academicJournals

Vol. 10(22), pp. 472-479, 15 June, 2016
DOI: 10.5897/AJPP2016.4558
Article Number: F5C35AA59186
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Omega 3-fatty acids, atorvastatin as modulators for inflammatory pattern versus diclofenac in osteoarthritis induced in experimental rats

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Received 8 March, 2016; Accepted 25 May, 2016

Antiinflammatory properties of statins and omega-3 fatty acids are well known and documented before. Present work aimed mainly to demonstrate their effects on inflammatory pattern of osteoarthritis (OA) induced in rats. Osteoarthritis was induced by single intraarticular injection of monosodium iodoacetate (MIA) in the right knee joints in a dose level of 24.6 mg/kg body weight. Omega 3 fatty acids and atorvastatin were applied topically(cream form) in a dose levels 1 g/kg and 10 mg/kg body weight respectively either individually or in combination versus diclofenac sodium in a dose level 5 mg/kg body weight for comparison. The treatment started after 24 h of OA induction, daily for 3 weeks. Collective results indicated that the drugs under study significantly decreased serum interleukine-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP) and total cholesterol (TC). Joint tissue contents showed significant decrease in myeloperoxidase (MPO), matrix metalloproteinase2 (MMP2) along with an increase in tissue inhibitor metalloproteinase2 (TIMP2). Combined form of atorvastatin and omega 3 fatty acids demonstrated marked effects than their individual use as compared to Diclofenac.

Key words: Osteoarthritis, monosodium iodoacetate, atorvastatin, omega-3 fatty acids, diclofenac.

INTRODUCTION

Osteoarthritis (OA) is a chronic joint disease, widely distributed all over the world. Joint is composed of articular cartilage and its mechanical properties are due to the integrity of extracellular matrix, which consists of proteoglycan and collagens. Degeneration of the joint cartilage is the main picture of OA, beside other features like changes in synovial and subchondral bone metabolism (Martel -Pelletier et al., 2008).

The clinical features of OA include joint pain, swelling, stiffness and loss of mobility (Goldring and Goldring, 2006) which may be progressed later to characteristic pathological form (Lee, 2003). Matrix metalloproteinases (MMPs) in general facilitate the breakdown of extracellular matrix of the connective tissue. On the other hand tissue inhibitor metalloproteinases (TIMPs) act as inhibitor for MMPs. Therefore during OA pathogenesis,

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TIMP shows significant decrease (Martel-Pelletier et al., 2008).

Proinflammatory cytokines are mediators of OA where interleukin (IL-1B) and tumor necrosis factor α (TNF-α) potentiate chondrocytes to induce matrix degradation factors and activate catabolic condition (Goldring, 2000). Monosodium iodoacetate (MIA) is a glycolytic pathway inhibitor which blocks the activity of glyceraldehydes 3-phosphate dehydrogenase in chondrocytes leading to disruption of metabolism and subsequent chondrocyte death (Grossin et al., 2006).

Diclofenac is an anti-inflammatory drug and has been used in treatment of OA (Burke et al., 2006). Its long term use is commonly associated with potential risk, however, testing other medications of marked therapeutic use and free of side effects may be better clinically (Farid et al., 2010). Last years, many studies referred to the anti-inflammatory effect of statins in chronic disease beside its potential as hypocholesterolemic agent (Youssef et al., 2002). Omega-3 fatty acids (FAs) are long chain polyunsaturated FAs which must be supplemented with diet, since the human body is unable to synthesize it in significant amount (Hussein et al., 2005), and its potential as anti-inflammatory agent is documented before (Simopoulos, 2002).

