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Hemolysin from *Escherichia coli* induces oxidative stress in blood

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ABSTRACT

Hemolysin (HlyA) produced by some stains of *Escherichia coli* is considered to be an important virulence factor of those bacteria. On the other hand, reactive oxygen species (ROS) have been reported to be involved in the pathogenesis of different diseases via oxidative stress generation. The purpose of this study was to analyze the capacity of HlyA to induce oxidative stress in whole blood cultures (WBCs). To this end, ROS production, the damage induced in lipids and proteins, and the antioxidant defense system was evaluated in blood cultures exposed to low concentrations of HlyA. We found that HlyA increased the level of free radicals detected by chemiluminescence assay. Moreover, lipid peroxidation and protein damage was significantly increased in cultures treated with HlyA in comparation with those found in control cultures. On the other hand, a decrease in total antioxidant capacity of plasma and in the activity of superoxide dismutase (SOD) was observed in plasma from blood treated with HlyA. Collectively, our data demonstrate that low concentrations of *E. coli* hemolysin induced oxidative stress in WBCs with the induction of different oxidative damage biomarkers.

1. Introduction

Escherichia coli is an important food-borne pathogen in Argentina and other parts of the world. The infections caused by these bacteria are responsible for widespread disease, including for example, hemolytic uremic syndrome (HUS), pyelonephritis, septicemia and gastroenteritis (Heffernan et al., 2009; Bentancor et al., 2007). During the infection, *E. coli* secretes different products, including shiga toxin (Stx) and Hemolysin (HlyA) (Proulx et al., 2001; Welch et al., 1995) with the latter being a pore-forming toxin which requires the *hlyCABD* operon for its correct synthesis and extracellular liberation (Welch et al., 1995). Numerous effects on different cellular populations have been attributed to sublytic concentrations of HlyA, including lipoxygenase product formation, liberation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-), and also of reactive nitrogen intermediates (RNI), for example nitric oxide (NO) (Grimminger et al., 1991; Bhakdi and Martin, 1991; Suttorp et al., 1993).

Oxidative stress is caused by an imbalance between the production of oxidants, such as the free radicals, peroxide and nitric oxide, and the levels of antioxidants present in the biological system. In this situation, the overproduction of ROS can lead to the damage of cellular components including lipids, protein, and DNA (Albesa et al., 2004; Baronetti et al., 2011). If this damage is not repaired, mutagenesis and cellular death can occur, which probably participate in the pathogenesis of different diseases such as Alzheimers, multiple sclerosis, diabetes and infectious diseases including Chagas disease, bacterial meningitis and

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hepatitis (Moreira et al., 2005; Gonsette, 2008; Hakim and Pflueger, 2010; Gupta et al., 2009; Kastenbauer et al., 2002; Pal et al., 2010).

Many different biomarkers have been used to follow the development of the oxidative stress in these diseases. For example, carbonyl residues and advanced oxidation protein products (AOPP) are the two parameters usually used to evaluate the level of oxidative damage to proteins (Fredriksson et al., 2005; Halliwell and Whiteman, 2004). In addition, with respect to the lipidic damage induced by oxidating stress, malonyldialdehyde (MDA) is also a well-known biomarker of this process (Niki, 2009). On the other hand, this oxidative imbalance, with over-production of ROS, can be caused by a reduction in the oxidative defenses which are insufficient to removal the free radicals and therefore the antioxidant system plays a very important role in the control of this process. The main enzymes of this system, involved in the detoxification of ROS, are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GSSGR) (Sardesai, 1995; Inal et al., 2002). With respect to SOD, which reduces O_2^- concentrations, a decrease in its activity has been observed in diseases associated with oxidative stress, such as chronic bacterial prostatitis and schizophrenia (Lou et al., 2006; Zhang et al., 2006).

In conclusion, considering that oxidative stress has been linked to illnesses associated with *E. coli* infections such as HUS and pyelonephritis (Celik et al., 2007), and that HlyA is a very important factor of virulence of this bacterium, the objective of this study was to analyze the capacity of this toxin to induce oxidative stress in whole blood cultures (WBCs), which could contribute to the understanding of the pathogenesis of infection by this pathogen.

