The anti-inflammatory and apoptotic effects of atorvastatin in combination with celecoxib in adjuvant-induced arthritis in rats

*[1]Rowaida Refaat, [1]Mona Salama, [1]Maher A Kamel and [2]Sherihan Salah EL Din

[1]Medical Research Institute, University of Alexandria, 165 Horreya Avenue, Alexandria, Egypt
[2]Faculty of Pharmacy, 6th October University, Cairo, Egypt

Statins seem to have anti-inflammatory effects independent of their lipid-lowering abilities. Previous studies demonstrated a strong synergy between statins and non-steroidal anti-inflammatory drugs in growth inhibition and apoptosis induction in cultured cancer cells. This study aimed at evaluating the combined anti-inflammatorty and apoptotic effects of atorvastatin and celecoxib in adjuvant-induced arthritis in rats. Adjuvant arthritis was induced in Sprague-Dawley rats by intradermal injection of 0.1 ml suspension of heat-killed *Mycobacterium butyricum* (12 mg/ml) in incomplete Freund's adjuvant. Rats were treated orally with atorvastatin (10 mg/kg/day), celecoxib (3 mg/kg/day) and their combination from day 12 to day 27 post-adjuvant injection. Arthritis progression was assessed by hind paw swelling and arthrogram scores. Serum levels of C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF) were measured. Caspase-3 activity and DNA fragmentation were determined in tibiotarsal joints tissue to evaluate apoptosis. Celecoxib proved to be more effective, than atorvastatin in suppressing clinical severity of arthritis, reducing serum levels of VEGF, CRP and TNF-α and increasing serum levels of IL-10. Caspase-3 activity and DNA fragmentation were more significantly enhanced by atorvastatin. Combining atorvastatin and celecoxib provided higher efficacy, in reducing inflammation and inducing apoptosis, than either agent alone.

Keywords: Adjuvant-induced arthritis, atorvastatin, celecoxib, apoptosis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease causing progressive joint destruction, deformity and disability (Lee and Weinblatt, 2001). The pathogenesis of the rheumatoid joint involves hyperplasia of the synovial lining cells, mononuclear cell infiltration and new blood vessel formation within the synovium. Moreover, destruction of the cartilage and underlying bone occurs as a consequence of pro-inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α) and proteases (Feldmann et al., 2005). Decreased apoptosis has been proposed as a possible factor that contributes to the hyperplasia of the synovial membrane and the accumulation of inflammatory cells observed in synovitis of patients with active rheumatoid arthritis (Hsu et al., 2006). Therefore, one strategy for treatment of RA is the design of drugs that can ameliorate inflammation and restore the normal apoptotic pathways in hyperproliferative cells (Ichikawa et al., 2003). Statins, competitive inhibitors of hydroxymethylglutaryl (HMG)-CoA reductase, were initially designed as inhibitors of cholesterol synthesis (Endo, 2004). However, statins seem to have anti-inflammatory effects that cannot be accounted for by their lipid-lowering abilities. These include the suppression of pro-inflammatory cytokine and chemokine production, immunomodulation and down-regulation of endothelial cell activation (Blanco-Colio et al., 2003; Palinski and Tsimikas, 2002). Statins have also been shown to modulate other biological processes, such as cell proliferation and apoptosis (McFarlane et al., 2002). Atorvastatin, a synthetic statin, was found to have a therapeutic effect in patients with rheumatoid arthritis

as well as beneficially influencing inflammatory markers (McCarey et al., 2004).

Cyclooxygenases (COXs) are key enzymes in the conversion of arachidonic acid to prostanoids which mediate mitogenesis, apoptosis, angiogenesis and inflammation (Harris, 2007). Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) that is a specific inhibitor of cyclooxygenase 2 (COX-2). It is indicated for a variety of chronic inflammatory conditions including RA (Alloza et al., 2006). Celecoxib has been reported to inhibit synovial hyperplasia by inducing apoptosis of human synovial fibroblasts (Kusunoki et al., 2008). Celecoxib has also been described as a pro-apoptotic factor in several human carcinoma cells (Liu et al., 2004; Maier et al., 2004). Previous studies demonstrated a strong synergy between the actions of atorvastatin and celecoxib in growth inhibition and apoptosis induction in cultured cancer cells (Xiao et al., 2008; Zheng et al., 2007). These observations provided a rationale for testing the combined anti-inflammatory and apoptotic effects of atorvastatin and celecoxib in chronic immune-mediated inflammatory diseases, such as RA, where impaired apoptosis has been proposed as a possible underlying mechanism.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 150-180 g were used in this study. Rats were obtained from the Medical Research Institute, Alexandria University, Egypt. All procedures used in this study complied with regulations of the National Research Council's guide for the care and use of laboratory animals.

Induction of adjuvant arthritis (AA)

Rats were divided into 5 groups of eight rats each. Adjuvant arthritis was induced in groups 1-4, as mentioned previously (Refaat et al., 2013), by injection of 0.1 ml suspension of heat-killed *Mycobacterium butyricum* (Difco Laboratories Co-USA), (12 mg/ml) in incomplete Freund's adjuvant (Sigma Aldrich Co-USA), intradermally at the base of the tail. The time of adjuvant injection was referred to as day 0. Treatment was initiated on day 12 post-adjuvant injection, when clinical signs of joint inflammation were noted, and was continued till day 27. Adjuvant arthritis rats in group 1 were treated orally with atorvastatin (Egyptian Int. Pharmaceutical Industries CO.), dissolved in phosphate buffered saline in a dose of 10 mg/kg/day (Barsante et al., 2005). Group 2 was treated orally with celecoxib (AMON Company, Giza, Egypt), in phosphate buffered saline in a dose of 3 mg/kg/day (Noguchi et al., 2005). Arthritic rats of group 3 received the combination of atorvastatin and celecoxib in the aforementioned doses. Group 4 (untreated adjuvant arthritis rats) and group 5 (normal control rats) received the vehicle (phosphate buffered saline), orally daily.

