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Omega 3 Fatty Acid and Vitamin A Ameliorate Carrageenan-induced Joint Inflammation in Wistar Rats

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ABSTRACT

This study investigates the effect of omega 3 and Vitamin A on joint inflammation in Wistar rats. Joint inflammation was induced by carrageenan and the animals were treated with omega 3, Vitamin A or a combination of both for 10 days. Changes in Knee diameter (KnD), Tumor Necrosis Factor-α (TNF-α) level and C-Reactive protein (CRP) level were assessed to determine treatment efficacy. Carrageenan caused significant increase in KnD, TNF-α and CRP compared to the control. Treatments with Omega 3 alone or in combination with Vitamin A significantly reduced the elevated KnD, TNF-α and CRP. Vitamin A alone produced similar effect on KnD and TNF-α but had no effect on CRP. It is thus concluded that though both treatments decreased knee diameter at the 10th week, Omega 3 alone and in combination with Vitamin A showed a better outcome with a higher decrease in TNF-α and CRP than Vitamin A alone.

Keywords: Joint inflammation, Omega 3 fatty acids, Vitamin A, knee diameter, TNF-α, C-reactive protein

INTRODUCTION

Inflammation of the joints is a known feature of arthritis disease affecting one or more joints[1]; it is strongly associated with age, joint trauma, altered biomechanics, and obesity[2,3] particularly osteoarthritis characterized by joint pain

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with loss of joint form and function secondary to articular degeneration[3,4]. The structural changes during arthritis was conclusively shown to be preceded by inflammation/synovitis[5,6] and prominent inflammatory mediators such as cytokines, Nitric Oxide, Reactive Oxygen Species and matrix degrading enzymes produced by chondrocytes and synoviocytes have been identified[7]. These mediators are activated by exogenous materials or autologous antigens in the case of autoimmune diseases like rheumatoid arthritis[8] while in the case of non-autoimmune arthritis like osteoarthritis, it could be activated by obesity, ageing or trauma[2,3]. As the joint is continuously exposed to these factors, degradation of collagen and proteoglycans in cartilage leads to fibrillation, erosion and cracking in the superficial cartilage layer which could later spread deeper.

A high prevalence of arthritis has been identified in the low- and medium-income countries[9] where 90 % of the global disease burden are found yet contributing 12 % in global spending on health[10]. In adults above 60 years of age, 9.6 % of men and 18 % of women in the world suffers varied degree of osteoarthritis with a 10-fold increased risk caused by farming for more than 10 years[11]. The multiplying effect of farming on osteoarthritis may account for a higher prevalence in the low-income country were obesity might not be rampant as obtained in high income countries. The United Nation's projection indicates that by the year 2050, 20 % of the world population will be above 60 years of age, 15 % of which would have symptomatic osteoarthritis with one third of them severely disabled. By this projection, when 130 million suffers osteoarthritis, 40 million of them will be disabled[12,13] in the year 2050. A clarion call was then made by WHO to intensify efforts at identifying a cost-effective, safe, and efficacious therapy for long-term management of osteoarthritis[13]. The current therapy ranges from biological (e.g. bone marrow transplant, gene therapy, nanotechnology etc) to chemical [e.g. non-steroidal anti-inflammatory drugs, corticosteroids, and Disease-modifying anti-rheumatic drugs (DMARDs)] which sometimes cause sustained remission with side effects that discourage long term usage yet they are expensive and the chances of infection are high[14].

Diet modification and food supplements have been documented to be beneficial in long-term prevention and treatment of chronic disease in the aging adults[15]. Omega 3 fatty acids are polyunsaturated fatty acids (PUFAs) which include α-linolenic acid (ALA) (found in plant oils), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) commonly found in marine oils. All are essential in the diet for normal metabolism, as there is no mechanism in humans for producing these fats from other substances[16,17]. Studies in humans and animals have shown a negative association between the ingestion of polyunsaturated

fatty acids and the incidence of cardiovascular diseases, diabetes, other autoimmune diseases and cancer; by decreasing the concentrations of proinflammatory cytokines, arachidonic acid derivatives and other inflammatory biomarkers[18]. Specifically, current evidences have shown that dietary intake of EPA and DHA as contained in omega 3 confers anti-inflammatory function by altering the production of proinflammatory markers prostaglandin E2, thromboxane B2, and leukotriene B4 toward a more anti-inflammatory profile in rheumatoid arthritis[19]. Also, omega 3 reduces the need for concomitant analgesic treatment when used as adjuvant to DMARD in rheumatoid arthritis patients[20].