Docosahexaenoic acid and eicosapentaenoic acid represent the main components of omega-3 FAs (Kris-Etherton et al., 2002). Eicosapentaenoic acid can activate the eicosanoid production in turn have anti-inflammatory and antiarteriosclerotic effect (Dwyer et al., 2004), as inhibitor of pro-inflammatory cytokines (Caughey et al., 1996). Systemic administration of these agents is well known (Raatz et al., 2009), however their application topically (with the except of diclofenac) is not reported before. The present work aimed mainly to study the therapeutic potential of the drugs under study versus Diclofenac (topically) on the inflammatory pattern of Osteoarthritis induced in joints of experimental rats.

MATERIALS AND METHODS

Animals

Adult male albino rats (150 to 200 g) were used in the present study. Animals were kept in a plastic cages at room temperature and on 12 h light-dark-cycle, were fed commercially available standard chow diet and water ad libitum. Experimental design, protocol of the study and animal handling were performed according to the guidelines of the Ethical Committee of the Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.

Induction of osteoarthritis

Forty two rats as supplied from Egyptian organization for biological products and vaccine (Cairo, Egypt), were divided into 6 groups (n = 7). First group was kept as normal, the remaining groups from (2 to 6) were anesthetized by thiopental 40 mg/kg. Right knees were shaved and disinfected, received a single intra-articular injection through the patellar ligament, of 24.6 mg/kg monosodium iodoacetate (MIA) in 0.6 ml saline (Sigma Aldrich).

Experimental design

Osteoarthritic rats were divided into five groups: The first one received no drugs and kept as OA control. Four groups received specific doses in the form of topical cream application of the following drugs separately for 3 weeks, diclofenac, (Novartis Pharma AG Basel, Switzerland) 5 mg/kg body weight, atorvastatin, (MUP pharmaceutical CO Isamelia, Egypt) in a dose level 10 mg/kg body weight, omega-3 fatty acids (eicosapentaenoic acid 54.5%,docosahexaenoic acid 45.5% ,Arab Co. for Gelatin and Pharmaceutical Products, Egypt) in a dose level 1 g/kg body weight . The last group received a combination of atorvastatin and omega-3 FAs using the above mentioned doses.

Cream preparation for atorvastatin and omega-3 FAs

Atorvastatin powder was finely ground in a glass morter to form very fine powder. Cream base was added in portions to atorvastatin and mixed thoroughly.Omega-3 FA and diclofenac sodium were similarly prepared like atorvastatin and were freshly prepared before their application.

Cream base formula

Cetyl alcohol 5 g, cetomacrogol 5 g and emulsifying wax, mineral oil 20 g, glycerin 10 g,methyl paraben 0.18 g, propyl paraben 0.02 g and purified water 59.8 g.

Dose incorporation

Diclofenac, atorvastatin, omega-3 FAs, in cream form were applied daily for 3 weeks in a constant weight 0.5 g cream/200 g body weight of the rat, corresponding to 5 and 10 mg, 1 g/kg body weight respectively.

Blood and tissue sampling

At the end of 1 day (after OA induction) and 3 weeks of treatment, 2 ml blood from retro-orbital vein were collected and centrifuged for serum preparation. Serum IL-6, C-reactive protein(CRP), and TNFα were evaluated by ELISA technique, following the instruction of their corresponding kits Ray Biotech Inc., DRG International Inc., USA and Koma Biotech Inc. Korea, respectively (Banerjee et al., 2003). Serum total cholesterol (TC) was determined colorimetrically using commercially available kit, xpress Bio and Biochain, CA, USA according to Allian et al. (1974). At the end of 3 weeks, right knee joints were isolated and rinsed in ice cold saline, divided into 2 parts; the first one was stored at -80°C for subsequent measurements of myeloperoxidase (MPO), TIMP2 and MMP2 using RT PCR technique to (Pfaffl, 2001). The second part was used for histopathological examination.

Histopathological study

Each knee joint was kept in 10% formalin, 1% HNO_3 for 24 h or more till they became soft, rinsed with running water, dehydrated in alcohol series, kept in xylene, paraffin 45°C, and lastly frozen. Five-micron tissue sections were cut by Leica Microtome, stained with haematoxylin and eosin (H&E) and subjected to histopathological examination.

