

Combined effect of α -lipoic acid and N-acetylcysteine: influence on the prooxidant state of Fanconi anemia cells

Ana Sofia Afonso Gomes

Mestrado em Bioquímica

Química e Bioquímica

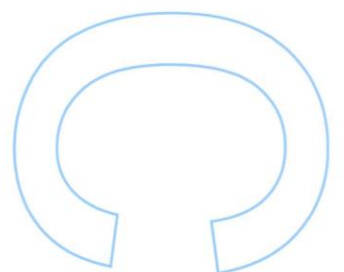
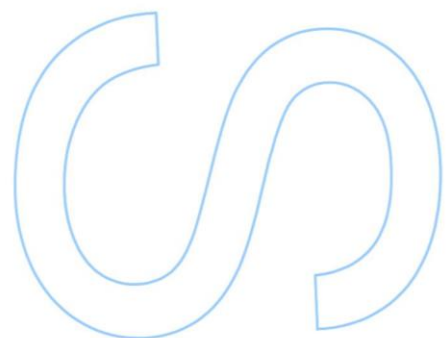
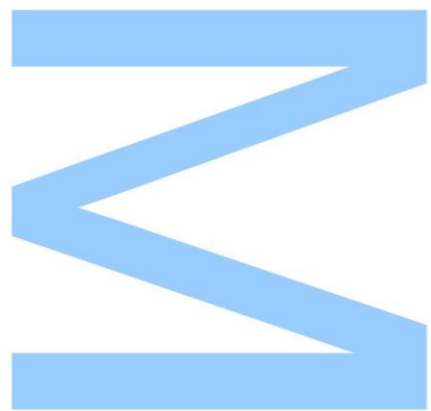
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Orientador

Beatriz Porto, Professora Auxiliar, Instituto de Ciências
Biomédicas de Abel Salazar

Coorientador

Pedro Fontes Oliveira, Professor Afiliado, Instituto de
Ciências Biomédicas de Abel Salazar



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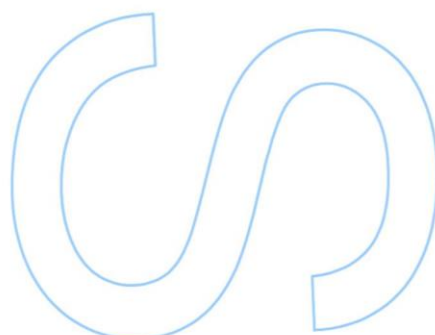
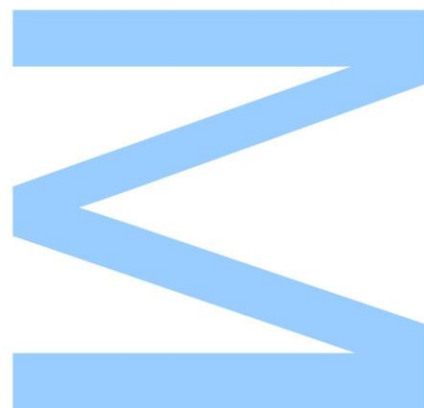


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Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____/____/____



**We cannot solve our problems with the same thinking
we used when we created them – Albert Einstein**

I. ACKNOWLEDGEMENTS

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II. ABSTRACT

Fanconi Anemia (FA) is a complex genetic disorder, with mutations in one of the 16 genes so far characterized that function in a common pathway for the maintenance of genomic stability. FA is commonly characterized by bone marrow failure, increased risk to malignancies and excessive sensitivity to DNA crosslink agents, such as diepoxybutane (DEB). A number of independent studies support the relationship between hypersensitivity to DEB and oxidative stress (OS), and emphasized that glutathione (GSH)-based pathways have an important role in this system.

It has been extensively described that FA cells are in a continuous cellular prooxidant state that results in chromosome instability (CI). It is also known that mutations in the FA pathway result in down regulation of the endogenous antioxidant defense, including the red blood cells (RBC), which are considered one of the most important endogenous antioxidant defense systems. Therefore, FA cells may benefit with an antioxidant therapy independent of the FA pathway control.

In a previous work, based on the hypersensitivity of FA cells to DEB, the authors searched for antioxidant molecules with ability to counteract the DEB related OS; this study lead to the selection of two orthodox antioxidant small molecules: N-acetylcysteine (NAC) and α -lipoic acid (ALA). Afterwards, *in vitro* studies were performed confirming that the ALA and NAC improve genetic stability, decreasing CI in cultured lymphocytes from FA patients.

The aim of the present study was to evaluate the influence of ALA and NAC in the redox state of RBC in blood cultures from FA patients, comparatively to the influence of the same antioxidants in DEB-induced cultures from healthy donors. For that purpose, the following OS parameters we evaluated: levels of GSH and glutathione peroxidase (GPx) activity; expression of protein oxidative damage, by the determination of carbonyl group content; the antioxidant potential, by the ferric reduction antioxidant potential (FRAP) assay.

The results showed that exposure to the antioxidants increased GSH levels and decreased GPx activity in RBC from FA patients. In RBCs from healthy donors no differences were observed both in GSH levels and GPx activity. ALA and NAC had no effect on the expression of protein oxidative damage and on the antioxidant potential, either in RBC from FA patients and healthy donors. Knowing that FA cells are in a

continuous prooxidant state, our results, based on GSH levels, suggest that the addition of ALA and NAC may improve the endogenous antioxidant defense and, consequently, improve the redox state of FA RBCs. The simultaneous increase in GSH levels and decrease in GPx activity may point to a combined redox dependent mechanism of ALA and NAC to improve antioxidant defense in FA RBC, by supplying an extra source of GSH and decreasing the requirement of GPx activity.

Based on these and previous results, we can hypothesize that NAC and ALA may be good prophylactic candidates to decrease the adverse effects of OS-related damage, having potential to be used as an extra source of exogenous antioxidant defense to FA patients.

KEYWORDS

Fanconi anemia; Oxidative stress; Red blood cells; Antioxidants; α -lipoic acid; N-acetylcysteine; Chromosome instability.

III. RESUMO

A anemia de Fanconi (FA) é uma doença genética complexa, com mutações em um dos 17 genes até agora caracterizados que funcionam numa via comum para a manutenção da estabilidade genómica. A FA é caracterizada por falência medular progressiva, um aumento do risco para neoplasias e uma sensibilidade excessiva a agentes indutores de DNA *crosslink*¹, tais como o diepoxibutano (DEB). Vários estudos independentes apoiam a relação entre a hipersensibilidade ao DEB e o *stress* oxidativo (OS), e salientam que as vias que envolvem glutathiona (GSH) têm um papel importante neste sistema.

Tem sido extensamente descrito que as células FA estão num contínuo estado prooxidante que resulta em instabilidade cromossómica (CI). É também sabido que mutações na via FA resultam numa regulação negativa na defesa antioxidante endógena, incluindo em eritrócitos, que são considerados um dos mais importantes sistemas de defesa antioxidante endógeno. Assim sendo, as células FA poderão beneficiar com uma terapia antioxidante independente do controlo da via FA.

Num trabalho prévio, com base na hipersensibilidade das células FA ao DEB, foram pesquisados antioxidantes com capacidade para proteger as células do OS relacionado com o DEB. Esse estudo levou à seleção de duas pequenas moléculas antioxidantes: o ácido α -lipoico (ALA) e a N-acetilcisteína (NAC). Mais tarde, estudos *in vitro* confirmaram que o ALA e o NAC melhoram a estabilidade genómica, diminuindo a CI em culturas de linfócitos de pacientes FA.

O objetivo do presente estudo foi avaliar a influência do ALA e NAC no estado redox dos eritrócitos de pacientes FA obtidos a partir de culturas de sangue com e sem tratamento antioxidante, comparativamente com a influência dos mesmos antioxidantes em eritrócitos de doadores saudáveis obtidos a partir de culturas de sangue induzidas por induzidas por DEB. Para essa finalidade foram avaliados os seguintes parâmetros de OS: níveis de GSH e atividade de glutathiona peroxidase (GPx); expressão de dano oxidativo em proteínas, quantificada pelo conteúdo de grupos carbonilo; o potencial antioxidante, medido pelo método do potencial antioxidante de ferro redutor (FRAP).