2. Material and methods

2.1. Culture conditions and purification of E. coli HlyA

The clinically isolated *E. coli* (associated with hemolytic uremic syndrome) was kindly provided by the Microbiology Laboratory of the Pediatric Hospital of Córdoba, Provincia de Córdoba, Argentina. Stock cultures were preserved at −80 °C using glycerol 1% (v/v) as the cryoprotectant and the *E. coli* strain was grown in tryptic soy broth at 37 °C. Bacteria were then pelleted by centrifugation at 16,000 *g* for 15 min at 4 °C, and the cell-free culture supernatants were obtained. The supernatant was precipitated with solid ammonium sulfate (55% w/v) at 4 °C and after 1 h the precipitate was collected by centrifugation (20,000 *g*, 30 min, 4 °C), redissolved in TCU buffer (20 mM Tris/HCL, 150 mM NaCl, 6 M urea; pH 7.0) and dialyzed. Then, the sample was subjected to gel chromatography in a Sephadex G-200 (40 cm long and 2 cm in diameter) and eluted with TCU buffer. The fractions obtained were assayed for proteins and for hemolytic activity and the toxin purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining (Paraje et al., 2005a, b).

2.2. Hemolytic activity

Erythrocytes were obtained from the heparinized blood of healthy individuals after University Human Ethics Committee protocol approval. Red cells were washed three times with phosphate-buffered saline (PBS)-albumin (0.1 mg/ml) plus $CaCl_2$ (20 mM) before suspension preparation. To 100 μl of diluted toxin, 100 μl of a 0.6% suspension of washed erythrocytes were added which was followed by incubation at 37 °C for 30 min. The percentage of hemolysis was determined by measuring the absorbance at 540 nm, and 100% of lysis was reached by incubation of erythrocytes with water. The hemolytic activity (hemolitic activity (HU/ml)) was taken to be the dilution of HlyA preparation able to produce 50% lysis in a 0.6% erythrocyte suspension (Paraje et al., 2005a, 2005b).

2.3. Detection of ROS by chemiluminescence (CL)

The capability of HlyA to generate ROS was examined in venous whole blood cultures (WBCs) using 3-aminophthalhydrazide 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) sensitized chemiluminescence (CL). All experiments were carried out following the guidelines of the Ethics Committee of Cordoba University with blood of normal volunteers. The production of ROS in 10 μl of whole blood was measured with 10 μl of Hanks'Balanced Salt Solution (HBSS) and 600 μl of reagent mixture, composed of 5 ml of 0.067% luminol in HBSS, 0.2 ml of 5% glucose, 1 ml of Ringer Lactate Solution and 3.6 ml of distilled water. Then, 10 μl of different concentrations of toxin were added and the CL was tested in a BioOrbit luminometer with the light emission results being expressed as relative light units (RLU). Controls were performed with 10 μl of PBS instead of toxin (Paraje et al., 2005a, 2005b).

2.4. Culture conditions

Two ml of whole blood were incubated with an equal volume of HlyA (0.4 and 0.2 HU/ml) or PBS negative control for 4 h. Then, the blood samples were centrifuged at 900 *g* for 10 min, and the plasma obtained was transferred into separate tubes.

2.5. Lipid peroxidation

150 μl of plasma sample was 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).

2.6. Determination of advanced oxidation protein products (AOPP)

One ml of plasma diluted 1/5 in PBS was analyzed with 0.1 ml of acetic acid and 50 μl of potassium iodide (IK)

(1.16 M) in test tubes. The final product of the reaction was evaluated by spectrophotometry at 340 nm, with Chloramine-T being used as the standard. The concentrations of AOPP were expressed as μmol/L of chloramine-T equivalents (Capeillere-Blandin et al., 2004).

2.7. Determination of carbonyl residues

Plasma (0.35 ml) was treated for 1 h with 1 ml of 0.1% 2,4-dinitro-phenylhydrazine solution in HCl 2 M. Then, proteins were precipitated from the solution by the use of trichloroacetic acid (10%), and the protein pellet was washed three times with ethanol and ethyl acetate (1:1) before being resuspended in 1 ml of 6 M guanidine at 37 °C for 30 min. The carbonyl content was determined by spectrophotometry from the absorbance at 364 nm (molar absorption coefficient, 22 mM^{-1} cm^{-1}) (Páez et al., 2011). The concentrations of carbonyl residues were expressed as nmol per mg of proteins, determined by the Bradford assay.

