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Original Paper

Characterization of Suicidal Erythrocyte Death (Eryptosis) in Dogs

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Key Words
Eryptosis • Dogs • Annexin • Cell volume • Calcium

Abstract
Background/Aims: Suicidal erythrocyte death (eryptosis) is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface following a Ca^{2+} entry in the cell. Eryptosis is stimulated by increased cytosolic Ca^{2+} ($[Ca^{2+}]i$), oxidative stress, energy depletion, or high osmotic shock. Eryptosis signaling includes p38 mitogen-activated protein kinase (MAPK), caspases, casein kinase 1 (CK1), janus kinase 3 (JAK3), and protein kinase C (PKC). Dog and human erythrocytes have different characteristics, for example, dog erythrocytes lack Na^+/K^+- ATPase activity. Whether eryptosis occurs in dog erythrocytes in an analogous way as that in humans remains unclear. Eryptosis in dogs has not been investigated. This study aimed to explore which stimulator and signaling molecules are involved in eryptosis in healthy dog erythrocytes. ***Methods:*** Erythrocytes were isolated from 10 dogs, and eryptosis was stimulated by oxidative stress with tert-butyl hydroperoxide (tBOOH), high osmotic shock with excessive sucrose condition, energy depletion with minus glucose condition, and high $[Ca^{2+}]i$ with ionomycin. Phosphatidylserine exposure was estimated using annexin V binding. Erythrocyte volume and $[Ca^{2+}]i$ were measured by forward scatter and Fluo3-fluorescence, respectively. In addition, the role of certain mediators was assessed using the following inhibitors to determine the detailed mechanisms of eryptosis in dog erythrocytes: p38MAPK, caspase family, CK1, JAK3, and PKC inhibitors. ***Results:*** All eryptosis-inducing factors resulted in phosphatidylserine exposures, except for ionomycin. In addition, the erythrocyte volume increased with ionomycin and tBOOH but decreased with excessive sucrose and minus glucose condition. All treatments increased $[Ca^{2+}]i$. Furthermore, WH1-P154 and chelerythrine significantly blunted the increase of annexin V binding erythro-

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cytes following the tBOOH treatment. ***Conclusion:*** Eryptosis in dogs is triggered by oxidative stress, hyperosmotic shock, and energy depletion. It is suggested that JAK3 and PKC play an important role in eryptosis following an oxidative stress in dog erythrocytes.

Introduction

Suicidal death of mature red blood cells (RBCs) or erythrocytes is commonly known as eryptosis. Analogous with apoptosis of nucleated cells, eryptosis typically leads to cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface [1-7]. Eryptosis is associated with many clinical conditions such as hemolytic anemia, sepsis, chronic renal failure, and coagulation abnormalities [3-10]. These diseases in humans and dogs are clinically comparable; thus, eryptosis is suspected to occur in dogs as it does in humans [8-13]. In most cases, eryptosis is triggered by the activation of Ca^{2+}-permeable channels and subsequent increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) [1-7]. Increased $[Ca^{2+}]i$ is followed by cell shrinkage due to activation of Ca^{2+}-sensitive K^+ channels, cell membrane hyperpolarization, increased electrical driving force for Cl^- exit, and cellular loss of KCl with osmotically obliged water. The activation of scramblases leads phosphatidylserine translocation to the erythrocyte membrane [1-7]. Eryptosis is triggered by oxidative stress, energy depletion, high osmotic shock, and diverse kinases and caspases such as p38 mitogen-activated protein kinase (MAPK), casein kinase 1 (CK1), janus kinase 3 (JAK3), and protein kinase C (PKC) [1-7, 14-18].

Some species differences are found between human and dog erythrocytes. Dog erythrocytes lack a Na^+/K^+-pump in their cell membranes, resulting in the absence of Na^+/K^+-ATPase activity that causes the cation composition in dog erythrocytes to be high in Na and low in K [19, 20]. By contrast, human erythrocytes do possess the Na^+/K^+-pump, with high intracellular K level and low Na [20, 21].

