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ORIGINAL ARTICLE

Erythropoietin Protects Erythrocytes Against Oxidative Stress-Induced Eryptosis *In Vitro*

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SUMMARY

Background: **Excessive eryptosis has been found in maintained hemodialysis or peritoneal dialysis patients. Signaling of triggering eryptosis includes oxidative stress, increased cytosolic Ca^{2+}-activity, and ceramide. Erythropoietin (EPO) possesses the property of an antioxidant. The aim of this study was to investigate the ability of hydrogen peroxide (H_2O_2) on erythrocytes *in vitro*, and to assess the possible effects of recombinant human erythropoietin (rhEPO) on eryptosis.**

Methods: **One percent erythrocyte suspension was cultured *in vitro* in three kinds of media: Control group (Group C), H_2O_2 group (Group H), and EPO group (Group E). Erythrocytes were sampled at 24 hours and 60 hours. Phosphatidylserine (PS) was estimated with annexin-V, reactive oxygen species (ROS) with 2',7'-dichlorodihydrofuorescein diacetate (DCFDA) and cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) with Fluo3.**

Results: **Eryptosis in Group C increased as the incubating time extended (2.05 ± 0.06 at 24 hours, and 10.00 ± 0.08 at 60 hours). Eryptosis increased in Group H compared with Group C (10.86 ± 0.06 at 24 hours, $p < 0.01$; 12.46 ± 0.14 at 60 hours, $p < 0.01$, respectively), while it decreased in Group E compared with Group H (8.80 ± 0.08 at 24 hours, $p < 0.01$; 11.29 ± 0.04 at 60 hours, $p < 0.01$, respectively). Meanwhile, ROS increased in Group H compared with Group C (9.37 ± 0.04 versus 5.49 ± 0.09 at 24 hours, $p < 0.01$;19.82 ± 0.05 versus 13.51 ± 0.10 at 60 hours, $p < 0.01$). $[Ca^{2+}]_i$ increased in Group H compared with Group C (10.91 ± 0.12 versus 2.53 ± 0.06 at 24 hours, $p < 0.01$;14.55 ± 0.05 versus 4.63 ± 0.08 at 60 hours, $p < 0.01$). ROS decreased in Group E compared with Group H (6.80 ± 0.05 at 24 hours, $p < 0.01$; 16.82 ± 0.06 at 60 hours, $p < 0.01$). $[Ca^{2+}]_i$ decreased in Group E compared with Group H (7.63 ± 0.14 at 24 hours, $p < 0.01$; 10.72 ± 0.07 at 60 hours, $p < 0.01$).**

Conclusions: **Our research showed eryptosis was triggered by H_2O_2 and paralleled by increased ROS and $[Ca^{2+}]_i$ which was partially reversed by EPO. It indicated that EPO could protect erythrocytes against oxidative stress-induced eryptosis.**

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KEY WORDS

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INTRODUCTION

Although many factors are involved in renal anemia, the predominant cause of renal anemia is a relative deficiency in erythropoietin (EPO) production [1]. The other related etiologies include iron deficiency and shortened life span of erythrocytes, etc. To date, exogenous re-

combinant human (rh) EPO has been widely used as a powerful drug for the treatment of patients with renal anemia [2].

During the last decade many new and exciting functions have been attributed to EPO, and many of these are related to non-erythropoietic effects. Several functions of EPO, i.e., inhibition of inflammation [3] and apoptosis [4], anti-oxidant effects [5], and stimulation of angiogenesis [6], may be of potential use in the treatment of pathological conditions.

Oxidative stress as a consequence of increased reactive oxygen species (ROS) and decreased antioxidant defenses is prevalent in many health problems like chronic kidney disease (CKD) [7], and it still exists after transplantation [8]. Oxidative compounds have physiologic defense mechanisms in the body, but imbalance in oxidant generation results in cell damage. Oxidative compounds have negative effects on the life span of erythrocytes.