Table 1. Serum levels of inflammatory markers and TC after 24 h of Osteoarthritis induction (OA control) as compared to normal rats (n=7).

Biochemical parameters	Normal group	OA control
IL-6 (pg/ml)	8.3±1.1	18.9 ±5.9*
TNF-α (pg/ml)	11.2±1	28.02±2.7*
TC (mg/dl)	69±0.9	75.8 ±11.7*

Values are expressed as mean ± SD, $p < 0.05$.

Table 2. Serum levels of inflammatory markers and TC in OA rats treated with diclofenac, atorvastatin, omega-3 FAs for 3 weeks as compared to OA control group (n=7).

Biochemical parameters	Normal	OA control	Diclofenac	Atorvastatin	Omega-3	Atorvastatin + omega-3
IL-6 (pg/ml)	7.8±0.8	27.4±1.4*	13.2±0.6*$^{\neq}$	13.7±0.5*$^{\neq}$	19±1*$^{\neq a}$	10.6±0.6*$^{\neq a}$
TNF-α (pg/ml)	9.8±0.8	29.4±1.3*	15.4±0.7*$^{\neq}$	15.9±0.7*$^{\neq}$	21.3±1.2*$^{\neq a}$	12.5±0.6*$^{\neq a}$
CRP (ng/ml)	1.8±0.1	13.1±0.7*	4.6±0.5*$^{\neq}$	6.8±0.3*$^{\neq a}$	7.3±0.2*$^{\neq a}$	2.9±0.3*$^{\neq a}$
TC (mg/dl)	86±2.3	97.5±1.9*	82.8±1.9*$^{\neq}$	89.2±1.7$^{\neq a}$	73±1.9*$^{\neq a}$	90±2.9$^{\neq a}$

Values are expressed as the mean ± SD, $p < 0.05$. ≠ versus the OA control group.

Table 3. Effect of diclofenac, atorvastatin, omega-3FAs for 3 weeks on joint tissue contents of TIMP2, MMP2 and MPO against OA control in Osteoarthritis rats (n=7).

Items	Normal	OA control	Diclofenac	Atorvastatin	Omega-3	Atorvastatin and Omega-3
TIMP2	25.5±4	11.5±2.7*	33.1±6.3$^{\bullet}$	29.6±6.1$^{\bullet}$	26.7±5.8$^{\bullet}$	27.7±3.6$^{\bullet}$
MMP2	1.2±0.2	10.3±1.8*	1.2±0.1$^{\bullet}$	1.3±0.1$^{\bullet}$	3.1±0.1*$^{\bullet x}$	3.4±0.1*$^{\bullet x}$
MPO	0.1±0.02	0.9±0.1*	0.3±0.03$^{*\bullet}$	0.4±0.1*$^{\bullet}$	0.4±0.1*$^{\bullet}$	0.2±0.03$^{\bullet}$

Values are expressed as the mean ± SD, $p < 0.05$. *versus the normal group. $^{\bullet}$versus the OA control group. ^{x}versus the diclofenac-treated group.

Statistical analysis

Statistical analyses of data were done by Prism 5, Graph Pad, CA, USA. Results were presented as mean ± Standard Deviation (SD). Statistical differences were compared using student's t-test or one-way Analysis Of Variance (ANOVA), followed by Tukey test, considering $p < 0.05$ as statistically significant.

RESULTS

Effect of MIA after 24 h

Rats which received MIA injection demonstrated after 24 h significant increase in serum IL-6, TNFα and TC as compared to normal rats ($p<0.05$, Table 1).

Effect of atrovastatin, Omega-3 FAs and diclofenac after 3 weeks

Topical application of diclofenac induced a significant decrease of all the inflammatory markers. Omega 3 FAs and atorvastatin application showed similar decrease, while their combination demonstrated marked anti-inflammatory effect. Serum IL-6 demonstrated significant decrease following diclofenac (54.8%), atorvastatin (59.1%), omega 3 FA (45.8%), combination of the last two achieved a marked decrease (69.4%), total cholesterol illustrated also significant decrease (Table 2). Matrix metalloproteinase 2 and MPO showed significant increase along with TIMP2 decrease in OA group. Drugs application induced the reverse effects (Table 3, Figures 1 to 3).