Oxidative stress is also implicated in the aetiology of arthritis thus, use of antioxidants such as Vitamin A is also beneficial in relieving joint inflammation. Vitamin A acts as a first line defence against free radical attack and lipid peroxidation by its ability to stabilize highly reactive free radicals either independently or as a part of large enzyme system[21]. Vitamin A has been postulated to improve joint inflammation by protecting against oxidative damage, and modulating inflammatory response, cellular differentiation and biologic actions related to bone and collagen synthesis[22]. Thus, both omega 3 fatty acids and Vitamin A possess anti-inflammatory activity that could be used as alternative medicine for the management of chronic joint inflammation, it is however not known if a combination of both could act synergistically to alleviate joint inflammation. This study was therefore designed to investigate the effect of omega 3 and Vitamin A on joint inflammation induced by carrageenan in female Wistar rats.

METHODOLOGY

Drugs

Omega-3 fish oil (containing EPA 180 mg and DHA 120 mg) and Vitamin A (10,000 IU [from fish liver oil and retinyl palmitate]) manufactured in USA for Mason Vitamins.

Animals

A total of 24 female Wistar rats weighing 150-200g were used for the study. The animals were obtained from Department of Human Physiology, Ahmadu Bello University Zaria and housed in cages under standard laboratory conditions and had access to food and water *ad libitum*.

Experimental design

The animals were randomly divided into 6 groups (n=4) and used for the ten weeks study. In the first 2 weeks, animals received CGN injection at their knee joint 3 times/week to induce inflammation (0.02ml of 1% CGN) as described

by Manole et al[23]. After establishment of inflammation, which was assessed by clinical signs such as swelling, redness, deformity and ankylosis in the knee joints, 200mg/kg omega 3 and Vitamin A (4000IU/kg) were orally administered daily, and treatment with CGN 3 times/week was continued for the remaining period. The experimental groups were as follows:

Group 1: Served as normal control and were given normal feed and water (NC);

Group 2: Served as injection control and were injected normal saline solution in their knee joint 3 times/week (NSi);

Group 3: Served as disease control and were injected 0.02 ml of 1% carrageenan solution in their knee joint 3 times/week (CGN);

Group 4: Oral administration of 200mg/kg omega 3 daily[24] + 0.02 ml of 1% carrageenan solution injected in their knee joint 3 times/week for 8 weeks (CGN+Omg3);

Group 5: Oral administration of 4000IU/kg Vitamin A daily[25] + 0.02 ml of 1% carrageenan solution injected in their knee joint 3 times/week for 8 weeks (CGN+VitA);

Group 6: Oral administration of 200mg/kg omega 3+ 4000IU/kg Vitamin A daily + 0.02 ml of 1% carrageenan solution injected in their knee joint 3 times/week for 8 weeks (CGN+Omg3+Vit A).

Induction of Joints inflammation and measurement of knee joint diameter

Female Wistar rats were subjected to knee joint inflammation by injecting 0.02ml of 1% CGN solution into the knee joint 3 times/week for 10 weeks[23]. Joint diameter was measured before first injection (at day 0), second week and thereafter, weekly for 8 weeks. Appearance of clinical symptoms such as swelling, redness, deformity and ankylosis in the knee joints indicates a sign of joint inflammation. Joint inflammation and its severity were measured weekly by measuring the diameter of the swelling at the knee of the hind limbs using veneer calliper[26,27].

Blood collection

At the end of the 10th week, the animals were anesthetized with 0.15ml/kg ketamine hydrochloride injection[28] and blood samples were collected by cardiac puncture into plane sample bottles for serum determination of tumour necrosis factor-α (TNF-α) and C-reactive protein (CRP) level.

