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Lipoteichoic acid reduces antioxidant enzymes in H9c2 cells

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ABSTRACT

Infective endocarditis (IE) is an illness where the heart is invaded by bacteria, like *Streptococcal* and *Staphylococcal* species that contain lipoteichoic acid (LTA) related to an essential role in this disease. This study is the first in evaluating antioxidant enzyme levels in embryonic cardiomyocyte cell line (H9c2) induced by LTA from *Streptococcus sanguinis*. LTA increased reactive oxygen species (ROS) and reduced the levels of the antioxidant enzymes glutathione peroxidase, superoxide dismutase (SOD)-1 and catalase (CAT) but did not affect glutathione content. At the highest LTA concentration (15 μg/ml), SOD-1 and CAT levels did not change, and this effect was related to the induction of mRNA levels of Nrf2 induced by LTA. These results suggest that low antioxidant enzyme levels and ROS production could be related to IE.

1. Introduction

Infective endocarditis (IE) is a pathology caused by infectious bacteria that lead to inflammation of the inner lining of the heart valves, causing damage to the valves, heart failure, chronic inflammation and even, thromboembolic disease, thus threatening patient's life [1–3]. *Streptococcus* and *Staphylococcus* are the main microorganisms associated with various types of IE [4].

Lipoteichoic acid (LTA) is the main constituent of the cell wall of Gram-positive bacteria [5]. It consists of a backbone of repeating glycerophosphate units with D-alanine or N-acetylglucosamine substituents and a lipophilic anchor [6]. Toll-like receptors (TLRs) are part of the innate immune system that recognize molecular patterns associated with pathogens such as LTA. TLR2 is mainly involved in LTA detection derived from Gram-positive bacteria [3,7]. LTA induces inflammation and contributes to the severe infections caused by Gram-positive bacteria [3,8].

In a normal cell, there is an adequate pro-oxidant/antioxidant balance. However, when the reactive oxygen (ROS) and nitrogen (RNS) species production increased, or there is a diminution in the activity of antioxidant enzymes, oxidative stress occurs [9]. Oxidative stress leads to activation of pro-apoptotic signal proteins, primarily through activation of mitogen-activated protein kinase (MAPK) cascade and c-Jun N-terminal kinases (JNK) [10]. Further, oxidative stress can damage biomolecules, such as DNA, lipids and proteins [11].

The erythroid nuclear factor 2-like 2 (Nrf2) is the master regulator of redox homeostasis; it is a transcription factor that induces the expression of antioxidant and detoxification enzyme genes [12,13]. Nrf2 can be activated by xenobiotics, oxidizing agents and electrophiles by regulating antioxidant defense systems through various mechanisms [14]. In basal conditions, Keap1 represses the transcription factor Nrf2 within the cytoplasm, directing it to ubiquitination and proteasome degradation. When oxidative stress occurs, Nrf2 is released from its repressor, which leads to its translocation to the nucleus and subsequent expression of its target genes [13,15]. Thus, Nrf2 confers cellular protection against the damaging effects of several insults [16].

Some studies have previously shown that LTA from *Streptococcus* induces ROS production, SOD activity reduction, moderate activation of inducible nitric oxide synthase (NOS), and subsequent nitric oxide (NO) production [6,17]. Nevertheless, LTA effects on superoxide dismutase-1

Abbreviations: AKT, protein kinase B; Carboxy-H_2DCFDA, 6-carboxy-2′,7′ dichlorodihydrofluorescein diacetate; CAT, catalase; DHE, dihydroethidium; ERK, extracellular signal-regulated kinases; FDA, fluorescein diacetate; GPx-1, glutathione peroxidase-1; GSH, glutathione; H_2O_2, hydrogen peroxide; IE, infective endocarditis; JNK, c-jun N-terminal kinases; NOS, nitric oxide synthase; LTA, lipoteichoic acid; $O_2^{\cdot-}$, superoxide radical; $OH^{\cdot}$, hydroxyl radical; $ONOO^{-}$, peroxynitrite anion; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; Nrf2, nuclear factor (erythroid-derived 2)-like 2; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD-1, superoxide dismutase-1

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(SOD-1), catalase (CAT), and glutathione peroxidase-1 (GPx-1) antioxidant enzymes levels have not been evaluated.

This work aimed to investigate the LTA effects on ROS and NO production, glutathione (GSH) content, levels of the antioxidant enzymes (SOD-1, CAT, and GPx-1) and Nrf2 mRNA expression, as well as to determine antioxidant enzymes role in cell protection.

