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Alteration of the aortic vascular reactivity associated to excessive consumption of *Hibiscus sabdariffa* Linnaeus: Preliminary findings

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ABSTRACT

The moderate production of reactive oxidative species (ROS) is important because ROS act as second messengers. However, their depletion through the over-activity of the antioxidant system may lead to reductive stress (RS) which is characterized by an increase in reducing equivalents and an elevation of some components of the antioxidant system disturbing redox homeostasis. *Hibiscus sabdariffa* Linnaeus (HSL) is a plant with antioxidant properties that provides compounds that favor the antioxidant system. However, excess chronic consumption could lead to the over expression of the antioxidant enzymatic system, and this could contribute to decrease ROS. Therefore, the objective of this study was to evaluate the alteration of the vascular reactivity associated to excessive and chronic consumption of HSL infusions at different percentages. 40 male Wistar rats were divided into 4 groups. Group 1 control (drinking tap water), group 2, 3 and 4, drinking water supplemented with 15, 30 and 60 g/L of HSL calyxes respectively. The systolic blood pressure (SBP), vascular reactivity, morphological changes, and different components of the enzymatic antioxidant system were evaluated in the thoracic aorta by spectrophotometry. We also determined glucose-6-phosphate dehydrogenase (G6PD), glutathione-S-transferase (GST), thioredoxin-reductase (TrxR), glutathione peroxidase (GPx) and glutathione reductase (GR) and some markers of the non-enzimatic system such as the NO_3^-/NO_2^- *ratio,* glutathione (GSH), selenium, thiols, lipoperoxidation (LPO), and 3-nitrityrosine (3-NT). Vasoconstriction was increased and vasorelaxation was decreased. These alterations were reversed by O_2^- and H_2O_2. There was an increase in the wall thickness and elastic fibers ($p = 0.004$ and $p = 0.02$, respectively) and in G6PD, GPX, TrxR ($p = 0.02$, $p = 0.03$, and $p = 0.01$ respectively). LPO, GSH ($p = 0.01$), and selenium ($p = 0.04$) were decreased. There was a decrease in thiols ($p < 0.001$), 3-NT ($p = 0.04$) and GST ($p = 0.0005$) in rats that received the infusion at 3 and 6%. The excess antioxidants provided by the HSL infusions at 3% and 6% modified vascular reactivity, increasing the enzymatic antioxidant system, and depleting ROS.

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1. Introduction

Redox balance is important for cellular homeostasis and a moderate production of reactive oxygen species (ROS) leads to beneficial effects [1,2]. ROS, such as nitric oxide (NO), anion superoxide (O_2^-) and peroxide hydrogen (H_2O_2) in small amounts act as second messengers that contribute to aortic vasodilation [3]. In this sense, H_2O_2 modulates signal transduction by the reversible oxidation of redox-active cysteines [4] and it elevates the expression of at least 40 gene products [5]. In contrast, overproduction of ROS has deleterious effects. Therefore, enzymatic and non-enzymatic antioxidant systems play an important role in maintaining the non-cytotoxic level of ROS (at picomolar concentrations) [6]. Among the enzymes that constitute the antioxidant enzymatic system, thioredoxin reductase (TrxR), superoxide dismutase (SOD) isoforms, the glutathione peroxidase (GPx) family, glucose-6-phosphate dehydrogenase (G6PD) and glutathione-S-transferase (GST) can be mentioned [7]. On the other hand, molecules that participate in the non-enzymatic antioxidant system include vitamins C, E, A, glutathione (GSH), and Selenium (Se), among others. The proper functioning of the enzymatic- and non-enzymatic antioxidant systems contributes to the balance of redox homeostasis [8]. In addition, much attention has been paid on the effects of excessive ROS and oxidative stress (OS), acting upon complex metabolic pathways in multiple organs and systems and on their involvement in physiology and pathology [1]; however, less emphasis has been placed on the effects of reduced levels of ROS by over expressions of the enzymatic or non-enzymatic antioxidant system that could result in reductive stress (RS).

RS is the counterpart of OS, and it consists of an abnormal increase in reducing equivalents in the presence of intact oxidation and reduction systems [9]. Depletion of ROS and/or overproduction of the enzymatic and non-enzymatic antioxidant systems lead to pathological consequences [9]. In this sense, the excess reducing equivalents in the form of GSH/GSSG, $NADPH/NADP^+$ and $NAD/NADH^+$ redox couples or overexpression of antioxidant enzymatic systems can deplete ROS [9,10]. Excess reducing equivalents may reduce mitochondrial function, cell growth responses, and cellular metabolism. They may induce alterations in the formation of protein disulfide bonds, and contribute to the development of some diseases that are closely associated with inflammatory conditions such as muscular dystrophy, protein deficiency, hypertrophic cardiomyopathy, pulmonary hypertension, rheumatoid arthritis, cancer, and metabolic syndrome (SM); Furthermore, chronic RS may induce OS by a positive feedback regulation [9–11]. However, the metabolic pathways or the ROS that participate in the regulation of RS remain unknown.

The aorta and other vessels are susceptible to the harmful effects of RS [9,11]. This vessel is the largest artery in the organism of animals and humans and any alteration in its structure and/or function disturbs the cardiovascular system [12]. The aortic wall has three layers: Intima (endothelium), media (smooth muscle cells, collagen, and elastic fibers), and adventitia. Both collagen and elastin determine the resistance to traction forces and stiffness of the aorta. The mechanical properties of the aorta depend on the amounts of the main components of these layers, and on the spatial organization and the mechanical interactions between these components [13, 14]. In addition, endothelial cells, located in the intima, are metabolically active and release various vasoactive mediators such as NO, prostaglandins, and endoperoxides [15,16] and may stimulate the synthesis metalloproteinase, growth factors and heparin [15]. Homeostasis of the production of these substances is atheroprotective, since an imbalance generates endothelial dysfunction, which predicts the development of atherosclerosis and hypertension [16]. Likewise, endothelial cells are constantly exposed to various stimuli, among which oxidized lipoproteins, frictional forces, inflammatory agents, cytokines, and ROS can be counted [17]. The alteration in the structure and/or function of endothelial cells leads to various pathologies such as cardiomyopathies [15].

