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Hiper-homocisteinemia leve promove dano por estresse oxidativo via Nrf2, perfil inflamatório e colinesterases no sistema cardiovascular de ratos machos envelhecidos

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel(a) em Biomedicina.

Orientador(a): Profª Drª Angela Terezinha de Souza Wyse

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RESUMO

A homocisteína (Hcy) é um fator de risco independente para doenças cardiovasculares (DCV), cujos mecanismos não são bem conhecidos. O objetivo do presente estudo foi investigar os efeitos da hiper-homocisteinemia crônica leve (HHcy) sobre parâmetros oxidativos, inflamatórios e colinesterásicos em coração e soro de ratos Wistar machos envelhecidos (365 dias de idade). Os animais receberam Hcy (0.03 μmol/g de peso corporal) por via subcutânea durante 30 dias, duas vezes ao dia e eutanasiados 12 horas após a última injeção; logo após o sangue foi coletado e o coração dissecado para as análises bioquímicas realizadas. Foram avaliados os níveis de fatores pró-oxidativos e do anti-oxidante glutationa (GSH). Também foram avaliadas as atividades das enzimas antioxidantes, o perfil inflamatório, o qual foi determinado através do imunoconteúdo para as proteínas e expressão para interleucinas, bem como as atividades das colinesterases. Resultados mostraram um aumento de diclorofluoresceína (DCF) no coração e no soro e uma diminuição das enzimas antioxidantes [superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx)] e do conteúdo de GSH no coração. No coração foi observado um aumento na fração citosólica de fator nuclear kappa B (NFκB) p65 e nos níveis de expressão das interleucinas IL-6 e IL-10. Quanto ás colinesterases, observamos uma diminuição na atividade da acetilcolinesterase (AchE) no coração e da butirilcolinesterase (BuchE) no soro. Estas alterações sugerem uma ligação entre o envelhecimento e a HHcy, envolvendo a redução no imunoconteúdo de Nrf2 e defesas antioxidantes, bem como alterações a fatores inflamatórios e colinesterases, as quais juntas podem contribuir a compreensão do impacto da Hcy como fator de risco e fisiopatologia das doenças cardiovasculares e na dinâmica circulatória.

Palavras-chave: homocisteína; estresse oxidativo; Nrf2; inflamação; colinesterase

ABSTRACT

Homocysteine (Hcy) is an independent risk factor for cardiovascular diseases (CVD), whose mechanisms are poorly understood. The aim of the present study was to investigate the effects of mild chronic hyperhomocysteinemia (HHcy) in oxidative and inflammatory parameters, and in cholinesterases on the heart and serum of aged male Wistar rats (365 days old). The animals received Hcy (0.03 μmol/g body weight) subcutaneously for 30 days, twice a day, and were euthanized 12 hours after the last injection; shortly after, the blood was collected and the heart was dissected with biochemical analysis performed. The levels of pro-oxidative factors and of the antioxidant glutathione (GSH) were evaluated; antioxidant enzymes activity was measured; inflammatory profile determined through immunocontent for proteins and expression for interleukins and the activity, for cholinesterases, assessed. Results demonstrated an increase in dichlorofluorescein (DCF) in the heart and serum, with a decrease in antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and GSH content on the heart. In the heart, an increase in the cytosolic fraction of nuclear factor kappa B (NFκB) p65 and in the expression levels of interleukins IL-6 and IL-10 was observed. As for cholinesterases, we observed a decrease in the activity of acetylcholinesterase (AchE) in the heart and butyrylcholinesterase (BuchE) in the serum. These changes suggest a link between aging and HHcy, involving a reduction in the immunocontent of Nrf2 and antioxidant defenses, as well as changes in inflammatory factors and cholinesterases, which together can contribute to understanding the impact of Hcy as a risk factor and pathophysiology of cardiovascular diseases and circulatory dynamics.