2.8. Ferric reducing antioxidant power (FRAP) assay

The total antioxidant capacity of plasma samples was evaluated by the FRAP assay. Briefly, 100 μl of plasma sample was mixed with 3 ml of the following mix work (10:1:1): (a) 300 mM acetate buffer pH: 3.6; (b) 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCL; and (c) 20 mM $FeCl_3 \cdot 6H_2O$. Then, the absorbance was measured at 593 nm after 4 min of incubation. The FRAP values were calculated using a $FeSO_4$ calibration curve and expressed as μmol/L (Benzie and Strain, 1996).

2.9. Assay of superoxide dismutase (SOD) activity

Total SOD activity was assayed photochemically based on the inhibition of nitro blue tetrazolium (NBT) reduction (Aiassa et al., 2010). The ability of SOD to inhibit the reduction of NBT by the O_2^- generated by the illumination of riboflavin in the presence of oxygen and the electron donor methionine, was evaluated in the plasma samples. One unit of SOD (U) was defined as the amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions. The results were expressed as U/ml.

2.10. Statistical analysis

Data were expressed as means ± standard errors of the means (SEMs), and were analyzed by a one-way analysis of variance with Tukey's post hoc test to determine the statistical significance for all pairwise multiple comparison procedures. A p value of <0.05 was considered significant. All experiments were repeated, and equivalent results were obtained for each experiment.

3. Results

3.1. ROS production in whole blood cultures

The generation of ROS in WBCs in response to HlyA was measured using a luminol-dependent chemiluminescence assay. This assay allowed the detection of oxidative stress in blood cells in the presence of plasma. Whole blood was cultured at 37 °C in the presence of sublytic concentrations of HlyA (0.4, 0.2 or 0.1 HU/ml) or PBS, and the production of free radicals was studied. Fig. 1 show that either the treatment of whole blood cells with 0.4 or 0.2 HU/ml of HlyA induced a significant increase in the levels of ROS production in these cells in comparison to the cell cultures exposed to PBS, although the highest levels of ROS were induced by the treatment with 0.2 HU/ml. These results indicate that this effect is not dependent of toxin concentration. On the other hand, WBCs in the presence of 0.1 HU/ml of HlyA did not trigger a significant increase in the generation of ROS with respect to the culture controls. Therefore, for our next experiments, only the concentrations of 0.4 and 0.2 HU/ml of HlyA were used.

3.2. Malondialdehyde (MDA) levels

To investigate the effect of HlyA on plasma lipid peroxidation, the levels of MDA in the WBCs subjected to different concentrations of this toxin were evaluated. As shown in Fig. 2, the treatment of blood cells with 0.2 HU/ml of HlyA induced a significant increase in the plasma MDA levels in comparison with those found in the cell cultures exposed to PBS. On the other hand, there were no significant statistical differences between the levels of MDA observed in cultures exposed to 0.4 HU/ml of HlyA or PBS.

3.3. Protein oxidation

In addition to lipid peroxidation, the overproduction of ROS can also lead to protein damage. Therefore, we investigated the presence of the two markers of protein oxidation, carbonyl residues and AOPP, in WBCs. In a similar way to MDA levels, a significantly greater level of carbonyl residues was detected in plasma from cultures subjected to 0.2 HU/ml of HlyA than in plasma from control cultures. In

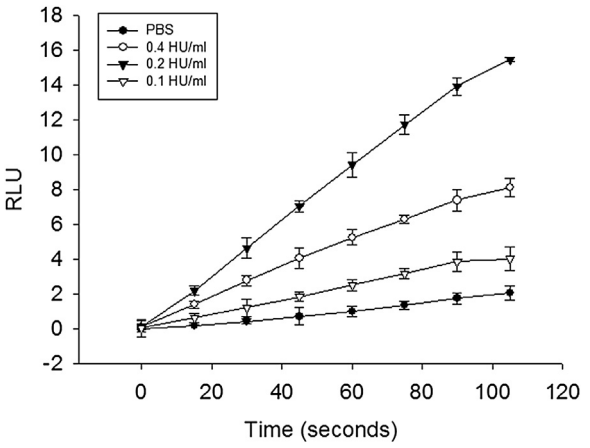

Fig. 1. Effect of HlyA on production of ROS by blood cells. Whole blood was exposed to different concentrations of HlyA (0.4, 0.2 or 0.1 hemolytic activity (HU)/ml) or PBS as negative control. Then, the production of ROS at various time points (0–120 s) was measured using luminol-dependent chemiluminescence. The results are expressed as relative light units (RLU). Data shown are the mean ± SEM from at least three independent experiments.

