Original Article

Docosahexaenoic Acid Promotes Eryptosis and Haemolysis through Oxidative Stress/Calcium/Rac1 GTPase Signalling

(DHA / eryptosis / haemolysis / calcium / Rac GTPase / anticancer)

FERYAL H. ALHARTHY, JAWAHER ALSUGHAYYIR, MOHAMMAD A. ALFHILI

Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

Abstract. Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid with promising anticancer potential. Anaemia is a frequent adverse effect of anticancer treatment caused in part by eryptosis and haemolysis. Thus, it is important to investigate the role of DHA in red blood cell (RBC) death. RBCs were treated with anticancer concentrations (10–100 μM) of DHA under different physiological conditions, and fluorescence-assisted cell sorting was employed to measure eryptotic markers. Cell membrane scrambling was detected by annexin-V-FITC labelling, cytoplasmic Ca^{2+} by Fluo4/AM, cell size by forward scatter (FSC), and oxidative stress by H_2DCFDA. Haemolytic markers were also assayed by photometric methods. DHA caused significant phospholipid scrambling with Ca^{2+} accumulation, loss of cellular volume, and oxidative stress. These changes were associated with dacrocyte formation, as revealed by electron microscopy. Moreover, DHA exhibited a dual effect on membrane integrity: it was haemolytic under isotonic conditions and anti-haemolytic in hypotonic environments. Importantly, inhibition of Rac1 GTPase activity with NSC23766 significantly reduced DHA-mediated haemolysis, as did co-administration of either sucrose or polyethylene glycol 8,000. Conversely, the presence of 125 mM KCl and urea without extracellular Ca^{2+} significantly exacerbated DHA toxicity. In conclusion, this is the first report that identifies key biochemical mechanisms underlying the cytotoxic effects of DHA in RBCs, promoting further development and validation of DHA in anticancer therapy.

Introduction

Chemotherapy is the mainstay of cancer treatment and poses serious risks to patients including myelosuppression, tumour lysis syndrome, anaemia and death. In fact, anaemic cancer patients have worse outcomes and worse survival rates than non-anaemic patients (Badheeb et al., 2023). Chemotherapy-induced anaemia may be caused by suicidal red blood cell (RBC) death known as eryptosis, as seen in the case of cisplatin and paclitaxel. This type of cell death is regulated by many signalling molecules such as p38 MAPK, Rac1 GTPase, caspases, casein kinase 1α (CK1α), mixed lineage kinase domain-like protein (MLKL) and protein kinase C (PKC) (Lang et al., 2012). When RBCs undergo eryptosis, certain features typical of apoptotic cell death are observed. These include cell membrane scrambling leading to loss of phospholipid asymmetry. In particular, the orientation of phosphatidylserine (PS) moieties changes to face outward, which facilitates the removal of eryptotic cells by phagocytes. Other features include accumulation of reactive oxygen species (ROS), loss of ionic regulation, changes in cellular volume, ATP exhaustion and ceramide abundance in the plasma membrane (Lang and Lang, 2015).

Docosahexaenoic acid (DHA), an ω-3 polyunsaturated fatty acid (PUFA) found in fish oil, has a range of anti-inflammatory effects. In fact, DHA and eicosapentaenoic acid (EPA) inhibit cyclooxygenase, which converts arachidonic acid to inflammatory eicosanoids (Calder, 2014; Smith and Malkowski, 2019). Several studies have found reverse associations between dietary intake of DHA or blood DHA and cytokines, C-reactive protein and adhesion molecules (Farzaneh-Far et al.,

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Corresponding author: Mohammad A. Alfhili, Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, King Khalid Street 12372, Riyadh, Saudi Arabia. E-mail: malfeehily@ksu.edu.sa

Abbreviations: AChE – acetylcholine esterase, ASA – acetylsalicylic acid, CK1α – casein kinase 1α, DHA – docosahexaenoic acid, EPA – eicosapentaenoic acid, ESR – erythrocyte sedimentation rate, MLKL – mixed lineage kinase domain-like protein, MTN – melatonin, NSA – necrosulphonamide, PS – phosphatidylserine, PKC – protein kinase C, PUFAs – polyunsaturated fatty acids, RBCs – red blood cells, ROS – reactive oxygen species.

2009; Grenon et al., 2013; Fontesa et al., 2015). In particular, clinical trials have revealed that consumption of DHA, with or without EPA, improves inflammatory states and redox balance. In sickle cell disease patients, DHA and EPA supplementation significantly reduces C-reactive protein and maintains redox homeostasis (Daak et al., 2013; Giriraja et al., 2023). Both acids also reduce sickle cell crisis rate and haemolysis (Okpala et al., 2011), peripheral leukocyte count, nuclear factor-kappa B gene expression and cell adhesion (Daak et al., 2015). Likewise, DHA improves the rheology of sickle cells, inflammation and haemolysis (Daak et al., 2020). In preterm infants, DHA supplementation, with or without arachidonic acid, diminishes circulating interleukin 6 levels (Skouroliakou et al., 2016) and improves the redox status and inflammation (Ramiro-Cortijo et al., 2020). Furthermore, dietary DHA reduces the expression of intercellular adhesion molecule 1 on the surface of monocytes (Hughes et al., 1996) and circulating levels of soluble vascular cell adhesion molecule 1 in the elderly (Miles et al., 2001).

