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The Effect of Curcumin on T Helper 1/T Helper 17 Balance in Rat Collagen-Induced Arthritis Model

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Abstract: Immunomodulatory imbalance is the characteristic feature of autoimmune diseases like rheumatoid arthritis (RA). T helper (Th) 17 plays a critical role in the pathogenesis of RA. The balance of Th1 and Th17 is important for normal immune response. In RA therapy, methotrexate (MTX) has an important use depending on its anti-inflammatory effects, however, is limited due to its side effects. Curcumin is a natural plant product used as an immunomodulator. This study aimed to investigate the impact of curcumin and MTX administration singly or combined on a rat model with collagen-induced arthritis. The results revealed that the level of serum interleukin (IL)-17 showed a positive correlation with IL-23, interferon-gamma (IFN-γ) and transforming growth factor-beta (TGF-β) and a negative correlation with IL-27 at the end of the experiment. Treatment with curcumin, MTX and their combinations also caused a significant ($P<0.05$) reduction in the pro-inflammatory cytokines (IL-17, IL-23, IFN-γ, and TGF-β) and improvement of the histopathological appearance of the hind paw as compared to arthritic rats at the end. However, single treatment with curcumin added more amelioration by induction of chondrocytes in the histopathology of the hind paw. Thereby, the combined treatment of curcumin and MTX is associated with suppression of Th1/Th17 cytokines and showed a significant production of IL-27 as a protective cytokine.

Key words: T Helper 17 · Cytokines · Curcumin · Methotroxate · Collagen-Induced Arthritis · Interleukin-27 · Transforming Growth Factor-Beta · Interferon-Gamma, Interleukin-23

INTRODUCTION

Rheumatoid arthritis (RA) is a major human autoimmune disease which is a chronic progressive disease but with unknown etiology [1,2]. RA generally affects various body joints being a chronic systemic inflammatory disorder [3,4]. Many previous studies documented that T cells, B cells and cytokines play a pivotal role in the pathogenesis of RA [5, 6].

The major cell type in the synovial infiltration in the joints is T cells [7-9]. Attention was drawn to the pro-inflammatory cytokines that may lead to the initiation and augmentation of chronic inflammation in RA. Thus, using of animal models to investigate cytokine-dependency in each phase of arthritis progression was taken into consideration [10]. An interrelationship between synovial fibroblasts and T helper (Th)-17 immunity was found in animal models of RA, , as they promote the migration of Th17 cells to the affected joints causing a profound increase in interleukin (IL)-17 production, leading to a chronic inflammation that characterizes RA [10].

The increase of IL-17 levels was found to promote the production of other cytokines that may mediate cartilage destruction such as IL-1β, tumor necrosis factor-alpha (TNF-α), IL-6 and receptor activator of nuclear factor kappa-beta ligand (RANKL) as well as chemokines such as macrophage inflammatory protein and IL-8 [11]. These pro-inflammatory cytokines involved in progression of RA via an upregulation of cyclooxygenase and metalloproteinases leading to activation of joint inflammation and destruction of synovial joints. IL-27 is a heterodimeric cytokine, produced by many cells including B cells, monocytes, macrophages and dendritic cells. It belongs to the IL-6/IL-12 cytokine family. It has been mentioned to be implicated in the pathogenesis of autoimmune diseases, with a pivotal role as both a pro- and anti-inflammatory cytokine. The immunosuppressive effects of IL-27 have been associated with inhibiting the development of Th17 cells and inducing IL-10 production.

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IL-27 has an influence on various B cell subsets and suppression of antibody production [12].

Methotrexate (MTX) is the most used RA therapies as an immunosuppressive agent [13]. MTX is the first line therapy for RA [14], as it acts on actively proliferating cells inhibiting the synthesis of DNA, RNA thymidine and proteins. In addition, MTX was shown to be related to the decrease of macrophages, T cells and plasma cells' number. It also may inhibit osteoclastogenesis by decreasing the production of TNF-a and IL-6 or by reducing RANTL secretions by synovial fibroblasts or macrophages [15]. In RA therapy, the used drugs expected to modulate the inflammation processes, suppress the joint destruction and are safe from side effects. Unfortunately, the majority of these drugs typically are accompanied by severe side effects including gastrointestinal bleeding, increased blood pressure, accelerated osteoporosis, myelosuppression, hepatotoxicity, ocular toxicity, hypersensitivity and allergic reactions, as well as increased risk of infections [16,17]. Therefore, there is a growing direction of using natural products from plant extracts as therapeutic agents for many diseases.

