Eicosapentaenoic Acid Triggers Phosphatidylserine Externalization in the Erythrocyte Membrane through Calcium Signaling and Anticholinesterase Activity

Feryal H. ALHARTHY[1], Jawaher ALSUGHAYYIR[1], Mohammad A. ALFHILI[1]

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

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Summary

Hemolysis and eryptosis contribute to anemia encountered in patients undergoing chemotherapy. Eicosapentaenoic acid (EPA) is an omega-3 dietary fatty acid that has anticancer potential by inducing apoptosis in cancer cells, but its effect on the physiology and lifespan of red blood cells (RBCs) is understudied. Human RBCs were exposed to anticancer concentrations of EPA (10-100 μM) for 24 h at 37 °C. Acetylcholinesterase (AChE) activity and hemolysis were measured by colorimetric assays whereas annexin-V-FITC and forward scatter (FSC) were employed to identify eryptotic cells. Oxidative stress was assessed by H_2DCFDA and intracellular Ca^{2+} was measured by Fluo4/AM. EPA significantly increased hemolysis and K^+ leakage, and LDH and AST activities in the supernatants in a concentration-dependent manner. EPA also significantly increased annexin-V-FITC-positive cells and Fluo4 fluorescence and decreased FSC and AChE activity. A significant reduction in the hemolytic activity of EPA was noted in the presence extracellular isosmotic urea, 125 mM KCl, and polyethylene glycol 8000 (PEG 8000), but not sucrose. In conclusion, EPA stimulates hemolysis and eryptosis through Ca^{2+} buildup and AChE inhibition. Urea, blocking KCl efflux, and PEG 8000 alleviate the hemolytic activity of EPA. The anticancer potential of EPA may be optimized using Ca^{2+} channel blockers and chelators to minimize its toxicity to off-target tissue.

Keywords

EPA • Eryptosis • Hemolysis • Calcium • Anticancer

Corresponding author

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

Introduction

Fatty acids (FAs), a primary energy source for certain cell types, are categorized based on the length and degree of unsaturation of their hydrocarbon chain. Omega-3 FAs (ώ-3 FAs) are long-chain polyunsaturated FAs that are particularly promoted to improve cardiovascular health [1]. These FAs have also been a source of interest owing to their other pharmacological activities including neuroprotective, anti-allergic, and antidiabetic properties [2]. It has previously been demonstrated, in several human populations, that consuming foods rich in ω-3 FAs is associated with a decreased risk of developing common cancers such as colorectal [3], prostate [4], and liver cancer [5]. In particular, experimental evidence for the anticancer activity of eicosapentaenoic acid (EPA) is presented in numerous investigations both *in vitro* and *in vivo* [6,7] which is attributed to cell apoptosis [8,9] and anti-angiogenic properties [10,11].

EPA induces an increase in pro-apoptotic proteins such as Bax and Bak in colorectal cancer cells [12] and Bax, caspase-3, and caspase-9, in neuroblastoma cells [8]. EPA has also been demonstrated to trigger cell cycle arrest and increase membrane polarity in breast cancer cell lines [13,14]. Oxidative stress also drives EPA-induced apoptosis in several cancer cell lines such as breast [13], bladder [15], and hepatoma cells [16]. Also, EPA reduces mitochondrial membrane potential leading to cytoplasmic cytochrome *c* release and the activation of caspases in various cancer cell lines including pancreatic [17], colorectal [18], esophageal

squamous carcinoma [19], lymphoma [20], and peripheral blood mononuclear cells of multiple myeloma patients [21]. Moreover, EPA reduces the expression of X-linked inhibitor of apoptosis [12] and promotes surface expression of FAs in breast cancer cells [22]. EPA also stimulates caspase-8 activation pancreatic cancer [23] and promyelocytic leukemia cells [24] and activates Bid and mitochondrial outer membrane permeabilization [24].

The burden of cancer on global public health is still very high. Cancer patients often exhibit cachexia, a condition manifesting in weight loss and muscle atrophy. Several clinical trials have revealed that ώ-3 FAs, especially EPA, may offer significant advantages for several cancer-related side effects. For instance, EPA-based nutritional therapy for cancer patients with cachexia reduces weight loss [25,26], regulates skeletal muscle protein turnover [27], improves energy and protein intake and body composition [28], and extends survival [6]. Importantly, anemia is a common adverse effect of chemotherapy observed in many different types of cancers and emerging evidence indicates that eryptosis contributes to chemotherapy-related anemia [29]. The principal characteristics of eryptosis are phosphatidyl-serine (PS) exposure on the outer cell surface, shrinkage, increased intracellular Ca^{2+} concentrations, inhibition of acetylcholinesterase (AChE), and membrane ruffling and blebbing [30]. Through PS-mediated phagocytosis, macrophages efficiently eliminate eryptotic erythrocytes [31]. Additionally, prostaglandin E_2 can accelerate the entry of Ca^{2+} [32] whereas ceramide, produced from sphingomyelin, functions as a significant second messenger facilitating the externalization of PS [33].

