What Should Be Responsible for Eryptosis in Chronic Kidney Disease?

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Keywords

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Abstract

Background: Renal anemia is an important complication of chronic kidney disease (CKD). In addition to insufficient secretion of erythropoietin (EPO) and erythropoiesis disorders, the impact of eryptosis on renal anemia demands attention. However, a systemic analysis concerning the pathophysiology of eryptosis has not been expounded. **Summary:** The complicated conditions in CKD patients, including oxidative stress, osmotic stress, metabolic stress, accumulation of uremic toxins, and iron deficiency, affect the normal skeleton structure of red blood cells (RBCs) and disturbs ionic homeostasis, causing phosphatidylserine to translocate to the outer lobules of the RBC membrane that leads to early elimination and/or shortening of the RBC lifespan. Inadequate synthesis of RBCs cannot compensate for their accelerated destruction, thus exacerbating renal anemia. Meanwhile, EPO treatment alone will not reverse renal anemia. A variety of eryptosis inhibitors have so far been found, but evidence of their effectiveness in the treatment of CKD remains to be

established. **Key Messages:** In this review, the pathophysiological processes and factors influencing eryptosis in CKD were elucidated. The aim of this review was to underline the importance of eryptosis in renal anemia and determine some promising research directions or possible therapeutic targets to correct anemia in CKD.

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Introduction

Anemia, as a universal complication of chronic kidney disease (CKD), is considered to be an independent risk factor of cardiovascular events and mortality especially for patients with end-stage renal disease (ESRD) [1]. Anemia is diagnosed when hemoglobin (Hb) falls below 13.0 g/dL for males and 12.0 g/dL for females. It first manifests in the early stages of CKD and gradually worsens as kidney function declines.

An epidemiological study showed that the prevalence of renal anemia was 8.4%, 12.2%, 17.4%, 50.3%, and 53.4%

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in patients with CKD stages 1–5, respectively [2]. Another study revealed that anemia was diagnosed in approximately 60% of nondialysis patients and 93% of dialysis patients [3]. Without timely management, anemia will cause serious problems such as decreased quality of life, increased risk of cardiovascular events, frequent hospitalizations, and hemodialysis [4, 5].

In 1980s, the development of erythropoietin (EPO)-stimulating agents (ESA) was a revolutionary breakthrough for the treatment of renal anemia as it not only improved the quality of life of patients with ESRD but also lessened the demand for blood [6]. To some extent, ESA can lessen the frequency of dialysis and burden of medical resources especially for CKD patients who require dialysis [7]. Follow-up studies have found that approximately 10% of patients who received ESA developed EPO resistance [6, 8], which is possibly related to iron deficiency and inflammation [9]. When the dose of ESA increases, the risk of cancer [10], all-cause mortality, and hospitalization would also dramatically increase [11, 12]. In recent years, the hypoxia-inducible factor prolyl hydroxylase inhibitors, which stimulates hematopoiesis by boosting the synthesis of endogenous EPO, have been approved for clinical use [13]. However, a comprehensive data evaluation of patients with cancer is required before administration. A number of issues regarding the management of renal anemia, including defining the reasonable Hb level for individuals, reducing adverse effects associated with the ESA dose, and relieving the patient's financial burden need to be addressed [14, 15].

Insufficient EPO synthesis and iron deficiency required for red blood cell (RBC) production are the main causes of renal anemia. In addition, an investigation found that mean RBC lifespan (RBCL) in CKD stages 1–5 are 122 ± 50 , 112 ± 26 , 90 ± 32 , 88 ± 28 , and 60 ± 24 days, respectively [16]. In early CKD, the elevation of EPO levels is a response to decreased Hb levels partly from RBC destruction. New RBCs cannot immediately replenish the amount of destroyed RBCs, which may aggravate renal anemia especially in uremic patients [16–18]. For ESRD patients undergoing hemodialysis or peritoneal dialysis [19, 20], eryptosis cannot be disregarded due to its role in decreasing the number of circulating RBCs [18, 21, 22]. Despite its benefits, ESA use is not the only solution for renal anemia. The results of several researches that clarified the mechanisms and factors influencing eryptosis may provide promise in ameliorating renal anemia. This review narrates the pathophysiological processes of eryptosis and its main influencing factors based on current research.