¹ *do inglês*, agente intercalante de DNA

Os resultados mostraram que a exposição aos antioxidantes aumentou os níveis de GSH e diminuiu a atividade de GPx nos eritrócitos de pacientes FA. Nos eritrócitos dos dadores saudáveis não foram observadas diferenças quer nos níveis de GSH quer na atividade da GPx. O ALA e o NAC não tiveram efeito na expressão de dano oxidativo em proteínas nem no potencial antioxidante, tanto em eritrócitos de pacientes FA como de dadores saudáveis.

Os resultados, baseados nos níveis de GSH, sugerem que a adição dos antioxidantes ALA e NAC podem melhorar a defesa antioxidante endógena e, consequentemente, melhorar o estado redox dos eritrócitos de pacientes FA. O efeito simultâneo de um aumento nos níveis de GSH e de uma diminuição da atividade GPx sugere a ocorrência de um mecanismo protetor do ALA e NAC que melhora a defesa antioxidante dos eritrócitos dos doentes FA, através de uma fonte extra de GSH e de uma diminuição no requerimento da atividade GPx. Com base nestes resultados e em estudos anteriores propõe-se que o ALA e o NAC podem ser bons candidatos profiláticos para diminuir os efeitos adversos relacionados com OS, tendo potencial para serem usados como uma fonte extra de defesa antioxidante para os pacientes FA.

PALAVRAS-CHAVE

Anemia de Fanconi; Stress oxidativo; Eritrócitos; Antioxidantes; Ácido α -lipóico; N-acetilcisteína; Instabilidade Cromossómica

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V. ABBREVIATIONS

AML – acute myelogenous leucemia

ALA – α -lipoic acid

CAT – Catalase

CHP – Hospital Geral de Santo António

CI – Chromosome instability

DEB – 1,2:3,4-Diepoxybutane

FA – Fanconi Anemia

FRAP – Ferric Reducing Antioxidant Power

GSH – Glutathione

GPx – Glutathione peroxidase

GR – Glutathione reductase

HD – Healthy donors

Hb – Hemoglobin

ICBAS – Instituto de Ciências Biomédicas Abel Salazar

ICL – Interstrand crosslink

metHb – Methemoglobin

NAC – N-acetylcysteine

OS – Oxidative Stress

ROS – Reactive oxygen species

RBC – Red blood cell

RT – Room temperature

SCT – Stem cell transplantation

SOD - Superoxide dismutase

UV – Ultraviolet

1.INTRODUCTION

1.1 The Fanconi Anemia (FA) Disease

Fanconi anemia (FA) is a rare autosomic or X-linked recessive disorder reported for the first time by Guido Fanconi, a Swiss pediatrician, who recognized siblings with “peculiar phenotypic abnormalities and anaemia” (Fanconi, 1927). Nowadays it is known that FA is clinically characterized by diverse congenital malformations, bone marrow failure, and predisposition to cancer, in particular acute myelogenous leukemia (AML) (Kutler et al., 2003). At molecular level, seventeen genes have been characterized so far (FANC-A, -B, -C, -D1 (BRCA2), -D2, -E, -F, -G, -I, -J (BRIP1), -L, -M, -N (PALB2), -O (RAD51C), -P (SLX4), -Q (ERCC4) and -S (BRCA1)) (Sawyer et al., 2015; Virts et al., 2015). It is known that these genes function together in an ordinary pathway, the FA-BRCA pathway, involved in the repair of interstrand DNA crosslinks (ICLs) Therefore, a mutation in one of those genes originates genomic instability, by lack of ICL repair (reviewed in Schneider, Chandler, Tischkowitz, & Meyer, 2014).

Although FA patients may be clinically and genetically variable, they are characterized by a common cellular feature: hypersensitivity to ICL agents. ICLs are very toxic lesions that block DNA replication and transcription. The lack of ICL repair results in increased genomic instability, represented, at cellular level, by increased chromosome instability (CI), including breaks, tri- and tetra-radial figures, that can be observed in metaphases obtained from cultured lymphocytes (Figure 1) (Auerbach, 1989).

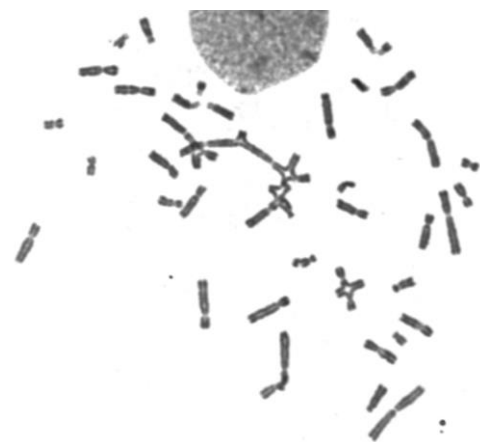


Figure 1: Diepoxybutane (DEB)-induced chromosome instability (CI) in Fanconi anemia(FA) patient.

Selected metaphase from a FA lymphocyte culture exposed to 0.1 $\mu\text{g/ml}$ of DEB for 48 h. Chromosomes were stained with 4% Giemsa solution. High levels of CI can be visualized; the presence of tri- and tetra-radial figures is particularly important for the diagnosis, since they can discriminate FA from the other CI syndromes. The image was captured with an optical microscope (500x amplification). Illustration from chapter II of Giovanni, Pagano. Fanconi Anemia and Oxidative Stress: Mechanistic Background and Clinical Prospects. New York United States: Nova Science Publishers Inc, 2015

1.2 Relationship between Oxidative Stress (OS) and Fanconi Anemia

Oxidative stress (OS) mediated by reactive oxygen species (ROS) is a major cause of DNA ICLs. Some of them produce or are free radicals. Free radicals are defined by any species containing unpaired electrons being alone in the orbital (Halliwell, 1991). Increased levels of OS are very prejudicial to the organism because they can damage membranes, proteins, and DNA. This effect is a factor involved in the development of many chronic and degenerative aging diseases (Packer, 1995).

Nordenson described for the first time the role of OS in FA cells damage (Nordenson, 1977), showing that superoxide dismutase (SOD) and catalase (CAT) decreased the frequency of spontaneous CI in cultured lymphocytes. Joenje and Oostra described for the first time a direct correlation between high tensions of oxygen and increased damage in FA lymphocytes (Joenje and Oostra, 1983). Another study demonstrated an overproduction of ROS by FA leukocytes, due to NADPH oxidase activation, decrease in cellular antioxidant defense systems, increase in cellular free iron levels and a deficiency in SOD measured in red blood cells (RBCs) (Korkina et al., 1992). Takeuchi and Morimoto suggested that a high sensibility of FA lymphoblastoid cells to oxidative DNA damage is possible, resulting from a decrease in CAT activity (Takeuchi and Morimoto, 1993). All these studies provide evidence that OS is an important element responsible for the FA cellular phenotype.

1.3 Relationship between Oxidative Stress and diepoxybutane (DEB) toxicity

The hypersensitivity of FA cultured lymphocytes to ICL agents, that leads to a an increase of CI, supplies a diagnostic test for the disease (D'Andrea and Grompe, 2003). Since 1989, the alkylating agent 1,2:3,4-diepoxybutane (DEB) is used as a certified diagnostic tool for FA (D'Andrea and Grompe, 2003); Kutler et al. 2003). Its mode of action implies alkylation of nucleic acid bases producing monoadducts (Gherezghiher et al., 2013; Wen et al., 2011). This monoadducts can suffer again alkylation or hydrolysis with neighboring nucleobases to form DNA-DNA cross-links (Gherezghiher et al., 2013). Evidence points to the association of DEB toxicity with redox mechanisms. In fact, it was described that the epoxide structure of DEB implies redox-mediated catalysis in the rearrangement of oxygen bonds (Bartók and Láng,

1980). Additionally, it became known that glutathione (GSH)-based pathways are involved in the metabolism and detoxification of DEB. Korkina and co-workers (2000) showed that DEB induces high mortality and modulates CAT activity, affecting hydrogen peroxide (H_2O_2) balance in sea urchin embryos (Korkina et al., 2000). All these studies support the association between DEB toxicity and OS, and emphasize that GSH-based pathways have an important role in this system. Assuming that, the increase of CI induced by DEB may be an indirect consequence of OS damage.