Further research is indeed needed to demonstrate the value of EPA in cancer treatment. This study aims to explore the potential for EPA to injure untargeted tissue by inducing eryptosis and the molecular mechanisms that may be involved.

Methods

Experimental design

The Institutional Review Board of King Saud University Medical City approved the protocol for this study (#E-23-7485). All chemicals were purchased from Solarbio Life Sciences (Beijing, China). EPA stock solution (10 mM; 3 mg/ml DMSO) was prepared, aliquoted, and stored at -80 °C. Whole blood samples were collected in lithium heparin (hemolysis and eryptosis studies) or EDTA (complete blood count (CBC)) vacutainer tubes from consented, healthy donors in line with the guidelines laid down by the Declaration of Helsinki. A total of 19 subjects (10 males and 9 females), aged 28–39 years, with normal BMI and CBC results, were recruited. Individuals with conditions known to accelerate eryptosis, including inflammation, were excluded.

RBCs were isolated by centrifugation at 2,500 RPM for 20 min at RT, washed twice and resuspended in phosphate-buffered saline (PBS) at 4 °C for a maximum of 24 h. Cells were treated with 10–100 μM of EPA at 37 °C for 24 h either in PBS or in Ringer solutions. In all experiments, a negative control and a positive control were prepared by suspending the cells in 0.1 % DMSO or in distilled water, respectively. In inhibitor assays, RBCs were co-treated with 40 μM EPA and Rac1 GTPase inhibitor NSC23766 (100 μM), p38 mitogen-activated protein kinases (p38 MAPK) inhibitor SB203580 (100 μM); protein kinase C (PKC) inhibitor staurosporine (StSp; 1 μM), cyclooxygenase inhibitor acetylsalicylic acid (ASA; 25 μM), mixed lineage kinase domain-like protein (MLKL) inhibitor necrosulfonamide (NSA; 0.5 μM), casein kinase 1α (CK1α) inhibitor D4476 (20 μM), vitamin C (100 μM), melatonin (MTN; 1 μM), or nitric oxide synthase (NOS) inhibitor L-NAME (20 μM) [34].

Hemolysis

The supernatants of control and treated cells were collected by centrifugation (13,000 RPM, 1 min, RT). Hemoglobin (Hb) was estimated in supernatants (λ_{max}=405 nm) as a measure of hemolysis by LMPR-A14 microplate reader (Labtron Equipment Ltd., Surrey, UK) [35] using the following formula:

$$\text{Hemolysis} = \frac{\text{Hb release triggered by EPA}}{\text{Hb release triggered by water}} \text{x100}$$

Hemolytic markers

Potassium in supernatants was measured by EXIAS e|1 electrolyte analyzer (EXIAS Medical GmbH, Graz, Austria) while enzymatic activities of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured in pooled supernatants by BS-240Pro clinical chemistry analyzer (Mindray Medical International Ltd., Shenzhen, China) [35].

PS externalization

Cells were stained with 1 % annexin-V-FITC and analyzed by Northern Lights flow cytometer (Cytek Biosciences, Fremont, CA, USA) [36]. In brief,

homogenous cell suspensions were collected from each treatment condition and stained with annexin-V-FITC for 10 min at RT in the dark. A total of 10,000 events were analyzed at Ex/Em = 488/521 nm.

Cellular morphology

Control and treated cell morphology were detected using flow cytometry. Forward scatter channel (FSC) measures the amount of light that passes through cells as an estimate of cell diameter (volume), whereas side scatter channel (SSC) captures light at a 90°-degree angle to the incident light which estimates cell surface complexity [37]. Cells used for annexin analysis were also tested for these two signals.

Intracellular Ca^{2+}

To measure intracellular Ca^{2+} by flow cytometry, control and treated cells were incubated with 2.5 μM Fluo4/AM in 5 mM $CaCl_2$ buffer for 30 min in the dark at 37 °C. Then, 10,000 cells were analyzed for Fluo4 fluorescence intensity (Ex/Em = 488/512 nm) which is proportional to Ca^{2+} content [38].

Oxidative stress

Cells were labeled with 5 μM 2',7'-dichloro-dihydrofluorescein diacetate (H_2DCFDA) and analyzed by flow cytometry [39]. In brief, homogenous cell suspensions were collected from each treatment condition and stained with H_2DCFDA for 30 min in the dark at 37 °C. A total of 10,000 events were analyzed at Ex/Em = 488/521 nm.