On the other hand, the high consumption by the population of antioxidants from plants such as *Hibiscus sabdariffa* Linnaeus (HSL) or antioxidant synthetic products is increasing without a proper assessment of the physiological impact that may be caused in the long-term, including RS. HSL is known as Jamaica flower in México and Karkade or Zoborodo in Sudan and Nigeria respectively [18,19]. It is used in the preparation of different drinks. In traditional medicine, it has been used as treatment for hypercholesterolemia, hypertension, liver damage, kidney stones, pyrexia, choleretic, diuretics, and hypotensive effects and for stimulating intestinal peristalsis [19–21]. It has been described that the HSL calyxes contain many chemical components including flavonoids, polyphenols, quercetin, vitamin C, selenium (Se) and protocatechuic, gallic, tannic acids that have been used against the OS present in pathologies such as the MS and hypertension [22]. Also, various chemical compounds present in the HSL calyxes are water soluble and can be extracted. Infusion of this plant contain considerable concentrations of these antioxidants. In this sense, polyphenols, may protect cellular components from oxidative damage, such as lipoperoxidation (LPO) [23], increase the activities of catalase (CAT), GPx and SOD isoforms, inhibit the activities of xanthine-oxidase, angiotensin converting enzyme, cyclooxygenase 2 and the inducible nitric oxide synthase (iNOS), and contribute to regeneration of vitamin C and E. They increase the concentration of GSH and the formation of thiol bridges between the cysteines of the proteins [24]. They may also prevent liver cell apoptosis by inhibiting the activation of *p*-JNK and p38 MAPK, which are transcription factors activated during inflammation [25]. However, polyphenols can show pro-oxidant activity when they are consumed at high doses [26]. The pro-oxidant activities of several polyphenols, such as quercetin, catechins, and gallic acid, have been reported [27]. Several studies have shown that cell survival and viability, thiol content, total antioxidant capacity (TAC), and activities of SOD isoforms, CAT, and glutathione-S-transferase (GST) are reduced by quercetin at 50 μM [28]. High levels of flavonoids (50–250 μM) produce cytotoxicity, DNA damage, apoptosis, and the presence of ROS by auto-oxidation [29]. In addition, polyphenolic acids at high concentrations exhibit pro-oxidant activities when transition metal ions are present, such as copper and iron, forming chelator agents and reducing the TAC [30]. The phenolic antioxidants are converted to phenoxyl radicals which can be the basis of a cascade of pro-oxidant events that are characterized by self-oxidation of diphenol or polyphenols concomitant with univalent reduction of molecular oxygen, followed by dismutation of O_2^- formation and subsequent $OH^\bullet$ formation in a Fenton-Weiss type reaction [31].

There is little experimental evidence to suggest that chronic consumption of high concentrations of antioxidants may lead to the development of aortic alterations. Therefore, the objective of this study was to evaluate the changes in the vascular reactivity

associated to excessive and chronic consumption of HSL infusions at different percentages. Our hypothesis was that the excess of antioxidant agents supplied by different percentages of HSL in infusion could modify vascular function by generating the over-expression of reducing equivalents and/or antioxidant systems that eliminate ROS, thus favoring RS.

2. Materials and methods

2.1. Animals

The study was designed and carried out in compliance with the Laboratory Animal Care Committee of the National Institute of Cardiology "Ignacio Chávez" in Mexico (SICUAE.DC.2019/3–4) approved for experiments in animals. Experiments were conducted in compliance with the Guide for the Care and use of laboratory animals of the National Institutes of Health (NIH). Exclusion criteria: Rats weighing less or more than required, and animals that presented clinical diseases such as respiratory problems, digestive problems, loss of appetite, aggressiveness, among others. 40 male Wistar rats were used to form 4 groups with 10 animals each, as follows: Group 1, control (water); Group 2, HSL at 1.5%; Group 3, HSL at 3%; Group 4 HSL at 6%. The animals were kept for 4 weeks under the following conditions: 12-h light/12-dark cycle, environment temperature, and relative humidity between the ranges of 18–26 °C and 40–70%, respectively. Commercial rodent feed that contained 6% crude fiber, 4.5% crude fat, 23% crude protein, 2.5% minerals and 8% ash, (Labdiet 5008; PMI Nutrition International, Richmond, IN.) was provided freely. The HSL infusion at different percentages was administered orally (ad libitum) in drinking water. The systolic blood pressure (SBP) determinations were taken at the end of the experimental period of 4 weeks and it was measured using a tail cuff attached to a pneumatic pulse transducer method [32], (Narco Bio-Systems Inc., Houston, TX, USA).

2.2. Preparation of the HSL infusion

The HSL calyces were acquired in Chilapa de Alvarez, México. To one liter of boiling water, 15, 30, and 60 g of HSL calyxes were added. The solution was kept boiling for 10 min, and was then left to cool, filtered and stored at 4 °C until consumption.

2.3. Determinations of polyphenols, total flavonoids, and total anthocyanins in the HSL infusion

The determination of total flavonoids was done by the Jia method [33]. This technique determines total flavones and flavonols that react with aluminum trichloride forming stable complexes such as apigenin, chrysin, and luteonin, as the flavonols morin, quercetin, myricetin, kaempferol, and galangin, which can be analyzed spectrophotometrically. The calibration curve was achieved using quercetin as a standard and the absorbance was read at 510 nm. Determination of total anthocyanins was performed by Lee's method [34,35]. This technique determines the total anthocyanins which have various monomeric pigments in their structure that manifest when the pH changes. At acidic pH, this molecule is in a stable form known as oxonium, which is of a deep red color, while at basic pH; the molecule loses a proton and adds water to its structure, giving rise to its unstable form known as hemicetal, which has no color. The absorbance was measured at 520 nm and 700 nm and compared with a blank cell containing distilled water. The absorbance difference was performed to calculate cyaniding-3-glucoside.

2.4. Vascular reactivity

The thoracic aorta (TA) was dissected and sectioned in rings of 2 mm that were suspended by metallic hooks in 5 ml glass chambers for isolated organ register according to the method described by Pérez-Torres et al. [17]. Concentration-response curves to acetylcholine (Ach) were obtained; the contraction of the rings was induced initially with 2×10^{-7} M, norepinephrine (NE) and then Ach was added in concentrations from 2×10^{-9} to 10^{-5} M accumulatively. For the contraction curves, NE was added at increasing concentrations; 2×10^{-9} to 2×10^{-5} M; when the maximal contraction response curve was reached for each concentration, the next concentration was added. After obtaining each series of contraction or relaxation curves, the aortic rings were washed three times with Krebs solution and allowed to recuperate for 30 min. Dose-response curves to assess the participation of ROS were obtained in the presence of potassium superoxide (KO_2). The aortic rings were incubated for 10 min with KO_2 at a concentration of 2×10^{-7} M (Half maximal effective concentration, EC_{50}). After this time, the relaxation curve was made by adding 2×10^{-7} M of NE (EC_{50}). Once the contraction curve reached its maximum, increasing and cumulative concentrations of Ach; 2×10^{-9} M to 2×10^{-5} M were added. After obtaining the relaxation and contraction curves in the presence of KO_2, dose-response curves were obtained with H_2O_2 at a concentration of 2×10^{-6} M (EC_{50}), in the same way as for the KO_2.

2.5. Histology of the aortic rings

The aortic rings of each of the rats were preserved in 10% formalin in a 1:20 ratio. Histological sections were made and stained with the Masson trichrome and Jones methylamine silver techniques. The histological sections were analyzed with a Carl Zeiss light microscope (66300 Model) equipped with a 9-megapixel Cool SNAP-Pro digital camera and analyzed with the Sigma Scan Pro 5® program.

2.6. Homogenization of the thoracic aorta

After obtaining the aortic rings, the rest of the TA was frozen in liquid nitrogen and homogenized, according to the methods that were previously described by Soto et al. [36]. The determination of total proteins from the homogenate was carried out according to the method described by Bradford.

2.7. NO_3^-/NO_2^- ratio

NO_2^- reacts in acid media with sulfanilic acid to form a diazonium cation which is then coupled to the aromatic amine 1-naphthylamine to produce a red-violet coloration that can be read by the spectrophotometer at 540 nm. The NO_3^- was reduced to NO_2^- by the nitrate reductase enzyme reaction (5 units), of 50 μg protein from the TA homogenate previously deproteinized with 0.5 N NaOH. The absorbance was measured at 540 nm, and detected by the Griess method [37].