Histopathological results

Joint tissues of normal rats exhibited normal articular surface, bone, synovium and chondrocytes (lesion score 0+) (Figure 4a). Osteoarthritic control group demonstrated intense pathological alteration in articular surfaces components (lesion score 3+) pyknotic chondrocytes, debris in articualr cavity, thickened synovial membrane by edema and inflammatory cells.

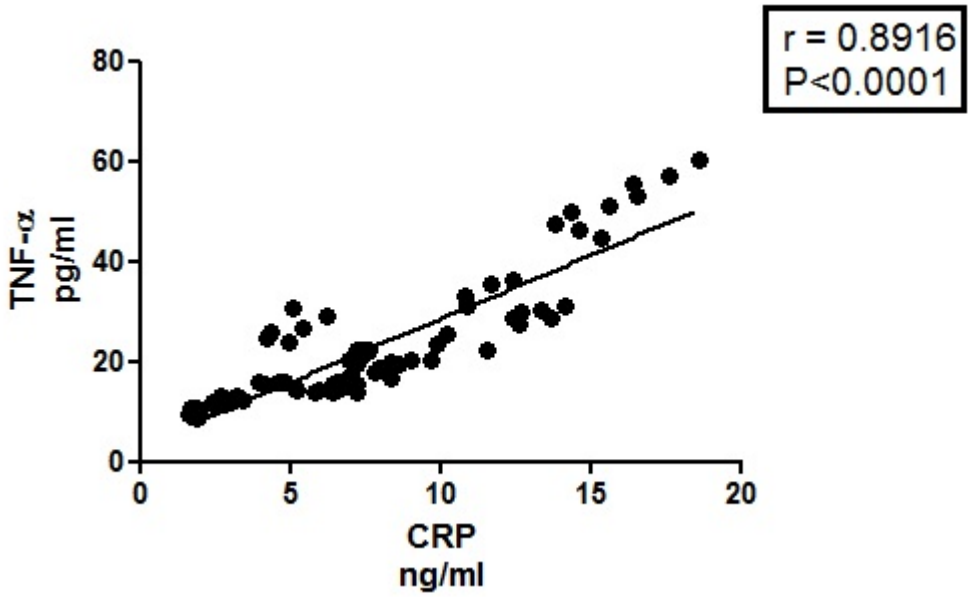

Figure 1. Correlation between CRP and TNF-α.