Accumulating evidence indicates that consumption of ω-3 PUFAs may aid in cancer prevention (Fu et al., 2015; Volpato and Hull, 2018), and many PUFAs have shown great promise in preclinical models (D'Eliseo and Velotti, 2016). DHA has been demonstrated to have anticancer properties through several mechanisms (D'Eliseo and Velotti, 2016; Volpato and Hull, 2018). DHA inhibits cell proliferation through cell cycle arrest (Newell et al., 2017), oxidative stress (Cavazos et al., 2011), caspase stimulation (Fukui et al., 2013) and mitochondrial damage (Yamagami et al., 2009; Sun et al., 2013; Park et al., 2018). Furthermore, DHA inhibits adhesion (Victory et al., 2007), migration (Brown et al., 2020), and angiogenesis (Spencer et al., 2009) and improves the nutritional state of cancer patients (Fietkau et al., 2013; McGlory et al., 2019).

The aim of this study was to evaluate the safety profile of DHA as a promising anticancer compound by examining its potential toxicity in RBCs.

Material and Methods

Chemicals

Solarbio Life Sciences (Beijing, China) provided all chemicals. A stock solution of DHA ethyl ester (10 mM) was prepared by dissolving 3.5 mg in 1 ml dimethyl sulphoxide, which was aliquoted, and stored at – 80 °C. Phosphate-buffered saline (PBS) contained 125 mM NaCl, 1 mM KH_2PO_4 and 5.6 mM Na_2HPO_4 (pH 7.4,) while standard Ringer solution contained 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose and 1 mM $CaCl_2$ (pH 7.4). In some experiments, the Ringer solution did not include $CaCl_2$ or was modified to include isosmotic urea (300 mM), sucrose (250 mM) or KCl (125 mM) instead of NaCl, or 10 % *w*/*v* PEG 8,000 (PEG) (Alghareeb et al., 2023b).

Experimental design

Ethical clearance for this study was granted from the Institutional Review Board of King Saud University Medical City (#E-23-7485). Lithium heparin and EDTA blood samples were collected from 24 healthy donors aged 22–35 years with normal BMI and CBC results. RBCs were isolated by centrifugation (1,500 × *g*, 20 min, RT). After two washes in PBS, cells were resuspended in either PBS or Ca^{2+}-free Ringer buffer and allowed to sit at 4 °C for no more than 24 h. Cells were subjected to 10–100 μM DHA treatment at 37 °C for 24 h under different physiological conditions. Also, cells were co-treated with DHA and p38 inhibitor SB203580 (100 μM), PKC inhibitor staurosporine (StSp; 1 μM), CK1α inhibitor D4476 (20 μM), cyclooxygenase inhibitor acetylsalicylic acid (ASA; 25 μM), Rac1 GTPase inhibitor NSC23766 (100 μM), MLKL inhibitor necrosulphonamide (NSA; 0.5 μM), nitric oxide synthase (NOS) inhibitor L-NAME (20 μM), melatonin (MTN; 1 μM), vitamin C (1 mM), or ATP (0.5 mM). Each experiment contained a negative and a positive control consisting of cells suspended in 1 % dimethyl sulphoxide and in distilled water, respectively (Alfhili et al., 2023).

Eryptosis

Eryptosis was detected by fluorescence-assisted cell sorting analysis using a Northern Lights™ flow cytometer (Cytek Biosciences, Fremont, CA). Externalization of PS, intracellular ROS and cytoplasmic Ca^{2+} were measured by 1 % annexin-V-FITC, 10 μM H_2DCFDA and 5 μM of Fluo4/AM, respectively. The cell size was determined from forward scatter (FSC). In all experiments, 10,000 events were excited by the blue laser (488 nm) to capture the emitted green fluorescence (520 nm) (Alamri et al., 2020).

Haemolysis

The absorbance of haemoglobin in the supernatants was measured photometrically at 405 nm using an LMPR-A14 microplate reader (Labtron Equipment Ltd., Camberley, UK) as follows:

$$\%\ Haemolysis = \frac{\text{DHA–induced Hb release}}{\text{water–induced Hb release}} \times 100$$

Lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and creatinine kinase (CK) were measured in pooled supernatants by a BS-240Pro clinical chemistry analyser (Mindray Medical International Ltd., Shenzhen, China). K^+ was measured by an EXIAS electrolyte analyser (EXIAS Medical GmbH, Graz, Austria) (Alfhili et al., 2023).

Cellular morphology

Standard protocols were followed to stain control and DHA-treated (80 μM) cells with Giemsa stain. To examine the cells via electron microscopy, cells were fixed in 2.5 % glutaraldehyde, stained with 1 % osmium tetraoxide, dried in 50–100 % ethanol and observed by a JSM-7610F

ultra-high resolution Schottky field emission scanning electron microscope (Alghareeb et al., 2023a).

Acetylcholine esterase (AChE) activity

AChE activity was assayed in haemolysates using the BS-240Pro analyser. In the reaction mixture, butyrylthiocholine is hydrolysed by AChE to butyrate and thiocholine, and hexacyanoferrate(III) is reduced to hexacyanoferrate(II), causing a decrease in absorbance (405 nm) that reflects the AChE activity.

