Omega 3 (n – 3) fatty acids down-regulate nuclear factor-kappa B (NF-κB) gene and blood cell adhesion molecule expression in patients with homozygous sickle cell disease


ABSTRACT

Chronic inflammation and reduced blood levels of omega-3 fatty acids (n – 3) are known characteristics of sickle cell disease (SCD). The anti-inflammatory properties of n – 3 fatty acids are well recognized. Omega-3 treated (n = 24), hydroxyurea (HU) treated (n = 18), and n – 3 untreated (n = 21) homozygous SCD patients (HbSS) and healthy (HbAA) controls (n = 25) matched for age (5 – 16 years), gender and socioeconomic status were studied. According to age (5 – 10) or (11 – 16) years, two or three capsules containing 277.8 mg docosahexaenoic (DHA) and 39.0 mg eicosapentaenoic (EPA) or high oleic acid placebo (41%) were assigned to n – 3 treated and n – 3 untreated groups, respectively. Hydroxyurea treated group was on dosage more than 20 mg/kg/day. The effect of supplementation on systemic and blood cell markers of inflammation was investigated. The n – 3 treated group had higher levels of DHA and EPA (p < 0.001) and lower white blood cell count and monocyte integrin (p < 0.05) compared with the n – 3 untreated. No difference was detected between the two groups regarding C-reactive protein, granulocytes integrin and selectin, plasma tumour necrosis factor-α and interleukin-10. The n – 3 treated group had lowered nuclear factor-kappa B (NF-κB) gene expression compared to n – 3 untreated and HU treated groups (p < 0.05). This study provides evidence that supplementation with n – 3 fatty acids may ameliorate inflammation and blood cell adhesion in patients with SCD.
The mechanisms by which omega-3 fatty acids exert their anti-inflammatory effects are not fully understood. Nevertheless, mounting evidence indicates the importance of nuclear factor-kappa B (NF-κB) gene [21,22], a key transcription factor involved in the up-regulation of inflammatory cytokines and adhesive molecules genes [23]. Therefore, we postulated that the observed therapeutic effect of omega-3 fatty acids supplementation on sickle cell disease could partly implicate suppression of NF-κB gene transcription and adhesive molecule expression in patients with SCD.

In this study, we investigated a) whether supplementation with n-3 DHA and EPA ameliorates systemic markers of inflammation and blood cells adhesion and b) modulates NF-κB gene expression.

2. Methods

2.1. Subjects

This study was conducted in the context of a single centre, two-arms, randomised, placebo-controlled, double blinded clinical trial [16]. Steady state homozygous (HbSS) sickle cell patients (who did not experience painful crisis or other acute medical condition for at least 1 month) were enrolled in Ibn-Aoaf Paediatric Hospital, Khartoum, Sudan. Haemoglobin phenotype of patients (HbSS) and healthy controls (HbAA) was confirmed by cellulose acetate electrophoresis. The patients were on regular folate supplement, before and after enrolment. The exclusion criteria were: other chronic diseases and receiving blood transfusion in the previous four-months. The group on omega-3 fatty acid received, according to age, a daily dosage of 500–750 mg capsules containing 277.8 mg DHA and 39.0 mg EPA. The placebo group received capsules containing high oleic acid (41%) oil blend. Vitamin E (1.5 mg/capsule) was added to both types of capsules to prevent fatty acid peroxidation. SCD patients on hydroxyurea treatment were on dosage more than 10 ml 1× PBS and washed twice by centrifugation at 250 × g for 5 min. Mononuclear cells and granulocytes were washed twice with 10 ml 1× PBS at 2000 × g for 5 min and resuspended in 450 μl serum obtained from the same blood sample and 50 μl dimethyl sulfoxide (DMSO). Red blood cells were washed three times with normal saline (0.85% NaCl) and centrifuged at 3000 rpm to remove traces of plasma anduffy coat. The resulting plasma and RBC were stored at −80 °C, whilst mononuclear cells and granulocytes were stored in liquid nitrogen, until the analysis.

2.3. Haematological parameters

Before sample processing, Hb concentration, white blood cell count (WBC) and platelet count were obtained by using an automated haematology analyser Sysmex KX-21N (Sysmex Corporation, Kobe, Japan).

2.4. Analysis of red blood cell fatty acids

Total lipids were extracted by the method of Folch et al. [1957]; the detailed methodology was described in our previous publication [14].