Eryptosis is a suicidal death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation. Signaling of triggering eryptosis includes oxidative stress, increased cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), and ceramide. We would observe the protective effect of EPO on erythrocytes under the stimulus of hydrogen peroxide (H_2O_2) *in vitro* and explore its mechanism. The aim of the present study was to examine the effect of exogenous EPO supplementation on (i) the expression of phosphatidylserine (PS) on membrane of erythrocyte, and (ii) the changes of ROS and $[Ca^{2+}]_i$ of erythrocytes *in vitro*. Our study may provide therapeutic strategies for renal anemia in clinic.

MATERIALS AND METHODS

Materials

Ringer's solution was purchased from Sigma. H_2O_2 was purchased from Biyuntian, China. EPO injection was purchased from SanSheng Pharmaceutical Industry, Shenyang, China (10,000 U/mL). Annexin V-FITC apoptosis detection kit was purchased from BD, USA. ROS (DCFH-DA) detection kit and calcium fluorescent probe (Flu-3-AM) were purchased from Abcam, Great Britain. A sterile operating platform was produced by ESCO Class II. Sterile twelve-well plates were produced by Greiner, Germany. High-speed centrifuge was made by Anting, Shanghai, China. Cell incubator was MCO-18AIC. The flow cytometry instrument was produced by BD LSRFortessa, USA. Ethylenediaminetetraacetic acid (EDTA) tubes were produced by BD, USA.

Methods

Preparation of healthy erythrocytes *in vitro*

Blood samples used in this study were collected from the Second Hospital of Shandong University. The study was approved by the Ethics Committee of the Second Hospital of Shandong University. Written informed consent was obtained from each participant before blood collection. EDTA anticoagulative tubes were used to collect 8 milliliters peripheral vein blood samples from 6 participants (male 4, female 2) with normal hepatic and renal function. The average age was 37.45 ± 1.78 years old. Fresh blood was centrifuged at 2,000 g for 5 minutes at room temperature, and plasma containing blood platelets and white cells were carefully removed. Erythrocytes were carefully removed into another tube and washed three times with Ringer's solution. Then 2 mL erythrocytes were suspended in 200 mL of Ringer's solution at 1% hematocrit. The suspension liquid was injected into 12 well culture plates and incubated in a 37°C, 5% CO_2 incubator.

Incubation of erythrocytes *in vitro*

After centrifugation and washing at least twice, erythrocytes were incubated under the specified condition. The suspension of erythrocytes was exposed to different media. Erythrocyte samples were randomly divided into 3 groups: Group C (only treated with Ringer's solution), Group H (treated with H_2O_2, the final concentration was 100 μmol/L), Group E (treated with H_2O_2 and EPO, the final concentration was 20 U/mL). Erythrocytes in Group E were pretreated with EPO (20 U/mL).

At 24 hours and 60 hours erythrocytes were sampled and centrifuged, then washed twice by Ringer's solution. All of the erythrocytes were examined within 1 hour.

Annexin V-binding

Annexin V-FITC is able to label the externalized PS on the outer layer of the cell membrane. To determine Annexin V-binding, erythrocyte suspensions were gently mixed with Annexin V-FITC and stored in the dark at 37°C for 15 minutes. After being centrifuged and washed three times in 200 μL Ringer's solution, Annexin V-FITC fluorescence intensity was detected by flow cytometry equipped with an excitation wavelength of 488 nm and an emission wavelength of 530 nm using a FACS Calibur.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihy-drofluorescein diacetate (DCFDA). Erythrocytes were stained with DCFDA in Ringer's solution at 37°C for 30 minutes. The cell suspension was centrifuged and then washed three times in 150 μL Ringer's solution. The DCFDA-loaded erythrocytes were resuspended in 200 μL Ringer's solution. ROS-dependent fluorescence intensity was detected with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Intracellular Ca^{2+}

To quantify intracellular Ca^{2+} concentration, erythrocyte suspensions were mixed in Ringer's solution containing 5 mM $CaCl_2$, stained with Flu-3/AM, and incubated at 37°C for 30 minutes. The suspension was centrifuged and then washed three times in 200 μL Ringer's solu-

Table 1. The results of ERYPTOSIS, ROS, $[Ca^{2+}]_i$ at different times (mean ± SEM, n = 6).