2.8. Glutathione concentration

This test consists of the oxidation of GSH by means of Ellman's reagent to form the yellow chromophore 5′-thio-2-nitrobenzoic (TNB), which can be quantified by using the spectrophotometer. The rate of TNB formation is proportional to the concentration of GSH in the sample. 100 μg of protein from the TA homogenate were used to make the determination according to a previous method by Ellman, and the absorbance was read at 412 nm [38].

2.9. Selenium determination

200 μg of protein from the TA homogenate were used to make this determination according to the method described by Soto et al. [37], and the absorbance was read at 600 nm.

2.10. Determination of total thiol groups

This test consists of the reaction of Ellman's reagent with a thiol, commonly a thiolate, producing thoil-nitrobenzoate. The technique used was previously described by Erel and Neselioglu [39], with some modifications carried out in our laboratory as previously reported [37]. 50 μg of protein from the TA homogenate were used for the determination and the absorbance was measured at 415 nm.

2.11. Determination of LPO levels and 3-nitrotyrosin

This test is based on the reaction of malondialdehyde, a secondary product of the oxidation of fatty acids with three or more bonds, with thiobarbituric acid in an acid medium and at high temperature, generating a pink-colored product [40]. 100 μl of protein from the TA homogenate were used for this determination and the absorbance was measured at 532 nm. The determination of 3-nitrotyrosin (3-NT) was made with a kit provided by LifeSpan BioSciencies, Seattle, WA, USA kit No. LS-40120). This assay is based on the competitive ELISA principle and is measured at a wavelength of 450 nm, using a visible light micro plate reader (Stat Fax 3200 Awareness Technology Palm City, FL, USA).

2.12. Determinations of the activities of TrxR, GPx, GST and GR

The activity of TrxR was determined using 100 μg of protein from the TA homogenate according to the method described by Soto et al. [38]. The sample was incubated and monitored at 412 nm for 6 min at 37 °C. To evaluate the GPx, GST and GR activities, 100 μg of protein from the TA homogenate were used as previously described [22]. The sample was incubated and monitored at 340 nm for 8 min at 37 °C.

2.13. Activity of glucose 6 phosphate dehydrogenase

To evaluate G6PD activity, 2.9 ml of TRIS-HCl buffer (0.1 M, pH 7.5) was used, plus 2 μl of G6P (2 mM) and 10 μl of $NADP^+$ (5 mM). The mixture was read in a spectrophotometer at 340 nm and allowed to stabilize for 1 min at 37 °C, after which 100 μg of protein from the TA homogenate were added and the absorbance was monitored for 8 min at 340 nm [41].

2.14. Statistical analysis

The Sigma Plot program (SigmaPlot® version 14.5, Jandel Corporation) was used for statistical analysis. The GraphPad-Prism 8 Software. Inc. (San Diego, CA, USA), 1995–2023, was used to generate the graphs. The data are presented as mean ± standard error. Statistical significance was determined with one-way ANOVA and Tukey's post hoc test. A $p \leq 0.05$ was considered as significant.

3. Results

3.1. Subsection guaranteed analysis

Table 1 shows the amounts anthocyanins, flavonoids and polyphenols contributed by the drinking water at the different percentages of HSL infusions. These infusions were administered to the rats from the different experimental groups.

3.2. Systolic blood pressure

Fig. 1 Shows the SBP of the experimental groups. The rats that consumed the HSL infusion at 3% and 6% showed a significant increase ($p = 0.02$ and $p = 0.01$ respectively) compared to the control group.

3.3. Vascular reactivity

Fig. 2 (A) shows the vasodilation response in the aortic rings of the experimental groups. There is a decrease in the vasodilation response compared to the control group in the rats that consumed the HSL infusion at 3% ($p = 0.05$) and 6% ($p < 0.01$). In Fig. 2B the vasoconstriction in the aortic rings of the rats that consumed the HSL infusion at 3% ($p = 0.3$) and 6%, $p = 0.001$) showed an increase when compared to the control group.

Fig. 3 (A) shows the O_2^- participation in the vasoconstriction and vasodilatation response in the thoracic aorta rings of the different experimental groups. The vasoconstriction response showed an increase in the control group in the presence of NE vs. NE plus KO_2 (30 μM), ($p < 0.001$). In addition, an increase in the vasoconstriction response in the aortic rings was observed between the control group in the presence of NE and the 3% and 6% HSL infusion groups ($p = 0.002$ and $p = 0.0003$ respectively).

Fig. 3 (B) shows that the vasodilatation response in the control group was lower ($p = 0.01$) with a significant difference in the presence of Ach vs. Ach plus KO_2 (30 μM). This same trend was present in the vasodilation response in the control group in the presence of Ach and in the group of rats that received the 6% HSL infusion ($p = 0.02$). Fig. 3 (C) shows there was a significant difference with the HSL infusion at 1.5%, 3% and 6% in the presence of NE vs. NE plus H_2O_2 ($p = 0.001$, $p = 0.007$ and $p = 0.02$ respectively). In addition, there was a significant increase in the vasoconstriction response in the presence of NE in the control group against the experimental groups with HSL infusion at 3% and 6% ($p = 0.002$ and $p = 0.0003$ respectively). There was also an increase in the vasoconstriction response in the presence of NE plus H_2O_2 between the groups with the HSL infusion at 6% ($p = 0.03$) against the control group. Fig. 3 (D) shows that the vasodilation response in the thoracic aorta ring was decreased significantly in the HSL group at 6% with Ach ($p = 0.01$) in comparison with the control group. In addition, when aortas were incubated with Ach plus H_2O_2, an increase was observed in the same group with the HSL infusion at 6% ($p = 0.04$).

3.4. Histology and densitophotometry analysis

Fig. 4 (A, B, C, and D) shows the representative photomicrographs of the Jones methylamine silver stain in thoracic aortas of the experimental groups. It can be observed that the HSL infusion at a percentage of 6% increases in the thickness of the elastic fibers in the TA in comparison with the control group. This observation was corroborated with the analysis by densitophotometry of the photomicrographs. Fig. 4 (I), shows a greater thicknesses of the elastic fibers with a significant difference between the same group ($p = 0.02$). Fig. 4 (E, F, G, and H) shows the representative photomicrographs of Masson's trichrome stain. The percentage at 6% of the HSL infusion increased the thickness of the tunica media and intima of the TA with respect to the control group. This observation was corroborated with the analysis and the densitophotometry of the photomicrographs. Fig. 4 (J), shows a greater thickness of the tunica media and intima of the TA with a significant difference against the same group ($p = 0.004$).

3.5. Enzymatic activities of the G6PH, GPx, TxrR, and GST

Fig. 5 (A, B, and C) shows the enzymatic activities of G6PD, GPX, and TxrR respectively in the TA homogenate. There were

Table 1
Analysis of the components and the amounts of anthocyanins, flavonoids and polyphenols, contributed by the drinking water at the different percentages of the HSL infusions.

