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Original article:

PROTECTIVE EFFECT OF CURCUMIN ON EXPERIMENTALLY INDUCED ARTHRITIC RATS: DETAILED HISTOPATHOLOGICAL STUDY OF THE JOINTS AND WHITE BLOOD CELL COUNT

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ABSTRACT

Curcuma longa (turmeric) rhizomes contains curcumin, an active compound which possesses anti-inflammatory effects. Collagen-induced arthritis (CIA) is an accepted experimental animal model of rheumatoid arthritis. The present study aimed to observe the histological changes in the joints of experimental arthritic rats treated with curcumin. Twenty four male Sprague-Dawley (approximately 7 weeks-old) rats were randomly divided into four groups. Three groups were immunized with 150 µg collagen. All rats with established CIA, with arthritis scores exceeding 1, were orally treated with betamethasone (0.5 mg/ml/kg body weight), curcumin (110 mg/ml/kg body weight) or olive oil (1.0 ml/kg body weight) daily, for two weeks. One remaining group was kept as normal control. Treatment with 110 mg/ml/kg curcumin showed significant mean differences in the average white blood cell (WBC) count ($p<0.05$), cell infiltration, bone and cartilage erosion scores ($p<0.05$) compared to the olive oil treated group. Pannus formation scores showed that curcumin supplementation successfully suppressed the pannus formation process that occurred in the articular cartilage of the CIA joints. The mean difference for histological scores for the curcumin group was insignificant compared to the betamethasone treated group. It is concluded that supplementation of curcumin has protective effect on the histopathological and degenerative changes in the joints of CIA rats which was at par with betamethasone.

Keywords: experimental, arthritis, collagen, curcumin, inflammation, histopathology

INTRODUCTION

Curcuma longa (CL) is a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae which is native in tropical South Asia. Research reports depict that traditionally it has been used to treat pain, inflammation, tumours and wound (Sharma et al., 2007; Bright, 2007). The powdered form of CL rhizome is commonly used as a spice in cooking curries at many South Asian restaurants and it is also used in the dyeing industry. Its active compound, curcumin possesses an earthy, bitter, peppery flavour (Jagetia & Aggarwal, 2007).

Rheumatoid arthritis (RA) is a chronic autoimmune disease of the joints. RA commonly involves the metacarpophalangeal and proximal interphalangeal joints and the joints of the hand, wrist, shoulder and knee. In Malaysia, RA affects about 5 in 1000 people and 75 % of the sufferers are women, according to reports released by the Arthritis Foundation of Malaysia 2007. The inflammatory changes that occurred in RA cause swelling (oedema), pain, stiffness

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and redness (erythema) of the affected joints. The inflammation reaction in RA also causes damage to the synovial membrane, and peri-articular cartilage and bone. The inflammation leads to ankylosis and destruction of the joints and this may result in disability in the movements (Paleolog, 2002).

Collagen-induced arthritis (CIA) is an experimental autoimmune mediated polyarthritis that is well accepted in different types of rodents. CIA is induced by immunization with type-II collagen, the major constituent protein of articular cartilage. Compared to other experimental arthritis models, CIA has a similar resemblance to human RA in terms of its clinical, histological and immunological features as well as genetic linkage (Trentham et al., 1978). In the present study, the CIA model was tested to be sensitive to betamethasone with expectation of the known anti-inflammatory response (Larsson et al., 2004). Current conventional medications such as NSAIDs and DMARDs have been reported to have various types of adverse effects (Miyake et al., 2008). Patients also seemed to prefer alternative medicine compared to the conventional ones (Tsai et al., 2007).

The present study aimed to study the role of curcumin as an anti-inflammatory agent. The white blood cell count and histopathological features were examined in detail in order to ascertain the protective role of curcumin in experimental arthritis. The results would help in better understanding of the role curcumin in the treatment of arthritis.

MATERIALS AND METHODS

Animals

We followed an earlier protocol for the present study (Taty Anna et al., 2011). Twenty four male *Sprague-Dawley* rats aged around 7 weeks (200–250 g) were obtained from Universiti Kebangsaan Malaysia. The animals were housed individually in each cage with food and water being provided *ad libitum*. The experimental animals were kept under standard laboratory conditions at room temperature with 12 hours light/dark cycle. Prior ethical approval was obtained from the institutional animal ethics committee (UKMAEC) for the study.

