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Different expression of acetylcholinesterase, caspase 3 and CD38 proteins and Amyloid β peptide in erythrocytes of β thalassemia major patients

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Abstract

Beta thalassemia (β thalassemia) major is a hereditary disease that makes blood transfusion dependency. These patients suffering from some physiological abnormalities like iron overload, oxidative stress and aggregated β-globin proteins that could also trigger early death in transfused red blood cells (RBCs). This study aimed to evaluated the possible changes which could facilitate the eryptosis and death process in RBCs related to β thalassemia major patients. For this purpose, RBCs were isolated from patients just before blood transfusion and healthy controls and the expression of some membrane bound proteins such as acetylcholinesterase (AChE), cyclooxygenase-2 (COX-2), CD38 and caspase 3 (Casp3) and also amyloid β (Aβ) were evaluated by using specific ELISA kits or immunoblotting methods. Obtained results showed COX-2 and CD38 contents increased significantly in RBCs originated form β thalassemia patients that could cause Ca^{2+} upregulation and inflammatory stress. According to the results, iron overload also induces AChE upregulation which participate in reactive oxygen species (ROS) production and cause oxidative stress that is hallmark of β thalassemia major. Oxidative stress could be caused by significantly increased Aβ peptides and its aggregated form. Overproduction of Aβ also induce cell death in RBCs is named eryptosis. Our results manifested that pathophysiological conditions in β thalassemia major trigger eryptosis machinery and reduce lifespan of transfused RBCs so decrease blood transfusion efficiency. According to our results and literature, blocking the oxidative stress, inhibition of the AChE catalytic and non-catalytic functions, quenching of the COX-2 and antioxidant agents could help to increase the RBCs lifespan and functionality, improve life quality of patients and also increase time interval between blood transfusion.

Keywords: Amyloid β; Iron toxicity; Oxidative stress; Ca^{2+} influx; Acetylcholinesterase; Eryptosis

1. Introduction

Beta thalassemia (β thalassemia) are a group of inherited blood disorders caused by reduced or absent synthesis of the beta chains of hemoglobin that result in variable outcomes ranging from severe anemia to clinically asymptomatic

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individuals [1]. This pathologic condition is caused by mutations in the HBB gene on chromosome 11, inherited in an autosomal recessive fashion[1] . Therefore, homozygosity or compound heterozygosity for β thalassemia mutations cause a more severe spectrum of anemias called β thalassemia major or Cooley's anemia that is life-threatening disease and patients with this condition require lifelong blood transfusion[1] . Children who born with Cooley's anemia, in addition to transfusion dependency, suffering from different symptoms including pale skin, enlargement of the spleen, fussy, slow growth, skeletal deformities, enlargement of bone marrow, yellowish skin and having a poor appetite and different infections[2]. According to the previous reports, the main problem in major β thalassemia is imbalance biosynthetic ratio of α-globin versus β + γ-globin that causes misfolded aggregates of globin proteins inside the red blood cells (RBCs)[3]. Precipitation of aggregated proteins leads to hemolysis, premature destruction of RBC precursor in the bone marrow and short lifespan of mature RBCs in the circulation [3]. Thus, blood transfusion to receive fresh and healthy RBC seems a reasonable treatment method. Since every 1 ml of transfused RBC deposits 1.08 mg of iron in tissues at the end of the cells' lifespan, this treatment causes iron accumulation in the body (iron overload) that catalyzes the generation of reactive oxygen species (ROS) and ferroptosis process, causing damage to vital organs such as the heart, liver and the endocrine system and also causes short lifespan of healthy RBCs [4, 5]. RBCs lifespan was estimated to be about 120 days in healthy people and cells age of standard blood for transfusion was assessed to be less than 60 days, with a perfect residual mean survival of 60 days [4].While in practice, transfused red cells generally survive much less than 60 days possibly due to early cell

death induced by ROS, iron toxicity and other unknown events.

