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Oxyphyllacinol reprograms red cell lifespan through calcium and p38 MAPK/CK1α signaling axis

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The medicinal value of *Alpinia oxyphylla* is attributed to numerous compounds with varied pharmacological activities. However, no reports on oxyphyllacinol (OPC), a major antitumor diarylheptanoid in the plant's capsular fruit, have been published to date. Since anemia is a common complication of chemotherapy, this work examines the hemolytic and eryptotic properties of OPC in human red blood cells (RBCs). RBCs were exposed to 10-100 μM of OPC for 24 h at 37°C and photometric assays were used to measure hemolytic markers whereas eryptosis was detected by flow cytometry using Annexin-V-FITC, Fluo4/AM, and H_2DCFDA to quantify phosphatidylserine (PS) translocation, intracellular Ca^{2+}, and oxidative stress, respectively. OPC caused significant hemolysis at 100 μM with elevations in lactate dehydrogenase and aspartate transaminase. Also, OPC significantly increased Annexin-V-FITC and Fluo4 but not DCF fluorescence. Importantly, OPC-induced hemolysis was significantly inhibited by PEG 8000, SB203580, and D4476. OPC depleted hemoglobin content in whole blood and increased mean corpuscular volume, fragmented RBCs, immature granulocytes, and large platelets. Altogether, this work shows that OPC stimulates hemolysis and eryptosis in human RBCs which may be ameliorated by blocking p38 MAPK or casein kinase 1α. These novel findings necessitate further safety assessment of OPC as an anticancer agent.

Keywords: Eryptosis, Hemolysis, Anticancer, *Alpinia oxyphylla*

The capsular fruits of the herbaceous perennial plant, *Alpinia oxyphylla*, have traditionally been known to improve gastrointestinal, urinary, and cognitive symptoms due to their anti-inflammatory, antioxidant, and neuroprotective properties[1,2]. Among the many secondary metabolites of *A. oxyphylla*, oxyphyllacinol (4-(3-hydroxy-7-phenylheptyl)-2-methoxyphenol; OPC) is a major diarylheptanoid (Fig. 1A) which shows anticancer activity against various cell types[3]. Moreover, in rats fed *A. oxyphylla* extracts, OPC was synthesized from other components, yakuchinone A and B, and was detected in plasma along with its glucuronidated form[4]. Since it demonstrates cytotoxic effects against tumor cells and seems to be metabolically well-tolerated, OPC thus holds great promise as an anticancer agent.

Chemotherapy-related anemia is a frequent side effect of both investigative and approved therapeutics being encountered in more than 75% of patients[5]. Myelosuppressive syndrome has been attributed to anemia of chemotherapy, but emerging evidence also points to a central role for eryptosis as an underlying mechanism[6]. Both biochemically and morphologically, eryptosis resembles apoptosis of nucleated cells with canonical features including phosphatidylserine (PS) translocation to the outer membrane leaflet, loss of ion transport regulation, intracellular Ca^{2+} overload, oxidative stress, metabolic shutdown, membrane blebbing, and cell shrinkage. Furthermore, eryptosis is orchestrated by various signaling mediators, most notably caspases, p38 MAPK, casein kinase 1α (CK1α), Rac GTPases, and ceramide[7,8].

Various compounds with antitumor activity have been shown to induce hemolysis and eryptosis[9] but the toxicity of OPC has as of yet not been investigated. Herein, our objective is to examine the hemolytic and eryptotic potential of OPC and identify the underlying biochemical mechanisms.

Materials and Methods

Blood collection, chemicals, and reagents

Ethical clearance was granted by the Institutional Review Board of King Saud University Medical City (E-20-4544). Blood samples were collected in EDTA-coated (1.5mg/mL) vacutainer tubes from 15 healthy participants (8 males and 7 females) aged 25-38 years

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with normal BMI and CBC and no history of chronic disease. All participants provided written informed consent in line with the Declaration of Helsinki. RBCs were isolated by density-gradient centrifugation and repeated washing in PBS (3,000 RPM, 20 min, RT). All chemicals were of analytical grade and were purchased from Solarbio Life Science (Beijing, China). Synthetic OPC (CAS #87657-77-0; purity 98.24%) was prepared by dissolving 10mg in 3.18ml of DMSO to obtain a 10mM stock solution. Ringer solutions were prepared as described elsewhere[10].