2.5. C-reactive protein (CRP) assay

C-reactive protein was assayed by using commercially available enzyme-linked immunosorbent i-Chroma hscRP test kits and an i-CHROMA reader (Boditech, Korea).

2.6. Measurements of plasma tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10)

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine TNF-α and IL-10 plasma concentration (Pierce Biotechnology, Rockford, USA). All samples were analysed simultaneously using the same kits under the same analytical conditions.

2.7. Assessing (52- integrin CD11b/C18 (CD-11b) and L-selectin (CD62L) expression in granulocytes and monocytes

Mononuclear cells and granulocytes were quickly defrosted at 37 °C. 200 μl of each defrosted sample was washed in 10 ml 1× PBS by centrifugation at 400 × g for 5 min. The supernatant was discarded, and the pellet was suspended in 1 ml 1× PBS/BSA 2 mg/ml (Sigma-Aldrich Co. UK, A7906). 50 μl of cell suspension was incubated on a shaker with 10 μl (used as supplied) of anti-human-CD11b fluorescein isothiocyanate (FITC) conjugated antibody, anti-human-CD62L-FITC, or mouse anti-IgG, at 4 °C for one hour in the dark. Cells were also incubated with no antibody at the same conditions, and used as negative control. After incubation, cells were washed three times in 1.5 ml 1× PBS, by centrifugation at 150 × g for 5 min. Cell pellet was resuspended in 200 μl 1× PBS, and used for flow cytometry analysis.

In order to standardise the positioning of monocytes, micro beads conjugated to anti-Human-CD14 antibody were used for monocyte purification (Miltenyi Biotec GmbH, Germany). 107 total mononuclear cells counted by microscope visualization using a counting chamber were suspended in 80 μl of incubation buffer (0.5% BSA, 2 mM EDTA in 1× PBS pH 7.2), and incubated with CD14 micro beads for 30 min followed by 3 ml of histopaque-1077 to form two distinct phases. Consequently, 5 ml of the collected whole blood was layered carefully over the histopaque and centrifuged at 700 × g for 50 min at 22 °C (room temperature). Separation of plasma, mononuclear cells/platelets, granulocytes and red blood cells was obtained. The top layer (plasma) was transferred to cryotubes and stored at − 80 °C. The mononuclear cells/platelets fraction was transferred to a new tube and re-suspended in 10 ml 1× phosphate buffer saline (1× PBS) (Sigma-Aldrich, UK) and centrifuged at 250 × g for 10 min at 22 °C. The supernatant (platelets) was transferred to a new 15 ml tube, whereas the remaining pellet was re-suspended with 10 ml 1× PBS and washed twice by centrifugation at 250 × g for 10 min. Mononuclear cells and granulocytes were washed twice with 10 ml 1× PBS at 2000 × g for 5 min and resuspended in 450 μl serum obtained from the same blood sample and 50 μl dimethyl sulfoxide (DMSO). Red blood cells were washed three times with normal saline (0.85% NaCl) and centrifuged at 3000 rpm to remove traces of plasma and Buffy coat. The resulting plasma and RBC were stored at − 80 °C, whilst mononuclear cells and granulocytes were stored in liquid nitrogen, until the analysis.

2.2. Preparation of the samples

The whole blood (5 ml) was fractionated into red blood cells (RBCs) and plasma by cold centrifugation at 3000 rpm for 15 min. The top plasma layer was carefully syphoned off and transferred into another tube. Theuffy coat was removed and transferred to a new tube containing 0.5 of RNA later (Sigma, UK) for RNA stabilisation. The lower red cell layer was washed three times with physiological saline (0.85% NaCl) and centrifuged to remove traces of plasma and Buffy coat. The resulting plasma and RBC pellet were thoroughly flushed under oxygen-free nitrogen and immediately stored at −80 °C until analysis.

10 ml of whole blood was used to obtain monocytes and granulocytes through a sequence of centrifugation steps using histopaque-1077 (density 1.077 g/ml) and histopaque-1119 (density 1.119 g/ml) (Sigma-Aldrich, UK). Firstly, 3 ml of histopaque-1119 was added to a 50 ml tube
at 4 °C, on a shaker in the dark. Subsequently, cells were washed three times in incubation buffer by centrifugation at 300 × g for 10 min. Cells were re-suspended in 500 μl of incubation buffer. Cells bound to CD14 micro beads were sheathed by the use of a column placed in a magnetic field separator (Miltenyi Biotec GmbH, Germany). Cells were eluted by washing the column with 1 ml of incubation buffer. Cells were precipitated at 300 × g, diluted in 1 × PBS>BSA 2 mg/ml, and labelled with anti-human-CD11b FITC conjugated anti-bodies. The fluorescent signal from the purified monocytes was compared to the same non-purified sample.