Determination of Tumor Necrosis Factor-α

The assay for TNF-α activity was carried out using Rat TNF-α ELISA kit purchased from Elabscience (Catalog No: E-EL-R0019 96T) USA.

The micro ELISA plate provided has been pre-coated with an antibody specific to Rat TNF-α and the kit uses sandwich-ELISA principle. Standards or samples were added to the micro ELISA plate well and combined with specific antibody. Then a biotinylated detection antibody specific for Rat TNF-α and Avidin-Horseradish peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contain Rat TNF-α, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in colour. The enzyme-substrate reaction was terminated by the addition of stop solution and the colour turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450nm. The concentration of Rat TNF-α in the samples was calculated by comparing the OD of the samples to the standard curves.

Determination of C-Reactive Protein

The assay for CRP was carried out using CRP rapid latex slides (REF 514002: Spectrum bioscience). Spectrum CRP latex reagent is a suspension of polystyrene particles sensitized with anti-CRP.

All reagents and specimens were brought to room temperature. Serum to be titrated was serially diluted (1:2, 1:4, 1:8 etc) in 0.9 g/ml saline solution. One drop of positive control was placed on slide. Each serum dilution (50µl) was placed individually in successive circles on the slide and (50µl) of the positive control into separate circles on the glass slide. CRP latex reagent was shaken gently and one drop (45µl) was added on each circle next to the sample to be tested and control. It was well mixed using a disposable stirrer spreading the mixture over the whole test area and the slide was tilted gently. Agitated for about 2 minutes with hand and the presence or absence of agglutination was observed.

Presence or absence of agglutination indicate positive (indicating a CRP level of more than 6 mg/L) or negative result respectively. The serum CRP titre can be defined as the highest dilution showing a positive result. The approximate CRP level (mg/L) present in the sample can be obtained by the following formula:

CRP Titre (mg/L) = Highest dilution with positive reaction x Reagent sensitivity (6 mg/lL)

Statistical analysis

The results were expressed as Mean ± Standard Error of Mean (SEM) and values were analysed using mixed analysis of variance (ANOVA) for knee diameter, one-way ANOVA for TNF-α and CRP followed by Tukey's post hoc test. The data were analysed using SPSS for windows (Version 22). *P*-values of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Effect of omega 3 and/or Vitamin A on Knee diameter

The effects of omega 3 and/or Vitamin A on Knee diameter (KnD) at the 2nd and 10th week are shown in table 1. At week 2, KnD was significantly increased in CGN (5.61 ± 0.17 mm), CGN+Omg3 (5.25 ± 0.17 mm), CGN+Vit A (5.12 ± 0.20 mm) and CGN+Omg3+VitA (5.91 ± 0.07 mm) compared to NC and NSi (4.08 ± 0.04 mm and 4.26 ± 0.10 mm, respectively). Progression of inflammatory response was confirmed by steady rise in KnD of CGN (7.06 ±0.21 mm) compared to NC and NSi (4.30 ±0.06 and 4.59 ±0.05, respectively) at the 10th week. Treatment with Omega 3 alone (CGN+Omg3) or in combination with Vitamin A (CGN+Omg3+Vit A) significantly decreased KnD at the 10th week (4.51 ± 0.15 mm and 4.51 ± 0.07 mm, respectively) compared to their respective week 2 values and that of CGN at week 10. While KnD of the animals in CGN+VitA group at week 10 (5.20 ± 0.18 mm) was not different from its value at week 2 (5.12 ± 0.20 mm), it was however significantly lower than the week 10 KnD of CGN (7.06 ±0.21 mm).

Table 1. Effect of omega 3 and/or Vitamin A on Knee diameter at the 2nd and 10th week of carrageenan-induced joint inflammation in female Wistar rats.