2. Material and methods

2.1. Reagents

Rat embryonic cardiomyocyte (H9c2) cell line was from American Type Culture Collection (Manassas, VA, USA). LTA (*Streptococcus sanguinis*), trichloroacetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco´s Modified Eagle´s Medium (DMEM), fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride, ethylenediaminetetraacetic acid, Tris–HCl, NaCl, Nonidet P-40, leupeptin, sodium orthovanadate, fluorescein diacetate (FDA), sodium fluoride, and sodium pyrophosphate were purchased from Sigma-Aldrich (St. Louis MO, USA). One-step reverse transcription-polymerase chain reaction (RT-PCR) and polyvinylidene difluoride (PVDF) membranes were purchased from Invitrogen (Carlsbad, CA, USA), 5-carboxy-2´,7´-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) and dihydroethidium (DHE), were purchased from Molecular Probes (Eugene, OR, USA). Antibodies against GPx-1 were from Abcam (Boston, MA, USA), antibodies against SOD-1 were from Stressgen (San Diego, CA, USA), against CAT were from Calbiochem (San Diego, CA, USA), and against vinculin from Santa Cruz Biotechnology (Dallas, TX, USA). DMEM without red phenol, sulphanilamide, phosphoric acid, sodium nitrite, tetramethoxypropane, acetonitrile, methanol, 1-methyl-2-phenylindole, HCl, monochlorobimane, glutathione-S-transferase (GST) and GSH were purchased from Enzo Life Sciences (Farmingdale, NY, USA). All other reagents were of analytical grade and commercially available.

2.2. Culture treatment

Studies were performed on H9c2 cell cultured in DMEM medium with 10 % FBS supplemented with streptomycin (100 mg/mL), penicillin (100 U/mL) and L-glutamine (2 mM). For measurement of NO production, DMEM without phenol red was used. A humidified incubator with 5 % CO_2 at 37 °C was used. The cells were used at 80 % of confluence. LTA Stocks (1 mg/ml) and their dilutions prepared with phosphate-buffered saline (PBS) sterile. Cells were treated with LTA at 0.1, 1, 5, 10 and 15 μg/ml for 24 h in DMEM-2 % FBS [18] to perform the following determinations.

2.3. Cell viability assay

The proportion of viable cells (percentage of control) was estimated using MTT and FDA assays. Formazan blue crystals are formed after the reduction of tetrazolium sales by mitochondrial dehydrogenase enzymes. Therefore, its absorption is directly proportional to viable cells. The cells were incubated with MTT during four h at 37 °C. Then, the medium was removed and the formazan crystals were dissolved with acid 2-propanol and quantified at 540 nm. The FDA fluorochrome is cell-permeable and is a substrate for viable cell esterases. After treatment, FDA (12 μM) was added to the cell culture, which was placed at 37 °C for 5 min in darkness. Later, cells were washed with PBS and DMEM-2 % FBS were added again. Fluorescence was quantified with Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VA, USA) at 528/20 nm emission and 485/20 nm excitation [19].

2.4. ROS determination

After LTA treatment, ROS production was quantified by using the fluorescent probes carboxy-H_2DCFDA and DHE, according to Hernández Fonseca et al. [20]. 15 μM carboxy-H_2DCFDA and 10 μM DHE were co-incubated in DMEM for 20 min at 37 °C in darkness. Next, they were washed with PBS and subsequently DMEM-2 % FBS was added. Cells were examined under an epifluorescence microscope, using fluorescent cubes B-2A/C and G-2A from Nikon Co. (Tokyo, Japan) using an excitation 488 nm and 530 nm emission for carboxy-H_2DCFDA, and 510 nm excitation and 560 nm emission for DHE. Fluorescence intensity was measured in five different fields for each well for each treatment employing the Imaging Software NIS-Elements (Nikon Co.).

2.5. Measurement of NO production

After LTA treatment, the nitrite released was measured using the Griess method, according to Gutiérrez-Venegas et al. [6]. Briefly, in a 96-well plate, 100 μL of 1 % sulphanilamide in 5 % phosphoric acid were mixed with 100 μL of culture medium without phenol red and incubated for 20 min at 27 °C. The diazo product was measured at 550 nm in a microplate reader, Biotek ELx808. Nitrite concentration was calculated from a standard curve of sodium nitrite. The experiments were conducted three times.

2.6. GSH content

The GSH content was measured by the formation of fluorescent adducts with monochlorobimane. The cells were treated with 1 mM monochlorobimane and 1 U/L GST, for 30 min. By other hand, a standard curve of known GSH concentrations was employed. Fluorescent adducts were measured using a Synergy HT multi-detection microplate reader (Ex/Em = 385/478 nm). The protein concentration was measured using the Bradford protein assay. Values expressed as mmol of GSH mg^{-1} protein [21].