Samples were loaded into 96-well plates in duplicates, and expressions of CD11b and CD62L were measured by flow cytometry using the GUAVA PCA-96 flow cytometer and GUAVA express plus software (Guava technologies, UK). An optical emission filter for 580–583 nm was used to detect FITC fluorescence. Over 5000 cells were submitted to analysis at each trial. Unlabelled cells and IgG labelled cells were used as the negative control for non-specific flow cytometry background. Monocytes and granulocytes cells were gated carefully based on forward scatter and side scatter. We could not measure expression of CD62L in monocytes because we did not have enough samples to conduct the analysis.

2.8. RNA isolation and Reverse Transcription-Polymerase Chain Reaction (PCR)

RNA was isolated from peripheral blood cells using RNAqueous Kit according to the manufacturer’s instructions (Ambion). RNA integrity was checked by using 2% agarose gel electrophoresis. First strand cDNA synthesis was performed using 1 μl total RNA and 300 ng/μl random primers and SuperScript III Reverse Transcriptase (Life Technologies). The resultant cDNA stored at −20 °C.

2.9. Quantitative Real-time Polymerase Chain Reaction

Real-time quantitative PCR of the cDNA template was performed in an ABI Prism 7000 SDS (Applied Biosystems) run by software SDS-2.3. The PCR reaction contained 4 μl of cDNA, 20 μl reaction mix (Rob mix, ABI). The PCR cycling conditions were: 95 °C (15 s), 58 °C (20 s), 72 °C (25 s) for 35 cycles. Results were calculated as expression of the target gene (NF-κB) relative to expression of the reference gene (GAPDH).

2.10. Data analysis

The data were described by mean ± SD or median and interquartile range (IQR) as pertinent. The three groups were compared for NF-κB gene expression, TNF-α and CRP level by using one-way analysis of variance (ANOVA). When statistical differences were indicated, Tamhane’s post hoc tests were performed. Non-parametric test related-sample Wilcoxon signed ranks was applied to compare differences in fatty acids, haematological parameters. The statistical significance was assumed at a p-value of less than 0.05. The statistical software, SPSS for Windows, Version 19 (SPSS Ltd., Surrey, UK) was used to analyse the data.

3. Results

3.1. Fatty acid composition of red blood cell phosphatidyl ethanolamine (PE) and choline (PC)

The fatty acid compositions of red blood cells PE and PC at baseline and after one year of supplementation with n − 3 treated and untreated (placebo) groups were included in Table 1. The fatty acid profile of the n − 3 treated and untreated groups was comparable at baseline (p > 0.05). In n − 3 treated group, DHA and EPA increased three-fold in red cell choline (PC) and ethanolamine (PE) phosphoglycerides compared with the baseline (p < 0.001), whereas arachidonic acid (AA) and total n − 6 FAs were significantly reduced in PE and PC (p < 0.001). Intervention had no effect on PE fatty acid profile after one year of supplementation (p > 0.05) in n − 3 untreated. However, significant increase in total n − 6 and decrease in EPA of PC phosphoglyceride were detected in n − 3 untreated after one year of supplementation in comparison to baseline.

3.2. Haematological parameters

Supplementation with n − 3 and placebo increased Hb concentration (p < 0.001). In contrast to the placebo group, n − 3 supplements caused significant reduction on total white blood cells (TWBC) (P < 0.05). Both n − 3 treated and untreated groups, intervention showed no effect on platelet count, Table 1.

3.3. Effect of n − 3 supplementation on plasma C-reactive protein levels (CRP)

Both treated and untreated groups of patients had higher concentrations of C-reactive protein compared with their healthy controls (p < 0.001). There was no significant difference in plasma CRP concentrations between the supplemented and un-supplemented patients (p > 0.05), Fig. 1.

3.4. Plasma levels of tumour necrosis factor-α (TNF-α) in hydroxyurea (HU) treated and untreated group

The HbSS treated with hydroxyurea had a significantly higher level (p < 0.05) of TNF-α in comparison to the untreated group (Median = 41.7 (IQR = 24.4) pg/ml vs Median = 14.7 (IQR = 19.6) pg/ml) and n − 3 treated group (Median = 41.7 (IQR = 24.4) pg/ml vs Median = 15.8 (IQR = 8.8) pg/ml), Fig. 2.

