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Nitrosylation: An adverse factor in Uremic Hemolytic Syndrome. Antitoxin effect of *Ziziphus mistol Griseb*

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ABSTRACT

Toxins of *Escherichia coli* (STEC) causing Uremic Hemolytic Syndrome (UHS) generate oxidative stress in human blood with more production of nitric oxide (NO) than reactive oxygen species (ROS). Shiga toxin (Stx) together with the hemolysin (Hly) increased lipid oxidation, as evaluated by malondialdehyde MDA and oxidation of proteins. The addition of *Ziziphus mistol Griseb* extracts decreased NO, ROS, MDA and simultaneously caused an increase in the degradation of oxidized proteins to advanced oxidation protein products (AOPPs) in controls and samples with toxins. Furthermore, the nitrosylated proteins/AOPP ratio was reduced, due to the increase of AOPP. *Z. mistol Griseb* extracts exhibited a high proportion of polyphenols and flavonoids, with evident correlation with ferrous reduction antioxidant potential (FRAP). The plasma of eight children with UHS showed oxidative stress and NO stimulus, comparable to the effect of toxins during the assays *in vitro*. UHS children presented high levels of nitrosylated proteins respect to control children of similar age. Although the degradation of oxidized proteins to AOPP rose in UHS children, the nitrosylated proteins/AOPP rate increased as a consequence of the elevated nitrosative stress observed in these patients.

1. Introduction

An altered production or distribution of reactive oxidant species (ROS) and reactive nitrosative species (RNS) generates oxidative and nitrosative stresses in humans. The maintenance of the redox equilibrium involves enzymatic and non-enzymatic reactions, which are indispensable during prolonged oxidative stress. The necessity of an equilibrium between ROS and nitric oxide (NO) has been described, with the latter being a small molecule that is very diffusible, highly reactive and with a short-life in biological systems (Svegliati-Baroni et al., 2001). NO directly reactivates catalase, a major antioxidant enzyme, thus suggesting a possible role for NO in defending cells against an oxidative attack. However, the cells can generate NO under different conditions, and a frequent consequence of this production is the nitrosylation of glutathione and proteins, which is a post-translational modification that adds a nitrosyl group to the proteins. Moreover, it is the conversion of thiol groups, including the cysteine residues of proteins to form S-nitrosothiols (RSNOs), which exerts a post-translational regulation of most proteins. These important biological reactions of NO reduce the velocity of the degradation of oxidized proteins, with adverse consequences for cell viability. Therefore, the denitrosylation or elimination of RSNOs is a necessary process that is very important for defense against oxidative stress (Steiner et al., 2002).

Some infectious pathology, such as Uremic Hemolytic Syndrome (UHS), has been found to be associated with oxidative stress. It has also been hypothesized that the increase in lipid peroxidation is a consequence of an enhanced production of endothelial cell-derived reactive oxidants, due to the reduction of the catalase levels in endothelial cells, with the resulting hydroxyl radical participating in endothelium injury through a marked enhancement of the lipid peroxidation in UHS (Matsunaga et al., 1999). The cause of this disease is *Escherichia coli* (STEC), the producer of Shiga-like toxin (Stx) of principally the serotype O157:H7, although other serotypes such as O104:H4 were also implicated in STEC outbreaks (Safadi et al., 2012).

Stx and hemolysin (Hly) are two of the principal virulence factors observed in *E. coli* (STEC), and cause thrombotic thrombocytopenia with microvascular occlusive disorders due to increased platelet aggregation and hemolysis. It has been accepted

Abbreviations: AOPP, advanced oxidation protein products; Hly, hemolysin; UHS, Hemolytic Uremic Syndrome; MDA, malondialdehyde; NO, nitric oxide; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSNOs, S-nitrosothiols; STEC, Shiga toxin producing *Escherichia coli*; Stx, Shiga toxin.

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that the alteration of the vascular endothelium plays a central role in the pathogenesis of thrombotic microangiopathy and the ischemic lesions characteristic of UHS (Salmenniemi and Remes, 2012).