The Maturation Processes of Erythrocytes

Erythrocyte maturation undergoes seven different stages of differentiation. During erythrocyte differentiation, various transmembrane proteins are synthesized or downregulated to form a spectrin-based membrane skeleton, providing stability of the membrane [23]. Ankyrin, protein 4.1, and band 3, along with spectrin and actin, collectively contribute to membrane-anchored protein cytoskeleton [24, 25]. Spectrin is mostly present during the burst-forming unit-erythroid stage, and ankyrin, band 4.1, band 3 and actin are gradually detected during the colony-forming unit-erythroid stage [26]. Regulated by EPO, the proerythroblast, which is regarded as the earliest recognizable erythrocyte, transforms into a basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, and reticulocyte [27–29]. During the period between the proerythroblast and orthochromatic erythroblast stages, two key changes occur: erythrocyte enucleation and change in membrane protein components. The nucleus is phagocytosed by macrophages in a way of DNase I-dependent, and the ratio changes in protein composition of the membrane were obvious. Meanwhile, three characterized changes occur. Band 3, Rh, and CD47 are highly expressed but that of CD44, ICAM-4, and $\beta 1$ integrin is markedly decreased [30]. Also, the landmark protein CD71 and XK are relatively unchanged.

During the transformation from reticulocyte to mature erythrocyte, deformability is achieved by reducing cell volume through secretory vesicles. Maturation to an adult erythrocyte is completed when reticular networks of polyribosomes are eliminated, and the biconcave shape of the plasma membrane develops [31].

Interestingly, membrane remodeling of erythrocytes proceeds individually, regardless whether the cells have completed enucleation or not [25]. During maturation, reticulocytes lose cell membrane material, content and volume, Na^+/K^+ -ATPase, and $\text{Na}^+/\text{K}^+ - 2\text{Cl}^-$ cotransport [32]. Hence, cytoskeleton changes and ion channel activity are uninterrupted throughout the process of RBC maturation.

Pathophysiological Changes in Eryptosis

Unlike apoptosis of other nucleated cells, eryptosis is erythrocyte-programmed suicide induced by exposure of phosphatidylserine (PS), which are located under the cell membrane surface. Eryptosis is characterized by membrane contraction, membrane blistering, ceramide for-

mation, PS exposure, and macrophage phagocytosis. The special structural characteristics of RBCs determine the difference between eryptosis and other cells' apoptosis [33]. In eryptosis, the activity of some enzymes that accelerate RBC removal, such as calpain, flippase, and other proteases increases [34]. Flippase and scramblase mainly contribute to the translocation of PS; the former shifts PS from the outer leaflet to the inner leaflet, while the latter plays a reverse role. These actions have a converse effect on PS movement. Aminophospholipid transferase can affect PS translocation from the outside to the inside [35]. Externalized PS, which is an early sign of eryptosis, identifies cells for phagocytosis by macrophages. The exposed PS can then combine with Tim-1/4, stabilin-2, or CD300 located on macrophages [36]. In some cases, CD47 on RBC combine with SIRP α , preventing phagocytosis, and maintaining the RBC homeostasis [37].

The accumulation of ceramide is another significant characteristic of eryptosis. Ceramide can regulate the cytoskeleton and endocytosis, and it is also involved in procedures such as protein transport, subcellular localization, cell cycle, and autophagy [38]. When RBCs adopted into a hyperosmotic environment, the sphingomyelin within the RBC membrane is hydrolyzed, leading to ceramide formation [39]. Erythrocyte shrinkage then activates phospholipase A2, triggering the formation of platelet-activating factors (PAFs) [40]. Abundant amounts of PAFs induce two processes: sphingomyelin breakdown and subsequent ceramide formation [40]. Ceramide can act with Ca^{2+} to synergistically increase the sensitivity of RBCs to changes in internal Ca^{2+} concentration, thus accelerating eryptosis. Meanwhile, ceramide can independently induce eryptosis when RBCs undergo osmotic stress. Sphingosine 1 phosphate, a degradation product of ceramide by ceramidase, could inhibit eryptosis [41]. Ceramide generated in the plasma membrane augments membrane rigidity by stabilizing smaller lipid platforms known as lipid rafts. Therefore, the accumulation of ceramide is an indication of increased membrane fragility [42].

Ion flow also causes the abnormal morphological state of RBC. During oxidative stress, osmotic stress, accumulation of prostaglandin E2, and removal of Cl^- processes, Ca^{2+} flows into the RBC through Ca^{2+} -permeable cation channels. Increased intracellular calcium concentrations and membrane changes activate calcium-sensitive potassium channels, which causes outflow of K^+ along the concentration gradient, causing hyperpolarization of the RBC. The decreased intracellular K^+ causes outflow of chloride ions, resulting in a decreased osmotic pressure

and cell shrinkage [43–45]. Meanwhile, Ca^{2+} activates calpain, triggering RBC membrane blebbing [46, 47]. Plasma membrane Ca^{2+} -ATPase and plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger are responsible for Ca^{2+} outflow, and both require ATP to overcome the enormous chemical gradient [48]. Plasma membrane Ca^{2+} -ATPase activity gradually attenuates with the degree of renal failure [49]. Additional research regarding the mechanism of Ca^{2+} flow may further elucidate the process of CKD.

