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CELLULAR AND MOLECULAR BIOLOGY

Effects of diet-induced hypercholesterolemia and gold nanoparticles treatment on peripheral tissues

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Abstract: Cholesterol is a lipid molecule of great biological importance to animal cells. Dysregulation of cholesterol metabolism leads to raised blood total cholesterol levels, a clinical condition called hypercholesterolemia. Evidence has shown that hypercholesterolemia is associated with the development of liver and heart disease. One of the mechanisms underlying heart and liver alterations induced by hypercholesterolemia is oxidative stress. In this regard, in several experimental studies, gold nanoparticles (AuNP) displayed antioxidant properties. We hypothesized that hypercholesterolemia causes redox system imbalance in the liver and cardiac tissues, and AuNP treatment could ameliorate it. Young adult male Swiss mice fed a regular rodent diet or a high cholesterol diet for eight weeks and concomitantly treated with AuNP (2.5 μg/kg) or vehicle by oral gavage. Hypercholesterolemia increased the nitrite concentration and glutathione (GSH) levels and decreased the liver's superoxide dismutase (SOD) activity. Also, hypercholesterolemia significantly enhanced the reactive oxygen species (ROS) and GSH levels in cardiac tissue. Notably, AuNP promoted the redox system homeostasis, increasing the SOD activity in hepatic tissue and reducing ROS levels in cardiac tissue. Overall, our data showed that hypercholesterolemia triggered oxidative stress in mice's liver and heart, which was partially prevented by AuNP treatment.

Key words: Antioxidant molecules, gold nanoparticles, hypercholesterolemia, oxidative stress, peripheral tissues.

INTRODUCTION

Cholesterol, a compound of the steroid family, is a lipid molecule with great biological importance for all animal cells. Because of that, cells need a continuous supply of cholesterol. Most of it is derived from endogenous synthesis, and only a small amount of the body cholesterol is provided from the diet (IQWiG 2006). Its metabolism occurs mainly in the liver, where cholesterol homeostasis maintenance is carried out by many mechanisms, such as biosynthesis, storage, and conversion into bile acids (Luo et al. 2020). In this regard, dysregulation of body cholesterol homeostasis can lead to raised blood cholesterol levels, a clinical condition called hypercholesterolemia (Ibrahim et al. 2021).

Regardless of their etiology, high levels of plasma cholesterol contribute to the development of diseases (Nelson 2013, Csonka et al. 2017). Hypercholesterolemia is a well-known risk factor for developing cardiovascular disease (CVD), mainly related to atherosclerosis (Gidding & Allen 2019). High blood cholesterol levels have

also been shown to exert direct heart negative effects in a process that occurs independently of atherosclerosis disease (Csonka et al. 2016). Experimental evidence has reported that dietary cholesterol and hypercholesterolemia appear to be significant risk factors for liver steatosis (Wouters et al. 2008). Importantly, epidemiological and clinical studies have shown a strong correlation between liver disease and the increase in several markers of subclinical atherosclerosis and carotid artery inflammation (Moon et al. 2015, Oni et al. 2013). Dyslipidemia, arterial hypertension, liver inflammation, and increased reactive oxygen species (ROS) production seem to be the connective factors between liver and heart diseases (Byrne & Targher 2015).

Oxidative stress is a condition characterized by a redox system imbalance that favors the ROS accumulation and oxidative damage to macromolecules (Burton & Jauniaux 2011), which is related to the initiation and progression of liver and heart diseases (Csonka et al. 2016, Polimeni et al. 2015). Since hypercholesterolemia contributes to oxidative stress, managing oxidative stress can be a promising therapeutic strategy to prevent the liver and heart diseases linked to hypercholesterolemia. In this regard, experimental studies have shown critical antioxidant properties of gold nanoparticles (AuNP) administration in many diseases (Tsai et al. 2007, Sumbayev et al. 2013, Muller et al. 2017), including therapeutic effects on the liver (Kabir et al. 2014) and heart diseases (Qiao et al. 2017). Scavenger action against ROS (Barathmanikanth et al. 2010, Razzaq et al. 2016) and improvement of antioxidant defenses (Ko et al. 2020) are among the mechanisms involved with the therapeutic effect of AuNP. Therefore, considering the peripheral consequences of hypercholesterolemia and the therapeutic effects of treatment with AuNP on the liver and cardiac tissues, this study aimed to investigate the effects of AuNP on the imbalance of the peripheral redox system associated with hypercholesterolemia.

MATERIALS AND METHODS

Animals

Male Swiss mice (three months old) weighing between 25 and 30g from the Central Animal House of Universidade do Extremo Sul Catarinense (UNESC, Brazil) were used. The animals were maintained in 9 to 10 animals per cage in plastic cages, with controlled temperature and light conditions. All animal procedures were performed following the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA) and with the Ethics Committee of the University of the UNESC under protocol 007/2018-1.

Gold nanoparticles synthesis

Gold nanoparticles (AuNP) of 20 nm were synthesized as described previously by Rodrigues et al. 2021, which involved the chemical reduction of the metallic precursor tetrachloroauric acid ($HAuCl_4$; Sigma-Aldrich, MO) using the reducing agent and stabilizing sodium citrate ($Na_3C_6H_5O_7.2H_2O$; Nuclear, São Paulo, Brazil). The physical-chemical properties of AuNP used in our study were previously described in the literature. The average size of AuNP was confirmed by UV-vis, in which the nanoparticles exhibited a purple color and an absorption maximum of 524 nm. This size was confirmed by transmission electron microscopy (TEM) analysis, which demonstrated that nanoparticles present an oblong morphology (nearly spherical), and an average size of 20 nm (Della Vechia et al. 2020). Regarding its Zeta potential and hydrodynamic size, Della Vechia et al. (2020) showed that AuNP exhibits a Zeta

potential of -42 ± 4 mv and hydrodynamic size of 26 ± 4nm. It is important to mention that the viability of AuNP at 70 mg/L was monitored every two days by UV-visible spectrometry. Before each administration, the AuNP solution at 70 mg/L was diluted in water to reach the working concentration of 0.25 µg/mL, resulting in a dose equivalence of 2.5 µg/kg body weight mice.