An enhanced NO production has been detected in mouse peritoneal macrophages (Yuhas et al., 1996). Although, the implication of NO in UHS pathogenesis has not yet been clarified, NO inactivation was observed in urine due to its binding to free hemoglobin released from lysed red cells (Siegler et al., 2005; Te Loo et al., 2006; Vareille et al., 2008). Subsequently, new attempts must be made to try to elucidate the NO role in the pathogenesis of UHS and its potential relation to damage and mortality.

Since antioxidants are used to protect cells from increased levels of ROS, in the present investigation the fruits of *Zizyphus mistol* were selected due to previous research in our laboratory indicating an elevated proportion of polyphenol in this native fruit (Albrecht et al., 2010; Aiassa et al., 2011). Additionally, numerous other studies have confirmed the antioxidant capacity of polyphenolic fractions in different natural products, with resulting potential pharmacological applications (Vallverdú-Queralt et al., 2011). In particular, the genus *Ziziphus* has been investigated to evaluate the preventive effect of the polyphenols against the myocardial injuries induced by the isoproterenol and biotoxicity of aluminum in rats (Cheng et al., 2012).

Flavonoids are a large group of polyphenolic compounds naturally occurring in several plants and fruits as glycosides or, less frequently as their aglycones, which seem to be absorbed best in the intestine, with some evidence of both passive and active transport. Polyphenols can exhibit various pharmacological properties, such as anti-inflammatory, anti-oxidant, anti-microbial, anti-mutagenic or anti-carcinogenic, and exhibiting cholesterol lowering and free radical scavenging (Serra et al., 2008).

The purpose of this work was to study the involvement of oxidative stress in the action of Stx and Hly, the role of NO in the development of UHS and the levels of nitrosylation occurring in this disease. In addition, we attempted to prevent oxidative consequences by the use of natural polyphenols.

2. Materials and methods

2.1. Culture conditions of STEC and purification of Stx and Hly toxins

The STEC stain was grown in tryptic soy broth for 48 hs at 37 °C, and the cell-free culture supernatant was obtained by a sterilizing filtration with a membrane of 0.22 μm-diameter pores. Stx toxin was separated from the culture supernatants by precipitation with a 60% saturation of *ammonium sulfate* $(NH_4)_2SO_4$ and then dialyzed for 24 h in phosphate buffer (pH:7). The Stx was purified by receptor-mediated affinity chromatography (Nakajima et al., 2001). Hly was purified by chromatography (Ostolaza et al., 1991). The toxin purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with silver staining. Then, 0.4 ml of 10 μg/ml Stx with Hly 10HU/ml Hly (Stx–Hly) were applied together in the assays of NO, ROS, carbonyls, AOPP and the nitrosylated proteins.

2.2. Preparation of fruit extracts

Flour of *Z. mistol Griseb* fruits deposited in TICA Laboratory of Nutrition School in Medicine Faculty, with Voucher specimen number three, were treated with different solvents for 24 h at 4 °C, with acetone, ethylic alcohol or water. Solids were separated by filtration and supernatants were dried until reaching constant weight in a rotatory extractor. The dry extracts were dissolved in water (50 mg/ml), before being conserved at −20 °C.

2.3. Phenolic compounds and flavonoid assays

The fruit extracts were analyzed by the assay described previously using Folin–Ciocalteu reactive and Na_2CO_3, and the results were expressed in μg gallic acid/mg dry extract (Dewanto et al., 2002). The flavonoid content was determined with $AlCl_3$ and CH_3COOK, using the assay described by Salamanca Grosso and Correa Carvajal (2007)), and these results were expressed in μg of quercetin/mg dry extract.