Hemolysis

Cells were centrifuged (13,000 ×*g*, 1 min) and the supernatants were photometrically assayed for hemoglobin (Hb) at 405nm using LMPR-A14 microplate reader (Labtron Equipment Ltd., Surrey, UK) with cells suspended in distilled water as a positive control[11]. Lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured in the supernatants by BS240-pro clinical chemistry analyzer (Mindray Medical International Ltd., Shenzhen, China).

PS translocation

Homogenous suspensions of vehicle control and treated RBCs were stained with 1% annexin-V-FITC for 10 min at room temperature in the dark and 10,000 events were analyzed using Northern Lights flow cytometer (Cytek Biosciences, Fremont, CA, USA)[12]. RBCs exposed to distilled water served as a positive control for eryptotic markers.

Cellular morphology

Forward scatter (FSC) and side scatter (SSC) were recorded as cell size and complexity indicators, respectively[13].

Intracellular Ca^{2+}

Homogenous suspensions of vehicle control and treated RBCs were stained with 5µM of Fluo4/AM for 30 min, at 37°C, away from light, washed in PBS to remove excess dye, and analyzed by flow cytometry[14].

Oxidative stress

Homogenous suspensions of vehicle control and treated RBCs were stained with 10µM of H_2DCFDA, at 37°C, in the dark and analyzed by flow cytometry following washing in PBS[15].

Systemic toxicity

Whole blood was diluted 1:5 with physiological saline and treated with 100µM of OPC for 24 h at 37°C. A CBC was then obtained using BC-6200 hematology analyzer (Mindray, Shenzhen, China) [7].

Statistical analysis

Results are shown as arithmetic means±SEM of at least three independent experiments each run in triplicates. FlowJo™ v10.7.2 (Becton, Dickinson and Company, Ashland, OR, USA) was used to analyze flow cytometry data. All statistical analyses were performed by GraphPad Prism v9.2.0 (GraphPad Software, Inc., San Diego, CA, USA). A threshold significance value of P <0.05 was tested using the unpaired, two-tailed t-test and one-way ANOVA followed by Dunnett's correction.

Results

OPC induces hemolysis

Significant hemolysis was detected at 100µM of OPC (1.65±0.26% to 23.20±3.15%, P<0.0001) as shown in Fig. 1B. A concurrent leakage of LDH (8.21±1.26 U/L to 70.69±12.50 U/L, P<0.0001) and AST (1.83±0.29 U/L to 5.92±0.76 U/L, P<0.0001) was also observed in Fig. 1C and 1D, respectively.

OPC stimulates eryptosis

In cells treated with 100µM of OPC, the geomean of Annexin-V-FITC fluorescence significantly increased from 2034±171.5 arbitrary units (a.u.) to 4686±614.1 a.u. (P<0.0001, Fig. 2A and 2B) as

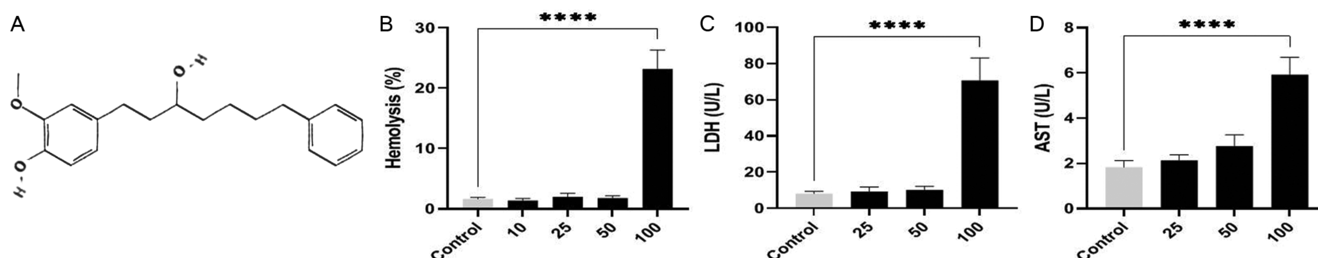

Fig. 1 — OPC induces hemolysis. (A) Molecular structure of OPC ($C_{20}H_{26}O_3$; 314.4 g/mol)[35]; (B) Hemolytic rate. (C) LDH. (D) AST. Results are shown as means ± SEM (n = 9). ***(P <0.0001).

did the percentage of annexin-V-positive cells (3.47±0.53% to 30.56±1.87%, P<0.0001, Fig. 2C). This was associated with a significant decrease in both FSC (2.13±0.02 a.u. to 1.41±0.07 a.u., P<0.0001) and SSC (169.3±3.25 a.u. to 108.3±14.58 a.u., P<0.0001) as seen in Fig. 2E and 2F, respectively.

