“BABES-BOLYAI” UNIVERSITY

FACULTY OF BIOLOGY AND GEOLOGY

DEPARTMENT OF EXPERIMENTAL BIOLOGY

SUMMARY OF DOCTORAL THESIS

Oxidative stress and Hemolytic anemia

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TABLE OF CONTENTS

Keywords:

Acetyl cysteine proline cysteine amide, Antioxidant, Auto-immune hemolytic anemia, Erythropoietin ,Fermented papaya preparation, Flow cytometry, Hemolysis, Hereditary spherocytosis, Howell-Jolly RBCs, Intra-vascular complement mediated hemolysis, *N*-acetylcysteine ,Oxidative stress, Paroxysmal nocturnal hemoglobinuria, *β*-thalassemia .

1. SUMMARY

Free radicals are chemical species composed of molecular fragments that possess an unpaired electron in the outer (valence) shell, which makes them highly reactive. The most important free radicals are those derived from oxygen, known as reactive oxygen species (ROS). They are produced continuously in cells as by-product of metabolism. The oxidative status of cells represents the balance between oxidants (e.g. ROS) and antioxidants (e.g. reduced glutathione, GSH). Disturbance of this balance towards oxidants results in oxidative stress. Their production can be grossly amplified in response to a variety of pathophysiological conditions such as inflammation, immunologic disorders, hypoxia, hyperoxia, metabolism of drugs or alcohol, exposure to UV or therapeutic radiation and deficiency in antioxidant vitamins (Chan et al. 1999). ROS formed within the cells can oxidize various molecules leading to cell death and tissue injury (Droge et al. 2002). Oxidative stress is suspected to play a role in the pathophysiology of many diseases, including inflammatory disorders, cardiovascular diseases, central nervous system injury, pulmonary diseases, etc. (Kohen and Nyska 2002). It may also be involved in hemolytic anemias(Rachmilewitz and Fibach, 2002). ROS contribute to the pathogenesis of several congenital and acquired hemolytic anemias, including thalassemia, hereditary spherocytosis, paroxysmal nocturnal hemoglobinuria (PNH) as well as autoimmune hemolytic anemia (AIHA).

In β-hemoglobinopathies such as (thalassemia and sickle cell anemia), although the basic lesion is in the globin genes, the pathology involves oxidative stress-mediated cell damage in the bone marrow (defective red cell production=ineffective erythropoiesis) due to apoptosis of early erythroid precursors and in the peripheral blood (short survival of mature RBC). To study the role of oxidative stress in hemolytic anemias, we developed a flow cytometry methodology for its measurement in various blood cells. Using this methodology, we showed that RBC obtained from these patients contain increased amounts of ROS, lipid peroxidation and external phosphatidylserine and decreased amounts of GSH than do RBC from normal donors, indicating a state of oxidative stress .Despite extensive research efforts in recent years, yielding promising results, there is still a need for additional research on oxidative stress in humans. In our study we investigated, for the first time, the oxidative status of other types of hemolytic anemias and the effect of other antioxidant compounds such as, FPP, EPO and CB3. Our result suggested that these antioxidants might be used as potential therapeutic modality.

We studied the oxidative stress in the following aspects of hemolytic anemias:
1-The involvement of oxidative stress in thalassemia (page 7).
2-The oxidative status of Thalassemic DNA-Containing Red Blood Cells (page 9).
3-The Antioxidant Effect of Erythropoietin on Thalassemic Blood Cells (page 9).
4-The contribution of Oxidative stress to hemolysis in patients with hereditary spherocytosis (page 11)
5-The involvement of oxidative stress in paroxysmal nocturnal hemoglobinuria (page 12).
6- The effect of new antioxidant on oxidative stress in hemolytic anemia (page 14).
7- The involvement of oxidative stress in Auto-immune hemolytic anemia (page 16)

Although the primary etiology is different in these anemias, oxidative stress mediates several of their pathologies, mainly hemolysis.

2. OBJECTIVES

We studied the possible involvement of oxidative stress in the pathology of various aspects of hemolytic anemias.

Specific Aims:

1- To measure oxidative stress in blood cells derived from patients with thalassemia, hereditary spherocytosis, paroxysmal nocturnal hemoglobinuria as well as autoimmune hemolytic anemia compared with cells from normal donors.

2- To measure the levels of reactive oxygen species and exposed phosphatidylserine in Howell-Jolly cells in the peripheral blood of thalassemic patients.

3- To investigate the in vitro effect of Erythropoietin on the oxidative status of RBC and platelets from β-thalassemic patients and the in vivo effect on these cells of Erythropoietin administration to β-thalassemic mice.

4- To investigate the oxidative status of RBCs in Hereditary Spherocytosis, and its contribution to hemolysis and the effects of an antioxidant, both in vitro and in vivo.

5- To analyze conditions and drugs that attenuate oxidative stress *in vitro* and *in vivo*.

6- To study the involvement of oxidative stress in the pathogenesis of complement-mediated hemolysis, and the possibility of using antioxidants for treatment.

3. METHODS

The methods used were described in detail in the articles attached. The most frequently used method is the flow cytometry.

3.1 Blood Samples

Peripheral blood (PB) samples were collected in EDTA-containing tubes .obtained from normal donors and hemolytic anemia patients (according to type of anemia). The blood was diluted with Ca^{2+}- and Mg^{2+}-free

Dulbecco's phosphate-buffered saline (PBS) and used within 2 h of collection. In some cases the samples were obtained from the counting vials after all diagnostic laboratory tests were completed. The research was approved by the Hadassah-Hebrew University Medical Centre Ethical Committee of Human Experimentation. Informed consent was obtained in all cases. In polytransfused patients, blood samples were obtained before transfusion, at least 3 weeks following the previous transfusion.

3.2 ROS (Reactive Oxygen Species) Assay

RBC were incubated with 2'-7'-dichlorofluorescin diacetate (DCF) (Sigma), dissolved in methanol (Bio Lab, Jerusalem, Israel), at a final concentration of 0.4 mM. After incubation at 37°C for 15 min in a humidified atmosphere of 5% CO_2 in air, the cells were washed and re-suspended in Dulbecco's phosphate-buffered saline (PBS) to the original cell concentration.

Upon crossing the membrane, DCF undergoes deacetylation by intracellular esterases producing a nonfluorescent compound that becomes highly green fluorescent following oxidation by ROS.

3.3 GSH (Reduced Glutathione) Assay

RBC were washed with PBS and then spun down. The pellet was incubated for min. at room temperature with 40 μM (final concentration) of mercury orange (Sigma). A 100 μM stock solution of mercury orange was made up in acetone and stored at 4°C. RBCs were then washed, resuspended in PBS, and analyzed by flow cytometry.

Mercury orange reacts with the SH group of GSH to produce red-orange fluorescence.

3.4 Lipid Peroxidation Assay

RBC suspensions (5×10^6 cells/ml) in PBS were labeled with 50 μM *N*-(fluorescein-5-thiocarbamoyl)-1, 2-dihexadecanoyl- *sn*-glycero-3-phosphoethanolamine, triethylammonium salt (fluor-DHPE) (Molecular Probes, Eugene, OR) dissolved in ethanol. The cells were incubated for 1 h at 37°C in a humidified atmosphere of 5% CO_2 in air with continuous agitation, centrifuged once to remove unbound label, and resuspended in PBS.

Fluor-DHPE is a lipophilic fluorescent probe that loses its fluorescence upon reaction with peroxyl radicals, especially after induction of lipid peroxidation.

3.5 PS (Phosphatidylserine) Assay

Cells of both normal and thalassemic or apoptosis-induced cells are washed in PBS. Pellet is resuspended in 45μl of binding buffer containing calcium. Five μl of AnnexinV-FITC is added and incubated for 15 min. at 37^0C. Cells were then washed and resuspended with PBS.