Moreover, the signal pathways that regulate the state of ion channels and apoptosis also affect the state of Ca^{2+} channels on RBC membranes, thereby regulating eryptosis. Phosphoinositide-dependent kinase 1 (PDK1) involves in PI3 kinase-dependent stimulation of SGK1 and activates transient receptor potential cation channel V5 [50, 51]. Both PDK1 and the transient receptor potential channel 6 contribute to the erythrocyte Ca^{2+} leak and PS exposure in eryptosis [52]. Whether TRPV5 is involved in Ca^{2+} flow during eryptosis remains unclear. However, a study about PDK1 gene deficiency and eryptosis found that the activation of Ca^{2+} channels on the RBC surface was significantly inhibited, and blunted eryptosis was consistent with the reduced Ca^{2+} channels [53]. PDK1 also activates K^+ channels, inducing erythrocyte shrinkage and eryptosis [53]. Annexin A7, as a Ca^{2+} /GTP sensor that regulates vesicle secretion, is an intracellular protein which can bind both Ca^{2+} and phospholipids [54]. The mice model experiment proved that the knockout of annexin A7 elevated K^+ channel sensitivity and PS exposure, thereby accelerating eryptosis [55]. The characteristics of eryptosis and relative measuring methods are shown in the Table 1.

RBCs with exposed PS, which are mainly recognized by macrophages in the limbic system of the spleen and CD207 $^+$ dendritic cells, are phagocytosed and cleared from the circulatory system [56, 57]. After phagocytosis, iron in the digested erythrocytes is recycled for erythropoiesis with the catalysis of hemoxygenase [58]. However, due to an inflammatory state, uremic toxins, and potential hypoxia in CKD, high levels of hepcidin suppress the absorption of iron, contributing to the disturbed iron homeostasis [59, 60].

Factors Influencing Eryptosis in CKD

There are many clinical and physical conditions related to eryptosis including iron deficiency [61], hemolytic anemia [62], antiphospholipid syndrome [63], glucose-6-phosphate dehydrogenase deficiency [64], sickle-

Table 1. The characteristics of eryptosis and corresponding investigation methods [144, 203–211]

Evidences of eryptosis	Detection reagents	Detection methods	Citation
Intracellular Ca^{2+} concentration	Fluo-3/4 fluorescence	Flow cytometry	[203, 204]
Ceramide abundance	Monoclonal antibody-based assay	Flow cytometry	[205]
PS exposure	Annexin V	Flow cytometry	[144, 206–209]
Cell volume		Confocal microscopy	[144]
Lipid peroxidation	Dye BODIPY 581/591 C11	Flow cytometry	[210]
Caspase-3	FAM-DEVD-FMK measurement	Flow cytometry	[211]

cell disease [65], and sepsis [66]. For CKD patients, the main pathophysiological factors affecting eryptosis include oxidative stress, energy depletion, uremic toxins, and vitamin D treatment.

Oxidative Stress and Eryptosis

As the sole oxygen carrier of the body, RBCs are periodically exposed to reactive oxygen species (ROS). In general, oxidative stress refers to oxidative damage from free radicals and redox molecules, which leads to intracellular and/or extracellular damage caused by the imbalance between oxidants and antioxidants [67, 68]. In physiological conditions, a state of dynamic equilibrium between the production and removal of ROS is maintained within cells, called redox homeostasis [69]. When oxidative stress increases beyond the antioxidant capacity, damage gradually accumulates within the erythrocyte, resulting in molecular changes and relevant pathophysiological states [70]. Due to the lack of mitochondria and lysosomes, RBCs cannot carry out reparative processes. To alleviate damage from ROS, RBCs maintain a balance between levels of oxidants and antioxidants through non-enzymatic antioxidant and enzymatic antioxidant systems that include superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxin 2 [71, 72]. Evidence has shown that oxidative stress is negatively associated with the glomerular filtration rate and duration of dialysis [73]. In ESRD patients, glutathione reductase activity is increased [74], while that of glutathione peroxidase is attenuated [75]. During HD, the dialysis membrane worsens oxidative stress, which is further exacerbated due to the loss of antioxidants [20].