RBCs were considered unable to undergo apoptosis previously due to lack of mitochondria and nuclei that are essential organelles in apoptosis process [6]. As shown recently, some events like Ca^{2+} upregulation, oxidative stress and exposure to xenobiotics triggers phosphatidylserine exposure, membrane blebbing, protease activation and shrinkage of RBCs, the typical properties of apoptosis in nucleated cells[7, 8]. This process named eryptosis that remove RBCs from circulation before its normal time of senescence[7]. It seems eryptosis is one of the main events that reduce RBCs lifespan in all types of thalassemia specially in major β thalassemia[8]. The molecular mechanism of eryptosis in human RBCs is not fully understood, but it's obvious that membrane bond proteins could regulate it in organelle-free cells. Previous studies reported dysregulation of the membrane bond enzymes including acetylcholinesterase (AChE), cyclooxygenase (COX)-2, cluster of differentiation 38 (CD38) and caspase 3 (Casp3) in RBCs of patients suffering from different types of anemia[8-11] that reinforce the possibility of their involvement in eryptosis process.

Acetylcholinesterase (AChE) is a glycosylphosphatidylinositol (GPI)-linked enzyme that could be considered as a marker for membrane integrity and play role in erythrocyte aggregation and other ageing-associated remodeling [12]. Enzyme-substrate complex of AChE has important role in nitric oxide (NO) signaling pathway in RBC through inhibition of the adenylyl cyclase enzyme [9]. Cox-2 is the inducible form of cyclooxygenase which is located in erythrocytes membrane and catalyzes the production of prostaglandins (PGs) by using arachidonic acid as substrate [10, 13] .According to previous reports, PGs specially PGE2 have crucial role in eryptosis process

through Ca^{2+} upregulation [14]. CD38 is a multifunctional membrane protein participate in Ca^{2+} influx and oxidative stress in RBC through synthesis of the cyclic ADP-ribose (cADPR) as secondary messenger [11, 15] . Therefore, these proteins and also Casp3 [8] play major role in eryptosis process. This study aimed to compare the mentioned proteins content in RBCs extracted from β thalassemia major patients and healthy controls to clarify the possible molecular events that reduce RBC's lifespan in β thalassemia major. We also studied amyloid β (Aβ) peptide that is hypothesized to play a vital role by oxidatively affecting the capacity of RBCs to transfer oxygen[16, 17]. This kind of researches could help to understand some of the ambiguities in eryptosis and also help to increase blood transfusion efficiency in transfusion dependent patients.

2. Material and methods

2.1. Experimental design and participant

This study aimed to evaluate the contents of AChE, CD38, COX-2, Casp3 and Aβ in erythrocytes isolated from blood samples of β thalassemia major patients and healthy people don't have thalassemia as control that were referred to the Center of the Genetic Blood Diseases in Thi-Qar city (Iraq) between 4 March to 27 June 2022. For this purpose, 30 patients with β thalassemia major disease and 30 healthy controls were included in the study. Informed and written consent is obtained from all of the patients and healthy samples. β thalassemia major was approved in patient by using genetic and biochemical test, all of them suffering from severe anemia and are transfusion dependent (regularly received blood). The blood samples were prepared before new blood transfusion. Patients with liver deficiency, kidney disorder, thyroid disorder, acute coronary syndromes, Alzheimer disease, different cancers, and people with family history of dementia were excluded from the

study. Control group was selected among age- and sex-matched healthy peoples that had not been diagnosed with different kinds of anemia and don't suffer from heart disease, thyroid disorder and Alzheimer disease or other neurological disorders. In this study, 5 ml of blood samples were taken from the cases and centrifuged at 10000 rpm for 5 min to separate RBCs from blood serum, serum samples stored at -20°C temperature for biochemical analysis. This study was approved by the health and ethics committee of the health center, and all the participants gave their informed consent in accordance with the Declaration of Helsinki. Relevant sociodemographic, clinical and laboratory data were obtained from the medical records of the patients including: age, gender, ferritin and time interval between transfusions. The range of age in β thalassemia major patients and control was estimated between 4 to 32 years. Ferritin content in patient group was evaluated to be 3820.11±587.27 ng/ml that is significantly higher than control who have 87.65±9.78 ng/ml ferritin in blood serum ($P<0.0001$). Pre-transfusion hemoglobin level was assessed between 9 to 10.5 g/dL that was reach to less around 15 g/dL after transfusion. Interval time between transfusions is different and ranged between 5 days to 2 weeks in patient group.