Drugs and chemicals

The CL extract (Curcumin C3 complex powder) was purchased from the Sabinsa Company, Malaysia. The HPLC method showed that the bisdemethoxycurcumin was not less than 2.5 % and not more than 6.5 %, demethoxycurcumin being not less than 15 % and not more than 25 % and curcumin content was not less than 70 % and not more than 80 %. Olive oil was used as vehicle. Betamethasone sodium phosphate powder was obtained from the Sigma Company. Bovine type-II collagen in 0.1 M acetic acid and incomplete Freund's adjuvant were purchased from Chondrex, USA.

Experimental arthritis induction

According to an earlier protocol, Bovine type-II collagen in acetic acid was emulsified with incomplete Freund's adjuvant (Brand et al., 2007). All the animals were divided equally into four different groups. Arthritis was induced systemically in three groups on day 0, by injecting 150 µg of bovine type-II collagen emulsified in incomplete Freund's adjuvant (IFA) through subdermal route at the base of the tail. This was based on a modified method by Chondrex, USA (Brand et al., 2007).

Oral supplements preparation

The CL extract (curcumin) and betamethasone were dissolved in olive oil. Care was taken to store the oral supplement solutions in sealed bottles wrapped with aluminium foil and they were kept in a refrigerator (4 ºC). All supplement solutions were replenished weekly.

Treatment of animals

Oral supplement solutions of curcumin, betamethasone and the vehicle (olive oil) were administered daily. It was initiated on the day after the onset of arthritis when the arthritis score exceeded 1 (on day 14) and

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were continued until day 28 of the experiment as per an earlier research protocol (Cai et al., 2005). Rats with established CIA were treated orally with betamethasone (0.5 mg/ml/kg body weight), olive oil (1.0 ml/kg body weight), curcumin (110 mg/ml/kg body weight) (Larsson et al., 2004; Taty Anna et al., 2011). The betamethasone treated and olive oil treated groups were used as positive and negative control groups, respectively.

White blood cell (WBC) count

Blood samples were collected from the retro-orbital sinus on days 0, 14 and 28. A modified method mentioned by Wallberg (2001) was used to determine the WBC count. Dilution factor of 1:20 was used for each of the blood sample together with 2 % glacial acetic acid as a diluent that lysed the red blood cells. Each of the diluted blood samples was loaded into a Neubauer haemocytometer slide chamber for cell counting, under the light microscope.

Histopathological study

Rats were sacrificed on day 28 by cervical dislocation following asphyxiation with diethyl ether. The ankles of the hind paws were dissected and preserved in 10 % buffered formalin solution. The preserved ankles were then decalcified in 10 % formic acid for 21 days, dehydrated and then processed and embedded in paraffin. Specimens were cut longitudinally to the midline, and 5µm sections mounted for staining with hematoxylin and eosin (H & E) (Shealy et al., 2002).

The stained joint sections were scored in blinded manner by two independent observers according to a semi-quantitative scoring system for the histopathological changes as described by earlier researchers (Zhu et al., 2005). Grading of cellular infiltration, synovial hyperplasia, pannus formation and bone and cartilage erosion of the ankle joints were performed using scores of 0 (normal), 1 (mild changes of 1–25 %), 2 (moderate changes of 26–75 %) and 3 (severe changes of 76–100 %). Histopathological scores were combined and expressed as the sum of both right and left ankle joints to give a maximum score of 6 for each histological parameter per rat.

Statistical analysis

Data were expressed as means ± SEM. Pearson's correlation test was used to measure the correlation between scores made by the two blinded observers. One-way analysis of variance with post hoc LSD and Tukey tests were used to analyze differences between treatment groups.

RESULTS

Changes in WBC count

The mean WBC count for the olive oil (olive) treated group showed continuously increasing trend from day 0 to day 28. The contrary was observed in the betamethasone (beta) and 110 mg/ml/kg curcumin (CL) treated groups, which showed an increase from day 0 to day 14 and then a decreased pattern from day 14 to day 28 (Figure 1).