OPC elevates intracellular Ca^{2+}

In cells treated with 100µM of OPC, the geomean of Fluo4 fluorescence significantly increased from 215.4±16.69 a.u. to 828.8±46.12 a.u. (P<0.0001, Fig. 3A and 3B) as did the percentage of cells with elevated Ca^{2+} (3.18±0.42% to 43.31±2.20%, P<0.0001, Fig. 3C).

OPC reduces reactive oxygen species

In cells treated with 100µM of OPC, the geomean of DCF fluorescence was significantly reduced from 645.1±31.20 a.u. to 476.7±19.48 a.u. (P<0.05, Fig. 3D and 3E).

OPC toxicity is mediated through p38 and CK1α

Significant inhibition of OPC-induced hemolysis was noted in the presence of SB203580 (31.41±5.33% to 15.04±3.14%, P<0.01, Fig. 4A) and D4476 (16.55±1.75% to 6.11±1.17%, P<0.0001, Fig. 4B), but not in the absence of Ca^{2+} (Fig. 4C) or the presence of high KCl (Fig. 4D), urea (Fig. 4E), or sucrose (Fig. 4F). PEG 8000 also offered significant protection (40.28 + 7.39% to 19.19 + 4.05%, p <0.01, Fig. 4G).

OPC exhibits distinct toxicity profiles in whole blood

Although no significant decrease in RBC count (Fig. 5A) or circulating hemoglobin (Fig. 5B) was noted, OPC significantly depleted intracellular hemoglobin stores (305.2±0.91 g/L to 291.4±2.33 g/L, P<0.0001, Fig. 5C) and increased cellular volume (96.61±0.18 fL to 101.5±0.75 fL, P<0.0001, Fig. 5D) and number of macrocytes ($88.0\times10^3\pm4.0\times10^3$ cells/µL to $159.0\times10^3\pm7.90\times10^3$ cells/µL, P<0.0001, Fig. 5E) and their proportion (7.41±0.30% to 12.84±0.81%,