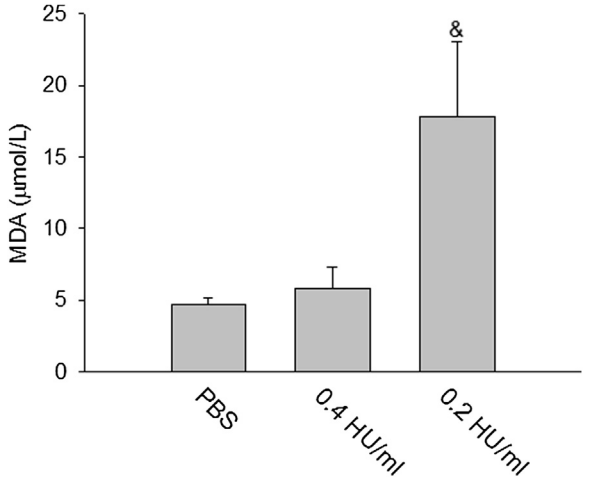

Fig. 2. Lipid peroxidation levels in plasma. Whole blood was exposed to different concentrations of HlyA (0.4 or 0.2 hemolytic activity (HU)/ml) or PBS for 4 h at 37 °C. After incubation, the levels of malonyldialdehyde (MDA) in the plasma were determined. The results are presented as the mean ± SEM from three separate experiments. $^{\&}p < 0.05$ (0.2 HU/ml vs. PBS).

contrast, no differences in the level of this marker were observed between plasmas from WBCs treated with 0.4 HU/ml of HlyA and those from control cultures (Fig. 3A). On the other hand, with respect to AOPP, the levels found in plasma from WBCs stimulated with 0.2 or 0.4 HU/ml of HlyA were higher than those observed in control cultures, with the treatment of blood cells with 0.2 HU/ml of HlyA resulting in a slightly greater increase in AOPP levels than in WBCs subjected to 0.4 HU/ml of HlyA (Fig. 3B).

3.4. Antioxidant system

As a reduction in the oxidative defenses can result in oxidative stress; the total antioxidant capacity of plasma and the SOD activity was evaluated in our system. The FRAP values found in WBCs treated with 0.2 and 0.4 HU/ml of HlyA were lower than those from cultives exposed to PBS (Fig. 4A). Similarly, the treatment of blood cells with 0.2 and 0.4 HU/ml of HlyA also resulted in a decrease in the activity of SOD (Fig. 4B).

4. Discussion

Under physiological conditions, a critical balance between the production of ROS and the antioxidant system exists (Aiassa et al., 2011). However when this balance is broken, through an increase in the ROS production or, a reduction in the antioxidant defenses (or by both), an overproduction of ROS is induced resulting in a process denominated oxidative stress. A link between oxidative stress and different diseases has been previously demonstrated since ROS overproduction was able to induce damage in important macromolecules, such as lipids, proteins and DNA (Moreira et al., 2005; Gonsette, 2008; Hakim and Pflueger, 2010; Gupta et al., 2009; Kastenbauer et al., 2002; Pal et al., 2010).