Erythrocyte sedimentation rate (ESR)

After vertical sedimentation in Westergren tubes for 1 h at RT in the dark, the ESR was measured in the control and treated whole blood (Zhbanov and Yang, 2015).

Osmotic resistance

Cells with and without 1 μM or 5 μM of DHA were suspended in saline solutions of decreasing tonicity (0.9–0.1 %) for 1 h at 37 °C and haemolysis was measured (He et al., 2008).

Statistical analysis

All statistical analyses were performed by GraphPad Prism v9.5.1 (GraphPad Software, Inc., San Diego, CA). Results are shown as means ± SEM (SD in the text) of three independent experiments, each conducted in triplicate. Student's *t*-test and one-way ANOVA were applied as appropriate. Statistical significance was established based on a P value of less than 0.05.

Results

First, we set out to examine whether DHA possesses a pro-eryptotic activity, and as demonstrated in Fig. 1B and C, DHA-treated cells showed a concentration-dependent increase in the percentage of cells exhibiting PS exposure from 3.3 ± 0.49 % (control) to 15.73 ± 5.86 % (80 μM, P = 0.0001) and 18.13 ± 3% (100 μM, P < 0.0001). Since eryptosis increases cell aggregation, we measured ESR in control and treated cells (100 μM), which showed no significant increase (Fig. 1D).

Next, to delineate the mechanisms through which DHA triggers PS exposure, we assessed ROS and Ca^{2+} levels. Fig. 1F shows significant elevations in DCF fluorescence from 1.00 ± 0.06 arbitrary units (a.u.) in control cells to 1.20 ± 0.13 a.u. in treated cells (100 μM, P = 0.0051). The percentage of oxidized cells (Fig. 1G) at 100 μM was also increased (1.58 ± 0.58 % to 5.98 ± 5.66 %, P = 0.0119). Significant elevations in Fluo4 fluorescence were also detected (Fig. 1I), from 538.3 ± 166.7 a.u. (control) to 786.7 ± 159.9 a.u. (80 μM, P = 0.0329) and to 913.2 ± 287.9 a.u. (100 μM, P = 0.001). Also, the percentage of cells with elevated intracellular Ca^{2+} (Fig. 1J) significantly increased at 100 μM (10.62 ± 7.94 %, P = 0.0005) compared to that of control cells (2.55 ± 0.59 %).

We also assessed the haemolytic potential of DHA, which increased from baseline readings of 1.46 ± 0.60 % to 4.87 ± 2.97 % (20 μM, P = 0.0105), 15.96 ± 5.164 %, (40 μM, P < 0.0001) and 62.35 ± 20.24 % (80 μM, P < 0.0001), as shown in Fig. 2A. Accordingly, AST activity increased significantly from 1.67 ± 0.58 U/l (control) to 22.00 ± 1.00 U/l (P < 0.0001) at 80 μM (Fig. 2C). In addition, CK activity was falsely elevated from 2.33 ± 1.53 U/l to 18.67 ± 3.06 U/l at 40 μM (P < 0.0001) and to 44.67 ± 2.08 U/l at 80 μM (P < 0.0001), as shown in Fig. 2D. However, this increase in activity reflects leakage of ATP-producing metabolites such as adenylate kinase that interfered with the CK assay. Furthermore, LDH (Fig. 2E) activity also significantly increased to 86.00 ± 3.464 U/l (P = 0.0002) at 40 μM and to 393.0 ± 19.31 U/l (P < 0.0001) at 80 μM, compared to the control values of 1.33 ± 0.57 U/l. Also, Fig. 2F shows significant elevations in K^+ levels from 0.13 ± 0.06 mmol/l (control) to 1.28 ± 0.07 mmol/l (40 μM, P < 0.0001) and to 1.46 ± 0.01 mmol/l (80 μM, P < 0.0001). Because many lipophilic compounds have been shown to modulate the haemolytic threshold of RBCs in hypotonic conditions, it was therefore of interest to examine whether low concentrations of DHA protect the cells from hypotonic lysis. As illustrated in Fig. 2G, at 0.5 % NaCl, cells treated with 1 (79.83 ± 4.3 %, P = 0.007) and 5 μM (74.15 ± 13.46 %, P = 0.017) of DHA displayed significantly increased osmotic resistance compared to untreated cells (92.61 ± 2.25 %).

Another feature of cell death is loss of volume following intracellular Ca^{2+} increase. FSC was thus measured (Fig. 3C) and showed significant decreases from 2.53 ± 0.21 a.u. to 1.88 ± 0.58 a.u. (P = 0.043) and to 1.60 ± 0.89 a.u. (P = 0.003) at 40 and 80 μM, respectively. AChE is a hydrolase that controls corpuscular morphology, but no significant changes in its activity upon DHA exposure were observed (Fig 3D).

We then set out to assess the importance of several physiological conditions on the toxicity of DHA. It was revealed that elimination of extracellular Ca^{2+} (Fig. 3E), increasing KCl to 125 mM (Fig. 3F), or addition of isosmotic urea (Fig. 3G) had no significant effect on the haemolytic activity of DHA. In contrast, addition of isosmotic sucrose (Fig. 3H) significantly decreased haemolysis (40.26 ± 21.13 % to 22.81 ± 13.35 %, P = 0.0158). Interestingly, only when Ca^{2+} was removed and both KCl and urea were added did the haemolytic activity of DHA significantly increase (23.36 ± 4.44 % to 28.67 ± 2.97 %, P < 0.0001, Fig. 3L).

