Letter to the Editor

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# Eryptosis is induced by hyperthermia in hereditary spherocytosis red blood cells

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To the Editor,

In pediatric hereditary spherocytosis (HS) patients, anemia frequently worsens throughout the course of infectious diseases, due either to increased hemolysis or to transitory erythropoietic aplasia [1]. Hemolysis may be so important that even asymptomatic individuals can incidentally be diagnosed with HS during a hemolytic crisis secondary to febrile disease [2]. It has been reported that HS children, especially those under 6 years old, frequently suffer hemolytic crises triggered by viral infections [2–4]. These reports, along with usual clinical practice, suggest a potential harmful effect of temperature on erythrocytes which may affect their survival.

Increasing evidence is now available to demonstrate that erythrocytes can also undergo premature self-destruction through a mechanism known as eryptosis, which shares several features with nucleated-cell apoptosis. Since this process may be accelerated by different environmental factors, we decided to study whether hyperthermia “per se” has a direct effect on HS erythrocytes.

The study protocol was approved by Committees of Ethics and Research, according to Helsinki international ethical standards on human experimentation. Informed consent was obtained from patients, parents (in case of children) or healthy blood donors before entering the protocol.

Red cells from HS patients and healthy donors were incubated at two different temperatures, mimicking either the physiological body temperature (36.5 °C) or a febrile state (38.5 °C). Fresh erythrocytes were incubated for 24 h at 1% hematocrit in HEPES buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2.5 mM $CaCl_2$, 10 mM glucose and 0.1% (w/v) bovine serum albumin, pH 7.4). Cell shrinkage, the main morphological characteristic of premature cell death, was analyzed by flow cytometry (FACSort, Becton Dickinson) through the forward scatter (FSC) parameter which correlates with cell volume. No changes in cell shrinkage were observed after exposing control erythrocytes to the higher temperature (Figure 1A). Otherwise, significantly increased cell shrinkage was observed after incubation of HS erythrocytes at 38.5 °C compared to incubation at 36.5 °C (Figure 1B; *$p<0.05$ $HS_{36.5\,°C}$ vs. $C_{36.5\,°C}$; **$p<0.01$ $HS_{38.5\,°C}$ vs. $HS_{36.5\,°C}$ and $HS_{38.5\,°C}$ vs. $C_{38.5\,°C}$; n=9). Results are presented throughout the whole work as mean±standard error (mean±SEM). Comparison between groups was carried out either with the Kruskal-Wallis test followed by the Mann-Whitney U-test, or with the Wilcoxon paired test when corresponding, while statistical significance was defined as $p<0.05$.

To investigate if the morphological changes in HS erythrocytes were related to premature cell death, we

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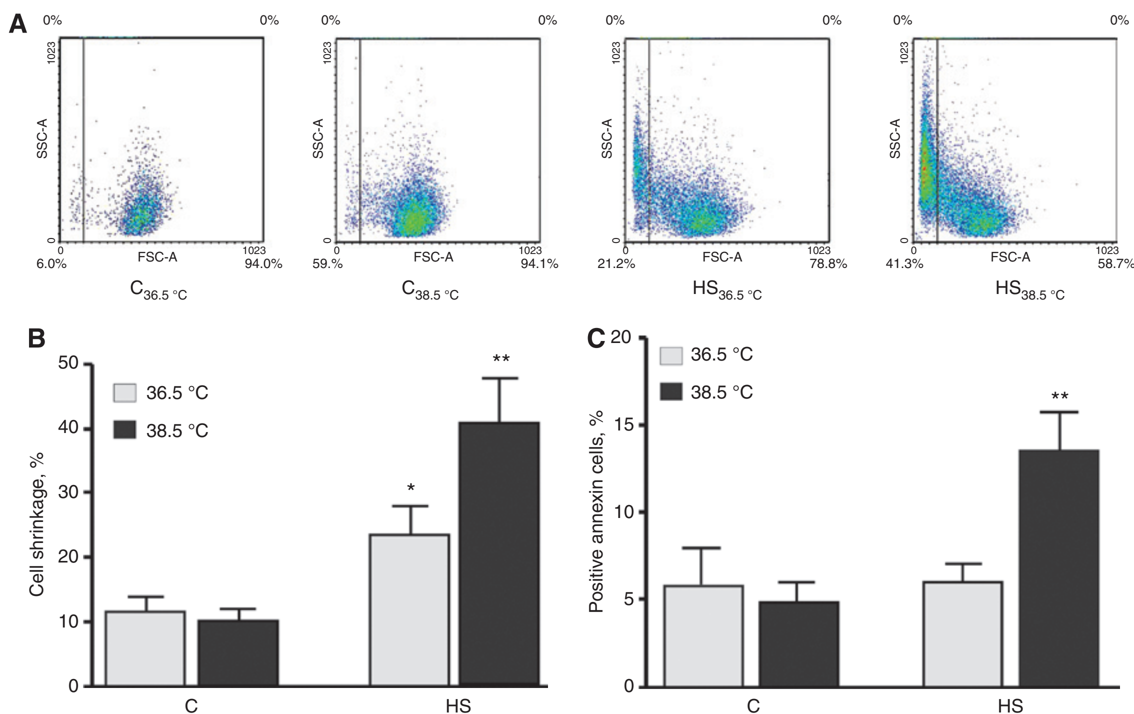