3.5. Plasma levels of tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10) in n − 3 treated, HU treated and untreated groups

Supplementation with n − 3 fatty acids had no effect on plasma TNF-α (18.6 ± 12.8 vs 17.8 ± 9.1) (p > 0.05), Fig. 3a) and IL-10 (11.0 ± 4.3 vs 11.3 ± 5.8) compared to baseline levels (p > 0.05), Fig. 3b.

3.6. Expression of j2-integrin CD11b/C18 (CD-11b) in monocytes at baseline

The level of adhesive molecules of placebo and active group were comparable at baseline (p > 0.05), Fig. 4a and b.

After one year of supplementation with n − 3, CD11b expression in monocytes were significantly lower compared to placebo and baseline (P < 0.05), Figs. 4a and 5, respectively.

3.7. Expression of j2-integrin CD11b/C18 (CD-11b) and L-selectin (CD62L) in granulocytes

The level of adhesive molecules of placebo and active group were comparable at baseline (p > 0.05). Supplementation with n − 3 or placebo caused no significant effect on CD11b and CD62L expression in granulocytes (p > 0.05), Figs. 4b and 5.

3.8. Effect of omega-3 fatty acids and HU treatments on gene expression of white blood cell NF-κB gene

Treatment with n − 3 fatty acid resulted in a significant reduction in NF-κB gene relative fold gene expression in comparison to n − 3
untreated patient (p < 0.05). The group on hydroxyurea treatments showed no significant difference when compared with untreated group (p > 0.05) or omega-3 treated group (p > 0.05), Fig. 6.

4. Discussion

The anti-inflammatory effect of n−3 fatty acids is well documented [24]. However, to the best of our knowledge, no study investigated the effect of fatty acid supplementation on genetic and molecular markers of inflammation in patients with sickle cell disease. Findings of this study provide evidence that the observed beneficial effects of n−3 fatty acid supplementation on patients with SCD could be partially due to amelioration of chronic inflammation and increased blood cell adhesion, the major causative factors of vaso-occlusion and complications characterizing the disease [25,26].

The SCD patients and healthy who participated in this study were from common ethnic and socio-economic backgrounds. The patients were under regular management protocols and similar quality of care. Moreover, baseline and one-year samples were collected at the same season during the year. Supplementation with n−3 fatty acids resulted in more than twofold increase in EPA and DHA both in PE and PC. On the other hand, fatty acids of the placebo group remained comparatively constant apart from slight increase in total n−6 and decrease in EPA of PC phosphoglyceride. Therefore it is unlikely to account the observed results to factor(s) other than n−3 intervention or treatment with hydroxyurea.

Total white blood count and CRP are known markers of systemic global inflammatory activity. Consistent with the previous studies, sickle cell patients have elevated baseline values of TWBC and CRP [27,28]. These results provide additional evidence that SCD is a disease with an important inflammatory component [1].

The observed decrease in TWBC owing to n−3 fatty acids supplementation is constituent with findings obtained from conditions other than SCD [29–31] and patients with SCD [16]. It is tenable; this
reduction in blood cell count was a manifestation of an improved inflammatory state [32,33] or diminished haemopoietic activity of the bone marrow [34]. On the other hand, treatment with n−3 fatty acids resulted in lower but not significant levels of CRP or platelet count compared to untreated patients. This finding is consistent with results reported from studies on healthy adults [35], patients with chronic non-autoimmune disease [36] and SCD patients [37]. It is possible that a larger sample size is needed to elucidate the effect of supplementation on variables of huge inter-individual variations such as platelet count.

TNF-α is a cytokine with a wide variety of actions that include activation of leukocytes, synthesis of acute phase proteins and the expression of adhesion molecules. Studies on the effect of HU treatment on plasma TNF-α level yielded controversial results [38,39]. In this study, HU treated patients had a high level of TNF-α. Considering the fact that patients assigned to HU are those with a relatively severe type of disease [40], the observed high levels of TNF-α could be a mere reflection of the well-known heterogeneous biological effects of HU treatment in SCD [41].

Fig. 3. Plasma TNF-α concentrations (a) and IL-10 concentrations (b) of patients with n−3 fatty acids before and after one year of supplementation with n−3 fatty acid.