A morphological assessment of Giemsa-stained treated (80 μM) cells (Fig. 4A) shows evidence of tear cell formation (dacrocytes), which is similarly seen with SEM examination (Fig. 4B).

Finally, we were interested in identifying the signalling pathways through which DHA exerts its haemolytic activity in RBCs by use of small molecule inhibitors. Our results show that in the presence of SB203580 (Fig. 5A), D4476 (Fig. 5B), NSA (Fig. 5C), ATP (Fig. 5E), StSp (Fig. 5F), MTN (Fig. 5H), ASA (Fig. 5I), vitamin C (Fig. 5J), or L-NAME (Fig. 5K), no statistically significant decrease in the haemolytic activity of DHA was ob-

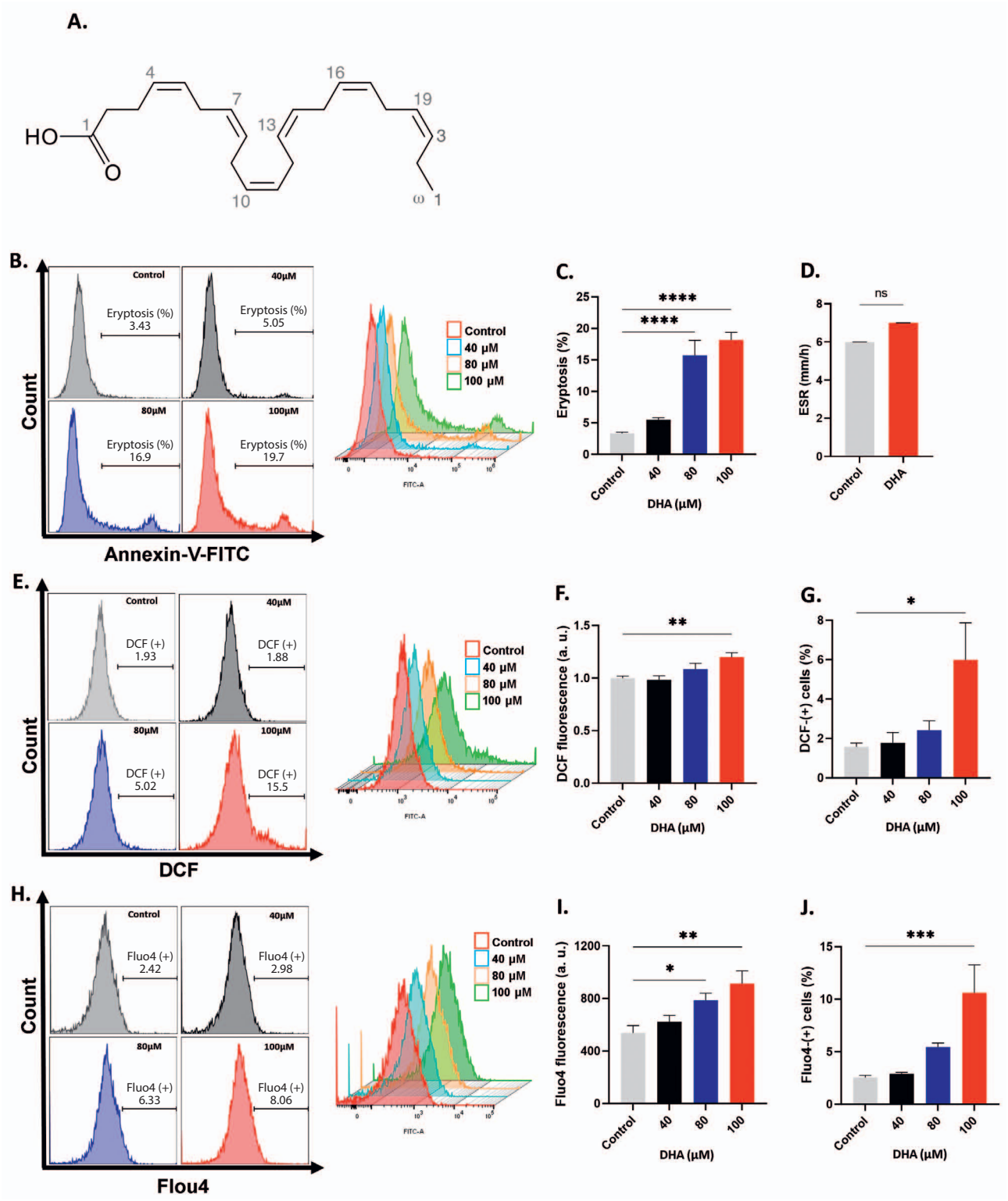

Fig. 1. Eryptotic activity of DHA. **(A)** Molecular structure of DHA ethyl ester. **(B)** Representative histograms of annexin-V-FITC fluorescence of control and treated cells (40–100 µM). **(C)** Percentage of eryptotic cells (40–100 µM). **(D)** ESR in control and treated cells (100 µM). **(E)** Representative histograms of DCF fluorescence of control and treated cells (40–100 µM). **(F)** DCF fluorescence of control and treated (40–100 µM) cells. **(G)** Percentage of oxidized cells (40–100 µM). **(H)** Representative histograms of Fluo4 fluorescence of control and treated cells (40–100 µM). **(I)** Fluo4 fluorescence of control and treated (40–100 µM) cells. **(J)** Percentage of cells with excess Ca^{2+} (40–100 µM). Results are shown as means ± SEM (N = 9). ns indicates no statistical significance, while $^{*}(P < 0.05)$, $^{**}(P < 0.01)$, $^{***}(P < 0.001)$ and $^{****}(P < 0.0001)$.