In other species, eryptosis in rats and mice appear to have identical characteristics to those of human eryptosis [3, 22, 23]. Eryptosis in dogs has not been investigated. Whether eryptosis occurs in dog erythrocytes in an analogous way as seen in human erythrocytes remains to be elucidated. To observe eryptosis in dog erythrocytes, herein we triggered eryptosis by oxidative stress using the oxidant tert-butyl-hydroperoxide (tBOOH), hyperosmotic shock with +550 mM sucrose, energy depletion with minus glucose, and increased $[Ca^{2+}]i$, all of which are the main triggers of eryptosis in human erythrocytes with or without respective inhibitors. We hypothesized that eryptosis in dogs parallels that of humans because they suffer clinically similar diseases as those seen in humans. Characteristics of eryptosis may be different especially because of the specie difference in the cellular electrolyte composition.

Materials and Methods

Erythrocytes, solutions, and chemicals

Blood was drawn into heparin-rinsed syringes from 10 healthy Beagles. The care and handling of the animals were in accordance with the Azabu University Animal Experiment Guidelines. All experiments were reviewed and approved by the Ethics Committee of Azabu University (approval number: 191205–1). Blood was centrifuged at 120 g for 20 min at room temperature, and the platelet- and leukocyte-containing supernatants were disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO4, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl2, at 37°C with the oxidant tBOOH (0.3 mM, Sigma Aldrich, Hamburg, Germany) for 25 min, with sucrose (550 mM, Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 60 min, without glucose for 48 h, and with Ca^{2+} ionophore ionomycin (1 μM, AdipoGen Life Science, San Diego, USA) for 60 min. In subsequent experiments, erythrocytes were exposed to 0.3mM tBOOH for 25 min, 550 mM sucrose for 60 min, and Ringer solution without glucose for 48 h, each in the absence or presence of inhibitors that significantly mediate eryptosis: p38 MAPK inhibitor SB203580 (2 μM, Tocris Bioscience, Bristol, UK), cas-

pase family inhibitor Z-VAD-FMK (10 μM, Enzo Life Science, Farmingdale, NY, USA), casein kinase 1 (CK1) inhibitor D-4476 (10 μM, Tocris Bioscience, Bristol, UK), Janus-associated kinase 3 (JAK3) inhibitor WHI-P154 (11.2 μM, Tokyo Chemical Industry, Tokyo, Japan), and protein kinase C (PKC) inhibitor chelerythrine (10 μM, Enzo Life Science).

FACS analysis of annexin V binding and forward scatter

After incubation under the respective experimental conditions, a 150 μL cell suspension was washed with Ringer solution containing 5 mM $CaCl_2$ and then stained with annexin V FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min in the dark. Using flow cytometry, the forward scatter of the cells was determined, and annexin V abundance at the erythrocyte surface was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on EC800 (Sony Biotechnology Inc., San Jose, CA, USA).

Measurement of intracellular Ca^{2+}

After incubation, erythrocytes were washed with Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 5 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca^{2+}-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on EC800 (Sony Biotechnology Inc.).

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was performed using ANOVA with Dunnett as post-test and t-test. n denotes the number of different erythrocyte specimens investigated. Since different erythrocyte specimens used in distinct experiments are differently susceptible to eryptosis triggers, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study aimed to clarify the features of eryptosis in dog erythrocytes. Phosphatidylserine-exposing erythrocytes were identified using detection of annexin V binding by flow cytometry. Other characteristics of eryptosis include decreased cellular volume and increased $[Ca^{2+}]i$, detected using forward scatter with flow cytometry or by Flo3-fluorescence, respectively.

As illustrated in Fig. 1A, exposure to tBOOH containing Ringer solution resulted in a significant increase of annexin V binding erythrocyte percentage, forward scatter (Fig. 1B), and Fluo3 fluorescence (Fig. 1C). After induction of hyperosmotic shock with 1 h exposure of dog erythrocytes to isotonic or 550 mM sucrose added to hypertonic extracellular fluid, a significant increase of the percentage of annexin V binding erythrocytes (Fig. 2A), significant decrease of forward scatter (Fig. 2B), and marked increase of fluo3 fluorescence (Fig. 2C) occurred. Energy depletion with incubation for 48 h in the absence of glucose produced a significant increase of the percentage of annexin V binding erythrocytes (Fig. 3A), significant decrease of forward scatter (Fig. 3B), and marked increase of fluo3 fluorescence (Fig. 3C). After incubation of dog erythrocytes for 60 min in 1 μM ionomycin containing Ringer solution (a Ca^{2+} ionophore increasing Ca^{2+} concentration in erythrocytes), no significant change in the percentage of annexin V binding erythrocytes (Fig. 4A), a marked increase of forward scatter (Fig. 4B), and marked increase of fluo3 fluorescence (Fig. 4C) were found.

