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Potential of Topical Curcumin in Reduction of TNF-α expression and Synovium Hyperplasia on Wistar Rats of Rheumatoid Arthritis Model

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ABSTRACT

Rheumatoid arthritis is a chronic inflammatory autoimmune disease associated with articular and systemic effects. This disease affects synovial joints covered by a special tissue called synovium. Curcumin has a potent antioxidant, antiinflammatory agent, antiangiogenic and anticarcinogenic. Curcumin can down regulate the expression of various proinflammatory cytokines and is reported beneficial effects in arthritis, but has a poor solubility and bioavailability as well. The purpose of this research was to study the potential of liposomes topikal curcumin in reducing athritis score, reducing the expression of TNF-α and histopathological synovium hyperplasia of hind paw on *Wistar* rats with CFA that had been treated with topical curcumin. In this study, rats were divided into 7 groups: positive control, negative control, rheumatoid arthritis with topical curcumin therapy of 90 mg/kg BW, rheumatoid arthritis with topical curcumin therapy of 110 mg/kg BW, rheumatoid arthritis with topical curcumin therapy of 200 mg/kg BW, rheumatoid arthritis with methotrexate therapy, rheumatoid arthritis with placebo therapy. Results from this experiment indicated that topical curcumin has no significant to the arthritis score, significantly effect to percentase expression of TNF-α ($p<0.05$) and could decrease synovium hyperplasia based on histophatology examination. It could be concluded that therapy of topical curcumin could decrease the expression of TNF- α and synovium hyperplasia in rheumatoid arthritisrat.

Keywords: Arthritis, CFA, Curcumin, TNF-α, synovium hyperplasia.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, progressive, inflammatory autoimmune disease associated with articular and systemic effects (Choy, 2012). RA patients experience swelling in the joints, synovial tissue inflammation and subsequent damage to the cartilage. This will result in significant disability and decrease in the quality of life (Zahidah *et. al.*, 2012; Lindqvist *et. al.*, 2003). Suppresion of progression in the early stages of the disease can result in substantial improvements in long-term outcomes.

The prevalence of RA is believed to range 0.4% to 1.3% of the population in the world, while in Indonesia there are from 0.1% to 0.3% of patients RA (1 between 1000-5000 people) (Nainggolan, 2009). RA is characterized by inflammation of the joints that can become joint damage and affect quality of life (Suwito *et. al.*, 2010; Wu, Zeng, *et. al.*, 2013).

There is no cure for RA, but treatment can help reduce inflammation in the joints, relieve pain dan prevent or slow joint damage, using non-steroidal antiinflammation drugs (NSAIDs), spesific inhibitors of proinflammatory mediators, glucocorticoids, disease-modifying

antirheumatic drugs (DMARD): synthetic chemical compounds (sDMARDs) and biological agents (bDMARDs) (Gaujoux-Viala *et. al.*, 2014; Thanusubramanian *et. al.*, 2014). However, most of these drugs posses a wide spectrum of untoward effects (Kaur & Sultana, 2012; Simadibrata, 2004; Sooriakumaran, 2006).

Curcumin (diferuloylmethane) is the most active component of Curcuma longa L. Several clinical trials have found curcumin to be a notable antiinflammatory, antioxidant, antiangiogenicand anticarcinogenic activity (Borashan, Ilkhanipoor, Hashemi, & Farrokhi, 2009; Goel, Kunnumakkara, & Aggarwal, 2008; Sonavane *et. al.*, 2012). Curcumin has been shown to inhibit many aspects of arthritis, including reducing the activation of T and B cells, macrophages, neutrophils and natural killer cells. In rheumatoid arthritis, curcumin exerts beneficial effects by inhibiting the expression of collagenase and stromelysin and the proliferation of synoviocytes (Jagetia & Aggarwal, 2007), induce apoptosis in synovial fibroblasts (Kim *et. al.*, 2007). Curcumin has limitations in terms of solubility and low bioavailability. Curcumin absorption from the gastro-intestinal tract is not satisfactory, and experiencing rapid metabolism in the liver (Anand, 2007). For it is necessary to study alternative route of administration other than oral, which may keep the bioavailability of curcumin and avoids hepatic metabolism process. The purpose of this research was to study the potential of liposomes topikal curcumin in reducing athritis score, reducing the expression of TNF-α and histopatho-logical synovium hyperplasia of hind paw on *Wistar* rats with CFA.