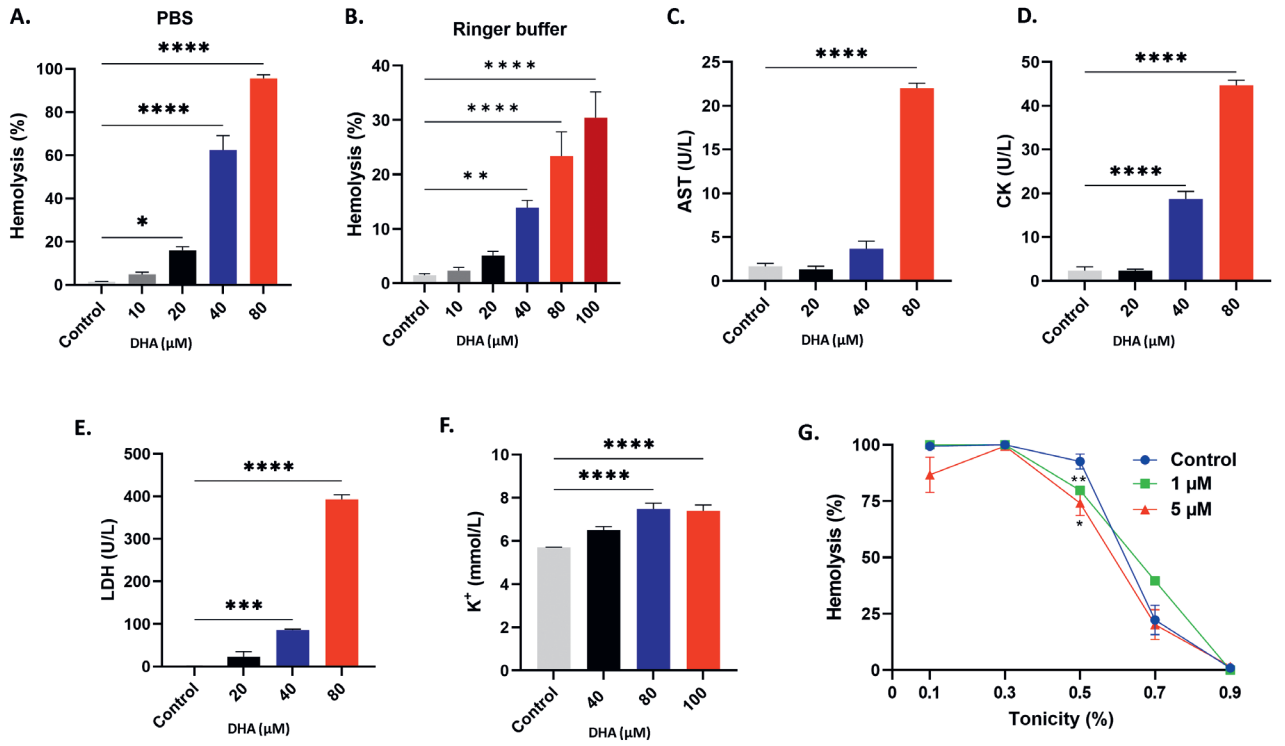

Fig. 2. DHA induces haemolysis. **(A)** Haemolytic activity of DHA (10–80 μM) in PBS and in **(B)** standard Ringer buffer (10–100 μM). Haemolytic markers **(C)** AST, **(D)** CK, **(E)** LDH and **(F)** K^+ in the supernatants (20–80 μM). **(G)** Osmotic fragility curves. Results are shown as means ± SEM (N = 9). ns indicates no statistical significance, while $^{*}(P < 0.05)$, $^{**}(P < 0.01)$, $^{***}(P < 0.001)$ and $^{****}(P < 0.0001)$.

served. Nonetheless, in cells co-treated with NSC23766 (Fig. 5D), a significant reduction in haemolysis was observed, from 66.98 ± 14.25 % to 18.30 ± 5.93 %, $P < 0.0001$). This was also the case when PEG (Fig. 5G) was added to Ringer buffer (32.68 ± 14.75 % to 7.09 ± 3.98 %, $P < 0.0001$).

Discussion

DHA exhibits anticancer effects in several cancer cells including pancreatic (Park et al., 2018), breast (Xue et al., 2014), prostate (Volpato and Hull, 2018) and hepatic (Sun et al., 2013) cells. The primary aim of this study was to investigate the impact of DHA on RBCs in order to determine whether the anticancer properties of DHA are associated with cytotoxicity to normal cells that could give rise to anaemia in patients. Our results indicate that antitumor concentrations of DHA induce PS exposure (Fig. 1B–C), which is consistent with previous reports in neuroblastoma (So et al., 2015), leukaemia (Yamagami et al., 2009) and breast cancer cells (Pizato et al., 2018). The premature exposure of PS moieties accelerates the clearance of circulating cells, leading to anaemia and increased risk for thrombotic episodes due to their enhanced aggregability within the endothelial lumen (Pretorius et al., 2016).