Inhibitors of main mediators of eryptosis were used to explore what signals participate in eryptosis in dogs induced by oxidative stress, hyper osmotic shock, and energy depletion. Inhibitors that significantly blunted the percentage of annexin V binding erythrocytes included WH1-P154 and chelerythrine only with the oxidative stress condition using tBOOH (Fig. 5A). No inhibitors significantly blunted the percentage of annexin V binding erythrocytes with osmotic stimulation or energy depletion (Fig. 5B, C).

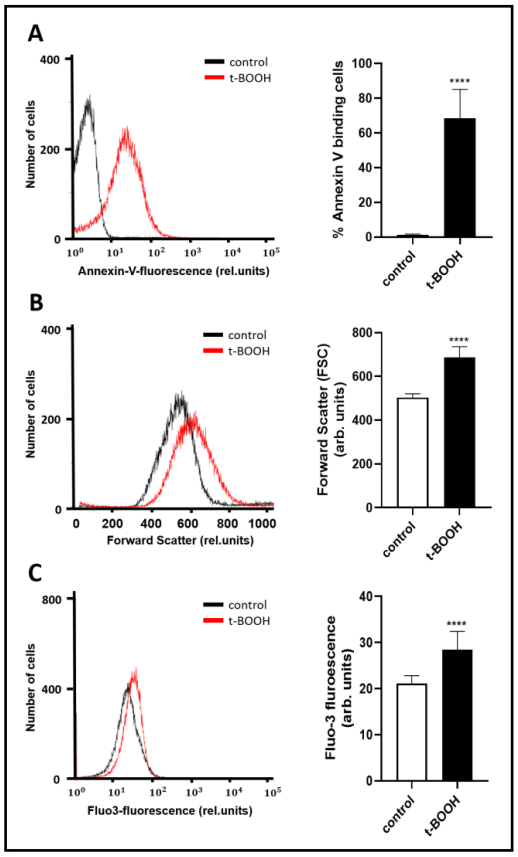

Fig. 1. A. Effect of oxidative stress on the percentage of annexin V binding erythrocytes. Original histogram and arithmetic means ± SEM (n = 10) of annexin V binding of erythrocytes following exposure to Ringer solution without (black line and white bar) and with (red line and black bar) the presence of 0.3mM tBOOH. ****(p<0.001) indicates significant difference from the absence of tBOOH (ANOVA). B. Effect of oxidative stress on erythrocyte forward scatter. Original histogram and arithmetic means ± SEM (n = 10) of forward scatter of erythrocytes following exposure to Ringer solution without (black line and white bar) and with (red line and black bar) presence of 0.3mM tBOOH. ****(p<0.001) indicates significant difference from the absence of tBOOH (ANOVA). C. Effect of oxidative stress on cytosolic Ca^{2+} concentration. Original histogram and arithmetic means ± SEM (n = 10) of Fluo3 fluorescence reflecting cytosolic Ca^{2+} concentration in erythrocytes following exposure to Ringer solution without (black line and white bar) and with (red line and black bar) the presence of 0.3 mM tBOOH. ****(p<0.001) indicates significant difference from the absence of tBOOH (ANOVA).