3.6 Flow cytometry

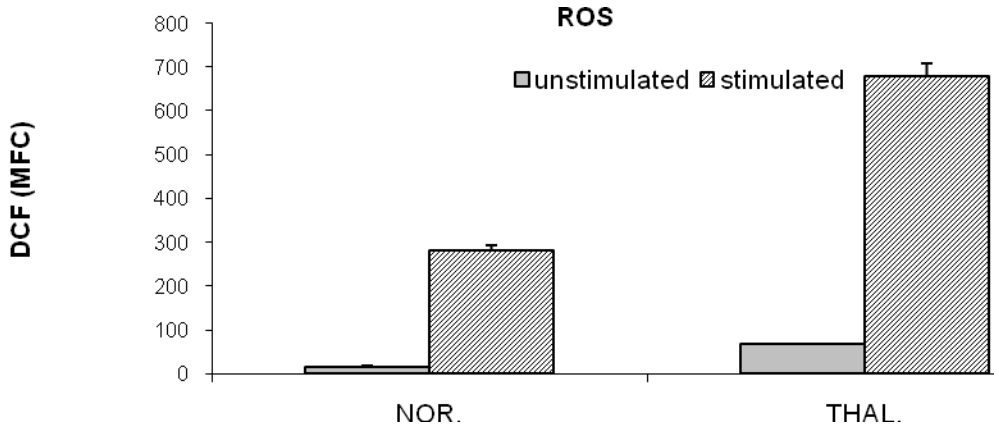
RBC treated as indicated above were analyzed by a fluorescence-activated cell sorter (FACScalibur, Becton Dickinson, Immunofluorometry systems, Mountain View, CA). Cells were passed at a rate of about 1,000 per second, using saline as the sheath fluid. A 488 nm argon laser beam was used for excitation. To exclude non-RBC from analysis, a two-parameter dot-plot of the side light scatter (SSC) and forward light scatter (FSC) of the population was first analyzed. A gate was set to include only RBC, and to exclude reticulocytes and white blood cells (WBC) .RBC labeled with DCF and fluor-DHPE were detected by the FL-1 PMT using linear amplification, while mercury orange-labeled RBC were detected by the FL-2 PMT using log amplification. For every assay, unstained cells, both treated and untreated, were used as controls. Instrument calibration and settings were performed using Cali- BRITE™-3 beads. The mean fluorescence channel (MFC) of the entire RBC population was calculated for DCF, GSH and lipid peroxidation by the FACS-equipped CellQuest software.

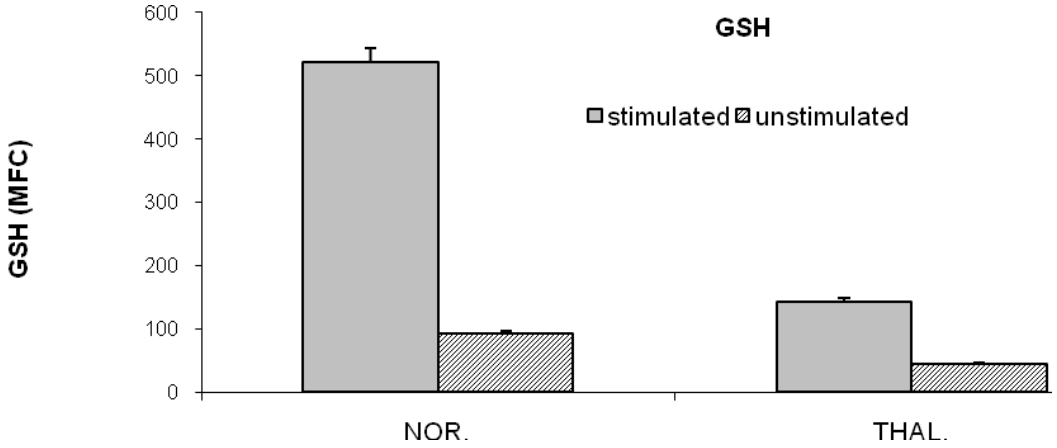
4. RESULTS

4.1. <u>The involvement of oxidative stress in thalassemia</u>

Flow cytometric methods were used for measuring oxidative status of normal and thalassemic RBC and for studying the effects of various oxidants and antioxidants. The following cellular parameters were measured(Fig. 1): (1) generation of ROS, including both the baseline capacity of cells to generate these oxygen species, as well as their response to oxidative stress (e.g., exposure to hydrogen peroxide); (2) the content of reduced GSH; and (3) peroxidation of membrane lipids.

The results indicated that ROS and lipid peroxidation were higher, and GSH lower, in thalassemic RBC compared with normal RBC, both at baseline as well as following oxidative stress, such as exposure to hydrogen peroxide.





Lipid peroxidation

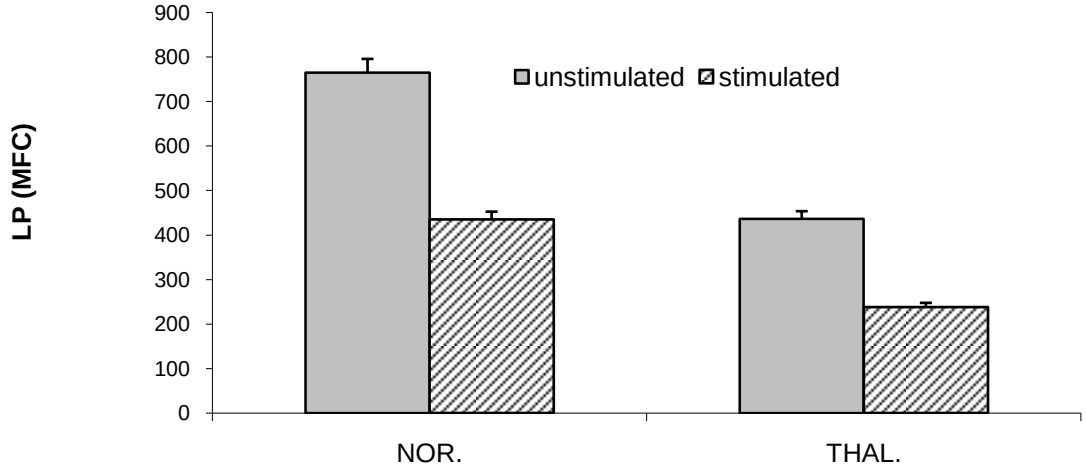


Figure 1. Flow cytometric analyses of (ROS), (GSH), and lipid peroxidation of H_2O_2-stimulated and unstimulated red blood cells (RBC) from representative normal and thalassemic donors. For the ROS and GSH assays, RBC were preincubated with and without 2 mM H_2O_2 for 1 h and then labeled with either 0.4 mM (DCF) for 15 min. or with 40 μM mercury orange for 3 min at room temperature. Both stains were then washed and the cells resuspended with PBS. In the lipid peroxidation assay, RBC were first labeled with 50 μM fluor-DHPE at 37°C for 1 h, then washed and either stimulated, or not, with 8 mM H2O2 for 1 h at room temperature. MFC results were shown.

Taken together, these results emphasized the inverse relationship between ROS and GSH. ROS and lipid peroxidation were higher, and GSH lower, in thalassemic RBC compared with normal RBC, both at baseline as well as following oxidative stress, such as exposure to hydrogen peroxide.

4.2 *Thalassemic DNA-containing red blood cells are under oxidative stress*

M. Dana, E. Prus, E. Fibach. Anemia, 2012 Article ID 943974, (2012).

(Appendix 1)

We studied the nature of enucleated RBCs containing DNA remnants, Howell-Jolly (HJ) RBCs and reticulocytes (retics) that are characteristically present in the circulation of thalassemic patients, especially after splenectomy. Using flow cytometry, we measured oxidative status parameters of these cells in patients with β-thalassemia. In each patient studied, these cells had higher content of reactive oxygen species and exposed phosphatidylserine compared with their DNA-free counterparts. These results suggest that oxidative stress in thalassemic developing erythroid precursors might, through DNA-breakage, generates HJ-retics and HJ-RBCs and that oxidative stress-induced externalization of phosphatidylserine is involved in the removal of these cells from the circulation by the spleen, a mechanism similar to that of the removal of senescent RBCs.