The build-up of Ca^{2+} (Fig. 1H-J) triggers the opening of Ca^{2+}-responsive K^+ channels. This leads to KCl and water efflux (Tkachenko and Onishchenko, 2023), resulting in a decrease in cellular volume (Fig. 3A–C). Additionally, the observed reduction in cell size can be attributed to the action of cytoskeleton-degrading enzyme calpain, which is controlled by Ca^{2+} (Maćczak et al., 2015). Shrinkage is thought to facilitate the phagocytic clearance of dead cells (Zelenak et al., 2012). Similarly, the activities of some enzymes that rely on Ca^{2+} and help stabilize the cell membrane, such as scramblases, become disrupted when there is an increase in Ca^{2+} levels (Sakuragi and Nagata, 2023). This subsequently leads to the loss of membrane asymmetry, as seen in Fig. 1B and C. However, the observed duality of DHA action on the cell membrane merits comment. Our findings show that despite being potently haemolytic in isosmotic conditions (Fig. 2A–F), DHA rather protected the cells from hypotonic lysis (Fig. 2G), which suggests that DHA exerts significant membrane distension that increases the total cellular volume and, ultimately, the haemolytic threshold. This finding is consistent with an established role of DHA in the modulation of membrane fluidity, permeability and elastic compressibility (Stillwell and Wassall, 2003).

In an attempt to further dissect the underlying mechanisms of DHA action on RBCs, we found that DHA tox-