2.7. Antioxidant enzymes levels

Cells were lysed in lysis buffer containing 0.05 M Tris − HCl, 0.5 M phenyl-methylsulfonylfluoride, 0.15 M NaCl, 1 % Nonidet P-40, 10 μg/ml leupeptin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate, pH 7.4. Bradford protein assay was used to quantify protein concentration. Thirty micrograms protein samples were used for SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes. Membranes were blocked in 5 % skim milk during 2 h. Membranes were incubated with primary antibody against SOD-1, CAT and GPx-1 at 4 °C overnight. Then, membranes were incubated with secondary antibody at room temperature for 2 h, as previously described [22]. Bands were visualized using the ECL Western blotting detection advance kit (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions. Films were visualized, scanned, and, quantified using Digi-Doc software.

2.8. Nrf2 mRNA levels by RT-PCR

After six h of LTA treatment, total cellular RNA was isolated [23]. Using the RT-PCR kit, total cellular RNA was reverse-transcribed. PCR was performed using the following oligonucleotides: 5´ TCT CCT CGC TGG AAA AAG AA 3´ (Nrf2 sense); 5´ AAT GTG CTG GCT GTG CTT TA 3´ (Nrf2 antisense) [24,25]. The reaction was performed at 94 °C for amplification, annealing at 55 °C and extension at 72 °C. PCR were carried out for 35 cycles. The amplified PCR products were visualized on an agarose gel by ethidium bromide staining. Data were analyzed with LabsWorks 4.0 (Upland, CA, USA) commercial software.

2.9. Statistical analysis

All the values are expressed as mean ± standard error of the mean

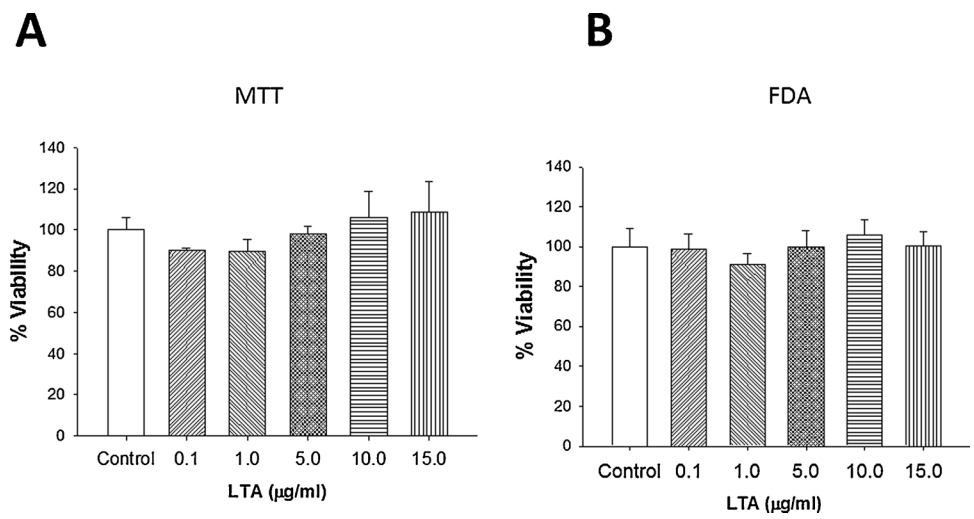

Fig. 1. Effect of lipoteichoic acid (LTA, 0–15 µg/ml) on viability in H9c2 cells determined with (**A**) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and (**B**) fluorescein diacetate (FDA) assays. Each bar represents mean ± SEM from three independent experiments.

(SEM). One-way ANOVA and *post-hoc* Bonferroni tests were used to compare the data using the statistical program Sigma Plot v 11.0 (Systat Software, San Jose, CA, USA). $p < 0.05$ was considered significant.

3. Results

3.1. Cell viability

In order to establish the LTA effect on cell viability, H9c2 cells were incubated at several ligand concentrations (0–15 µM) for 24 h. After treatment, the determination of cell viability was performed by the MTT and FDA methods (Fig. 1). At the concentrations evaluated, LTA exhibited no cytotoxic effect with any of both methods employed. A slight non-significant increase in viability with MTT was observed at higher LTA concentrations (10 and 15 µM).

3.2. ROS determination

ROS production was evaluated using carboxy-H_2DCFDA and DHE. In ROS presence, these compounds oxidized to the fluorescent compounds carboxy-DCF and ethidium, respectively. Both compounds were oxidized in a concentration-dependent fashion with LTA treatment in H9c2 cells (Fig. 2A). The increase of fluorescence was statistically significant at 10 µM LTA (Fig. 2B). It was found that LTA increases ROS levels in a concentration-dependent manner.

3.3. NO production

Treatment with LTA increased NO production in a concentration-dependent manner, getting a maximum effect at 15 µg/ml for 24 h (Fig. 3). However, NO production continued until 72 h (data not shown).

3.4. GSH levels

GSH content was employed as an oxidative stress marker (Fig. 4). Treatment with LTA showed a tendency to reduce GSH at 0.1 and 1 µM LTA; however, these changes were not statistically significant.