Assessment of arthritis progression

Hind paw swelling was assessed by caliper measurement of ankle (tibiotarsal) joint width (Bendele, 2001), every other day from day 0 till onset of the disease on day 12 then twice a week till the end of the study. The severity of arthritis was scored on a 4-point scale, in which 0 = normal, 1 = slight edema of the small digital joints, 2 = edema of the digital joints and footpad, 3 = gross edema of the entire footpad below the ankle or wrist, 4 = gross edema of the entire footpad including the ankle joint or wrist joint. The sum of the scores for all 4 limbs was calculated as the arthritic index, with a maximum possible score of 16 per rat (Baggott et al., 1998). Arthrogram scores were evaluated again on day 20 and on day 28 just before sacrifice.

Serum parameters

On day 28, animals were anesthetized with diethyl ether and blood was collected from the posterior vena cava through a laparotomy incision. Sera were separated and stored at -80 ˚C for determination of C-reactive protein (CRP) (Otsuji et al., 1982), TNF-α, interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF) by rat enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen Corporation, USA). All ELISA test kits were used according to the manufacturers' instructions.

Tissue parameters

The subcutaneous tissue of the hind paw and surrounding the tibiotarsal joints of all rats were removed (Barsante et al., 2005), and stored at -80 °C for determination of caspase-3 activity by using caspase-3 Colorimetric Protease Assay kit (Invitrogen Corporation, USA**)** and DNA fragmentation by DNA Purification Kit (Fermentas, Thermo Scientific, USA) (Broom et al., 1990). Fragmented DNA was run in a 1.5% agarose gel containing ethidium bromide. Gels were examined and photographed under UV light.

Statistical analysis

Data are presented as mean ± SEM of 8 observations. One-way analysis of variance (ANOVA) was used for normally distributed data when more than two popula-

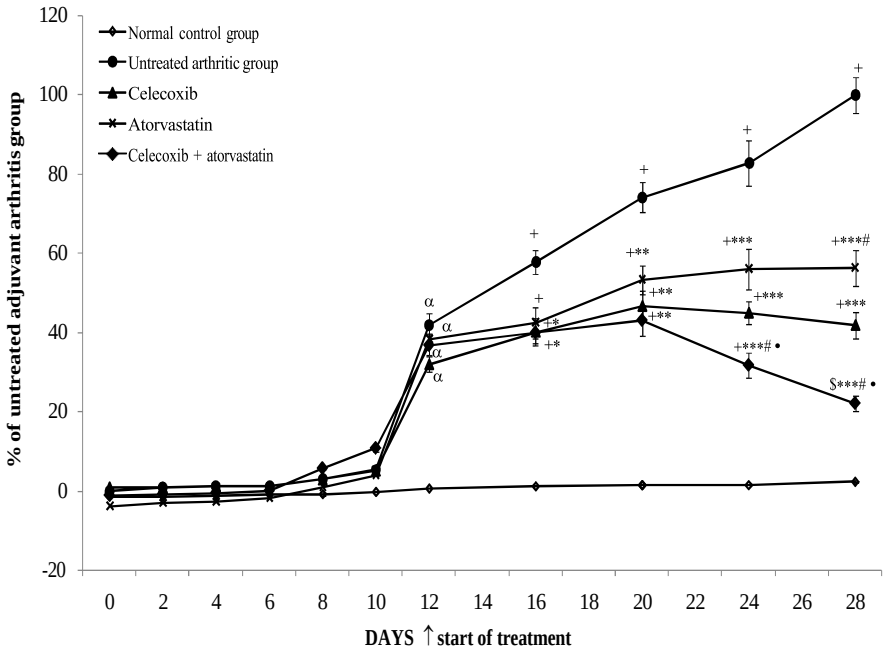

Figure 1. Effect of treatment with atorvastatin, celecoxib and their combination on hind paw swelling in adjuvant-induced arthritis rats. Each point represents the mean ± SEM of 8 rats. Data are expressed as percent of control with the untreated adjuvant arthritic group on day 28 being set to 100%. Drug treatment reduced paw swelling in a time-dependent manner. As early as day 16, celecoxib alone and in combination with atorvastatin caused significant reduction in hind paw swelling. By day 20, all drug-treated rats showed significant reductions in paw swelling. The reduction in paw swelling continued to increase significantly along the rest of the study where the lowest values were observed on day 28 in the combined therapy group. $^{\alpha}P < 0.001$ versus day 0, $^{\$}P < 0.05$, $^{+}P < 0.001$ versus normal control rats, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus untreated adjuvant arthritis rats, $^{\#}P < 0.01$ versus celecoxib-treated rats, $^{\bullet}P < 0.01$ versus atorvastatin-treated rats.

tions were analyzed. Also paired t-test was used to analyze two paired data. For abnormally distributed data, Mann-Whitney test was used to analyze two independent populations. If more than two populations were analyzed, Kruskal Wallis test was used. $P < 0.05$ was considered as the significance limit for all comparisons.