Osmotic fragility

Control and treated (1 and 1.5 μM EPA) cells were suspended in NaCl solutions of decreasing tonicity (0.9–0 % NaCl) and observed for hemolysis following incubation for 1 h at 37 °C [37].

Anticholinesterase activity

The activity of AChE was determined in pooled hemolysates by Ellman's method using AChE Activity Assay Kit (Solarbio). In short, thiocholine is hydrolyzed by AChE to yield 5-mercaptonitrobenzoic acid (TNB) from 2-nitrobenzoic acid (DTNB) which has maximum absorbance at 412 nm. The enzyme activity of AChE is directly correlated with absorbance [40].

Extracellular acidity and electrolytes

The pH of control and treated cell supernatants were assessed using EXIAS e|1 electrolyte analyzer [37].

Cellular aggregation

Erythrocyte sedimentation rate (ESR) was measured in Westergren tubes as per standard protocols [41]. Briefly, cells were loaded into Westergren tubes placed in an upright position and the distance travelled under the force of gravity is noted.

Statistical analysis

All analyses were carried out by GraphPad 9.0 (GraphPad Software, Inc., San Diego, CA, USA) and data are shown as means ± SD of two or three independent experiments each conducted on cells isolated from three different volunteers (n = 6-9). Student's t-test and one-way ANOVA followed by Dunnett's test were used as appropriate. Significance threshold was defined by a p value of <0.05.

Results

Dual effect of EPA on hemolysis

As shown in Fig.1A, the rate of hemolysis in PBS increased significantly at 20 μM, 40 μM, 80 μM, and 100 μM. In Ringer solution (Fig. 1B), EPA elicited a significant but weaker increase in hemolysis starting at 40 μM. Hemolytic markers, namely K^+, LDH, and AST significantly increased as shown in Fig. 1C, 1D, and 1E, respectively. Fig. 1F illustrates that treatment of cells with 2.5 μM of EPA significantly inhibited hypotonic hemolysis at 0.1 % and 0.3 % NaCl compared to the negative control.

Eryptotic activity of EPA

In Fig. 2B, EPA-treated cells demonstrated a concentration-dependent increase in the geometric mean of annexin-V-FITC fluorescence and the percentage of cells exhibiting PS exposure at 20 μM and 40 μM as shown in Fig. 2C. AChE activity, a marker of cellular aging, was significantly diminished in EPA-treated cells (Fig. 2D) starting at 10 μM as was extracellular pH (Fig. 2E) while no effect on ESR was noted.

EPA increases Ca^{2+} levels to induce cell shrinkage

Significant elevations in the geomean of Fluo4 fluorescence were noted upon exposure to EPA (Fig. 3B) at 20 μM and 40 μM. Additionally, the percentage of cells with high intracellular Ca^{2+} significantly increased at 40 μM as seen in Fig. 3C. Notably, removal of extracellular Ca^{2+} (Fig. 3D) did not rescue the cells from EPA-induced hemolysis.

