesis. On the other hand, B cells produce autoantibodies such as rheumatoid factor and anticyclic citrullinated peptide antibody (2,5,6).

Oxidative stress is associated with the pathogenesis of RA (7,8). Free radicals such as superoxide and hydrogen peroxide are released from inflammatory cells in the synovial fluid of RA patients. They cause joint damage and pannus formation (7,8). However, antioxidant enzymes play a role in the defense system against free radicals. Nuclear factor erythroid 2-related factor 2 (Nrf2), a cytoprotective transcriptional factor for the upregulation of the antioxidant response element (ARE) pathway, enhances the expression of several antioxidants (9).

Gemcitabine (GEM) is a nucleoside antimetabolite. It is an analog of deoxycytidine, which blocks deoxyribonucleic acid synthesis. GEM is converted to active metabolites including difluorodeoxycytidine-diphosphate and -triphosphate after being transported into the cell. The triphosphate analog replaces one of the
nucleic acid building blocks during DNA replication. Moreover, GEM inhibits the ribonucleotide reductase enzyme. Therefore, it is used to treat several malignant tumors (e.g., ovarian and breast cancers) (10). In addition, GEM possesses antiproliferative and apoptotic effects on T and B lymphocytes (11).

The purpose of the present study is to evaluate the potential therapeutic efficacy of GEM in an in vivo experimental model of RA, collagen-induced arthritis (CIA).

2. Materials and methods

2.1. Animals

The study was started after approval was obtained from the Ethics Committee of Firat University. The experiment was conducted with 40 female albino Wistar rats of 10 weeks old weighing from 200 to 250 g. The rats were kept in separate cages at a humidity of 55 ± 5% and 22 ± 2 °C for a 12-h photophase and a 12-h scotophase. Standard rat feed was used for their nutrition, and they were given ad libitum access to water.

2.2. Experimental design

The study was conducted by dividing the rats into four groups: control, arthritis (placebo), low-dose GEM, and high-dose GEM. Each group consisted of 10 subjects. The first group was the control group. Arthritis was induced via collagen injection in the other three groups. Type 2 collagen, obtained from Sigma-Aldrich (St. Louis, MO, USA), was diluted with 0.1 M acetic acid to reach 1 mg in 1 mL. The collagen solution was emulsified with an equal amount of incomplete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA). The prepared solution was injected intradermally into the dorsal tail (100 μg) and back paws (50 μg to each paw and a total of 200 μg to each rat) on the first day. A volume of prepared solution of 100 μg was applied to the dorsal tail for booster injections 7 days later. Development of arthritis after the collagen injection was individually evaluated for each rat by clinical scoring. Arthritis scorings for the study groups were done according to a previously described method (12). According to the clinical scoring, prominent arthritis was observed on the 12th and 13th days in the collagen-injected groups (Figure 1); hence, GEM treatment was started on the 14th day.

GEM dosages of 5 and 20 mg/kg were given intraperitoneally to the 3rd and 4th groups, respectively, twice a week after the 14th day, which was the beginning of arthritis. The GEM dosages were determined according to a previous study (13). The same volume of physiological serum was injected intraperitoneally into the control and arthritis (placebo) groups twice per week from the 14th to the 29th day.

2.3. Sample acquisition

Rats were euthanized on the 29th day. Back paws were amputated under the knee joint for further examination, and blood samples were taken for biochemical analysis. The blood samples were centrifuged and harvested sera were stored at ~20 °C until analysis. Joint samples were divided into two sections for western blot (WB) analysis and histopathological examination. The section for the WB analysis was kept at ~80 °C and did not undergo any manipulation. The remaining section was put into 10% formaldehyde for histopathological examination.

2.4. Laboratory analyses

Serum malondialdehyde (MDA) levels were measured by the high-performance liquid chromatography (Shimadzu, Tokyo, Japan). Serum superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) levels were analyzed using the appropriate commercial kits (Cayman Co., Ann Arbor, MI, USA) in accordance with the enzyme-linked immunosorbent assay (ELISA) method. Serum TNF-α (Invitrogen, Camarillo, CA, USA) and IL-17 (Wuhan USCN Business Co., Ltd., Wuhan, China) levels were measured using the ELISA method.