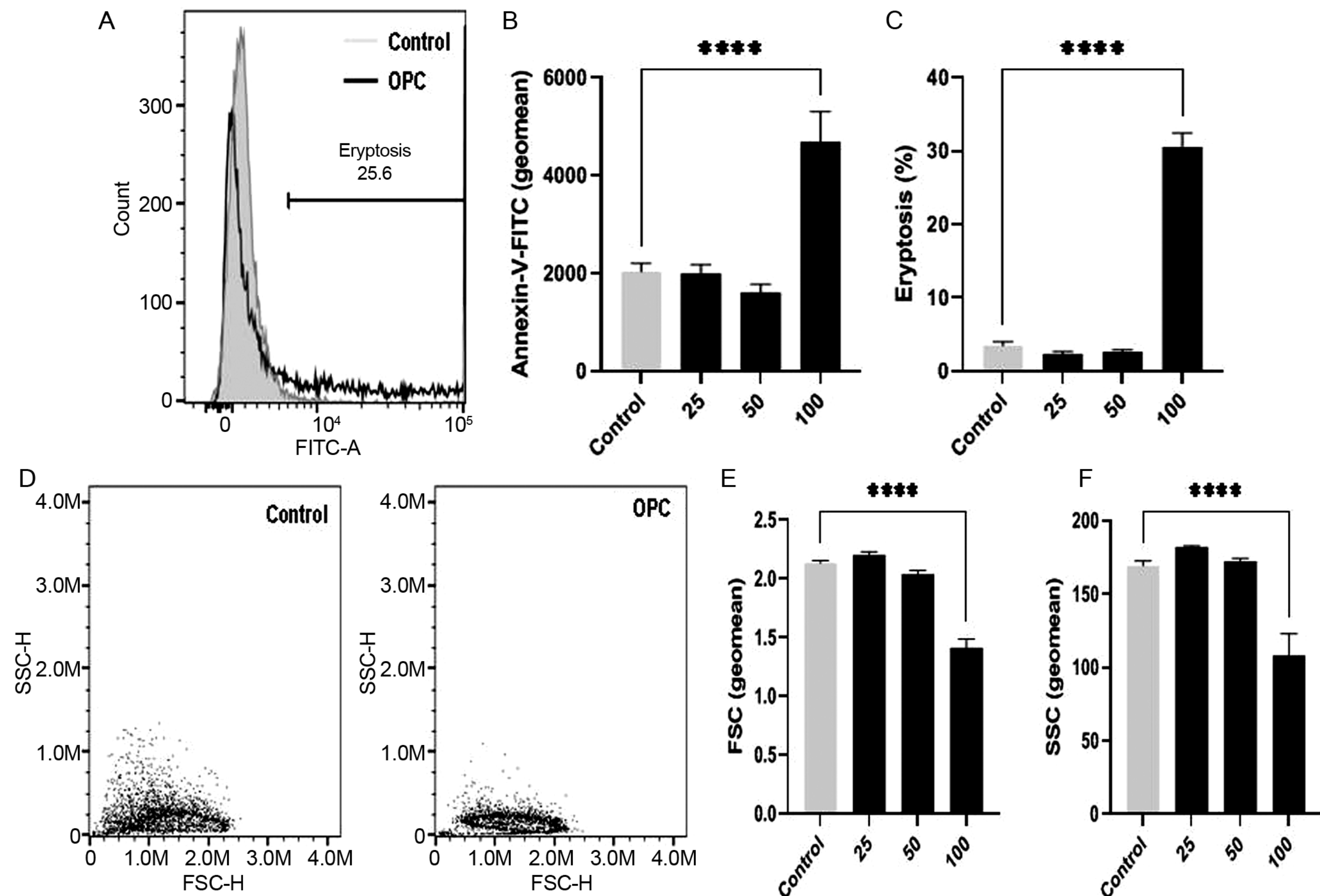

Fig. 2 — OPC stimulates eryptosis. (A) Original histograms of Annexin-V-FITC fluorescence in control and treated cells. (B) Geometric means of Annexin-V-FITC fluorescence. (C) Percentage of Annexin-V-positive cells. (D) Original dot plots of SSC and FSC in control and treated cells. (E) Geomean of FSC. (F) Geomean of SSC. Results are shown as means ± SEM (n = 9-12). ***(P <0.0001).

P<0.0001, Fig. 5F). Fragmented cells were also increased upon OPC exposure (4.83±0.19 cells/μL to 6.1±0.22 cells/μL, *P*<0.001, Fig. 5G) corresponding to an increase from 0.41±0.017% to 0.49±0.02% (*P*<0.01, Fig. 5H).

While WBCs remained unchanged (Fig. 5I), significant elevations in the number (11.11±2.60 cells/μL to 21.11±2.60 cells/μL, Fig. 5J, *P*<0.05) and percentage (0.01078±0.002308% to 0.01944±0.002316%, Fig. 5K, *P*<0.05) of immature granulocytes were noted upon OPC treatment.

Platelet count ($51.33\times10^3\pm1.19\times10^3$ cells/μL to $104.30\times10^3\pm6.89\times10^3$ cells/μL, *P*<0.0001, Fig. 5L) and large platelets ($14.11\times10^3\pm0.82\times10^3$ cells/μL to $28.56\times10^3\pm2.51\times10^3$ cells/μL, *P*<0.0001, Fig. 5M) significantly increased, whereas immature platelet indices were significantly diminished including immature platelet fraction ($3.89\times10^3\pm0.26\times10^3$ cells/μL to $2.0\times10^3\pm0.17\times10^3$ cells/μL, *P*<0.0001, Fig. 5N) and its percentage (10.20%±0.56% to 4.74%±0.42%, *P*<0.0001, Fig. 5O), along with immature platelets with high fluorescence intensity (1.61%±0.10% to 0.72%±0.06%, *P*<0.0001, Fig. 5P).

Discussion

Chemotherapy increases the incidence of anemia[16,17]; a common complication[18] caused, at least in part, through eryptosis[19]. In this report, we reveal that OPC reprograms RBCs to stimulate premature eryptosis and hemolysis. Although no studies have been published to date on the antitumor concentrations of OPC, Huo *et al*. reported IC_{50} values ranging from 46 to 63 μM against a panel of murine and human cancer cells[3]. More studies are required to determine the effective concentration range against tumor cells without appreciable toxicity to off-target tissue. Determination of the bioavailability of OPC in animal models is also encouraged.