Keywords: homocysteine; oxidative stress; inflammation; cholinesterase; deepeningcardiovascular system.

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1 INTRODUÇÃO COMPREENSIVA

A homocisteína (Hcy) é um aminoácido sulfurado não proteico. Ela faz parte do ciclo da metionina, um aminoácido essencial obtido através da dieta (ŠKOVIEROVÁ *et al.*, 2016). A metionina é convertida a S-adenosilmetionina (SAM) através da metionina adenosiltransferase (MAT), que por sua vez é convertida a S-adenosilhomocisteína por ação da enzima metiltransferase. Posteriormente S-adenosilhomocisteína é hidrolisada pela S-adenosilmetionina hidrolase (SAMH), formando Hcy e adenosina, como produtos (KATKO *et al.*, 2012). O controle biológico dos níveis plasmáticos de Hcy, portanto, dá-se majoritariamente pela regulação de duas vias metabólicas: remetilação à metionina ou transulfuração formando cistationa (WYSE *et al.*, 2021).

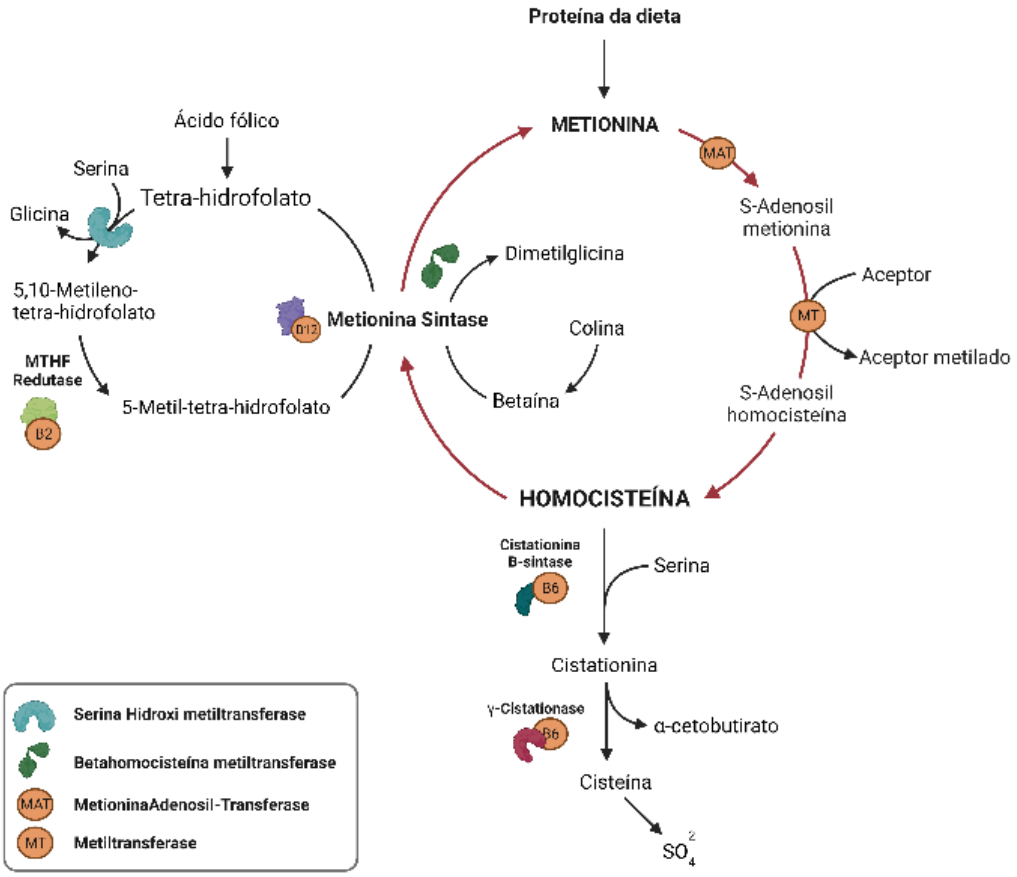


Figura 1: Rota metabólica de conversão da homocisteína

Fonte: adaptado de ((BLAISE *et al.*, 2005))