2.4. Determination of ferrous reduction antioxidant potential (FRAP)

The ability of *Z. mistol* extract to reduce Fe^{+3} to Fe^{+2} was tested by using a reaction of 1.12 ml of 300 mM acetate buffer (pH:3.6); 0.14 ml of 20 mM $FeCl_3.6H_2O$; 0.14 ml of 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl and 0.1 ml of 50 mg/ml *Z. mistol* extracts. After 20 min, the OD at 593 nm was determined. Standard $FeSO_4$ diluted into different concentrations was employed to express the results in mM of $FeSO_4$.

2.5. Nitric oxide assay

Blood (1 ml) was incubated for 3 h with 0.4 ml of Stx–Hly, and in some assays *Z. mistol Griseb* extracts (1.59, 0.64, 0.12 or 0.04 mg/ml) were applied together with the toxins. Separated plasma samples were deproteinized with $ZnSO_4$ (final concentration 1.5 *g/l*). Then, NO converted to nitrite (NO_2^-) in plasma was assayed by reduction of NO_2^- using granulated cadmium: 90 μL of the deproteinized plasma was mixed with 90 μL of Griess's reagent 1% (w/v) sulfanilamide and 0.1% (w/v) of N-(1-naphthyl) ethylenediamine dihydrochloride in 2% H_3PO_4. This was followed by a spectrophotometric analysis of total NO_2^- at 540 nm, with sodium nitrite being used as the standard (Aggarwal and Mehta, 1996). The results were expressed in μM NO_2^-/mg of protein, with the quantities of protein being determined by a Bradford assay.

2.6. Detection of ROS by chemiluminescence (CL)

The capability of Stx and Hly to generate ROS was examined in 10 μl venous whole blood with 10 μl of Hanks'Balanced Salt Solution (HBSS) and 600 μl of reagent mixture, this last composed of 5 ml of 0.067% 3-aminophthalhydrazide 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) in HBSS, 0.2 ml of 5% glucose, 1 ml of Ringer Lactate Solution and 3.6 ml of distilled water. Then, 0.4 ml of Stx–Hly was added, and in some assays *Z. mistol Griseb* extracts (1.59 or 0.64 mg/ml) were applied to the CL test (Bromme et al., 1999). Controls were performed using 10 μl of HBSS instead of toxin. Light emission was tested in a BioOrbit luminometer, and the results were expressed as relative light unities (RLUs).

2.7. MDA determination

Blood (1 ml) was incubated with 0.4 ml of Stx–Hly for 4 h, or with *Z. mistol Griseb* extracts (1.59 or 0.64 mg/ml) in some assays, then 150 μl of plasma was separated and mixed with 300 μl of a TCA–TBA–HCl reagent [Trichloroacetic acid (TCA): 15% w/v, thiobarbituric acid (TBA) 0.375%, hydrochloric acid (HCl) 0.25 N] and heated in boiling water for 30 min. An ice bath was then used to cool the samples, after which they were centrifuged at 1500 *g* and the absorbance of the supernatant was determined by spectrophotometry at 535 nm. The reference standard used was 1,1,3,3 tetraethoxypropane, and MDA levels were expressed in μmol/L (Becerra et al., 2006).

2.8. Determination of advanced oxidation protein products (AOPPs)

Protein oxidation was measured samples of plasma separated from 1 ml of blood previously incubated for 4 h with Stx–Hly, or with *Z. mistol Griseb* extracts in some assays, by determining the AOPP levels spectrophotometrically. Then, 1 ml of plasma diluted 1/5 in PBS was analyzed with 0.1 ml of acetic acid and 50 μl of 1.16 M potassium iodide in test tubes, and 1 ml of 0–100 μM chloramine T was added in standard tubes (Correa-Salde and Albesa, 2009). The absorbance of the reaction mixture was read at 340 nm to calculate the chloramine-T equivalents in μM.