Variables	HSL (%)		
	1.5	3	6
Cyanidin-3-glucoside (mg/L)	105.8 ± 19.48	551.44 ± 9.64	603.94 ± 10.03
Quercetin (mg/day)	0.29 ± 0.01	0.97 ± 0.00	1.24 ± 0.01
Polyphenols (mmol/day)	0.38 ± 0.00	0.70 ± 0.01	0.78 ± 0.00
Corresponds equivalence the waters consumption and of the antioxidants per rat/day			
Water consumption (mL/day)	50.6 ± 1.9	48.2 ± 1.3	46.4 ± 0.5
Cyaniding-3-glucoside (mg/day)	0	$5.09 \pm 2 \times 10^{-2}$	$25.57 \pm 4 \times 10^{-3}$
Quercetin (mg/day)	0	$0.01 \pm 1 \times 10^{-5}$	$0.04 \pm 9 \times 10^{-6}$
Polyphenols (mmol/day)	0	$0.01 \pm 2 \times 10^{-5}$	$0.03 \pm 4 \times 10^{-6}$

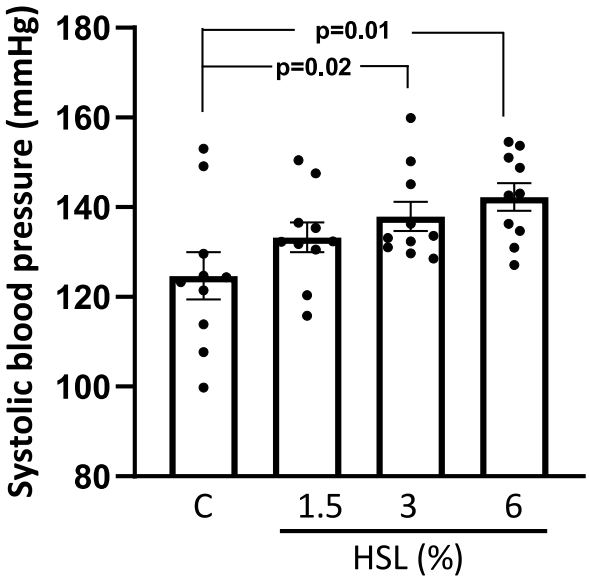

Fig. 1. Systolic blood pressure of the experimental groups. Values are expressed as mean ± standard error; (n = 10). Abbreviations: HSL= *Hibiscus Sabdariffa* Linnaeus.

significant increases in the HSL group at 6% in comparison with the control group (p = 0.02, p = 0.03 and p = 0.01 respectively). Moreover, in the HSL group at 1.5% the G6PH activity showed an increase in comparison to the control group (p = 0.01). However, the GST activity showed a significant decrease in the HSL group at 3% (p = 0.0009), and 6% (p = 0.0005) with respect to the control group (Fig. 5 (D).

3.6. Oxidative stress markers

Fig. 6 (A, B, C, and D) shows the concentration of NO_3^-/NO_2^- ratio, LPO, Se and 3-NT in the TA, respectively. There was a tendency to increase in the NO_3^-/NO_2^- ratio without there being a significant difference (p = 0.09, Fig. 6 (A). The LPO levels showed an increase in the HSL group at 6% in comparison with the control group (p = 0.01, Fig. 6 (B). In the groups that received the HSL infusion at percentages of 3% and 6%, there was an increase in the Se concentration (p = 0.03 and p = 0.05 respectively, Fig. 6 (C)). However, the 3-NT in the group that consumed the HSL infusion at 6% showed a significant decrease in comparison with the control group (p = 0.04, Fig. 6 (D).

Fig. 7 (A and B) shows the concentration of total thiols and the concentration of GSH in the TA homogenate. There was an increase in the total thiols in the HSL group that received the infusion at 1.5% (p = 0.005, Fig. 7A). However, in the HSL groups at 3% and 6% there were significant decreases (p = 0.002 and p < 0.001) in comparison with the control group. The GSH concentration showed an increase in the HSL group that received the percentages at 3% and 6% (p = 0.03 and p = 0.01, respectively, in comparison with the control group Fig. 7 (B).

4. Discussion

HSL is considered a "functional" food that provides various organic components with biological activity such as polyphenols, flavonoids, anthocyanins, delphinidin, hibiscetin, quercetin and gossypetin, protocatechuic acid, alkaloids, L-ascorbic acid, carotenoids, anisaldehyde, galactose, mucopolysaccharides, pectins, polysacchrarides and stearic acid. At low concentrations, this food acts against OS and diabetes, it decreases hight blood pressure, obesity, hypertriglyceridemia, hypercholesterolemia, hyperglycemia, insulin resistance, hyperinsulinemia and LDL oxidation. HSL can also provide different minerals and essential amino acids that form part of the antioxidant system. It also fights cancer and modulates the immune response [42–44]. However, these "beneficial" characteristics could become detrimental if consumed in excess or for a prolonged lapse of time, increasing antioxidant enzymes and excessively depleting ROS [42–44]. This could lead to RS and to a redox imbalance [11,43,44]. Therefore, the objective of this study was to evaluate the alteration of vascular reactivity associated to excessive and chronic consumption of HSL infusions at different percentages.

Our results show a significant increase in the elastic fiber thickness of the tunica media and intima of the thoracic aorta when HSL is consumed. These changes suggest higher rigidity of the vessel and hypertrophy, which was reflected in the increase in SBP in the groups with HSL infusion at 3% and 6%. Structural alterations and hypertrophy in the conductive arteries is associated with stiffness, inflammation, and increased blood pressure [12,44]. In this sense, a study carried out in male "Carworth" rats was associated with an increase in collagen and elastic fibers in the renal artery and the presence of renovascular hypertension [45]. The same effect was observed in another study when analyzing the structure of the aorta in hypertensive "Wistar" rats, where the hypertrophy caused in the

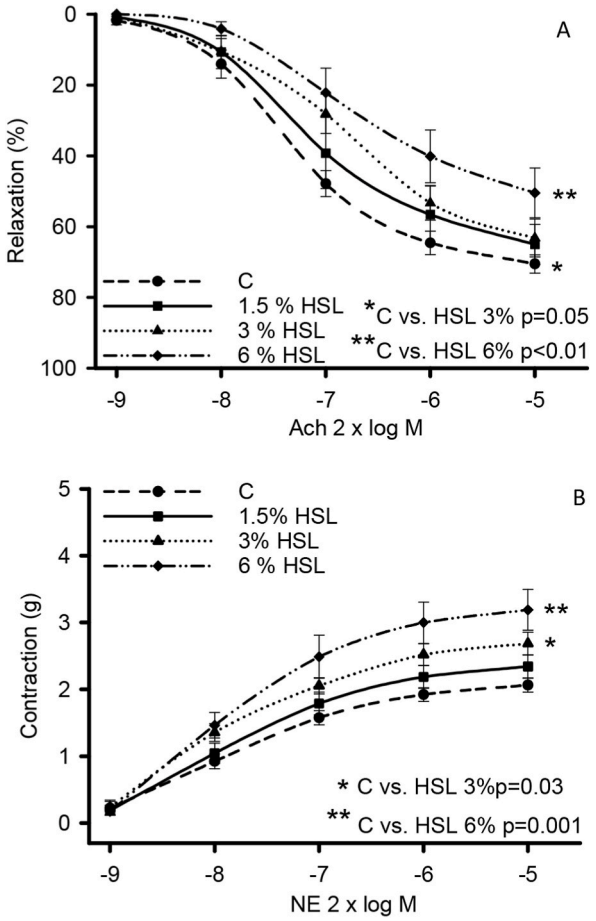

Fig. 2. Vasorelaxation (A) and vasoconstriction (B) responses in the aortic rings of the experimental groups of the rats that consumed the HSL infusion at different percentages. Values are expressed as mean ± standard error; (n = 10). Abbreviations: HSL= *Hibiscus Sabdariffa* Linnaeus, NE = norepinephrine, Ach = acetylcholine.

wall decreased the elasticity of fibers and collagen biosynthesis leading to increased SBP [12,44]. The hypertrophy of the aorta may stimulate the synthesis of angiotensin II and produce vasoconstriction favoring O_2^- production through the activation of NAD/NADH$^+$. This leads to inactivation of eNOS in the endothelium, causing, in turn, over-expression of iNOS that contributes to an inflammatory process [17,44,46]. Furthermore, HSL contains tyrosine, and an excess of this amino acid could lead to an increased synthesis of NE by the sympathetic nerve endings, which could contribute to increase SBP in the groups with HSL infusions at 3% and 6% [44,47].