4.3. The Antioxidant Effect of Erythropoietin on Thalassemic Blood Cells

Amer J, **Dana M**, Fibach E. ***The antioxidant effect of erythropoietin on thalassemic blood cells***. Anemia 2011; 2010(978710).

(Appendix 2).

EPO effect on ROS generation

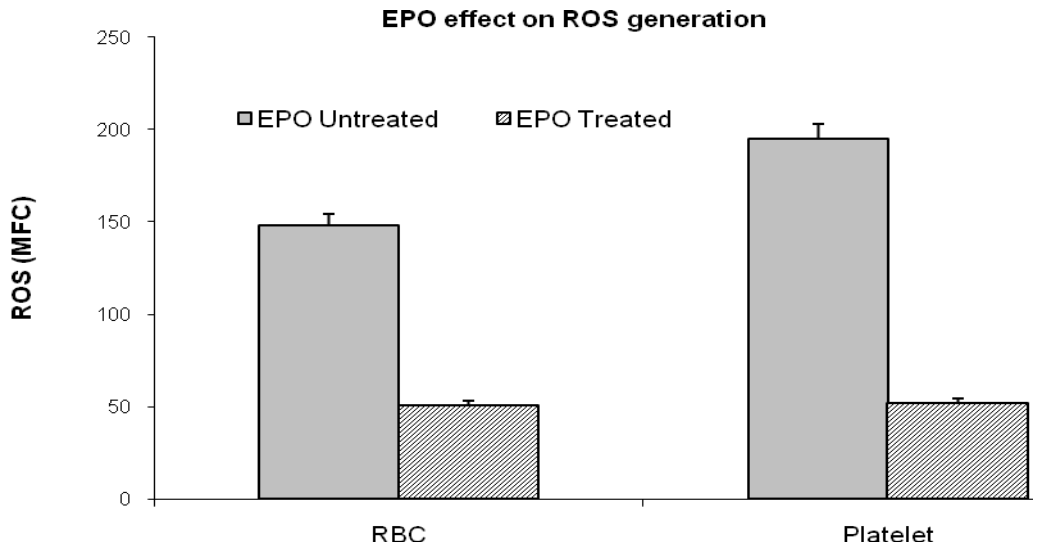


Figure 2: The Epo effect on ROS generation by RBC and platelets. A diluted blood sample obtained from a thalassemia patient was treated for 2 hrs with Epo (1 U/ml) at 37◦C, stained with DCF and then stimulated with 1mM H2O2 for 15min.ROS was measured by flow cytometry following gating of RBC and platelet.

The in vivo effect of Epo in thalassemic mice

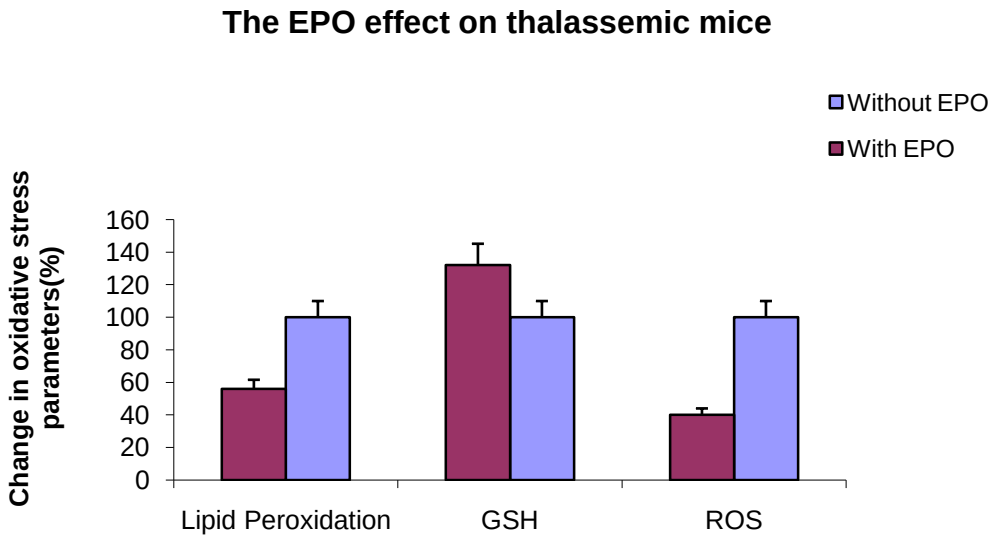


Figure 3. Effect of Epo on thalassemic mice. Heterozygous *β*- thalassemic intermedia (Hbb*th*3/+) mice (*N* = 4) with low hemoglobin levels (7–9 g/dL) were inoculated (i.p) with or without Epo (5000 U/kg). After 2 hrs, blood was drawn and RBC were assayed. The changes in the indicated parameters are shown. Values of untreated mice (Without Epo) were taken as 100%.

4.4. <u>The contribution of Oxidative stress to hemolysis in patients with hereditary spherocytosis</u>

Ghoti H, Fibach E, **Dana M**, et al. ***Oxidative stress contributes to hemolysis in patients with hereditary spherocytosis and can be ameliorated by fermented papaya preparation.*** Ann Hematol 2010; 90(5):509-13

(Appendix 3).

In vitro study

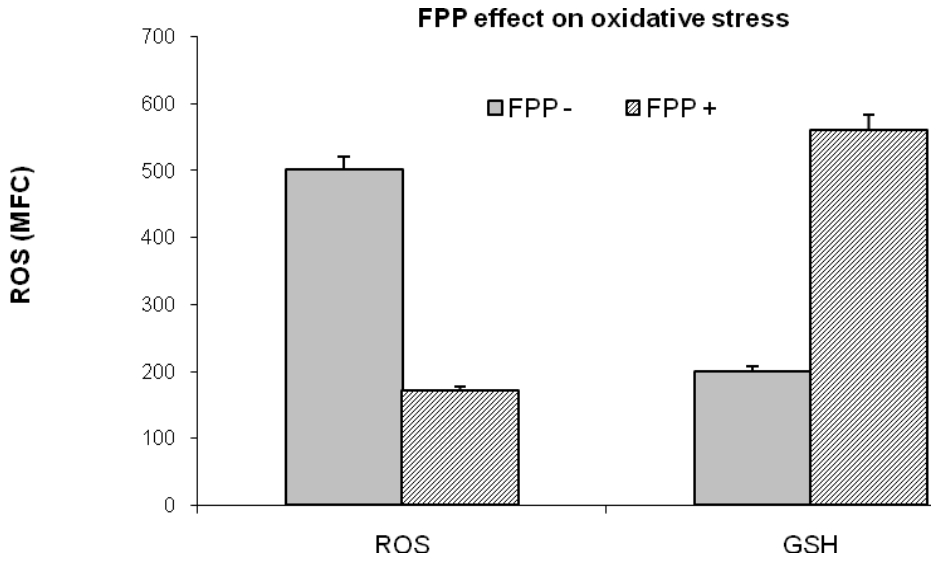


Figure 4. The in vitro effect of FPP on oxidative stress of HS-RBC. RBC from HS patients were diluted in PBS and incubated for 2 h with or without 0.1 mg/ml FPP. The cells were then assayed for ROS and GSH. The results are expressed as the average MFC of 17 patients.