1.4 Importance of red blood cells (RBCs) in measurement of oxidative stress (OS)

The most abundant cell in the human body is the red blood cell (RBC). RBCs are very important and highly specialized in the transport of O_2 from lungs to tissues through hemoglobin (Hb). Because of the elevated amount of O_2 in contact with Hb, specifically to ferrous iron, oxidate Hb to methemoglobin (metHb) which is incapable to bind O_2 . MetHb levels can reach toxic levels under OS conditions. For that reason, RBCs have strong enzymatic and non-enzymatic antioxidant machineries, that are capable to move and scavenger free radicals from any tissue. So, they protect themselves, other tissues and organs (Arbos et al., 2008; Siems et al., 2000). Reduced GSH, ascorbic acid, α -tocopherol and other thiol groups are the major non-enzymatic antioxidants. Some of the main endogenous enzymatic protectors are SOD, glutathione peroxidase (GPx), glutathione reductase (GR) and CAT (Pandey and Rizvi, 2011).

Previous reports have shown that FA patients present RBCs damage by deficiency in SOD activity, although other enzymes such as GPx and CAT, may present normal activities (Joenje et al., 1979; Okahata et al., n.d.; Yoshimitsu and Kobayashi, 1984). In other study led by Gille and co-workers (1987) it was reported that FA fibroblasts exhibit normal CuZn-SOD activities and normal levels of GSH, but in the case of Mn-SOD, CAT, and GPx activities were consistently higher. They hypothesized that the increase of these activities might reflect a cellular prooxidant state in FA caused by an increased formation of endogenous oxidizing molecular species that induce an increased synthesis of enzymatic antioxidant defenses (Gille et al., 1987).

The biconcave disc form of mature RBC is also essential for its normal function. It is determined by membrane protein and minimal changes in the surface may manage to morphological and functional abnormalities. Lipid peroxidation caused by ROS can injury RBC shape. In a morphometric study presented by Malorni and co-workers

(2000) it was shown that RBCs from FA patients were significantly altered, and they hypothesized that those morphologic changes could be the result of the redox levels alterations (Malorni et al., 2000).

Another study by Porto and co-workers (2010) showed that normal RBC had a significant protective effect when compared with FA RBC, by testing the effect of normal RBC on the protection against DEB-induced CI in FA primary lymphocytes (Porto et al., 2010).

1.5 Link between FA pathway and antioxidant defense system

The influence of OS in the FA pathway has been extensively studied. Du and co-workers (2012) have reported that this pathway is involved in safeguarding the cellular antioxidant defense system. Specifically, FA patients showed a down-regulation of antioxidant defense genes, correlated with increased DNA damage in the promoters of those genes. They proposed that, in the presence of OS damage, the function of FA proteins is to regulate gene expression of antioxidant defense in order to protect the promoters from oxidative DNA damage. Accordingly, the loss of protective gene function causes an increment of OS damage (Du et al., 2012). These findings may suggest that the hypersensitivity of FA cells to OS is due to a down-regulation of genetic antioxidant control. Therefore, other enzymatic and non-enzymatic antioxidant defenses, working apart from the FA genetic control, may be important in protecting cells from OS damage.

In summary, all these studies supply direct and indirect evidence that OS damage involved in FA phenotype is a direct cause for the increased CI, which may be counteracted by antioxidant agents other than the endogenous systems dependent of the FA pathway control.

1.6 Antioxidant defense

Some antioxidants are included in food as vitamin E, vitamin C, beta-carotene, leutin, α -lipoic acid (ALA), coenzyme Q10, lycopene, zeaxanthines, and selenium (I. Jialal and Devaraj, 2005; Lonn et al., 2002; Pruthi et al., 2001). Several of *in vitro* studies have been done to test the use of antioxidants to decrease chromosome damage in FA cells. Dallapiccola and co-workers (1985) described a partial correction of CI in FA lymphocytes treated with several antioxidants, like L-cysteine, 2-mercaptoethanol, α -

mercaptopropionyl-glycine and GSH, all of them known to be donors of the SH group (Dallapiccola et al., 1985). Later, the same group (Porfirio et al., 1989) obtained a 50% reduction of the spontaneous CI with desferoxamine, an iron chelator.

Researchers have found that combinations of antioxidants can be profitable in cases of pathology associated with increased OS levels (Age-Related Eye Disease Study Research Group, 2001; Milton et al., 2006; Rodriguez et al., 2007). In the last few years, several studies have been performed with a combined treatment of N-acetylcysteine (NAC) with α -lipoic acid (ALA), suggesting a powerful antioxidant effect (Zembron-Lacny et al., 2009). Some of them have shown an increased cellular viability and also in GSH and ATP percentages (Columbaro et al., 2014). Due to the unique hypersensitivity of FA cells to DEB, Ponte and co-workers (2012) have used NAC and ALA to reduce the clastogenic effect of DEB and prevent spontaneous CI. These antioxidants have shown protective roles against GSH depletion (Ponte et al., 2011) and, mostly, they demonstrated an increased capacity to reduce CI in DEB-induced primary lymphocytes of both FA patients and healthy individuals (Ponte et al. 2012). This group have also found that both molecules could act together, as a cocktail ALA+NAC, and it was shown a protective effect reducing significantly CI in spontaneous and DEB-induced FA lymphocyte cultures (between 60% and 80%). Presuming that this *in vitro* studies could occur also *in vivo*, it can be suggested that this cocktail ALA+NAC is a promising prophylactic way to decrease the adverse effects of oxidative damage in FA patients. This supposition is based on the particular properties of both antioxidants.

1.6.1 Alpha Lipoic Acid (ALA)

ALA is a disulphide natural and endogenous compound that occurs in mitochondria (Ponte et al., 2012) as a cofactor in metabolic reactions involving enzyme complexes such as pyruvate dehydrogenase, 2-oxo-glutarate dehydrogenase and branched chain oxo acid dehydrogenase (Tarnopolsky, 2008). ALA has beneficial effects against OS because of its synergistic action with other antioxidants (Prahalathan et al., 2006). This antioxidant exhibits a cyclic disulfide moiety which exists with its reduced form, dihydrolipoic acid (Ponte et al., 2012). This dihydrolipoic acid can reduce glutathione (GSH), vitamin C, vitamin E and coenzyme Q10 (Ponte et al., 2012; Tarnopolsky, 2008; Zembron-Lacny et al., 2009). It has been reported as well that ALA can increase these reduced GSH and restore these levels in GSH deficient cells (Mantovani et al., 2003).

1.6.2 N-acetylcysteine (NAC)

NAC is a sulphydryl group donor which acts as a precursor of reduced GSH and as direct scavenger of ROS. As a consequence of OS and inflammation, intracellular reduced GSH is exhausted. NAC can reverse this situation by regulating the redox equilibrium (Columbaro et al., 2014; Mantovani et al., 2003). A study demonstrated that NAC can inhibit ROS production and apoptosis induced by DEB in human lymphoblasts (Ponte et al., 2012).

Considering the above mentioned properties of ALA and NAC, we suggest that these two antioxidants may be good candidates to counteract the endogenous antioxidant defense systems dependent of the FA pathway control.