The balance between the levels of oxidants and antioxidants first develops during since the early stages of CKD. In ESRD, oxidative stress is exacerbated because of the suppressed antioxidant systems (decreased levels of vitamin C and selenium and disorder of the glutathione

[GSH] scavenging system) and factors that increase oxidant levels (advanced age, diabetes, and uremia) [76]. GSH, an oxyradical scavenger, is the main nonenzymatic antioxidant system within RBCs [77]. Studies have shown that PS exposure was positively correlated with lipid peroxidation, although it was negatively correlated with the total plasma antioxidant capacity and GSH/GSSG ratio in patients with diabetic nephropathy [78]. This highlights oxidative stress and the impaired antioxidant capacity as partly responsible for eryptosis in CKD patients with diabetes. SOD1, the only form of superoxide dismutase present in mature RBCs [79], may attenuate the eryptosis. When the gene of SOD1 was knocked out, the RBCL in mice is shortened [79]. In another study, SOD1 gene knockout resulted in an increase in the number of auto-antibodies and RBCs that combined with IgG as well as immune deposits in the glomerulus [80, 83]. Meanwhile, antioxidant N-acetylcysteine administration significantly alleviated this phenomenon [80, 83]. Under the oxidative stress, eryptosis is inhibited through cGMP-dependent protein kinase type 1, which is stimulated by nitric oxide (NO) [81]. RBCs enzymatically produce NO from RBC-NO synthase. Distinctly high levels of basal enzymatic NO and accumulation of cGMP resist the decline of NOS expression within uremic RBCs, which is a promising method to extend RBCL [82]. The cGKI-deficient mice developed anemia and splenomegaly due to the shortened lifespan of erythrocytes [212].

Oxidative stress causes the impairments in the membrane structure, permeability, and fragility of RBCs in CKD patients. ROS from H_2O_2 and hydroxyl free radicals injure RBC membrane proteins and lipids, resulting in the rearrangement of the erythrocyte membrane skeleton and the reduction of cell membrane stability and deformability [84]. The pivotal of these phenomena laid in the activity of microvesicles (MVs) and band 3 [85]. Generally, oxidative stress initiates eryptosis via Ca^{2+} -depend-

Fig. 1. Hypothesis of the eryptosis process induced by oxidative stress. PS, phosphatidylserine; PAF, platelet-activating factors.

dent or Ca^{2+} -independent pathways. The elevated inward Ca flux mediates the PS externalization and shedding [86]. The cholesterol/phospholipid ratio is essential in maintaining the membrane fluidity. However, PS shedding disrupts this ratio, resulting in decreased membrane fluidity that influences the lifespan of RBCs [86]. In addition to activate Ca^{2+} -permeable cation channels, oxidative stress induces eryptosis via Cl^- channels [87], subsequently leading to erythrocyte membrane shrinkage [88]. PS exposure induced by oxidative stress, independently of Ca^{2+} concentration, is consistent with the accumulation of MV and band 3 in RBC, which further drives the process of eryptosis. MV release is part of the physiological RBC aging process in vivo and represents the structural change of the lipid bilayer and cytoskeleton. The increased MV release from RBCs of HD patients disturbs the ultrastructure related to membrane stability [89]. Lactadherin, a PS inhibitor, limits MV release, thus strengthening micro-vesiculation in eryptosis [90]. Band 3, also named anion exchanger 1, allows the $\text{HCO}_3^-/\text{Cl}^-$ exchange across plasma membrane, thereby contributing to cell morphology and deformability [91]. Compared with the RBCs in healthy participants, band 3 protein content in RBCs of patients with acute renal failure and CKD is markedly decreased [92]. Band 3 may undermine the adhesion between the cytoskeleton and lipid bilayer [92],

thus inducing the vesiculation and affecting RBC properties [89], making the erythrocyte vulnerable to hemolysis [85]. Oxidative damage has been shown to modify the rheological properties of circulating erythrocytes, shortening their lifespan through the monocyte-macrophages system [93, 94]. To prolong their survival, RBCs eliminate the oxidized Hb in the form of vesicles [84, 95].

Oxidative stress also triggers the cysteinyl-aspartate-specific proteinase (caspases). Cysteinyl-aspartate-specific proteinase-3 (caspase-3) activity in erythrocyte is considered as indirect evidence for eryptosis [96]. A published experiment showed that caspase-3 may exert effects on eryptosis by mediating flippase activity [97]. Since blocking the activity of caspases could not reverse the PS exposure, the caspases is not the only way mediating this change [97]. Notably, the Ca^{2+} -dependent membrane scrambling does not depend on the activation of caspases [98]. When triggered by oxidative stress, caspases cleave band 3; thus, band 3 interacting with the protein 4.2 of cytoskeletal network of erythrocyte is decreased, which promotes PS translocation from the lobules to the surface of the RBC membrane and band 3 clustering [99, 100]. Additionally, PS exposure would accelerate band 3 aggregation, and the eryptotic erythrocyte would subsequently be engulfed by macrophages [57, 100]. High caspase-3 erythrocytes may contribute to the shortened life-

span of uremic erythrocytes in CKD [101]. Caffeine contributes to inhibit caspase-3 activation due to its antioxidative capacity [102]. Oxidative stress disturbs erythrocyte membrane structure, increases MV, and activates caspases, all of which aggravate eryptosis that eventually leads to renal anemia. The hypothesis of eryptosis process induced by oxidative stress is shown in Figure 1.