3.5. Levels of antioxidant enzymes

Levels of the antioxidant enzymes SOD-1, CAT, and GPx-1 were evaluated by Western blot (Fig. 5). SOD-1, CAT and GPx-1 levels were significantly decreased at 0.1-1.0 µg/ml, 0.1-5.0 µg/ml and 0.1-15 µg/ml, respectively. Thus, our data evidence that LTA treatment induces oxidative stress.

3.6. Nrf2 mRNA levels

LTA treatment induced a significant increase of Nrf2 mRNA levels at 15 µg/ml (Fig. 6), suggesting that Nrf2 and perhaps some phase II enzymes are induced after 6 h treatment with LTA.

4. Discussion

This study is the first demonstration of LTA-induced oxidative stress, evidenced by decreased antioxidant enzymes (SOD-1, GPx-1, and CAT) levels in H9c2 cells.

IE is characterized by bacteria colonization in the heart valves and the vegetations formation composed of bacteria microcolonies, immune cells, fibrin, and blood platelets [1,2]. LTA plays an important role in diverse biological functions. Toll-like receptors (TLR) 2 and TLR 6 are key receptors that recognize components of Gram-positive bacteria, such as LTA [26]. LTA is involved in signal transduction pathways and was shown to induce extracellular signal-regulated kinases (ERK1/2), JNK, p38 MAPK, protein kinase B (AKT) phosphorylation and interleukin-1 beta gene expression in H9c2 cells [1,6].

In this study, LTA did not show a cytotoxic effect in H9c2 cells and confirms what was previously found by Gutierrez-Venegas et al. [27]. However, Liu et al. [28] found a reduction in cell viability, probably due to the usage of DMSO to dissolve LTA [29] and a different LTA source (which was not indicated).

The primary sources of intracellular ROS are the mitochondrial electron transport chain, the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and cytochrome P450. The major ROS are superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) [30,31]. The antioxidant system maintains redox balance in the body. It consists of non-enzymatic antioxidants, such as bilirubin and GSH, and enzymatic antioxidants, such as SOD, CAT and GPx, among others [32]. When there is an excessive ROS and RNS production or a reduction in the antioxidant system, oxidative stress is generated.

Oxidative stress has been related to physiological processes like aging and atherosclerosis, cardiovascular diseases, cancer, diabetes mellitus and kidney damage [10]. In fact, Ostrowski et al. [33] have proposed increased ROS production in IE as a clinical indicator. ROS production and inflammatory markers, including procalcitonin, C-reactive protein (CRP), leukocytosis and erythrocyte sedimentation rate

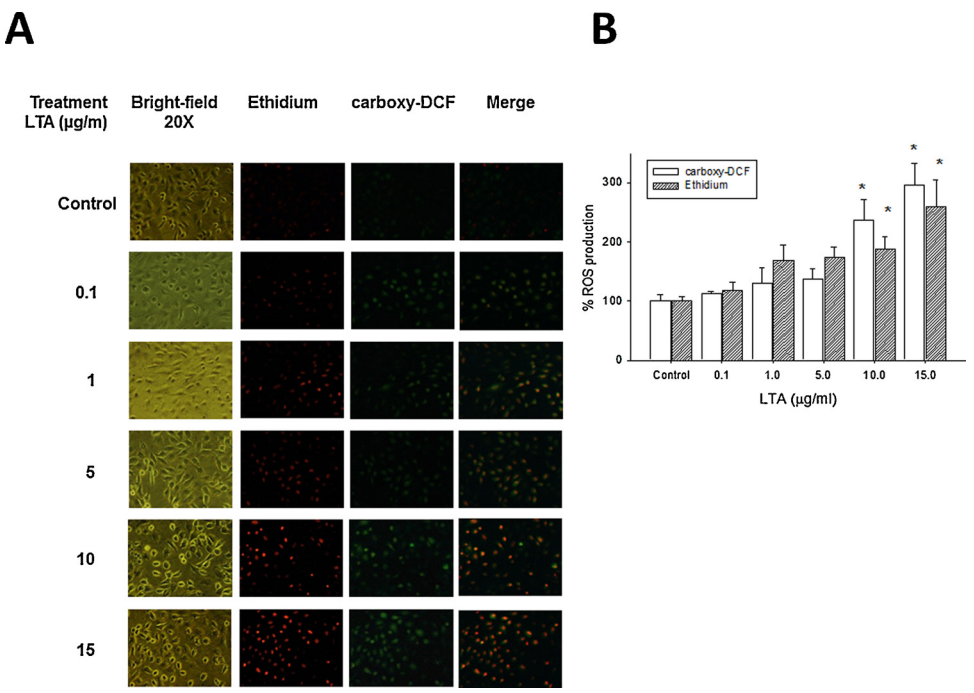

Fig. 2. Lipoteichoic acid (LTA, 0–15 µg/ml) induces reactive oxygen species (ROS) production in H9c2 cells. **A**: Representative micrographs show that LTA treatment increases ROS production in a concentration-dependent manner employing ethidium (in red) and carboxy-DCF (in green). Merge images shown in orange. **B**: Fluorescence intensity measured in five different fields per well per condition of three independent experiments. Fluorescence changes in ethidium and carboxy-DCF expressed as a percentage of ROS production relative to the control group. Each bar represents mean ± SEM from at least three independent experiments. * $p \leq 0.05$ vs. control group.