Group	Treatment	Knee Diameter (mm)	
		WK 2	WK 10
1	NC	4.08 ± 0.04	4.30 ±0.06
2	NSi	4.26 ± 0.10	4.59 ±0.05
3	CGN	5.61 ± 0.17*	7.06 ±0.21*
4	Omg3+CGN	5.25 ± 0.17*	4.51 ±0.15#
5	Vit A+CGN	5.12 ± 0.20*	5.20 ±0.18#
6	Omg3+Vit A+CGN	5.91 ± 0.16*	4.51 ±0.07#

*P<0.05 compared with control, #P<0.05 compared with CGN

Effect of omega 3 and/or Vitamin A on Tumor Necrosis Factor-Alpha

As shown in figure 1, serum TNF-α was significantly increased (P<0.01) in the CGN group (228.81 ± 25.74 mg/L) compared to NC (51.27 ± 4.57 mg/L) and NSi (61.39 ± 8.45 mg/L), this was however reduced by treatment with omega 3 and/or Vitamin A in the CGN+Omg3 (27.13 ± 3.33 mg/L), CGN+VitA (123.99 ± 17.92mg/L) and CGN+Omg3+VitA (31.48 ± 1.55 mg/L). In fact, Omega 3 either alone or in combination with Vitamin A significantly reduced TNF-α to values below the control levels.

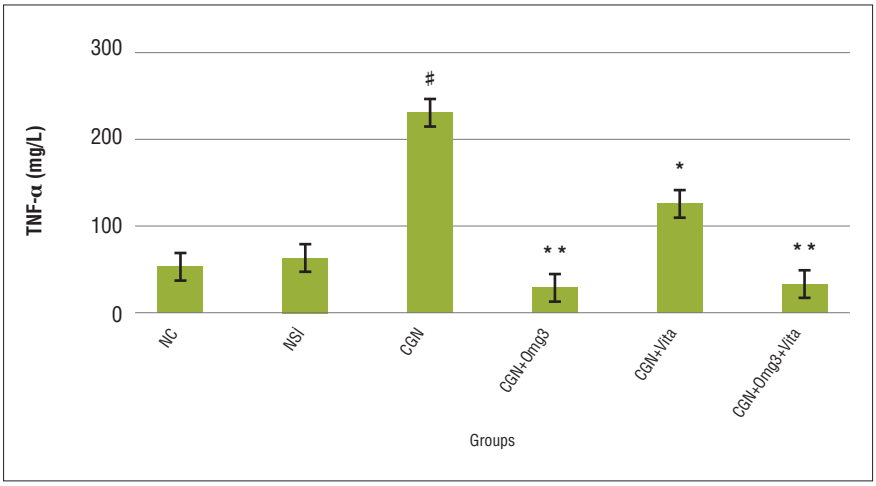

Figure 1. Effect of omega 3 and Vitamin A on Tumor Necrosis Factor-alpha following carrageenan-induced joint inflammation in female Wistar rats. # P<0.05 compared to control, *P<0.05, **P<0.01 compared to CGN.

Effect of omega 3 and/or Vitamin A on C-Reactive Protein

The serum CRP concentration was significantly increased (P<0.01) in CGN compared to NC and NSi, signifying an elevated inflammatory activity in response to carrageenan injection at the 10th week, this was however significantly reduced by omega treatment in the CGN+Omg3 and CGN+Omg3+VitA animals. Treatment with Vitamin A alone in the CGN+VitA animals produced no significant difference in the CRP compared to CGN animals (figure 2).