Scientists are heading to study the impact of complementary and alternative medicine for treatment trying to avoid the side effects and high cost of conventionally used anti-inflammatory drugs. Curcumin, a hydrophobic polyphenol (an active constituent of turmeric) proved to have a supposed effect on the regulation of multiple molecular targets [18]. It has anti-oxidant, anti-inflammatory and pro-apoptotic properties [19, 20]. The present study aimed to induce rat collagen-induced arthritis model (CIA) which is somewhat more analogues to human RA. To assess the influence of curcumin and/or MTX administration on Th17 and Th1 related cytokines during the pathogenesis of the disease. To address the relationship between IL-17 and IL-27. To evaluate the effect of curcumin and/or MTX administration on the histology of hind paw.

MATERIALS AND METHODS

Animals: Male albino rats of Wistar strain, weighing 180-200 g, were obtained from the animal house of National Research Center, Egypt. Rats were allowed to acclimate for one week prior to initiation of experimentation. The environmental conditions were properly standardized with a 12-h dark/light cycle, a constant temperature of 20°C and relative humidity of 50-55%. Rats were fed on a standard rodent diet (Formula diet 5008; Lab diet, St Louis, MO) with water *ad libitum.* The animals were treated and cared according to the National Health and Medical Research Council guidelines and approved by the Institutional Animal Ethics Committee.

Drugs and Chemicals: Complete Freund's adjuvant (CFA), dimethyl sulfoxide (DMSO) and curcumin were purchased from Sigma, St. Louis, Mo, USA. Lyophilized bovine type II collagen was purchased from BioCol GmbH, Michendorf, Germany and MTX was purchased from Ebewe, Austria. Interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β), IL-17, IL-23 and IL-27 were purchased from WKEA MED Supplies Corp, NY, USA. Curcumin was dissolved in DMSO at a dose of 100 mg/kg body weight (b.w) and administered intraperitoneally (i.p) thrice a week [21]. MTX was dissolved in phosphate buffered saline at a dose of 1.0 mg/kg (b.w) once a week, administered i.p [21,22]. DMSO was administered i.p at a dose of 0.2 ml/100 mg b.w thrice a week [23].

Induction of Collagen-Induced Arthritis: Lyophilized bovine type II collagen was dissolved at 4 mg/ml 0.05M acetic acid by gentle stirring overnight at 4°C. In an ice bath, CFA and collagen solutions were mixed by using a homogenizer with a small blade (5mm in diameter). One volume of collagen solution was added to an equal volume of CFA drop-wise while mixing at low speed. Mixing was continued until a stiff emulsion resulted at maximum speed (approximately 30,000 r.p.m. for 2-3 min). The emulsion was cooled on ice bath prior mixing. The emulsion was transferred to Hamilton glass 1 ml syringe. 0.2 ml (200 µg collagen) of the emulsion was injected subcutaneously at the base of the tail. A booster injection (0.1 ml of the emulsion) was administered on day 7 after initial immunization. All treatments were initiated from the 20th day and continued till day 69.

Experimental Design: Animals were divided into 7 groups, 10 rats each: Group N: normal rats were left without any treatment as control. Group D: (vehicle control) injected with 0.2 ml/100 g b.w DMSO, i.p route thrice a week for 7 weeks. Group A: (arthritis control) rats were immunized with lyophilized bovine type II collagen in CFA at the base of the tail to induce arthritis and then were injected with a booster dose on day 7. Group Cb: (curcumin+arthritis+curcumin) rats were injected with curcumin by a dose of 100 mg/Kg b.w thrice a week for 7 weeks by i.p route prior to induction of arthritis and as a treatment after induction for another 7 weeks. Group Ca: (arthritis+curcumin) arthritic rats were injected with curcumin by a dose of 100 mg/kg b.w thrice a week for 7 weeks by i.p route. Group M: (arthritis+MTX) arthritic rats were injected with 1 mg/Kg b.w MTX by i.p route once a week for 7 weeks. Group CM:

(arthritis+MTX+curcumin) arthritic rats were injected with 1 mg/ Kg b.w MTX by i.p route once a week for 7 weeks. After 30 min of MTX treatment, the same rats were injected with 100 mg/kg b.w curcumin thrice a week by i.p route for 7 weeks. Blood samples were collected on days 0, 20, 41, 48, 61 and 69 and collected sera were used for assaying different cytokines.