About 10% of HD patients suffer from hypoxia during a dialysis session. This is defined as prolonged intradialytic hypoxemia, a state wherein arterial oxygen saturation falls below 90% [103]. However, the effect of hypoxia on eryptosis is controversial. Hypoxia causes transformation of the oxygenated Hb into deoxygenated Hb. Deoxygenated Hb removes ankyrin from band 3, resulting in increased RBC deformability [104]. However, Hb binding to the ligand is reduced when RBCs is incubated with acute hypoxic stimulation [105]. In a rat model of chronic hypoxia, eryptosis inhibition was attributed to the decreased intracellular Ca^{2+} rather than the PAF/ceramide pathway [106, 107]. CKD patients, especially those suffering from respiratory distress syndrome, usually develop hypoxemia during dialysis, stimulating ROS generation [108, 109]. The formation of redox oligomers of GAPDH and PRDX2, which is induced by over-oxidized sulfinic acid, ultimately leads to a decline in RBC antioxidant capacity, subsequently decreasing RBCL, and worsening eryptosis [110]. If accelerated eryptosis exceeds reactive erythropoiesis, anemia develops [111]. An in vitro study revealed the independent synergistic relationship between uremic toxins and hypoxia on eryptosis [103]. Uremic toxins and hypoxia condition exacerbate eryptosis and disturb the redox balance. Studies have also showed that both hypoxia and uremic toxins shorten RBCL and exacerbate anemia in HD patients [112].

Decreased antioxidant levels and HD treatment can exacerbate oxidative stress in CKD patients [113, 114]. In uremic patients undergoing PD or HD treatment, oxidative stress and eryptosis were found to be enhanced [21]. Renal anemia has been shown to exacerbate oxidative stress especially in HD patients, whereas improvement of anemia may decrease oxidative stress status [113]. While several studies have found that improvement of anemia with ESA use could significantly inhibit the oxidation process [115, 116], another study found that ESA use had no noticeable effect on improving oxidative parameters (including plasma thiobarbituric acid-reactive substances, plasma total thiol, erythrocyte nonprotein thiol, erythrocyte glutathione peroxidase, and erythrocyte catalase) compared with the non-EPO administration group [117].

Recombinant human EPO (rhEPO) therapy could prolong erythrocyte lifespan in dialysis patients by inhibiting Ca^{2+} channels, thus attenuating eryptosis [118]. rhEPO also mitigates oxidative stress, which lessens the risk of cardiovascular events such as atherosclerosis [119]. However, the existing experiment indicated that mice with chronically overexpressed EPO would be more sensitive to eryptosis despite a stronger resistance against osmotic shock [120]. Therefore, rhEPO not only mitigates the oxidative stress-induced eryptosis but also weakens the rhEPO dose-related sensitivity to eryptosis. Notably, as an antioxidant, EPO exerts its effects via the NO synthase pathway in RBCs, by enhancing GSH oxidation, which is hinged on L-arginine availability [121]. This effect was also observed in ESRD patients treated with ascorbic acid [122]. These show the necessity of exploring the actual influence of antioxidant treatments.

At present, several studies have verified that antioxidants can inhibit or blunt eryptosis. These antioxidants include DMTD [123], EPO [124–126], nitroprusside [127], NO [128], vitamin C [129, 130], resveratrol [131], and cinnamaldehyde [132] (the two can have a synergistic effect), hydroxytyrosol [133], salidroside [134], thymol [135], apigenin [136], L-carnitine [137], phlorizin [138], naringin [139], temsirolimus [140], pyrogallol [141], reversine [142], sonidegib [143], chelerythrine [144], xanthohumol [145], teriflunomide [146], and ASP3026 [147]. The substances, their effective concentrations and the possible mechanisms suppressing oxidative stress have been shown in Table 2. Although in vitro experiments have proven that these substances blunt eryptosis due to their antioxidant properties, whether they alleviate eryptosis in a uremic environment still needs further investigations.

Osmotic Stress and Eryptosis

Erythrocytes are frequently exposed to the hyperosmotic environment of the renal medulla. However, contact time between erythrocytes and the renal medulla is too short to induce eryptosis [148]. Furthermore, the widely high Cl^- level prevents the opening of nonselective cation channels, and urea in the medulla disturbs the sphingomyelinase [35]. These conditions protect the erythrocyte from damage when passing through the kidney.