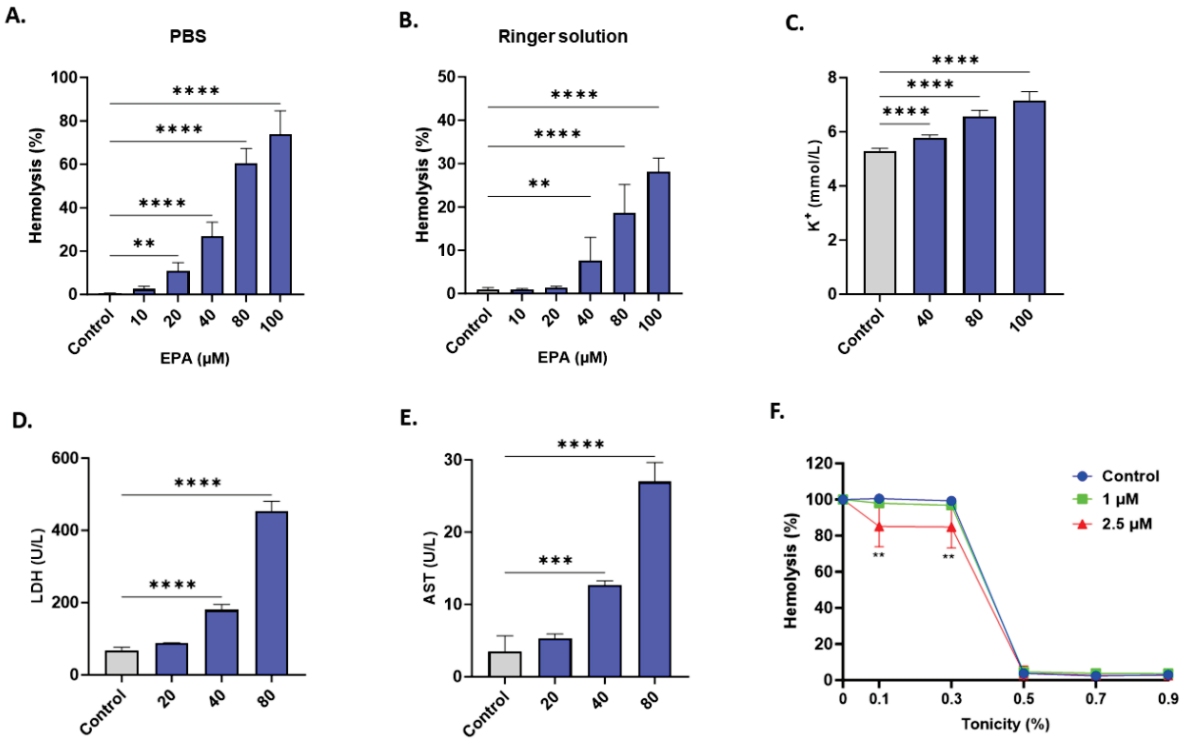

Fig. 1. EPA induces hemolysis. **(A)** concentration-responsive hemolytic activity of EPA (10-100 μM) in PBS and in **(B)** Ringer buffer. **(C)** K^+ and **(D)** LDH and **(E)** AST activity. **(F)** Effect of EPA on hypotonic hemolysis. ns indicates no statistical significance, while $^{**}(p<0.01)$, $^{***}(p<0.001)$, and $^{****}(p<0.0001)$. Data were analyzed by one-way ANOVA followed by Dunnett's correction.

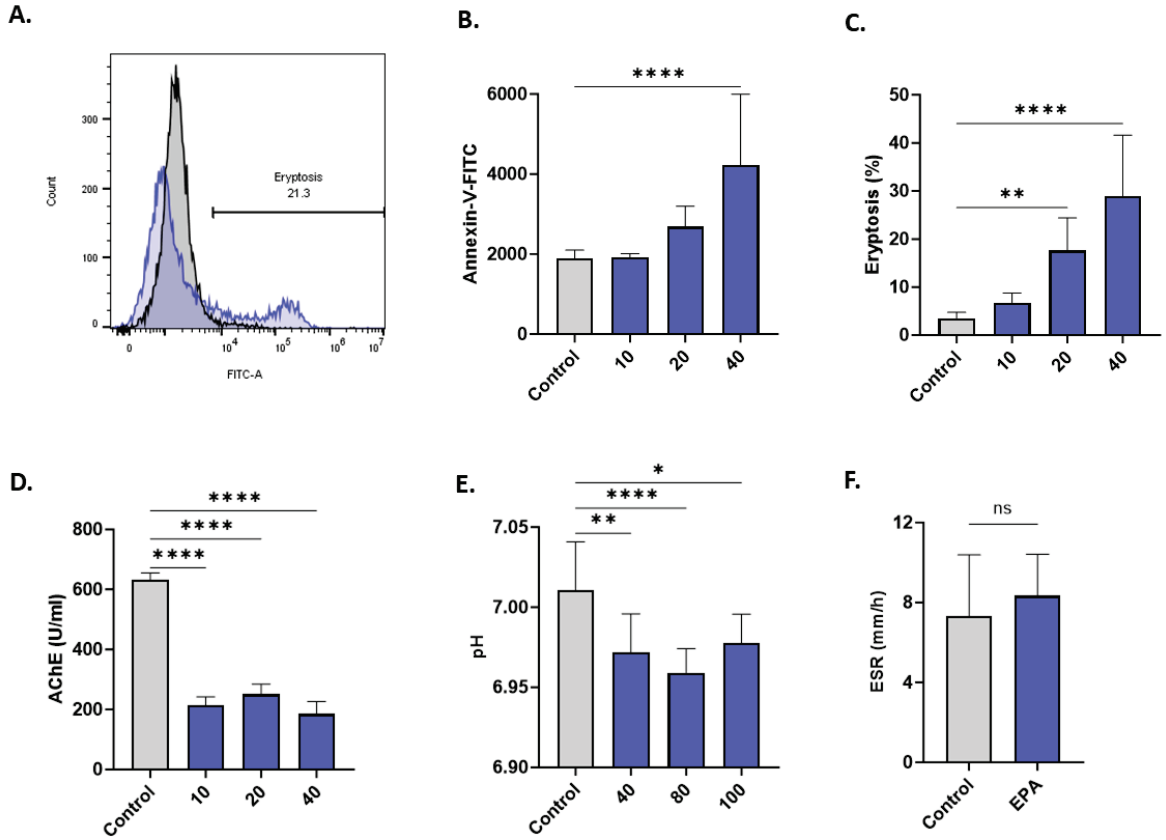

Fig. 2. EPA elicits eryptosis. **(A)** Representative histograms of annexin-V-FITC fluorescence of control (black line) and treated cells (40 μM; blue line). **(B)** Annexin-V-FITC fluorescence. **(C)** Percentage of eryptotic cells. **(D)** AChE activity. **(E)** Extracellular pH. **(F)** ESR. ns indicates no statistical significance, while $^{*}(p<0.05)$, $^{**}(p<0.01)$, and $^{****}(p<0.0001)$. Data were analyzed by one-way ANOVA followed by Dunnett's correction. ESR data were analyzed by Student's *t*-test.