2.9. Nitrosylated protein assay

Nitrosylated proteins in human plasma were quantified. In all tests the blood was incubated with toxins or toxins more extracts, and then samples of plasma were separated to perform nitrosylated protein assay. Briefly, 20 μl of plasma was acidified by the addition of 100 μl of 0.2 N H_2SO_4. After 2 min, 100 μl of a 0.5% solution of ammonium sulfamate was added and the mixture was allowed to react for 3 min. The mixture was then supplemented first with 80 μl of a mixture of 0.25% HgCl2 and 2.55% sulfanilamide in 0.4 N HCl and then with 80 μl of a solution of 0.38% N-1-naphthylethylenediamine in 0.4 N HCl. After 5 min, the absorbance at 540 nm was measured. The concentration of *S*-nitrosothiol was calculated using the extinction coefficient of 50,000 M^{-1} cm^{-1} and proteins were normalized to the total protein amount determined by the Bradford assay. Results were expressed as ηmoles of S-nitrosylated proteins per mg of protein (Stamler et al., 1992).

2.10. Statistical determinations

The statistical analysis was performed using ANOVA, with $p < 0.05$ taken as being statistically significant. The experiments were repeated at least three times, and the means and standard deviations were calculated.

Table 1
Polyphenols and flavonoids in *Z. mistol* extracts.

Extracts	Polyphenols (μg eq. gallic acid/mg dry extract)	Flavonoids (μg eq. quercetin/mg dry extract)
Z. mistol acetonic extract	34.58 ± 0.12	2.82 ± 0.02
Z. mistol ethanolic extract	31.86 ± 0.01	2.41 ± 0.01
Z. mistol aqueous extract	34.83 ± 0.02	2.75 ± 0.01

Table 2
NO/ROS ratio in plasma obtained from blood incubated *in vitro* with toxins and ethanolic extract of *Z. mistol*.

	NO/ROS
Blood	1.04
Blood + 1.59 mg/ml Z. mistol ethanolic extract	0.40
Blood + 0.64 mg/ml Z. mistol ethanolic extract	0.98
Blood + toxins	1.41
Blood + toxins + 1.59 mg/ml Z. mistol ethanolic extract	1.21
Blood + toxins + 0.64 mg/ml Z. mistol ethanolic extract	1.33

3. Results

In this study of NO production, Stx and Hly toxins of *E. coli* were applied *in vitro* to whole blood of normal humans, with the purpose of investigating if these toxins were able to increase NO in the plasma. Simultaneously, different extracts of *Z. mistol Griseb* were prepared to determine whether they could conteract the effect of *E. coli* toxins. Aqueosus, ethanolic and acetonic extracts showed a high content of polyphenols and flavonoids, considered to be important antioxidants (Table 1); however, we selected ethanolic extract because it presented a better linear correlation of ROS with FRAP (0,9947, $y = 0{,}4118x + 0{,}8085$) than aqueosus and acetonic extracts.

Fig. 1A exhibits the results of the NO generated in the plasma of normal blood by *E. coli* toxins, and the benefical action of *Z. mistol Griseb* which decreased the normal production of NO and also reduced the rise in NO generated by Stx and Hly toxins. The ethanolic extract selected for these assays, showed a higher antioxidant effect in the concentration 1.59 mg/ml than in that of 0.64 mg/ml, and the ethanolic extract of *Z. mistol Griseb* was also able to reduce the ROS generation in samples of blood during quimioluminiscente assay with toxins (Fig. 1B).

The toxins elevated the NO/ROS ratio, whereas *Z. mistol Griseb* decreased this relation (Table 2) in the *in vitro* assay with normal blood incubated simultaneously with Stx–Hly toxins. The prooxidant capacity of Stx–Hly and the antioxidant property of *Z. mistol Griseb* were confirmed by assays that demonstrated the an increase in lipid oxidation (Fig. 2) by *E. coli* toxins in the plasma of normal blood, with a significant reduction occurring through the addition of *Z. mistol Griseb* extract. The degradation of oxidized proteins to AOPP increased in the assays with toxins, and the addition of ethanolic *Z. mistol Griseb* increased this even more (Fig. 3). This unexpected result was reinforced by assays with extracts obtained using other solvents, which also provoked the degradation of oxidized proteins by *Z. mistol*. The acetonic extract was included, which confirmed the rise in of AOPP due to this fruit.