Experimental design

Young adult (three-month-old) male Swiss mice fed a normal rodent diet (22.53 % of protein, 41.02 % of carbohydrate, 8.75 % of fat, and calorie value of 3.3 kcal/g) or a high cholesterol diet (14.79 % of protein, 50.19 % of carbohydrate, 23.64 % of fat, 1.25% of cholesterol and calorie value of 4.7 kcal/g) continuously for eight weeks (Moreira et al. 2014, Rodrigues et al. 2021). Also, mice were, since the beginning of the experimental period, concomitantly treated with AuNP (2.5 µg/kg, in an aqueous solution) or with a vehicle solution (water) by oral gavage on alternate days for eight weeks, totalizing four experimental groups (n = 6 animals per experimental group). The animal's body mass was measured every two days during all experimental period (eight weeks). Twenty-four hours after the last treatment section, the animals were food-deprived for 6 hours, and the blood was collected by cardiac puncture to determine plasma total cholesterol levels. After that, the mice's cardiac and liver tissue were dissected to determine oxidative stress parameters (Figure 1a). It is important to mention that the dosage of 2.5 µg/kg used in our study does not have toxic effects on mice, evidenced by no changes in the levels of ALT in plasma and the levels of MTT in the liver, an essential marker of cell death. Other parameters were not modified, like the levels of γ-GT and creatinine on plasma, which reinforces the safe use of AuNP in the dose of 2.5 µg/kg in our study (Rodrigues et al. 2021).

Plasma total cholesterol levels

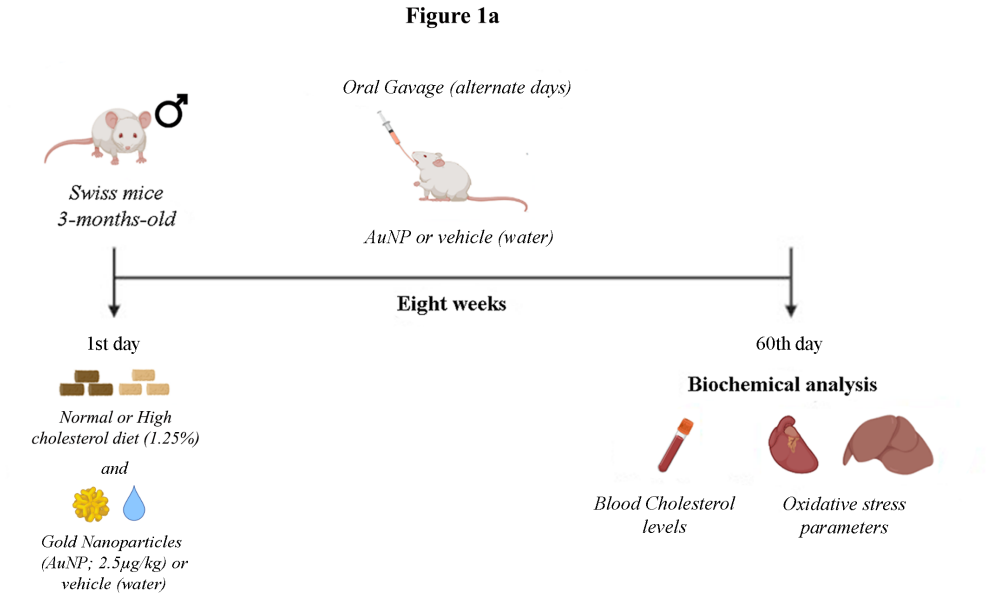
The animals' blood was collected from the animals' hearts under previous anesthesia with ketamine and xylazine (75 and 10 mg/kg, respectively, i.p.). The blood was immediately centrifuged, and the plasma was collected. According to the manufacturer's instructions, total cholesterol levels were measured in plasma using a commercial kit (Gold Analisa Diagnostica Ltda, Minas Gerais, Brazil). The data are expressed as mg/dL.

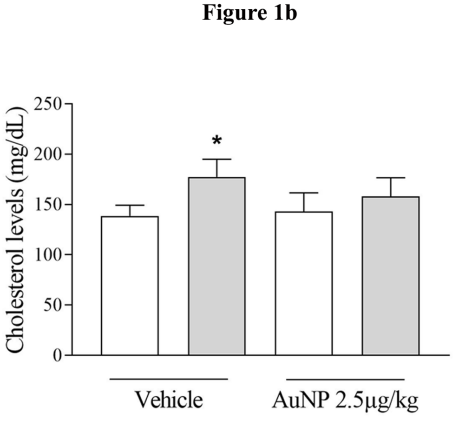
Determination of intracellular reactive oxygen species (ROS)

The intracellular levels of oxidized 2,7-dichlorodihydrofluorescein (DCF) were measured based on the oxidation of the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe to a 2',7'-dichlorodihydrofluorescein (DCF) fluorescent compound, as previously described (Hempel et al. 1999). The sample was incubated with 10 mM DCFH-DA at 37 °C for 30 minutes. DCF-DA is de-esterified inside cells by endogenous esterases to the ionized free acid, DCFH. DCFH is oxidized to 2,7-dichlorofluorescein (DCF) by reactive species. The formation of this oxidized fluorescent derivative was monitored with the excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer. A DCF standard curve was constructed using DCF (concentration of 10 nM) as an internal control in the experiment. Data are expressed as U of fluorescence/mg of protein.