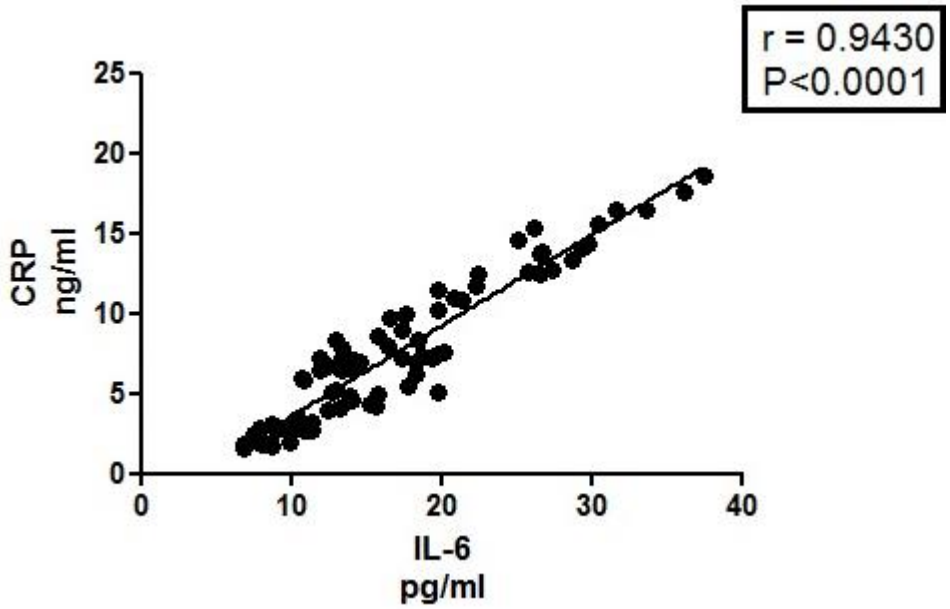

Figure 2. Correlation between IL-6 and CRP.

Moreover fragmentation of bony trabeculae with partial replacement by fibrous tissues accompanied by numerous osteoclasts (Figure 4b, c). Diclofenac treated group showed slight improvement in pathological changes (lesion score 2+). The articular surface showed loosing of chondrocytes from their lacunae, mild spindle cells proliferation together with normal synovium and articular cartilage (Figure 4d). Atorvastatin treated group had moderate improvement in OA (lesion score2+) and usually showed inflammatory cells aggregation inside the joint cavity, mild thickening in synovial membrane and early necrotic changes in bony tissues (malacia) (Figure 4e). Omega-3 FA group illustrated great improvement in OA changes of joints (lesion score 1+), softening and disorganised bone trabeculae, disorganized lacunae and absence of inflammatory cells (Figure 4f). The

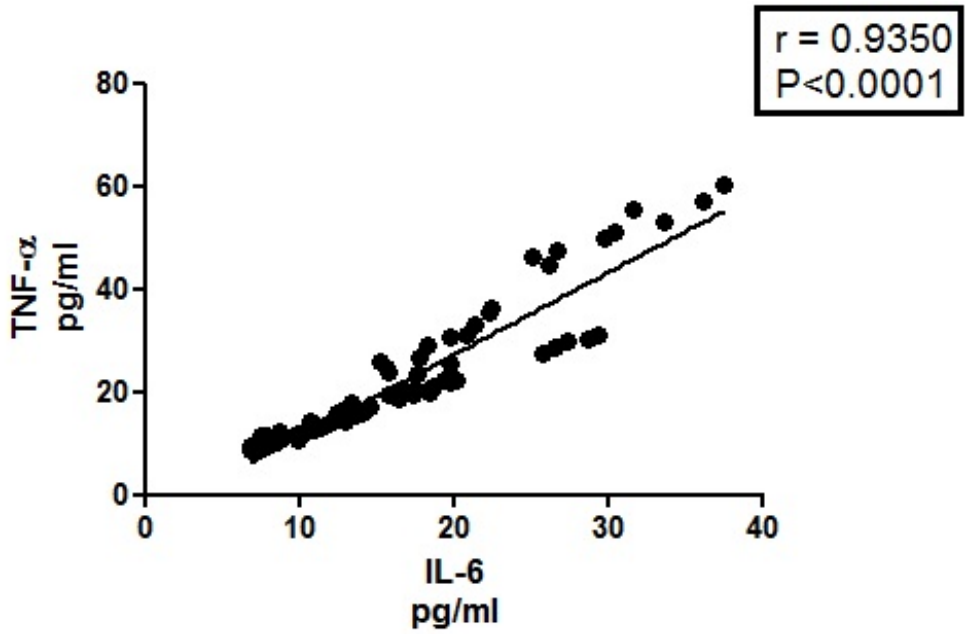

Figure 3. Correlation between IL-6 and TNF-α. Positive correlations were recorded between the inflammatory markers (CRP-TNF-α), (IL-6, CRP) and (IL-6, TNF-α).

combination treatment demonstrated great amelioration in the pathological alteration in the articular surfaces (lesion score 1+). Mild changes characterized by disorganized cartilageneous lacunae and articular cartilage, with a few pyknotic chondrocytes and normal synovial without inflammatory changes (Figure 4g).