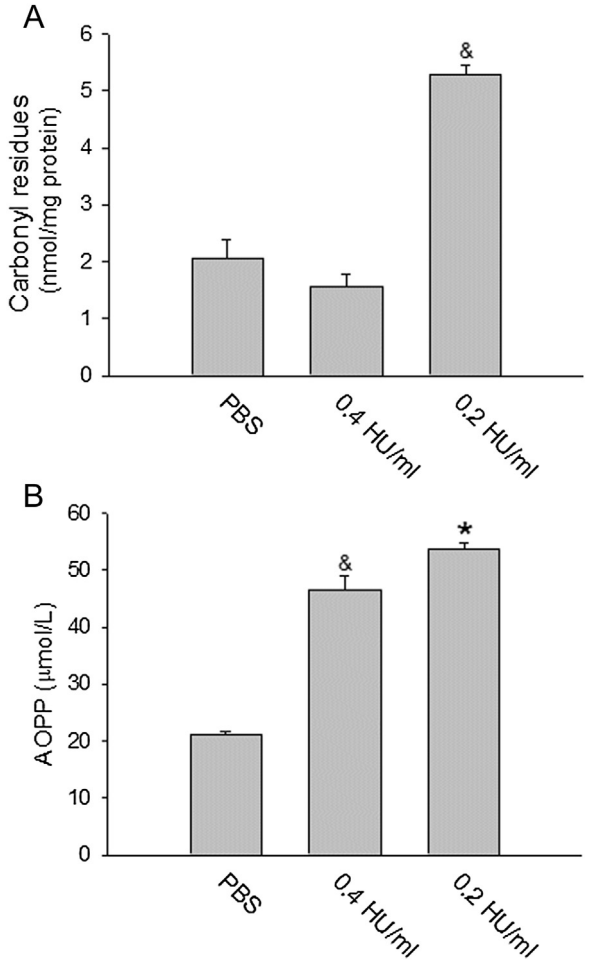

Fig. 3. Effect of HlyA on protein oxidation. Whole blood was exposed to different concentrations of HlyA (0.4 or 0.2 hemolytic activity (HU)/ml) or PBS for 4 h at 37 °C. After incubation, the levels of carbonyl residues (A) and advanced oxidation protein products (B) present in the plasma were determined. Each column shows the mean ± SEM and the results are representative of three independent experiments. A) $^{\&}p < 0.006$ (0.2 HU/ml vs. PBS); B) $^{\&}p < 0.009$ (0.4 HU/ml vs. PBS), $^{*}p < 0.002$ (0.2 HU/ml vs. PBS).

E. coli infections such as pyelonephritis and septicemia have been shown to be related to oxidative stress. In a model of pyelonephritis in rats, the infection of these animals with *E. coli* resulted in an oxidative injury to renal tissue, with elevated levels of ROS and MDA (Sener et al., 2006). Furthermore, the induction of septicemia by the intracecal injection of rats with *E. coli* suspension also produced in oxidative stress (Ninković et al., 2006), with the cerebral capillaries of these animals showing in an increase in the levels of lipid peroxidation, NO and O_2^- production, but a lower activity of SOD with respect to control group.

An important virulence factor secreted by the bacteria involved in these two diseases is a pore-forming cytotoxin called HlyA (Cavalieri et al., 1984; Smith et al., 2007), and it has been estimated that approximately 50% of all cases of pyelonephritis are caused by infection with HlyA-producing *E. coli* (Siegfried et al., 1994). In agreement