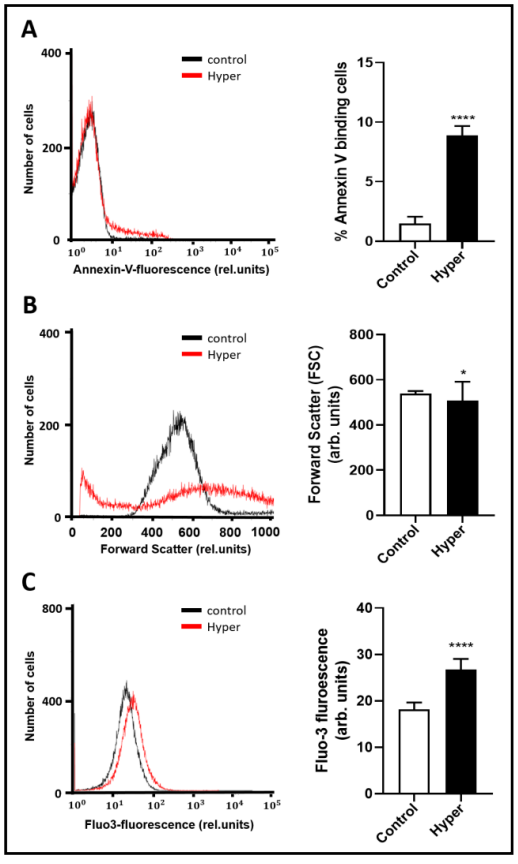

Fig. 2. A. Effect of hyperosmotic shock on the percentage of annexin V binding erythrocytes. Original histogram and arithmetic means ± SEM (n = 10) of annexin V binding of erythrocytes following exposure to isosmotic Ringer solution (black line and white bar) and hyperosmotic Ringer (550 mM sucrose added) (red line and black bar). ****(p<0.001) indicates significant difference from the group in the presence of isotonic Ringer (ANOVA). B. Effect of hyperosmotic shock on erythrocyte forward scatter. Original histogram an arithmetic means ± SEM (n = 10) of forward scatter of erythrocytes following exposure to isosmotic Ringer solution (black line and white bar) and hyperosmotic Ringer (550 mM sucrose added) (red line and black bar). *(p<0.05) indicates significant difference from the presence of isotonic Ringer (ANOVA). C. Effect of hyperosmotic shock on cytosolic Ca^{2+} concentration. Original histogram and arithmetic means ± SEM (n = 10) of Fluo3 fluorescence reflecting cytosolic Ca^{2+} concentration in erythrocytes following exposure to isosmotic Ringer solution (black line and white bar) and hyperosmotic Ringer (550 mM sucrose added) (red line and black bar). ****(p<0.001) indicates significant difference from the absence of isotonic Ringer (ANOVA).

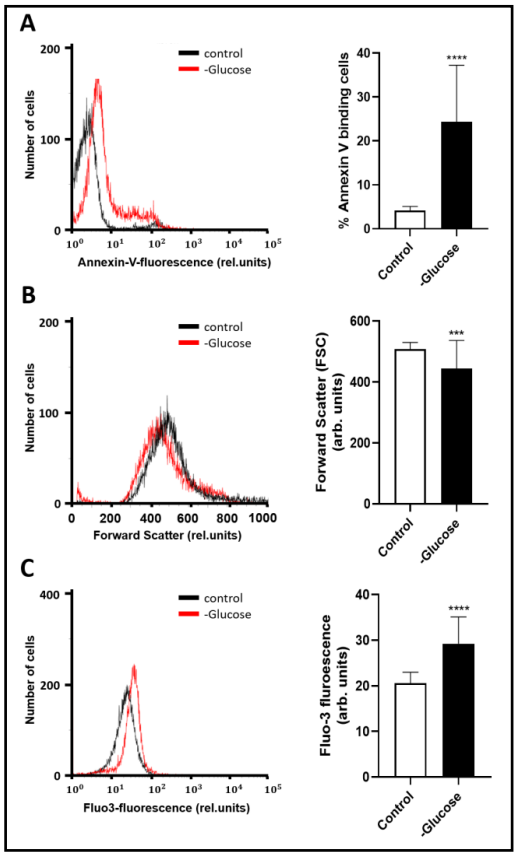

Fig. 3. A. Effect of energy depletion on the percentage of annexin V binding erythrocytes. Original histogram and arithmetic means ± SEM (n = 10) of annexin V binding of erythrocytes following exposure for 48 h to glucose containing glucose Ringer solution (black line and white bar) and Ringer solution without glucose (red line and black bar). ****(p<0.001) indicates significant difference from the presence of glucose (ANOVA). B. Effect of energy depletion on erythrocyte forward scatter. Original histogram and arithmetic means ± SEM (n = 10) of forward scatter of erythrocytes following exposure for 48 h to glucose containing Ringer solution (black line and white bar) and Ringer solution without glucose (red line and black bar). ***(p<0.005) indicates significant difference from the presence of glucose (ANOVA). C. Effect of energy depletion on cytosolic Ca^{2+} concentration. Original histogram and arithmetic means ± SEM (n = 10) of Fluo3 fluorescence reflecting cytosolic Ca^{2+} concentration in erythrocytes following exposure for 48 h to glucose-containing glucose Ringer solution (black line and white bar) or Ringer solution without glucose (red line and black bar). ****(p<0.001) indicates significant difference from the absence of glucose (ANOVA).