Our results show that vasoconstriction and vasodilation increased and decreased, respectively, in the rats that consumed the HSL infusion at 3% and 6%. This suggests that, the structural changes, associate to the excess consumption of HSL infusion may alter vascular function. This effect could be due to an increase in the antioxidants provided by the HSL infusion such as cyanidin-3-glucoside, quercetin and polyphenols that increase the activities of the SOD isoforms, CAT, GPx enzymes of the antioxidant system. However, decreased LPO, and the increase in these enzymes can, in turn, decrease the ROS [42–44] which could modulate vascular reactivity. In this sense, our results showed that the rats that consumed the 6% infusion of HSL, showed an increase in the ratio NO_3^-/NO_2^- are secondary metabolites of the oxidation of nitric oxide (NO). The presence of a high ratio of these secondary metabolites is associated with over-expression/activity of iNOS or by uncoupled eNOS. This could also influence the thickening of elastic fibers, in the middle muscle zone, and the loss of the elasticity in the thoracic aorta. The structural alterations and hypertrophy in conductive arteries such as the aorta may lead to chronic inflammation, increased SBP and hypertension [43]. In this sense, it is essential to maintain the redox balance in endothelial cells, and a decrease in ROS could contribute to the alteration of the vascular function and consequently lead to various vascular diseases such, endothelial dysfunction and hypertension [2]. The effects of ROS in the thoracic aorta are not uniform since they depend on which ROS molecule is acting and at what concentration [48]. In the thoracic aorta, the most important ROS are O_2^-, H_2O_2, NO and peroxynitrite ($ONOO^-$), [49].

The excess of antioxidants and precursors of the antioxidant systems provided by HSL infusions such flavonoids and polyphenols could decrease O_2^- and H_2O_2, which, at physiological concentrations, act as second messengers [44,48]. Our results show that the vasoconstriction response in the experimental groups with infusions with different HSL percentages eliminated the significant difference found in the control group after being incubated with KO_2. KO_2 reacts violently with water to form KOH and, in this process, O_2^-

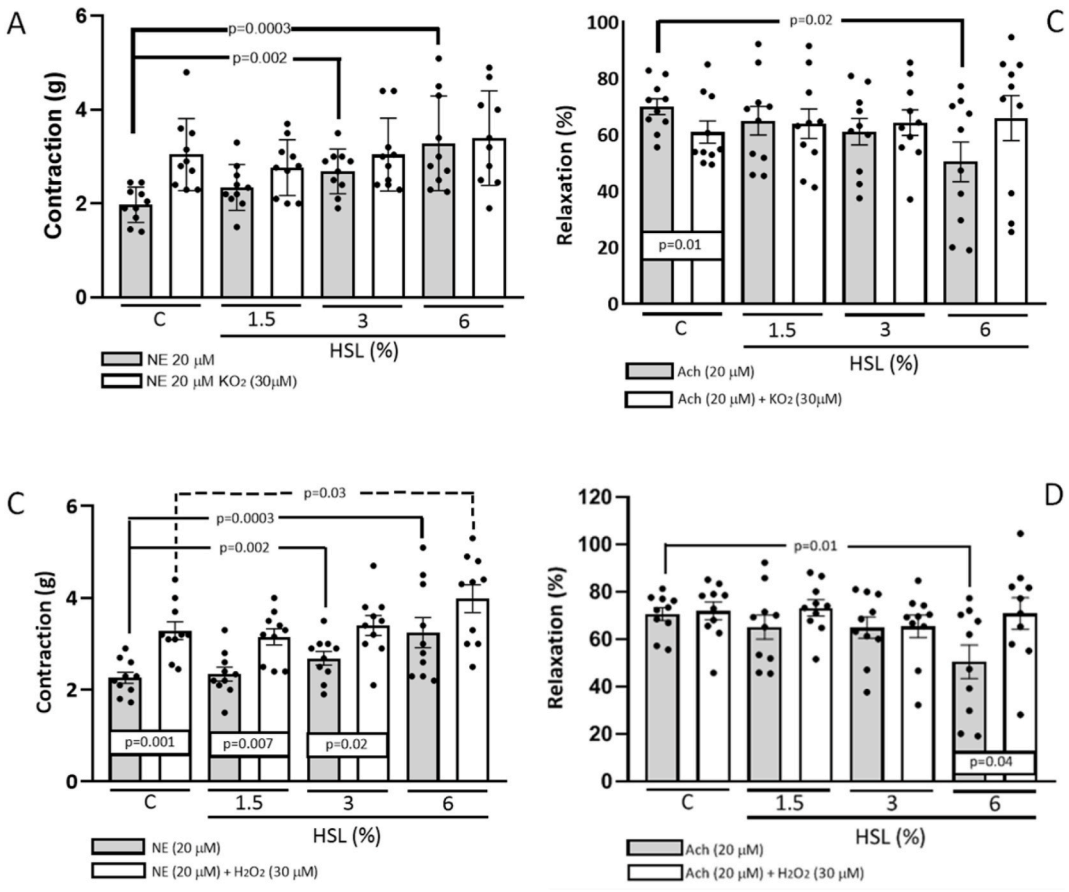

Fig. 3. Vasoconstriction responses in presence of the KO_2 (**3** A) and H_2O_2 (3C) respectively. Vasorelaxation responses in presence of the KO_2 (3 B) and H_2O_2 (3 D) respectively in the aortic rings of the experimental groups of the rats that consumed the HSL infusion at different percentages. Abbreviations: HSL= *Hibiscus Sabdariffa* Linnaeus, KO_2= Potassium superoxide, H_2O_2=Hydrogen peroxide, NE = norepinephrine, Ach = acetylcholine. Values are expressed as mean ± standard error; (n = 10).

is formed. This suggests the participation of O_2^- in the vascular response, and it also shows that an excess of antioxidants provided by HSL decreases the concentration of O_2^-. This result can be associated with the alteration of the vascular reactivity and with increased SBP.