2.5. Western blot measurements

Joint tissue samples were analyzed for the expression of Nrf2 and heme oxygenase-1 (HO-1), using the WB technique. The hind paws of all rats were rapidly excised from the euthanized rats and then immediately frozen at ~80 °C. Small pieces of the paw joints in each group of animals were pooled together for WB analysis. Homogenates were prepared in an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.26% sodium deoxycholate, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 10 μg/mL leupeptin, and 50 μg/mL phenylmethylsulfonyl fluoride and then incubated on ice for 40 min. Next, 80 μL of 10% Nonidet P-40 solution was added to the homogenates, and the mixture was then centrifuged for 2 min at 14,000 × g at 4 °C to remove cellular debris and isolate total protein. Concentration of the protein was determined according to the procedure described by Lowry et al. (14) using a protein assay kit supplied by Sigma-Aldrich. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing 2% β-mercaptoethanol was added to the supernatant. Equal amounts of protein (50 μg) were electrophoresed and subsequently transferred to nitrocellulose membranes (Schleicher and Schuell Inc., Keene, NH, USA). Nitrocellulose blots were washed twice for 5 min each in phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin in PBS for 1 h prior to application of the primary antibody. The antibodies against Nrf2 and HO-1 were purchased from Abcam Inc. (Cambridge, UK). The primary antibody
was diluted (1:1000) in the same buffer containing 0.05% Tween-20. The nitrocellulose membrane was incubated overnight at 4 °C with protein antibody. The blots were washed and incubated with horseradish peroxidase-conjugated goat antimouse IgG (Abcam). Specific binding was detected using diaminobenzidine and H₂O₂ as substrates. Protein loading was controlled using a monoclonal mouse antibody against β-actin antibody (A5316; Sigma-Aldrich). Blots were performed at least three times to confirm the reproducibility of the results. Bands were analyzed densitometrically using an image analysis program (Image J; National Institutes of Health, Bethesda, MD, USA).

2.6. Histopathological evaluations
Tissue samples fixed in formalin solution were decalcified with 10% nitric acid (30 days) to prepare paraffin blocks. Cross-sections taken from the blocks were stained with hematoxylin and eosin (H&E). They were then examined by a specialist pathologist under 40×, 100×, 200×, and 400× magnifications with a light microscope to assess inflammatory cell infiltration, pannus formation, and bone destruction around the joint. The samples were scored on a scale between 0 and 4 points for histopathological scoring (Tables 1 and 2) as previously described (15,16).

2.7. Statistical analysis
Data were analyzed using IBM SPSS 21.0 (IBM Corp., Armonk, NY, USA). Data were expressed as mean ± standard deviation. Data were analyzed with Kruskal–Wallis variance analysis. The Mann–Whitney U test was used for dual comparisons. Categorical data were analyzed by the chi-square test. The differences in the arthritis scoring from the 14th to the 29th day were evaluated with the Wilcoxon rank-sum test. All P-values of less than 0.05 were accepted as statistically significant.

3. Results
3.1. Clinical scoring of arthritis
Paws were slightly erythematous after the first injections of collagen in the arthritis (placebo) and GEM-treated groups. From 12th to the 13th day, there was permanent arthritis in the collagen-injected groups (arthritis and GEM-treated groups) compared to the control group (P < 0.05 for all). In the arthritis group, the mean arthritis score on the 29th day was higher than the score of the same group on the 14th day (P < 0.05). However, the mean arthritis scores on the 29th day were lower compared to the 14th day scores in the low- and high-dose GEM-treated groups (P < 0.001 for both). On the other hand, the mean arthritis scores of the 29th day were significantly lower in both GEM-treated groups compared to the arthritis group (Table 3; Figure 1).

None of rats in the control or arthritis group died. One rat in the low-dose GEM group and two rats in the high-dose GEM group died. However, these increased mortality rates (10% and 20%, respectively) were not statistically significant compared to the control and arthritis groups (P > 0.05 for both groups). On the other hand, diarrhea was detected in two rats (22.2%) and three rats (37.5%) in the low- and high-dose GEM-treated groups, respectively, while diarrhea was not found in the control or the arthritis group. Similar to the mortality rates, the increase noted in diarrhea (dose-dependent) in the GEM-treated groups compared to the control and arthritis groups was not statistically significant (P > 0.05 for both groups).