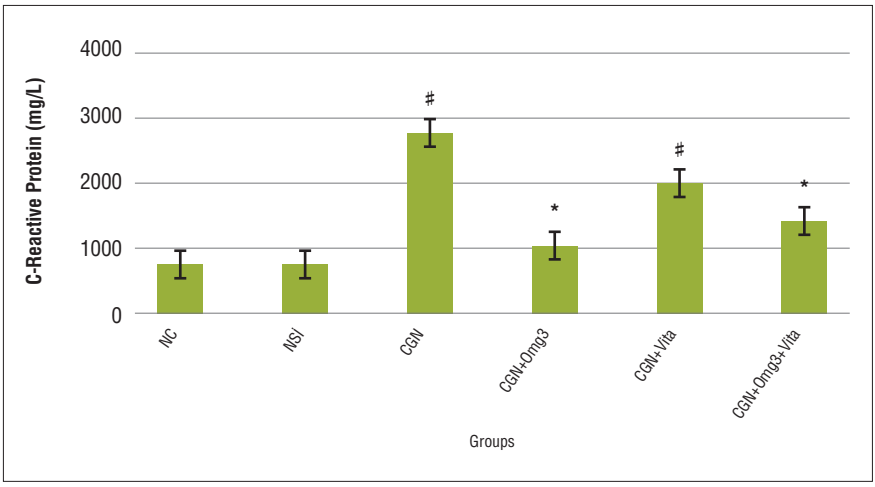

Figure 2. Effect of omega 3 and Vitamin A on C-Reactive Protein following carrageenan-induced joint inflammation in female Wistar rats. # P<0.05 compared to control, *P<0.05compared to CGN.

This study examined the influence of omega 3 fatty acids, Vitamin A and their coadministration on TNF-α and CRP following injection of carrageenan on knee joints of female Wistar rats using the method of Manole *et al* [23]. An elevated inflammatory activity in response to carrageenan injection was observed, which indicates a successful induction of sustained inflammation throughout the period of treatment[23,29]. Assanga *et al.*[30] reported an increase in rat paw oedema and CRP concentration after injection of (0.1ml/paw) of carrageenan solution.

The significant decrease in KnD presented within the groups treated with omega 3 FAs alone and its coadministration with Vitamin A indicate a high degree of anti-inflammatory activity, which was observed by suppression or reversion of inflammatory changes with duration of treatment. That is, decrease in levels of TNF-α and CRP. The effect observed in coadministration of these supplements is assumed to be mostly contributed by omega 3 FAs since Vitamin A alone could not show a similar outcome. Report by El-Seweidy *et al.*[31] supports the finding of the present study, that omega 3 FAs exerted a significant improvement in knee joint of osteoarthritic rats which may imply a significantly decreased pathological change in joint articular surface. A possible mechanism through which Omega 3 fatty acids reduced inflammation may be proposed as due to the fact that omega 3 reduces the formation of eicosanoids with inflammatory characteristics by competing with omega-6 fatty acids for the same enzymatic pathway that leads to the inhibition of TNF-α, IL-1, IL-6 synthesis, cartilage-degrading enzymes and reducing the intercellular adhesion molecule-1 (ICAM-1) expression[32,33].

The above report is in support of the present finding in animals treated with omega 3 FAs where they showed more effectiveness in lowering inflammatory activity by reverting the concentration of TNF-α to the lowest concentration. El-Seweidy *et al.*[34] who reported a significant decrease in TNF-α level following administration of omega 3 FAs in murine osteoarthritic rats. They also observed that omega 3 FAs can reduce serum soluble TNF-α receptor p55 and production of pro-inflammatory cytokines induced via the NFκB system. Nobre *et al.*[29] also reported a decrease in TNF-α along with a decreased paw swelling after treatment with omega 3 FAs in carrageenan-induced rat paw oedema. Omega 3 FAs was able to mediate its effect due to it being an important precursor for resolvins and protectins which are lipid-derived modulators of cell inflammatory processes. These lipid mediators have anti-inflammatory and inflammation resolving capabilities as they inhibit migration of neutrophils from capillaries and limit neutrophil infiltration at sites of inflammation with consequent inhibition of the production of TNF-α[35,36,37].

The positive outcome observed in coadministration of these supplements could be due to TNF-α reverting effect mainly mediated by omega 3 FAs. This implies that omega 3 FAs is more effective in decreasing TNF-α concentration than Vitamin A in CGN-induced joint inflammation and combination of these supplements did not show a better outcome than omega 3 FAs alone. This could be due to potential implication of retinoic acid in cytotoxicity induced NrF2 target genes at some concentrations; NrF2 participate in adaptive cellular defence against retinoid toxicity by enhancing transcription of antioxidant gene[38]. With chronic administration of Vitamin A this mechanism would have been depleted and retinoic acid toxicity outweighed the NrF2 activity. It could also be considered that anti-inflammatory and bone growth supporting effect of Vitamin A at the concentration used (4000IU/kg) is improved when co-administered with omega 3 FAs rather than Vitamin A alone. This could explain the reason why decrease in KnD was more pronounced in Omg3 and Omg3+Vit A than Vit A when compared to CGN, also, when considering response within groups, omega 3 FAs alone and in combination with Vitamin A had a linear decreasing effect across the weeks of experimental period.