**Figure 1:** Effect of temperature on the development of eryptosis.
Control and HS erythrocytes were incubated for 24 h at either 36.5 °C or 38.5 °C and eryptosis was then analyzed by flow cytometry. (A) A representative experiment showing the forward scatter (FSC) parameter used to evaluate cellular size. (B) Percentage of cell shrinkage during temperature exposure. (C) Annexin V-FITC binding to PS exposed in the outer cell membrane determined by flow cytometry.

further analyzed phosphatidylserine (PS) translocation, which is a specific sign of eryptosis. PS exposure was detected by measuring binding of the phospholipid to annexin V by flow cytometry (15 min in the dark using Annexin V-FITC apoptosis detection Kit II, BD Biosciences) [5]. In agreement with the abovementioned results, HS erythrocytes but not control cells showed significantly increased PS externalization at the higher temperature used for cell incubation (Figure 1C; *$p<0.01$ $HS_{38.5\,°C}$ vs. $HS_{36.5\,°C}$ and $HS_{38.5\,°C}$ vs. $C_{38.5\,°C}$; n=9).

PS translocation of HS red cells incubated for 24 h at 36.5 °C did not differ from that of the control cells. In a previous report, Jong et al. showed that PS exposure could not be demonstrated in either normal or HS red cells in assays without temperature increment [6]. Moreover, Boas et al. suggested that exposed PS might not be the cause of reduced red cell life span in patients with hemolytic anemia, with the possible exceptions of those with unstable hemoglobins or sickle cell anemia [7]. Interestingly, our results showed that in vitro incubation of HS red blood cells at 38.5 °C significantly increased eryptosis, characterized by cell shrinkage and PS externalization, in comparison with control cells. These results show that red blood cells from HS patients are more sensitive to high temperature than those from normal individuals and suggest that hyperthermia produces direct cellular damage. Föller et al. reported that normal erythrocytes showed significantly decreased FSC values when exposed to high temperatures [8], but in disagreement with their results, we did not find hyperthermia-induced eryptosis in red blood cells from healthy donors. This discrepancy could be ascribed to the different experimental conditions employed in both trials, given that they only exposed normal erythrocytes which showed changes at higher temperature values than those used in our study. Other authors demonstrated that release of ATP from erythrocytes is sensitive to physiological increases in temperature, whereas red blood cell count, mean cell volume, mean cell hemoglobin and mean corpuscular hemoglobin concentration did not change, further suggesting that the rise in temperature did not affect the volume and integrity of human red blood cells from healthy individuals [9].

Taking into account that high levels of oxidative compounds which appear during the process of oxidative stress may induce eryptosis, we evaluated the presence of reactive oxygen species and glutathione content by flow cytometry (probes: DCFH-DA 15 min at 37 °C and Mercury Orange 3 min at 0 °C, respectively) in erythrocytes previously incubated at both experimental temperatures. However, no significant differences were observed either