Determination of intracellular nitric oxide

Nitric oxide was estimated spectrophotometrically from the stable metabolite nitrite production, derived from the action of nitric oxide (NO) on O_2^-. At room temperature, the samples were incubated with Griess reagent (1% sulfanilamide in 0.1 mol/L and 0.1% N-[1-naphthyl] ethylene diamine dihydrochloride) for 10 minutes, and





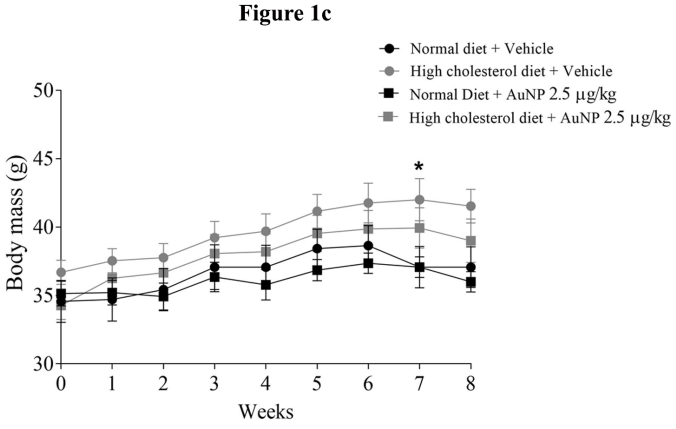


Figure 1. Experimental protocol and the effects of high cholesterol diet and AuNP treatment on plasma cholesterol levels. (a) Experimental protocol. The animals were exposed for eight weeks to a normal diet (3.3 kcal/g) or a high cholesterol diet (1.25% cholesterol and 20% saturated fat; calorie value of 4.7 kcal/g). Concomitantly, the animals were treated with AuNP (2.5 µg/kg, in aqueous solution) or vehicle solution (water) by oral gavage every two days. Twenty-four hours after the last treatment section, the animals were food-deprived for 6 hours, and the blood was collected by cardiac puncture to determine plasma total cholesterol levels. After that, the mice's cardiac and liver tissue were dissected for further determination of oxidative stress parameters. (b) Plasma cholesterol levels. (c) Evaluation of body mass throughout the experimental period. Data are shown as mean ± SD (n = 6 animals per group). * p <0.05 versus normal diet and vehicle group (Two-way ANOVA followed by Duncan post hoc test).

the absorbance was measured at 540 nm using a microplate reader. The nitrite content was calculated based on a standard curve from 0 to 100nm performed with the sodium nitrite metabolite ($NaNO_2$). The results are expressed as µmol Nitrite/mg of protein (Chae et al. 2004).

Protein carbonylation content

Measurement of protein carbonyl content has been used as a marker of oxidative damage to proteins. Protein oxidation was measured using labeled protein-hydrazone derivatives with 2,4-dinitrophenylhydrazine. These derivatives

were extracted with trichloroacetic acid, followed by ethanol/ethyl acetate treatment, and dissolved in urea hydrochloride. This reaction generates the formation of incorporated 2,4-dinitrophenylhydrazine, which was determined spectrophotometrically at 370 nm, as previously described by Levine et al. (1990). Values are expressed as nmol/mg of protein.

Superoxide dismutase (SOD) activity

The SOD activity was measured by inhibiting adrenaline's oxidation adapted from Bannister & Calabrese (1987). For this, the samples were previously homogenized in PBS buffer, and from this homogenate, three different sample volumes (5, 10, and 15 µL) were removed. 5 µL of catalase (0.0024 mg / mL of distilled water) was added to these volumes. Then, a glycine buffer in variable volume was added according to the number of samples. After these processes, a baseline reading was performed. Then, 5 µL of adrenaline (60 mM in distilled water + 15 mL/mL of steaming hydrochloric acid (HCl) was added, and the kinetics were visualized. The readings were taken for 180s at 10s intervals and measured in an ELISA reader at 480nm. Results are expressed as U of SOD/mg of protein.

Reduced glutathione (GSH) levels

The GSH content was measured in homogenate after protein precipitation with 0.6M perchloric acid and centrifugation at 4 °C for 10 minutes. The resulting supernatant was incubated with 800 mM phosphate buffer (pH 7.4) and 1 mg/mL of ortho-phthalaldehyde (OPT) for 15 minutes at room temperature. The resulting color development from OPT and thiols' reaction reaches a maximum of 5 minutes and remains stable for more than 30 minutes. After 10 minutes, the fluorescence intensity was determined (emission wavelength of 420 nm and 350 nm of excitation). A standard, reduced glutathione curve was used to calculate the samples' GSH levels (Hissin & Hilf 1976). The results are expressed as U fluorescence/mg of protein.

Determination of protein content

The protein content was quantified using bovine serum albumin as a standard, as Lowry et al. (1951) described previously. Absorbance was measured in a SpectraMax M5 microplate reader at a wavelength of 700nm.

Statistical analysis

All results were presented as mean ± SD. The statistical analysis was carried out using the two-way analysis of variance (ANOVA), followed by the Duncan test when appropriate. The results were considered significant when $p \leq 0.05$. Statistical tests were performed using the Statistica® program.