Histological Examination of Hind Paws: Rats were euthanized on the 69th day, the hind paws were harvested and fixed for 3-7 days with 10% phosphate-buffered formalin (Fisher Scientific, Fair Lawn, NJ), washed in slowly running tap water for 30 min Thereafter, paws were decalcified in HCI/Formic acid (1:1) working solution (Fisher Scientific), the solution was changed each day until decalcification was complete [1]. Once decalcification was complete, the paws were rinsed in water briefly and transferred to ammonia solution and left for 30 min to neutralize acids left in the specimens, washed in running tap water thoroughly up to 24 h, embedded in paraffn. Transverse and longitudinal serial sections of 6 to 12 µm were stained in alum haematoxylin and eosin (H&E) (Sigma-Aldrich), examined under the microscope for morphological and cellular infiltrations assessment in the joints.

Cytokine Assays: The concentrations of IFN-γ, TGF-β, IL-17, IL-23 and IL-27 in serum samples were determined by sandwich Enzyme Linked Immunosorbont Assay (ELISA). The assays were performed as suggested by the manufacturer's instructions (WKEA MED Supplies Corp, NY, USA). The levels of cytokines were expressed as pg/ml.

Statistical Analysis: Data were represented as mean ± standard error of mean. The present data were analyzed using one-way ANOVA to demonstrate the effect of different treatments on the concentration of cytokines. In addition, post-HOC was applied to compare the significant difference among the examined groups at $\alpha = 0.05$. Correlation coefficient was used to fit the relationship between various cytokines using SPSS version 20.0.

RESULTS

The onset of arthritis occurred between days 10-15, it was characterized by redness and swelling of the hind paw and was confirmed by histological examination.

Cytokines Assays: We investigated the immunological changes associated with the severity of CIA in Wistar rats at different time points following curcumin and/or MTX treatment including the kinetics of Th17 and Th1 responses. The levels of different cytokines were monitored in the various groups utilized in the study.

***Interleukin-17*:** As depicted in Table (1), there was a profound significant increase in the level of IL-17 in group A compared to groups N and D at all the time points. However, the IL-17 levels dropped significantly in groups Ca, M and CM as compared to group A from day 41 until day 69. In group Cb, the IL-17 level gradually decreased after day 20 compared to groups N, D and A.

***Interleukin-23*:** Data presented in Table (2) demonstrated that the level of IL-23 in group A was significantly higher than groups N and D along the period of experiment except at day 41. At day 20, the level of IL-23 in group Cb was significantly higher than group N and significantly lower than group A (Table 2). At day 41, groups CM and Ca exhibited significant elevation regarding all other groups, while the lowest level was found in group M. At day 48, the mean level of IL-23 was significantly elevated in group Ca in comparison to all other groups. On the other hand, the other groups showed no significant changes as the level ranged between (16.85±0.23) and (17.48±1.59). At days 61 and 69, IL-23 level was significantly elevated in group A compared to most other groups, whereas the lowest levels were found in groups M and CM.

***Interferon-Gamma*:** At day 20, level of IFN-γ was significantly elevated in group Cb compared to groups N and D (Table 3). In contrast, groups CM and M showed no significant changes with restoration of normal levels from day 41 till the end of the experiment.

***Interleukin-27*:** At day 20, the serum level of IL-27 was significantly increased in group A compared to groups N and Cb (Table 4). At day 41, the IL-27 level in group A dropped profoundly compared to its level in day 20 and continued to decrease till the end of the experiment. However, the level of IL-27 was significantly increased (5.25 ± 0.23) in group M at day 41 in comparison to other groups under the study, but at the end of the experiment (day 69) the level dropped (2.83 ± 0.22) profoundly. The least level of IL-27 appeared in group CM and exhibited restoration as compared to group N. At day 48, the mean level of IL-27 was elevated in groups A, Cb, M and CM regarding groups N and D. At day 61 the serum level of IL-27 was significantly increased in group CM, followed by group M, whereas the other groups showed no significant changes compared to groups N and D. While at day 69, the level of IL-27 in group CM followed by Ca

Table 1: Interleukin -17 level in normal and arthritic rats before and after treatment with curcumin and methotrexate singly or combined