No processo de remetilação, a Hcy recebe um grupo metil do N-5-metiltetrahidrofolato ou da betaína, sendo, no primeiro caso, dependente de vitamina B12 e folato como cofatores. Já na transulfuração de Hcy condensa-se com a serina para formação de cistationa, que tem como cofator a vitamina B6 (ŠKOVIEROVÁ *et al.*, 2016). Dessa forma,

processos que interrompam o funcionamento dessas vias metabólicas, seja por defeito genético em enzimas ou nutrição deficiente de vitaminas que são cofatores nessas vias, podem levar a um aumento de Hcy plasmática (CLARKE *et al.*, 2010; WIERZBICKI, 2007).

Níveis plasmáticos elevados de Hcy são definidos como hiper-homocisteinemia (HHcy). Enquanto níveis normais de Hcy são definidos entre 4 e 15 uL/mol, valores entre 16 e 30 uL/mol já se enquadram em uma condição de HHcy leve, podendo ainda ser caracterizada como moderada (30-100 uL/mol) e severa (>100 uL/mol) (MARON; LOSCALZO, 2009). A HHcy leve é uma condição associada como fator de risco a múltiplas doenças, incluindo as cardíacas, como o infarto do miocárdio (DOS SANTOS *et al.*, 2019; PACANA *et al.*, 2015).

Quando ocorre o acúmulo de Hcy intracelular, esse aminoácido tende a ser exportado, intuindo evitar um possível efeito citotóxico, entretanto, como consequência, essa ação expõe o endotélio a grandes quantidades de Hcy (JACOBSEN, 1998). O transporte da Hcy ao endotélio é mediado por múltiplos sistemas transportadores de aminoácidos componentes da superfamília dos transportadores de soluto (KAPLAN *et al.*, 2020). Efeitos dessa exposição podem induzir o estresse oxidativo (PERNA; INGROSSO; DE SANTO, 2003), e complicações ateroscleróticas (SRECKOVIC *et al.*, 2017). Destaca-se ainda que, através do sistema colinérgico, associado ao óxido nítrico (regulador do tônus vascular) (SARTORI; LEPORI; SCHERRER, 2005), a Hcy pode influir na frequência cardíaca (KHANDOKER *et al.*, 2022). Dados de nosso laboratório mostram que que a HHcy leve está associada a um aumento na atividade da AchE (RAMIRES JUNIOR *et al.*, 2022) no cérebro de ratos e redução da butirilcolinesterase (BuchE) no soro de humanos (SCHERER *et al.*, 2007).