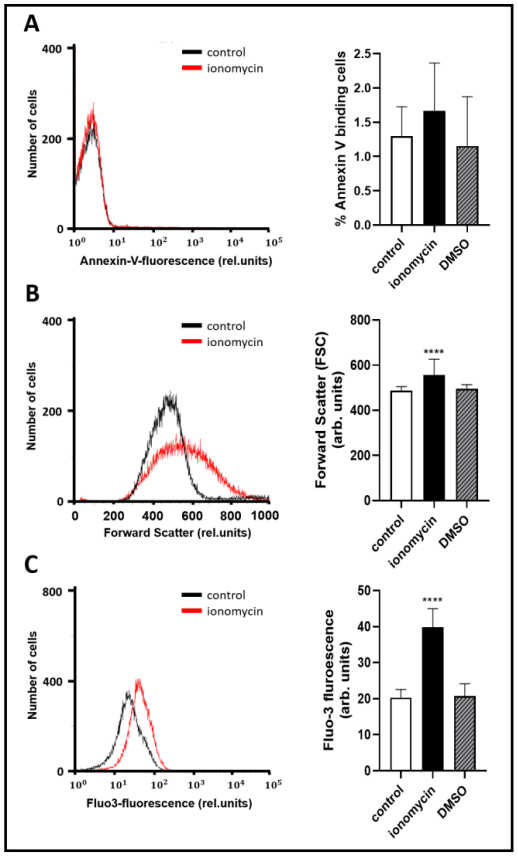

Fig. 4. A. Effect of extracellular calcium overload on the percentage of annexin V binding erythrocytes. Original histogram and arithmetic means ± SEM (n = 10) of annexin V binding of erythrocytes following exposure to Ringer solution without (black line and white bar) or with (red line and black bar) the presence of 1 µM ionomycin or with solvent alone (striped grey bar). B. Effect of extracellular calcium overload on erythrocyte forward scatter. Original histogram and arithmetic means ± SEM (n = 10) of forward scatter of erythrocytes following exposure to Ringer solution without (black line and white bar) and with (red line and black bar) presence of 1 µM ionomycin or with solvent alone (striped grey bar). ****(p<0.001) indicates significant difference from the absence of ionomycin (ANOVA). C. Effect of extracellular calcium overload on cytosolic Ca^{2+} concentration. Original histogram and arithmetic means ± SEM (n = 10) of Fluo3 fluorescence reflecting cytosolic Ca^{2+} concentration in erythrocytes following exposure to Ringer solution without (black line and white bar) and with (red line and black bar) presence of 1 µM ionomycin or with solvent alone (striped grey bar). ****(p<0.001) indicates significant difference from the absence of ionomycin (ANOVA).