Group	Day 0	Day 20	Day 41	Day 48	Day 61	Day 69
N	$14.57^{a} \pm 0.36$	$14.62^{b} \pm 0.04$	$14.66^{c} \pm 0.32$	$14.68^{b} \pm 0.33$	$14.73^{b} \pm 0.36$	$14.79^{b} \pm 0.02$
D			$13.86^{c} \pm 0.38$	$14.76^{b} \pm 0.63$	$14.98^{b} \pm 1.04$	$14.85^{b} \pm 0.04$
Cb	$12.27^{b} \pm 0.36$	$17.63^{a} \pm 0.03$	$16.34^{b} \pm 0.16$	$16.27^{ab} \pm 0.1$	$14.76^{b} \pm 0.15$	$13.31^{c} \pm 0.82$
A		$16.73^{a} \pm 0.94$	$16.99^{a} \pm 0.45$	$17.34^{a} \pm 1.29$	$17.37^{a} \pm 0.32$	$17.61^{a} \pm 0.08$
Ca			$16.05^{b} \pm 0.11$	$15.51^{b} \pm 0.53$	$15.45^{b} \pm 0.69$	$14.44^{b} \pm 0.44$
M			$16.03^{b} \pm 0.86$	$15.73^{b} \pm 0.5$	$15.58^{b} \pm 0.90$	$15.27^{b} \pm 0.69$
CM			$16.48^{b} \pm 0.12$	$15.79^{b} \pm 0.39$	$15.74^{b} \pm 0.63$	$15.47^{b} \pm 0.52$

Data are represented as mean ± standard error of mean. In columns: Means with the same superscript letters are similar (insignificant, $P > 0.05$), whereas others are significant ($P < 0.05$) at α=0.05. N: normal, D: rats injected with DMSO, Cb: rats injected with curcumin before induction of arthritis then treated with curcumin after induction, A: arthritic nontreated rats, Ca: arthritic rats treated with curcumin, M: arthritic rats treated with MTX and CM: arthritic rats injected with both curcumin and MTX

Table 2: Interleukin-23 level in normal and arthritic rats before and after treatment with curcumin and methotrexate singly or combined

Group	Day 0	Day 20	Day 41	Day 48	Day 61	Day 69
N	$16.74^{a} \pm 0.17$	$16.8^{b} \pm 0.19$	$16.76^{bc} \pm 0.59$	$16.85^{bc} \pm 0.23$	$16.82^{b} \pm 0.10$	$16.87^{b} \pm 0.41$
D			$16.52^{bc} \pm 1.26$	$17.45^{bc} \pm 1.37$	$16.63^{b} \pm 3.43$	$16.85^{b} \pm 0.62$
Cb	$16.25^{a} \pm 1.02$	$22.26^{a} \pm 0.55$	$18.77^{b} \pm 2.26$	$17.48^{bc} \pm 1.59$	$14.97^{bc} \pm 0.97$	$14.56^{b} \pm 1.79$
A		$12.30^{c} \pm 1.21$	$14.95^{c} \pm 0.72$	$18.49^{b} \pm 1.46$	$19.56^{a} \pm 1.67$	$22.62^{a} \pm 0.48$
Ca			$20.79^{a} \pm 0.99$	$20.42^{a} \pm 0.93$	$17.64^{ab} \pm 1.21$	$15.13^{b} \pm 0.83$
M			$13.47^{c} \pm 0.80$	$13.46^{c} \pm 1.63$	$13.41^{c} \pm 1.32$	$11.74^{c} \pm 0.99$
CM			$22.32^{a} \pm 1.94$	$18.53^{b} \pm 1.49$	$13.76^{c} \pm 1.77$	I 1. $93^{c} \pm 1.46$

Data are represented as mean ± standard error of mean. In columns: Means with the same superscript letters are similar (insignificant, $P > 0.05$), whereas others are significant ($P < 0.05$) at α=0.05. N: normal, D: rats injected with DMSO, Cb: rats injected with curcumin before induction of arthritis then treated with curcumin after induction, A: arthritic nontreated rats, Ca: arthritic rats treated with curcumin, M: arthritic rats treated with MTX and CM: arthritic rats injected with both curcumin and MTX

Table 3: Interferon-gamma level in normal and arthritic rats before and after treatment with curcumin and methotrexate singly or combined