In contrast, H_2O_2 favors vasodilation and produces NO through the activation of different signaling pathways such as PI3K/Akt, Erk1/2, and p38MAPK [49]. H_2O_2 not only stimulates eNOS but it can also increase its expression. Another protein that participates in the regulation of vasodilation is PKG1α. This protein kinase is sensitive to oxidation of H_2O_2 through the formation of a disulfide bond. The vasodilation that occurs is independent of the levels of cyclic guanosine monophosphate [48]. The results obtained in our study show that vasodilation increases after incubation with H_2O_2 in the group that consumed the HSL infusion at 6%, thus suggesting the participation of H_2O_2 in the vasodilatation response. Our results also suggest that the excess antioxidants provided by the HSL infusion at 6% decreases the H_2O_2 concentration, which is reflected in the alteration of the arterial function, probably due to a decrease of the eNOS pathway. In this sense, the tendency, and the increase in the NO_3^-/NO_2^- ratio in the groups that consumed the HSL infusion at 3% and 6% could indirectly reflect the over-expression/activity of iNOS [44,50]. Some reactive nitrosative species such as $ONOO^-$ may participate in the inflammatory processes due to an increase in the availability of NO provided by the iNOS pathway [50,51]. However, our results showed that NO_3^-/NO_2^- ratio had a tendency to increase, but that the 3-NT was decreased. These results could be paradoxical because an increase in protein nitrosylation was expected, through the iNOS metabolic pathway that would probably be increased in the aorta. However, the excess of antioxidants provided by the 6% HSL infusion could decrease the $ONOO^-$, which participates in the nitrosylation process, thus explaining the decrease in 3-NT in the aortic homogenate. This decrease is also associated with the low concentration of thiol groups in the cysteines of proteins, by excessive consumption of HSL that decreases the concentration of thiol groups [50,51]. However, more studies are required to corroborate this hypothesis.

In our study we analyzed the activity of several enzymes including G6PD which is responsible for providing reducing equivalents such as NADPH and of controlling ROS production through of the antioxidant enzymes that are dependent on NADPH [52]. Overexpression of G6PD decreases the excess ROS in endothelial cells treated with H_2O_2 or TNFα. Moreover, the G6PD overexpression can increase the concentration of GSH through the GR pathway [52,53]. Furthermore, in vascular cells, some processes that generate

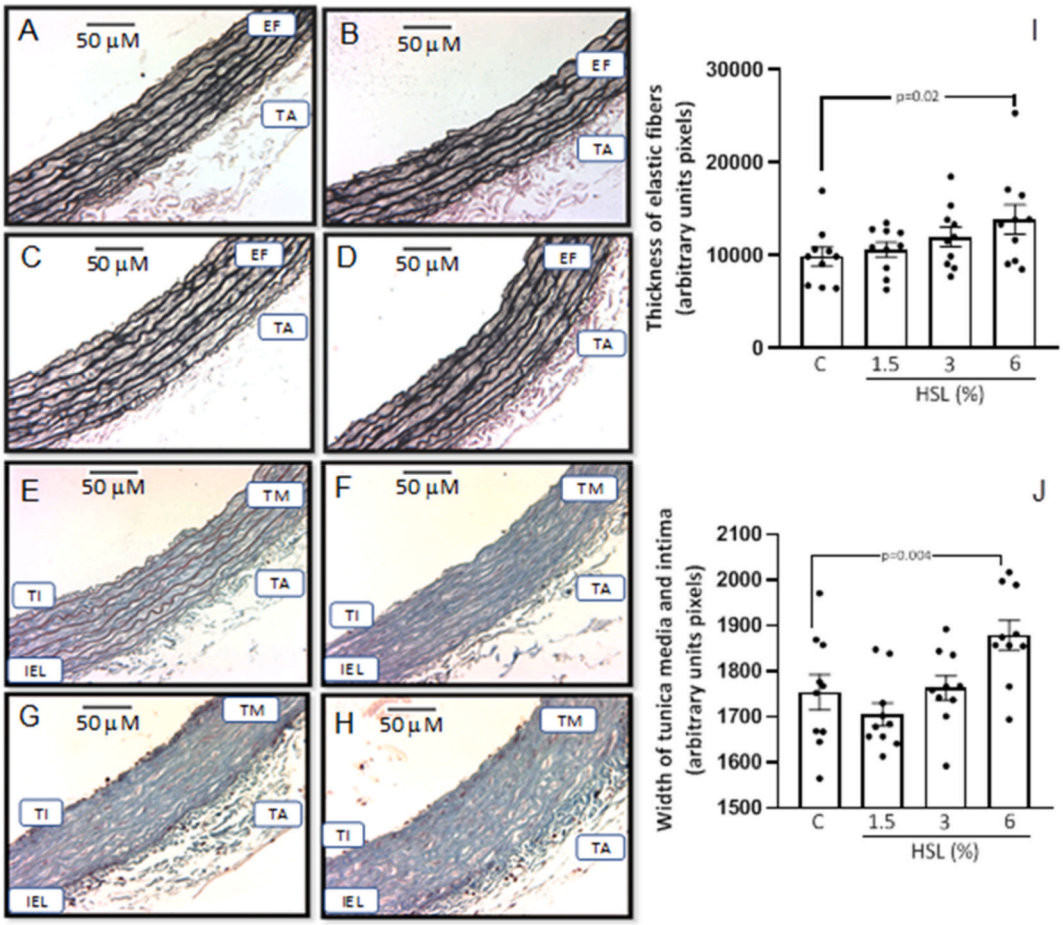

Fig. 4. Representative photomicrographs of the histology of the TA of rats that received the different percentages of the HSL infusion (A control, **B**, **C,** and **D** different percentages of (HSL). The tissue was processed according to conventional histological procedures, and histological sections were made and stained by Jones methylamine silver stain at 16x. In figures**E**, **F**, **G,** and **H** the representative photomicrographs of the histology of the aorta rings stained with Masson's trichrome stain at 16x are shown. Likewise, figures **I** and **J** show the densitophotometric analysis for each stain. Abbreviations: TI = Tunica intima, IEL= Internal elastic lamina, TM = Tunica media, TA = Tunica adventitia, EF = Elastic fibers.Values are expressed as mean ± standard error; (n = 10).

inflammation, such as the hypertrophy of the vascular wall in MS and type 2 diabetes, could increase the expression or activity of G6PD and favor high levels of NADPH as a compensatory mechanism [53]. Our results showed an increase in the activity of G6PD in the aorta homogenate. This could be attributed to the alteration of the vascular function and structural changes associated with inflammation and hypertrophy of the aortic wall. The hypertrophy may cause activation of NADPH oxidase and the release of some interleukins that favor the pro-inflammatory state. These processes can stimulate the activation of G6PD and favor the production NADPH that is a cofactor for some antioxidant enzymes such as GPx, TrxR, and iNOS. HSL could also favor the G6PD overexpression or activity, leading to an increase in NADPH [54].

In addition to the fact that G6PD overexpression can increase GSH concentration, HSL can contribute to increase GSH by itself, since it contains cysteine, glycine, and glutamic acid, which are precursors of GSH. Therefore, this could increase the synthesis of GSH [19,44]. In this sense, the results showed a significant increase in the concentration of GSH in the groups that received the HSL infusion at 3% and 6%.

HSL in various concentrations also provides Se and the amount provided depends on the crop, type of soil, pH, type of plant species, and salinity [55,56]. Se is considered as an essential micronutrient and its excess or depletion in the body favors the appearance of some diseases such as hypothyroidism, cardiovascular disorders, cognitive impairment, and weakening of the immune system [56]. Furthermore, Se is indispensable for 25 seleno enzymes such as GPx and TrxR, which contribute to maintain the redox homeostasis [56]. Our results show that the Se concentration was increased in the groups of rats that received HSL infusion at 3% and 6%. This suggests that the excessive consumption the HSL could lead to selenosis, which is caused by excess intake of Se. However, more studies are required to corroborate this hypothesis.