Treatment	ERYPTOSIS		ROS		$[Ca^{2+}]_i$	
	24 hours	60 hours	24 hours	60 hours	24 hours	60 hours
Group C	2.05 ± 0.06	10.00 ± 0.08	5.49 ± 0.09	13.51 ± 0.10	2.53 ± 0.06	4.63 ± 0.08
Group H	10.86 ± 0.06 **	12.46 ± 0.14 **	9.37 ± 0.04 **	19.82 ± 0.05 **	10.91 ± 0.12 **	14.55 ± 0.05 **
Group E	8.80 ± 0.08 ##	11.29 ± 0.04 ##	6.80 ± 0.05 ##	16.82 ± 0.06 ##	7.63 ± 0.14 ##	10.72 ± 0.07 ##

** - $p < 0.01$ indicates significant difference to Group C, ## $p < 0.01$ indicates significant difference to Group H.

Table.2. The correlation analysis of different index at different observation time.

Observation Time	ERYPTOSIS vs. ROS		ERYPTOSIS vs. $[Ca^{2+}]_i$		ROS vs. $[Ca^{2+}]_i$	
	R value	p-value	R value	p-value	R value	p-value
24 hours	0.88	0.000	0.91	0.000	0.93	0.000
60 hours	0.89	0.000	0.87	0.000	0.84	0.000

tion. The Flu-3/AM loaded erythrocytes were resuspended in 200 μL Ringer's solution. Ca^{2+}-dependent fluorescence intensity was detected with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Three parallel contrast wells were equipped at the observation time. Since erythrocytes were differently susceptible to the triggers of eryptosis, erythrocyte specimens from the same participant were used in the three groups. So, the same experiment was repeated six times. The results were averaged at the same observation time.

Statistics

All data were dealt with SPSS 18.0. Results were expressed as arithmetic means ± standard error (mean ± SEM). ANOVA with LSD as the post-test was performed as appropriate to determine statistical significance between two groups. n denotes the number of individuals. For all correlations, Spearman's nonparametric analysis was used. Least significant difference with $p < 0.05$ was considered as the criterion for statistical significance.

RESULTS

The present study aimed to quantify the eryptosis induced by H_2O_2 and to elucidate whether eryptosis was inhibited by EPO. Eryptosis was reflected by the percentage of erythrocytes exposing PS at the surface. As illustrated in Table 1 and Figure 1 (Figure A and Figure B), eryptosis increased with extended incubating time (2.05 ± 0.06 at 24 hours, 10.00 ± 0.08 at 60 hours) in Group C. Eryptosis was significantly higher in Group H than in Group C (10.86 ± 0.06 at 24 hours, $p < 0.01$; 12.46 ± 0.14 at 60 hours, $p < 0.01$). H_2O_2 led to a significant increase in eryptosis. On the contrary, EPO significantly decreased eryptosis. Eryptosis was significantly lower in Group E (8.80 ± 0.08 at 24 hours, $p < 0.01$; 8.80 ± 0.08 at 60 hours, $p < 0.01$) than in Group H. Eryptosis is further induced by oxidative stress, which will be enhanced in ESRD. Therefore, oxidative stress in erythrocytes was determined utilizing 2', 7'-DCFDA. As illustrated in Table 1 and Figure 1 (Figure C and Figure D), DCFDA fluorescence was significantly higher in Group H than in Group C (8.80 ± 0.08 vs. 5.49 ± 0.09 at 24 hours, $p < 0.01$; 19.82 ± 0.05 vs. 13.51 ± 0.10 at 60 hours, $p < 0.01$). Significant difference was observed between Group E and Group H (6.80 ± 0.05 at 24 hours, $p < 0.01$; 16.82 ± 0.06 at 60 hours, $p < 0.01$). A major trigger of eryptosis is an increase of cytosolic Ca^{2+} activity. Fluo3 fluorescence was employed to estimated $[Ca^{2+}]_i$. As illustrated in Figure 1 (Figure E and Figure F), Fluo3 fluorescence was significantly higher in Group H than in Group C (0.91 ± 0.12 vs. 2.53 ± 0.06 at 24 hours, $p < 0.01$; 14.55 ± 0.05 vs. 4.63 ± 0.08 at 60 hours, $p < 0.01$). Significant difference was observed between Group E and Group H (7.63 ± 0.14 at 24 hours, $p < 0.01$; 10.72 ± 0.07 at 60 hours, $p < 0.01$).