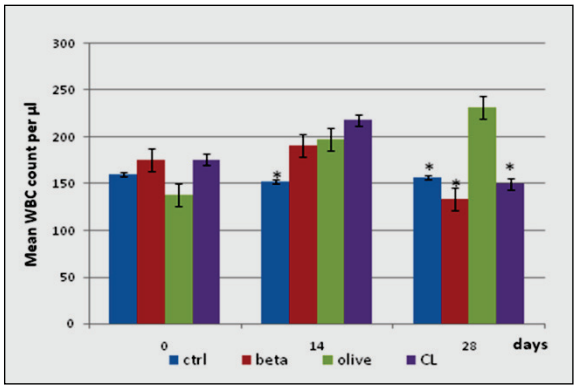

Figure 1: Bar chart of mean white blood cell (WBC) count at day 0, 14 and 28. Each bar represents mean ± SEM. Ctrl – control, beta – betamethasone, olive – olive oil, CL – curcumin groups.
* statistically significant difference compared to the olive oil (negative control) group

Histological scores

The histological changes that occurred due to inflammatory reactions in CIA were observed and scored at the end of the experiment. The common histological characteristics observed were: 1) inflammatory cells infiltration; 2) synovial hyperplasia; 3) pan-

nus formation and 4) cartilage and bone erosion.

Cell infiltration

The mean cell infiltration score for all groups was higher than the normal score of 0. The olive oil group recorded maximum cell infiltration score of 3. The curcumin treated group recorded a mean score which was not significantly different from the betamethasone treated group. The mean score of the control (ctrl), curcumin and betamethasone groups were significantly different compared to the olive oil groups, respectively (Figure 2A).

Synovial hyperplasia

The highest mean synovia hyperplasia score was recorded by olive oil group, followed by curcumin group, betamethasone group and lastly, the control group. Unlike the curcumin group, both the betamethasone and control group scores were significantly different compared to the olive oil group, respectively (Figure 2B).

Pannus formation

The mean pannus formation scores for all treatment groups exceeded the normal score of 0. Betamethasone group recorded the lowest pannus formation score amongst the treated groups. It was followed by curcumin group, and the higest score was recorded by the olive oil group (Figure 2C). Pannus formation score recorded by the betamethasone group differed significantly compared to the olive oil group, but it was not significantly different from the curcumin treated group.

Cartilage and bone erosion

The mean scores for bone and cartilage erosion were generally lower than other histological features mentioned supra. The control group recorded a normal score of 0, and the betamethasone treated group recorded the lowest score amongst the treatment groups. Curcumin treated group recorded a score that was significantly different compared to the olive oil group, but it was not significantly different from the betamethasone group. The olive oil group recorded the highest cartilage and bone erosion score, which was significantly different compared to the other groups (Figure 2D).

DISCUSSION

Previous findings reported the anti-inflammatory effect of betamethasone in the treatment of arthritic symptoms (Larsson et al., 2004). Additionally, glucocorticoids were also reported to be able to reduce the edema and inflammation in rats with CIA) (Sharma et al., 2004). Researchers also reported the decrease in arthritic symptoms due to CL supplementation that caused a reduction in the formation of new blood capillaries (angiogenesis), which commonly occurs in the untreated arthritic joints (Earp et al., 2008; Fassbender, 1988). The angiogenesis process worsens the inflammatory process as the fluid from the blood vessels are filtered out into the affected tissue area and causing oedema. This process is very common in any inflammatory reactions, including the inflammation that occurs in the ankle joints of the CIA rats. Earlier researchers reported that curcumin could reduce the expression of angiogenesis-linked genes, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) that reduces the new blood vessels formation (Aggarwal et al., 2005). Jackson et al. (2006) reported that curcumin could prevent angiogenesis (Maheshwari et al., 2006). Even in one of our earlier studies, we had reported the changes in erythrocyte sedimentation rate and radiological changes as a result of curcumin ingestion in arthritic state (Taty Anna et al., 2011).