RESULTS

Hypercholesterolemic diet caused an increase in total plasma cholesterol levels

As shown in Figure 1b, the animals' exposure to a hypercholesterolemic diet for eight weeks caused a significant increase in the total plasma cholesterol levels. The AuNP treatment did not change the cholesterol levels in mice fed either a normal diet or high cholesterol diet. In addition, analyzing the animals' body weight over the weeks, we noticed that hypercholesterolemic animals had higher body weight in the seventh week than other experimental groups. No effect of the AuNP administration was observed in the animal's body weight, regardless of the diet (Figure 1c).

Effects of hypercholesterolemic diet and AuNP treatment on oxidative stress parameters in hepatic tissue

The effects of high cholesterol diet and AuNP treatment on oxidative stress parameters in hepatic tissue are shown in Figure 2. Levels of ROS and nitrite were used as oxidative parameters. High cholesterol diet significantly increased the ROS and nitrite levels in the liver, which was not prevented by AuNP treatment (Figure 2a, 2b).

The GSH levels and SOD activity were measured to evaluate the antioxidant system. Hypercholesterolemic diet significantly reduced the SOD activity in the hepatic tissue of mice. Notably, AuNP treatment ameliorated the SOD activity in mice's liver exposed to a high cholesterol diet (Figure 2c). We observed that a high cholesterol diet induced a significant increase in the hepatic GSH levels of animals, which was not changed by AuNP treatment (Figure 2d). Furthermore, carbonyl levels were used as a measure of oxidative protein damage. No diet and AuNP treatment effects were observed regarding the carbonyl levels in hepatic tissue (Figure 2e).

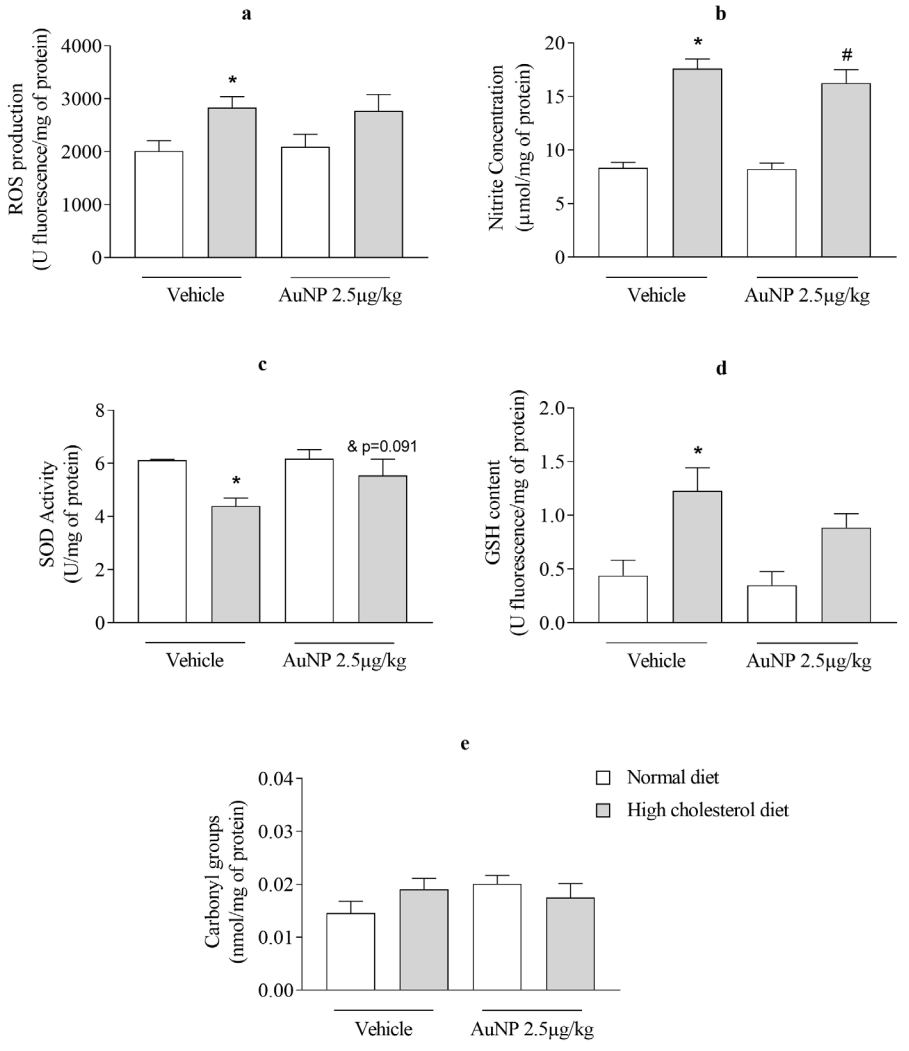


Figure 2. Effects of hypercholesterolemia and AuNP treatment on oxidative stress parameters in hepatic tissue. (a) ROS levels, (b) Nitrite concentrations, (c) SOD activity, (d) GSH content, and (e) carbonyl levels. Data are shown as mean ± SD (n = 6 animals per group). * p <0.05 versus normal diet and vehicle group; # p <0.05 versus normal diet and AuNP group; & p <0.05 versus high cholesterol diet and vehicle group (Two-way ANOVA followed by Duncan post hoc test).