1.7 Aim of the study

FA cells are in a continuous prooxidant state, which results in increased CI, possibly due to a down regulation of the endogenous antioxidant defense, including the RBC system. Therefore, protection of FA cells against CI may be improved with an antioxidant therapy independent of the FA pathway control. Considering this, the aim of the present study is to evaluate the influence of the two selected antioxidants, ALA and NAC, in the redox state of FA RBCs. For that purpose, OS parameters will be evaluated in RBCs obtained from FA and healthy donors blood cultures with and without ALA and NAC treatments. Blood cultures from healthy donors will be exposed to DEB, in order to induce OS damage and consequently, a prooxidant state.

For the accomplishment of these objectives, the following studies will be performed:

- Effect of ALA and NAC on the frequency of CI in lymphocytes from healthy donors. This effect was already evaluated in in lymphocytes from FA patients.
- Effect ALA and NAC on GSH levels, GPx activity, antioxidant potential and protein oxidative damage in RBCs from healthy donors and FA patients

2. MATERIAL AND REAGENTS

See all the material and reagents used in the table 42 in appendix.

3. METHODS

3.1 Subjects

In this study 11, healthy donors (HD) recruited from the blood bank of Hospital Geral de Santo António (CHP) and 5 FA outpatients from CHP, with the diagnosis confirmed by DEB test in the Laboratory of Cytogenetics, ICBAS, were included in this study. All procedures were performed with the informed written consent of the participants.

For each experiment, 6-12 ml of blood was collected by venipuncture into vacuum tubes with lithium heparin, from each patient or control.

3.2 Experimental procedures

3.2.1 Isolation of RBC

HD RBC from day 0 and day 3 were isolated from peripheral blood and blood cultures, respectively, by a gradient density centrifugation method, using Histopaque solution 1077 in polypropylene centrifuge tubes. Briefly, 3 ml of collected blood was carefully layered on top of 3 ml of Histopaque 1077 in each 15 ml polypropylene tube. The tube was centrifuged at 890 \times g for 30 min at room temperature (RT). The RBC layer was removed to a separate tube. FA RBCs were previously isolated by the same method described and stored at -80 °C.

3.2.2 Whole blood cultures

Whole blood (0.5 mL) was cultured in RPMI 1640 complete medium supplemented with 15% fetal calf serum, antibiotics (10,000 units/mL of penicillin and 10,000 μ g/mL of streptomycin) and 29 mg/mL of L-glutamine. Cultures were stimulated with 5 μ g/mL of

phytohemagglutinin and placed in an incubator at 37°C with 5% CO₂ atmosphere, for 72 h.

3.2.3 Exposure to DEB

DEB ((±)-1,2:3,4-diepoxybutane, prepared in RPMI 1640, was added to appropriate cell cultures from HD, 24 h after their initiation, thus exposing cells to the chemical for 48 h. DEB was added at the final concentration of 0,4 µg/mL. Since DEB is a genotoxic agent specific precautions were taken. All culture procedures were handled using appropriate gloves and in a vertical laminar flow hood.

3.2.4. Antioxidant incorporation

Fresh solutions of the antioxidants ALA and NAC, at the final concentrations of 20 µM (Sigma) and 500 µM (Sigma) respectively, were added to cell cultures as described by Ponte et al 2012 (Ponte et al., 2012).

3.2.5. Antioxidant treatments

Three sets of peripheral blood cultures were performed for each condition (HD and FA patients). In the first set, cultures were pre-treated with α -LA 20 µM. In the second set, cultures were pre-treated with NAC 500 µM. In the third set, both antioxidants were added simultaneously, at the same concentrations. In all experiments from HD the antioxidants were added 1.5 h before DEB exposure. In FA experiments no DEB was added to the cultures, and the antioxidants were added 24h after culture initiation.

All cultures were performed in duplicate for cytogenetic evaluation and study of OS parameters.

3.3 Cytogenetic evaluation

After 3 days, cultures for cytogenetic evaluation were harvested after 1 h of incubation with colcemid (4 µg/mL), followed by hypotonic treatment with 75 mM KCl and fixed 3 times in a 1:3 iced solution of acetic acid : methanol. The resulting suspensions were dropped onto microscope slides and stained for 5 min in a 4% Giemsa solution diluted in phosphate buffer saline solution.

Analysis was performed on 50–100 metaphases from each experiment, by an independent scorer and in a blinded manner. Each cell was scored for chromosome number and the number and type of structural abnormalities. Gaps (acromatic areas less than a chromatid in width) were excluded in the calculation of chromosome breakage frequencies, and rearrangements (triradials and quadriradials, dicentrics and ring chromosomes) were scored as two breaks.

3.4 Characterization of oxidative stress parameters

3.4.1 Red blood cell processing for the study of oxidative stress parameters

In an aliquot with 250 μ L of isolated RBC it was added 1 mL of RBC lysis solution (1 M Tris-base HCL in a pH of 7.2, 5 M NaCl and 1 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) during 15 min. This suspension was centrifuged at 180 \times g for 5 min at RT. The RBC pellet was dispensed and the supernatant (hemolysate) was transferred to another aliquot. The hemolysates were immediately stored at -20 $^{\circ}\text{C}$ for posterior determination of OS parameters (GSH and GPx quantifications, ferric reducing antioxidant power (FRAP) and carbonyl group levels).

3.4.2 Sample processing for glutathione

Cell suspension aliquots were de-frozen and spun at 13000 \times g for 10 s at 4 $^{\circ}\text{C}$ and the supernatants discarded. The pellet was then treated with HClO_4 10%. After a brief vortexing, the homogenates were centrifuged 13000 \times g for 10 min at 4 $^{\circ}\text{C}$. Aliquots of the resulting supernatants were diluted 20x with HClO_4 5% and used for the determination of GSH levels.

3.4.3. Glutathione quantification

GSH quantification was performed by the DTNB-GR recycling assay, based on the oxidation of GSH by 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB), as described by Vandeputte and coworkers (Vandeputte et al., 1994) with some modifications (Carvalho et al., 2004). KHCO_3 0,76 M (200 μ l) was added to the supernatant (200 μ l) and centrifuged 13,000 \times g, 1 min at 4 $^{\circ}\text{C}$. Standard GSH corresponding to concentrations ranging between 0,5 and 15 nmoles were also prepared. Freshly prepared reagent solution (DTNB 0,7 mM and NADPH 0,24 mM in sodium phosphate-

EDTA buffer, pH 7,5) was added and incubated at 30 °C during 15 min, in the dark. Glutathione reductase (GR) was added immediately before measuring the plate at 412 nm by spectrophotometry. The concentration of total GSH was calculated using a standard curve. GSH levels were measured as μmol of GSH *per* mg of protein present in RBC. GSH percentage was calculated relatively to basal levels (day 0).

3.4.4 Protein quantification

Protein quantification from RBC was measured by BCA protein assay kit (Pierce) based on biuret reaction. Standard and RBC samples were added into a microplate well (2 μL of each). 200 μL of working reagent was added to each well and mixed for 30 sec and incubated in the dark at 37°C for 30 min. Protein concentration was measure at 595 nm by spectrophotometry. The concentration of total proteins was calculated using a standard curve. Proteins concentrations were measured as μg *per* mL of RBC.

3.4.5 Glutathione peroxidase activity determination

Glutathione peroxidase (GPx) assay kit directly measures NADPH consumption in the enzyme coupled reactions. The working reagent was prepared by mixing 84 μL assay buffer, 5 μL glutathione, 3 μL NADPH (35 mM) and 8 μL GR enzyme. 90 μL of working reagent was added to 10 μL of samples and controls wells and mixed. 100 μL of substrate solution, containing 1:10 peroxidase solution in distilled H_2O , was added to all samples and controls wells and mixed. The optical density was measure immediately (0 min) and again at 4 min at 340 nm. The measured decrease of NADPH on optical density at 340 nm is directly proportional to the enzyme activity in the sample. GPx percentage was calculated relatively to basal levels (day 0).