Fig. 4. Monocyte integrins CD11b/C18 (CD-11b) at baseline and after one year intervention (Fig. 4a) and granulocyte integrins CD11b/CD18 (CD-11b) at baseline and after one year of intervention (Fig. 4b).
In accordance with the studies conducted on patients with diabetes mellitus [42,43], rheumatoid arthritis [44,45] and Crohn’s disease [46], supplementation with n-3 fatty acids showed no significant change on plasma levels of TNF-α or IL-10 [47]. Conversely, some studies that measured the effect of supplementation on cytokine production by monocytes [12,13] or local rather than systemic levels of cytokines showed significant effects [48]. This discrepancy between the systemic and cell or tissue-specific levels is intriguing. Though, it gives some indication that systemic plasma cytokine levels are not sensitive enough to reflect the tissue-specific immunological response to supplementation with n-3 fatty acids [47]. Moreover, as previously suggested [11], very high dose of n-3 fatty acids might be necessary to achieve detectable systemic anti-inflammatory response.

It has been shown that the adhesive molecules involved in increased adhesiveness of granulocytes and monocytes and leukocytes interaction with endothelial cells are the cytokine-induced L- and P-selectin (CD62L), β2-integrin CD11b/CD18 (CD-11b) and LFA-1 (CD11a/CD18) [49-52]. L-selectin (CD62L) facilitates rolling and temporary arrest of circulating leukocytes on endothelium surface [53], whereas β2-integrin (CD11b) is involved in subsequent stable adhesion [54,55]. Therefore, therapeutic approaches to reduce expression of CD62L and CD11b have been important strategies to prevent vaso-occlusion in sickle cell disease [50]. In vitro [56,57] and in vivo [13] studies in conditions other than SCD have revealed that supplementation with n-3 resulted in significant reduction in blood cell adhesive molecules. Interestingly, we observed a significant reduction of adhesive molecules in monocytes in the group supplemented with n-3 fatty acids, the finding which denotes improvement in SCD-associated chronic inflammatory state.

Experimental studies [10], and pilot clinical trials on patients with disease have shown that NF-κB inhibition is associated with significantly reduced leukocyte adhesion and improved microvascular blood flow [7]. Strikingly, in the current study supplementation with n-3 fatty acids, but not hydroxyurea, resulted in significant reduction in white blood cells’ NF-κB gene expression in the patients with SCD. This result is in line with whole genome gene expression studies in adults supplemented with n-3 fatty acids [58,59]. Knowing that NF-κB pathway mediates oxidative stress response [60], the observed reduction in NF-κB gene expression could be a reflection to the observed improvements in the patients’ oxidative stress status after supplementation with n-3 fatty acids [19]. The results of the present study might also suggest pathways other than NF-κB gene involved in hydroxyurea well-documented anti-adhesive effects [9].

Chronic inflammation, intravascular haemolysis, imbalanced vascular nitric oxide (NO), oxidative stress are known factors that underlie vasculopathy-related complications in SCD [61,62]. Among these complications, pulmonary hypertension (PHT) and stroke stand out as the most prevalent and devastating. Interestingly, SCD patients with PHT have higher levels of markers of endothelial activation, coagulation activation and other inflammatory markers than SCD patients without PHT. Hence the clinical importance of the findings of this study, besides supporting previous reports about the positive therapeutic effect of n-3 fatty acids in SCD [16,18,19,37], that it suggests high DHA n-3 supplement as a potential intervention to prevent PHT, stroke and other SCD vasculopathy-related complications.

One limitation of our study is that we did not measure the blood cells’ cytokine levels. Therefore, it would be a justifiable analysis interest to focus on the effect of supplementation on tissue-specific pro- and anti-inflammatory cytokines, and fatty acids derived active metabolites of the orthodox and novel pathways [63]. Based on the results of the present study we might wish to suggest a relatively higher dosage of n-3 for patients with SCD to achieve more potent systemic anti-inflammatory effect.

In conclusion, the current study demonstrates that supplementation with n-3 fatty acids mitigate some measures of inflammation and blood cell adhesion in patients with sickle cell disease. The results of this study provide some biological plausibility to n-3 therapeutic effects on patients with sickle cell disease, and support its use as a safe and effective treatment for acute and chronic complications of the disease.

**Conflict of interest**

The funding bodies had no influence on the study design, collection and analysis of data, interpretation of results or writing. The authors do not have a conflict of interest or financial relationships with the funding bodies.

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