Goup	Day 0	Day 20	Day 41	Day 48	Day 61	Day 69
N	$92.14^{b} \pm 1.11$	$92.18^{c} \pm 0.82$	$92.31^{d} \pm 1.07$	$92.29^{c} \pm 1.4$	$92.25^{d} \pm 1.10$	$92.0^{bc} \pm 0.50$
D			$92.35^{d} \pm 0.25$	$92.6^{c} \pm 2.75$	$92.75^{d} \pm 3.25$	$92.5^{bc} \pm 1.0$
Cb	$154.17^{a} \pm 9.97$	$179.5^{a} \pm 16.27$	$162.50^{a} \pm 8.1$	$158.33^{a} \pm 15.15$	$144.17^{a} \pm 5.8$	$133.0^{a} \pm 4.25$
A		$121.0^{b} \pm 2.52$	$125.83^{b} \pm 1.69$	$128.33^{b} \pm 4.67$	$135.5^{b} \pm 5.19$	$135.33^{a} \pm 4.68$
Ca			$136.67^{ab} \pm 3.93$	$118.67^{bc} \pm 0.93$	$116.5^{bc} \pm 5.84$	$106.0^{b} \pm 3.01$
M			$102.67^{cd} \pm 3.34$	$97.0^{c} \pm 6.33$	$96.25^{cd} \pm 3.25$	$87.75^{c} \pm 7.25$
CM			$104.83^{cd} \pm 1.59$	$99.0^{c} \pm 3.5$	$97.83^{cd} \pm 10.46$	$91.50^{bc} \pm 6.79$

Data are represented as mean ± standard error of mean. In columns: Means with the same superscript letters are similar (insignificant, $P > 0.05$), whereas others are significant ($P < 0.05$) at α=0.05. N: normal, D: rats injected with DMSO, Cb: rats injected with curcumin before induction of arthritis then treated with curcumin after induction, A: arthritic nontreated rats, Ca: arthritic rats treated with curcumin, M: arthritic rats treated with MTX and CM: arthritic rats injected with both curcumin and MTX

Table 4: Interleukin -27 level in normal and arthritic rats before and after treatment with curcumin and methotrexate singly or combined

Group	Day 0	Day 20	Day 41	Day 48	Day 61	Day 69
N	$2.61^{b} \pm 0.13$	$2.55^{c} \pm 0.36$	$2.59^{c} \pm 0.02$	$2.56^{b} \pm 0.17$	$2.62^{c} \pm 0.06$	$2.67^{c} \pm 0.31$
D			$3.21^{bc} \pm 0.41$	$2.86^{b} \pm 0.27$	$2.63^{c} \pm 0.25$	$2.87^{c} \pm 0.06$
Cb	$3.38^{a} \pm 0.21$	$3.46^{b} \pm 0.35$	$3.48^{b} \pm 0.16$	$3.41^{a} \pm 0.2$	$3.46^{bc} \pm 0.44$	$3.65^{b} \pm 0.66$
A		$4.15^{a} \pm 0.63$	$3.88^{b} \pm 0.39$	$3.49^{a} \pm 0.06$	$3.35^{b} \pm 0.15$	$3.32^{bc} \pm 0.2$
Ca			$3.04^{bc} \pm 0.17$	$3.14^{ab} \pm 0.34$	$3.24^{bc} \pm 0.11$	$3.84^{b} \pm 0.27$
M			$5.25^{a} \pm 0.23$	$3.37^{a} \pm 0.25$	$3.65^{b} \pm 0.39$	$2.83^{c} \pm 0.22$
CM			$2.83^{c} \pm 0.42$	$3.22^{a} \pm 0.22$	$4.47^{a} \pm 0.17$	$4.59^{a} \pm 0.13$

Data are represented as mean ± standard error of mean. In columns: Means with the same superscript letters are similar (insignificant, $P > 0.05$), whereas others are significant ($P < 0.05$) at α=0.05. N: normal, D: rats injected with DMSO, Cb: rats injected with curcumin before induction of arthritis then treated with curcumin after induction, A: arthritic nontreated rats, Ca: arthritic rats treated with curcumin, M: arthritic rats treated with MTX and CM: arthritic rats injected with both curcumin and MTX

Table 5: Transforming growth factor-β level in normal and arthritic rats before and after treatment with curcumin and methotrexate singly or combined Effect of curcumin and methotrexate treatment on TGF-β levels in normal and arthritic rats