3.2. Histopathological scoring
According to the scoring results, a significant decrease was found in inflammation and destruction in both GEM-treated groups (P < 0.001) (Table 3). However, the destruction score in the group with the high dose of GEM had more significant improvement (P < 0.05), while the improvements of the inflammation score were similar in both of the GEM-treated groups (P > 0.05) (Table 3; Figure 2).

3.3. Serum cytokine levels
Serum TNF-α and IL-17 concentrations were found to be higher in the placebo group than in the control group (P < 0.01 for both). However, their levels were lower in the GEM-treated groups than in the placebo group (P < 0.01 for all). In the GEM-treated groups, serum TNF-α levels were similar in the control group (P > 0.05), while serum

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<td>PT inflammation, no aggregates</td>
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<tr>
<td>PT inflammation, occasional small focal aggregates</td>
<td>2</td>
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<tr>
<td>Moderate PT inflammation, many small aggregates</td>
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<tr>
<th>Arthritis severity</th>
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IL-17 levels were still higher than in the control group (P < 0.01 for both) (Table 3). Serum TNF-α and IL-17 levels were similar in both the GEM-treated groups.

### 3.4. Oxidants and antioxidants

Serum MDA levels were higher in the placebo group than in the control group (P < 0.01). However, its level was lower in the GEM-treated groups than in the placebo group (P < 0.01 for both) (Table 3). No significant difference was observed for MDA levels between the GEM-treated groups (P > 0.05).

SOD, CAT, and GPx levels were lower in the placebo group than in the control group (P < 0.01 for all). However, CAT and GPx levels were higher in the GEM-treated groups compared to the placebo group (Table 3). SOD levels were significantly higher in the low-dose GEM-treated group, but it was relatively higher in the high-dose GEM-treated group.

The results of WB analysis are presented in Figure 3. The tissue Nrf2 and HO-1 levels were lower in the placebo group when compared to the control group (P < 0.05 for both).
Figure 2. Histopathological sections of joints in the study groups (H&E, 400×). Synovial and perisynovial tissue was normal in the control group (A). Perisynovial inflammation and destruction of cartilage/bone were obvious in the placebo group (B). Perisynovial inflammation and synovial hyperplasia were ameliorated in the GEM-treated groups with low (C) and high (D) doses.

Figure 3. Western blot analysis and densitometric quantifications of Nrf2 (A) and HO-1 (B). Representative blots, repeated at least 3 times (n = 4), are shown. Actin was included to ensure equal protein loading. The densitometric quantifications were normalized to actin densities for each sample and expressed as mean ± SD.

*The tissue Nrf2 and HO-1 levels were lower in the arthritis (placebo) group compared to the control group (P < 0.05). ‡On the other hand, they were higher in the GEM-treated groups compared to the arthritis group (P < 0.05). Nrf2: Nuclear factor erythroid 2-related factor 2, HO-1: heme oxygenase-1, GEM: gemcitabine, GEM-5: low-dose gemcitabine group, GEM-20: high-dose gemcitabine group.
Both GEM-treated groups had increased Nrf2 and HO-1 levels compared to the placebo group (P < 0.05 for all).

4. Discussion
RA primarily affects small- and medium-sized joints (17,18). Systemic involvements such as the respiratory, cardiovascular, and hematopoietic systems may also occur. The typical lesion is synovitis, leading to the chronic inflammation of the synovial membrane and formation of pannus, which ultimately leads to joint destruction (4). In RA, joint destruction causes deformities and reduces the quality of life in patients. Thus, these patients experience a high rate of work-related disabilities. Moreover, the treatment of RA patients puts a heavy economic burden on many countries, and today’s basic goal is to minimize or fully remove this burden. Although great improvements have occurred in current RA treatment modalities, side effects and the ineffectiveness of treatment remain limitations of the current medications. Therefore, new therapeutic agents are required in the treatment of RA.

Gemcitabine is a pyrimidine antimetabolite that is anabolized to a defluorodeoxycytidine-triphosphate, which is incorporated into DNA and is used to treat several cancers. Nowak et al. (11) showed that GEM causes a profound depletion of lymphocytes and impairs antigen-specific cellular and humoral immunity. Conversely, it has been reported that GEM therapy decreases the count of memory T cells but does not deplete the activation of inflammatory cells (19). GEM treatment has resulted in the upregulation of IL-23R in the A549 cell line (20). On the other hand, Landi et al. (21) reported that GEM attenuated psoriasis, an inflammatory disease. In our study, GEM ameliorated arthritis in a CIA model.