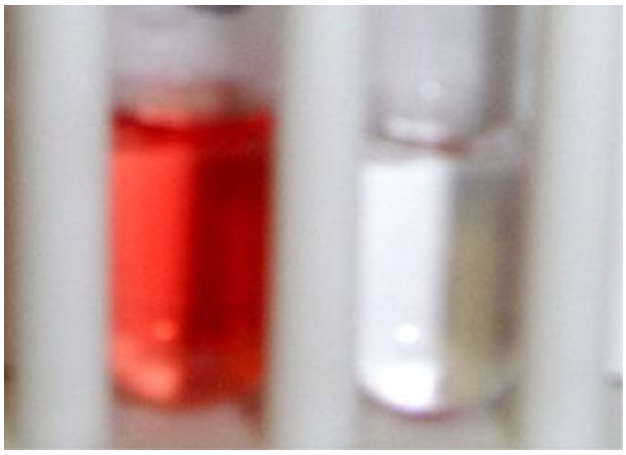


Figure 5 .The in vitro effect of FPP on hemolysis of HS-RBC .RBC from one HS patient were diluted in PBS and incubated overnight with or without 0.1 mg/ml FPP. Hemolysis is present in the absence of FPP (left tube) but not following incubation with FPP (right tube)

4.5 The involvement of oxidative stress in paroxysmal nocturnal hemoglobinuria (PNH)

4.5.1 Oxidative status of PNH-RBC cells

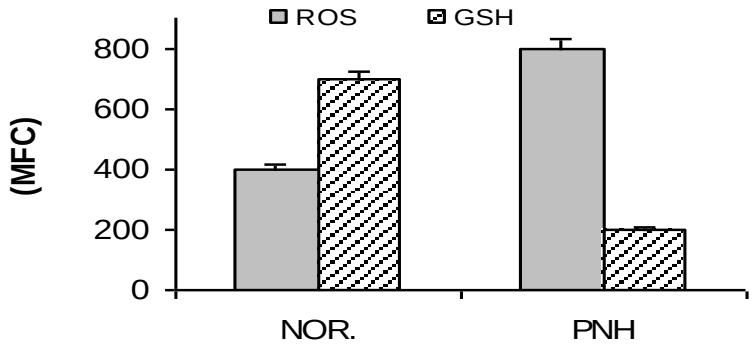


Figure 6. **The oxidative status of RBC from PNH patients compared to RBC from normal donors**. RBC were assayed for ROS and GSH. ROS was measured following exposure for 15 minutes to 1 mM H_2O_2. The MFC are shown.

4.5.2 The oxidative status of CD55/CD59 negative RBC

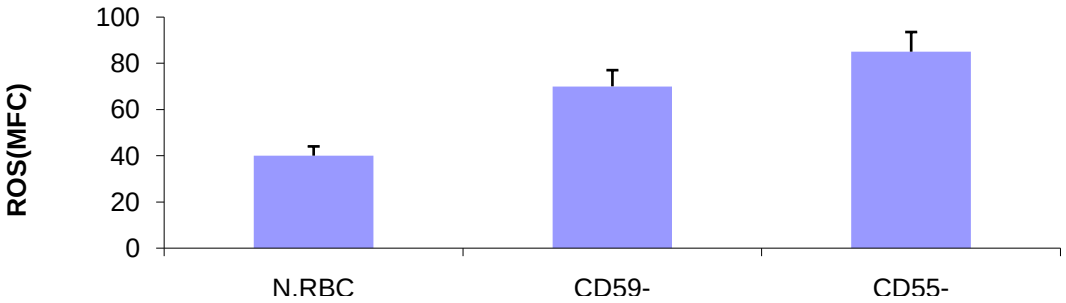


Figure 7. **The oxidative status of CD55/CD59 negative RBC.** Blood samples obtained from 5 normal donors and 5 PNH patients were stained for CD55 and CD59 and ROS. Gates were set on RBC, and the intensity of CD55, CD59 and ROS fluorescence was determined. The proportion of CD55– and CD59– cells in the patients' blood ranged from 20% to 27%. The results with cells from PNH patients show higher ROS levels in CD55/CD59-negative cells than CD55/CD59 positive cells (normal cells).

4.5.3 Effect of complement on ROS of PNH-RBC

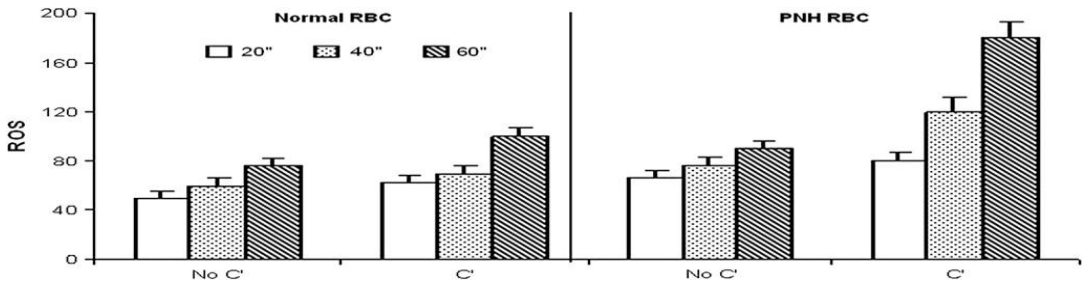


Figure 8. Effect of complement on ROS of RBC. Normal and PNH-RBC were incubated at 37 ºC with complement-containing serum (C') or complement inactivated serum (heated at 56 ºC for 30 minutes) (No C'). The sera were ABO compatible with the treated RBC. At the indicated times, aliquots of RBC were assayed for ROS. Results are expressed as the average 6 SD of the mean fluorescence channel of four experiments carried out with RBC from different normal donors and PNH patients (having 20–27% CD55/ CD59-negative cells).

4.5.4 PNH-like RBC

4.5.4.1 Oxidative status of PNH- like RBC cells

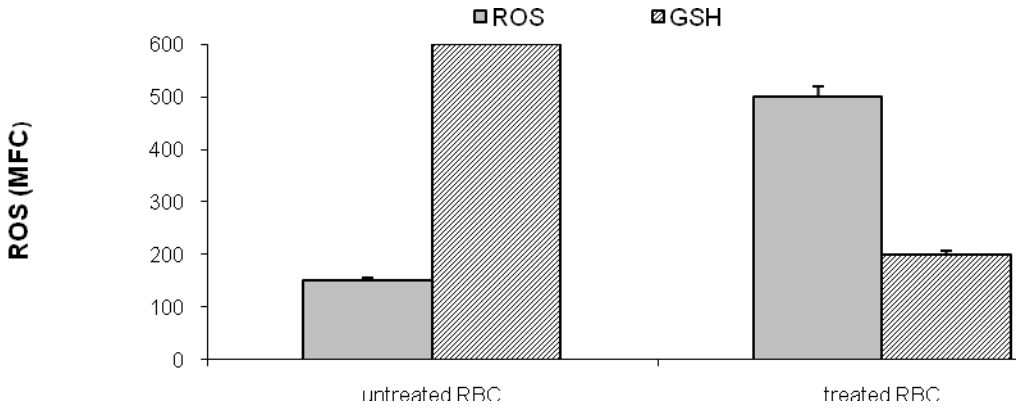


Figure 9.The oxidative status of AET treated-RBC. RBC were assayed for ROS, and GSH. The MFC are shown.