Group	Day 0	Day 20	Day 41	Day 48	Day 61	Day 69
N	$20.99^{a} \pm 1.58$	$21.02^{b} \pm 0.29$	$21.12^{b} \pm 0.51$	$21.07^{b} \pm 1.31$	$21.11^{b} \pm 0.95$	$21.19^{b} \pm 0.27$
D			$19.31^{b} \pm 0.16$	$19.47^{b} \pm 1.16$	$19.58^{b} \pm 0.50$	$20.68^{b} \pm 0.70$
Cb	$24.03^{a} \pm 0.89$	$27^{a} \pm 1.09$	$26.95^{ab} \pm 4.02$	$26.26^{ab} \pm 1.16$	$25.92^{ab} \pm 1.96$	$25.23^{ab} \pm 0.51$
A		$27.1^{a} \pm 2.21$	$28.54^{a} \pm 2.77$	$28.56^{a} \pm 0.74$	$28.81^{a} \pm 3.50$	$29.96^{a} \pm 0.50$
Ca			$23.23^{b} \pm 2.04$	$22.69^{b} \pm 2.32$	$21.08^{b} \pm 1.86$	$21.04^{b} \pm 1.03$
M			$22.04^{b} \pm 1.58$	$20.46^{b} \pm 0.8$	$20.25^{b} \pm 0.94$	$18.48^{b} \pm 1.61$
CM			$21.93^{b} \pm 0.38$	$19.87^{b} \pm 1.31$	$19.89^{b} \pm 0.86$	$18.66^{b} \pm 1.52$

Data are represented as mean ± standard error of mean. In columns: Means with the same superscript letters are similar (insignificant, $P > 0.05$), whereas others are significant ($P < 0.05$) at α=0.05. N: normal, D: rats injected with DMSO, Cb: rats injected with curcumin before induction of arthritis then treated with curcumin after induction, A: arthritic nontreated rats, Ca: arthritic rats treated with curcumin, M: arthritic rats treated with MTX and CM: arthritic rats injected with both curcumin and MTX

Table 6: Correlation between of the level of IL-17 and levels of IL-23, Il-27, IFN-γ and TGF-β at day 69 in all groups of the study

Group	IL-23	IL-27	IFN-γ	TGF-β
Cb	+0.93	-0.93	+0.84	+0.65
A	+0.97	-0.41	+0.97	+0.98
Ca	+0.93	-0.81	+0.95	+0.96
M	+0.83	-0.94	+0.98	+0.75
CM	+0.95	-0.98	+0.93	+0.78

Cb: rats injected with curcumin before induction of arthritis then treated with curcumin after induction, A: arthritic non¬treated rats, Ca: arthritic rats treated with curcumin, M: arthritic rats treated with MTX and CM: arthritic rats injected with both curcumin and MTX

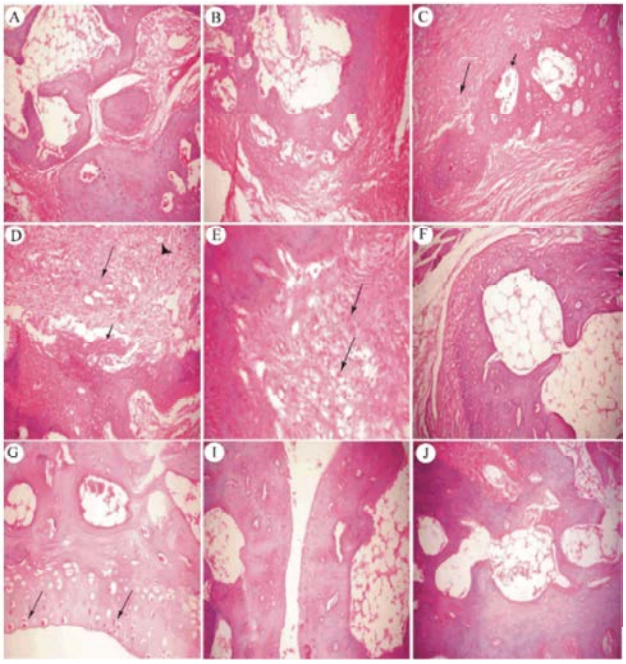

Fig. 1: Histological features of hind paw among various studied groups at the end of the experiment. (A) Hind paw sections from normal group (group N) showed normal histopathological architecture. (B) Hind paw sections from DEMSO treated group (group D) showed normal hispathology. (C, D, E) Hind paw sections from group A (arthritis rats) showed; microfractures of articular cartilage (small arrow) associated with fibroblasts proliferation (large arrow) (C), necrosis of articular cartilage (small arrow) and focal haemrrhage (arrow head) (D), leukocytic cells infiltration (arrows) (E). (F) Group Cb (curcumin 100 mg/kg +CIA) showed no hispathological changes. (G) Group Ca (arthritis+curcumin 100 mg/kg) showed proliferation of chondrocytes (arrows). (H, I) Group M (arthritis+methotrexate) and group CM (arthritis+methotrexate +curcumin) showed normal histological appearance. All tissues stained with hematoxylin and eosin H&E. (Figs. A, C, D, I, J X200; Figs. B, E, F, G X400)

and Cb exhibited higher significant levels compared to groups N and D. In group CM, the IL-27 level was elevated throughout the experiment from days 48 to 69 in comparison to group N.