MATERIAL & METHODS

Animals.

Male *Wistar* rats (10-12 weeks old) weighing 150-200 g were purchased from The Malang Murine Farm, Singosari, Malang. The animals were housed each one per plastic cage. The animals were maintained under 12-h light/12-h dark cycles each day. All animals were fedpellet diet and water *ad libitum*. The study was approved by the Brawijaya University Ethics Committee with the ethical clearance no. 236/EC/KEPK-S2/03/2015.

Experimental Design.

A total of 28 rats were divided equally into seven groups. The grouping details are as follows: (A) negative control rats, (B) positive control rats, (C) treatments rats (CFA induction then given 90 mg/kg/day of curcumin starting from day 14 until day 42), (D) treatments rats (CFA induction then given 110 mg/kg/day of curcumin starting from day 14 until day 42), (E) treatments rats (CFA induction then given 200 mg/kg/day of curcumin starting from day 14 until day 42), (F) treatments rats (CFA induction then given 150 µg/kg/week/oral of methotrexate on day 14, 21, 28, 35, and 42), (G) treatments rats (CFA induction then given placebo).

Preparation of curcumin liposomes.

Briefly 25 g curcumin was dissolved in 70% ethanol. The curcumin solution was added in the mixture soya lecithin-cholesterol (7:3) with constant stirring by over head stirrer at 1500 rpm for about 30 min, and then evaporated in a rotary evaporator. The thin film formed in the round-bottomed flask was sonicated for an hour.

Preparation of curcumin gel.

Various Gel formulations were prepared using carbopol 940 and HPMC as gelling agents (see Table 1). Required quantity of gelling agent was weighted and dispersed in a small quantity of water to form a homogeneous dispersion. Curcumin

liposomes was dissolved in propylene glycol and added to the above solution with continuous stirring. In carbopol gels, pH of the gel was brought to skin pH by triethanolamine. The final weight of the gel was adjusted with water.

Table 1. Gel Formulations Each Dose.

Ingredients	Dose 90 mg/kg	Dose 110 mg/kg	Dose 200 mg/kg
Carbopol 940	15	15	15
HPMC	15	15	15
Curcumin liposomes	5.06	6.19	11.25
Propylene Glycol	7.52	7.52	7.52
Triethanolamine	q.s	q.s	q.s
Water	q.s	q.s	q.s

Induction of CFA.

CFA was induced in male *Wistar* rats using the method described by Bush *et. al.* (2002). Rats were intradermally injected at the base of the tail with 100 µl of completed Freund's adjuvant (Sigma Aldrich, USA) on the day 0.

Assessment of arthritis score.

The incidence and severity of arthritis were evaluated using a system of arthritic scoring every 2 days beginning on the day after CFA injection by two independent observers. Arthritis score was used as a semi quantitative parameter of polyarthritis severity through a well established, widely used scoring system. Paws were examined and graded for severity of erythema and swelling using a 5-point scale: 0=no signs of inflammation, 1=erythema and mild swelling confined to the tarsal or ankle joint, 2=erythema and mild swelling extending from the ankle to the mid-foot, 3=erythema and moderate swelling extending from the ankle to the metatarsal joints, 4=erythema and severe swelling encompassing the ankle and foot (Banda et al., 2009).

Immunohistochemical detection of tissue TNF-α.