3.4.6 Ferric reducing antioxidant power assay

The FRAP of the cellular pellets was determined using the colorimetric method described by Benzie and Strain (1996). In brief, working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3,6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM in 40 mM HCl) and FeCl_3 (20 mM) in a 10:1:1 ratio (v:v:v). The reduction of the Fe^{3+} -TPTZ complex to a colored Fe^{2+} -TPTZ complex by the samples was monitored immediately after adding the sample and 40 min later, by measuring the absorbance at 595 nm using an Anthos 2010 microplate reader. The antioxidant potential of the

samples was determined against standards of ascorbic acid, which were processed in the same manner as the samples. Absorbance results were corrected by using a blank, with distilled water instead of sample. The changes in absorbance values of test reaction mixtures were used to calculate FRAP value as described elsewhere (Benzie and Strain, 1996).

3.4.7 Carbonyl Group levels

Protein carbonyl content is commonly used as a marker for protein oxidation. The content of protein carbonyl groups in RBCs from the different experimental groups was evaluated using the Blot technique and specific antibodies. For carbonyl groups evaluation, protein samples were derivatized using 2,4-dinitrophenylhydrazine (DNPH) to obtain dinitrophenyl (DNP) according to the method developed by Levine and collaborators (1990) (Levine et al., 1990). The slot-blot technique was performed using a Hybri-slot manifold system and the resulting PVDF membranes were incubated overnight (4°C) with a rabbit anti-DNP (1:5000). Samples were visualized using rabbit anti-goat IgG-AP (1:5000). Membranes were then reacted with ECFTM substrate and read using a BioRad FX-Pro-plus. Densities from each band were quantified using the BIO-PROFIL Bio-1D Software from Quantity One. Carbonyl group levels were measured compared to the control.

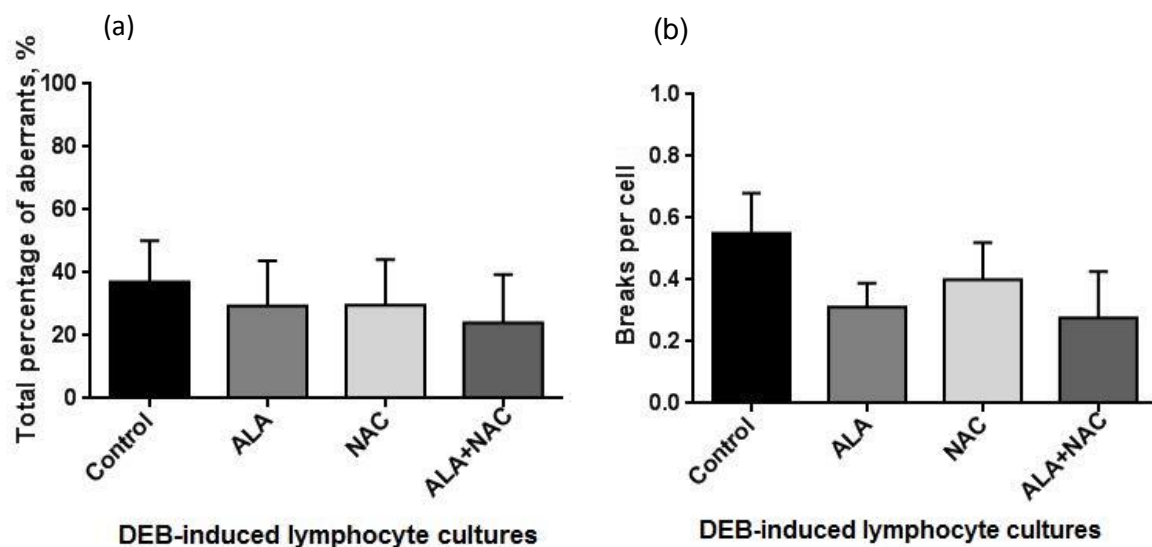
3.5 Statistical analysis

Results are expressed as mean \pm SEM. Statistical comparison among groups was estimated using one-way ANOVA, followed by the Bonferroni post hoc test or using paired t-test, both with GraphPad Prism 5. P values lower than 0.05 were considered as statistically significant.

4. RESULTS

4.1 Effect of α -lipoic acid and N-acetylcysteine on the frequency of chromosome instability in lymphocytes from healthy donors

The CI was evaluated by the percentage of aberrant cells (Graphic 1(a)) and the number of breaks per cell (Graphic 1(b)) in cultured lymphocytes from healthy donors. In Graphic 1(a) we can observed a decrease of the percentage of aberrant cells in ALA, NAC or ALA + NAC (29,5%, 29,7%, 24,0%, respectively) treatments comparability to the control group (37,0%). In the Graphic 1(b) we can see a reduction of the number of breaks per cell in the treatments ALA, NAC and ALA + NAC (0,31, 0,40, 0,28, respectively) comparability to the control group (0,55). In both cases, the observed reduction of CI was not statistically significant, either in the cultures treated with ALA, NAC or ALA+NAC.

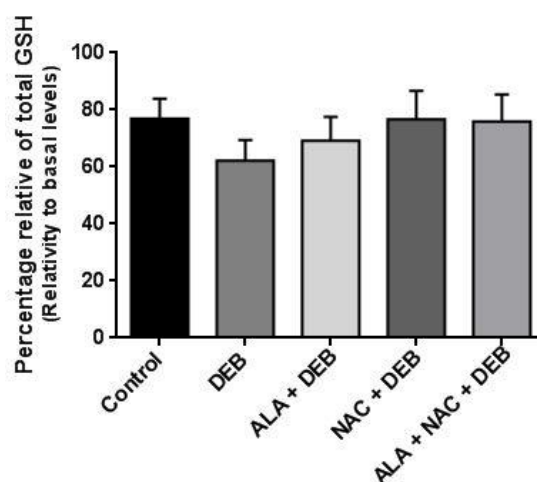


Graphic 1: Effect of α -lipoic acid (ALA) and N-acetylcysteine (NAC) on the frequency of 1,2:3,4-diepoxybutane (DEB)-induced (0,4 μ g/mL) chromosome instability in cultured lymphocytes from healthy donors.

Panel (a) – Percentage of aberrant cells; Panel (b) – Number of breaks per cell; Control - cultures without antioxidants; ALA – cultures treated with 20 μ M ALA; NAC – cultures treated with 500 μ M NAC; ALA+NAC – cultures treated with 20 μ M ALA and 500 μ M NAC. The observed reduction of CI was not statistically significant, either in the cultures treated with ALA, NAC or ALA+NAC Results are presented as the mean \pm SEM of 6 independent experiments.

4.2 Effect of α -lipoic acid and N-acetylcysteine on glutathione levels in red blood cells from healthy donors and Fanconi anemia patients

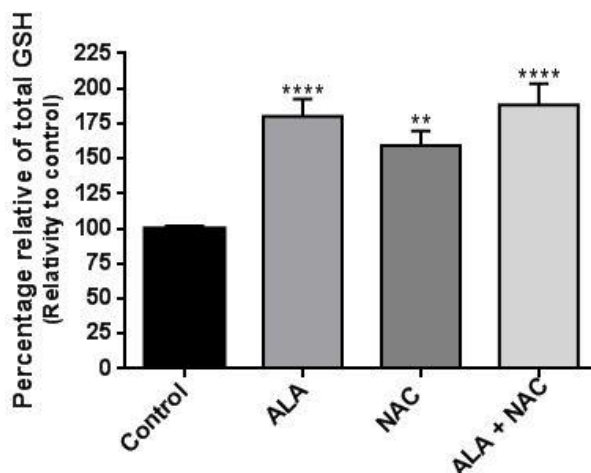
Total GSH concentration was measured on RBC present in DEB-induced cell blood cultures from healthy donors. As shown in Graphic 2, the decrease of the GSH in DEB-induced cultures comparatively to cultures in control conditions (21,64) was partially recovered with the addition of ALA, NAC and ALA+NAC (19,06; 21,17; 20,87) although no statistical significance was reached.



Graphic 2: Effect of α -lipoic acid (ALA) and N-acetylcysteine (NAC) on glutathione (GSH) levels in red blood cells from healthy donors blood cultures.