Our results showed that eryptosis was positively correlated with ROS ($r = 0.88$ at 24 hours, $p < 0.01$; $r = 0.89$ at 60 hours, $p < 0.01$), as well as $[Ca^{2+}]_i$ ($r = 0.91$ at 24 hours, $p < 0.01$; $r = 0.87$ at 60 hours, $p < 0.01$). $[Ca^{2+}]_i$ was positively correlated with ROS ($r = 0.93$ at 24 hours, $p < 0.01$; $r = 0.84$ at 60 hours, $p < 0.01$). The data were shown in Table 2.

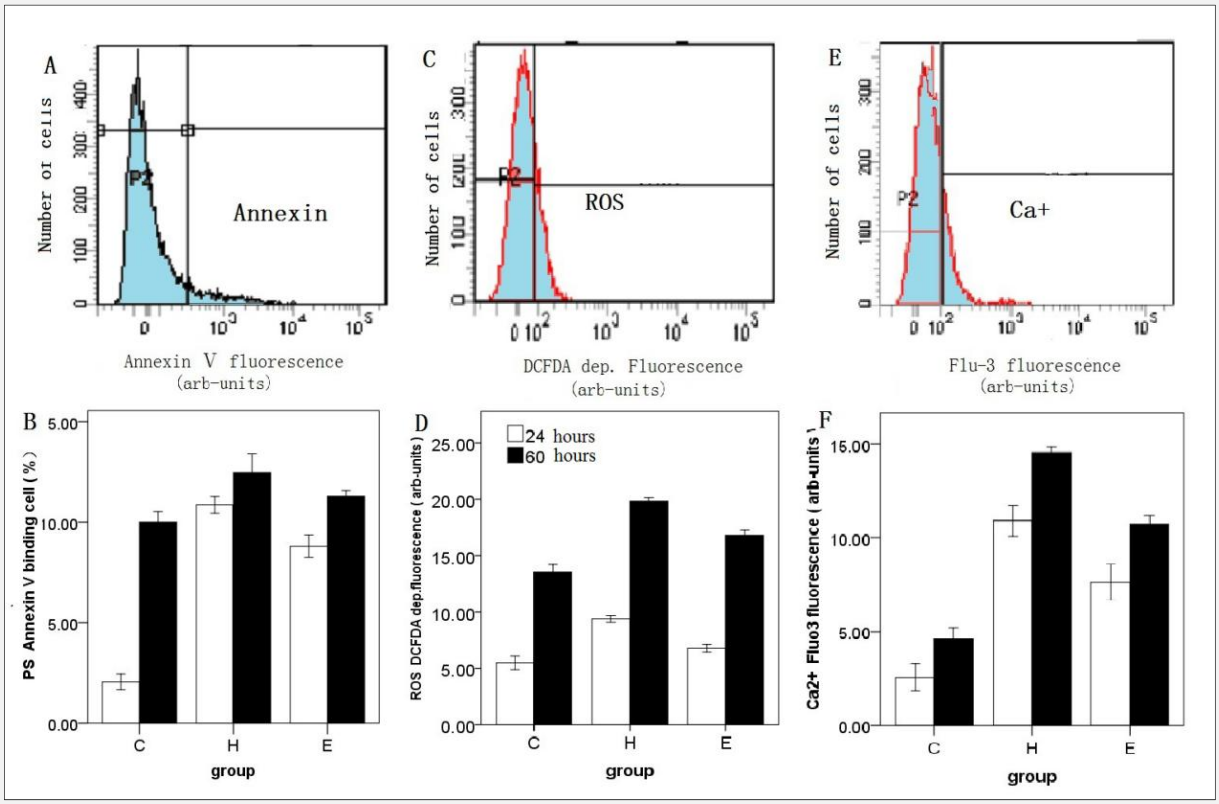

Figure 1. PS, ROS, $[Ca^{2+}]_i$ in erythrocytes from fresh blood of participants.

Figure A, C, E. PS-exposure (Figure A), DCFDA-fluorescence (Figure B) and Fluo3 fluorescence (Figure C) were diagrams of flow cytometry. Figure B, D, F. MEAN ± SEM (n = 6) Representative histograms of PS-exposure (Figure A), DCFDA-fluorescence (Figure B) and Fluo3 fluorescence (Figure C). White bars express the mean value of 24 hours. Black bars express the mean value of 60 hours.

DISCUSSION

In the present study, H_2O_2 and EPO were used to stimulate healthy erythrocytes. By detecting the exposure of PS on erythrocyte, ROS and $[Ca^{2+}]_i$ in different media, we observed that EPO could reverse PS exposure by decreasing ROS and $[Ca^{2+}]_i$.

EPO can reliably cure the anemia of chronic renal failure and may be effective in the treatment of anemias secondary to chronic inflammation [9], malignancy [10], and marrow suppression [11]. Further investigation regarding the mechanisms of EPO to cellular antioxidative responses would provide a better insight to the cytoprotective action of EPO.

Eryptosis is stimulated in a wide variety of diseases such as malignancy [12], hemolytic uremic syndrome [13], sepsis [14], heart failure [15], etc. Oxidative stress increases with the progressive decline in renal function, and remains elevated on renal replacement therapy. In our study, the effect of oxidative stress is mimicked by H_2O_2, which is a common inducer in experimental models. Our data indicated that H_2O_2 at 100 μmol/L strongly increased the exposure of PS in erythrocytes. The result was consistent with Shan et al's research [16]. Our results indicated that the ability of H_2O_2 to increase the exposure of PS in erythrocytes is associated with ROS and $[Ca^{2+}]_i$.