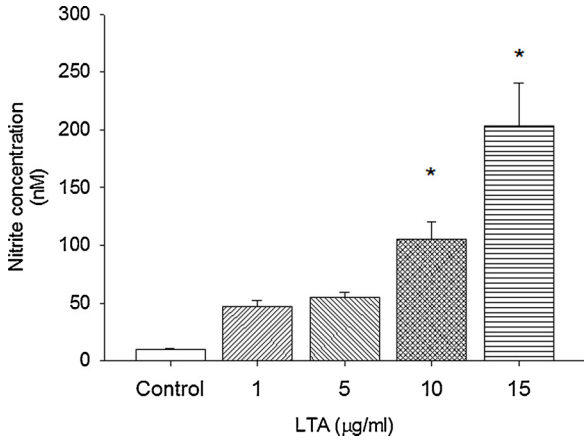

Fig. 3. Nitric oxide released to the culture medium of H9c2 cells. Lipoteichoic acid (LTA) treatment increases nitric oxide production in a concentration-dependent manner. Each bar represents mean ± SEM from at least three independent experiments. * $p \leq 0.05$ vs. control group.

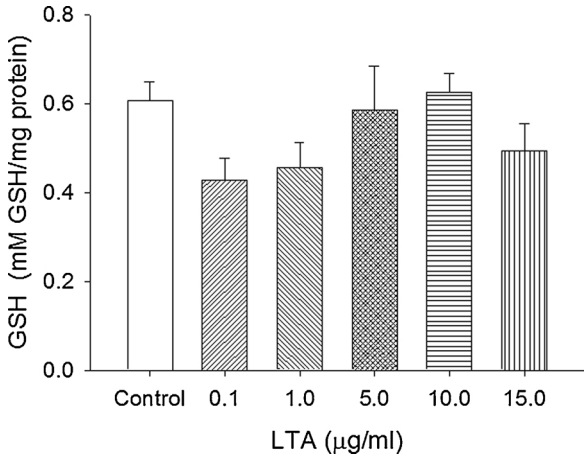

Fig. 4. Effect of lipoteichoic acid (LTA) on glutathione (GSH) content in H9c2 cell line. Each bar represents mean ± SEM from at least three independent experiments.

(ESR), were evaluated on patients operated for IE. Noteworthy, it was found a positive correlation between ESR, CRP, and ROS in the preoperative period, indicating that ROS assessment could be a clinical parameter in patients with IE [33].

In the present study, carboxy-H_2DCFDA and DHE were used to evaluate ROS production. DHE is considered a specific test for the detection of $O_2^{\cdot -}$ production, but it also can react with other species such as peroxynitrite anion ($ONOO^-$) and hypochlorous acid, providing an index of RNS and ROS production [34]. Ethidium is the responsible fluorophore generated after its oxidation by these species and is inserted into DNA emitting bright red fluorescence [20,34]. Carboxy-H_2DCFDA, a cell membrane-permeable dye, accumulates in the cytosol where intra-cellular esterases remove acetates producing the impermeable and non-fluorescent compound carboxy-H_2DCF, which is oxidized by several ROS including H_2O_2, $O_2^{\cdot -}$, $OH^{\cdot}$, nitrogen dioxide, NO, $ONOO^-$, peroxyl radical, alcoxyl radical and carbonate and is converted to the fluorescent compound 2′,7′-carboxydichloro fluorescein (carboxy-DCF), which emits a green fluorescence [34,35]. This compound employed as an indicator of the degree of cellular oxidation, and it is considered a marker of cellular oxidative stress [34,36]. Our results indicate that LTA induces oxidative stress in a concentration-dependent manner.

LTA treatment induced a significant increase with carboxy-DCF and DHE at 10 µg/ml concentration, indicating that LTA ligand enhances ROS and RNS production in H9c2 cells. Results of this work agree with other studies in which it has been shown that LTA increases ROS production using carboxy-H_2DCFDA in brain astrocytes (RBA-1), human dermal fibroblasts (HDF), human tracheal smooth muscle cells (HTSMC), rat lung microvessel endothelial cells (RLMVEC), spinal cord microglia [8,37–39] and recently in H9c2 cells [28] or using DHE in RLMVEC cells [8,37–39]. It has described that NADPH oxidase [8,37,39], mitochondrial dysfunction [28] and NOS [24] are responsible for ROS and RNS production, respectively.