4.5.4.2 The effect of complement on AET-treated red cells

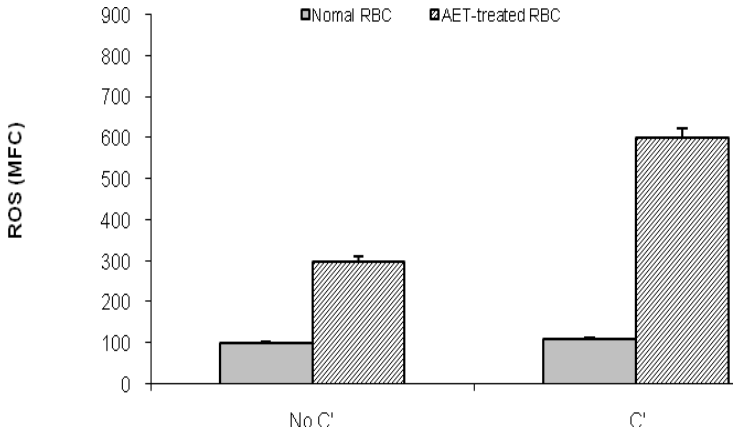


Figure 10. Effect of complement on ROS of PNH-likeRBC. Normal and AET treated RBC were incubated at 37 ºC with C'-containing serum or C' inactivated serum (heated at 56 ºC for 30 minutes) (No C'). The sera were ABO compatible with the treated RBC. The results indicated an increase in fluorescence in non-heated serum, but not in heated serum.

4.6 The Antioxidant Effect of Thioredoxin Mimetic Peptides (Trx) on oxidative status of PNH and PNH-like RBC

4.6.1 CB3 and NAC effect on ROS and GSH within blood cells

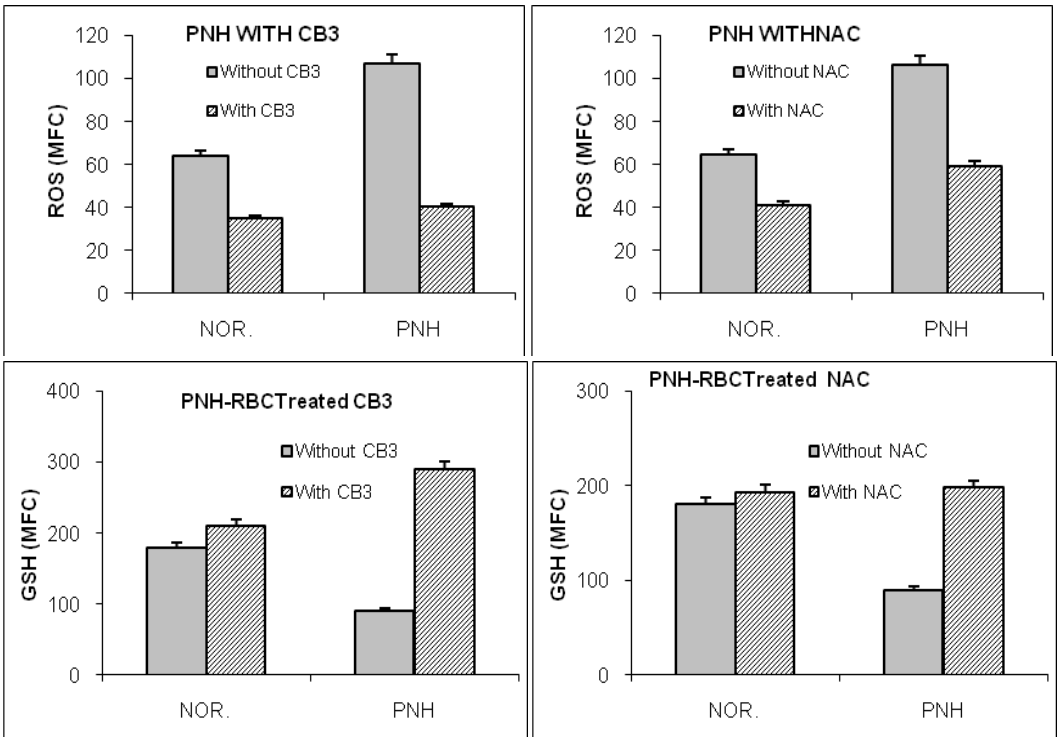


Figure11. **CB3 and NAC effect on ROS and GSH within RBC.** PNH-RBCs diluted in PBS were incubated for 30 min with 1mM of CB3 or NAC. ROS and GSH were measured following staining with DCF and mercury orange, respectively. Our results show that CB3 significantly reduced ROS production and increased GSH content, and that it was more effective than NAC.

4.6.2 The effect of in vitro treatment with CB3 on hemolysis

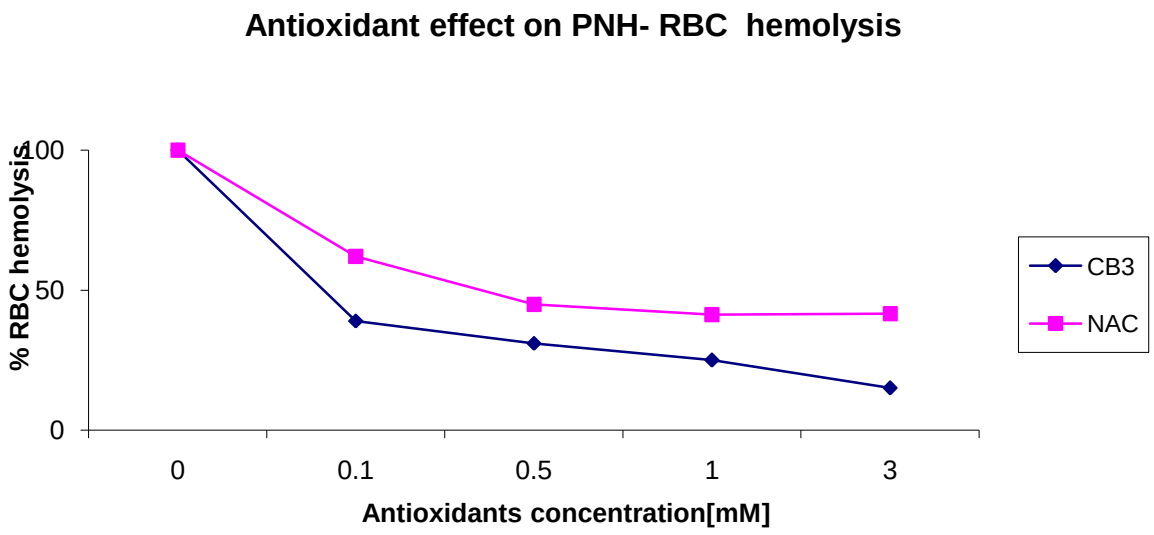


Figure 12. **The effect of in vitro treatment with CB3 on hemolysis.** PNH-RBC diluted in PBS were incubated overnight with various concentrations of CB3 or NAC. The cells were then centrifuged, the Hb were measured by Drabkin's Reagent. Complete hemolysis was detected by lysing the RBC solution by osmotic shock in water and measuring the Hb which represents 100% hemolysis (blank), then the results expressed as absorbance of tested sample versus the blank as the reference at 540 nm. The results indicate severe hemolysis of the untreated PNH-RBC while NAC and CB3 treatment inhibited lysis, in a dose-dependent manner

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4.6.3 The effect of in vitro treatment with CB3 on PNH-like RBC hemolysis