Hyperosmotic stress is another important predisposing factor for eryptosis. First, it may enhance cyclooxygenase and increase production of prostaglandin E2, leading to the activation of calcium-sensitive cation channels [149]. Second, it stimulates phospholipase A2 with or

Table 2. The antioxidative reagents’ effective concentrations and their possible mechanisms relieving eryptosis [128, 129, 132–134, 137, 139, 142–147]

Substance	Concentration	Possible mechanism	Citation
Chelerythrine	10 μM	Inhibiting the PKC- α	[144]
NO	0.1/1 μM	Simulation of cGMP	[128]
Vitamin C	0.11; 0.28 mM	Inhibiting PS exposure and Ca^{2+} -permeable erythrocyte cation channels	[129]
Cinnamaldehyde, resveratrol	CYA (40 mg/mL); CIN (40 mM)	Decreasing the generation of ROS; enhancing the antioxidative activity	[132]
Hydroxytyrosol	1; 5 μM	Restoring ATP and GSH content	[133]
Salidroside	100 μM or 300 μM	Reducing the high cellular level of Ca^{2+}	[134]
L-Carnitine	200 $\mu\text{mol/L}$	Blocking the activation of caspase-3	[137]
Naringin	40 μM	Inhibiting oxidative stress	[139]
Reversine	1–10 μM	Blunting the increase of in cytosolic Ca^{2+}	[142]
Sonidegib	2–6 $\mu\text{g/mL}$	Blunting the cell membrane scrambling	[143]
Xanthohumol	0.5–1 μM	Blunting the effect of energy depletion, hyperosmotic shock, and oxidative stress	[143]
Teriflunomide	5 or 10 $\mu\text{g/mL}$	Blunting the increase in cytosolic Ca^{2+} and cell membrane scrambling	[145]
ASP3026	1–4 $\mu\text{g/mL}$	Blunting the increase in cytosolic Ca^{2+} and cell membrane scrambling	[146]

Fig. 2. Possible mechanisms of eryptosis provoked by main factors. Generally, the microenvironment of CKD, including the enhanced oxidative stress, the energy depletion, hyperosmotic stress, the uremic toxins (phosphate, IS, and acrolein), and so on, disturb erythrocyte membrane structure and accelerate the PS exposure of the erythrocyte through formatting ceramide, elevating intracellular Ca^{2+} concentration, and activating caspase-3 and finally accelerate the eryptosis. (created with BioRender.com). cGK1, cGMP-dependent protein kinase type 1; COX, cyclooxygenase; GC, Gardos channel; IS, indoxyl sulfate; NO, nitric oxide; NSC, nonselective cation channels; PAF, platelet-activating factors; PLA, phospholipase; PGE2, prostaglandin E2; SCR, scrambling enzyme; SM, sphingomyelinase.

without PAF release [149], which accelerates the synthesis of ceramide by activating sphingomyelinase [34]. This process is shown in Figure 2. In a hyperglycemic environment, a series of events related to eryptosis have been observed in RBCs [123], such as the accumulation of advanced glycosylation products, augmented oxidative stress, and subsequent changes in morphology and structure [78]. Experiments have shown that a hyperosmotic environment could enhance the Ca^{2+} -dependent cation channel activity of the RBC membrane [36]. The changes above were also confirmed in patients with diabetes mellitus [150], which seems to partly explain why diabetes patients are more prone to renal anemia. The microenvironments in patients with type 2 diabetes mellitus alters lipid-protein interactions, which induces oxidative stress and enzyme peroxidation that attenuates erythrocyte membrane fluidity [151]. Additionally, the corresponding oxidative stress will reduce the lifespan and properties of erythrocytes [152]. Generally, the oxidative stress in diabetes patients intensifies damage caused by Hb glycation of band 3 protein [153]. Therefore, hyperglycemic environment will destroy band 3 on the surface of erythrocytes, which will not only alter their shape and rheological properties but also reduce the lifespan. In addition, a significantly elevated concentration of methylglyoxal has been observed in patients with hyperglycemia, which results in PS exposure on the surface of erythrocytes [154].

Energy Depletion and Eryptosis

Due to the lack mitochondria, mature RBCs obtain energy through glycolysis and the pentose phosphate pathway. A low ATP supply will activate AMP-activated protein kinase (AMPK) to rapidly regulate the corresponding signal transduction pathways, including fatty-acid oxidation and autophagy [155].

AMPK, an energy-sensing enzyme, is a heterotrimer composed of $\alpha\beta\gamma$ subunits [156], of which, the α and β subunits can alleviate damage caused by oxidative stress to the RBC [157]. Its γ subunit detects the energy state in cells through a competitive combination with AMP and ATP, among which the $\gamma 1$ subunit is a necessary structure to maintain the elasticity of the RBC membrane [158]. The limited capability to synthesize energy makes erythrocytes more sensitive to a deficient energy supply. When the ratio of ATP/AMP declines, the AMPK activation seems to inhibit eryptosis caused by energy depletion [159]. During hypoxia, the ADORA2B-AMPK signal causes an increase in 2,3-BPG, which promotes oxygen transport to attenuate the hypoxia environment, delaying the progress of CKD progression [160].