The investigation of nitrosylated proteins in plasma from assays of normal blood incubated for 4 h *in vitro* with toxins and *Z. mistol*, indicated no significant variations during this time (Fig. 4). However, the nitrosylated proteins/AOPP ratio of blood + toxin decreased from 0.0067 to 0.046 with *Z. mistol Griseb* due to the increase in AOPP.

The *in vivo* consequences of toxins during infection with *E. coli* STEC were also investigated in UHS patients. NO in the plasma of children with UHS was compared with NO in plasma of normal or control children of similar age, with the results indicating elevated NO in UHS patients (3.45 ± 1.48 μM NO_2^-/mg proteins) whereas control exhibited a significant lower mean value of NO (1.31 ± 0.71 μM NO_2^-/mg proteins) (Fig. 5A). Likewise, UHS patients exhibited a significantly increased nitrosylation of proteins, with a mean value of 0.82 ± 0.03 μM nitrosylated proteins/mg proteins, while in controls this value was 0.55 ± 0.02 μM. Together with the increase in NO, there was a rise in nitrosylated proteins in UHS children, as a consequence of the prolonged action of toxins during the infection (Fig. 5B).

In UHS children, the AOPP were significantly higher (91.11 mM eq.chloramineT/ml) than in controls (73.9 mM eq.chloramineT/ml), and the nitrosylated proteins/AOPP ratio in UHS children rose to a mean value of 0.009 ± 0.001, significantly ($p < 0.05$) higher than the 0.007 ± 0.001 in normal children. Similarly, the nitrosylated proteins/AOPP ratio increased by incubation *in vitro* with Stx–Hlys toxins, although normal blood did not exhibit any significant rise in nitrosylated proteins under the conditions applied during the *in vitro* assays.

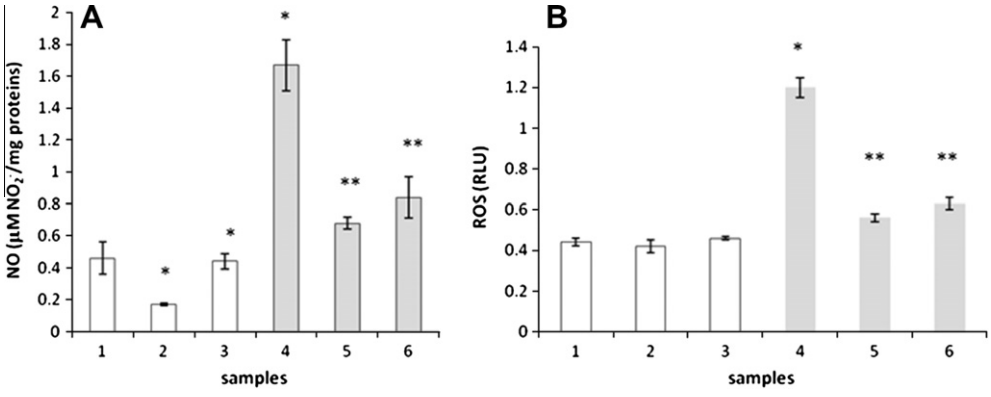

Fig. 1. (A) Production of NO and (B) ROS in samples of plasma obtained from assays with: (1) blood alone, (2) blood with 1.59 mg/ml of *Z. mistol* ethanolic extract, (3) blood with 0.64 mg/ml of *Z. mistol* ethanolic extract, (4) blood plus toxins, (5) blood plus toxins and 1.59 mg/ml of *Z. mistol* ethanolic extract, (6) blood plus toxins and 0.64 mg/ml of *Z. mistol* ethanolic extract.*$p < 0.05$ respect sample 1, **$p < 0.05$ respect sample 4.