Briefly, slides from paraffin-embedded hind pawtissue sections of 5 micron thickness were dewaxed first in xylol and then in graded ethanol solution, rehydrated, washed in phosphate buffered saline pH 7.4. The sections were then immune-stained with rabbit monoclonal IgG to rat TNF-α primary antibody (Santa cruz Biotech, inc., USA). After washing the slides with the appropriate buffer, the sections were incubated incubated with anti-mouse Horseradish Peroxidase (HRP) conjugated secondary antibody for 40 min. Sections were then washed again and incubated for 10 min in a solution 3.3 di-amino-benzidine tetrahydrochloride. Counter staining was performed using Mayer Hematoxylin and incubated for 10 min. The slides were visualized under a light microscope.

Histopathology of hind paw.

At day 42, after euthanasia the left hindpaws including the paw, ankle and knee were surgically removed from all rats fixed immediately in 10% formalin, embedded in paraffin and 5 µm sections were cut and stained with hematoxylin and eosin stain. The tissue sections were observed under a research light microscope for hyperplasia in sinovium cell. All tissue sections were analysed by a trained observer.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software version 20.0 *for Windows* (SPSS Inc., Chicago, IL, USA). The

results were expressed as mean ± standard deviation (SD). Normality of distribution was verified by Shapiro-Wilk test. Homogeneity was verified by Levene Statistic. The TNF-α expression were analyzed by One-Way ANOVA and followed by LSD and Tukey post hoc test to compare the difference among groups. The differences among groups were considered to be significant at $p<0.05$.

RESULT

Arthritis score on fore paw and hind paw of CFA induced arthritis rats.

Table 2 shows the arthritis score of knee joint before the injection of CFA on the 0th day, after the injection of CFA on the 14th day (pre-treatment), and on the 42th day (post-treatment started on the 14th day after CFA). There was no sign of inflammation either erythema or swelling on the 0th, 14th, and 42th day.

Table 2. Mean arthritis score on 0 day (before injection), 14th day (pre-treatment), and 42th day (post-treatment).

Groups	Right fore paw (Mean±SD)			Left fore paw (Mean±SD)			Right hind paw (Mean±SD)			Left hind paw (Mean±SD)		
	0 d	14 d	42 d	0 d	14 d	42 d	0 d	14 d	42 d	0 d	14 d	42 d
Positive control	0	0	0	0	0	0	0	0	0	0	0	0
Placebo	0	0	0	0	0	0	0	0	0	0	0	0
Dose of 90 mg	0	0	0	0	0	0	0	0	0	0	0	0
Dose of 110 mg	0	0	0	0	0	0	0	0	0	0	0	0
Dose of 200 mg	0	0	0	0	0	0	0	0	0	0	0	0
Methotrexate	0	0	0	0	0	0	0	0	0	0	0	0
Negative control	0	0	0	0	0	0	0	0	0	0	0	0

Area Percentage of TNF-α.

Result area percentage of TNF- α which is done with the stereology method, gives a positive reaction with immunohistochemical staining can be seen in figure 1.