Impact of hypercholesterolemic diet and AuNP treatment on oxidative stress parameters in cardiac tissue

The effects of hypercholesterolemia and AuNP administration in the heart were also investigated. The hypercholesterolemic diet significantly increased the ROS levels in the cardiac tissue, which were reduced by the AuNP treatment (Figure 3a). No effect of the high cholesterol diet and AuNP treatment was observed in nitrite levels in this same tissue (Figure 3b).

Evaluation of the antioxidant system showed that the hypercholesterolemic diet induced an enhancement in the levels of GSH in the cardiac tissue, while the AuNP treatment reduced these antioxidant levels in hypercholesterolemic mice (Figure 3d). Moreover, no effect of diet and AuNP treatment was observed regarding the SOD activity in the heart (Figure 3c). Finally, as shown in figure 3e, the hypercholesterolemic diet tended to increase carbonyl levels in cardiac tissue (p= 0.149), which was not modified by AuNP treatment.

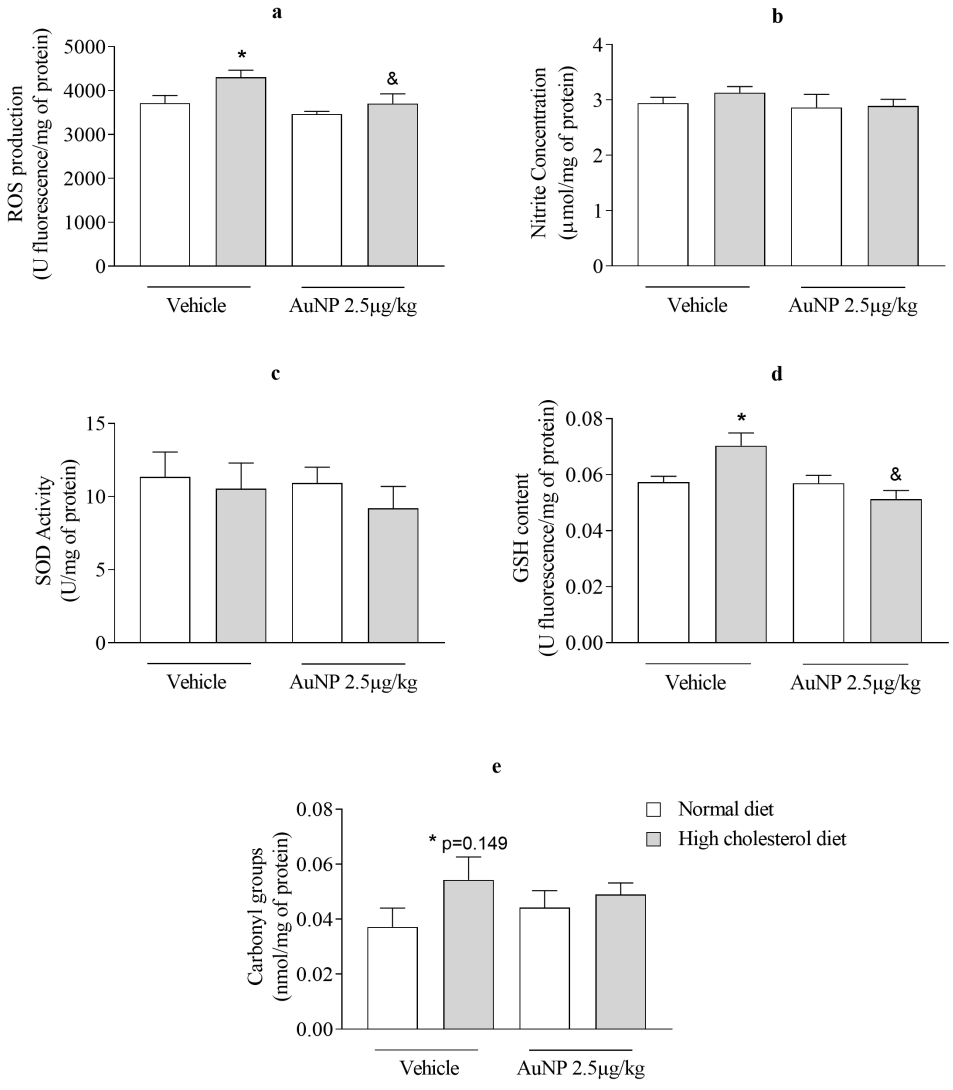


Figure 3. Effects of hypercholesterolemia and AuNP treatment on oxidative stress parameters in cardiac tissue. (a) ROS levels, (b) Nitrite concentrations, (c) SOD activity, (d) GSH content, and (e) carbonyl levels. Data are shown as mean ± SD (n = 6 animals per group). * p <0.05 versus normal diet and vehicle group; & p <0.05 versus high cholesterol diet and vehicle group (Two-way ANOVA followed by Duncan post hoc test).

DISCUSSION

Alterations in cholesterol metabolism can lead to the development of pathologies (Ibrahim et al. 2021). Hypercholesterolemia exerts direct negative effects on the heart, being a well-known cardiovascular risk factor (Gidding & Allen 2019, Csonka et al. 2016). High serum cholesterol levels can also impair liver function and contribute to liver steatosis occurrence (Wouters et al. 2008). Oxidative stress appears to be the connective factor between hypercholesterolemia and heart and liver diseases (Csonka et al. 2016, Polimeni et al. 2015). Since oxidative stress contributes to endothelial dysfunction (Elahi & Matata 2015), cardiac injury (Wilson et al. 2002), atherosclerosis (Mangge et al. 2014), and liver steatosis progression (Wouters et al. 2008), the management of oxidative stress in these organs is of great relevance. In this context, the AuNP, due to its significant antioxidant properties (Tsai et al. 2007, Sumbayev et al. 2013, Muller et al. 2017), might be an important therapeutic intervention to mitigate the peripheral consequences of hypercholesterolemia. In this regard, we aimed to investigate AuNP treatment's effects on peripheral consequences of hypercholesterolemia, focusing mainly on changes in the redox system in cardiac and liver tissues.