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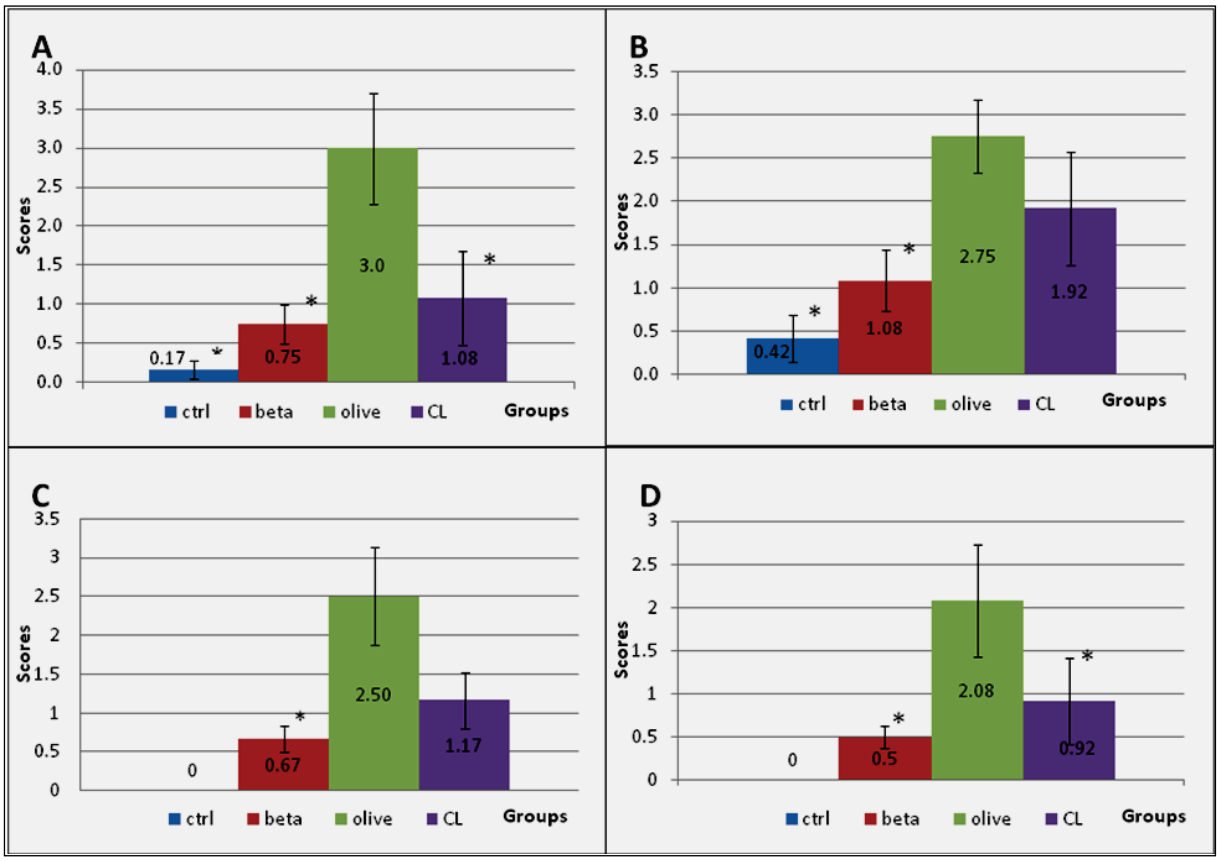

Figure 2: Bar charts for histological scores of the rats' ankle joints. A – cell infiltration, B – synovial hyperplasia, C – pannus formation, D – cartilage and bone erosion scores. Each bar represents mean ± SEM. Ctrl – control, beta – betamethasone, olive – olive oil, CL – curcumin groups.
* statistically significant difference compared to olive oil (negative control) group

White blood cell count increase is an implication of systemic inflammatory reaction within the body (Kumar et al., 2004). It involves the production of more white blood cells such as T cells, B lymphocytes, monocytes and neutrophils. All these cells have their own important role in an inflammatory reaction (Antony et al., 1999). Some of the factors or proteins produced during the reaction, e.g. inflammatory cytokines may further induce the maturity and activation of the white blood cells especially those involved in the cell mediated immune response (VanderBorght et al., 2001). Marked decrease of white cell count after betamethasone or CL extract supplementations suggested that each of those supplements possesses some anti-inflammatory effects towards CIA. These findings are in accordance with an earlier research which stated that treatment with curcumin reduced the proliferation of lymphocytes following the inductive action of IL-2 (Ranjan et al., 2004).

However, Sharma et al. (2005) found that a low dose of curcumin did not have any significant effect on the white cell count in colorectal cancer cases. Therefore, the exact mechanisms involved in those anti-inflammatory effects that caused the decrease in white cell count were still poorly understood. The decrease may be mostly due to the direct effect from the decrease in disease processes as a whole, following the 14 days oral supplementation of 110 mg/kg of curcumin or betamethasone. The histopathological scores for cell infiltration showed that the olive oil (negative control) group recorded the highest score (3.0). This signifies that severe cell infiltration had occurred (76–100 % changes). On the other hand, treatment with 110 mg/kg of curcu-

min had successfully reduced the cell infiltration effect, even though it was not at par with the betamethasone treated group (dose of 0.5 mg/kg).