Nitrate and nitrite concentration also evaluated as the stable products of NO. Treatment with LTA increased in the production of these metabolites in a concentration-dependent manner. Data are consistent with those of Gutiérrez-Venegas et al. [6] and Chatterjee et al. [17]

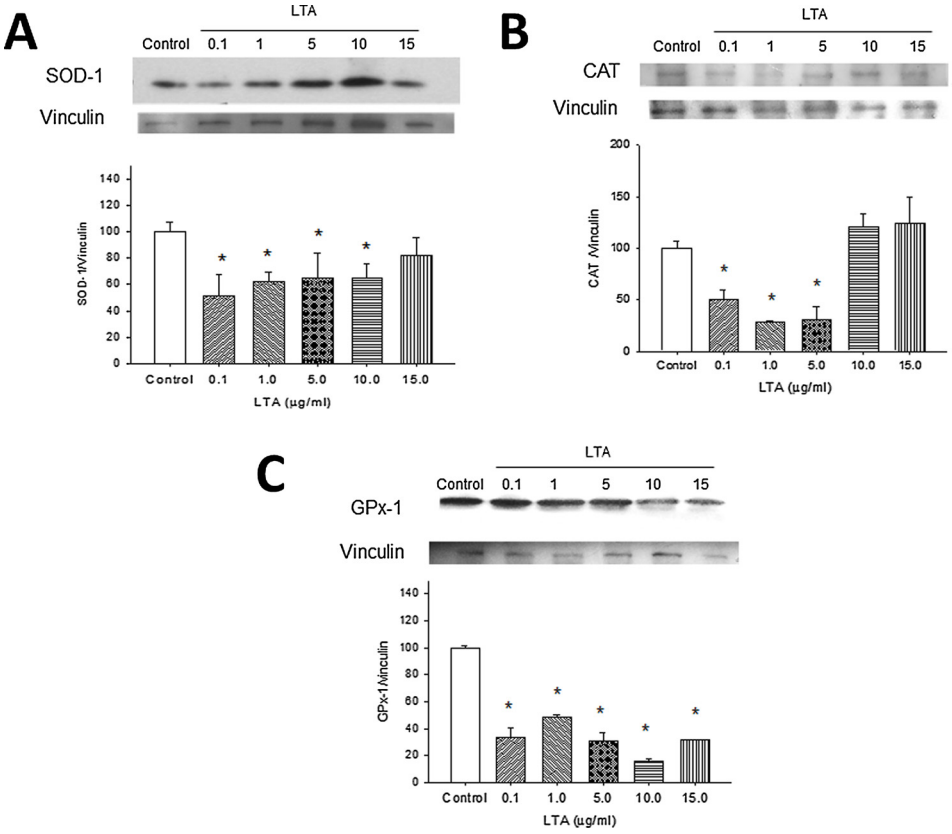

Fig. 5. Effect of lipoteichoic acid (LTA) on antioxidant enzyme levels measured by Western blot. **A**: Superoxide dismutase-1 (SOD-1) was reduced with LTA (0.1–10 µg/ml) treatment. **B**: Catalase (CAT) was reduced with LTA (0.1–5 µg/ml). **C**: Glutathione peroxidase-1 (GPx-1) was depleted with LTA treatment (0.1–15 µg/ml). Vinculin (a cytoskeletal protein) used as a loading control. Graphs show the densitometric analysis (protein/vinculin) from each band from three independent experiments. Data are means ± SEM, *p ≤ 0.05 vs. control group.

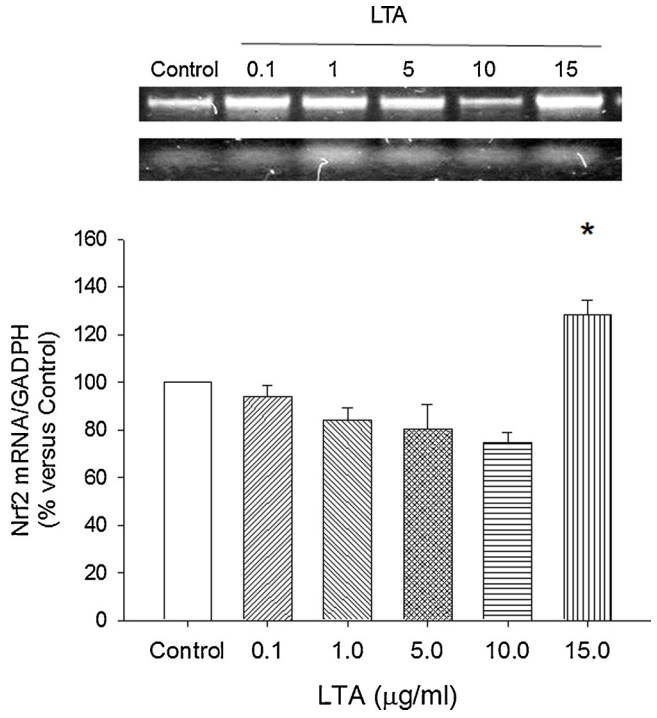

Fig. 6. Effect of lipoteichoic acid on the induction of mRNA Nrf2 expression. LTA (15 µg/ml) increased mRNA levels of Nrf2. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) used as a control. Graphs show densitometric analysis (expressed as Nrf2/GADPH ratio) from each band from three independent experiments. Data are means ± SEM. *p ≤ 0.05 vs. control group.