Hemolysis results from direct membrane damage that causes cellular contents to leak into the

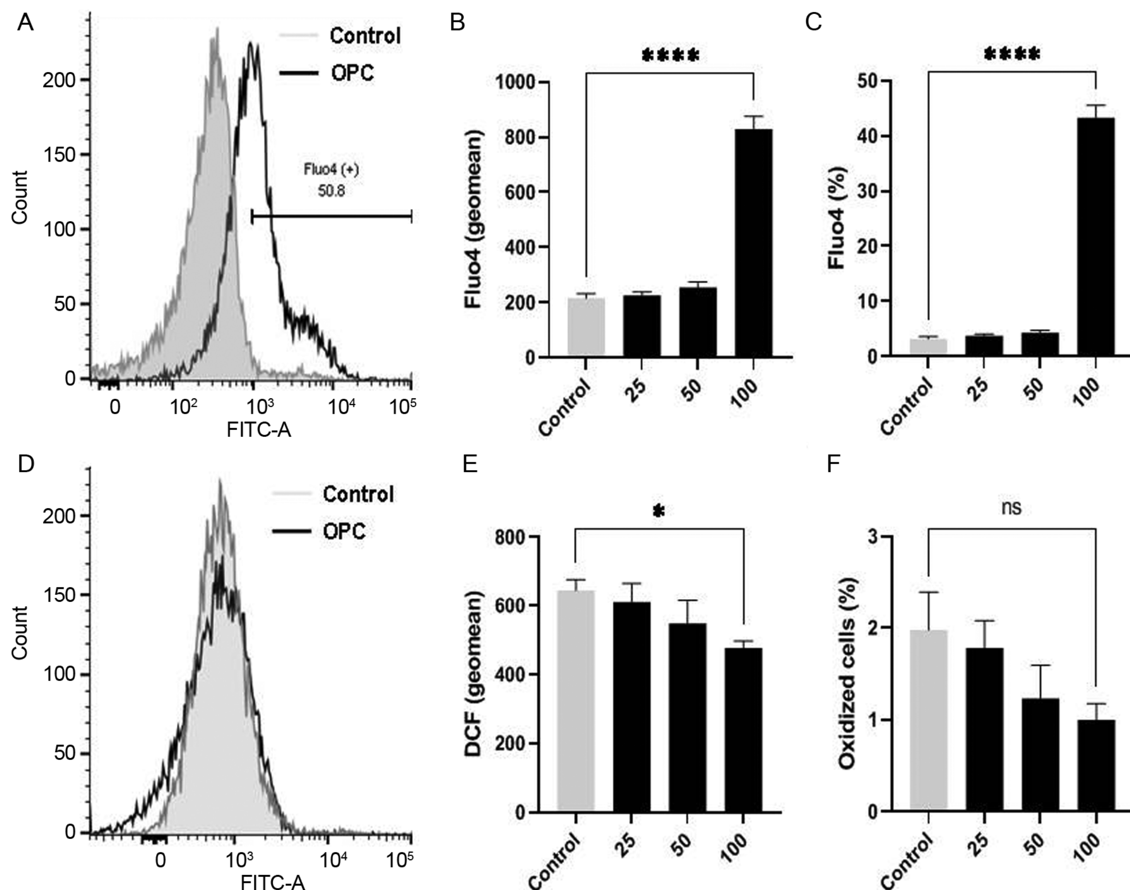

Fig. 3 — OPC elevates intracellular Ca^{2+}. (A) Original histograms of Fluo4 fluorescence in control and treated cells. (B) Geometric means of Flu4 fluorescence. (C) Percentage of Fluo4-positive cells. (D) Original histograms of DCF fluorescence in control and treated cells. (E) Geometric means of DCF fluorescence. (F) Percentage of DCF-positive cells. Results are shown as means ± SEM (*n* = 9). ns = not significant, *(*P* <0.05), and ***(*P* <0.0001).

extracellular space. Hb constitutes up to 95% of the dry mass of RBCs, and, as such, heavily contributes to the pathophysiological consequences of intravascular hemolysis. The large amount of Hb released into the bloodstream during hemolysis overwhelms the binding capacity of haptoglobin. As potent oxidants, free Hb dimers precipitate in the tubular lumen following glomerular filtration causing acute and chronic kidney disease due to the release of the heme ring and free iron in the presence of inflammatory mediators[20]. Furthermore, free Hb sequesters the nitric oxide pool leading to loss of vascular tone and ischemic lesions[21].