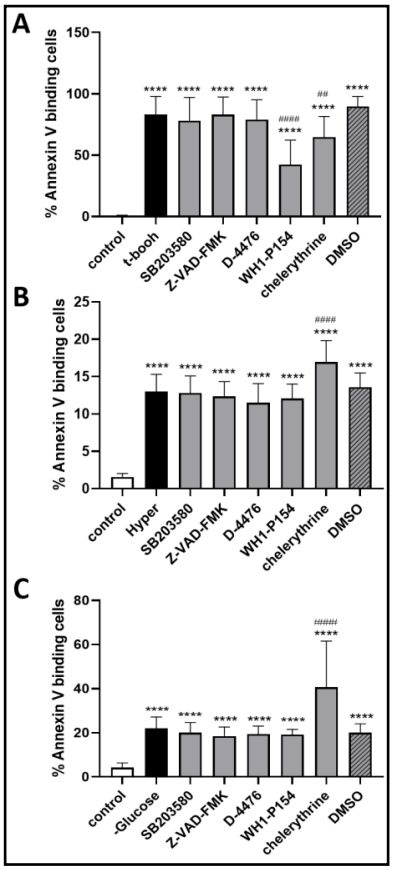

Fig. 5. A. Eryptotic inhibitors sensitivity of phosphatidylserine exposure following oxidative stress. Arithmetic means ± SEM (n = 10) of the percentage of annexin V binding erythrocytes following incubation for 25 min in Ringer solution without (white bar, control) or with (black and gray bars) 0.3 mM tBOOH in the absence (black bar) or presence of inhibitors (gray bar) or of solvent alone (Striped bar). ****(p<0.001) indicates significant difference from the absence of tBOOH, ####(p<0.001), ##(p<0.01) indicates significant difference from the absence of inhibitors. B. Eryptotic inhibitors sensitivity of phosphatidylserine exposure following hyperosmotic shock. Arithmetic means ± SEM (n=10) of the percentage of annexin V binding erythrocytes following incubation for 60 minutes in isosmotic Ringer solution (white bar, control) or hyperosmotic Ringer solution (black and grey bars) in the absence (black bar) and presence of inhibitors (grey bar) or of solvent alone (Striped bar). ****(p<0.001) indicates significant difference from the presence of isotonic Ringer, ####(p<0.001) indicates significant difference from the absence of inhibitors (ANOVA). C. Eryptotic inhibitors sensitivity of phosphatidylserine exposure following energy depletion. Arithmetic means ± SEM (n=10) of the percentage of annexin V binding erythrocytes following incubation for 48-hours in glucose containing Ringer solution (white bar, control) or Ringer solution without glucose (black and grey bars) in the absence (black bar) and presence of inhibitors (grey bar) or of solvent alone (Striped bar). ****(p<0.001) indicates significant difference from the presence of isotonic Ringer, ####(p<0.001) indicates significant difference from the absence of inhibitors (ANOVA).

Discussion

To our knowledge, this is the first report of eryptosis in canine erythrocytes. Eryptosis is characterized by cell shrinkage, cell membrane scrambling with increased phosphatidylserine-exposing erythrocytes by detection of annexin V [1-7]. In this study, treatment of canine erythrocytes with Ringer solution with tBOOH, sucrose and without glucose resulted in increase of annexin V binding erythrocytes, which indicate that eryptosis was stimulated in dog erythrocytes by oxidative stress, hyperosmotic shock, and energy depletion as found in human erythrocytes. Increased phosphatidylserine-exposing erythrocytes likely to adhere vascular wall and may impede microcirculation [1, 4, 5, 24]. Microcirculation blockage may eventually result in disseminated intravascular coagulation (DIC). No sensitive marker for early detection of DIC currently exists for dogs as well as humans; therefore, eryptosis may become a new marker for early DIC diagnosis [25-27].

Interestingly, eryptosis in dogs is not induced solely by an increase of cytosolic Ca^{2+} concentration in dogs. Exposure to ionomycin did not produce a marked increase of phosphatidylserine exposure in dog erythrocytes even though $[Ca^{2+}]$i was significantly increased $[Ca^{2+}]$i in erythrocytes was significantly increased following treatment with all three stimulators.

Ca^{2+} entry stimulates sphingomyelinase to form ceramide. Ca^{2+} and ceramide then activate a scramblase [4]. Phosphatidylserine is usually confined to the inner leaflet of the RBC. Its externalization requires both inhibition of the flippase and activation of the scramblase, and both may follow from elevation of intracellular Ca^{2+} [28, 29]. Our results suggest stimulation of sphingomyelinase, inhibition of flippase, and/or activation of scramblases could be less effective at causing phosphatidylserine externalization in dog erythrocytes. Oxidative stress, hyperosmotic shock, and energy depletion all increase $[Ca^{2+}]$i-like ionomycin treat-

ment, and phosphatidylserine exposure was also found under these conditions. To induce phosphatidylserine translocation of dog erythrocytes, an additional mechanism is suggested to be important in addition to increased $[Ca^{2+}]i$.

Surprisingly, it is likely that eryptotic erythrocytes in dogs will swell in contrast to other species. Eryptosis in humans is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at erythrocyte cell surface [6]. Cell volume was decreased with hyperosmotic shock and energy depletion treatment in dog eryptosis, and shrinkage did not accompany oxidative stress and ionomycin. This phenomenon is different from human eryptosis. Eryptosis in humans is mainly caused by an increase in cytosolic Ca^{2+} ion levels with several stimulations including oxidative stress and osmotic shock [3, 5, 30, 31]. An increase of $[Ca^{2+}]i$ following activation of the cation channels leads to activation of Ca^{2+}-sensitive K^+ channels called Gardos channels, eventually resulting in erythrocyte cell shrinkage due to the loss of water as it osmotically follows the loss of potassium chloride (KCl) from the erythrocytes [32]. Dog erythrocytes have low levels of $[K^+]i$, due to lack of Na^+/K^+-ATPase [33]. We hypothesize that the low level of $[K^+]i$ in dog erythrocytes suppresses the activation of Ca^{2+}-sensitive K^+ channels, which inhibit K^+ efflux and subsequent cell membrane hyperpolarization, resulting in no loss of the cellular KCl with osmotically obliged water. Further investigation is required for cell volume regulation in dogs because canine erythrocytes cation composition is primarily Na instead of K, and Na is suspected to be the main cation to regulate cell volume.