2.2. Erythrocyte preparation

Fresh venous blood samples were obtained from β thalassemia major patients and control cases by using venipuncture and heparin was used as anticoagulant. Erythrocytes were purified from blood samples were treated via anticoagulant by centrifugation for 10 min at 1300 rpm and 4°C. The erythrocyte pellet was suspended in a filtrated phosphate buffered saline (PBS) containing 5 mM glucose. Separated RBCs were washed twice by repeat centrifugation and resuspension in glucose containing PBS buffer to prevent the false results due to plasma proteins.

2.3. Measurement of Aβ content by using ELISA method

Separated RBCs (200 mL) in PBS were mixed with 200 mL of water and one mL of 70 % formic acid according to previous experiment [16]. A 40 mL aliquot of suspension was mixed with 760 mL of Tris-HCl 1 M with protease inhibitors, and the mixture (1 mL) was used to evaluate the $A\beta_{40}$ using the specific ELISA kit and normalized against the hemoglobin content in each sample. Human Amyloid beta 40 Elisa kit (MBS760432) from My BioSource Company was used and Aβ amount in each experimental group was expressed as nmol/mg hemoglobin.

2.4. COX-2 activity assay

Erythrocyte samples in PBS prepared as described above were used to COX-2 assay. The catalytic activity of COX-2 enzyme was evaluated by using a fluorometric kit prepared from Abcam Company, UK (ab204699) in the presence of COX-1 inhibitor according to the manual. The Conversion of substrate to the product was normalized against hemoglobin content in each sample and reported as pmol/min mg hemoglobin.

2.5 SDS PAGE and western blotting

The concentration of AChE, CD38 and Casp3 proteins in RBCs were separated from β thalassemia major patients and control samples was evaluated by using specific antibodies in an immunoblotting method. For this purpose, extracted erythrocyte ghosts were solubilized in 1 M Tris–HCl, pH 6.8, 2 % glycerin, 10 % SDS, 5 % 2-mercaptoethanol, 0.1 % bromophenol blue. After centrifugation in 4 °C, proteins were separated from insoluble debris. Supernatants' total protein concentration was also measured using the Bradford protein assay method. Then resulted protein mixtures were subjected to SDS-PAGE gel electrophoresis. After separation of proteins by using SDS-PAGE, the proteins band

transferred to the PVDF membrane in a semi dry western blotting system. To prevent nonspecific protein binding, PVDF membranes were blocked for 2 hours at room temperature with 5 % skim milk or 1 % BSA in Tris-buffered saline with 0.1 % Tween 20 (TBST). Then diluted (1:1500) primary antibodies were used to treat the membranes at 37 °C for two hours. Anti-AChE (ab31276) and anti-Casp3 (ab4051) were prepared from Abcam Company, USA and anti-CD38 (sc-374650) was purchased from Santa Cruz Biotechnology, USA. The membranes were then exposed to the suitable secondary antibodies coupled with horseradish peroxidase enzyme (HRP) (1:3000) in TBST for 2 hours at room temperature after three washes (3–5 min) in the buffer [18]. PVDF membranes were then exposed to Pierce ECL detection kit and protein bands visualized by the Alliance 4.7 Gel Doc (UK) and quantified by using imag J software. β-actin was used as the reference protein to control the protein concentration was loaded in each lane and at the end of experiment was used to normalize bands intensity.

2.6 Statistical Analysis

All of the experiments repeated at least three times independently and the data were reported as the mean ± standard deviation (SD) using the Graph Pad in Stat version 10.0.2 program (Graph Pad Software, San Diego, CA). By computing values with the Student's *t*-test at a 5 % significance level, statistical significance was set at $p < 0.05$.