3+------intense pathological changes in articular surfaces.
2+------moderate pathological changes in articular surfaces.
1+-----mild pathological changes in articular surfaces.
0+-----no pathological changes in articular surfaces.

DISCUSSION

Present study demonstrated that topical application of omega-3 FAs and atorvastatin either individually or in combination induced marked anti-inflammatory effect as compared to diclofenac. Histopathological examination of the knee joints of OA treated rats showed evident correlation with the biochemical findings. It was previously reported that MIA produces a rapid, technically, straight forward OA model which mimics the pathological and pharmacological features associated with human OA (Guzman et al., 2003).

In addition, it induces chemical injury and inflammation of chondrocytes or synovial membrane or both. Mechanical loading that induces OA causes the synovial cells to induce signals which can mediate the production of pro-inflammatory cytokines and cartilage degradation (Goldring and Goldring, 2007). Our histopathological findings present clear evidence of this and are in agreement with previous studies (Al-saffar et al., 2009). Induction of OA is associated with flactuation of TC. Gierman et al. (2014) indicated that hypercholesterolemia may have a role in the development of OA.

Tissue inhibitor metalloproteinase is an endogenous protease inhibitor which bind to active MMPs. MMPs derived from chondrocytes, synovium and polymorpho-nuclear leukocytes, play a major role in cartilage degradation in OA. The balance between TIMPs and MMPs is completely controlled in healthy joint, however in OA, MMP levels exceeds TIMPs leading to degradation of cartilage extracellular matrix (Alam et al., 2011).

Significant increase in MMP2 along with TIMP2 decrease in synovial fluid as observed in control group may suggest a disturbance in the balance of the enzymes, leading to high rate turnover in articular cartilage (Lee et al., 2008). Joint tissue of osteoarthritic rats demonstrated high levels of MPO. Presence of the later in syonvial fluid and neutrophils and/or macrophages within the affected joint can exaggerate the inflammatory response (Benito et al., 2005). Tissue MPO and MMP2 showed significant decrease after diclofenac treatment along with TIMP2 increase, as compared to OA control group.

Mahdy et al. (2002) demonstrated that decreased IL-6 may be attributed to reduced cyclic adenosine monophosphate and prostaglandin (PG) production. Interleukine-10 represents a responsive anti-inflammatory agent to PG effect and subsequent suppression of the