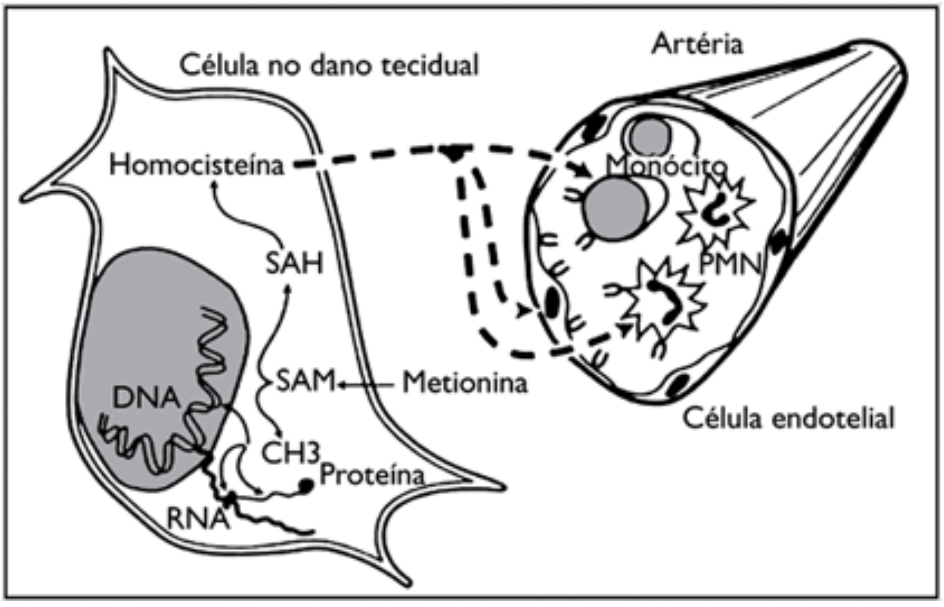


Figura 2: Relação homocisteína e dano endotelial

Fonte: (NEVES; MACEDO; LOPES, 2004)

As espécies reativas (ERs) podem ser definidas como moléculas instáveis com propriedades oxidantes, sendo essas produto natural da ação celular para realização de atividades biológicas necessárias a seu funcionamento (BRIEGER *et al.*, 2012). As ERs podem incluir os radicais livres, categorizados como átomos ou moléculas as quais possuem um elétron não pareado em sua última camada (GUTOWSKI; KOWALCZYK, 2013). As ERs são classificadas em dois grupos, as espécies reativas de oxigênio (EROs), e as espécies reativas de nitrogênio (ERNs), destas quais as de maior relevância e efeito deletério são: o radical ânion superóxido (O2•-), o radical hidroxil (•OH), o óxido nítrico (•NO), o peróxido de hidrogênio (H2O2), o peroxinitrito (ONOO-) e o ácido hipocloroso (HOCl) (HALLIWELL, 2006; SIES *et al.*, 2022).

Estudos experimentais em modelos animais com homocistinúria conduzidos pelo nosso grupo, observaram que ratos neonatos apresentaram uma mudança no perfil de estresse oxidativo no coração, com aumento das EROs e redução nas enzimas superóxido dismutase (SOD) e catalase (CAT) (KOLLING *et al.*, 2011), bem como em modelos para HHcy leve, no qual observou-se diminuição nas enzimas antioxidantes SOD, CAT e glutationa peroxidase (GPx) no coração (LONGONI *et al.*, 2017).

A enzima SOD é catalizadora da dismutação do radical ânion superóxido (O2 •−) a peróxido de hidrogênio (H2O2) e oxigênio (O2) (SAXENA *et al.*, 2022) enquanto a CAT metaboliza o H2O2 a H2O e O2 (HALLIWELL, 2006) e a GPx reduz o H2O2 a H2O enquanto converte glutationa (GSH), antioxidante celular mais abundante, de sua forma reduzida para a oxidada (GSSG) (WEISS, 2005).

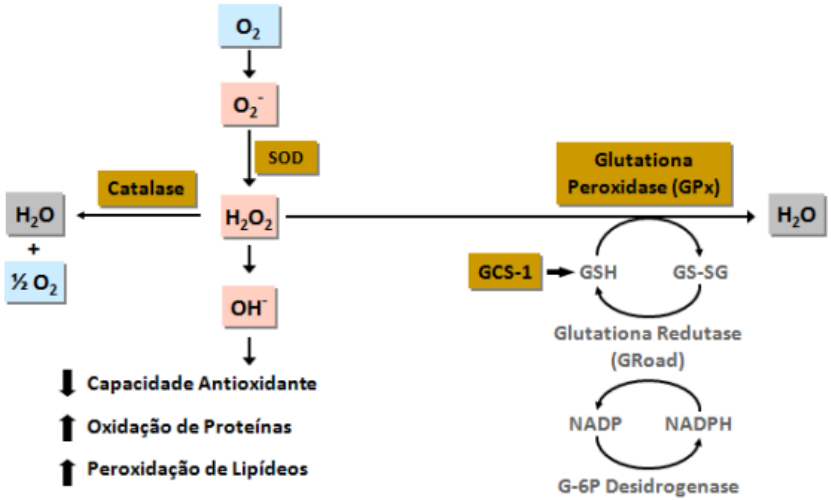


Figura 3: Esquema representativo das enzimas antioxidantes e conversão de espécies reativas de oxigênio