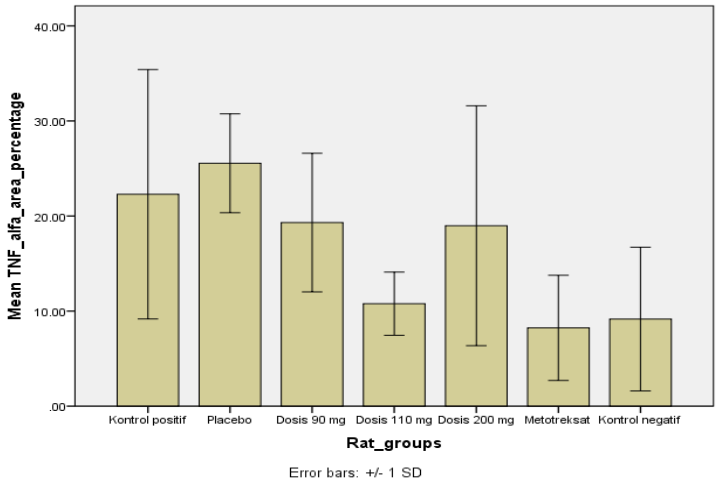

Figure 1. Effect of topical liposomes curcumin on the expression of TNF-α in the joint arthritis rats. Rats induced by CFA injection (i.d). Rats were treated with liposomes curcumin (90 mg/kg, 110 mg/kg, and 200 mg/kg, topical) once daily, methotrexate (150 µg/kg, po) once weekly, and placebo once daily from the 14th to the 42th dayafter CFA injection. On day 42th after CFA injection, the rats were terminated and left leg joint are collected. Tissue sections were stained with immunohystochemical. Percentage of area the expressionof TNF-α calculated using stereology methods. Data represent the mean±SD (n=4). $P<0.05$.

Expression of Tumor Necrosis Factor (TNF-α) on knee joint of CFA induced arthritis rats.

Expression of TNF-α is expressed as a percentage of the area, the area that gives a positive reaction to TNF-α antibody with immunohistochemical staining, are shown in figure 2.

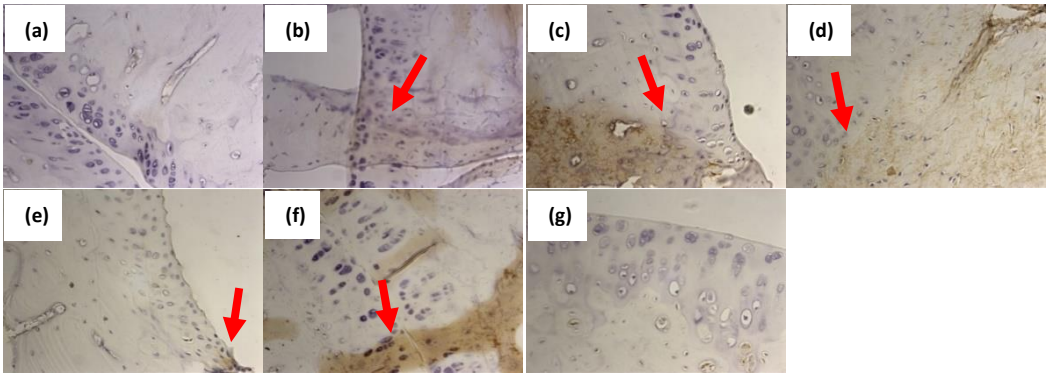

Figure 2. Expression of TNF-α on the ankle joint of CFA induced RA rats. Rats induced by CFA injection (i.d). Rats were treated with liposomes curcumin (90 mg/kg, 110 mg/kg, and 200 mg/kg, topical) once daily, methotrexate(150 µg/kg, po) once weekly, and placebo once daily from the 14th to the 42th day after CFA injection. On day 42 after CFA injection, the rats were terminated and left leg joint are collected. Tissue sections were stained with immunohystochemical. (a) negative control: no expression of TNF-α; (b) positive control: expressionof TNF-α is brown (red arrow); (c) placebo; (d) liposomes curcumin 90 mg/kg; (e) liposomes curcumin 110 mg/kg; (f) liposomes curcumin 200 mg/kg; (g) methotrexate: no expression of TNF-α. (Optical microscopy, x400).

Histopathological changes

Representative histopathological lessions in the hind knee joint from all of groups are shown in Figure 3. Synovial hyperplasia and cartilage destruction were observed in the model group. Histopathological lessions were ameliorated in the treated groups to a different extent. The histopathological lessions of the curcumin liposomes dose 110 mg/kg group improved well. However, following the treatment with curcumin liposomes, a significant and dose depedent improvement was observed in joint histology. It is shows that only moderate proliferation of the synovium occurred.