When treated without the corticosteroid (betamethasone) or curcumin, the inflammatory reactions in CIA progressed quickly (Figure 3b). This was especially observed in the infiltration of inflammatory cells into the joint tissues. The cell infiltration occurred due to the attraction of various immune cells toward the chemokines produced by various activated cells within the synovium. Seemayer et al. (2005) reported that arthritic synovial fibroblasts produced and released chemokines to attract immune cells towards the joint areas (Oelzner et al., 2006).

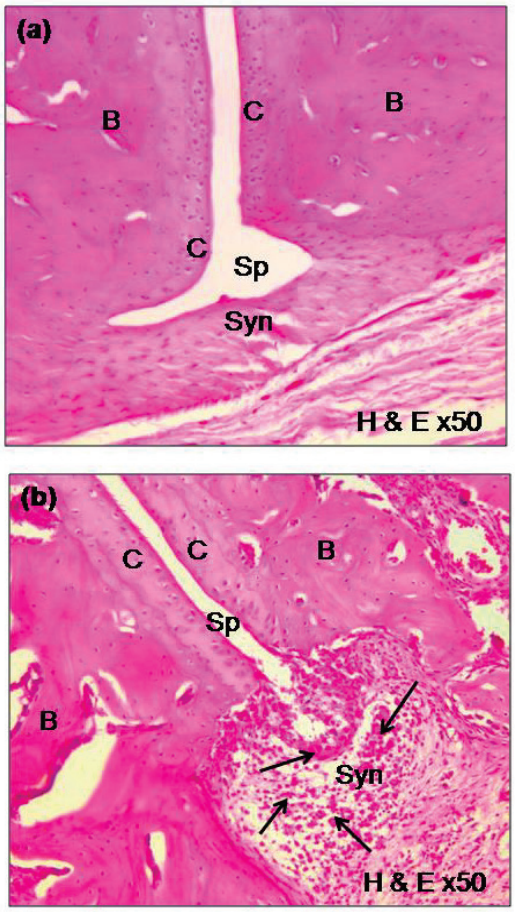

Figure 3: Photomicrographs of (a) normal joint structure and (b) joint with inflammatory cells infiltration. Infiltration of inflammatory cells into the synovial tissue were shown by the black arrows. B – bone, C – cartilage, Sp – joint space, Syn – synovial tissue

Suppression of cell infiltration by curcumin in this experiment are similar to earlier research findings which reported that curcumin could lower the expression of adhesion molecules on the surface of monocytes (Seemayer et al., 2005). This prevents the adhesion of monocytes activating factor and the monocytic inflammatory effects in the joints. Furthermore, these inactive monocytes may not produce pro-inflammatory cytokines or other chemokines that could attract other inflammatory cells into the joint areas. Additionally, curcumin was also reported to lower the pro-inflammatory cytokines expression produced by cells such as activated synovial fibroblasts, macrophages and neutrophils in the joints of CIA (Shakibaei et al., 2007). This effect may cause lesser cell migration from the blood circulation into the joint areas.

Normal synovial tissue consists of an acellular structure with synoviocytes within it (Funk et al., 2006). Synovial hyperplasia occurred due to an increase in the proliferation and activation of synoviocytes that mimics the fibroblasts and macrophages. This further increased the production of cytokines, such as IL-1, TNF-α, IL-8 and many others in the synovium (Funk et al., 2006). Decreased synoviocytes apoptotic rate was also reported to cause synovial hyperplasia, even without the increase in cell proliferation (Sweeney & Firestein, 2004).

In the present study, the synovial hyperplasia scores showed that curcumin supplementation could not suppress the synovial hyperplasia effectively (Figure 4b). Interestingly, these findings contradict earlier findings by Joe et al. (2004) who reported that curcumin could suppress cell proliferation at low dose and could cause cell apoptosis at higher dose (Joe et al., 2004). The betamethasone treated group showed low hyperplasia score, which might be due to the strong anti-inflammatory action of the corticosteroid (Larsson et al., 2004).