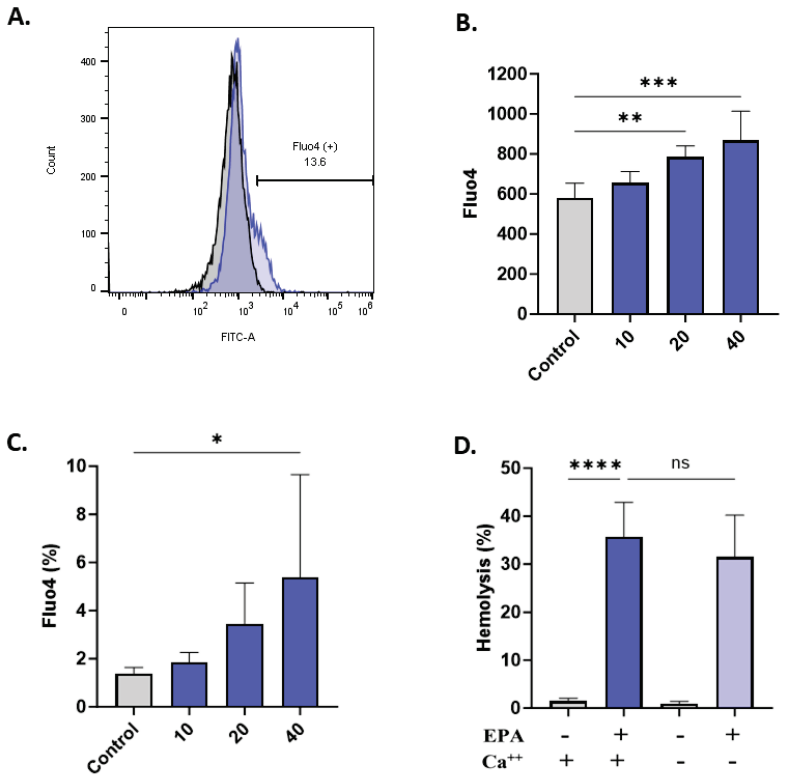

Fig. 3. EPA elevates Ca^{2+} levels. **(A)** Representative histograms of Fluo4 fluorescence of control (black line) and treated cells (40 μM; blue line). **(B)** Fluo4 fluorescence. **(C)** Percentage of cells with excess Ca^{2+} accumulation. **(D)** Effect of Ca^{2+} availability on hemolysis. ns indicates no statistical significance, while $^{*}(p < 0.05)$, $^{**}(p < 0.01)$, $^{***}(p < 0.001)$, and $^{****}(p < 0.0001)$. Data were analyzed by one-way ANOVA followed by Dunnett's correction.