Se may be oxidized in the presence nitrogen species and GSH [56] ,[57].Se compounds such as selenite and selenium dioxide can react with the thiol groups present in GSH to produce O_2^-. This has been observed in experiments with isolated mitochondria treated

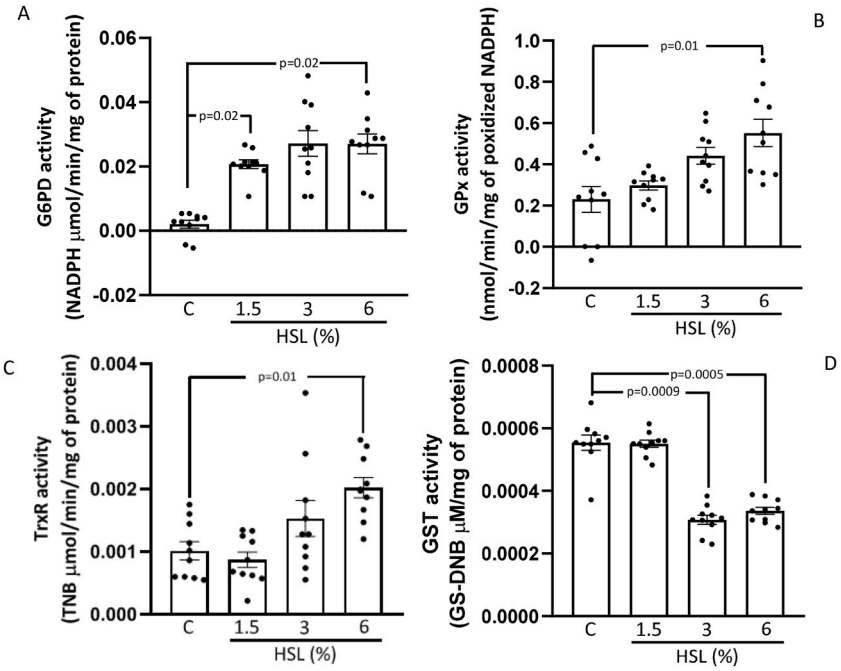

Fig. 5. Enzymatic activities of the G6PD (**A**), GPx (**B**), TxrR (**C**), and GST (**D**) in the TA aorta homogenate. Values are expressed as mean ± standard error; (n = 10).

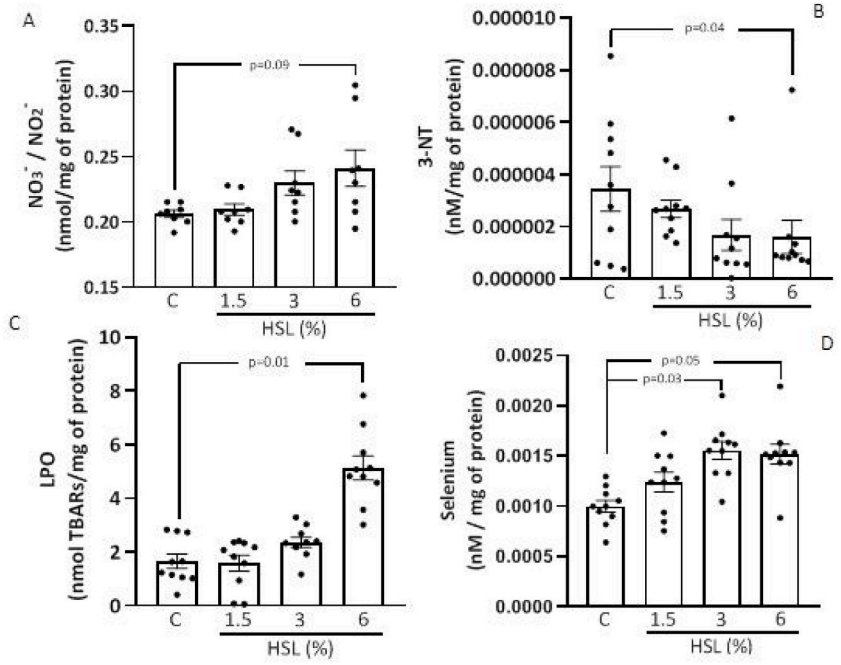

Fig. 6. Oxidative stress markers. Concentration of $NO3^{-}/NO_2^{-}$ ratio (**A**), LPO (**B**), Se (**C**) and 3-NT (**D**) in the TA of the rats. Values are expressed as mean ± standard error; (n = 10).

with selenium-containing components, including selenite and selenocysteine [57].This suggests that excess Se could lead to generation of O_2^{-} and $ONOO^{-}$and that the excessive antioxidants provided by the HSL infusion can decrease it.

A high level of Se concentration favors LPO, which causes DNA damage and favors oxidation of polyunsaturated fatty acids and degradation of proteins [57],[58]. A high Se concentration could cause thiol oxidation. Our results showed that the concentration of

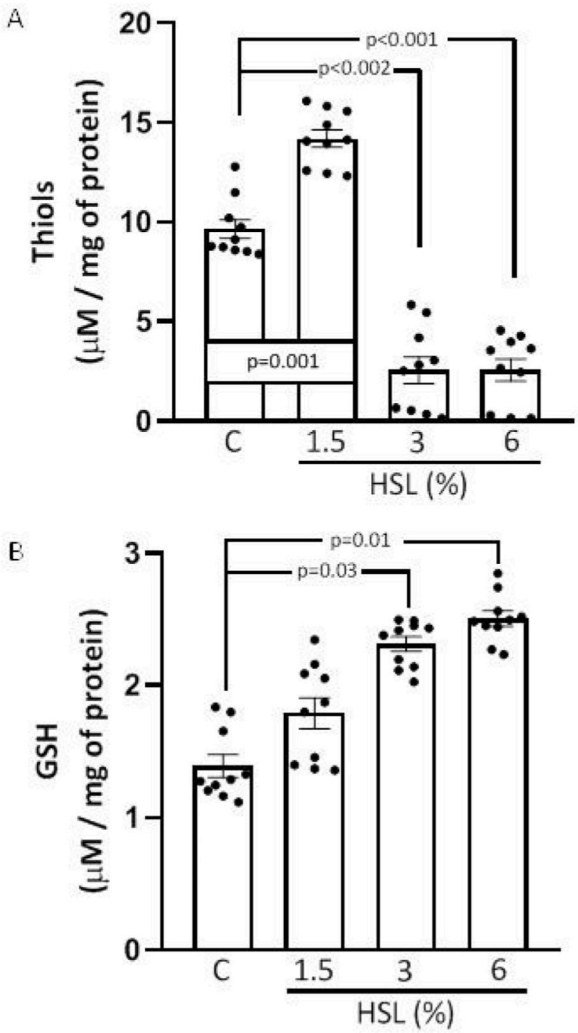

Fig. 7. Concentration of total thiols (A) and GSH (B) in the TA homogenate in rats that received different percentages of the HSL infusion. Values are expressed as mean ± standard error; (n = 10).

total thiol was decreased but that LPO was increased in the rats that received the HSL infusion at 3% and 6%. The low levels of thiols may contribute to the alteration of the vascular function and to structural changes in the thoracic aorta in these rats and are linked to cardiovascular events [59]. Another study associated the decrease in thiols and the increase in LPO with the severity of artery coronary disease lesions [60].