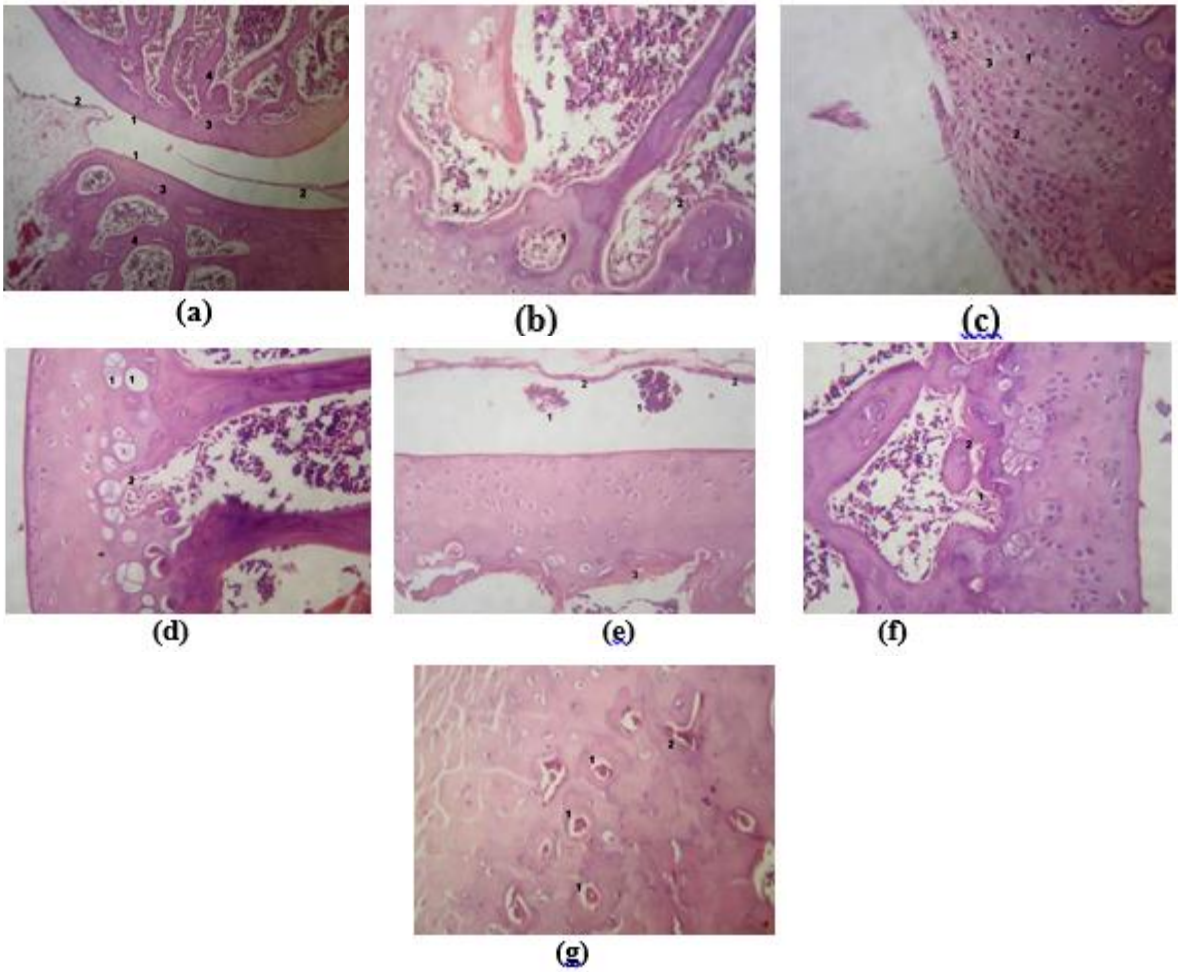

Figure 4. Photomicrographs of knee joints of normal (H&E x 300) and OA rats of the tested groups (H&Ex 1200). (a) Normal knee showing normal articular surface[1], synovium[2], chondrocyte[3] and bone[4]. (b) OA knee showing pyknotic chondrocytes[1], disorganized lacunae[2] and lysis of matrix proteoglycan with little debris[3], inside articular cavity. (c) OA knee showing fragmentation of bony trabeculae replaced by fibrous tissues[1], numerous osteoclasts[2], and inflammatory cell[3]. (d) Dic. OA treated knee showing losing of chondrocytes from lacunae[1], mild spindle cells[2], synovium and normal articular cartilage. (e) Atorvastatin OA treated knee showing aggregation of inflammatory cells in joint cavity[1], mild thickening in synovial membrane[2] and early necrotic changes malasia[3]. (f) Omega-3 FA OA treated knee showing softening of bone trabeculae[1], disorganized bone lacunae[2] and absence of inflammatory cells (g) Atorvastatin and omega-3 FA OA treated knee showing disorganized lacunae of cartilage[1], pyknotic chondrocytes[1] and disorganization of articular cartilage[2]

later resulted in IL-10 increase (Mitchell and Warner, 2006).

Non-steroidal anti-inflammatory drugs as diclofenac exhibit their action through inhibition of cyclo-oxygenase (COX) enzymes, and PG production (Barrios-Rodiles et al., 1999). COX-1 and COX-2 enzymes can be induced by cytokines as TNFα (Wahane and Kumar, 2010), although COX-1 and COX-2 were not measured. In the present study, it seems likely that they were involved and so, reduced TNF-α following diclofenac treatment may have involved COX metabolites. The current study demonstrated that topical application of atorvastatin inhibited the tested inflammatory cytokines (IL-6 and TNFα), confirming the anti-inflammatory propereties of statins that have been reported before (Maher et al., 2009).