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within or between experimental groups. An increased influx of calcium, which is among the mechanisms that can induce cell shrinkage and subsequent PS translocation [10], was also evaluated by flow cytometry (Fluo-4 AM 30 min). After incubation at 38.5 °C, the intracellular calcium content was significantly increased in erythrocytes from HS patients compared to normal controls and even to HS erythrocytes incubated at the lower temperature (Figure 2A; *$p<0.05$ with respect to $C_{38.5\,°C}$ and $HS_{36.5\,°C}$; n=6). The results suggest that hyperthermia might be more likely associated to calcium influx than to oxidative stress. Then, erythrocytes from HS patients were analyzed in media without calcium. Remarkably, calcium deprivation decreased PS exposure and the percentage of erythrocyte shrinkage from HS patients, suggesting that programmed erythrocyte death induced by hyperthermia is an active process requiring $Ca^{2+}$ entry into the cells (Figure 2B; each bar indicates percentage of variation between results observed at 38.5 °C and 36.5 °C. Significant differences between assays performed in medium with and without calcium *$p<0.05$; n=6). We did not observe changes in calcium levels of red blood cells from healthy donors due to the incubation temperature. Instead, Föller et al. reported that hyperthermia stimulates calcium increase in normal erythrocytes, thus inducing eryptosis [8]. These apparently conflictive results could be explained on the basis that Föller et al. observed increased intracellular calcium only at incubation temperatures above 39 °C.

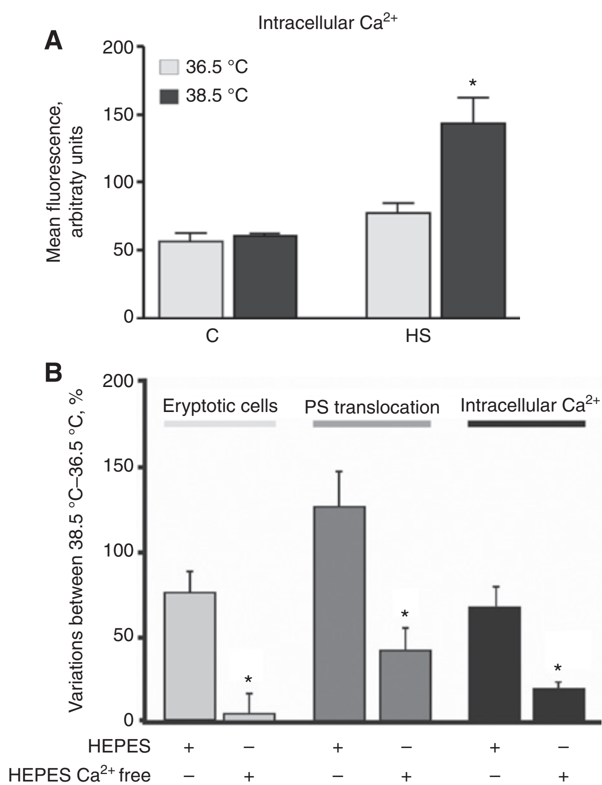


**Figure 2:** Role of calcium in the effect of temperature on HS erythrocytes.
(A) Intracellular calcium content was measured by flow cytometry in erytrocytes loaded with 2.5 μM Fluo-4 AM. (B) Cell shrinkage (eryptotic cells), PS translocation and intracellular calcium content were determined by flow cytometry in HEPES buffer with or without $Ca^{2+}$ (HEPES buffer $Ca^{2+}$ free).

We propose that at least one mechanism involved in the hemolytic crisis observed in HS patients may be related to an altered cell calcium influx. To our knowledge, this is the first report showing the relationship between PS externalization and intracellular calcium levels in HS erythrocytes exposed to high temperature. Interestingly, it has been demonstrated that HS animal models show high sensitivity to the activation of the Gardos channel mediated by calcium [11]. This stimulation of the Gardos channel induces loss of $K^+$ and water which may, consequently, lead to cell shrinkage. Besides, other mechanisms may account for PS translocation induced by cell calcium overload. It is known that, under physiological conditions, calpain is activated by $Ca^{2+}$ in human erythrocytes. Previously, in a model of eryptosis induced by cell calcium loading we found that protein phosphorylation and dephosphorylation are related to PS externalization. Particularly, the finding of a highly activated protein tyrosine phosphatase 1B (PTP1B) suggested the involvement of the calcium-dependent protease calpain in the mechanism of eryptosis [10].

It has to be noted that PS exposure on the cell surface may signal the sequestration of circulating red cells by macrophages. It is expected that temperature-induced eryptosis causes a disturbance of the fine equilibrium between erythrocyte production and destruction in vivo. Erythrocytes may then turn into abnormal cells, more prone to be eliminated by the reticuloendothelial system. This would lead to premature erythrocyte destruction, thus reducing red blood cell survival and, consequently, causing anemia.

In conclusion, based on the present results we propose that hyperthermia during febrile processes may have a direct effect upon HS erythrocytes, inducing eryptosis mediated by calcium influx, thus contributing to the worsening of the preexisting anemia in HS patients or otherwise triggering an acute hemolytic crisis in still non-diagnosed individuals.

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