3. Results

Regarding the short lifespan of the RBCs in circulation of β thalassemia major patients, this study aimed to examine the possible alterations of AChE, Aβ, Casp3, CD38 and COX-2 in RBCs related to β thalassemia patients that need to blood transfusion and compare them with the control. Because the published literatures reported the possible role of the proteins in eryptosis process,

suicidal cell death of erythrocytes. Early elimination of RBCs before normal lifespan (120 days) seems to be due to eryptosis which induced by abnormal conditions such as oxidative stress and iron overload.

3.1. $A\beta_{40}$ peptide was upregulated in RBCs extracted from β thalassemia major patients

$A\beta_{40}$ is a pathogenic peptide and its progressive accumulation in brain tissue associated with the neurodegenerative and dementia related disease[19-21]. While recent studies confirmed the essential role of the $A\beta_{40}$ in peripheral blood and RBCs function [16]. According to them, $A\beta_{40}$ could bond erythrocytes to each other and limit the oxygen transferring capacity (18). It could induce oxidative damages in RBCs with phospholipid hydroperoxidation that is a specific marker for RBC membrane oxidative damages [16].$A\beta_{40}$ also triggers the binding of RBCs to endothelial cells and causes oxidative and inflammatory stress [16, 22] . According to the results RBCs extracted from β thalassemia patients showed remarkable increase in $A\beta_{40}$ content in comparison with control. The concentration of $A\beta_{40}$ in control samples evaluated to be 5.21±1.14 nmol/mg hemoglobin while its content in patient's erythrocyte measured as 8.56±1.08 nmol/mg hemoglobin (Fig 1). P value calculated to be <0.0001 that manifested Aβ content difference between β thalassemia major and control is significant.

3.2. RBCs related to β thalassemia showed increased COX-2 activity

Cyclooxygenase is a prostaglandin-endoperoxide synthase enzyme the key enzyme for the generation of prostaglandins from arachidonic acid as substrate[23]. COX-2 play important role in erythropoiesis and eryptosis processes through synthesis of prostaglandins and intracellular Ca^{2+} regulation[24]. According to our results, iron overloud condition in β thalassemia causes over activation of COX-2 enzyme in RBCs in

comparison with control. Catalytic function of COX-2 was assessed to be 729.39±171.11 pmol/min.mg hemoglobin in control RBCs in the presence of COX-1 inhibitor. While the COX-2 enzyme extracted form β thalassemia patient's RBCs could convert 1215.34±210.07 nmol substrate to the product in time unite and mg of hemoglobin. Statistical analysis confirmed increased COX-2 activity in RBCs extracted from β thalassemia patients rather than control sample is significant ($P<0.0001$) (Fig 2).

3.3. Impact of β thalassemia major on membrane bond proteins in RBCs

By considering important role of AChE, CD38 and Casp3 in erythrocytes' shape, senescence and eryptosis, this study aimed to evaluate the possible changes in proteins content which could imposed by abnormal microenvironment of RBCs in β thalassemia major circulation. AChE content in RBCs of patients is significantly more than control, this increase was estimated around two-fold. According to Fig 3, the expression of CD38 increased more than two-folds in RBCs extracted from β thalassemia major patients in comparison with control RBCs. While evaluation of Casp3 expression by using specific antibody confirmed the content of Casp3 is equal in both types of RBCs (originated from control sample and β thalassemia major). Statistical analysis could not detect significant change between experimental groups ($P>0.05$).

4. Discussion

Erythrocytes or RBCs are organelle free cells packed with hemoglobin, play a crucial role in the oxygen transference[6] . While RBCs possess no nucleus or mitochondria, they accomplish numerous metabolic functions in cytosol and membrane[7, 8] . β thalassemia patients could not produce healthy RBCs due to impaired β-globin gene therefore are dependent to blood transfusion regularly [2, 3]. RBCs have an average lifespan of about 120 days in human body and after this time