In a similar fashion to Hb, eryptotic cells are recognized as important contributors to compromised blood rheology. Translocation of PS allows RBCs to interact with endothelial cells through transmembrane CXC chemokine ligand 16 forming cellular aggregates that interfere with blood flow. This mechanism may be behind the impaired microcirculation characteristic of cardiovascular disease and related conditions[22]. Moreover, membrane changes that occur in eryptotic cells cause reduced deformability leading to hemodynamic stasis and thromboembolic events[23]. Eryptotic cells are also rapidly disposed of by phagocytes, further increasing the risk for anemia despite accelerated erythropoiesis. It is important to mention that enhanced eryptosis has been recognized in a plethora of other pathological conditions[24].

OPC-induced eryptosis was accompanied by notable corpuscular shrinkage, which indicates cellular dehydration. The fall in cell volume is secondary to Ca^{2+} accumulation and the escape of KCl and water[8]. It is thought that macrophages for efficient elimination of aged, infected, and damaged erythrocytes more readily engulf smaller cells and cell fragments. It remains elusive, however, why rather significant RBC swelling was observed when cells are exposed to OPC in whole blood, but the presence of plasma proteins is a possible contributory factor. It seems plausible as well that Na^+ entry outweighs K^+ exit thereby leading to increased cellular volume[25]. Thus, examination of the influence of OPC on the activity of the Na^+/K^+-ATPase pump may provide useful insights. Cell swelling was observed for some