RESULTS

Hind paw swelling

In all adjuvant arthritis rats hind paw edema reached significantly higher values by day 12 compared to day 0 (Figure 1). In the untreated arthritic rats, there was a continuous increase in paw swelling till the end of the study on day 28, $P < 0.001$, compared to day 12. All drug regimens reduced paw swelling in a time-dependent manner. As early as day 16, celecoxib alone and in combination with atorvastatin caused a significant reduction in the progression of hind paw swelling compared to untreated arthritic rats. Atorvastatin alone caused a reduction in hind paw swelling but without statistical difference from untreated adjuvant arthritis rats. A more significant reduction in paw swelling was observed in all drug-treated groups, on days 20 and 24, as compared to untreated arthritic rats, $P < 0.01$ and $P < 0.001$, respectively. By the end of the study the inhibition of swelling was more evident in the celecoxib-treated rats as joint width values reached 41.9% of untreated arthritic rats compared to 56.3% in the atorvastatin group. The combined therapy caused a more significant inhibition of hind paw swelling reaching only 22.1% of untreated control values (Figure 1).

Arthrogram scores

On day 12, all arthritic rats attained arthrogram scores ranging from 8.88 ± 0.48 to 9.37 ± 0.12 with no significant difference among experimental groups (Table 1). In the untreated adjuvant arthritis group arthrogram scores continued to increase gradually to a maximum of 13.51 ± 0.33 on day 28. Treatment with celecoxib or atorvastatin caused significant reductions in clinical scores on days 20 and 28 as compared to untreated arthritic rats.

Table 1. Effect of treatment with celecoxib, atorvastatin and their combination on arthrogram scores in adjuvant-induced arthritis rats.

Experimental groups	Day 12	Day 20	Day 28
Untreated arthritic rats	9.25 ± 0.25	12.75 ± 0.45	13.51 ± 0.33
Celecoxib	8.88 ± 0.48	9.63 ± 0.26[b]	9.25 ± 0.45[b]
Atorvastatin	9.37 ± 0.12	10.75 ± 0.31[a]	11.25 ± 0.83[ad]
Celecoxib and atorvastatin	9.00 ± 0.38	8.50 ± 0.33[be]	7.62 ± 0.28[bce]

Data presented as mean ± SEM of 8 rats.
[a]$P < 0.01$, [b]$P < 0.001$ versus untreated adjuvant arthritis rats
[c]$P < 0.05$, [d]$P < 0.01$ versus celecoxib-treated rats
[e]$P < 0.001$ versus atorvastatin-treated rats

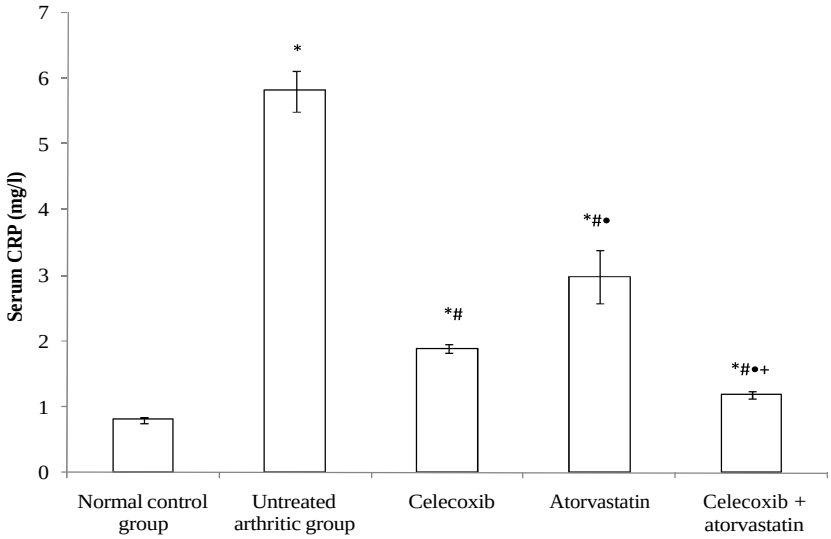

Figure 2. Changes in serum CRP concentration following treatment of adjuvant arthritis rats with celecoxib (3 mg/kg/day), atorvastatin (10 mg/kg/day) and their combination from day 12 to day 27 post-adjuvant injection. [*]$P < 0.01$ versus normal control rats, [#]$P < 0.01$ versus untreated adjuvant arthritis rats, [•]$P < 0.01$ versus celecoxib-treated rats, [+]$P < 0.01$ versus atorvastatin-treated rats.

However, on day 28, atorvastatin-treated rats exhibited less significant reduction in arthrogram scores relative to the celecoxib-treated group, $P < 0.01$. The combination therapy proved a better significant suppression of clinical arthrogram scores as compared to either atorvastatin or celecoxib alone, $P < 0.001$ and $P < 0.05$ respectively.

Serum C-reactive protein (CRP)

Untreated adjuvant arthritis rats exhibited a significant increase in serum CRP concentration compared to normal control rats (Figure 2). Celecoxib caused a more significant decrease in the elevated CRP concentration than atorvastatin, $P < 0.01$. Treatment of arthritic rats with both drugs in combination resulted in a more significant decrease in the elevated CRP values as compared to either drug alone, $P < 0.01$.

Serum TNF-α and IL-10

Serum levels of TNF-α were significantly elevated in adjuvant arthritis rats over the normal control (Table 2). In all drug-treated groups, rats exhibited significant reductions in serum TNF-α compared to untreated arthritic control. Arthritic rats treated with celecoxib showed more significant reductions in serum TNF-α compared to rats treated with atorvastatin. Further significant reductions in TNF-α were observed in the combined therapy group relative to rats treated with either drug alone, $P < 0.01$. On the other hand, serum levels of the anti-inflammatory cytokine IL-10 were significantly decreased in adjuvant arthritis rats as compared to normal control rats, $P < 0.01$. All treatment regimens caused significant elevations in serum levels of IL-10 with the highest increase observed in the combined therapy-treated group (Table 2).