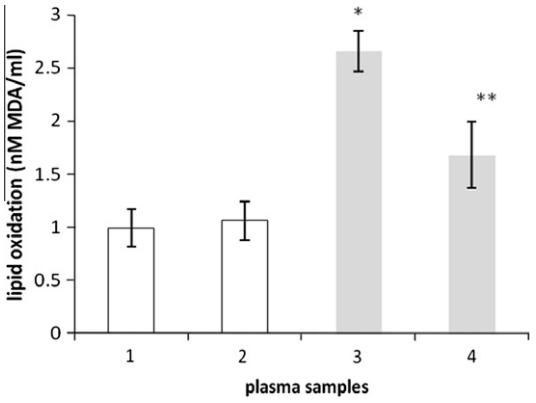

Fig. 2. Oxidation of lipids to MDA in samples of plasma obtained by *in vitro* assays with: (1) blood alone, (2) blood with 0.64 mg/ml of *Z. mistol* ethanolic extract, (3) blood plus toxins, (4) blood plus toxins and 0.64 mg/ml of *Z. mistol* ethanolic extract. $^{*}p < 0.05$ respect sample 1, $^{**}p < 0.05$ respect sample 3.

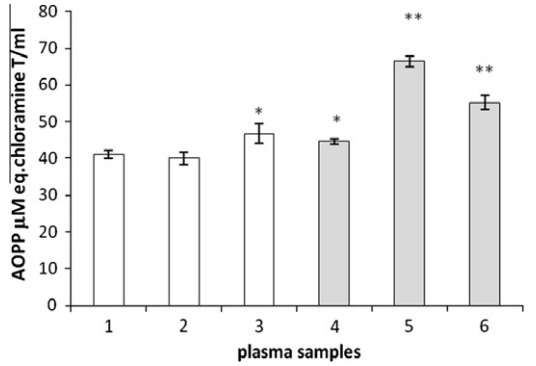

Fig. 3. Degradation of oxidized proteins to AOPP in samples of plasma obtained by *in vitro* assays with: (1) blood alone, (2) blood with ethanolic 0.64 mg/ml extract of *Z. mistol*, (3) blood with acetonic extract of *Z. misol*, (4) blood plus toxins, (5) blood plus toxins and ethanolic extract of *Z. mistol*, (6) blood plus toxin and acetonic extract of *Z. mistol.* $^{*}p < 0.05$ respect sample 1, $^{**}p < 0.05$ respect sample 4.

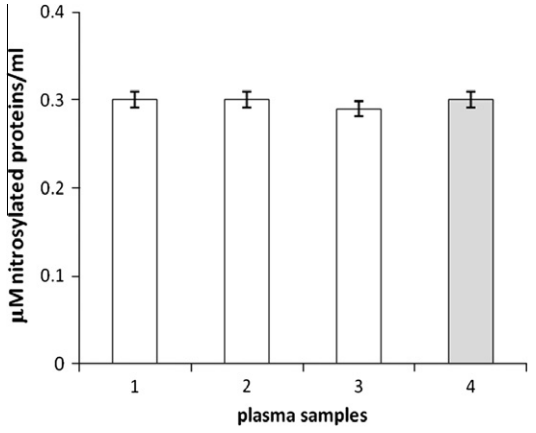

Fig. 4. Nitrosylated proteins in samples of plasma obtained by assays with: (1) blood alone, (2) blood with 0.64 mg/ml ethanolic *Z. mistol*, (3) blood plus toxins, (4) blood plus toxins and ethanolic *Z. mistol.*

4. Discussion

The results obtained "*in vitro*" with Stx–Hly toxins were in agreement with the previous publications of our group that indicated an increase in ROS by these toxins (Albrecht et al., 2010;