Fonte: (Franciny Aparecida Paiva [s. d.], 2015)

O conhecimento do mecanismo por trás da ação dos biomarcadores enzimáticos na HHcy leve, bem como não enzimáticos como vitamina C, sulfidrilas e GSH, é majoritariamente derivado de modelos animais em jovens-adultos. Não obstante, níveis elevados de Hcy foram demonstrados estarem relacionados com o envelhecimento (BOSTOM *et al.*, 1999), de forma que, da população geral, com prevalência de 5 a 7% para a condição, a mais afetada é a população idosa (SMITH; REFSUM, 2016). Ademais, em idosos, a HHcy leve pode ser relacionada a uma função enzimática reduzida, hábitos alimentares condutores de uma nutrição deficiente de vitaminas, a exemplo da B12 e B6, bem como o uso de drogas como tabaco e ao sedentarismo (DE BREE *et al.*, 2001; JACQUES *et al.*, 2001). Assim, conforme geram consequente elevação plasmática desse aminoácido no decorrer do tempo, suscita-se avaliar em modelos biológicos o efeito característico da HHcy leve sobre essa faixa etária. De forma que ratos da colônia Wistar constituem um ótimo modelo para replicação desse quadro vista semelhança entre estruturas e etapas do desenvolvimento (GHASEMI; JEDDI; KASHFI, 2021).

O dano tecidual é um passo sinalizador para o início do processo inflamatório, sendo o fator nuclear kappa B (NFκB) um mediador desse processo (SAHA *et al.*, 2020) o qual pode apresentar um aumento de sua expressão ou imunoconteúdo com a elevação dos níveis de Hcy (JI; KAPLOWITZ, 2004). Este aumento, por fim, pode acarretar no aumento de citocinas pró-inflamatórias como fator de necrose tumoral-alfa (TNF-α) e contribuir a formação de um microambiente pró-inflamatório contributivo a eventos cardiovasculares.

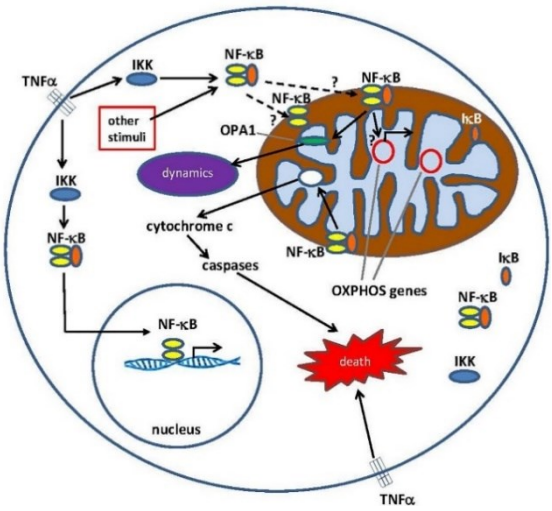


Figura 4: Rota pró-inflamatória do NFκB e resposta celular

Fonte: (ALBENSI, 2019)

Ainda, conforme a HHcy leve pode acarretar em uma diminuição do fator 2 relacionado ao fator nuclear eritroide 2 (Nrf2) (TAN *et al.,* 2013), uma proteína importante na promoção de expressão de enzimas antioxidantes, esse dano tecidual pode ser agravado, e levar a perda de tecido funcional. Portanto, considerando que HHcy leve representa um fator de risco para o sistema cardiovascular, o objetivo do nosso estudo foi avaliar alguns parâmetros de estresse oxidativo, do perfil inflamatório e das colinesterases em coração e/ou soro ratos machos envelhecidos submetidos à adminitração crônica de Hcy com a finalidade de elucidar ligações bioquímicas e biomoleculares entre o envelhecimento, HHcy leve e alterações no tecido cardíaco e séricos.