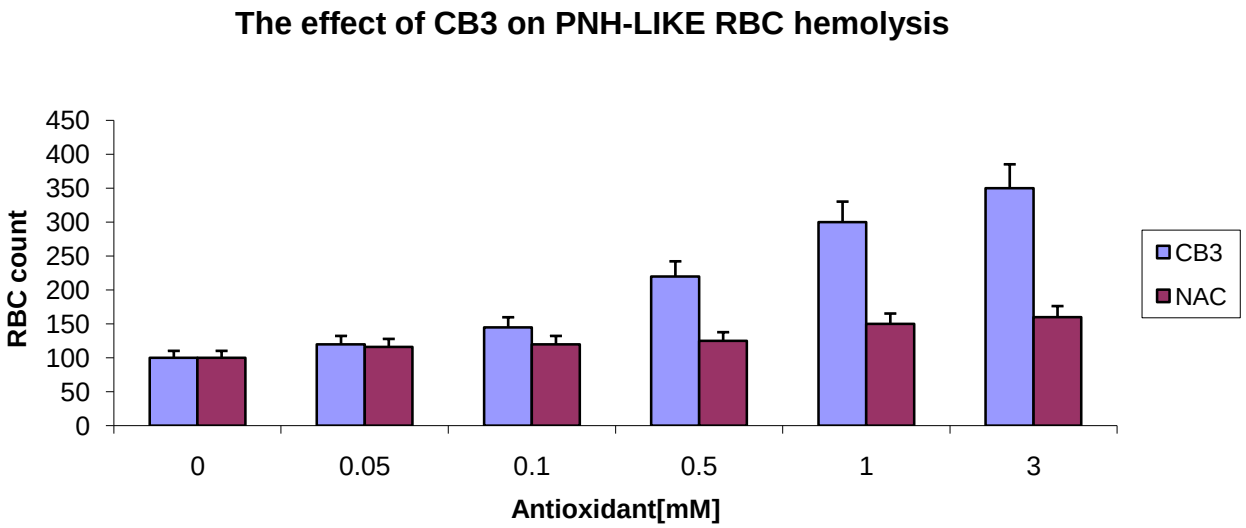

Figure 13. **The effect of in vitro treatment with CB3 on PNH-like RBC hemolysis.**
PNH-like RBC diluted in PBS were incubated overnight with various concentrations of CB3 or NAC. The cells were then centrifuged, and the sediment of RBC were counted by hematocytometer.

4.7 The involvement of oxidative stress in Auto-immune hemolytic anemia (AIHA)

4.7.1 In vitro AIHA Model

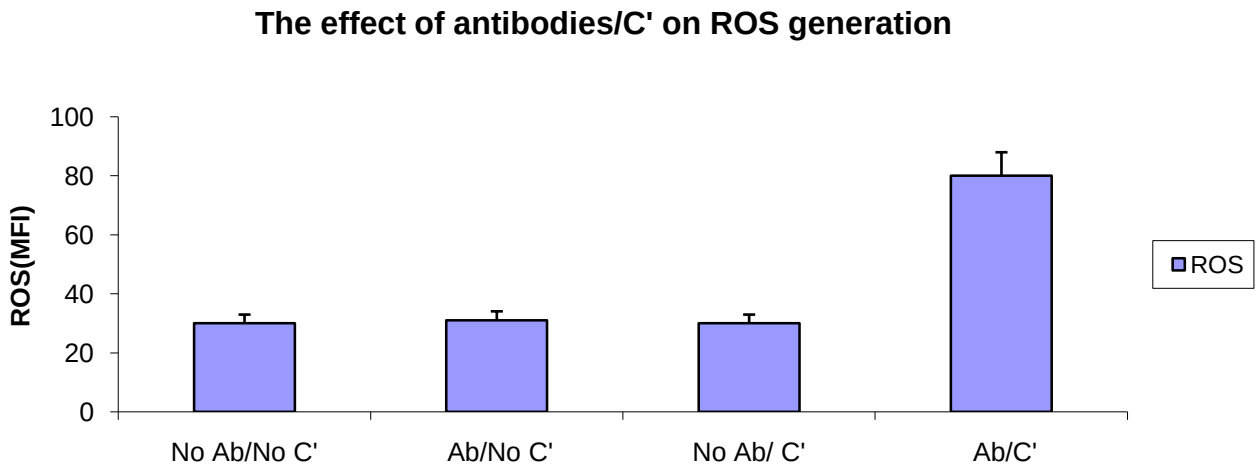

Figure 14A. The effect of Antibodies and Complement on ROS generation
The MFC of human A-type normal RBCs incubated with anti-A monoclonal antibodies and C'. The results indicated that neither antibodies nor C' alone induced ROS.

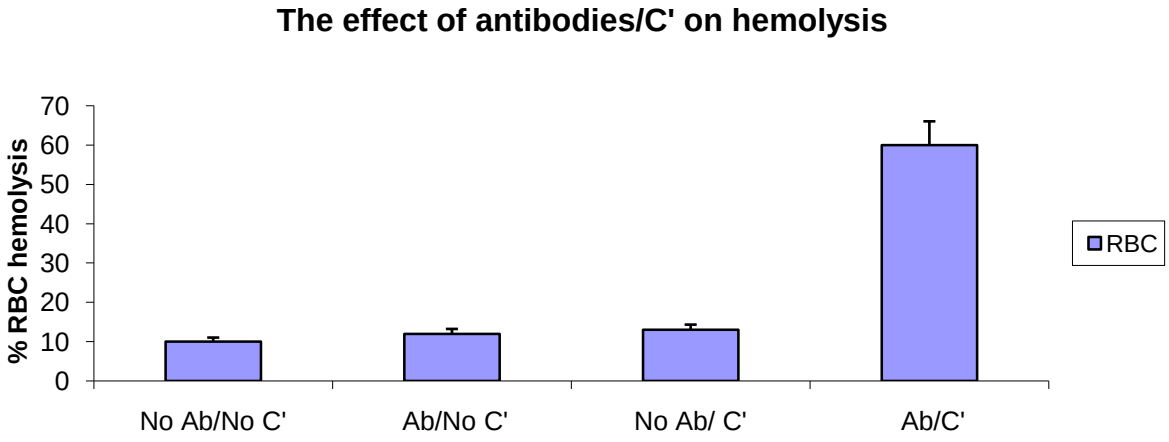

Figure 14B. The effect of Antibodies and Complement on hemolysis.
Human A-type normal RBCs incubated with anti-A monoclonal antibodies and C'. Hemolysis was observed within 30 min, and was measured by determining the free hemoglobin content of the supernatant .The results indicated that neither antibodies nor C' alone induced hemolysis.

These results suggested that neither antibodies nor C' alone induced hemolysis.

Effect of antioxidants on Antibodies/Complement -induced lysis of RBC

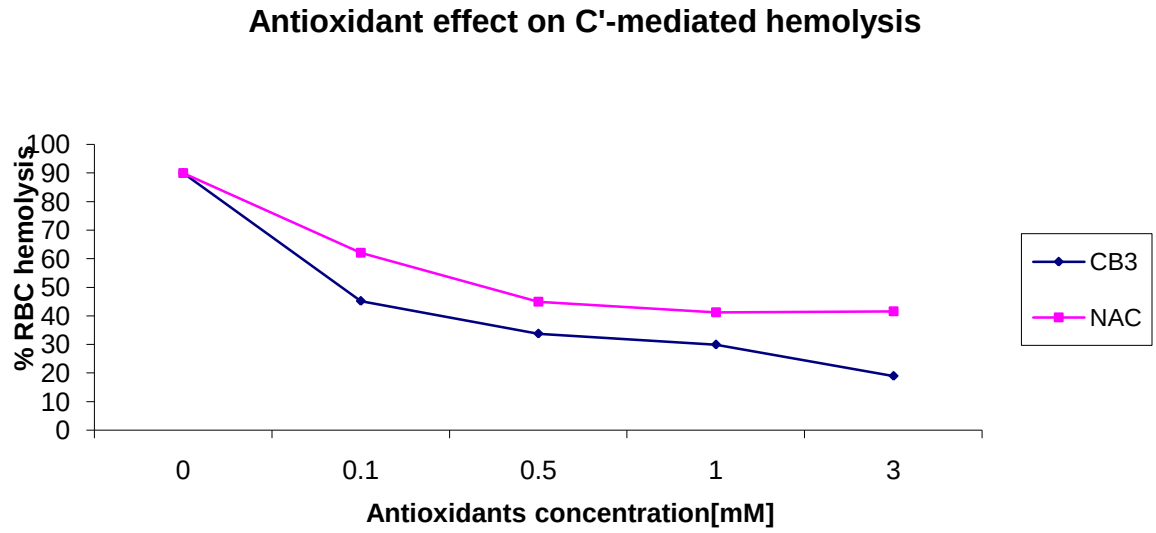

Figure 15.Antioxidants Effect on Antibodies /Complement -induced lysis of RBC. Human A-type RBC were incubated with autologous serum, at ratio 1:5 with 2µL mouse monoclonal anti-A antibodies (IgM) at different concentration of CB3, NAC at 37°Cfor 40 Min. Serum hemoglobin was tested by the Drabkin's solution. Our results show that CB3 significantly inhibited lysis of blood cells comparing with NAC.