In the present study, JAK3 and PKC were suggested to play an important role in eryptosis triggered by oxidative stress in dog erythrocytes. WH1-P154 and chelerythrine significantly blunted the increase of the percentage of annexin V binding erythrocytes following tBOOH treatment. However, we could not find any marked mediator in eryptosis following hyperosmotic shock and energy depletion. Energy depletion activates JAK3 and PKC as well as CK1 [16-18]. JAK3 is a tyrosine kinase enzyme, and activation of JAK3 ensuing from energy depletion results in scrambling of the erythrocyte cell membrane [17]. PKC is a protein kinase enzyme, and activation of PKC contributes to phosphatidylserine exposure and erythrocyte cell shrinkage after energy depletion [18]. Both JAK3 and PKC are strong stimulators of eryptosis [24]. In dogs, JAK3 and PKC were suggested to be involved in eryptosis following oxidative stress in contrast to humans, in which they contribute to eryptosis following energy depletion. These phenomena represent specie difference of eryptosis between human and dogs. Increased annexin V binding erythrocytes with chelerythrine following hyperosmotic shock and energy depletion may be independent from PKC activity, rather solely depend on chelerythrine induced phosphatidylserine exposure [34].

Furthermore, inhibitors of eryptosis revealed another species difference. In our study P38 MAPK, CK1, and caspases were not involved in eryptosis in dogs. A stress-activated serine/threonine protein kinase, P38 MAPK is expressed in erythrocytes, with activation by hyperosmotic shock participating in machinery triggering eryptosis in humans [14, 15]. CK1 participates in the regulation of a wide variety of cellular functions such as membrane trafficking, cell cycle progression, chromosome segregation, apoptosis, and cellular differentiation [6, 16]. CK1α is one of the isoforms of CK1, a monomeric serine/threonine protein kinase, and it is found within the cytosol as well as the membrane-bound form in human erythrocytes [35]. Caspases and CK1α are activated in human erythrocytes following oxidative stress. Caspases trigger erythrocyte cell membrane scrambling [36, 37], and CK1α is pharmacologically implicated in the increase of Ca^{2+} ions, both leading the consequent stimulation of eryptosis in humans [6]. Further study is required to discover what other molecules, for example those activated in cellular apoptosis, participate in eryptosis in canine eryptosis.

The major limitation of this study is that we have not compared canine and human erythrocytes in the same study, although we have found distinct phenomena in dogs under the same experimental conditions reported in the literature. In addition, we included only beagle dogs as healthy dogs, so further study is warranted to investigate whether the phenomenon found in this study is comparable in other breeds or species.

Conclusion

In conclusion, we found that eryptosis in dog erythrocytes are triggered by oxidative stress, hyperosmotic shock, and energy depletion but not with $[Ca^{2+}]i$ overload. In addition, JAK3 and PKC play important roles in eryptosis following oxidative stress in dog erythrocytes. Further studies are warranted to characterize eryptosis from multiple dog species including patients.

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Author Contributions

Ichiro Katahira and Sakurako Neo: co-first authors who primarily contributed to the conception, design of the work, interpretation of the data drafting the work, and revising it critically for important intellectual content.

Masahiro Naganeand Abdulla Al Mamum Bhuyan: contributed to interpretation of the data and drafting the work.

Saki Miyagi and Masaharu Hisasue: contributed to interpretation the data and revising it critically for important intellectual content.

All authors approved of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Statement of Ethics

The care and handling of the animals were in accordance with the Azabu University Animal Experiment Guidelines. All experiments were reviewed and approved by the Ethics Committee of Azabu University (approval number: 191205–1).

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Disclosure Statement

The authors have no conflicts of interest to declare.

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