We exposed Swiss mice to a high cholesterol diet (1.25% of cholesterol) for eight weeks to study hypercholesterolemia's peripheral consequences to cardiac and hepatic tissues. As expected, the hypercholesterolemic diet intake significantly increased the animals' total plasma cholesterol levels compared to the animals fed a regular diet, thus characterizing an experimental hypercholesterolemia model. Our findings follow previous studies, which used the same experimental protocol to induce hypercholesterolemia in mice (Moreira et al. 2014, Rodrigues et al. 2021). Similarly, mice fed a high cholesterol diet containing 1.5% of cholesterol for eight weeks showed an increase of about 30% in plasma cholesterol levels (de Souza et al. 2019). In fact, the experimental strategy of hypercholesterolemia induced by diet to mimic human hypercholesterolemia is well described in the literature (Thirumangalakudi et al. 2008, de Oliveira et al. 2011, Moreira et al. 2014).

The liver is the central organ of cholesterol and lipid metabolism in the body (Luo et al. 2020, Ponziani et al. 2015). Importantly, high cholesterol intake from the diet is related to the induction of advanced liver disease stages (Ichimura et al. 2017). High cholesterol content and accumulation led to hepatic oxidative stress and inflammation, favoring liver disease (Püschel & Henkel 2019). Here, to better assess the impact of dietary cholesterol and consequent hypercholesterolemia on liver function, we evaluate oxidative stress parameters in the hepatic tissue of mice exposed to a high cholesterol diet. We observed a significant increase in nitrite levels in the liver of hypercholesterolemic mice. The stable and measurable metabolite nitrite was evaluated as an indicator of nitric oxide (NO) production. Also, NO reaction with superoxide anion (O_2^-) produces the peroxynitrite ($ONOO^-$) anion, a molecule with strong oxidizing properties. Previous studies have pointed out that this reaction is exacerbated by clinical conditions that lead to endothelial and mitochondrial disorders, such as hypercholesterolemia (Madamanchi & Runge 2007, Knight-Lozano et al. 2002). Evidence showed that a cholesterol-rich diet induced an increase of NO synthase inducible (iNOS) mRNA expression in rat liver and of NO content in hepatic cells (Kim et al. 2002), which could trigger an important role in the pathology of liver diseases (Iwakiri & Kim 2015). The effect of high cholesterol levels in

hepatic dysfunction became more evident by reducing liver fibrosis and nitric oxide synthase expression in response to simvastatin in rats, a drug with hypocholesterolemic action (Wang et al. 2013). To better assess the effect of hypercholesterolemia in reactive species production, we performed analyses of ROS levels. Our data showed a significant effect of hypercholesterolemia on hepatic ROS levels. Likewise, Enríquez-Cortina et al. (2017) showed higher ROS content, measured by the DCFH technique, in response to a high cholesterol diet in mice's liver (Enríquez-Cortina et al. 2017). Since increased levels of nitrite and ROS lead to oxidative stress, our results showed that ingestion of a hypercholesterolemic diet causes increased oxidative stress in the liver tissue of mice.

One of the primary cellular defenses against the increase in ROS and nitrite is a group of oxidoreductases called SOD, critical antioxidant enzymes produced in the early stage of free radical generation (Chung 2017). Here, we observed a SOD activity reduction in response to hypercholesterolemia in the hepatic tissue of mice. Corroborating with our findings, rats fed a high cholesterol diet exhibited worsened antioxidant defenses, evidenced by the low activity of the enzymes SOD and catalase in hepatocytes (Zou et al. 2005). Three weeks of consumption of a cholesterol-rich diet was already able to reduce the activity of SOD in the rat's liver (Amin & Abd El-Twab 2009). Also, hypercholesterolemic rabbits exhibited reduced SOD activity in the liver in response to high cholesterol diet exposure for four weeks (Ashry et al. 2020). Another important molecule of the antioxidant system is GSH. The reduced GSH is the co-substrate of glutathione peroxidase, which catalyzes, through the glutathione cycle, the reduction of hydrogen peroxide previously produced by SOD (Gaucher et al. 2018). The antioxidant system is composed of enzymatic and non-enzymatic antioxidants (Birben et al. 2012). In our study, the hypercholesterolemic animals exhibited an increase in GSH levels in hepatic tissue. This data is according to a previous work in which rats showed an increase in hepatic GSH levels in response to a hypercholesterolemic diet (Manjunatha & Srinivasan 2007). In this same study, hypercholesterolemia has been associated with increased total thiol levels and decreased oxidative damage in the liver (Manjunatha & Srinivasan 2007), suggesting that GSH plays a vital role in protecting liver tissue from oxidative damage induced by hypercholesterolemia. We found that the increase in hepatic GSH content was related to the absence of oxidative damage to the liver proteins, evidenced by no change in carbonyl group content in the liver of hypercholesterolemic animals. A similar compensatory effect was observed in the GSH content in brain structures of low-density lipoprotein receptor knockout ($LDLr^{-/-}$) mice, an animal model to study familial hypercholesterolemia (de Oliveira et al. 2014). These facts reinforce the role of GSH in maintaining redox balance in response to hypercholesterolemia, including in the liver.