Table 2. Effect of treatment with celecoxib, atorvastatin and their combination on serum TNF-α, IL-10 and VEGF in adjuvant-induced arthritis rats.

Experimental groups	IL-10 (pg/ml)	TNF-α (pg/ml)	VEGF (pg/ml)
Normal control	628.72 ± 10.05	24.66 ± 0.48	41.93 ± 0.57
Untreated arthritic rats	257.84 ± 7.60[a]	125.00 ± 1.29[a]	221.58 ± 1.81[a]
Celecoxib	468.70 ± 3.87[ab]	59.11 ± 0.71[ab]	130.00 ± 0.85[ab]
Atorvastatin	349.97 ± 3.44[abc]	88.34 ± 1.98[abc]	176.08 ± 2.47[abc]
Celecoxib and atorvastatin	591.33 ± 7.01[abcd]	48.81 ± 0.83[abcd]	91.41 ± 1.08[abcd]

Data presented as mean ± SEM of 8 rats.
[a]$P < 0.01$ versus normal control rats
[b]$P < 0.01$ versus untreated adjuvant arthritis rats
[c]$P < 0.01$ versus celecoxib-treated rats
[d]$P < 0.01$versus atorvastatin-treated rats

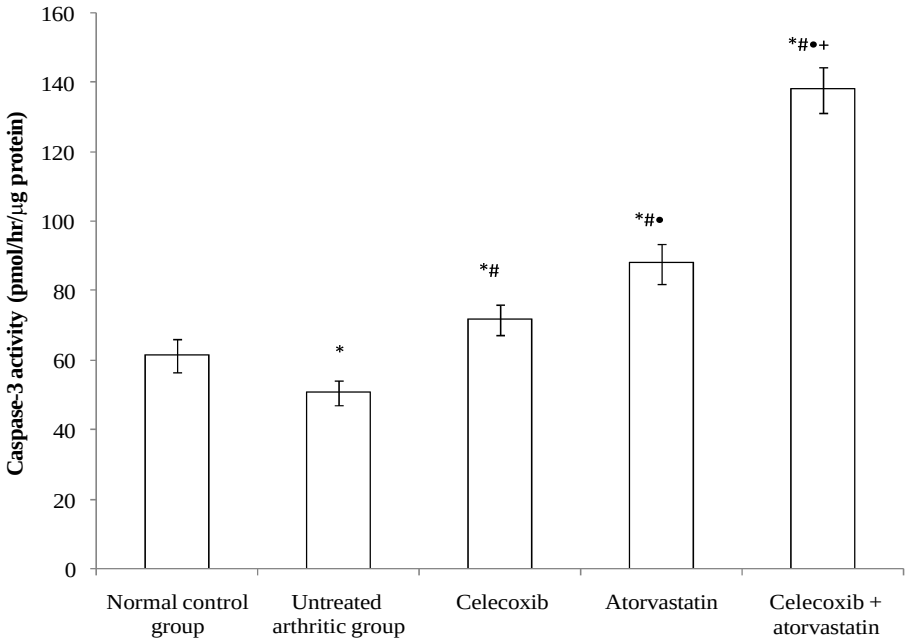

Figure 3. Changes in tibiotarsal tissue caspase-3 activity following treatment of adjuvant arthritis rats with celecoxib (3 mg/kg/day), atorvastatin (10 mg/kg/day) and their combination from day 12 to day 27 post-adjuvant injection. [*]$P < 0.01$ versus normal control rats, [#]$P < 0.01$ versus untreated adjuvant arthritis rats, [•]$P < 0.01$ versus celecoxib-treated rats, [+]$P < 0.01$ versus atorvastatin-treated rats.

Serum VEGF

The effects of drug treatment on serum VEGF are demonstrated in Table 2. Untreated adjuvant arthritis rats showed significantly raised VEGF concentrations compared to normal control values, $P < 0.01$. Administration of atorvastatin resulted in a less significant decrease in the elevated VEGF concentration as compared to the celecoxib-treated rats. The combination of both drugs resulted in a more significant decrease in VEGF values as compared to both drugs given alone, $P < 0.01$.

Caspase-3 activity

Untreated arthritic rats showed significant inhibition of capase-3 activity compared to normal control rats (Figure 3). Such inhibition was opposed significantly by the administration of celecoxib and atorvastatin either alone or in combination. In contrast to previously mentioned results, atorvastatin therapy resulted in a more significant elevation in capase-3 activity as compared to celecoxib-treated rats. The combined therapy showed a more marked elevation in tissue capase-3 activity as compared to both drugs given alone indicating a synergistic