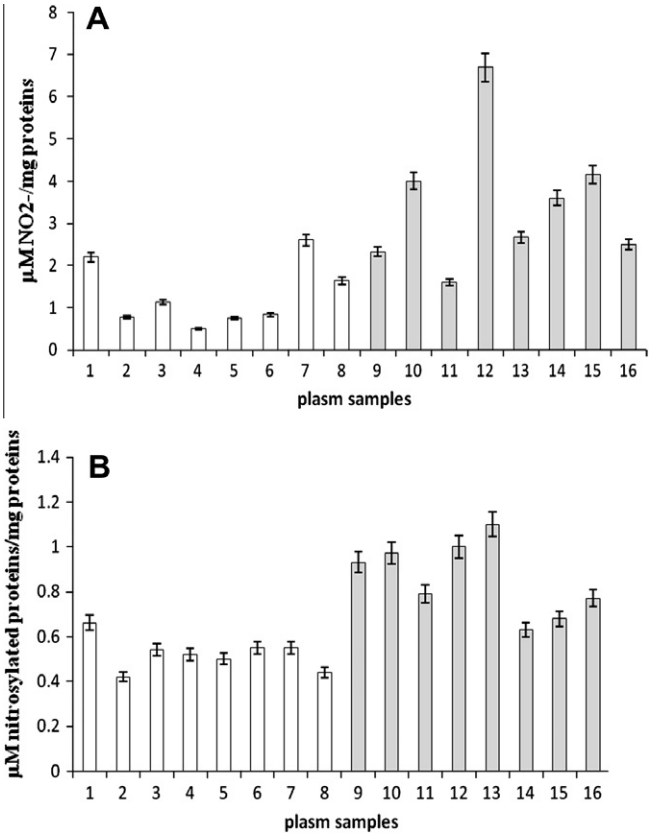

Fig. 5. (A) Nitric oxide and (B) Nitrosylated proteins in plasma of control children (□) and UHS children (■).

Aiassa et al., 2011). At the present, it should be emphasized that NO is the trigger of nitrosative stress, which occurs *via* S-nitrosylation by the interaction of NO with thiols of proteins. S-nitrosylation regulates the apoptotic signal cascade by activation of mitochondrial apoptotic pathways, and the cellular redox equilibrium determines whether nitrosylation will induce or reduce apoptosis (Kim et al., 2002). In the present work, an increment of NO in plasma together with stimuli of ROS was observed with a higher production of NO respect to ROS found by incubation with Stx–Hly in normal blood, and also in SUH patients without a previous incubation with these toxins.

The participation of oxidative/nitrosative stress in chronic disease is nowadays generally accepted, with nitrosative stress being involved in the pathogenesis of both Parkinson's and Alzheimer's diseases (Pal et al., 2010). The rise in nitrosative stress resulting from an increase of NO could to be one of the causes of damage generated by STEC, taking into consideration that nitrosative stress can contribute to diverse diseases, such as cardiovascular pathologies (Elahi et al., 2007).

The increase in NO observed *in vitro* in plasma from blood treated with Stx and Hly was coherent with the elevated levels of NO in UHS children, but in these the elevation of NO was prolonged during the infectious process. It should be pointed out according with previous knowledge, that NO can exert an early cytoprotective response, since initially NO directly reactivates catalase, a major antioxidant enzyme, which is suggestive of a role for NO in defending against oxidative stress. However, this fast metabolic change is followed by adverse consequences, since NO suppresses the enzymatic reduction of free cysteine, which stimulates a damaging Fenton reaction. Furthermore, during prolonged stress, the increase of NO leads to a high nitrosylation of proteins, which are eliminated more slowly than carbonyls (Gusarov and Nudler,

2005). Finally, the prolongation of NO stimuli as an adaptation to oxidative stress causes irreversible nitrosative damage. Recently, it was observed that higher cellular NO levels coincided with a higher accumulation of S-nitrosothiols and a defensive rise in intracellular polyphenols in fungus which was accompanied by an increase in the activity of denitrosylated S-nitrosoglutathione reductase and thioredoxin reductase. In addition, the reduction of S-nitrosothiols by means of inhibition of these enzymes down-regulated the accumulation of intracellular polyphenols (Zheng et al., 2011).