***Transforming Growth Factor-β*:** The level of TGF-β throughout the experimental intervals is illustrated in Table 5. At day 20, TGF-β level was significantly increased in groups A and Cb compared to groups N and D. In group A, the serum level of TGF-β was significantly higher than groups N and D at all the time points. From day 41 to day 69, the level of TGF-β in groups Ca, M and CM was not significantly changed from group N and group D.

***Correlation Analysis Between IL-17 and the Other Cytokines*:** Relation between the level of IL-17 and other cytokines in the experimental groups at day 69 is represented in Table 6. Serum level of IL-17 showed a positive correlation with the levels of IL-23, IFN-γ and TGF-β. However, serum level of IL-17 showed a negative correlation with IL-27 level including correlation coefficients ranging between -0.41, -0.81 -0.93, -0.94, -0.98 in groups A, Ca, Cb, M and CM respectively. Additionally, group CM revealed the highest negative correlation value than all other groups.

Histology of Hind Paws: Histological examination of hind paws at the end of the experiment was demonstrated in Fig. 1. Hind paws from group N showed normal architecture with no inflammatory cells infiltrations (Fig. 1A). Also, most sections from group D revealed no histopathological changes (Fig. 1B). Paws from group A showed widening of synovial cavity, thickening of the synovial membrane, disruption of the cartilaginous tissue and evidence of bone damage as compared to normal joint structure. Sections revealed microfractures of articular cartilage associated fibroblasts proliferation (Fig. 1C), necrosis of articular cartilage associated with fibrosis and focal haemorrhge (Fig. 1D) and leukocytic cells infiltration (Fig. 1E). Normal histological structure was noticed in most sections from groups Cb, M and CM (Figs. 1F, 1H and 1I). Group Ca revealed proliferation of chondrocytes in the paw of some sections (Fig. 1G).

DISCUSSION

The cellular mechanism of bone and cartilage destruction in RA still remains unclear [17]. Synovial tissues of RA joints produce various inflammatory cytokines, such as IL-lα and TNF-α, which are believed to play important roles in joint destruction. One key property of IL-I7A is its orchestral role in mediating the migration of inflammatory cells, which takes a central place in RA pathogenesis [24]. It is thought that Il-23 plays an important role in the survival and expansion of pathological Th17 cells [25-27]. IL-23 is involved in the differentiation of Th17 cells in a pro-inflammatory complex, especially in the presence of TGF-β and IL-6 [25]. Notably, the increase in IL-17 and IL-23 appears to be specific for RA [26].

MTX is the central drug in the management of RA, known to suppress joint destruction in RA and other immune mediated inflammatory diseases. However, MTX induces significant adverse events in a considerable number of patients [28]. Considering these toxicological aspects, combination therapy of curcumin and MTX may provide beneficial therapeutic strategy with reduced side effects in RA. In the present study, we assessed the level of IL-17 as the signature cytokine of Th17 and other cytokines during the course of CIA model and to investigate the effect of MTX singly or combined with curcumin.

Curcumin comprises 2-8% of most turmeric preparations and is generally regarded as its most active component, having potent antioxidant, anti-inflammatory and anti-carcinogenic properties [29]. Here, the immunologic pattern of arthritic rats (group A) at the onset of the disease at day 20 characterized with significant lower level of IL-23 and higher levels of IL-17, IL-27, IFN-γ and TGF-β compared to group N. However, pre-treatment with curcumin in group Cb altered this pattern, causing raised levels of IFN-γ between days 20 and 61 and reduced levels of IL-17 between days 41 an 69 compared to group A. The observed increase in IFN-γ level was matched with the reduction in the level of IL-17. It has been reported by other investigators that IFN-γ can downregulate the IL-17 response [30,31]. These results explain its ability to regulate differentiation of CD4+ T cells to Th17 and Th1 in normal state.