Our results show that CB3 significantly inhibited lysis of RBC comparing with NAC.

4.7.2 CB3 and NAC effect on ROS and GSH within AIHA-blood cells

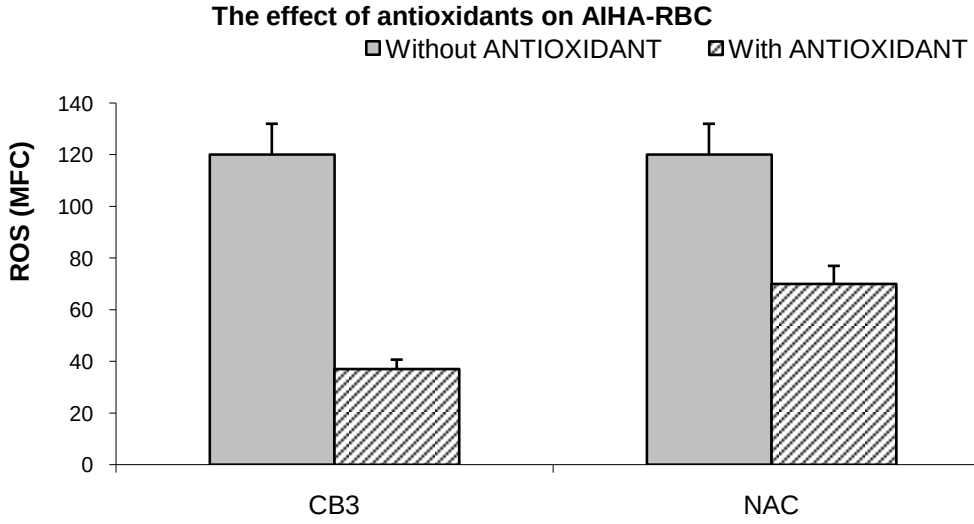

Figure 16 CB3 and NAC effect on ROS and GSH within AIHA-blood cells
Human A-type normal RBC were treated with 1 mM of CB3and NAC, for 20 minute, followed by incubation with autologous serum (C') and anti-A mouse monoclonal antibodies. RBC were washed and assayed for ROS.

Our results suggested that these Trx-mimetic peptides are potentially excellent candidates for treating oxidative stress-related disorders.

5. DISCUSSION

Oxidative stress arises when the balance between oxidants and antioxidants is tipped in favor of the former. This phenomenon may be influenced by exogenous agents (such as radiation, trauma, drug activation, oxygen excess), but also by endogenous factors which are associated with many pathological states including inflammatory disorders, cardiovascular diseases, injury to the central nervous system, pulmonary diseases, diabetes disorders, etc. (Kohen and Nyska, 2002). Antioxidants have been tried clinically in some oxidative-related disorders (e.g., diabetes disorders) (Giacco et al., 2010). Although not the primary etiology, oxidative stress plays an important role also in hemolytic anemias, mainly by causing damage to RBC. Antioxidants can neutralized the oxidative stress, but clinical trials have been carried out mainly in β-hemoglobinopathies but not in other types of hemolytic anemia. For examples, it has reported that antioxidants, such as the thiol compound N-acetylcysteine (NAC), vitamin C and the vitamin E derivative, tocotrienol, reduced oxidative stress in blood cells derived from thalassemic patients (Amer et al.,2004,Amer et al.,2005). Despite extensive research efforts in recent years, yielding promising results, there is still a need for additional research on oxidative stress in humans. In our study we investigated, for the first time, the oxidative status of other types of hemolytic anemias and the effect of other antioxidant compounds such as, FPP, EPO and CB3. Our result suggested that these antioxidants might be used as potential therapeutic modality.

5.1 Measurement of oxidative stress by flow cytometry

The technical aspects of the assays present a serious limitation. For example, methods for ROS measurements, such as electron spin resonance and spin trapping, are complicated and of poor sensitivity. A technique that demonstrates, identifies and quantifies free radical species applicable to human studies is of fundamental importance. Such technique should enable routine clinical applications such as screening and identifying subjects with oxidative stress and monitoring the effects of pharmacological treatments, antioxidant supplementations or lifestyle changes.

We developed a methodology based on flow cytometry techniques to measure multiple aspects of oxidative stress in various blood cells. Flow cytometry offers several advantages: (1) the ability to quantify single cell rather than the mean of the total population, (2) Using a gating strategy, specific sub-populations can be measured, while contaminating cells are excluded. The data is expressed in arbitrary fluorescence units rather than weight or molar concentrations, but it is useful for comparative purposes. Recently, an automatic and robotic high throughput technology has been utilized for screening of thousands of compounds for their activities.
Several fluorescent probes have been used in flow cytometry for measuring oxidative stress. Such probes are cell permeable and undergo cell sequestration, so that significant intracellular concentrations can be achieved. These probes are able to change their fluorescence properties upon interaction with the tested compound have high specificity and low rate of spontaneous conversion with minimal cellular toxicity.

The flow techniques enabled us to measure ROS generation by 2'-7'-dichlorofluorescin diacetate (DCF-DA), GSH content by staining with mercury orange, Lipid Peroxidation by Fluor-DHPE and Phosphatidylserine (PS) apoptosis marker by Annexin V stain. Using these mentioned approaches, we demonstrated that in β-thalassemia, hereditary spherocytosis, paroxysmal nocturnal hemoglobinuria (PNH) as well as autoimmune hemolytic anemia (AIHA) RBC's are under oxidative stress. These cells generate increased amounts of ROS, PS as well as membrane lipid peroxidation and have decreased amounts of GSH compared to normal cells.

5.2 Oxidative stress in thalassemia

We showed that ROS and lipid peroxidation were higher, and GSH lower, in thalassemic RBC compared with normal RBC, both at baseline as well as following oxidative stress, such as exposure to hydrogen peroxide.

In thalassemia patients, and especially following splenectomy, nucleated RBC precursors (normoblasts) and mature RBCs and retics that contain DNA remnants (Howell-Jolly bodies, HJ) are detectable in large numbers in the circulation. Normally, such cells do not exist in the peripheral blood. Using flow cytometry, we showed that HJ-RBCs and retics are under oxidative stress and carry exposed PS, which may present the trigger for their phagocytosis by macrophage and removal in the spleen.

We demonstrated that several oxidative stress-induced abnormalities can be shown to be ameliorated by antioxidant treatment. In *β*-thalassemia, Epo treatment was shown to improve the state of anemia. The rational of this treatment in thalassemia is twofold: to stimulate erythropoiesis and to elevate the production of fetal Hb; the latter compensates for the lack or reduced content of HbA. Epo is known to have a protective effect in non erythroid cells, such as neuronal cells and cardiomyocytes (Joyeux-Faure et al., 2007). Several reports attributed the protection by Epo in non erythroid cells to its anti-oxidative effect (Katavetin et al., 2007, Wu et al., 2007). We reported a direct effect of Epo on RBC, which is unrelated to erythropoiesis. The effects were observed in vitro by incubating peripheral blood RBC with Epo as well as shortly (3 hrs) after injection of Epo into *β*-thalassemic mice. Our results suggest that Epo might alleviate symptoms of hemolytic anemias as an antioxidant.