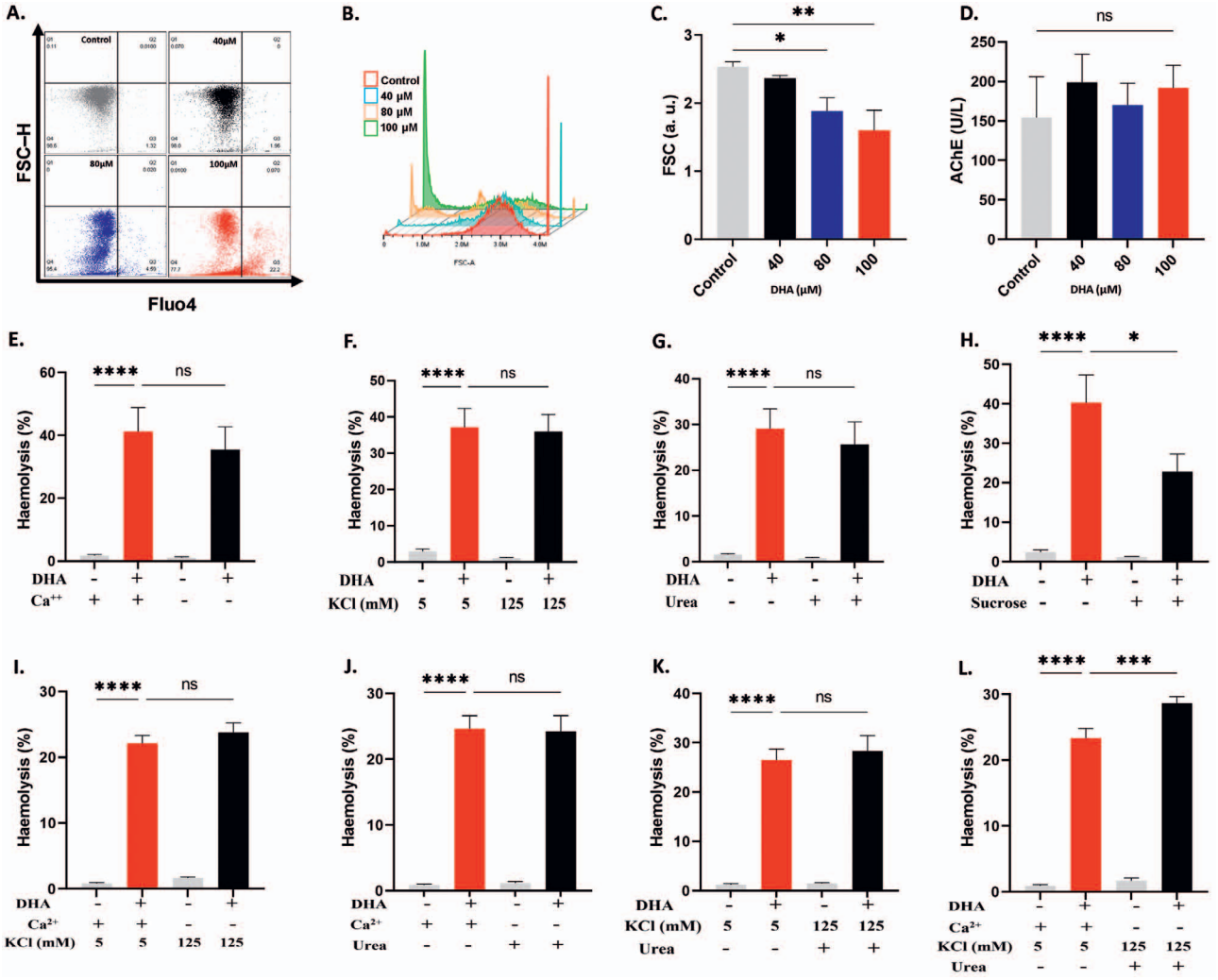

Fig. 3. Effect of DHA on cellular volume. **(A)** Dot plots of FSC-H and Fluo4 fluorescence. **(B)** Representative histograms of FSC (40, 80 and 100 μM). **(C)** Geomean FSC. **(D)** AChE activity. Modulation of DHA-induced haemolysis by **(E)** extracellular Ca^{2+} (1 mM) removal, **(F)** KCl increase (125 mM), **(G)** addition of urea (300 mM) or **(H)** sucrose (250 mM), and **(I–L)** combinations thereof. Results are shown as means ± SEM (N = 9). ns indicates no statistical significance, while $^{*}(P < 0.05)$, $^{**}(P < 0.01)$, $^{***}(P < 0.001)$ and $^{****}(P < 0.0001)$.

icity was significantly attenuated by sucrose (Fig. 3H) and PEG 8,000 (Fig. 5G). Sucrose can reduce colloid osmotic swelling by inhibiting the outflow of Cl^- ions and the entry of water; an event known to be promoted by DHA (Stillwell and Wassall, 2003). PEGylation in pharmaceutical preparations serves to aid in ingredient solubility, retention time and targeting while significantly decreasing antigenicity (Gupta et al., 2019). This must be taken into consideration for further therapeutic development of DHA. Interestingly, the haemolytic rate of DHA was significantly increased when KCl and urea were introduced together in the absence of Ca^{2+} (Fig. 3L), as opposed to when each was present alone (Fig. 3F–G). It seems that exclusion of Ca^{2+}, blocking KCl-induced water efflux, and the presence of urea altogether divert the cellular machinery to bring about haemolysis through other mechanisms. Also, the fact that this was observed only when Ca^{2+} was removed (Fig. 3K–L) argues that Ca^{2+} may suppress those mechanisms.

Another important finding is that the haemolytic activity of DHA significantly declined when Rac1 GTPase was inhibited by NSC23766 (Fig. 5D). The erythrocyte membrane skeleton is typically arranged in a hexagonal lattice structure, located underneath the cell membrane, the arrangement of which is preserved by Rac1 GTPases (Kalfa et al., 2006). It was found that erythrocytes lacking Rac1 GTPases have a disordered membrane skeleton characterized by holes in the meshwork structure (Konstantinidis et al., 2010). Also, previous studies have shown that Rac1 GTPase is essential for intracellular ROS formation by activating NADPH oxidases (George et al., 2013; Zimmer et al., 2021). Therefore, the reduction in DHA-mediated haemolysis upon Rac1 GTPase inhibition suggests a crosstalk with ROS (Fig. 1E–G).

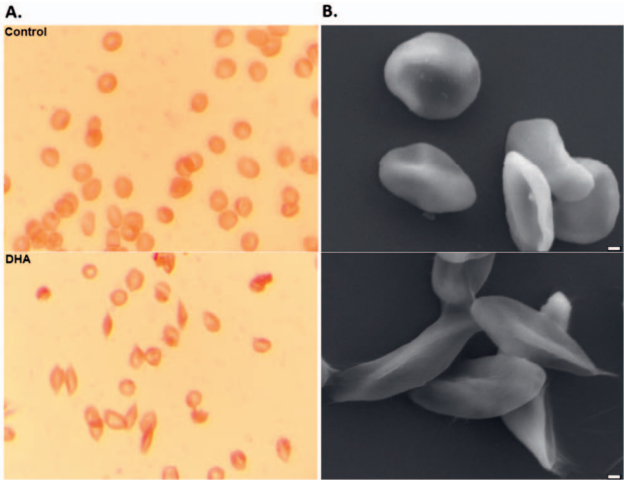

Fig. 4. Effect of DHA on RBC morphology. **(A)** Bright-field micrographs (×1000) and **(B)** SEM micrographs (×5000; scale bar: 1 μm) of control and treated (80 μM) cells.

The redox imbalance modifies proteins and lipids essential for cell survival and thus accelerates the scrambling of cell membranes (Fig. 1B–D) (Föller and Lang, 2020). This is in agreement with the oxidant activity of DHA in ovarian cancer cells (West et al., 2020). Notably, we have recently found that eriocitrin (Alghareeb et al., 2023b) and α-mangostin (Alghareeb et al., 2023c) similarly stimulate RBC death by modulating the Rac1 GTPase activity.

In addition to cancer treatment, DHA has shown promise against several other conditions. It has been reported that consumption of Manuka honey along with DHA and EPA confers anti-haemolytic and antioxidant protection in β-thalassemic patients evident as a decrease in serum iron, F2-isoprostane (8-iso-PGF2α), peripheral lipids, and the need for blood transfusion (Gamaleldin et al., 2023). In Alzheimer's disease, ώ-3 PUFAs may reduce cognitive impairment, improve memory and prevent brain atrophy. Similarly, DHA and EPA re-