The mechanism of action of statins in arthritis may be generated from their ability to suppress 3-hydroxy 3-methyl glutaryl coenzyme A reductase (HMG-CoA) reductase enzyme, and subsequent inhibition of isoprenoid intermediates synthesis which control many inflammatory pathways (Kwak et al., 2003). In turn cholesterol level determination may be relevant to inflammatory pattern of OA in the present study. Reduced TC here may be attributed to an inhibition of HMG-CoA and cholesterol biosynthesis (McCarey et al., 2004).

McCarey et al. (2004) demonstrated that statin can inhibit the levels of IL-6 and ameliorate endothelial dysfunction in rheumatoid arthritis. Previous study indicated that atorvastatin can shift the balance of cytokines milieu in the joints towards the production of anti-inflammatory cytokine IL-10, away from pro-inflammatory cytokines IL-6 and TNFα (Barsante et al., 2005). Topical application of omega-3 FAs significantly reduced serum IL-6, TNFα, and CRP as compared to OA control group. Omega-3 FAs can also reduce arachidonic acid metabolites and decrease the formation of pro-inflammatory compounds like leukotrienes and PGs (Chapkin et al., 1992; Joe and Lokesh, 1997).

Pischon et al. (2003) demonstrated an inverse relationship between omega-3 FAs intake and plasma level of soluble TNF receptors 1 and 2. The later encourage formation of complexes, which preserve the active trimeric form of TNF, preventing TNFα turn out into inactive monomeric forms. The receptors represent a binding protein and/or a slow release reservoir for TNF-α, indeed prolonging its half life.

Topical omega-3 FAs application resulted in hypocholesterolemic effect after 3 weeks of treatment in comparison to osteoarthritic group. Cell membrane FAs play a critical role in signal transduction where omega 3 FAs is able to modify gene expression, and change lipid level via this mechanism (Lapillonne et al., 2004). Omega-3 FAs modulate the function of sterol regulatory binding protein and peroxisome proliferation-activated receptors, both of which are involved in lipid homeostasis (Xu et al., 1999).

Yang et al. (2011) postulated that diclofenac down regulates MMP2 and MMP9 expression and their upstream enzymes of plasminogen activator urokinase and plasminogen inhibitor, both are associated with destruction of articular cartilage. Present study demonstrated also that atorvastatin significantly decreased MPO, due to an inhibition of neutrophil migration exerted Okouchi et al. (2003), and neutrophil influx to the joint of arthritic rats. This reflects a modification of tissue destruction (Joe and Lokesh, 1997). The current histopathology may present significant support to the biochemical one. Dalcico et al. (2012) reported that IL-6, IL-1 and TNFα activate the expression of metalloproteinase, so that cytokines inhibition by atorvastatin is associated with MMP2 reduction. Omega - 3 fatty acids suppresses also MMP and increases TIMP2 production via reduction of TNF-α and PGE2 (Curtis et al., 2002), while its association with atorvastatin showed significant results as compared to diclofenac treatment.

CONCLUSION

We conclude that topical application of omega 3 FAs and atorvastatin either individually or in combination induces anti-inflammatory and hypocholesterolemic effect in OA rats as compared to Diclofenac. This represents a new topical candidate for OA treatment in experimental rats but clinical trials for long term use may be recommended to confirm the present findings.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

We wish to thank Dr. Heba Khalil Mohammed, Theodor Belhars Research Institute, Egypt and Prof. Dr. Abd Al-Monem Ahmed Ali Mohamed, Head of Pathology Department, Veterinary Medicine College, Zagazig University, Egypt for their scientific help and performance of the histopathological study of the current work.

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