T cells are widely known to orchestrate the inflammatory process in RA, even though the pathogenesis of RA has not yet been fully elucidated (2,3). Th17 cells and IL-17 are two important agents in RA pathogenesis (22). It was reported that IL-17 provoked inflammations through other proinflammatory cytokines, including TNF-α and IL-1, and it leads to massive cartilage and bone destruction (23–25). Another study reported that IL-17 antibody treatment prevented cartilage and bone destruction (26).

In our study, GEM applications decreased IL-17 levels.

TNF-α is a cytokine with a significant function in RA pathogenesis, and it was previously reported that it exists in high amounts in synovial liquids and tissues. In addition, the serum level of soluble TNF receptors has been shown to be associated with increased inflammation and activity of the disease in RA patients (27,28). Currently, anti-TNF agents are used widely and successfully to treat RA. In our study, the mean TNF-α level was decreased in the GEM-treated groups in addition to the IL-17 level. According to these results, it can be concluded that the antiarthritic effect of GEM is a consequence of its antiinflammatory effects. Previous studies have demonstrated the antiinflammatory potential of GEM in vitro settings (11,19).

Oxidative stress increases in patients with RA (7). The radical derivatives of oxygen, such as superoxide and hydrogen peroxide, are the most important free radicals. Reactive oxygen radicals are released from inflammatory cells in the synovium and they contribute to pannus formation and joint damage (8). SOD, CAT, and GPx are antioxidant enzymes that play prominent roles in the defense system against reactive oxygen species. Increased free radical products can cause decreased antioxidant enzyme levels. Previous studies have reported decreased activity of antioxidant enzymes in patients with RA (29–31). In another study, Iyama et al. (32) showed that SOD treatment reduces cartilage/bone destruction and proinflammatory cytokines in experimentally induced arthritis. In our study, we determined that SOD, CAT, and GPx levels were increased in the GEM-treated groups.

Malondialdehyde, one of the reactive aldehydes, is a product of lipid peroxidation and it plays a role in oxidative stress. Reactive aldehydes cause damage in the membrane structure and other cell components (28). Previous studies found an increase in MDA levels in the synovial liquids and serums of RA patients (33,34). Our study documented that the MDA level was increased in the arthritis group compared to the control group, and it was decreased in the GEM-treated group compared to the arthritis group.

Nrf2, a redox-sensitive transcription factor, binds to ARE. ARE enhances genes encoding many detoxifying or antioxidant enzymes. Moreover, Nrf2 is related to stress-responsive proteins including glutathione S-transferase, GPx, and HO-1. Thus, Nrf2 regulates the redox status and plays a key role in cellular defense by enhancing the removal of reactive oxygen species (35). It was documented that Nrf2 knockout mice had more severe cartilage injuries and more oxidative damage in an experimental arthritis group (36). It was also reported that the expressions of Nrf2 target genes are enhanced in Nrf2 wild-type mice, but not in knockout mice (36,37). These results support a protective role of Nrf2 against joint inflammation.

In our study, Nrf2 and HO-1 expressions were decreased in the arthritis group compared to the control group, and it was decreased in the GEM-treated group compared to the arthritis group.

In our study, Nrf2 and HO-1 expressions were decreased in the arthritis group compared to the control group, and it was decreased in the GEM-treated group compared to the arthritis group. In vitro, Nrf2 and HO-1 expressions were decreased in the arthritis group, while GEM treatment increased their expressions. Duong et al. (38) documented that in vitro GEM applications increased the expressions of Nrf2 and HO-1. GEM can activate Nrf2 and HO-1. These positive effects of GEM may also be a consequence of its direct antiinflammatory effect. However, in both situations, the antioxidant potential of GEM may ultimately contribute to its antiarthritic effects.

The limitations of our study are as follows. First, it documents the early effects of GEM treatments on arthritis, but not its long-term effects, which also need to
be researched. Second, radiographic progressions need to be evaluated.

In conclusion, GEM, a nucleoside antimetabolite, decreases the levels of TNF-α and IL-17, increases antioxidant activity, and prevents cartilage/bone destruction in the CIA model. The present study suggests that GEM may be a viable candidate for research on the treatment of RA.

References