Previous evidence demonstrated that hypercholesterolemia increases oxidative stress and can affect the heart's functioning (Csont et al. 2007, Csonka et al. 2016). Herein, a significant effect of hypercholesterolemia was observed in the ROS levels, but not in nitrite formation, in mice's cardiac tissue. Similarly, hypercholesterolemic animals presented increased ROS generation in many tissues, including the heart (Oliveira et al. 2005, Csont et al. 2007). Our data also agrees with Csont et al. (2007), which did not observe changes in the cardiac content of NO and nitric oxide synthase activity in mice fed a high cholesterol diet (Csont et al. 2007). During hypercholesterolemia,

the balance between ROS and NO in the cardiovascular system is altered in a process that favors an increase in ROS and a decrease in NO biosynthesis (Stokes et al. 2002). Therefore, ROS-dependent mechanisms predominate compared to NO-dependent, which are inhibited. This phenomenon may partially explain the absence of an effect of hypercholesterolemia on nitrite levels in the heart and the presence of an effect of hypercholesterolemia on cardiac ROS levels observed in our study. In addition, like hepatic tissue, we observed that hypercholesterolemia increased GSH levels in cardiac tissue but did not alter SOD activity. Literature data showed that the effect of hypercholesterolemia on GSH levels in the heart directly depends on the time of exposure of the animals to the high cholesterol diet. Reduced levels of GSH were found in the heart of hypercholesterolemic animals exposed to a high cholesterol diet for 28 days (Deori et al. 2016) and six weeks (AlSaad et al. 2020). Evaluating the effect of high cholesterol diet on GSH levels in cardiac tissue of $LDLr^{-/-}$ mice, Girod et al. 1999 observed that animals exposed to the diet for twelve weeks showed a significant increase in GSH levels in this tissue (Girod et al. 1999). Interestingly, our study observed that a shorter exposure time of the animals to the diet could raise the GSH content in the heart of hypercholesterolemic mice. An increase of GSH levels was also observed in cardiac tissue of $LDLr^{-/-}$ treated with an antioxidant compound (Diphenyl diselenide), which reinforces the effect of hypercholesterolemia in the GSH levels in cardiac tissue of hypercholesterolemic mice (Mancini et al. 2013).

Despite the increased GSH content in the heart of hypercholesterolemic mice, this improvement in antioxidant defense did not translate into a decrease in oxidative damage, as evidenced by a trend of increasing carbonyl levels in cardiac tissue of mice fed a high cholesterol diet. This finding agrees with previous studies, in which animals with diet-induced hypercholesterolemia exhibit high carbonyl levels in heart tissue (Sozen et al. 2018, Sozer 2015). Hypercholesterolemia was also related to increased oxidative damage to lipids (AlSaad et al. 2020, Sozer 2015) and DNA (Chtourou et al. 2015) in cardiac tissue. Of particular interest, some of these negative effects of hypercholesterolemia on oxidative stress have been prevented by treatment with substances with antioxidant properties (AlSaad et al. 2020, Sozer 2015, Chtourou et al. 2015).

An antioxidant substance reduces or prevents macromolecules' oxidation by ROS, acting as an inhibitor of ROS production or as a positive regulator of antioxidant defense (Mut-Salud et al. 2016). In this context, nanoparticles' study as an antioxidant compound has grown due to their biocompatibility, high stability, and target delivery (Kumar et al. 2020). Among these nanoparticles with antioxidant action are AuNP. The experimental use of AuNP has demonstrated important antioxidant and therapeutic properties in many diseases, including metabolic diseases (Barathmanikanth et al. 2010, Chen et al. 2018a). Scavenger action against ROS (Barathmanikanth et al. 2010, Razzaq et al. 2016) and improvement of antioxidant defenses (Ko et al. 2020) are among the mechanisms involved in the antioxidant action of AuNP. However, little is known about the effects of AuNP on oxidative stress induced by hypercholesterolemia in peripheral tissues. To verify the antioxidant effects of AuNP on peripheral tissues of hypercholesterolemic mice, we administered AuNP in mice by oral gavage every two days for eight weeks. Regarding the administration route, it is worth mentioning that AuNP is subjected to metabolization by the gastrointestinal system when administered by the oral route. The stomach's gastric acid is likely to degrade the surface coatings of nanoparticles

(Pan et al. 2007, Schleh et al. 2012), resulting in the formation of ions from AuNP. However, the number of particles increased again in the gut, where the ions resulting from gastric acid digestion were deposited as particles in tissues. In this sense, the oral administration of AuNP with 28 nm in size to mice does not result in accumulation of AuNP in the stomach, but high levels of AuNP were easily found in the small intestine of the animals (Hillyer & Albrecht 2001). This is because of the ability of colloidal AuNP to remain trapped in the gastrointestinal mucosa (Hillyer & Albrecht 2001).

The choice of dose and administration period was made based on previous studies, which demonstrated that the administration of AuNP in a similar dose and every two days is safe and non-toxic to peripheral tissues (Chen et al. 2018b, Muller et al. 2017, Rodrigues et al. 2021). Using this safety administration strategy, we observed that AuNP treatment promotes redox homeostasis and displays important therapeutic effects in both cardiac and hepatic tissues, reducing ROS levels in the heart and increasing the SOD activity in the liver of hypercholesterolemic mice. On the other hand, long-term administration of AuNP in a higher dose (70 μg/kg) increased oxidative damage to the rat's heart and liver (Ferreira et al. 2015) This finding illustrates the importance of studying the AuNP dose in its therapeutic effect on peripheral tissues, mainly in the liver and in the heart.