The present work showed that treatment with curcumin for seven weeks after arthritis induction (group Ca) induced a significant reduction in the levels of IL-17, IL-23, IFN-γ and TGF-β compared to non-treated arthritic rats (group A). The suppression in the levels of these inflammatory cytokines within curcumin treated arthritic (group Ca) rats suggested an anti-inflammatory effect of curcumin. The elevated expression of the levels of IL-17 and IFN-γ in experimental autoimmune encephalomyelitis model was decreased following *in vivo*

treatment with curcumin [32]. Moreover, Arthritic rats treated with curcumin showed significant inhibition of serum proinflammatory cytokine (IL-1β) when compared with non-treated arthritic [33].

It was stated that IL-17 upregulates the production of pro-inflammatory cytokines especially IFN-γ [34] which indicated the positive correlation between IL-17 and IFN-γ. Th17 cells are developed from naïve CD4+T cells under the influence of a network of inflammatory cytokines including IL-1, IL-6 and TGF-β, which support the commitment to this lineage [26]. Thus, this relation could explain the positive correlation between both IL-17 and TGF-β in the present study. Additionally, we demonstrated a negative correlation between IL-17 and IL-27. This negative correlation could be caused by the inhibitory effects of IL-27 on Th1, Th2 and Th17 subsets of T cells as well as the expansion of inducible regulatory T cells [35-37]. It also inhibits the development of proinflammatory Th17 cells by suppressing the expression of Th17 master transcription factor ROR-γt, thereby preventing the production of IL-17A and IL-17F in naive T cells and up-regulation of IL-l0 expression [38]. In context of the above cytokine balance in the pathogenesis of autoimmune arthritis, the potential anti-arthritic activity of curcumin might skew the cytokine milieu towards reducing the pathogenic Th1 (IFN-γ)/Th17 (IL-17) response. These findings highlight that curcumin differentially regulates CD4+ Th cell responses in CIA.

In the current work, post-treatment with MTX (group M) ameliorates CIA rats, causing a reduction in the levels of IL-17, IL-23, IFN-γ and TGF-β significantly compared to non-treated arthritic rats (group A) throughout the experiment. However, IL-27 level was not significantly changed from days 48 to 69 in comparison to group A. This reduction in the serum levels of the measured cytokines can be attributed to the anti-inflammatory properties and an additional anti-proliferative activity of MTX. Also, the immunosuppressive activity of MTX, mediated by apoptosis of selective activated, but not resting, T cells of peripheral blood in the S/G2 phase of the cell cycle, even after short-term exposure to MTX [39].

The present data demonstrated that combined treatment of MTX and curcumin has the ability to ameliorate the clinical severity of CIA rats at the end of the experiment, causing a significant decrease in the levels of IL-17, IL-23, IFN-γ and TGF-β and an increase in the level of IL-27 compared to group A. Comparing with curcumin or MTX single used groups, combined treatment of curcumin and MTX showed a significant increase in the level IL-27 between days 61 and 69 in comparison to group A. Thus, IL-27 plays an important role as a protective cytokine in the pathogenesis and proposed as a potential treatment for arthritis [40,41].

RA is a systemic disease that affects a multitude of organs. Previously, it has been shown that RA induction caused drastic histopathological changes in thymus, liver, lung and kidney sections [42]. Additionally, the destructive effects accompanied with MTX treatment on these secondary organs were minimized and ameliorated by combined treatment of both curcumin and MTX [43]. Our data illustrated that the induction of arthritis caused a severe and drastic damage in the hind paws as observed in the examined sections from group A. MTX and/or curcumin treatment suppressed and ameliorated the drastic damage caused by the induction of arthritis. MTX has a powerful anti-inflammatory effect *in vivo* and inhibits human synovial fibroblast RANKL production and osteoclastogenesis in a dose-dependent manner [15]. Also, in case of group Ca curcumin treatment caused the proliferation of chondrocytes. These results are in agreement with previous studies which demonstrated that curcumin is a promising therapeutic agent for the treatment of RA as it has anti-inflammatory and anti-apoptotic effects in chondrocytes [44]. Curcumin has activities similar to the anti-TNF drugs, but without their serious side-effects [3].

CONCLUSION

The findings of the study indicate that curcumin has an ameliorative effect on CIA rats, involving downward trend in pro-inflammatory cytokines, including IL-17, IL-23, IFN-γ and TGF-β. Notably, curcumin treatment induced the proliferation of chondrocytes. An inverse interaction is considered between IL-17 and IL-27. Therefore, the combined treatment with both curcumin and MTX could modulate the cytokines production through upregulation of the protective cytokine represented by IL-27, caused the restoration of the normal structure of the hind paw.

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