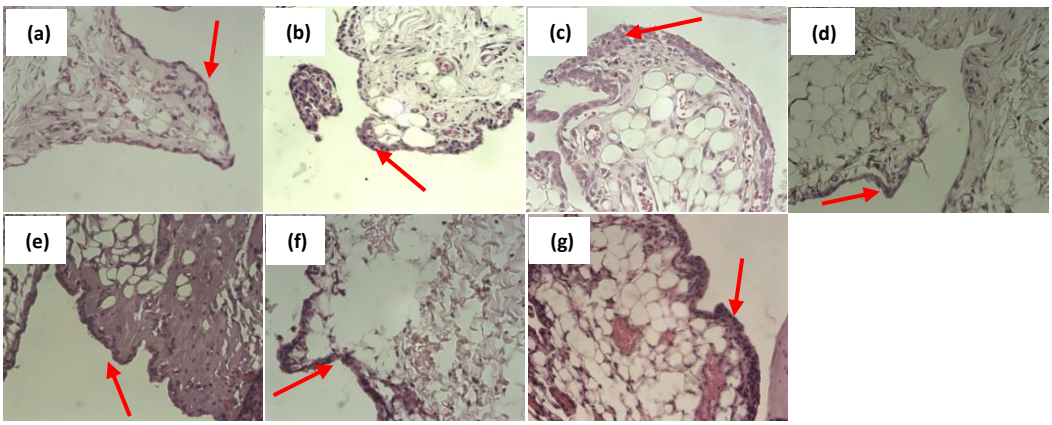

Figure 3. Effects of post treatment with liposomes curcumin on histological alternations of synovium. Rats were treated with liposomes curcumin (90 mg/kg, 110 mg/kg, and 200 mg/kg, topical) once daily, methotrexate (150 µg/kg, po) once weekly, and placebo once daily from the 14th to the 42th day after CFA injection (id). On day 42 after CFA injection, the rats were terminated and left leg joint are collected. Tissue sections were stained with hematoxylin and eosin (HE). (a) negative control: normally synovium; (b) positive control: hyperplasia synovium (red arrow); (c) dose of 90 mg/kg: hyperplasia synovium; (d) dose of 110 mg/kg: hyperplasia synovium; (e) dose of 200 mg/kg: hyperplasia synovium; (f) methotrexate: hyperplasia synovium (g) placebo: hyperplasia synovium. [HE, x400].

DISCUSSION

Rheumatoid Arthritis (RA) is a chronic disease that results from dysregulation of pro-inflammatory cytokines (e. g., tumor necrosis factor and interleukin-1β), and proinflammatory enzymes that mediate the production of prostaglandin (e. g., lipoxy-genase), together with the expression of adhesion molecules and matrix metalloproteinase, and hyperproliferation of synovial fibroblasts (Kapoor B. *et. al*, 2014) (Sahebari M, 2011).

Pro-inflammatory cytokines that are the focus of this research is tumor necrosis factor (TNF)-α, are important cytokines in inflammation and are considered to be the most important mediators involved in the pathogenesis of RA (Chu K. *et. al.*, 2013).

CFA induced rats model has been widely used to evaluate candidate compounds as anti-inflammatory. CFA models can cause some of the characteristics commonly found in RA (Sahebari *et. al.*, 2011). Adjuvant induced arthritis is a T-cell-mediated, chronic inflammatory stress. Following a single intradermal injection of ground, the animals develop a hind paw inflammation 13-15 days later (Harbuz *et.al.*, 1996).

In this study, liposomes curcumin with topical administration not showed erythema and joint swelling on CFA induced Wistar. Observation were made on both the front and rear legs of each rats by 2 observer shown zero score on all group from the 1st to the 42th day after CFA injection.

AIA is initiated in Lewis rats by intradermal injection of CFA at the base of the tail. The genetic background of rats is important, in that both MHC and non-MHC genes contribute to their susceptibility to AIA. Specific trait loci are associated with the severity of disease. AIA is characterized by a rapid on set and progression to polyarticular inflammation. Signs of arthritis usually develop between day 10 and day 14 following the injection of CFA. Usually the disease is severe and leads to permanent joint malformations, including ankylosis. Joint swelling, lymphocyte infiltration, and cartilage degradation are shared feature with human RA (Bevaart *et. al.*, 2010). Signs of arthritis in the joints that did not seem possible due to genetic factors.