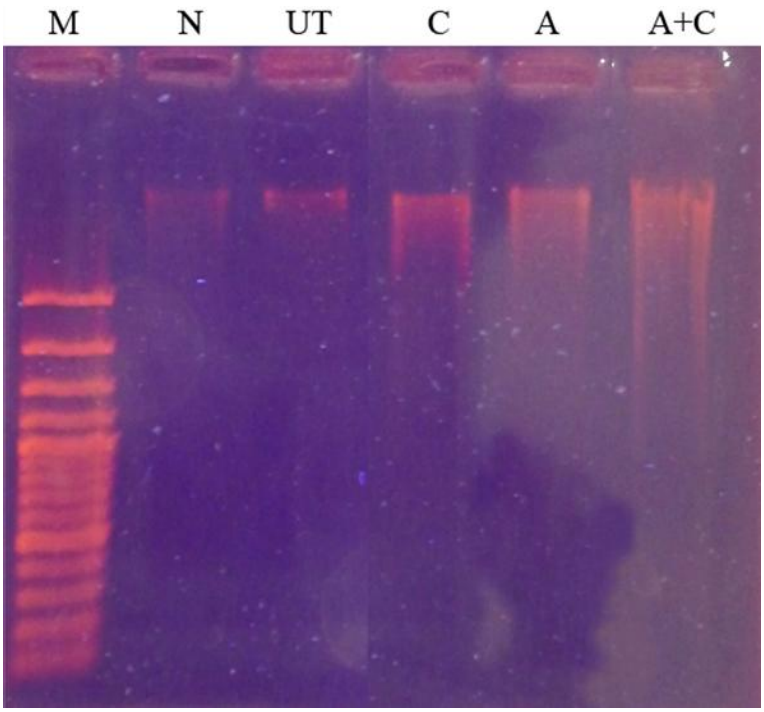

Figure 4. Effect of treatment with atorvastatin, celecoxib and their combination on DNA fragmentation. Fragmented DNA was extracted, run in 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. The DNA intact band appeared to be condensed with no or very low DNA smearing in the untreated adjuvant arthritis rats suggesting low level of DNA fragmentation. Treatment with atorvastatin resulted in a slightly more DNA fragmentation compared to celecoxib. The highest level of DNA fragmentation was observed with the combination of the two drugs. M: 100 bp DNA ladder N: normal control group, UT: untreated adjuvant arthritis group, A: atorvastatin-treated group, C: celecoxib-treated group, A+ C: combined therapy-treated group.

apoptotic effect.

DNA fragmentation

The agarose gel electrophoresis showed very low DNA fragmentation in the tibiotarsal tissues of untreated adjuvant arthritis rats as the DNA intact band appeared to be condensed with no or low DNA smearing (Figure 4). Treatment with atorvastatin was associated with slightly more DNA fragmentation compared to celecoxib. The highest level of DNA fragmentation was observed with the combination of the two drugs. These results confirm the results of caspase-3 activity which showed the same pattern of changes.

DISCUSSION

The adjuvant model of arthritis resembles several aspects of human RA and appears to be useful in evaluating potential anti-arthritic drugs (Bendele, 2001). Celecoxib significantly suppressed arthritis progression, as evidenced by the reduction in arthrogram scores and hind paw swelling, which confirmed previous observations demonstrating the potency of celecoxib in reducing clinical arthritis progression (Noguchi et al., 2005). Atorvastatin substantially decreased the arthrogram scores and hind p w swelling but to a lesser extent than celecoxib. Atorvastatin has been previously reported to produce a dose-d pendent reduction in joint inflammation, with relief of hyperalgesia in adjuvant-induced monoarthritis, and to effectively inhibit hind paw swelling in the same model of arthritis (Barsante et al., 2005; Wahane and Kumar, 2010). The combination therapy caused more suppression in the intensity of joint inflammation by causing more significant reduction in arthrogram scores and paw swelling.

Elevated blood levels of CRP have been found during virtually all diseases associated with active inflammation or tissue destruction. In the current study, arthritic rats showed a significant increase n serum CRP which could be due to the increase in pro-inflammatory cytokines; IL-1 and TNF-α that stimulate the hepatic surge of CRP

(Calabro et al., 2005; Kushner et al., 2010). Treatment with atorvastatin caused a significant reduction in serum CRP which is in line with previous reports in the same model of arthritis and in patients with cardiovascular diseases (Abdin et al., 2012; Dimitrow and Jawień, 2010). This reduction could be due to the pleiotropic effect of atorvastatin that decreases the isoprenylation of Rac-1 which is a mediator of the IL-6 signaling in hepatocytes ((McCarty, 2003). In addition, statins were found to reduce mRNA expression of IL-6 and increase peroxisome proliferator-activated receptor alpha (PPAR-α) and gamma (PPAR-γ) mRNA expression in hepatocytes and endothelial cells (Inoue et al., 2000). PPAR-α and PPAR-γ negatively interfere with transcription factors; nuclear factor-kb (NF-kb), signal transducer and activator of transcription (STAT) and activator protein-1 (AP-1), causing a decrease in the inflammatory cytokines that are important regulators of CRP expression (Chinetti et al., 2001).

In the present work, celecoxib caused a more significant decrease in serum CRP. A similar decrease in CRP was reported after oral administration of celecoxib to rabbits with rheumatoid cachexia (Romero et al., 2010). This effect, mediated by celecoxib, could be due to its ability to decrease prostaglandin E_2 (PGE_2), which in turn decrease the production of IL-6 and consequently the hepatic production of CRP (Inoue et al., 2002). The reduction of serum CRP was more pronounced in the combined therapy group reflecting a better anti-inflammatory effect.

In addition to the crucial role of TNF-α in inflammation, it is also capable of signaling both cell survival and cell death signals (Gupta and Gollapudi, 2005). The observed increase in serum TNF-α, in arthritic rats, is consistent with previous studies in the same model of arthritis and in RA patients (Chen et al., 2011; Refaat et al., 2013). Our results proved celecoxib to be more effective, in reducing serum TNF-α, than atorvastatin and both drugs being less efficacious than the combined therapy. The significant decrease in serum TNF-α, observed with atorvastatin, could be due to its ability to repress the induction of class II major histocompatibility complex (MHC) protein on antigen presenting cells, thus decreasing T-cell activation and inflammatory cytokines production (Danesh et al., 2003). Atorvastatin was also reported to inhibit TNF-α regulated rheumatoid arthritis synovial fibroblasts (RASFs) survival enabling TNF-α to function as an apoptotic signal rather than a survival signal (Connor et al., 2006). Moreover, treatment with atorvastatin, in the present work, was found to increase the concentration of the anti-inflammatory cytokine IL-10. It has been suggested that IL-10 is an important factor in resolving chronic inflammation (Van Roon et al., 2001). Therefore the observed beneficial effects of atorvastatin could be due to shifting the balance of cytokines away from the pro-inflammatory cytokines and towards the production of the anti-inflammatory ones.