To maintain thiol homeostasis in cysteines of proteins, mammalian cells depend on the TrxR system [55,61]. This system also participates in the regulation of the vascular tone, and when the TrxR is overexpressed, vascular reactivity is improved by increasing NO bioavailability [62]. A study in mice showed that the TrxR deletion generated a lower response to vasodilation and a greater $ONOO^-$ production [63,64]. Our results showed an increase in the activity of this enzyme in the group of rats that consumed the HSL infusion at 6%. These results suggest that an increase in the TrxR activity could be attributed to the excess Se and the increase of the activity of G6PD [62–64]. This could be a compensatory mechanism that is aimed to increase the levels of thiols [61] and could be associated with the excessive consumption of antioxidants such as polyphenols, quercetin, flavonoids, Se, and gallic, protocatechuic, tannic acids, which may be provided by the HSL infusion.

Another enzyme that increased its activity in this study was GPx. This enzyme contains a selenium-cysteine at its catalytic site and was overactive in the aorta homogenate from the rats of the group that consumed the HSL infusion at 6%. This increased activity could occur because of substrate surplus such as GSH and NADPH that were provided by the G6PD and to the Se elevation. Furthermore, moderate ROS are required for keeping the formation of disulfide (thiols) in the cell and the GPx overexpression can lead to a decrease of protein disulfides that is related to reduce signaling from growth factors and decreased mitochondrial function [65].

On the other hand, GST is an antioxidant enzyme that participates in the detoxification process caused by oxidative damage, xenobiotic agents, and oxidized lipids, and it may prevent the effects of LPO [66]. This enzyme conjugates GSH with electrophilic and xenobiotic agents such as drugs, toxins, carcinogens, and detoxifies the organism [67].Our results showed a decrease in the GST

activity in the group of rats that consumed the HSL infusion at 3% and 6%. The tannic acid administration at doses of 80 mg/kg reduces the activity of the "mu" and "alpha" subunits of the GST in the liver [66]. It also decreases the "pi" subunit of the GST when protocatechuic acid is provided [66,67]. Another study demonstrated that high flavonoid concentrations could inhibit GST activity and favor an increase in LPO [67]. This suggests that the excess consumption of flavonoids, tannic and protocatechuic acids, which are provided in the HSL infusion at 3% and 6%, favored the decrease in the GST activity in the aorta and contributed to increase LPO [67].

Fig. 8 summarizes the physiological alterations and anatomical changes in the thoracic aorta in response to different percentages HSL infusions.

5. Conclusions

The excessive and chronic consumption of antioxidants such as polyphenols, quercetin, flavonoids, Se and gallic, protocatechuic, tannic acids provided by the HSL infusion at 3% and 6% may deplete the ROS thus deteriorating vascular function in healthy rats. These effects were not observed at low concentrations such as 1.5%.

The deterioration of vascular reactivity is accompanied by an increase in the thickness of the thoracic aorta wall and with rupture of elastic fibers that contribute to increase the systolic blood pressure and favor hypertension.

These changes are accompanied with the over-activity of the antioxidant system that may eliminate ROS and alter the redox homeostasis, generating RS and contributing to hypertension.

Author contributions

Linaloe Manzano-Pech, PhD:. Conceived and designed the experiments; analyzed and interpreted the data; wrote the paper.

Verónica Guarner-Lans, PhD:. Contributed reagents, material, analysis tools or data; Wrote the paper.

María Elena Soto, PhD:. Conceived and designed the experiments; analyzed and interpreted the data.

Eulises Díaz-Díaz, PhD:. Conceived and designed the experiments; analyzed and interpreted the data.

Israel Pérez-Torres, PhD:. Conceived and designed the experiments; analyzed and interpreted the data; contributed reagents, material, analysis tools or data; Wrote the paper.

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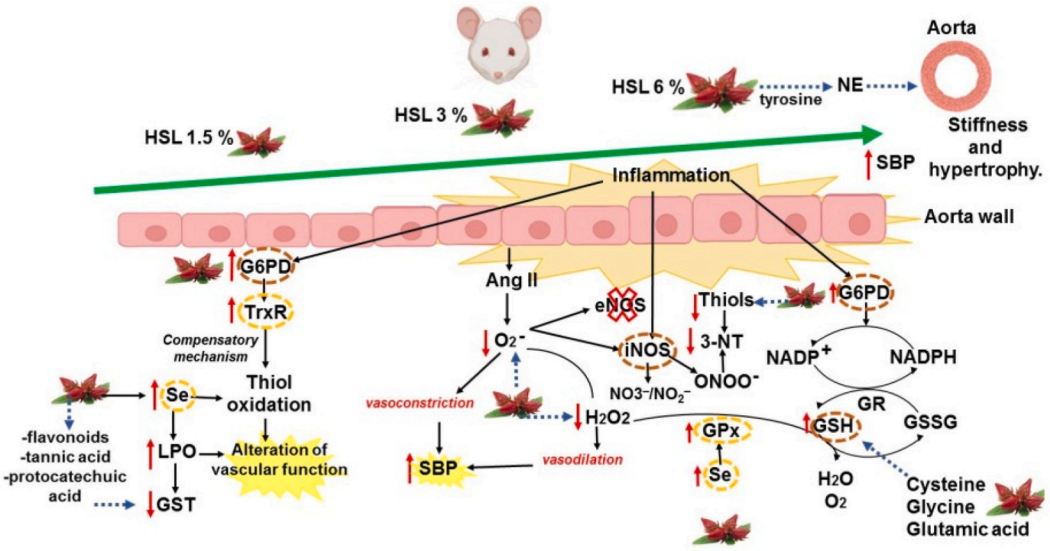

Fig. 8. Summary of the alteration in physiological and anatomical changes within the thoracic aorta in response to different percentages HSL infusions. These changes are accompanied by alterations of the enzymatic and non-antioxidant systems that contribute to the alteration of the redox homeostasis and generation of RS, which results in elevated blood pressure in rats. Abbreviations: eNOS = endotelial nitric oxide synthase, G6PD = Glucose-6-phosphate dehydrogenase, GR = glutathione reductase, GSH = glutathione, GSSG = oxidized glutathione, GST = glutathione-S-transferase, H_2O_2 = hydrogen peroxide, HSL= *Hibiscus Sabdariffa Linnaeus,* iNOS = inducible nitric oxide synthase, LPO = lipoperoxidation, NADPH = nicotinamide adenine dinucleotide phosphate, NE = norepinephrine, NO_3^-/NO_2^- = nitrate/nitrite ratio, 3-NT = 3-nitrityrosine, O_2^- = superoxide anion, SBP = systolic blood pressure, Se = selenium, TrxR = thioredoxin reductase.

Institutional review board statement

The Laboratory Animal Care Committee of the National Institute of Cardiology "Ignacio Chávez" in México (SICUAE.DC.2019/3–4) approved the experiments in animals, and they were conducted in compliance with the Guide for the Care and use of laboratory animals of the National Institutes of Health (NIH).

Informed consent statement

Not applicable.

Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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