AMPK is not only involved in the energy supply modulation of RBC but also prolongs their lifespan, probably by attenuating the effects of oxidative stress. AMPK $\alpha 1$ deletion leads to the clearance of premature erythrocytes with abnormal structures and disturbs the oxidant-antioxidant balance. The significantly reduced osmotic fragility of RBCs was observed in AMPK $\alpha 1$ deficient mice, which may have prevented RBCs from passing through the splenic sinus, leading to its premature clearance [161].

In addition to the above functions, AMPK also contributes to the differentiation and maturation of RBCs and maintains their integrity and survival [162, 163]. After energy depletion, activated AMPK inhibits nonselective cation channel, impeding Ca^{2+} influx and preventing changes in erythrocytes. AMPK disturbs the scrambling enzyme, which aids in the translocation of PS that subsequently results in the elimination of the eryptotic erythrocyte. Notably, AMPK does not prevent eryptosis but only blunts it [47]. Unlike AMPK, levels of phosphorylated Janus kinase 3 on the RBC surface increase when there is energy depletion, resulting in the downregulation of Na^+ - K^+ -ATPase activity and reduces ATP consumption to regulate energy balance [164]. The above mechanisms were shown in the Figure 2.

CK1 α is a positive regulator of eryptosis, AMPK alters the expression of CK1 α in erythrocyte progenitor cells. An experiment revealed that decreased CK1 α levels in AMPK $\alpha 1$ -deficient murine erythrocytes would augment Ca^{2+} influx together with membrane scrambling when stimulated by glucose depletion and oxidative stress [165]. Since erythrocytes lack nuclei, it is unknown whether AMPK regulates the CK1 α before erythrocyte maturation or alters the conformation of CK1 α in mature erythrocytes.

Uremic Toxins and Eryptosis

In addition to oxidative stress and energy depletion, the accumulation of multiple toxins in CKD accelerates eryptosis. The uremic toxins, mainly derived from self-metabolism and protein digestion, are responsible for eryptosis in CKD patients. Generally, uremic toxins are divided into water-soluble toxins, protein-bound toxins, and intermediate molecules [166]. The combination with albumin may prevent the clearance of uremic toxins by dialysis [167]. In ESRD patients, eryptosis is largely influenced by the plasma composition. This was revealed when RBCs of healthy individuals in the plasma of ESRD patients significantly increased the percentage of PS externalization [168]. The mean RBCL in healthy and HD patients were 128 ± 28 and 89 ± 28 days, respectively

[169]. At present, various studies have found that some uremic toxins trigger eryptosis, such as indoxyl sulfate (IS) [170], phosphate [171], acrolein [172], vanadate [173], methylglyoxal [154], and indole-3-acetic acid (IAA) [95].

Tryptophan is metabolized by intestinal bacteria into indole, which is oxidized in the liver and combined with sulfate to synthesize IS [174]. IS, a protein-bound uremic toxin, binds so tightly to plasma proteins that their combination cannot be effectively eliminated through the glomeruli. Therefore, IS mainly relies on organic anion transporter 1 (OAT1) and 3 (OAT3) of the renal tubular epithelial cells for their transport from the blood to the renal tubular cells [175]. The transport process from the renal tubular cells to the renal tubule lumen depends on ABCG2 [176]. Clinical trials have shown that IS is associated with cardiovascular events [177, 178], which are probably related to the high level of oxidative stress within endothelial cells [179].

An advanced study found that the transport efficiency of protein-bound uremic toxins (both influx and efflux processes) increased according to the following sequence: HA < IS < pCS < IAA [180]. After HD, IS concentration retains at a high level compared to healthy [112]. IS increases cytosolic Ca^{2+} concentration and stimulates erythrocyte cell membrane scrambling, resulting in PS exposure on the cell surface [170]. Additionally, IS is speculated to increase levels of ceramide, which is a contributing factor that aggravates eryptosis [170]. The present research shows that IS seems to initiate eryptosis through GSH-dependent mechanisms [181]. The transport and metabolism of several uremic toxins, including IS, is carried out by the OAT1 and OAT3 of renal tubular epithelial cells [175, 182]. Evidence has shown that IS and ROS synergistically induce eryptosis [103]. IS also activates p38 MAP kinase [183], an apoptosis-related protein, which further exacerbates eryptosis [184].

Interestingly, both IS and IAA induce PS externalization and release of RBC-derived microparticles (MPs), which enhances the thrombus formation [95]. During apoptosis, PS translocation is accompanied by MPs, which not only expose PS but also express membrane antigens on their surfaces [185]. FXa and prothrombinase complexes bind to RBC-derived MPs and the externalized PS, acting on the coagulation cascade, enhancing thrombin generation [95]. Consequently, the microcirculation in CKD is exacerbated, and the cardiovascular risk increases.