When observing the findings of CRP concentration, it could be seen that omega 3 FAs and its coadministration with Vitamin A had a significant decrease compared to CGN control while Vitamin A did not show significant decrease. This confirms omega 3 FAs ability to suppress inflammatory response which is also supported with the decrease in KnD as well as a fall in TNF-α concentration that was earlier mentioned. Hepatic production of CRP could have been decreased

through a mechanism involving omega 3, by the protectin D1 pathway. Protectin D1 is a bioactive product of DHA generated from 17S-hydroperoxy DHA (a metabolic intermediate). It potently regulates critical events related to inflammation and its resolution which involves inhibition of polymorphonuclear cells (PMN) infiltration, T-cell migration and decreased TNF-α level. PD1 has also been shown to decrease COX-2 mRNA expression and block NFκB activation[39]. These could most likely lower the production of proinflammatory cytokines, subsequently decreasing hepatic production of CRP.

It could be suggested that Vitamin A can depress progression of inflammation without necessarily reverting the already formed inflammatory response. This shows a lesser anti-inflammatory activity in Vit A group with duration of treatment, indicating that Vitamin A could suppress but not revert inflammatory changes caused by CGN injection. This finding supports the work of Nagai *et al.*[40] who reported that Am80 (a newly synthesized retinol) significantly reduced the development of foot pad swelling and bone damage in collagen induced arthritis, implying that Vitamin A has short-term anti-inflammatory activity which could be through its ability to modulate inflammatory response, cellular differentiation and biologic actions related to bone and collagen synthesis[41].

Treatment with Vitamin A showed lower anti-inflammatory activity by having a higher TNF-α concentration than that of omega 3 FAs alone and in combination, this coincide with the failure of Vitamin A to show significant decrease in KnD across experimental periods. This implies that Vitamin A could suppress, but not revert inflammatory activity following CGN injection. This contradicts the findings of Nagai *et al.*[40] who reported that Am80 (a synthetic derivative of retinoid) which exhibit specific biological activities of retinoic acid did not inhibit lipopolysaccharide induced TNF-α production in mice. The differences in the results may be due to the fact that in the present study, a different model of inflammation was used, there was a longer duration of treatment, accompanied with continuous feeding on standard nutritive diet. Also, differences in geographical location which could account for varying environmental condition can contribute to the contrasting outcome of these experiments. It could also be proposed that Vitamin A was able to decrease TNF-α through its ability to inhibit translocation of the transcription factor NFκB and interrupt the secretion of inflammatory cytokines[42].

The C-reactive protein concentration observed after treatment with Vitamin A could be the reason why the KnD of Vit A at week 10 could not show significant decrease when compared to week 2, signifying higher inflammatory activity

than omega 3 FAs and its combination, on knD. However, treatment with Vitamin A showed significant decrease in KnD when compared to CGN control; this at least shows a degree of anti-inflammatory activity which supports its lower CRP level compared to CGN. The finding that Vitamin A could not show significant decrease in CRP is in contrast with that of Cha *et al.*[25] who reported that 4000IU/kg of Vitamin A supplementation might be considered as adequate level that should show favourable effect in rats. In this study however, Vitamin A at 4000IU/kg showed a weak response to decrease CRP concentration; this could probably be due to difference in geographical as well as laboratory conditions or model of inflammation used. Nonetheless, Vitamin A has been reported likely to be toxic to cells even at levels considered safe affecting cell survival and function[43].

The measure of inflammatory activity through the knee diameter, inflammatory markers: TNF-α and CRP have been shown to be regulated at various level in response to the administration of omega 3 and Vitamin A either alone or in combination. From the result, these supplements show a promising outcome in reverting and suppressing carrageenan-induced joint inflammation in Wistar rats; but omega 3 alone or in combination with Vitamin A had a greater effect on knee diameter and other parameters investigated than Vitamin A alone.

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