5.3 Oxidative stress in hereditary spherocytosis

Using flow cytometry, we documented the presence of oxidative stress in 17 HS patients from seven Palestinian Arab families. This was demonstrated by increased generation of ROS and lipid peroxides and lower levels of GSH in their RBC, compared with healthy control donors. The finding of oxidative stress in RBC from patients with HS can be explained in part by the higher rate of Hb auto-oxidation and methemoglobin formation (Cadet et al., 2010), which may be caused by the increased intracellular Hb concentration (MCHC) due to RBC dehydration. Accumulation of oxidized

Hb close to the RBC membrane promotes localized oxidant damage to skeletal proteins and phospholipids, ultimately disrupting the membrane structure and function (Cadet et al., 2010). It has also been reported that spectrin in HS-RBC is highly sensitive to oxidative stress, which may contribute to membrane damage (Belloni et al., 2011).

We demonstrated that treatment with FPP ameliorated the oxidative parameters in HS and decreased hemolysis both in vitro and in vivo. The antioxidant properties of FPP (Keerthivasan et al., 2011) could be attributed to its high content of glutamic acid, glycine, and methionine, which serve as a substrate for glutathione synthesis. Amelioration of oxidative stress parameters by FPP has also been reported in thalassemia but without significant changes in hematological parameters (Khandelwal et al., 2007). A possible explanation could be the fact that free iron species such as labile plasma iron and intra cellular labile iron are higher in thalassemia, resulting in increased generation of ROS, compared with HS or PNH (see below), where improvement in hematological parameters has been found (Galili et al., 1958).

5.4 Oxidative stress in complement-mediated hemolysis (PNH and AIHA)

Using flow cytometry methodology, we found that PNH-RBC are under oxidative stress: their ROS is higher and their GSH is lower than normal RBC. In PNH, both pathological and normal stem cells coexist, giving rise to a mosaic of normal blood cells, having the CD55+CD59+ phenotype, and abnormal cells with a CD55–CD59– phenotype. In double-staining experiments with fluorescent antibodies to CD55/CD59 and oxidative stress markers, we demonstrated a higher oxidative status in cells derived from the pathological clone compared with cells derived from normal clones in the same patient. Indeed, we noticed that normal cells (with the CD55/CD59-positive phenotype) in the blood of PNH patients were at higher oxidative status than cells from blood of normal donors. Complement (C')-mediated hemolysis due to the deficiency in CD55/CD59 is the major feature of PNH. We showed that in vitro treatment of PNH-RBC with complement resulted in oxidative stress prior to any signs of hemolysis and that anti-oxidants such as CB3 and NAC reduced hemolysis, suggesting the involvement of oxidative stress as a mediator or an adjuvant. PNH is a rare disease, to study various aspects of PNH; we used an artificial PNH-like RBC. Normal RBCs were incubated with sulphydryl compound 2-aminoethylisothiouronium bromide (AET). AET induces susceptibility to complement-mediated lysis by disrupting the structural and functional integrity of membrane constituents such as decay accelerating factor (DAF), a membrane inhibitor of reactive lysis (MIRL), and complement receptor type 1 (CR1) that regulate the activity of both the C3 convertases and the membrane attack complex of complement (Sirchia, Ferrone and Mercuriali, 1965; Sirchia, Ferrone, Milani and Mercuriali, 1966).

AIHA is another form of C'-mediated hemolytic anemia, caused by auto-antibodies against antigens expressed on the surface of RBCs. Once formed, these antibodies bind to the surface of RBCs marking them for destruction through C'-mediated lysis (intravascular hemolysis) and/or Fc-mediated phagocytosis

(extravascular hemolysis). It is a serious systemic autoimmune disease for which there are no workable therapies other than splenectomy and general immunosuppressive and anti-inflammatory drugs.

To simulate an AIHA situation, two AIHA in vitro models were developed: human A-type normal RBCs were incubated with (a) anti-A monoclonal antibodies and C' (b) O sera (containing C' and Ab's). In the two models, hemolysis was observed. This was preceded by an abrupt increase in ROS generation. By themselves, neither antibodies nor C' induced hemolysis.

Antioxidants such as NAC and CB3 reduced the C'-mediated increase in ROS as well as the subsequent hemolysis. These Thioredoxin Mimetic Peptides (Trx) are blocked at both the N-and the C-termini, enabling them to freely traverse cell membranes and gain access to the cytosol. They were examined for their ability to improve the redox state of cells during oxidative stress (Bartov et al., 2006, Bachnoff et al., 2011). These peptides are expected to chemically reduce Trx-target proteins such as oxidoreductase, reversing oxidative stress by mimicking Trx's activity attributed to the consensus CxxC motif present at the catalytic site. As dithiols, these peptides could, in addition to the more selective and specific catalytic action on Trx substrates, act also directly as antioxidants, by interacting with and neutralizing noxious oxidant species. Acting as ROS scavengers and glutathione precursors, some of these peptides have been shown to effectively attenuate mitogen-activated protein kinase (MAPK) phosphorylation/activation and reverse NF-κB translocation to the nucleus under conditions of oxidative stress in vitro (Bartov et al., 2006 ,Lee et al.,2007).

The Trx peptides exhibited a significantly higher efficacy compared to the conventional thiol-containing antioxidants, including *N*-acetylcysteine (NAC), GSH, dithiothreitol, and ascorbic acid. It was shown that a novel thiol N-acetylcysteine amide (AD4), the amide form of NAC, was much more effective than the parental compound in its antioxidant and protective effects on oxidative stressed human RBCs (Grinberg et al.,2005). In our study, we investigated the antioxidant properties of a new hydrophobic thiol compound (**CB3**) compared to the known antioxidant *N*-acetylcysteine (NAC). Our results provide evidence for the superiority of CB3 in elevation of GSH levels and in inhibition of RBC lysis, supporting its potential clinical use in oxidative stress related disorders.

In summary, oxidative stress plays an important role in hemolytic anemias, although it is not the primary etiology of these diseases but it participates in causing damage in RBC, and it is effect can be neutralized by antioxidants.

6. CONCLUSIONS

- β-thalassemia is wildly spread in Israel and the Palestinian Authority. Treatment with anti-oxidants decreases ROS levels and ameliorates clinical symptoms associated with oxidative stress.

- Flow cytometry, a standard technology in most hematological and immunological labs, can be useful for measuring the oxidative status of RBC, platelets and PMN in various diseases, such as SCA and thalassemia and for studying various chemical agents as potential anti-oxidants.

- Oxidative stress in developing erythroid precursors might generate HJ-retics and HJ-RBCs and that oxidative stress induced externalization of PS might be involved in their removal from the circulation by the spleen, a mechanism similar to that of the removal of aging (senescent) RBCs.

- Epo might alleviate symptoms of hemolytic anemias as an antioxidant.

- Oxidative stress plays an important role in the pathophysiology of HS which can be ameliorated by an antioxidant such as FPP.

- In Intra-vascular complement mediated hemolysis such as PNH as well as AIHA, neither antibodies nor C' alone induced hemolysis.

- Thioredoxin Mimetic Peptides (Trx) such as CB3 are potentially excellent candidates for treating oxidative stress-related disorders.

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9. APPENDICES

Appendix 1. Dana M, Prus E, and Fibach E, "*Thalassemic DNA-Containing Red Blood Cells Are under Oxidative Stress*" *Anemia*, vol. 2012, pp. 1–5, 2012.

Appendix 2. Amer J, **Dana M**, Fibach E. *The antioxidant effect of erythropoietin on thalassemic blood cells*. Anemia 2011; 2010(978710).

Appendix 3. Ghoti H, Fibach E, **Dana M**, et al. *Oxidative stress contributes to hemolysis in patients with hereditary spherocytosis and can be ameliorated by fermented papaya preparation*. Ann Hematol 2010; 90(5):509-13