Meanwhile, eryptosis is a reversible process [17]. Our study showed that the exposure of PS was significantly lower in Group E which suggested that eryptosis was reversed by the addition of EPO. Then EPO extends the life span of circulating erythrocytes. In theory, anemia due to excessive eryptosis could be alleviated by treatment with inhibitor. The extroversion of PS on erythrocyte membranes could contribute to blood coagulation and thrombogenesis [18]. EPO obviously block the formation of ROS in the process. These results indicated that EPO could be an efficient oxygen free radical scavenger in the treatment of anemia.

Ca^{2+}-permeable cation channels is one signaling path-

way to trigger eryptosis [19]. Cytosolic Ca^{2+} activity is increased by oxidative stress and lead to membrane scrambling with subsequent PS exposure. Eryptosis is closely related with calcium ion concentration of erythrocytes. Our data showed the exposure of PS was paralleled by a significant increase of $[Ca^{2+}]_i$. At different observation times, the exposure of PS has positive correlation with $[Ca^{2+}]_i$. These results confirmed EPO counteracts eryptosis in part by inhibiting the Ca^{2+}-permeable cation channels.

CONCLUSION

Our research showed eryptosis is triggered by H_2O_2 and paralleled by increased ROS and $[Ca^{2+}]_i$ which was partially reversed by EPO. It indicated that EPO could protect erythrocytes against oxidative stress-induced eryptosis. Further research in this field would contribute to new therapeutic strategies and might improve the outcome of anemia.

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Declaration of Interest:
We have read and understood Clinical Laboratory's policy on disclosing conflicts of interest, and we declare that we have none.

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