Previously, NO has been demonstrated to have a biphasic action on oxidative stress, with low concentrations protecting against cell death, whereas higher concentrations are cytotoxic and elevated levels of NO can induce injury to the endothelium (Joshi et al., 1999). Therefore, it is possible to consider the effects of the NO response to be varied, with beneficial consequences and noxious effects on proteins both being observed.

In general it is accepted that NO induces apoptosis and the impact of the inhibition of mitochondrial protein synthesis during apoptosis is largely known. Related to this, mutations in mitochondrial DNA affecting the activity of respiratory complexes have been implicated in many chronic degenerative diseases. Likewise, mitochondrial proteins coded by mitochondrial and nuclear genes, are known to have important signaling roles in apoptosis (Ramachandran et al., 2002).

The results obtained in the present study indicated that the noxious consequences of NO increase over 4 h, with subsequent increases of MDA, as a consequence of oxidative damage of lipids. AOPP increased, although the stimuli of nitrosylated proteins was not significant after this time in the blood of normal persons. In contrast the rise in nitrosylated proteins was significant in UHS children respect to control patients, probably as a result of a prolonged time of NO increase during contact with *E. coli* toxins throughout the infection. Thus, it is probable that this pathology can be associated to a high nitrosylation or to a low denitrosylation, which was enhanced in the presence of the stressing toxins Stx–Hly.

Another result from this study was the increase of nitrosylated proteins/AOPP ratio in UHS children, which can be considered to be an adverse aspect because the nitrosylation of proteins enhanced the oxidative stress damage by opposing to the AOPP degradation. In fact, degradation of oxidized proteins to AOPP helps the synthesis of new proteins, and numerous systems have been described that protect against oxidative stress, with some antioxidant defenses depending on newly synthesized proteins. Thus, anti-stress protection is a result of the degradation of oxidized proteins to AOPP, which is indispensable during prolonged oxidative stress.

The observed capacity of *Z. mistol Griseb* to counteract the oxidative stress caused by Stx–Hly toxins seems to be promising. The assays with *Z. mistol Griseb* extracts were adequate to detect antioxidant effects, and the flavonoids may be one of the antioxidants of this fruit, with a potential application in the therapy of UHS. Another fruit of the *Ziziphus* genus (Cardozo et al., 2011) has been previously described to have an antioxidant capacity, but this was not investigated in UHS (Zhang et al., 2010).

The long administration of antioxidants is a possible way to reduce the effects of excessive macromolecule oxidation as a consequence of prolonged stress. Previously, it was demonstrated that polyphenols can avoid *S*-nitrosylation, since certain polyphenols intervene to prevent the formation of misfolded protein that accumulates upon exposure to oxidative stress, such as in neurodegenerative disease. Fortunately, polyphenols are important antioxidant which are ubiquitous in plants, with several thousand of them being contained in normal diet. However, the absorption and metabolism of polyphenols can be very different in humans (Frade et al., 2005).

Summing up, the reduction of NO, ROS and MDA obtained *in vitro* with *Z. mistol Griseb* extract, together with the decrease of NO/ROS and the nitrosylated proteins/AOPP ratio by activation of the degradation of oxidized proteins to AOPP, are all indicative of a protective effect probably due to polyphenols such as flavonoids. The reversal of the stressing effects of Stx–Hly obtained *in vitro* suggests a possible protection from toxicity and death, since it is generally accepted that oxidative stress can be attenuated by antioxidant vitamins or chelators that can change the disturbance in lipid peroxidation and even reduce the apoptosis provoked by oxidant agents (Sukumar Chattopadhya et al., 2002).

5. Conclusions

The results obtained permit *Z. mistol Griseb* to be included among the natural anti-stress products that can reduce the adverse factors of toxins in UHS by means of its antioxidants decreasing the production of NO, which is a beneficial effect in patients with elevated levels of nitrosylated proteins such as those observed in the UHS children during the present work.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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