should be phagocytized by macrophages of splenic and hepatic sinusoids [4].Therefore, transfusion treatment should be repeated in a regular time scale in patients. 1.08 mg iron are deposited as a result of 1 ml RBCs phagocytosis that finally led to iron overloud in β thalassemia major patients [4]. According to the previous reports the transferred RBCs usually survive less than 60 days that increases the number of blood transfusions and intensifies iron toxicity [4]. Early suicidal cell death of RBCs may be caused by eryptosis process that is programmed cell death in organelle-free erythrocytes was induced by a wide variety of contributors including hyperosmolarity, oxidative stress, and exposure to xenobiotics or heavy metals and energy depletion [7, 8]. This process is similar to the apoptosis in nucleated cells that characterized by cell shrinkage, membrane blebbing, activation of caspases and phosphatidylserine exposure [7, 8]. It seems reduced lifespan of RBCs in β thalassemia major patients is due to eryptosis induced by oxidative stress and iron toxicity. Here we investigated the possible changes of some proteins content and enzymes activity in erythrocytes isolated from β thalassemia major patients in comparison with control, the candidate proteins have important role in RBCs programmed death in different types of anemia [8-11, 13] . Despite the low complexity of erythrocytes, membrane proteins regulate cellular process such as oxidative response, erythropoiesis and eryptosis [24, 25] . According to our results changing microenvironment of RBCs in β thalassemia patients causes overactivation of COX-2 enzyme that led to increased production of the interleukins (ILs), prostaglandins (PGs) and cytokines [23]. As previous experiments, increased amounts of PGs specially PGE2 in RBCs trigger eryptosis through extra Ca^{2+} influx [25](Fig 4). Upregulation of intracellular free Ca^{2+} during eryptosis process was confirmed

latterly [7, 8, 10]. Our results also confirmed CD38-mediated signaling was activated in RBCs extracted from β thalassemia major patients. According to the Fig 3, CD38 expression increased more than 2-fold in patients' RBCs rather than control. CD38 is type II membrane glycoprotein that involves in cell adhesion, migration, and signal transduction [11]. This ectoenzyme catalytically converts NAD^+ to cADPR that is a secondary messenger in Ca^{2+} influx pathway [15]. The early experiments have been found a remarkable increase of RBCs CD38 concentration and catalytic activity in patients suffering from cancers and anemia [11]. They also reported a positive coloration between increased level of CD38 and degree of anemia and tumor progression [11]. It seems increased CD38 is upstream signaling that trigger eryptosis through upregulation of cytosolic Ca^{2+} that was schematically manifested in Fig 4. We also evaluated Casp3 protease enzyme due to its involvement in apoptosis in nucleated cells[26]. Previous reports confirmed Casp3 presence in normal RBCs that assumed to be have important role in erythropoiesis [27]. While our results revealed iron overload condition could not change Casp3 level in erythrocytes originated from β thalassemia major. It should be noted that this study measured the Casp3 concentration without considering its catalytic activity, its possible protease function of Casp3 changed in β thalassemia major that need to further investigation.

Presence of Exovesicules enriched with AChE protein originated from membranes of aged erythrocytes in plasma and also changes in kinetic properties of the membrane bound AChE in old RBCs confirmed involvement of this enzyme in eryptosis machinery[9]. AChE is a multifunctional enzyme which was previously considered as membrane integrity biomarker in erythrocytes[9]. This glycosylphosphatidylinositol (GPI)-anchored protein is involved in the nitric

oxide (NO) signal pathway in RBCs that decreased in aged cells[9, 28]. Our results approved AChE level increased in β thalassemia major's RBCs rather than erythrocytes originated from healthy controls. Obtained data confirmed that most of the RBC's population in β thalassemia major patients are young and were removed before they have a chance to reach their maximum lifespan (120 days). Increased exposure of membrane bound AChE also could change structural integrity of RBC's membrane and attach them to each other that lead to eryptosis, increased AChE expression was observed in different types of anemia such as microcytic or hemolytic[29]. Similarly, previous study reports its contribution in oxidative stress and potential to be an effective indicator of erythrocytes toxicity by pesticides[30]. AChE could also bind to the Aβ peptides and help to amyloidogenesis process through a non-enzymatic function[31]. In addition to aggregation, cytosolic Aβ also facilitate binding of RBCs to endothelial cells and declines endothelial capability, possibly by oxidative stress and inflammation. Our results showed a harsh increase in Aβ content of RBCs originated from β thalassemia major patients in comparison with control but we don't have enough document to presence of the insoluble amyloid fibrils. According to the experiments, increased monomeric Aβ oxidatively impairs RBC's function in oxygen delivery and decreases their survival remarkably [16].