suggests that inducible NOS produces NO. The antioxidant system were evaluated through the enzymes SOD-1, CAT, and GPx-1. SOD-1 is the intracellular isoform of SOD containing Cu/Zn at its catalytic center [40,41]. It is an enzyme that catalyzes $O_2^{\bullet-}$ dismutation producing the non-radical molecule H_2O_2 [32]. CAT is an enzyme that catalyzes the H_2O_2 decomposition into water and molecular oxygen, avoiding the damage to cellular components important for cell survival [9,32,42]. GPx-1 contains selenium as a cofactor, and it is the cytosolic isoform of GPx that metabolizes H_2O_2 or organic peroxides to water or alcohols, by coupling their reaction with GSH oxidation. GSH is found in the cytoplasm in mM concentrations [43]. LTA treatment depleted GPx-1, CAT and SOD levels at 0.1 to 5 and 0.1 to 15 µg/ml concentrations, respectively. LTA treatment decreased SOD-1 and might attenuate conversion of $O_2^{\bullet-}$ into H_2O_2; our data agree with those obtained by Liu et al., [28]. Besides, the decreased expression of GPx-1 and CAT enzymes may enhance H_2O_2 levels that may lead to increased $OH^{\bullet}$ production in the presence of free iron or $O_2^{\bullet-}$. The half-life time of $OH^{\bullet}$ is 10^{-9} s; it quickly attacks molecules that are around it, causing irreversible damage [44]. In fact, the reduction in the antioxidant enzyme system levels suggest a reduction in ROS scavenging [9]. The best way to prevent its damage is to avoid its production, therefore enzymes GPx-1 and CAT are extremely important.

GSH is a potent reducing agent and a major antioxidant that maintains the cell's antioxidant status, it is involved in cell signaling (cell cycle modulation, proliferation, and apoptosis) and metabolism of xenobiotics and it is a reservoir of cysteines [45]. The intracellular levels of GSH are a result of synthesis, consumption, and transport. Surprisingly, treatment with LTA did not significantly modify its content in H9c2 cells and only showed a tendency to reduce it. Oxidative stress may induce damage to antioxidant enzymes. Probably, ROS production, especially $OH^{\bullet}$, affected SOD-1, CAT, even GPx-1 enzymatic activity; for that reason, CAT increased their levels to high concentrations of LTA; however, ROS production continued to increase. These ideas are supported by Liu et al., which found SOD activity reduction induced by LTA [28]. Besides, the LTA concentration range employed in this research is biologically relevant; it can be achieved in the dead space of an intravascular catheter with biofilm (10^7 to 10^9 colony-forming units)