Control– cultures without treatments; DEB – cultures with DEB 0,4 μ g/mL; ALA+DEB - cultures with DEB 0,4 μ g/mL and treated with 20 μ M ALA; NAC+DEB - cultures with DEB 0,4 μ g/mL and treated with 500 μ M NAC; ALA+NAC+DEB - cultures with DEB 0,4 μ g/mL, treated with 20 μ M ALA, plus 500 μ M NAC. GSH percentage was calculated relatively to basal levels (day 0). Absolute values were converted in relative values; control value corresponds to 21,64. The observed recovery with the addition of ALA, NAC and ALA + NAC treatments was not statistical significant. Results are presented as the mean \pm SEM of 6 independent experiments.

Total GSH was also measured on RBC present in cultures from FA patients. As shown in Graphic 3, a significant increase of the GSH concentration was obtained after treatment with ALA, NAC or ALA+NAC when compared with the control treatment (0,27 vs. 1,20, $P \leq 0,0001$; 1,12, $P \leq 0,01$; and 1,34, $P \leq 0,0001$, respectively).

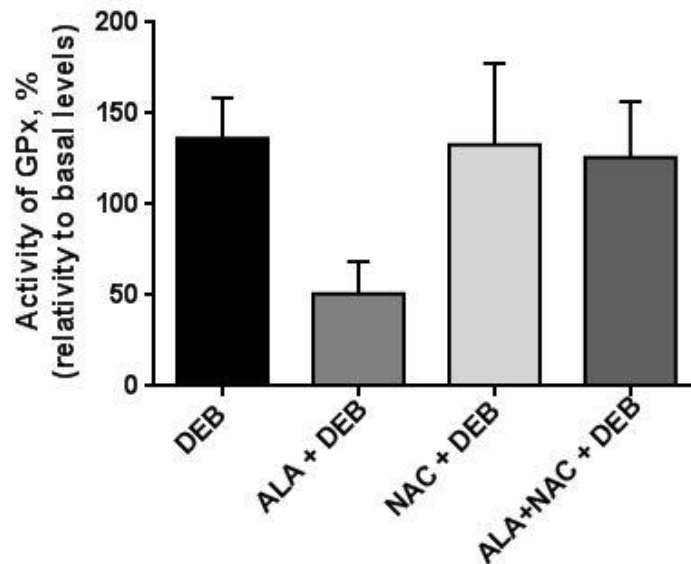


Graphic 3 Effect α -lipoic acid (ALA) and N-acetylcysteine (NAC) on glutathione (GSH) levels in red blood cells (RBCs) obtained from Fanconi anemia patients blood cultures.

Control– cultures without treatments; ALA - cultures treated with 20 μ M ALA; NAC - cultures treated with 500 μ M NAC; ALA+NAC - cultures treated with 20 μ M ALA, plus 500 μ M NAC. Absolute values were converted in relative values; 100% corresponds to 0,27 (control value). A significant increase of the GSH concentration was obtained after treatment with ALA, NAC and ALA+NAC. Results are presented as the mean \pm SEM of 6 experiments:**** $P < 0.0001$ and ** $P < 0.01$.

4.3 Effect of α -lipoic acid and N-acetylcysteine on glutathione peroxidase activity in red blood cells from healthy donors and Fanconi anemia patients

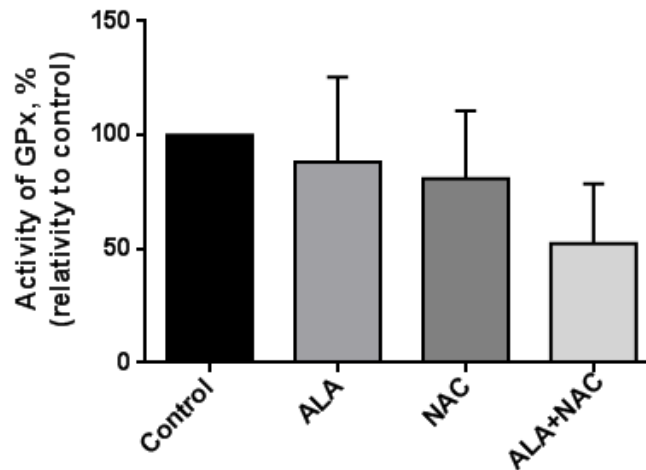
The activity of GPx was measured on DEB-exposed RBC from healthy donors blood cultures. As shown in Graphic 4, the addition of ALA decreases the activity of GPx (3,76) comparatively to that observed in cells from the DEB group (4,79 U/L), although no statistical significance was reached. NAC and ALA+NAC treatments remains with no alterations comparatively to the DEB group (4,79 U/L vs. 5,22 U/L; 6,04 U/L; respectively).



Graphic 4: Effect of α -lipoic acid (ALA) and N-acetylcysteine (NAC) on glutathione peroxidase (GPx) activity of 1,2:3,4-diepoxybutane (DEB)-induced (0,4 μ g/mL) red blood cells (RBCs) obtained from healthy donors blood cultures.

DEB– control culture with DEB 0,4 μ g/mL; ALA + DEB– cultures with DEB 0,4 μ g/mL and treated with 20 μ M ALA; NAC + DEB – cultures with DEB 0,4 μ g/mL and treated with 500 μ M NAC; ALA+NAC+DEB – cultures with DEB 0,4 μ g/mL, treated with 20 μ M ALA, plus 500 μ M NAC. Absolute values were converted in relative values; 100% corresponds to 2,82 U/L (basal levels value – day 0). As shown, ALA decreases the GPx activity and NAC and ALA + NAC treatments remains with no alterations, although no statistical significance was reached. Results are presented as the mean \pm SEM of 9 experiments.

The activity of GPx was also measured in cultured RBC obtained from FA patients. As shown in Graphic 5, a decrease of activity of GPx was observed after the treatment with ALA, NAC and ALA + NAC when compared with the control group, although it was not statistically significant (6,18 U/L vs. 4,61 U/L; 5,96 U/L; 3,56 U/L; respectively).

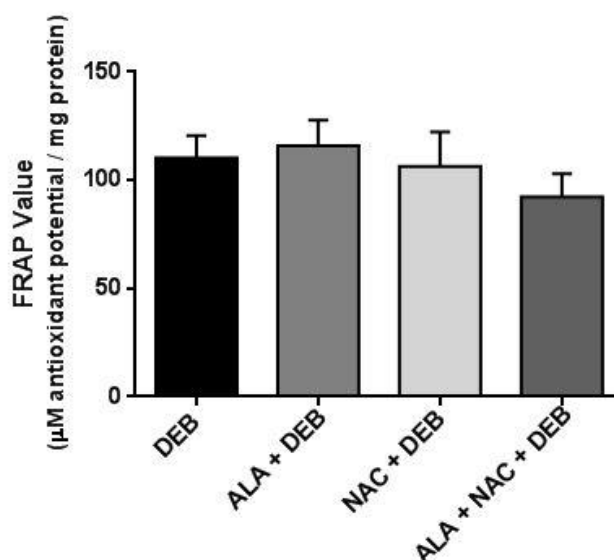


Graphic 5: Effect α -lipoic acid (ALA) and N-acetylcysteine (NAC) on glutathione peroxidase (GPx) activity in red blood cells (RBCs) obtained from Fanconi anemia patients blood cultures.

Control– cultures without treatments; ALA – cultures treated with 20 μ M ALA; NAC – cultures treated with 500 μ M NAC; ALA+NAC – cultures treated with 20 μ M ALA, plus 500 μ M NAC. Absolute values were converted in relative values; 100% corresponds to 6,18 U/L (control value). As shown, all the treatments decrease GPx activity, although no statistical significance was reached. Results are presented as the mean \pm SEM of 4 experiments.