The celecoxib-treated group exhibited a more significant decrease in serum TNF-α and a more significant increase in serum IL-10 compared to rats treated with atorvastatin. These results could be attributed to the ability of celecoxib to decrease PGE_2 levels (Boniface et al., 2009). However, the reduction in PGE_2 by celecoxib was reported to increase the expression of TNF-α in rheumatoid synovium, isolated peripheral blood monocyte populations and at the systemic level in the whole blood (Page et al., 2010). The combination of atorvastatin and celecoxib provided a better anti-inflammatory effect as evidenced by the more significant increase in IL-10 values than either drug alone.

VEGF and its receptors are the best characterized system in the regulation of RA by angiogenesis. It protects rheumatoid synoviocytes from apoptosis and can act as a direct pro-inflammatory mediator during the pathogenesis of RA (Yoo et al., 2008). In the present work, atorvastatin caused a significant decrease in the elevated VEGF serum levels as compared to untreated arthritic rats. This effect could be due to inhibition of isoprenylation of Rho GTPases which leads to inhibition of NF-kb activation causing a decrease in VEGF gene expression and serum levels (Alber et al., 2002). It has also been reported that atorvastatin could significantly inhibit VEGF expression, in non-small cell lung carcinomas, via inhibition of reactive oxygen species (ROS) production (Chen et al., 2012). However, atorvastatin was shown to increase significantly the expression of VEGF in ischemic hind limbs of rats (Matsumura et al., 2009). Therefore, the effect of atorvastatin on VEGF expression may differ in cancer or inflamed cells and normal cells.

Treatment with celecoxib caused a more remarkable decrease in VEGF serum levels. This decrease could be through the reduction of PGE_2, which was reported to stimulate the production of VEGF in human synovial fibroblasts, and by increasing serum levels of endostatin which is a very potent inhibitor of angiogenesis (Inoue et al., 2002; Ma et al., 2002). The combined therapy resulted in a stronger anti-angiogenic effect by causing more reduction in serum VEGF levels than either drug alone. As angiogenesis, apoptosis and inflammation are interdependent processes that may be regulated by VEGF (Yoo et al., 2008), the combination of atorvastatin and celecoxib may be an effective strategy that can ameliorate RA symptoms and reverse its fundamental pathology.

In the present study, an obvious decrease in caspase-3 activity with very low DNA fragmentation was observed in untreated arthritic rats. A similar decrease in the expression of procaspase-3 in synovial tissue of adjuvant arthritis rats was previously reported (Perera et al., 2010). Such decrease may be due to the activation of signaling pathways such as NF-kb and phosphatidylinositol 3 kinase/ protein kinase Akt-1 in the RA joint causing the

expression of a variety of anti-apoptotic molecules (Liu and Pope, 2003). In contrast to the previous results, the apoptotic effect of atorvastatin was more significant than that of celecoxib as it caused more significant increase in caspase-3 activity and DNA fragmentation. The apoptotic effect of atorvastatin, could be through modulation of the synthesis of the isoprenoids; geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), that become post translationally incorporated into Ras and Rho proteins which are responsible for cell growth, survival and differentiation. This phenomenon is considered to be mitochondrial and caspase-3 and caspase-9 dependent (Lazzerini et al., 2011). However, atorvastatin was found to improve renal ischemia reperfusion injury via direct inhibition of caspase-3 in rats (Haylor et al., 2011).

The exact mechanism by which celecoxib induces apoptosis is not well known. Celecoxib was reported to inhibit the proliferation of RASFs and induce their apoptosis through COX-2 and PPARγ-independent mechanisms. Previously reported induction of DNA fragmentation in RASFs, by celecoxib, was abolished after the addition of caspase-3 and caspase-8 inhibitors. Accordingly, the caspase cascade may play an important role in induction of apoptosis by celecoxib (Kusunoki et al., 2002).

In the present work, the combination therapy was much more efficient in inducing apoptosis, as illustrated by the synergistic increase in caspase-3 activity and DNA fragmentation than either drug alone. An additive induction in intestinal tumor caspase-3 activity was previously reported in mice fed with the both drugs in combination (Swamy et al., 2006). Mechanisms by which atorvastatin and celecoxib synergistically induce apoptosis are not fully known. Selective modification of membrane bound Rho proteins, Akt inactivation and c-Jun N-terminal kinases (JNK) activation likely contribute to this synergistic interaction (Xiao and Yang, 2008). However, our results also suggest that induction of caspase-3 activity, which is an effector molecule in apoptosis, could play an important role in down-streaming the apoptotic signaling pathway by both drugs in combination.

In conclusion the aforementioned promising findings strongly support the therapeutic potential of the combination of atorvastatin and celecoxib in treatment of RA. As the introduction of COX-2 inhibitors can increase the risk for cardiovascular events. Treatment with atorvastatin would be considered a dual strategy to tackle the cardiovascular risk by focusing on both the cardiovascular system and the inflammatory state. However, further trials may reveal more evidence to support this notion.

REFERENCES

Abdin AA, Abd El-Halim MS, Hedeya SE, El-Saadany A (2012). Effect of atorvastatin with or without prednisolone on Freund's adjuvant induced-arthritis in rats. Eur. J. Pharmacol.; 676:34-40.

Alber HF, Dulak J, Frick M, Dichtl W, Schwarzacher SP, Pachinger O, Weidinger F (2002). Atorvastatin decreases vascular endothelial growth factor in patients with coronary artery disease. J. Am. Coll. Cardiol. 39:1951-1955.