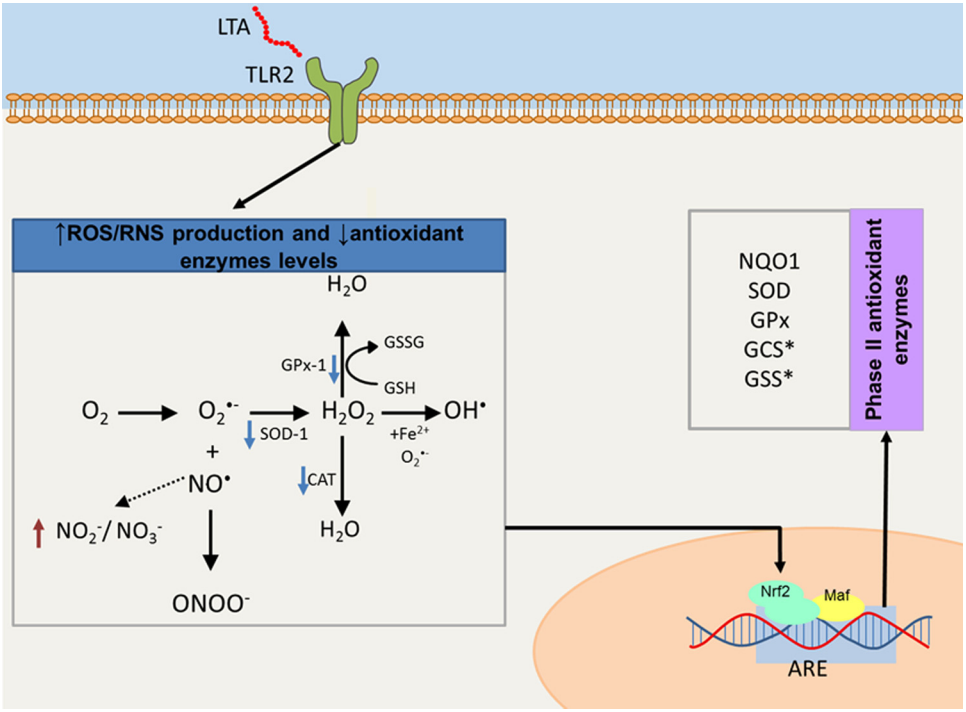

Fig. 7. Infective endocarditis (IE) is an inflammation of the inner lining of the heart valves caused by infectious bacteria such as *Staphylococcus aureus*. These Gram-positive bacteria contain lipoteichoic acid (LTA) that plays an important role in IE in which, Toll-like receptors (TLR) 2 are the main receptors that recognize it. It was found that LTA treatment induces ROS production. LTA treatment depleted glutathione peroxidase (GPx)-1 at all concentration employed and reduced catalase (CAT) and superoxide dismutase (SOD)-1 expression at 0.1 to 5 and 0.1 to 10 μg/ml concentrations, respectively. The LTA-induced decrease in SOD-1 expression at 0.1 and 1 μg/ml concentrations may attenuate the conversion of superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). Also, the decrease in the expression of GPx-1 and CAT enzymes may attenuate the H_2O_2 degradation and thus enhancing its concentration that might increase hydroxyl radical ($OH^{\cdot}$) production in the presence of free iron or $O_2^{\cdot-}$. Its half-life time is 10^{-9} s and quickly attack molecules that are around it, causing irreversible damage. Besides it was found that LTA induced nuclear factor erythroid 2-related factor 2 (Nrf2) related with the synthesis of enzymes of phase II as SOD, CAT, and the enzymes involved in glutathione (GSH) production and regeneration, gamma-glutamylcysteine synthetase (GCS*) and glutathione synthetase (GSS*), probably explaining why these proteins are not decreased at some LTA concentrations. Further studies should be performed to evaluate an "*in vivo*" model.

[38] that can induce IE in an "*in vivo*" model [46]. In fact, it has been described that patients with meningitis have LTA and teichoic acid concentrations in a range of 0.2 – 27 μg/ml of a sample of cerebral spinal fluid taken by lumbar puncture [47]. Similar doses to those used in this work have been used to induce uveitis (230.8 μg/ml blood) [48] and intestinal diseases (4.5 mg of LTA / ml blood) in rats [49]. In addition, the concentrations used in this work are the effective concentrations used in *in vitro* studies of LTA in H9c2 cells previously published [6,27,50]. Besides, it can induce chronic inflammation, thrombolytic problems [2,3,51,52] and trigger a full activation of monocytes [7]. On the other hand, Nrf2 induces the transcriptional activation of several antioxidants that contains the ARE sequence, including SOD, GPx, GST, glutathione synthetase and glutamate-cysteine ligase, enzyme that catalyzes the first and rate-limiting step in GSH synthesis [53,54]. Treatment with 15 μg/ml of LTA increased Nrf2 mRNA levels, and this could explain why SOD and CAT expression is normal at the highest LTA concentration in H9c2 cells compared with the other LTA concentrations, which by the way, decreased SOD and CAT levels (Fig. 5A and B). However, GPx-1 expression decreased at any LTA concentration (Fig. 5C). Besides, GSH content did not modify due to low GPx-1 expression (low consumption) and because it modulated by Nrf2 (induces its synthesis). Fig. 7 depicts the resume of data found in this work.

The present study differs from the previous report [28] in cell viability, GSH content, and Nrf2 evaluation. This difference may be secondary to the DMSO use to dissolve LTA and to the differences in the employed methodologies. Our results obtained in an *in vitro* model warrant studies in animal models.

5. Conclusion

In conclusion, LTA treatment-induced oxidative stress, which was characterized by ROS production, depletion of GPx-1, decreased levels of SOD-1 and CAT enzymes in H9c2 cells. Further studies should be performed to evaluate the antioxidant enzymes status in an "*in vivo*" model of IE. These data provide new information into the LTA mechanism related to ROS and antioxidant enzyme status exerted by bacteria that contain LTA in IE *in vitro* model. Further, the use of antioxidants would protect against LTA in IE.

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Transparency document

The Transparency document associated with this article can be found in the online version.

CRediT authorship contribution statement

Berenice Fernández-Rojas: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Gustavo I. Vázquez-Cervantes:** Investigation, Formal analysis. **José Pedraza-Chaverri:** Resources, Writing - review & editing. **Gloria Gutiérrez-Venegas:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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