Indeed, the antioxidant effect of AuNP in the liver and heart has been described in the literature. AuNP treatment exerts important antioxidant activity through the downregulation of Kupffer cells and hepatic stellate cell activities in rat's liver (de Carvalho et al. 2018). The AuNP treatment downregulated proinflammatory and improved lipid metabolic markers in the liver of obese mice (Chen et al. 2018a, b). In addition, diabetic mice treated with AuNP showed a significant decrease in ROS generation in the liver (Barathmanikanth et al. 2010). Reducing cellular damage markers to the liver and heart, restoring histopathological changes related to myocardial infarction, and reducing damage to the heart's DNA in mice are other effects related to the AuNP treatment in peripheral tissues (Ibrar et al. 2018). Also, AuNP significantly improved the antioxidant activity of GSH and ameliorated the redox and inflammatory profile on the rat's heart (Abdelhalim et al. 2015, Tartuce et al. 2020). These findings contrast in parts with the data obtained in our study, in which we do not observe the effect of AuNP treatment on GSH levels in the cardiac tissue of hypercholesterolemic mice. We hypothesized that these contrasting effects on GSH levels between our data and the result obtained by Abdelhalim et al. (2015) are due to AuNP's size and administration route differences. In other words, the AuNP with 10 nm in size and the intraperitoneal administration performed by Abdelhalim et al. (2015) and colleges facilitates the visualization of the antioxidant effect of AuNP in the cardiac tissue of Wistar-Kyoto rats. This is because AuNP smaller in size tends to cross the corporal barriers easily and could be found in more significant amounts in tissues (Hillyer & Albrecht 2001). Also, it was shown that AuNPs administration, when performed by oral route, only a small amount was absorbed by the tissues, whereas the intraperitoneal administration results in greater amounts of AuNP in tissues (Zhang et al. 2010).

Taken together, our data show that diet-induced hypercholesterolemia significantly altered the redox balance in the liver and heart in a process that favors oxidative damage. The treatment of hypercholesterolemic mice with AuNP promoted the redox balance in both cardiac and hepatic tissues, increasing the antioxidant defense and neutralizing the

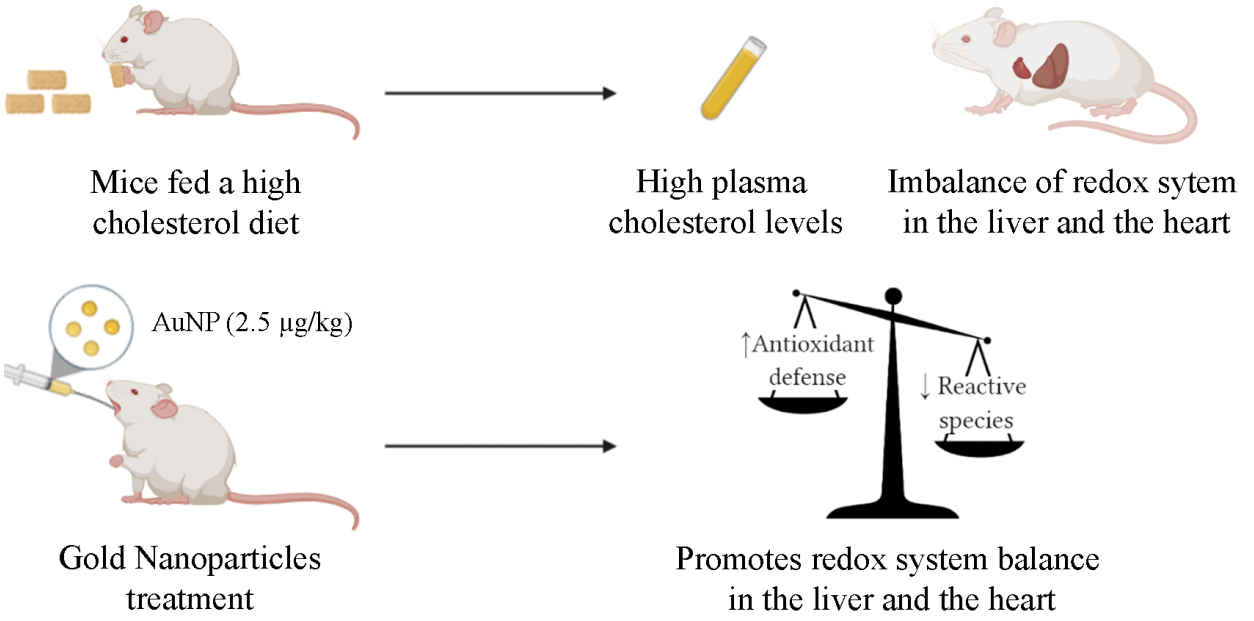


Figure 4. The illustration summarizes the effects of high cholesterol diet exposure and AuNP treatment on metabolic and oxidative stress parameters in the liver and heart of mice. Briefly, animals fed a high cholesterol diet exhibited high plasma cholesterol levels, i.e., hypercholesterolemia, which was not affected by AuNPs treatment. Also, hypercholesterolemia caused an imbalance of the redox system in the liver and the heart in a process that favors oxidative damage. AuNP treatment promoted redox system balance in both liver and heart, increasing antioxidant defense and reducing ROS production.

production of reactive species in these tissues (Figure 4). It is worth mentioning that our study is the first to demonstrate a significant antioxidant effect of AuNP in the peripheral tissues, mainly in the heart, of hypercholesterolemic mice. In this sense, additional biological studies must be performed to elucidate better the mechanisms involved in the antioxidant effect of AuNP in peripheral oxidative stress related to hypercholesterolemia.

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