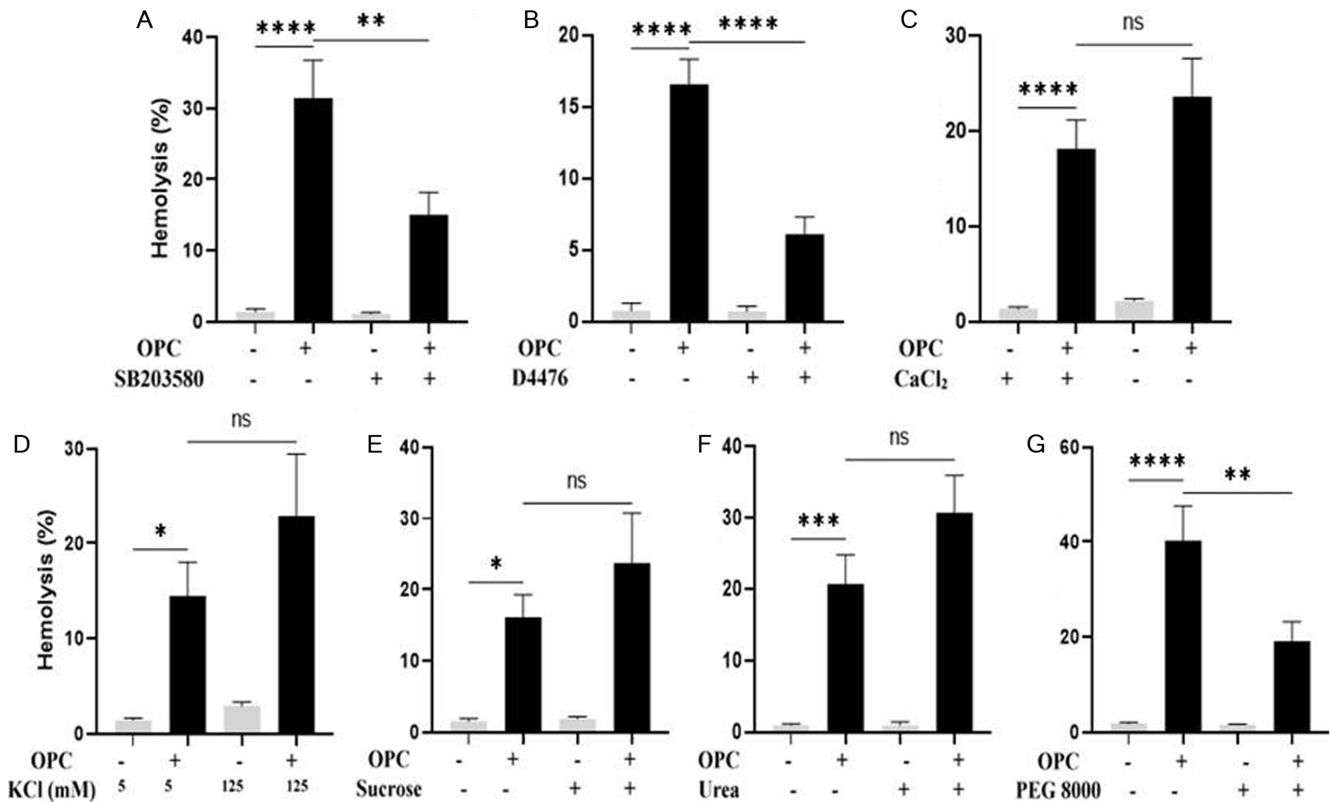

Fig. 4 — Inhibitors of OPC-induced hemolysis. Hemolytic rate of OPC in the presence and absence of (A) SB203580, (B) D4476, (C) 1 mM of extracellular Ca^{2+}, (D) 125 mM of KCl, (E) 250 mM of sucrose, (F) 300 mM of urea, and (G) PEG 8000. Results are shown as means $\pm$ SEM (n = 9). ns = not significant, *(P <0.05), **(P <0.01), ***(P <0.001), and ***(P <0.0001).

eryptosis inducers such as emodin[26] and bacterial lipopeptides[27], as was a dual effect on cellular volume[28].

Increased intracellular Ca^{2+} is a defining feature of eryptosis. The maintenance of physiological Ca^{2+} levels within the RBC is essential for survival, and it includes preservation of the asymmetrical arrangement of phospholipid species, the regulation of ion transport and gas exchange, and metabolic turnover. This is because many enzymes involved in these cellular functions are Ca^{2+}-dependent and are therefore vulnerable to fluctuations in Ca^{2+} activity. Relevant to eryptosis are scramblases, which aid in phospholipid organization, and calpains, which digest the cytoskeleton causing membrane blebbing[8].

Compared with pro-eryptotic compounds, OPC decreased free radicals. When cells undergo eryptosis, reactive oxygen species build up due to a failed antioxidant system, leading to damaged macromolecules. Eryptosis can, nonetheless, take place in the absence of appreciable redox imbalance, as is the case with micafungin[29]. However, this observation is not surprising for OPC since several studies have reported the antioxidant activity of plant extracts rich in OPC [2]. For instance, the water fraction of *Synedrella nodiflora* enhances the antioxidant capacity of the yeast *Schizosaccharomyces pombe*[30].

Our results revealed that p38 MAPK is required for the hemolytic activity of OPC. In nucleated cells, p38 is recognized as a stress-sensing enzyme that is also