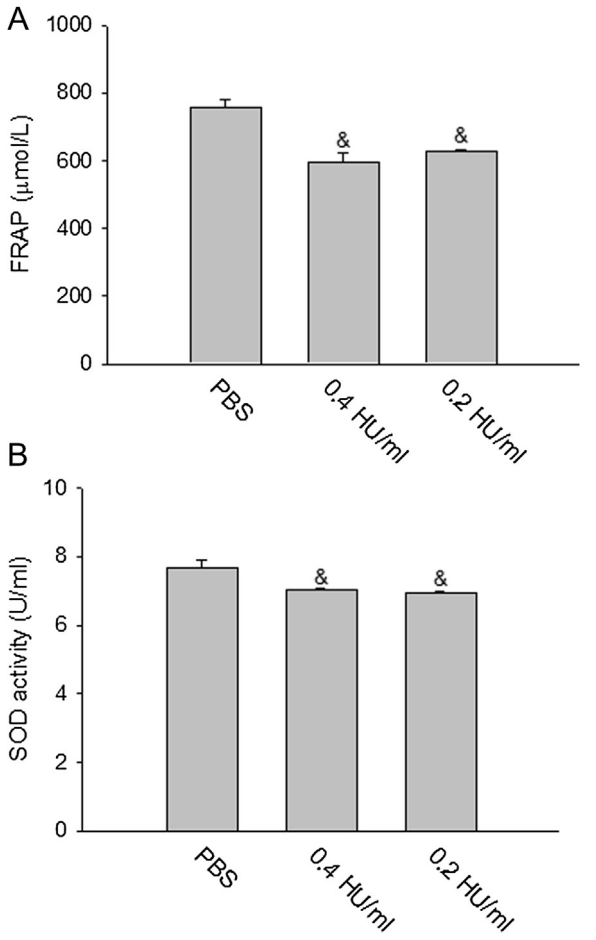

Fig. 4. Total antioxidant capacity of plasma and SOD activity. Whole blood cultures in the absence (PBS) or presence of HlyA (0.4 or 0.2 hemolytic activity (HU)/ml) were incubated for 4 h at 37 °C. Then, the plasma was obtained by centrifugation and the total antioxidant capacity of plasma (A) and the activity of SOD (B) were determined. Each column shows the mean ± SEM of three independent experiments. A) $^{\&}p < 0.006$ (0.4 and 0.2 HU/ml vs. PBS); B) $^{\&}p < 0.05$ (0.4 and 0.2 HU/ml vs. PBS).

with this, Trifillis et al. reported that *E. coli* strains mutants unable to express hemolysin activity induced significantly less damage in the human renal proximal tubular epithelial cells (HRPTEC), target cell in the pyelonephritis, than the pyelonephritogenic parent strain (Trifillis et al., 1994). Moreover, purified HlyA has been shown to stimulate the production of oxidative stress-related products, such as O_2^- and NO (Bhakdi and Martin, 1991; Suttorp et al., 1993; Valeva et al., 2005).

In agreement with these data, the results of our studies clearly show that HlyA exposure led to an oxidative stress response in blood cells, with the induction of different oxidative damage biomarkers.

In WBCs, where plasma and different types of cells are present, the presence of HlyA resulted in a rise in the production of ROS. For the range of concentrations tested in this study (0.1–0.4 HU/ml), both the treatments with 0.4 HU/ml or 0.2 HU/ml of HlyA induced an increase in ROS production, although the highest levels were observed using of 0.2 HU/ml. On the other hand, no significant differences in the production of these species were found between WBCs exposed to 0.1 HU/ml of HlyA and control cultures. This behavior has also been previously observed in the production of other substances by the stimulation with HlyA. For example, the dose-dependent liberation of lipoxygenase products by human neutrophils subjected to HlyA is similar to that observed in our study (Grimminger et al., 1991).

Elevated levels of ROS production are known to promote oxidative damage in different molecules. Therefore, the presence of these modified molecules has been used as biomarkers of oxidative stress. In this way, MDA levels are routinely used as an index of the peroxidation of lipids induced by the overproduction of ROS. In the present study, the plasma MDA levels were significantly increased in cultures treated with 0.2 HU/ml of HlyA in comparison with those found in control cultures. However, the exposition of WBC to 0.4 HU/ml of HlyA did not induce significant changes in the levels of this biomarker with respect to the cultures controls. Other macromolecules affected by an excess in ROS production are the proteins. In our system, the oxidative stress generated by the treatment with HlyA also resulted in damage to these molecules. The levels of carbonyl residues and AOPP in plasma from WBCs exposed to 0.2 HU/ml of HlyA were higher than those observed in cultures incubated in the presence of PBS. In addition, the treatment with 0.4 HU/ml of HlyA also resulted in an increase in the plasma values of AOPP. However, this concentration of HlyA did not induce a significant change in the plasmatic levels of carbonyl residues in comparison to the culture controls. It is known that, oxidative stress can also result from a decrease in the antioxidant levels. In this study, a reduction in the total antioxidant capacity of plasma and in the activity SOD was observed in plasma from blood treated with 0.2 or 0.4 HU/ml of HlyA.

In summary, we have shown that HlyA from *E. coli* induces an oxidative imbalance in WBCs, with an overproduction of ROS and a decrease in the antioxidant defenses. Moreover, this oxidative stress generated in the cultures resulted in oxidative damage to macromolecules such as proteins and lipids. This oxidative phenomenon, without being the main mechanism, could contribute to a better understanding of the infections by *E. coli*.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Aiassa, V., Barnes, A.I., Albesa, I., 2010. Resistance to ciprofloxacin by enhancement of antioxidant defenses in biofilm and planktonic *Proteus mirabilis*. Biochem. Biophys. Res. Commun. 393, 84–88.