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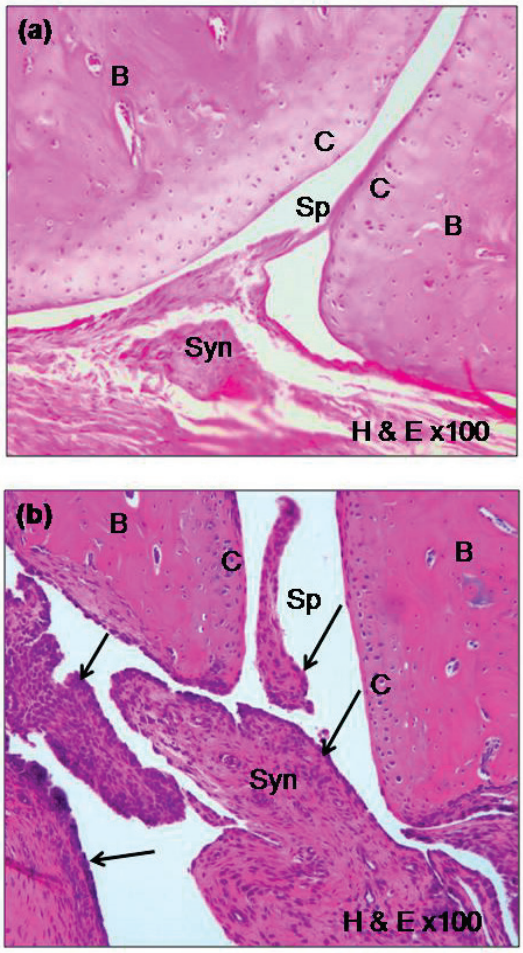

Figure 4: Photomicrographs of (a) normal joint structure and (b) joint with synovial hyperplasia. Synovial hyperplasia is shown by the black arrows. B – bone, C – cartilage, Sp – joint space, Syn – synovial tissue

The pannus formation occurred as a result from the collection of the inflammatory cells such as macrophages and lymphocytes as well as the immune complexes within the synovial space that contained the synovial fluid, on the articular cartilage surface of an arthritic joint (Romas et al., 2002; Arend, 2001). Increased chemokines and pro-inflammatory cytokines levels produced by synovial fibroblasts in the joint space may attract more T cells and macrophages to enter the joint space. This in turn, causes the release of matrix metalloproteinase (MMP) by the synovial fibroblasts (Karouzakis et al., 2006).

Pannus formation scores showed that curcumin supplementation with the dose of 110 mg/kg had successfully suppressed the pannus formation process that occurred on the articular cartilage of the CIA joints. The average score for the curcumin group was not significantly different compared to the betamethasone treated group. Olive oil treated group showed a higher score that represents 26–100 % changes that occurred in the joints (Figure 5b). Pannus formation scores obtained by both the betamethasone and curcumin (dose of 110 mg/kg) suggested that the prevention of pannus formation process is in need of the supplementation of potent anti-inflammatory agent such as curcumin.

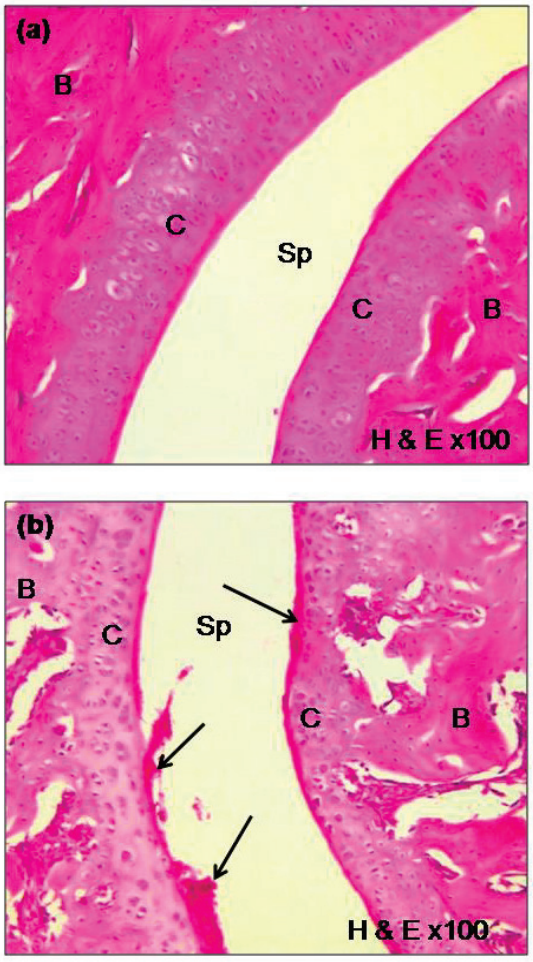

Figure 5: Photomicrographs of (a) normal articular cartilage and (b) articular cartilage with pannus. Pannus is formed on articular surface of cartilage (black arrows). B – bone, C – cartilage, Sp – joint space