4.4 Effect of α -lipoic acid and N-acetylcysteine on the antioxidant potential in red blood cells from healthy donors and Fanconi anemia patients

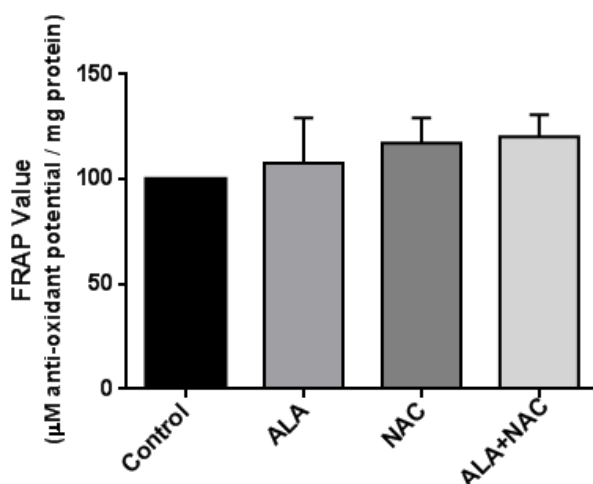
The antioxidant potential of RBC obtained from healthy donors was measured by FRAP assay. As shown in Graphic 6 no alteration on the antioxidant potential of the cells was observed, either in the cultures treated with ALA, NAC or ALA+NAC as compared with cells from the control group (110,3 μ M/mg vs. 115,9 μ M/mg, 106,4 μ M/mg, 92,3 μ M/mg, respectively).



Graphic 6: Effect of α -lipoic acid (ALA) and N-acetylcysteine (NAC) on the antioxidant potential of 1,2:3,4-diepoxybutane (DEB)-induced (0,4 μ g/mL) red blood cells (RBCs) obtained from healthy donors blood cultures.

DEB – control culture with DEB 0,4 μ g/mL; ALA + DEB – cultures with DEB 0,4 μ g/mL and treated with 20 μ M ALA; NAC + DEB – cultures with DEB 0,4 μ g/mL and treated with 500 μ M NAC; ALA+NAC+DEB – cultures with DEB 0,4 μ g/mL, treated with 20 μ M ALA, plus 500 μ M NAC. As described, antioxidant potential has no alteration in all the treatments. Results are presented as the mean \pm SEM of 11 experiments.

The antioxidant potential was also measured in cultured RBC obtained from FA patients. As shown in Graphic 7, an increase of the antioxidant potential was observed after treatment with ALA, NAC and ALA+NAC (107,6 μ M/mg, 117,3 μ M/mg, 120,2 μ M/mg, respectively), although no statistic significant was achieved.

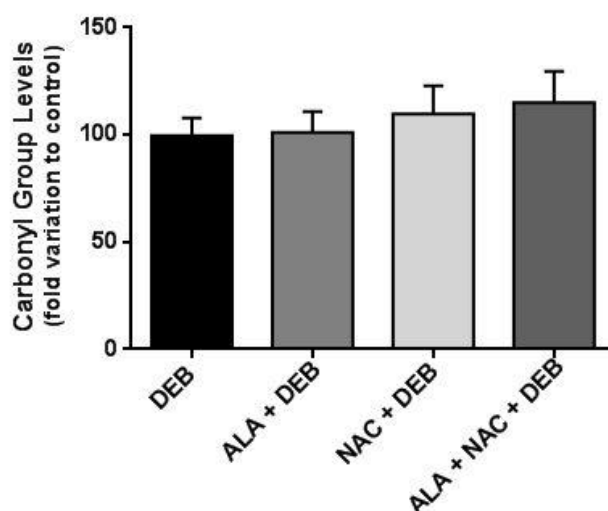


Graphic 7: Effect α -lipoic acid (ALA) and N-acetylcysteine (NAC) on the antioxidant potential of red blood cells (RBCs) obtained from Fanconi anemia patients blood cultures.

Control– cultures without treatments; ALA - cultures treated with 20 μ M ALA; NAC - cultures treated with 500 μ M NAC; ALA+NAC - cultures treated with 20 μ M ALA, plus 500 μ M NAC. As shown, all the treatments increase the antioxidant potential, although no statistical significance was reached. Results are presented as the mean \pm SEM of 5 experiments.

4.5 Effect α -lipoic acid and N-acetylcysteine on protein oxidative damage in red blood cells from healthy donors and Fanconi anemia patients

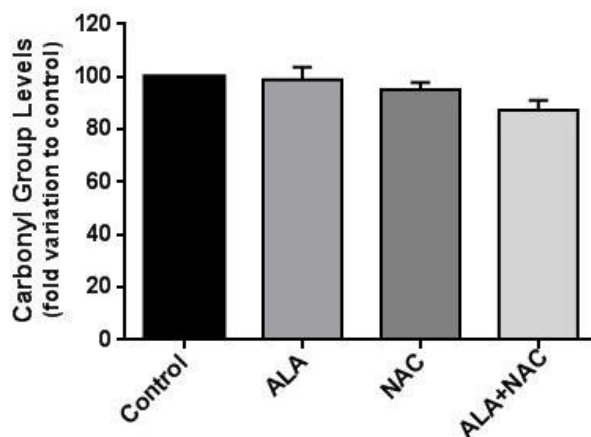
The protein oxidative damage was evaluated by measuring the carbonyl group content. As shown in Graphic 8 the addition of the different antioxidants ALA, NAC and ALA+NAC (101,0 %, 109,7%, 115,1 %, respectively), had no effect in RBC obtained from healthy donors (, 2.023.987, 2.164.255, 2.280.720, respectively).



Graphic 8: Effect α -lipoic acid (ALA) and N-acetylcysteine (NAC) on protein oxidative damage in 1,2:3,4-diepoxybutane (DEB)-induced (0,4 μ g/mL) red blood cells (RBCs) obtained from healthy donors blood culture.

DEB – control culture with DEB 0,4 μ g/mL; ALA+ DEB – cultures with DEB 0,4 μ g/mL and treated with 20 μ M ALA; NAC + DEB – cultures with DEB 0,4 μ g/mL and treated with 500 μ M NAC; ALA+NAC+DEB – cultures with DEB 0,4 μ g/mL, treated with 20 μ M ALA, plus 500 μ M NAC. Absolute values were converted in relative values; 100% corresponds to 1.962.805 (DEB value). As described, the antioxidants treatments have no influence on the protein oxidative damage. Results are presented as the mean \pm SEM of 11 experiments.

The carbonyl group content was also evaluated in cultured FA RBCs. As shown in Graphic 9, in the treatments with ALA and NAC no alterations were observed when compared with the control group (25.389.623, 24.729.067, respectively). A small decrease on protein oxidative damage was observed after treatment with ALA+NAC, although it was not statically significant (24.332.892).



Graphic 9: Effect α -lipoic acid (ALA) and N-acetylcysteine (NAC) on protein oxidative damage of red blood cells (RBCs) obtained from Fanconi anemia patients blood cultures.

Control– cultures without treatments; ALA - cultures treated with 20 μ M ALA; NAC - cultures treated with 500 μ M NAC; ALA+NAC - cultures treated with 20 μ M ALA, plus 500 μ M NAC. Absolute values were converted in relative values; 100% corresponds to 26.193.500 (control value). As shown, the protein oxidative damage remains with no alterations in ALA and NAC treatments, and a small decrease in ALA+NAC treatment, although no statistical significance was reached. Results are presented as the mean \pm SEM of 5 experiments.

5. DISCUSSION

It has been extensively described that FA patients are in a continuous cellular prooxidant state, which results in increased CI, possibly due to a down regulation of the endogenous antioxidant defense, including the RBC system (Pagano et al., 2012). Therefore, protection of FA cells against CI may be improved with an antioxidant therapy independent of the FA pathway control. Considering this, the aim of the present study was to evaluate the influence of the antioxidants ALA and NAC in the redox state of RBC in blood cultures from FA patients, comparatively to the influence of the same antioxidants in DEB-induced cultures from healthy donors. DEB was used to potentiate CI and, consequently, to induce a prooxidant state.