Alloza I, Baxter A, Chen Q, Matthiesen R, Vandenbroeck M (2006). Celecoxib inhibits interleukin-12 αβ and β_2 folding and secretion by a novel COX2-independent mechanism involving chaperones of the endoplasmic reticulum. Mol. Pharmacol. 69: 1579-1587.

Baggott JE, Morgan SL, Koopman WJ (1998). The effect of methotrexate and 7-hydroxymethotrexate on rat adjuvant arthritis and on urinary aminoimidazole carboxamide excretion. Arthritis Rheum. 41:1407-1410.

Barsante MM, Roffê E, Yokoro CM, Tafuri WL, Souza DG, Pinho V, Castro MS, Teixeira MM (2005). Anti-inflammatory and analgesic effects of atorvastatin in a rat model of adjuvant-induced arthritis. Eur. J. Pharmacol. 516:282-289.

Bendele AM (2001). Animal models of rheumatoid arthritis. J. Musculoskel Neuron Interact. 1: 377-385.

Blanco-Colio LM, Tunon J, Martin-Ventura JL, Egido J (2003). Anti-inflammatory and immunomodulatory effects of statins. Kidney Int. 63:12-23.

Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, McKenzie BS, Kastelein RA, Cua DJ, de Waal Malefyt R (2009). Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. J. Exp. Med. 206: 535-548.

Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, Van der Noordaa J (1990). Rapid and simple method for purification of nucleic acid. J. Clin. Microbiol. 28: 495-503.

Calabro P, Chang DW, Willerson JT, Yeh ET (2005). Release of C-reactive protein in response to inflammatory cytokines by human adipocytes: linking obesity to vascular inflammation. J. Am. Coll. Cardiol. 46: 1112-1113.

Chen DY, Chen YM, Chen HH, Hsieh CW, Lin CC, Lan JL (2011). Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF-α therapy. Arthritis Res. Ther. 13: 126.

Chen J, Liu B, Yuan J, Yang J, Zhang J, An Y, Tie L, Pan Y, Li X (2012). Atorvastatin reduces vascular endothelial growth factor (VEGF) expression in human non-small cell lung carcinomas (NSCLCs) via inhibition of reactive oxygen species (ROS) production. Mol. Oncol. 6: 62-72.

Chinetti G, Fruchart JC, Staels B (2001). Peroxisome proliferator-activated receptors (PPARs): nuclear receptors with functions in the vascular wall. Z. Kardiol. 90: 125-132.

Connor AM, Berger S, Narendran A, Keystone EC (2006). Inhibition of protein geranylgeranylation induces apoptosis in synovial fibroblasts. Arthritis Res. Ther. 8: 94.

Danesh FR, Anel RL, Zeng L, Lomasney J, Sahai A, Kanwar YS (2003). Immunomodulatory effects of HMG-CoA reductase inhibitors. Arch. Immunol. Ther. Exp. 51: 139-148.

Dimitrow PP, Jawień M (2010). Anti-inflammatory effect of atorvastatin in patients with aortic sclerosis or mild aortic stenosis independent of hypercholesterolemia. Pharmacol. Rep. 62: 1250-1254.

Endo A (2004). The origin of the statins. Atheroscler. Suppl. 5: 125-130.

Feldmann M, Brennan FM, Foxwell BM, Taylor PC, Williams RO, Maini RN (2005). Anti-TNF therapy: Where have we got to in 2005? J. Autoimmun. 25: 26-28.

Gupta S, Gollapudi S (2005). Molecular mechanisms of TNF alpha-induced apoptosis in aging human T cell subset. Int. J. Biochem. Cell. Biol. 37: 1034-1042.

Harris RE (2007). Cyclooxygenase-2 (cox-2) and the inflammogenesis of cancer. Subcell. Biochem. 42: 93-126.

Haylor JL, Harris KP, Nicholson ML, Waller HL, Huang Q, Yang B (2011). Atorvastatin improving renal ischemia reperfusion injury via direct inhibition of active caspase-3 in rats. Exp. Biol. Med. 236: 755-763.

Hsu HC, Wu Y, Mountz JD (2006). Tumor necrosis factor ligand-receptor superfamily and arthritis. Curr. Dir. Autoimmun. 9: 37-54.

Ichikawa K, Liu W, Fleck M, Zhang H, Zhao L, Ohtsuka T, Wang Z, Liu D, Mountz JD, Ohtsuki M, Koopman WJ, Kimberly R, Zhou T (2003). TRAIL-R2 (DR5) mediates apoptosis of synovial fibroblasts in rheumatoid arthritis. J. Immunol. 171: 1061-1069.

Inoue H, Takamori M, Shimoyama Y, Ishibashi H, Yamamoto S, Koshihara Y (2002). Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. Br. J. Pharmacol. 136: 287-295.

Inoue I, Goto S, Mizotani K, Awata T, Mastunaga T, Kawai S, Nakajima T, Hokari S, Komoda T, Katayama S (2000). Lipophilic HMG-CoA reductase inhibitor has an anti-inflammatory effect: reduction of MRNA levels for interleukin-1beta, interleukin-6, cyclooxygenase-2, and p22phox by regulation of peroxisome proliferator-activated receptor alpha (PPAR alpha) in primary endothelial cells. Life Sci. 67: 863-876.

Kushner I, Samols D, Magrey M (2010). A unifying biologic explanation for "high-sensitivity" C-reactive protein and "low-grade" inflammation. Arthritis Care Res. 62: 442-446.

Kusunoki N, Yamazaki R, Kawai S (2008). Pro-apoptotic effect of non-steroidal anti-inflammatory drugs on synovial fibroblasts. Mod. Rheumatol. 18: 542-551.