Aiassa, V., Baronetti, J.L., Páez, P.L., Barnes, A.I., Albrecht, C., Pellarin, G., Eraso, A.J., Albesa, I., 2011. Increased advanced oxidation of protein products and enhanced total antioxidant capacity in plasma by action of toxins of *Escherichia coli* STEC. Toxicol. In Vitro 25, 426–431.

Albesa, I., Becerra, M.C., Battán, P.C., Páez, P.L., 2004. Oxidative stress involved in the antibacterial action of different antibiotics. Biochem. Biophys. Res. Commun. 317, 605–609.

Baronetti, J.L., Angel Villegas, N., Paraje, M.G., Albesa, I., 2011. Nitric oxide-mediated apoptosis in rat macrophages subjected to Shiga toxin 2 from *Escherichia coli*. Microbiol. Immunol. 55, 231–238.

Becerra, M.C., Páez, P.L., Larovere, L.E., Albesa, I., 2006. Lipids and DNA oxidation in *Staphylococcus aureus* as a con sequence of oxidative stress generated by ciprofloxacin. Mol. Cell. Biochem. 285, 29–34.

Bentancor, A., Rumi, M.V., Gentilin, i M.V., Sardoy, C., Irino, K., Agostini, A., Cataldi, A., 2007. Shiga toxin-producing and attaching and effacing *Escherichia coli* in cats and dogs in a high hemolytic uremic syndrome incidence region in Argentina. FEMS Microbiol. Lett. 267, 251–256.

Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70–76.

Bhakdi, S., Martin, E., 1991. Superoxide generation by human neutrophils induced by low doses of *Escherichia coli* hemolysin. Infect. Immun. 59, 2955–2962.

Capeillere-Blandin, C., Gausson, V., Descamps-Latscha, B., Witko-Sarsat, V., 2004. Biochemical and spectrophotometric significance of advanced oxidized protein products. Biochim. Biophys. Acta 1689, 91–102.

Cavalieri, S.J., Bohach, G.A., Snyder, I.S., 1984. *Escherichia coli* alpha-hemolysin: characteristics and probable role in pathogenicity. Microbiol. Rev. 48, 326–343.

Celik, S., Gorur, S., Aslantas, O., Erdogan, S., Ocak, S., Hakverdi, S., 2007. Caffeic acid phenethyl ester suppresses oxidative stress in *Escherichia coli*-induced pyelonephritis in rats. Mol. Cell. Biochem. 297, 131–138.

Fredriksson, A., Ballesteros, M., Dukan, S., Nystrom, T., 2005. Defense against protein carbonylation by DnaK/DnaJ and proteases of the heat shock Regulon. J. Bacteriol. 187, 4207–4213.

Gonsette, R.E.J., 2008. Neurodegeneration in multiple sclerosis: the role of oxidative stress and excitotoxicity. Neurol. Sci. 274, 48–53.

Grimminger, F., Scholz, C., Bhakdi, S., Seeger, W., 1991. Subhemolytic doses of *Escherichia coli* hemolysin evokes large quantities of lipoxygenase products in human neutrophils. J. Biol. Chem. 266, 14262–21429.

Gupta, S., Wen, J.J., Garg, N.J., 2009. Oxidative stress in Chagas disease. Interdiscip. Perspect. Infect. Dis., 190354.

Hakim, F.A., Pflueger, A., 2010. Role of oxidative stress in diabetic kidney disease. Med. Sci. Monit. 16, 37–48.

Halliwell, B., Whiteman, M., 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br. J. Pharmacol. 142, 231–255.

Heffernan, E., Chatur, N., Zwirewich, C., 2009. *Escherichia coli* 0157 enterohaemorrhagic colitis associated with pyelonephritis: CT findings. Br. J. Radiol. 82, 63–66.

Inal, M., Altinişik, M., Bilgin, M.D., 2002. The effect of quercetin on renal ischemia and reperfusion injury in the rat. Cell. Biochem. Funct. 20, 291–296.

Kastenbauer, S., Koedel, U., Becker, B.F., Pfister, H.W., 2002. Oxidative stress in bacterial meningitis in humans. Neurology 58, 186–191.

Lou, J.G., Dong, J., Zheng, Y.C., Zhang, S.M., Xiao, W.Q., Zhou, J.F., 2006. Increased oxidative stress and damage in patients with chronic bacterial prostatitis. Biomed. Environ. Sci. 19, 481–486.