The cartilage erosion is largely caused by matrix degradation enzymes production, such as MMPs and cathepsin by synoviocytes (Oelzner et al., 2006). Additionally,

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oxidative stress and altered DNA repair process could also cause the increase in synoviocytes proliferation and cartilage invasion by those cells (Funk et al., 2006). Osteoclasts have also been reported to play an important role in cartilage and bone erosion process (Gravallese, 2002). Van den Berg et al. (1999) reported that IL-1 and TNF are the mediators that could directly activate the chondrocytes on the surface of articular cartilage. The activation of chondrocytes causes the destruction of enzymes (such as MMPs) to be released and matrix synthesis is being suppressed (Karouzakis et al., 2006; van den Berg et al., 2001).

Cartilage destruction process that is left untreated will worsen and later cause bone erosion on the affected joints. Infiltration of inflammatory cells that act as the mother cells for osteoclasts, into the cartilage and bone cause changes in normal activities involved in the maintenance of normal structure and functions of the bones (Schett, 2009). Osteoclasts are also reported to be the main cell that cause bone erosion in CIA (Joe et al., 2004). Cartilage and bone erosion scores showed that, unlike the olive oil, (Figure 6b) the supplement of curcumin could suppress or prevent the cartilage and bone erosion processes that occurred during the inflammatory reaction due to CIA. Similar event occurred in betamethasone treated conditions, which has been proven to be a potent anti-inflammatory agent in arthritis treatment, especially in the CIA (Larsson et al., 2004).

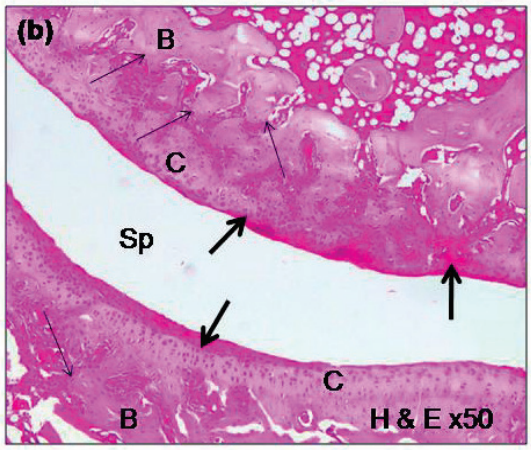

Figure 6: Photomicrographs of (a) normal cartilage and bone and (b) eroded cartilage and eroded bone. Erosion on cartilage (thick arrows) and erosion bone (thin arrows) were shown. B – bone, C – cartilage, Sp – joint space

As stated earlier, pannus formation is also one of the causative factors that initiate cartilage erosion. Funk et al. (2006) reported that *Curcuma longa* extract supplementation could suppress the formation of periarticular osteoclasts and inflammatory cells influx, as well as suppressing the increase of prostaglandin E_2 level within the joint space (Shakibaei et al., 2007). Collagenase expression suppression by curcumin also suppresses the cartilage erosion in RA (Maheshwari et al., 2006). Suppressive effects of curcumin in this experiment might be due to the combination of its action as an anti-oxidant agent in the high oxidative stress condition, and due to its role in decreasing the pro-inflammatory cytokines production by the activated synoviocytes in the CIA joints.

In an earlier study, we have already shown that treatment with curcumin influenced arthritic scores, erythrocyte sedimentation rate along with radiological scores ($p<0.01$) (Taty Anna et al., 2011). In the present study, our results showed that the supplementation of curcumin had a protective effect on the inflammatory and degenerative changes in the bone and joints of the CIA rats, as observed histologically.

CONCLUSION

The results of the present study depict that curcumin may prove to be beneficial as an effective antioxidant agent for the inflammatory and degenerative states like arthritis. This opens the door to try curcumin as an effective supplement for arthritis in future.

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