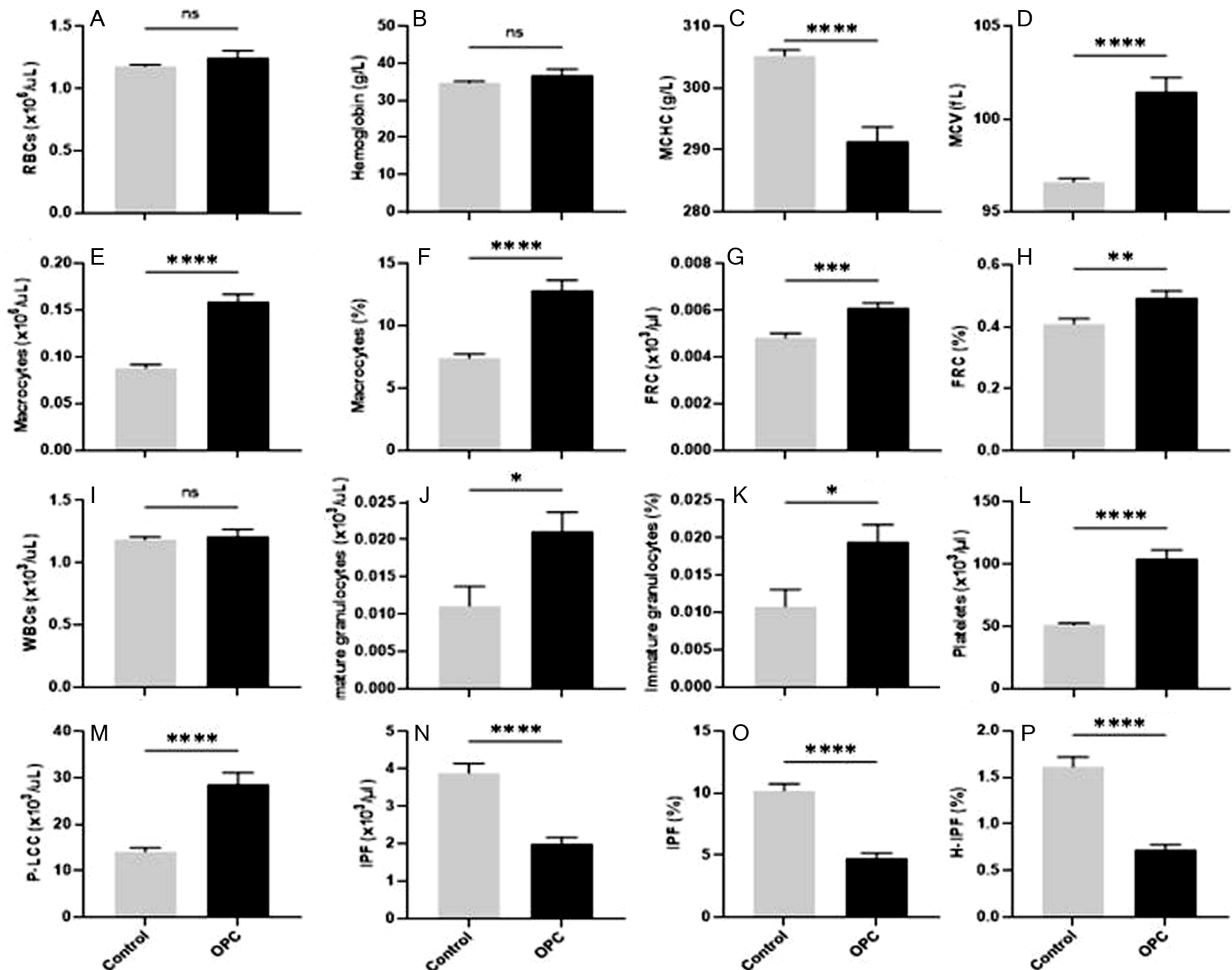

Fig. 5 — Effect of OPC on peripheral blood cells. (A) RBC count, (B) hemoglobin, (C) MCHC, (D) MCV, (E) macrocytes, (F) percent macrocytes, (G) fragmented red cells (FRC), (H) percent FRC, (I) WBC count, (J) immature granulocytes, (K) percent immature granulocytes, (L) platelet count, (M) platelet-large cell count (P-LCC), (N) immature platelet fraction (IPF), percent IPF (O), and (P) percent IPF with high fluorescence (H-IPF). Results are shown as means ± SEM (n = 9). ns = not significant, *(P <0.05), **(P <0.01), ***(P <0.001), and ***(P <0.0001).

essential for various cellular processes including proliferation and apoptosis[31]. Previous studies have unequivocally demonstrated that p38 is present in RBCs and that it mediates eryptosis following hyperosmotic shock and exposure to a host of toxic compounds[32]. In particular, p38 regulates cell volume, Ca^{2+} trafficking, and membrane asymmetry; all shown to be targeted by OPC in the current study. Likewise, inhibition of CK1α rescued the cells from OPC toxicity in a similar fashion to allicin, inauhzin, and gingerol, among others. In fact, CK1α promotes Ca^{2+} entry under conditions of metabolic stress which may explain its role in the present study[9]. Importantly, the antihemolytic protection provided by PEG 8000 indicates that membrane pores created by OPC are smaller in size than the diameter of PEG 8000[33]. Alternatively, OPC molecules may be trapped by the excipient through micellar solubilization as we have previously reported in the case of triclosan[34].

The present report also characterizes the toxicity of OPC to other blood cells. The increase in immature granulocytes reflects delayed or arrested differentiation which suggests that OPC may interfere with the transcriptional regulation and response to growth factors essential for granulopoiesis. OPC also increased platelets and decreased immature platelet indices, which could be due to platelet activation and the formation of cellular clumps that falsely elevate the count. Immature platelets are indicators of residual platelet reactivity which seems to be inhibited by OPC. Indeed, the current observations warrant further examination of the antiplatelet function of OPC.

Conclusion

In conclusion, this study constitutes the first evidence of the detailed cellular mechanisms of OPC. It was demonstrated that OPC stimulates eryptosis in human RBCs characterized by PS translocation, cell shrinkage, and Ca^{2+} buildup. OPC also elicits hemolysis through p38 MAPK and CK1α which may be reversed by PEG 8000. These findings advance our understanding of the bioactive properties of OPC and guide future efforts in the development and validation of OPC as an anticancer agent.

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Ethical statement:

This work was approved by the Ethics Committee of King Saud University Medical City (E-20-4544) and all participants provided written informed consent.

Conflict of Interest

The authors declare that there is no conflict of interest.

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