Conclusion

Consequently, this study evaluated cell death machinery in RBCs isolated from β thalassemia major patients that occurs before the erythrocyte has had a chance to be naturally removed from the circulation after its 120 days lifespan. Here, we present the main pathways leading to eryptosis (Fig 4), the programmed death of erythrocytes, with special focus on upregulation of AChE,

COX-2, Aβ and CD38 that finally causes oxidative stress and Ca^{2+} influx. It seems iron overload and other toxic agents in microenvironments of transfused RBCs induce eryptosis and reduces transfusion efficiency via early eryptosis of RBCs. This study tried to clarify some ambiguities in eryptosis process and also introduce key pathways and hub molecules that targeting them could improve survival of newly received RBCs and reduce transfusion dependency in patients with β thalassemia.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure Legends

Fig 1. Changes of the $A\beta_{40}$ in erythrocytes isolated from patients suffering from β thalassemia major in comparison with control. All data were expressed as mean ± SD. P value was indicated on the column graph in significant changes.

Fig 2. Cox-2 enzyme activity in total proteins extracted from RBCs isolated from patients suffering from β thalassemia major in comparison with control. All data were expressed as mean ± SD. P value was indicated on the column graph in significant changes.

Fig 3. Immunoblotting analysis to compare membrane bound proteins between RBCs isolated from patients and health controls. Western blot results display increased expression of CD38 and AChE in RBCs originated from β thalassemia major patients. The first three lanes are related to β thalassemia major (indicated by T) and 4, 5 and 6 lanes are biological replicates of healthy control samples (C). β-actin was used to control of protein concentration in lanes. Intensity of each band was quantified by using imageJ software and showed in column plot.

Fig 4. Schematic overview of eryptosis process in β thalassemia patients' RBCs with

special emphasis on AChE, Casp3, CD38 membrane bond proteins and Aβ peptide.

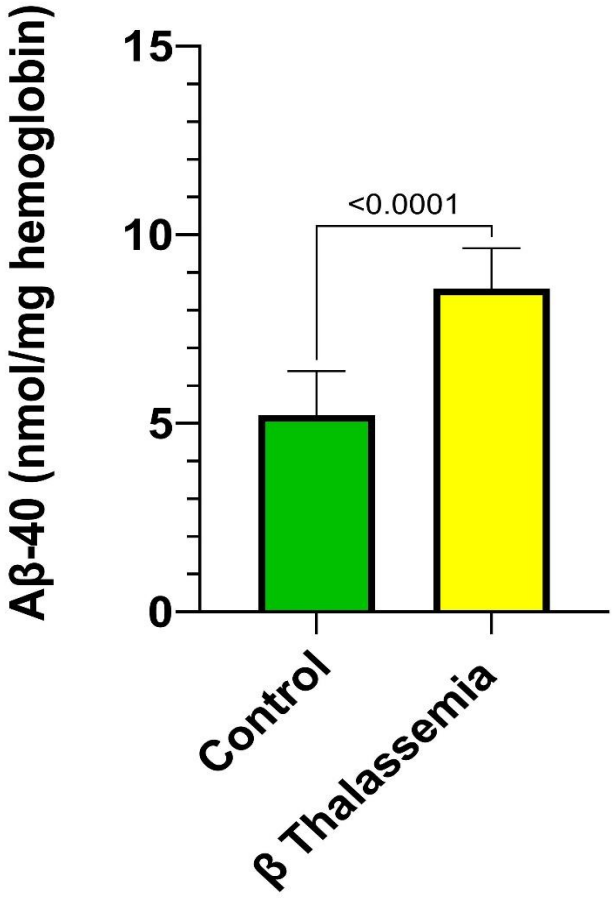
Aβ-40 (nmol/mg hemoglobin)
15
10
5
0
<0.0001
Control
β Thalassemia