Although phosphate is necessary for cell metabolism and growth, excess amounts are harmful especially when

there is dysfunction of renal phosphate excretion [186]. A dysfunctional kidney cannot effectively excrete discharge phosphates, resulting in its accumulation [187]. P38 MAP kinase, one of the downstream target signals of phospholipase 2, may participate in the process above. The deposited calcium phosphate crystals stimulate the activity of p38 kinase, which further stimulates the production of PAFs [111].

Similar to IS, high levels of vanadate in CKD patients promote the effect of Ca^{2+} on eryptosis. Vanadate inhibits ATP production, which results in an energy-deficient state that induces eryptosis [188]. Vanadate also regulates the activity of glyceraldehyde-3-p dehydrogenase by exchanging the redox state of the enzyme [189]. This inhibits the glycolysis within RBCs, resulting in an energy-deficient state [189]. Acrolein stimulates the suicidal death of erythrocytes by stimulating ceramide formation with subsequent sensitization of the erythrocytes to cytosolic Ca^{2+} concentrations [172]. The possible mechanisms of eryptosis induced by uremic toxins are also shown in Figure 2.

Other Factors Influencing Eryptosis

Aside from maintaining calcium levels and regulating phosphorus metabolism, vitamin D plays a role in promoting RBC proliferation and improving iron utilization [190]. Actually, anemia has been noted in 49% of 25-hydroxyvitamin D-deficient subjects [191]. Clinical studies have consistently confirmed the correlation between 25-hydroxyvitamin D and/or 1,25-dihydroxyvitamin D deficiency and the reduction of Hb levels in early stages of CKD [192]. FGF23, in cooperation with the membrane protein klotho, downregulates the formation of 1 α ,25-dihydroxyvitamin D. Anemia in klotho-deficient mice was attributed to the severely impaired erythropoiesis and enhanced sensitivity to eryptosis [193, 194]. Mice with a high-vitamin D diet also manifested elevated eryptosis when erythrocytes were incubated in an energy-depleted microenvironment, or hyperosmotic shock was induced [195].

Intriguingly, although 1,25-hydroxyvitamin D promoted Ca^{2+} influx in nucleated cells [196], vitamin D treatment did not show any correlation with increasing intracellular Ca^{2+} concentration in RBCs, which subsequently triggers eryptosis [195]. The same conclusion was proved in Klotho deficiency situation, which in some extents speculated the role of relative genomic regulation [193]. Vitamin D does not directly regulate eryptosis, but it participates by influencing signal molecules related to eryptosis, which presumably affects the regulation of ce-

Fig. 3. Overview of eryptosis in CKD. PS, phosphatidylserine; caspase-3, cysteinyl-aspartate-specific proteinase-3; EPO, erythropoietin.

ramide synthesis and activity of protein kinase C [197]. Vitamin D increases RBC sensitivity to stressful environments (such as osmotic shock and energy depletion).

Iron deficiency stands out among the mechanisms that contribute to anemia in the setting of a reduced kidney function [59]. Long-term EPO therapy without iron supplement can aggravate iron depletion. In this condition, enhanced programmed cell death of erythrocytes is observed [198]. The mechanism of iron deficiency-induced eryptosis partly induces the activity of the Ca^{2+} -permeable cation channel. Iron-deficient erythrocytes, with lower level of Hb, have reduced cell volumes, resulting in an enhanced activity of the non-selective cation channels [199]. Subsequent PS exposure scrambles the cell membrane [200]. Meanwhile, cellular KCl loss and cell shrinkage occur due to the activation of Ca^{2+} -sensitive potassium channel (Gardos channels) [45, 201]. In one study, the ROS level in iron-deficient mice was significantly higher than that of the control group [202], which contributed to the clearance of eryptotic erythrocytes.

Conclusion

In CKD, various factors, including energy depletion, high concentrations of uremic toxins environment, and oxidative stress increase the permeability of Ca^{2+} channels, dysregulate the cellular framework, and expose PS, resulting in eryptosis, as it was shown in Figure 3. Once these eryptosis evidences are recognized, the elimination of abnormal RBCs will be accelerated. This review highlights the role of eryptosis in exacerbating the destruction of erythrocytes, worsening renal anemia. From this point of view, targeted drugs that extend the RBCLs are promising treatments for correcting anemia in CKD patients.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Dongxin Li drafted the manuscript, and Xujuan Zheng helped to draft the manuscript. Xujuan Zheng, Yunxia Zhang, Xiangling Li, Xuexun Chen, and Yonghua Yin analyzed the current literature and revised the manuscript. Dongxin Li, Jingwen Hu, and Jialin Li designed the figures and tables. Min Guo and Xiangming Wang conceived the idea, carried out the implementation, and supervised the drafting.

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