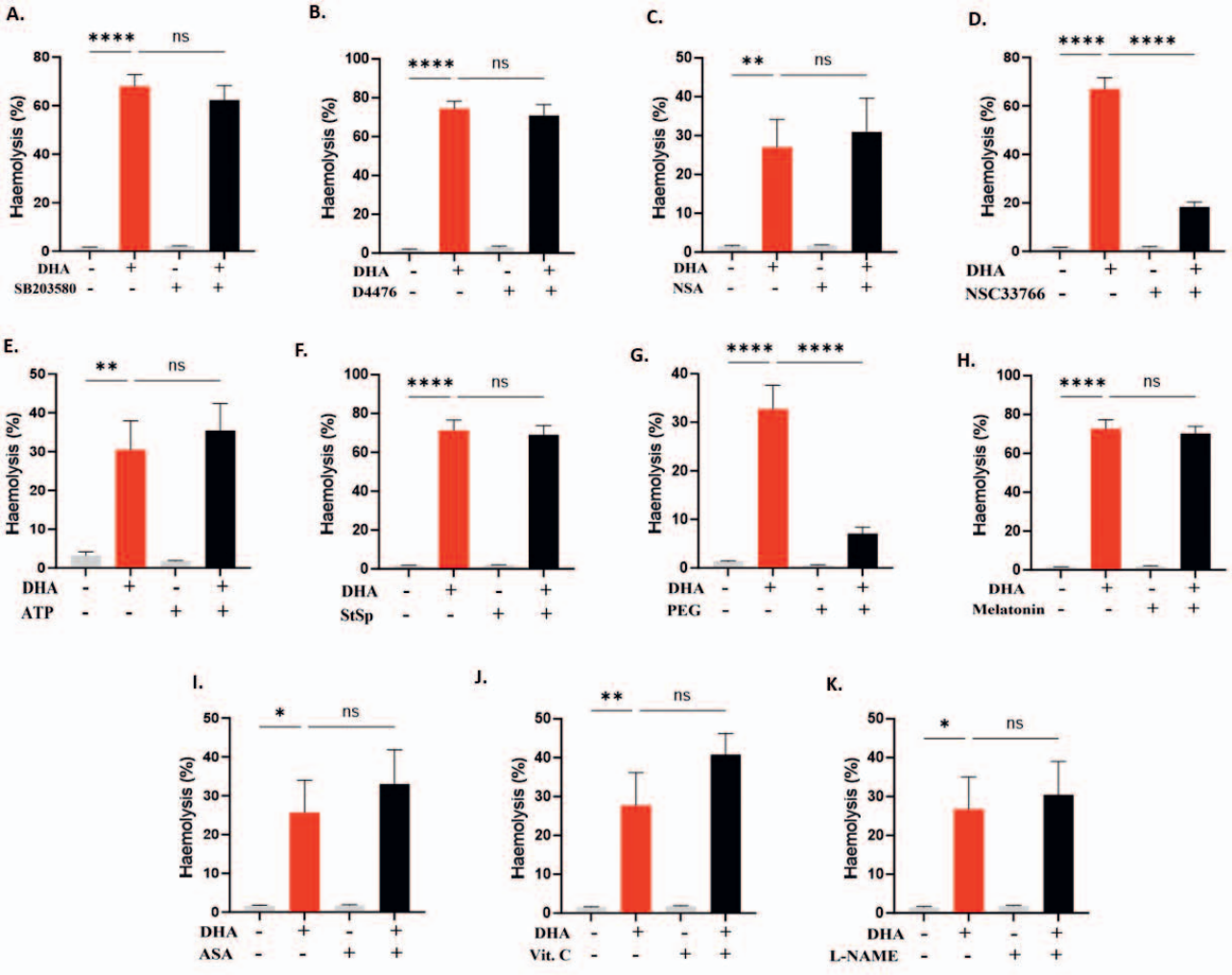

Fig. 5. Inhibitors of DHA-induced haemolysis. Effect of **(A)** SB203580 (100 μM), **(B)** D4476 (20 μM), **(C)** NSA (0.5 μM), **(D)** NSC23766 (100 μM), **(E)** ATP (500 μM), **(F)** StSp (1 μM), **(G)** PEG (10 % *w*/*v*), **(H)** MTN (1 μM), **(I)** ASA (25 μM), **(J)** vitamin C (1 mM), and **(K)** L-NAME (20 μM). Results are shown as means ± SEM (N = 9). ns indicates no statistical significance, while $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ and $^{****}(P < 0.0001)$.

duce depression and lessen the severity of Parkinson's disease (Kousparou et al., 2023). In cardiovascular disease, ώ-3 PUFAs have been reported to reduce revascularization, myocardial infarction and mortality (Khan et al., 2021), while increased blood DHA in particular lowers serum triglycerides and the risk of coronary atherosclerosis and sudden cardiac death (Holub, 2009).

In conclusion, this work is the first to show that anticancer concentrations of DHA trigger haemolysis and eryptosis in human RBCs, characterized by PS translocation, cellular dehydration, dacrocyte formation, oxidative stress and Ca^{2+} mobilization. These harmful effects were induced by Rac1 GTPase and can be mitigated by sucrose and PEG.

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Conflict of interest

The authors declare no conflict of interest.

Author contribution

Conceptualization: M.A.A., methodology: all authors, software: M.A.A., validation: F.H.A. and M.A.A., formal analysis: all authors, investigation: F.H.A. and J.A., resources: M.A.A., data curation: all authors, writing – original draft preparation: all authors, writing – review and editing: all authors; visualization: J.A., supervision: M.A.A., project administration: M.A.A., funding acquisition: M.A.A. All authors have read and agreed to the published version of the manuscript.

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