Moreira, P.I., Honda, K., Liu, Q., Santos, M.S., Oliveira, C.R., Aliev, G., Nunomura, A., Zhu, X., Smith, M.A., Perry, G., 2005. Oxidative stress: the old enemy in Alzheimer's disease pathophysiology. Curr. Alzheimer Res. 2, 403–408.

Niki, E., 2009. Lipid peroxidation: physiological levels and dual biological effects. Free Radic. Biol. Med. 47, 469–484.

Ninković, M., Malicević, I., Jelenković, A., Jovanović, D.M., Dukić, M., Vasiljević, I., 2006. Oxidative stress in the rats brain capillaries in sepsis-the influence of 7-nitroindazole. Acta Physiol. Hung. 93, 315–323.

Páez, P.L., Becerra, M.C., Albesa, I., 2011. Comparison of macromolecular oxidation by reactive oxygen species in three bacterial genera exposed to different antibiotics. Cell. Biochem. Biophys. 61, 467–472.

Pal, S., Polyak, S.J., Bano, N., Qiu, W.C., Carithers, R.L., Shuhart, M., Gretch, D.R., Das, A., 2010. Hepatitis C virus induces oxidative stress, DNA damage and modulates the DNA repair enzyme NEIL1. J. Gastroenterol. Hepatol. 25, 627–634.

Paraje, M.G., Eraso, A.J., Albesa, I., 2005a. Pore formation, polymerization, hemolytic and leukotoxic effects of a new *Enterobacter cloacae* toxin neutralized by antiserum. Microbiol. Res. 160, 203–211.

Paraje, M.G., Barnes, A.I., Albesa, I., 2005b. An *Enterobacter cloacae* toxin able to generate oxidative stress and to provoke dose-dependent lysis of leukocytes. Int. J. Med. Microbiol. 295, 109–116.

Proulx, F., Seidman, E.G., Karpman, D., 2001. Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. Pediatr. Res. 50, 163–171.

Sardesai, V.M., 1995. Role of antioxidants in health maintenance. Nutr. Clin. Pract. 10, 19–25.

Sener, G., Tuğtepe, H., Velioğlu-Oğünç, A., Cetinel, S., Gedik, N., Yeğen, B.C., 2006. Melatonin prevents neutrophil-mediated oxidative injury in *Escherichia coli*-induced pyelonephritis in rats. J. Pineal Res. 41, 220–227.

Siegfried, L., Kmetová, M., Puzová, H., Molokácová, M., Filka, J., 1994. Virulence-associated factors in *Escherichia coli* strains isolated from children with urinary tract infections. J. Med. Microbiol. 41, 127–132.

Smith, J.L., Fratamico, P.M., Gunther, N.W., 2007. Extraintestinal pathogenic *Escherichia coli*. Foodborne Pathog. Dis. 4, 134–163.

Suttorp, N., Fuhrmann, M., Tannert-Otto, S., Grimminger, F., Bhadki, S., 1993. Pore-forming bacterial toxins potently induce release of nitric oxide in porcine endothelial cells. J. Exp. Med. 178, 337–341.

Trifillis, A.L., Donnenberg, M.S., Cui, X., Russel, R.G., Utsalo, S.J., Mobley, H.L., Warren, J.W., 1994. Binding to and killing of human renal epithelial cells by hemolytic P-fimbriated *E. coli*. Kidney Int. 46, 1083–1091.

Valeva, A., Walev, I., Kemmer, H., Weis, S., Siegel, I., Boukhallouk, F., Wassenaar, T.M., Chavakis, T., Bhakdi, S., 2005. Binding of *Escherichia coli* hemolysin and activation of the target cells is not receptor-dependent. J. Biol. Chem. 280, 36657–36663.

Welch, R.A., Bauer, M.E., Kent, A.D., Leeds, J.A., Moayeri, M., Regassa, L.B., Swenson, D.L., 1995. Battling against host phagocytes: the wherefore of the RTX family of toxins? Infect. Agents Dis. 4, 254–272.

Zhang, X.Y., Tan, Y.L., Cao, L.Y., Wu, G.Y., Xu, Q., Shen, Y., Zhou, D.F., 2006. Antioxidant enzymes and lipid peroxidation in different forms of schizophrenia treated with typical and atypical antipsychotics. Schizophr. Res. 81, 291–300.