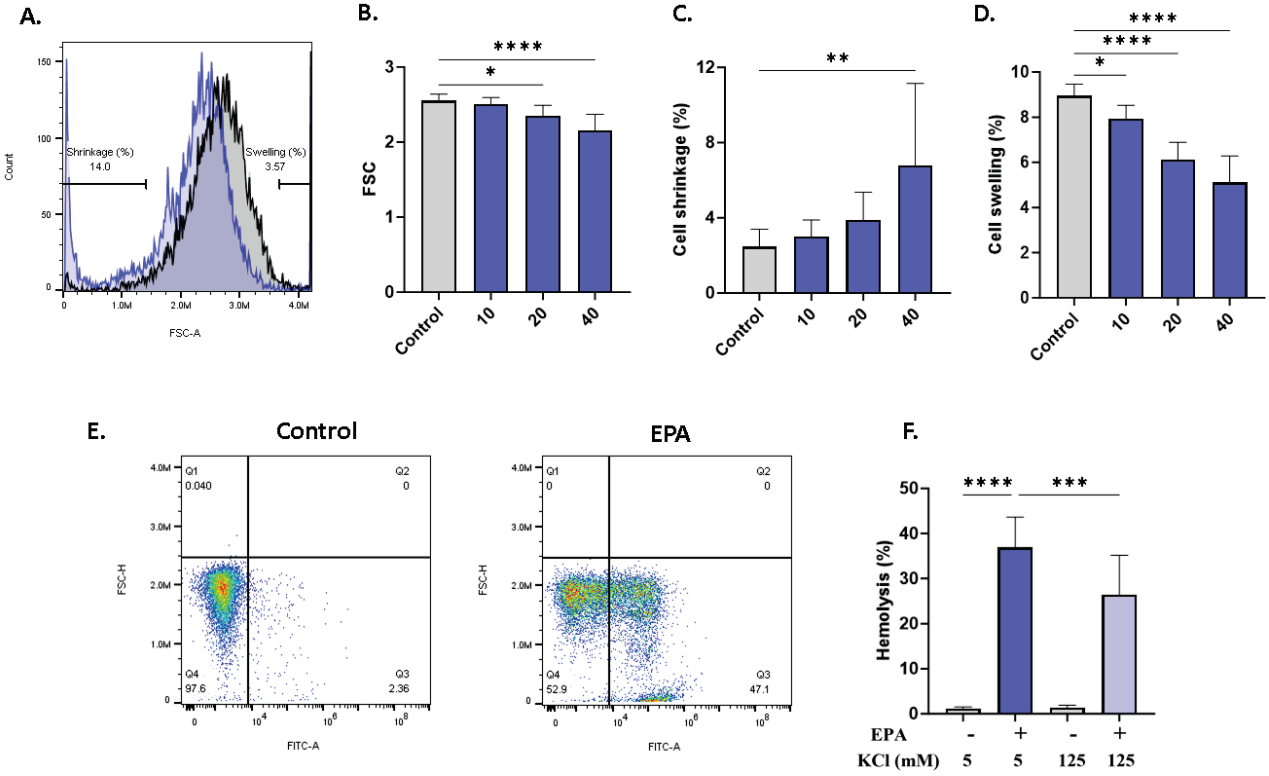

Fig. 4. Effect of EPA on RBC morphology. **(A)** Representative histograms of FSC of control (black line) and treated cells (40 μM; blue line). **(B)** Geomean FSC. **(C)** Percentage of cell shrinkage. **(D)** Percentage of cell swelling. **(E)** Dot plots of control and treated (40 μM) cells relative to FSC-H and annexin-V-FITC (annexin-V-positive cells are shown in Q3). **(F)** Effect of KCl (125 mM) on hemolysis. ns indicates no statistical significance, while $^{*}(p < 0.05)$, $^{**}(p < 0.01)$, and $^{****}(p < 0.0001)$. Data were analyzed by one-way ANOVA followed by Dunnett's correction.

Fig. 4B shows a significant decrease in geomean FSC at 20 µM and 40 µM. Accordingly, the percentage of cell shrinkage at 40 µM was significantly increased as shown in Fig. 4C. This was accompanied by a significant decrease in the percentage of cell swelling (Fig. 4D). Furthermore, hemolysis was significantly decreased by increasing KCl to 125 mM in the extracellular space as seen in Fig. 4F.

Protective effect of vitamin C on EPA toxicity

As seen in Fig. 5A, B, and C, EPA did not cause appreciable oxidative stress. Interestingly, cells cotreated with EPA and vitamin C showed significantly less hemolysis compared to those exposed to EPA alone. Likewise, in Fig. 6A, PEG 8000 at 10 % *w/v* protected the cells from the hemolytic activity of EPA as did isosmotic urea in Fig. 6B.

Discussion

The current study reveals that antitumor concentrations of EPA induce hemolysis and eryptosis in RBCs. Membrane rupture leads to the release of intracellular elements including Hb, K^+, LDH, and AST which cause oxidative damage to vital organs. However, it was also noted that EPA has an antihemolytic effect in a hypotonic environment (Fig. 1F). This biphasic action suggests that, under hypotonic conditions, low concentrations of EPA expand the RBC membrane to allow more water entry which delays hemolysis. This is in agreement with a prior study reporting that EPA increases membrane fluidity [42].

Our results show that EPA significantly decreased extracellular pH (Fig. 2E), indicating acidification of the extracellular environment, which appears to be associated with the accumulation of lactate. It is known that AMP-activated protein kinase (AMPK) is the main sensor of cellular energy status [43], increasing ATP levels by promoting glucose and lipid breakdown and inhibiting their synthesis and storage. Prior reports have found that EPA activates AMPK to prevent vascular endothelial function *in vivo* [45]. Additionally, in skeletal muscle cells, EPA promotes the activation of AMPK [46]. This evidence suggests that EPA may target AMPK-related pathways in RBCs [47]. Lactate levels rise because of AMPK-enhanced glycolysis, which might explain lactate buildup and acidification after EPA treatment.