Fig 1

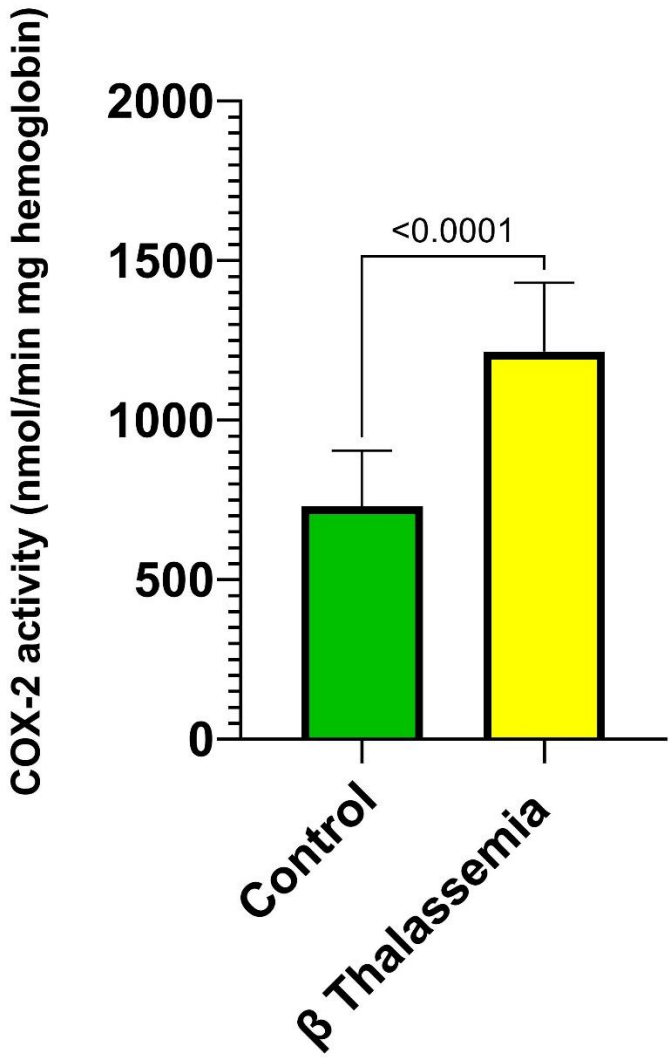
COX-2 activity (nmol/min mg hemoglobin)
2000
1500
1000
500
0
<0.0001
Control
β Thalassemia

Fig 2

Different expression of acetylcholinesterase, caspase 3 and CD38 proteins and Amyloid β peptide in erythrocytes of β thalassemia major patients