TNF-α is important proinflammatory mediators implicated arthritis. TNF-α regulates fibroblast proliferation, recruitment of leukocytes to arthritic tissue, stimulation of PGE_2 production, collagen synthesis by synovial cells, bone resorption contributing to pathogenesis of arthritis (Kaur and Sultana, 2012). Inhibition of TNF-α production supresses cartilage destruction significantly impending the pregression of athritis (Kaur and Sultana, 2012). In the present study, liposomes curcumin treatment was found to significantly ameliorate the expression of TNF-α. The percentage of TNF-α area represents the area ratio between the expression of TNF-α area with overall area of cartilage.

Negative control group rats were used as standards of normal condition wihout CFA induced. The doses of 100 mg groups showed a decrease in the percentage area of TNF-α the lowest compared to the other groups. These results reinforce the results of research conducted by Zahidah *et. al.*, 2012, that the curcumin at the dose of 110 mg/kg/day for 28 days has a potential to delay and improve joint abnormality and injury in *Sprague-Dawley* rats with collagen induced arthritis (CIA).

Tumor Necrosis Factor (TNF-α) is a cytokine that is secreted by macrophages that have an important role in inflammation. CFA injection triggers the synovial membrane damage causes vascularization and infiltration of inflammatory cells increases into the synovium, then activates macrophages to produce cytokines TNF-α that causes

inflammation. Expression of TNF-α characterized by their brown color on joint tissue performed immunohistochemical staining (Green and Flavell, 2000). High levels of TNF-α present in RA synovium playan important role in the synovial hyperplasia of RA by suppresing apoptosis and promoting proliferation of synoviocytes through NF-κB depending signaling patways mediated by up-regulated TNFR2 and RAF1-6 molecules (Youn et al, 2002). Observations carried out on the back of the leg joints, because many AR attack the hind paws of rats. In this study, it was observed synovial hyperplasia in joint histopathology rats using Hematoxylin eosin (HE) staining shown in Figure 3.

Rheumatoid arthritis is a chronic disease characterized by inflammation polyarticular marked by hyperplasia of cells lining the synovium and pannus formation, along with angiogenesis and infiltration of mono nuclear cells of inflammation, which play a major role in the immune response mediated cell (Choy, 2001; Volin and Koch,2000). Synovial tissue fibroblast hyperplasia is reminiscent of tumor-like proliferation and is a major cause of cartilage destruction in the RA joint (Volin and Koch, 2000). In the early rheumatoid arthritis, the synovial membrane becomes thickened because of hyperplasia and hypertrophy of the synovial-lining cells. The synovial membrane begins to invade the cartilage (Choy and Panayi, 2001). Erosion of articular cartilage occurs mainly in areas adjacent to cell proliferation, suggesting that these cells release proteolytic enzymes that degrade collagen and proteoglycans capable of cartilage and bone.

The results of histopathological obtained from this research as indicated in figure 3, hyperplasia synovium seen in positive control group (figure 3.b). In the treatment group therapy with liposomes curcumin dose 110 mg/kg bw (figure 3.d) looks only slightly hyperplasia synovium, the same thing happened at methotrexate therapy group (figure 3.f).

According to Moon *et. al.* (2010), the reduction and inhibition of TNF-α production cause disturbance to the cyclooxygenase-2 activity expression and reduction in the prostaglandin secretion. Hence, this suggests that curcumin has the capability in delaying the inflammatory response and reducing the occurence of the joints inflammation symptoms (Zahidah *et. al.*, 2012).

CONCLUSION

In this study, curcumin liposomes showed the capability to reduce TNF-α expression and a direct inhibitory effect on sinovium hyperplasia in RA rats. Therefore, it may be a useful curcumin liposomes in the treatment of completed freund adjuvant induced arthritis.

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