Kusunoki N, Yamazaki R, Kawai S (2002). Induction of apoptosis in rheumatoid synovial fibroblasts by celecoxib, but not by other selective cyclooxygenase 2 inhibitors. Arthritis Rheum. 46: 3159-3167.

Lazzerini PE, Capecchi PL, Selvi E, Lorenzini S, Bisogno S, Baldari CT, Galeazzi M, Laghi-Pasini F (2011). Statins and the joint: multiple targets for a global protection? Semin. Arthritis Rheum. 40: 430-446.

Lee DM, Weinblatt ME (2001). Rheumatoid arthritis. Lancet. 358: 903-911.

Liu H, Pope RM (2003). The role of apoptosis in rheumatoid arthritis. Curr. Opin. Pharmacol. 3: 317-322.

Liu X, Yue P, Zhou Z, Khuri FR, Sun SY (2004). Death receptor regulation and celecoxib induced apoptosis in human lung cancer cells. J. Natl. Cancer Inst. 96: 1769-1780.

Ma L, del Soldato P, Wallace JL (2002). Divergent effects of new cyclooxygenase inhibitors on gastric ulcer healing: Shifting the angiogenic balance. Proc. Natl. Acad. Sci. U S A. 99: 13243-13247.

Maier TJ, Schilling K, Schmidt R, Geisslinger G, Grosch S (2004). Cyclooxygenase-2 (COX-2)-dependent and –independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. Biochem. Pharmacol. 67: 1469-1478.

Matsumura M, Fukuda N, Kobayashi N, Umezawa H, Takasaka A, Matsumoto T, Yao EH, Ueno T, Negishi N (2009). Effects of atorvastatin on angiogenesis in hindlimb ischemia and endothelial progenitor cell formation in rats. J. Atheroscler. Thromb. 16: 319-326.

McCarey DW, McInnes IB, Madhok R, Hampson R, Scherbakov O, Ford I, Capell HA, Sattar N (2004). Trial of atorvastatin in rheumatoid arthritis (TARA): double-blind, randomised placebo-controlled trial. Lancet. 363: 2015-2021.

McCarty MF (2003). Reduction of serum C-reactive protein by statin therapy may reflect decreased isoprenylation of Rac-1, a mediator of the IL-6 signal transduction pathway. Med. Hypotheses. 60:634-639.

McFarlane SI, Muniyappa R, Francisco R, Sowers JR (2002). Pleiotropic effects of statins: lipid reduction and beyond. J. Clin. Endocrinol. Metab. 87: 1451-1458.

Noguchi M, Kimoto A, Kobayashi S, Yoshino T, Miyata K, Sasamata M (2005). Effect of celecoxib, a cyclooxygenase-2 inhibitor, on the pathophysiology of adjuvant arthritis in rat. Eur. J. Pharmacol. 513: 229-235.

Otsuji S, Shibata H, Umeda M (1982). Turbidimetric immunoassay of serum C-reactive protein. Clin. Chemi. 28: 2121-2124.

Page TH, Turner JJ, Brown AC, Timms EM, Inglis JJ, Brennan FM, Foxwell BM, Ray KP, Feldmann M (2010). Nonsteroidal anti-inflammatory drugs increase TNF production in rheumatoid synovial membrane cultures and whole blood. J. Immunol. 185: 3694-3701.

Palinski W, Tsimikas S (2002). Immunomodulatory effects of statins: mechanisms and potential impact on arteriosclerosis. J. Am. Soc. Nephrol. 13: 1673-1681.

Perera PK, Li Y, Peng C, Fang W, Han C (2010). Immunomodulatory activity of a Chinese herbal drug Yi Shen Juan Bi in adjuvant arthritis. Indian. J. Pharmacol. 42: 65-69.

Refaat R, Salama M, Abdel Meguid E, El Sarha A, Gowayed M (2013). Evaluation of the effect of losartan and methotrexate combined therapy in adjuvant-induced arthritis in rats. Eur. J. Pharmacol. 698: 421-428.

Romero FI, Martínez-Calatrava MJ, Sánchez-Pernaute O, Gualillo O, Largo R, Herrero-Beaumont G (2010). Pharmacological modulation by celecoxib of cachexia associated with experimental arthritis and atherosclerosis in rabbits. Br. J. Pharmacol. 16: 1012-1022.

Swamy MV, Patlolla JM, Steele VE, Kopelovich L, Reddy BS, Rao CV (2006). Chemoprevention of familial adenomatous polyposis by low doses of atorvastatin and celecoxib given individually and in combination to APCMin mice. Cancer Res. 66: 7370-7377.

Van Roon JA, Lafeber FP, Bijlsma JW (2001). Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis Rheum. 44: 3-12.

Wahane VD, Kumar VL (2010). Atorvastatin ameliorates inflammatory hyperalgesia in rat model of monoarticular arthritis. Pharmacol. Res. 61: 329-333.

Xiao H, Yang CS (2008). Combination regimen with statins and NSAIDs: a promising strategy for cancer chemoprevention. Int. J. Cancer. 123: 983-990.

Yoo SA, Kwok SK, Kim WU (2008). Proinflammatory role of vascular endothelial growth factor in the pathogenesis of rheumatoid arthritis: prospects for therapeutic intervention. Mediators Inflamm. 2008: 129873

Zheng X, Cui X, Avila G, Huang M, Liu Y, Patel J, Kong AN, Paulino R, Shih WJ, Lin Y, Rabson AB, Reddy BS, Conney AH (2007). Atorvastatin and celecoxib inhibit prostate PC-3 tumors in immunodeficient mice. Clin. Cancer Res. 13: 5480-5487.