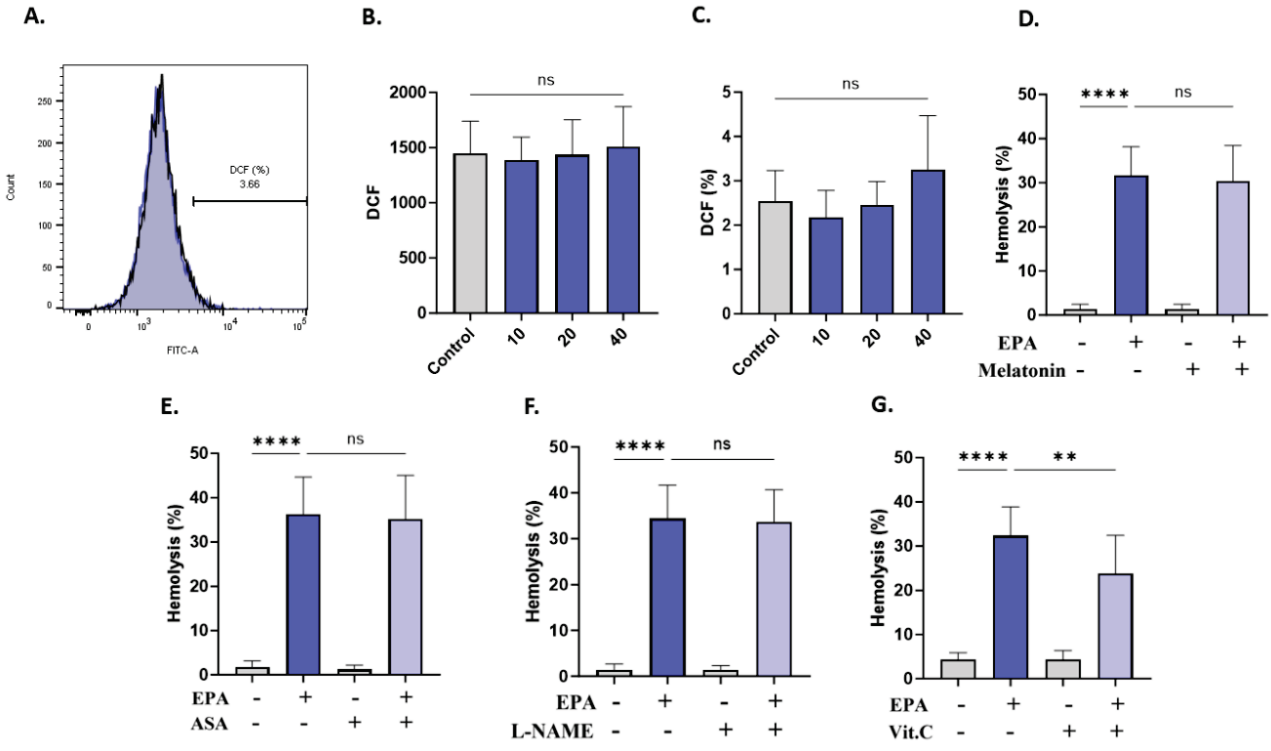

Fig. 5. Protective effect of vitamin C. **(A)** Representative histograms of DCF fluorescence of control (black line) and treated cells (40 µM; blue line). **(B)** DCF fluorescence. **(C)** Percentage of oxidized cells. Effect of **(D)** melatonin, **(E)** ASA, **(F)** L-NAME, and **(G)** vitamin C on EPA-induced hemolysis. ns indicates no statistical significance, while **(p <0.01), and ****(p <0.0001). Data were analyzed by one-way ANOVA followed by Dunnett's correction.