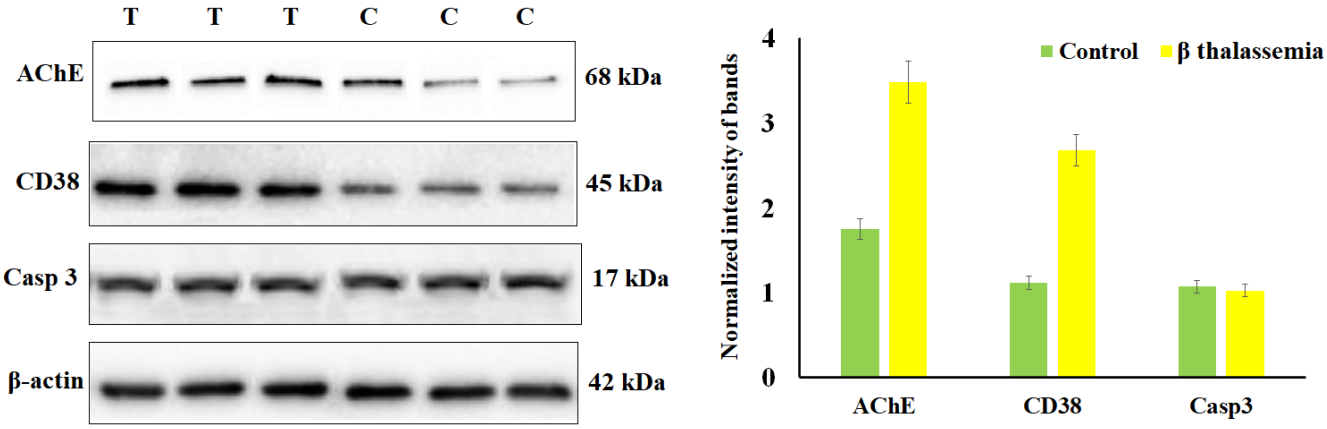

Fig 3

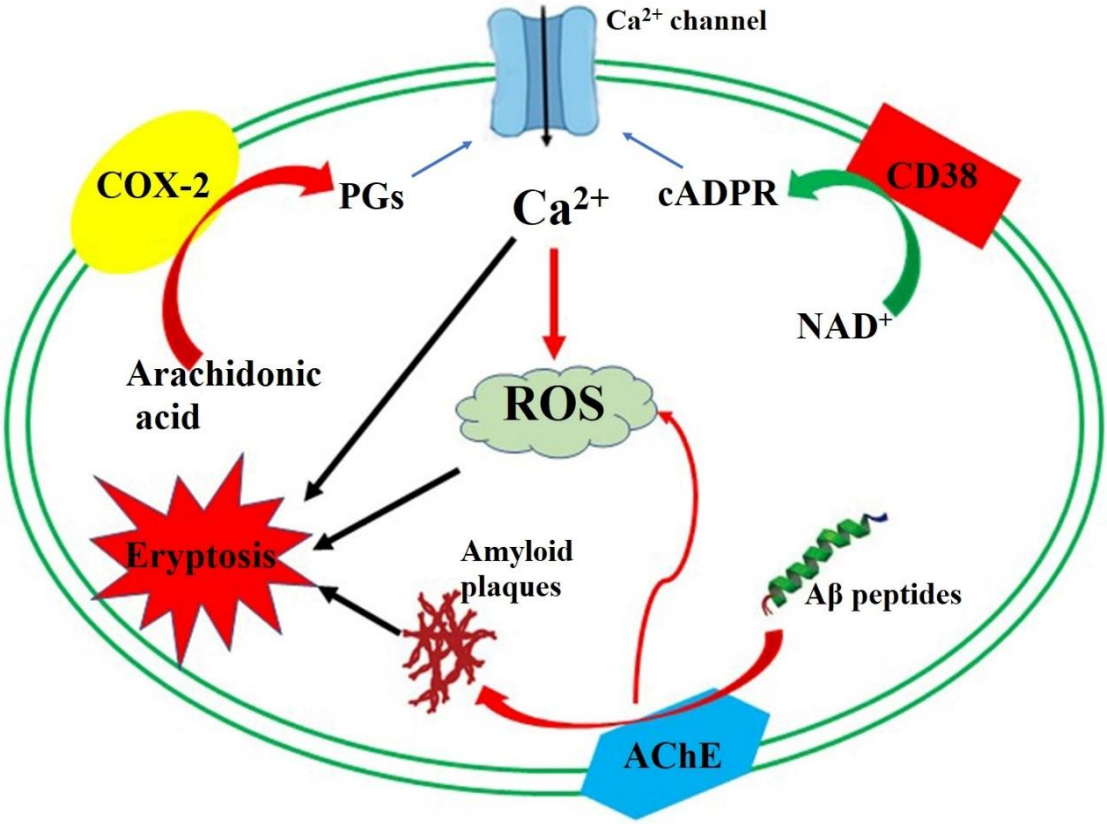
Ca2+ channel
COX-2
PGs
Ca2+
cADPR
CD38
NAD+
Arachidonic acid
ROS
Eryptosis
Amyloid plaques
Aβ peptides
AChE

Fig 4