In a previous work, based on the hypersensitivity of FA cells to DEB, it was already shown that NAC and ALA improved genetic stability, decreasing CI in cultured lymphocytes from FA patients (Ponte et al., 2012). In the present study we confirmed this effect in the DEB-induced lymphocyte cultures from healthy donors. In fact, a reduction of CI, although not statistically significant, was observed in the cultures treated with ALA and NAC. This suggests that the effect of ALA and NAC is not exclusive of FA cells, but to any cell in a prooxidant state. This will be most important to apply to other OS-related diseases.

As one of the first cells to be affected by changes in the redox state of the body, alterations in RBCs are widely used to study OS related pathological conditions (Pandey and Rizvi, 2011). To cope with OS RBCs has several types of enzymatic and non-enzymatic antioxidant mechanism. GSH, a non-enzymatic system, is considered to be a first degree protection against OS. In fact, it has been reported that cells with low levels of GSH are more sensitive to OS. By the other hand GPx, an enzymatic defense system, gives protection by directly scavenging superoxide radicals and H_2O_2 , converting them to less reactive species (Milic et al., 2009). In the present study we evaluate the influence of ALA and NAC in the redox state, measuring total GSH concentration and GPx activity in RBCs obtained from FA and HD blood cultures with and without ALA and NAC treatments. Our results showed an increment of GSH levels in FA RBCs in the presence of ALA and NAC. Knowing that FA cells are in a continuous prooxidant state (Pagano et al., 2012), our results suggest that the antioxidant therapy with ALA and NAC improved the endogenous antioxidant defense and, consequently, improved the prooxidant state of FA RBCs. RBCs from HD were

exposed to DEB in order to induce a prooxidant state. Our results showed that ALA and NAC had no effect on GSH levels of HD RBCs. We postulate that their normal mechanism of endogenous antioxidant defense was sufficient to counteract DEB toxicity, therefore ALA and NAC had no additional effect. Concerning GPx levels, our results showed that ALA and NAC had no effect on its activity in the HD RBCs. As postulated in the previous results, we suggest that normal mechanism of endogenous antioxidant defense was sufficient to counteract DEB toxicity. Our results showed that ALA and NAC decreased GPx activity in the FA RBCs. The simultaneous increase in GSH levels and decrease in GPx activity may point to a combined redox dependent mechanism of ALA and NAC to improve antioxidant defense in FA RBC. In fact, it was already described that NAC improves GSH levels by the supply of cysteine and ALA preserves the structural and functional integrity of RBCs by adjusting the redox disturbances (AL Abdan, 2012). We suggest that this combined effect may lead to a decrease of the requirement of GPX activity to the detoxification mechanism.

To better understand the effect of ALA and NAC in FA RBCs redox state, we used the FRAP assay in order to evaluate the combined antioxidant effect of non-enzymatic defenses (Benzie and Strain, 1996). To indirectly evaluate the lipid peroxidation in FA RBCs with and without ALA and NAC treatments, we measured the protein oxidative damage by carbonyl group levels, a hallmark test for oxidative modification (Levine et al., 1990). Our results showed that ALA and NAC had no effect on FRAP and carbonyl group levels either in FA and HD RBCs. The use of these tests have never been addressed in FA RBCs. Knowing that there is a considerable variation in the basal levels of protein carbonyls in certain tissues, depending on the carbonyl assay used (Dalle-Donne et al., 2003), further studies must be performed, with more samples, in order to confirm the obtained results.

Disclose realistic, for a rare disease like FA insuperable obstacles must be faced during research studies, such as the scanty number of patients susceptible to be recruited and the amount and type of sample (Pagano et al., 2012). Nevertheless, whether with a new study design or with more samples, and based on previous works, we hypothesize that NAC and ALA may be good prophylactic candidates to decrease the adverse effects of OS-related damage. Therefore, we can speculate that these two antioxidants, after a more complete investigation, have potential to be a first choice exogenous antioxidant therapy to apply to FA patients.

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7. APPENDIX

Table 1: Material and Reagents.

Detailed informations about the material and reagents used in this study.

Material and Reagents	Provider	Country	Reference	Quantity
Subject				
Vacuum tubes with lithium heparin	Vacurette	Portugal	456088	6 mL
Isolation of RBC				
Centrifuge	Hettich zentrifugen, Rotofix 32 A	Germany		
Histopaque solution 1077	Sigma-Aldrich	Germany	10771	100 mL
Graduate tubes	BD	Portugal	352095	15 mL
Whole blood cultures				
RPMI 1640	GIBCO	Portugal	0883	500 mL

Fetal calf serum	Sigma-Aldrich	Germany	7524	100 mL
L-glutamine	Sigma-Aldrich	Germany	G-5763	25 g
Penicillin-Streptomycin (10,000 U/mL)	GIBCO	Portugal	15140-148	20 mL
Phytohemagglutinin, M form (PHA-M)	GIBCO	Portugal	10576-015	10 mL

Exposure to DEB

1,2:3,4-diepoxybutano (DEB)	Sigma-Aldrich	Germany	202533	1 g
Vertical laminar flow hood	Gelair, BSB4A			

Antioxidant incorporation

N-acetylcysteine (NAC)	Sigma-Aldrich	Germany	A7250	10 g
α -lipoic acid (ALA)	Sigma-Aldrich	Germany	T5625	5 g
Dimethyl Sulfoxide	Sigma-Aldrich	Germany	D8418	50 mL
Dulbecco's phosphate buffered saline 10x (PBS)	Sigma-Aldrich	Germany	D1408	500 mL

Cytogenetic Evaluation

Colcemid	GIBCO	Portugal	15212-012	10 mL
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Potassium chloride (KCl)	Merk	Portugal	1.04936.0500	500 g
Methanol	Merk	Portugal	1.06009.1011	1 L
Acetic acid	Merk	Portugal	1.00062.1000	1 L
Giemsa	Merck	Portugal	1.09204.0500	500 mL
Dulbecco's phosphate buffered saline 10x (PBS)	Sigma-Aldrich	Germany	D1408	500 mL
Microscope	Olympus, CX31RBSF/DP20			
Red blood cell processing for the study of oxidative stress parameters				
Hydrochloric acid (HCl)	PanReacAppliChem	Portugal	131020.1211	1 L
Sample processing for glutathione				
Eppendorf thermomixer	Eppendorf, confort			
Perchloric acid (HClO ₄)	Sigma-Aldrich	Germany	244252	500 mL
Glutathione quantification				
5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich	Germany	D8130	
KHCO ₃	Merk	Portugal	1048730250	

β -Nicotinamide adenine dinucleotide phosphate, reduced tetra(cyclohexylammonium) salt (NADPH)	Sigma-Aldrich	Germany	N5130
Glutathione reductase (GR)	Sigma-Aldrich	Germany	G3664
Sodium phosphate monobasic monohydrate (Na_2HPO_4)	Sigma-Aldrich	Germany	S9638
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma-Aldrich	Germany	E5134
96-well plate reader	PowerWaveX; Bio-Tek	USA	
Protein quantification			
BCA protein assay kit	Pierce	USA	23225
Glutathione peroxidase activity determination			
GPx assay kit	Abnova	Portugal	KA0882
Ferric reducing antioxidant power assay			
2,4,6-Tripyridyl-s-Triazine (TPTZ)	Sigma-Aldrich	Germany	93285
Anthos 2010 microplate reader	Biochrom	Germany	
Carbonyl Group levels			
Hybri-slot manifold system	Biometra	Germany	

Rabbit anti-DNP (1:5000)	Sigma-Aldrich	USA	D9656
Rabbit anti-goat IgG-AP (1:5000)	Sigma-Aldrich	USA	A4187
ECF TM substrate	GE Healthcare	UK	
BioRad FX-Pro-plus	Bio-Rad	UK	
BIO-PROFIL Bio-1D Software	Quantity One	France	
Statistical analysis			
GraphPad Prism 5	GraphPad Software	USA	