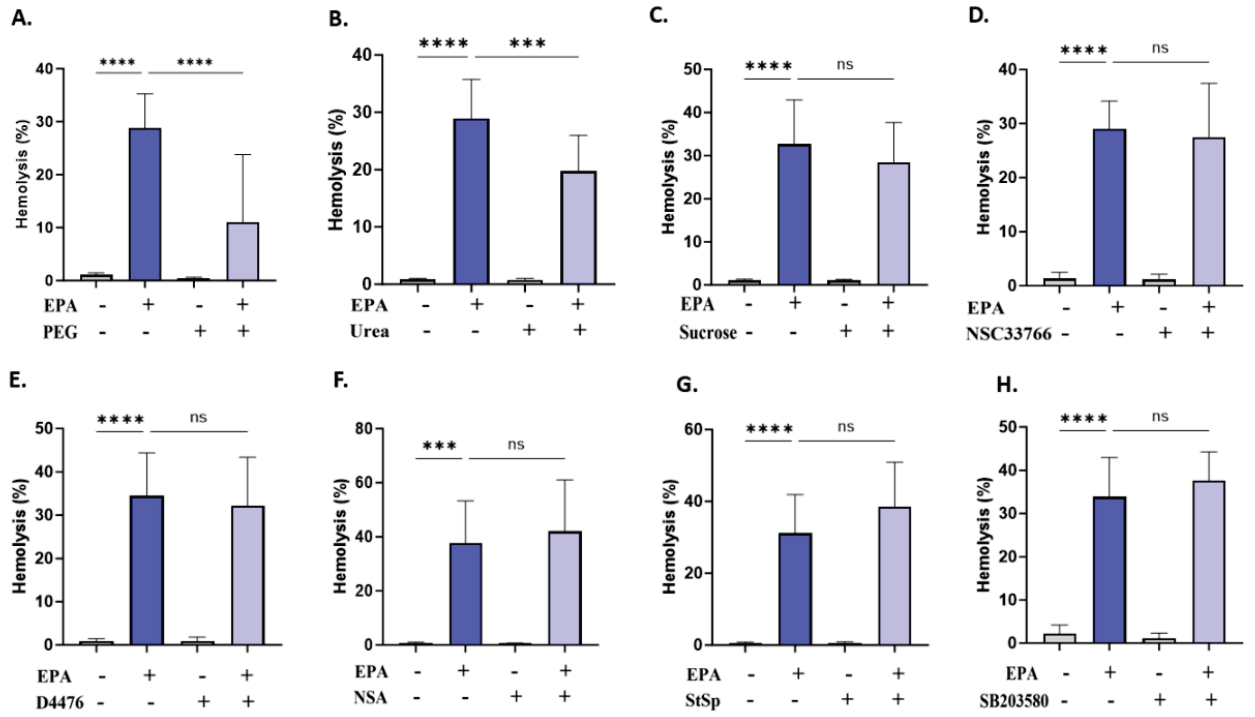

Fig. 6. Inhibitors of EPA-induced hemolysis. Effect of **(A)** PEG, **(B)** urea, **(C)** sucrose, **(D)** NSC23766, **(E)** D4476, **(F)** NSA, **(G)** StSp, and **(H)** SB203580 on EPA-induced hemolysis. ns indicates no statistical significance while $^{***}(p < 0.001)$, and $^{****}(p < 0.0001)$. Data were analyzed by one-way ANOVA followed by Dunnett's correction.

Loss of membrane asymmetry and PS externalization (Fig. 2B) is a hallmark of eryptosis and serves to identify senescent and damaged cells for removal by macrophages. Excessive eryptosis, thus, leads to anemia and is manifested in several clinical conditions including diabetes mellitus, hypertension, dyslipidemia, and hemolytic anemias [47]. Our result is consistent with a previous study reporting EPA induced apoptosis in human neuroblastoma cells [8]. In congruence, we also show that EPA significantly reduced AChE activity (Fig. 2D). A marker of membrane integrity and cellular aging [48], AChE is a glycosylphosphatidylinositol-linked membranous enzyme that appears to aid in maintaining the RBC size and shape. As such, the anticholinesterase activity of EPA may underlie the morphological changes observed in treated cells (Fig. 4).

Ca^{2+}-responsive K^+ channels open in response to increased Ca^{2+} activity in the cytosol (Fig. 3C), which in turn causes loss of KCl and water [32]. Blocking KCl efflux by adding 125 mM KCl to the extracellular space significantly inhibited EPA-induced hemolysis, which supports the thesis that KCl loss is required for the cytotoxicity of EPA in RBCs.

Inhibition of EPA toxicity by PEG (Fig. 6A) indicates that EPA and cells were physically segregated. Therefore, the anticancer efficacy and bioavailability of PEGylated EPA preparations require further testing. Urea was also effective in lowering EPA-induced hemolysis (Fig. 6B). Sphingomyelin is hydrolyzed by sphingomyelinases, activated by urea, to produce ceramide which increases membrane fragility. Thus, increased ceramide concentration is an indicator of eryptosis [50] which might reflect suppression of sphingomyelinase activity [50].

The lack of oxidative stress upon EPA treatment is consistent with the established role of ώ-3 FAs as enhancers of the antioxidant defense system [51,52]. Surprisingly, among all oxidative stress inhibitors used in this study, only vitamin C showed a significant diminishment in EPA toxicity (Fig. 5G) which suggests that the protection offered by vitamin C is unrelated to its antioxidant functions. Along those lines, a role for vitamin C in modulating Ca^{2+} channels has been described [53]. It follows, then, that inhibition was mediated through the action of vitamin C on Ca^{2+} channel activity independent of the redox status.

Conclusion

In conclusion, this report shows that EPA causes premature RBC death characterized by PS translocation, cell shrinkage, loss of ionic transport, and Ca^{2+} buildup

and that these toxic effects may be ameliorated by PEG 8000 and isosmotic urea. Further research must be directed toward investigating the toxicity of EPA in animal models and optimize the identified inhibitors for use as possible adjuvants of EPA in anticancer therapy.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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