1.1 JUSTIFICATIVA

Muitos estudos experimentais e epidemiológicos destacam o papel tóxico da Hcy sobre parâmetros de estresse oxidativo, inflamação e sistema colinérgico, em ratos submetidos ao modelo experimental de HHcy leve crônica. Estes parâmetros são considerados fatores de risco para o desenvolvimento de doenças cardiovasculares como aterosclerose e infarto, conforme vem sendo mostrado em nosso grupo de pesquisa, não obstante, pouco é compreendido dos mecanismos bioquímicos pelos quais o quadro contribui ao surgimento de DCV em associação a idade.

Nesse sentido, suscita-se a investigação destes mecanismos sobre a condição de HHcy leve no tecido cardíaco e soro de animais envelhecidos, conforme a HHcy é mais frequente na população idosa, com expansão desta, em grande parte, relacionada ao aumento da expectativa de vida mundial. Nossa hipótese é que a HHcy leve possa causar danos oxidativos, inflamatórios e da colinesterase em coração de ratos Wistar machos envelhecidos, os quais possam estar relacionados ao fator de risco em doenças cardíacas.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Este projeto tem como objetivo avaliar o perfil inflamatório, estresse oxidativo e colinesterase em coração e soro de ratos Wistar machos envelhecidos submetidos à HHCy leve.

1.2.2 Objetivos específicos

A. Avaliar o Imunoconteúdo de proteínas associadas a inflamação e síntese de enzimas antioxidantes: Nrf2, NFκB e p-NFκB.
B. Investigar as atividades das enzimas antioxidantes: SOD, CAT e GPx.
C. Avaliar a atividade das enzimas colinesterases: butirilcolinesterase (BuChE) para o soro e AchE (celular).
D. Investigar marcadores de estresse oxidativo: níveis de sulfidrilas, nitritos, DCFH, TBARs e GSH.
E. Avaliar a expressão dos genes: IL-6, IL-10, TNFRSF1 e NFκB1

A metodologia, os resultados e a discussão serão apresentados na forma de manuscrito científico, que será submetido à publicação em revistas da área.

2 ARTIGO CIENTÍFICO

Mild hyperhomocysteinemia promotes oxidative stress damage via Nrf2, inflammatory profile and cholinesterases in aged male rats cardiovascular system

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Abstract

Homocysteine (Hcy) is an independent cardiovascular disease (CVD) risk factor, whose mechanisms are poorly understood. We aimed to explore mild hyperhomocysteinemia (HHcy) effects on oxidative, inflammatory, and cholinesterase parameters in aged male Wistar rats (365 days old). Rats received subcutaneous Hcy (0.03 μmol/g body weight) twice daily for 30 days, followed by euthanasia, blood collection and heart dissection 12 hours after the last injection. Results revealed increased dichlorofluorescein (DCF) levels in the heart and serum, alongside decreased antioxidant enzyme activity (superoxide dismutase, catalase, glutathione peroxidase), reduced glutathione (GSH) content, and diminished acetylcholinesterase (AchE) activity in the heart. Serum butyrylcholinesterase (Buche) levels also decreased. Furthermore, nuclear factor erythroid 2-related factor 2 (Nrf2) protein content decreased in both cytosolic and nuclear fractions, while cytosolic nuclear factor kappa B (NFκB) p65 increased in the heart. Additionally, interleukins IL-6 and IL-10 showed elevated expression levels in the heart. These findings suggest a connection between aging and HHcy in CVD. Reduced Nrf2 protein content and impaired antioxidant defenses, combined with inflammatory factors and altered cholinesterases activity, may contribute to understanding Hcy's impact on cardiovascular dynamics. This study sheds light on the complex interplay between HHcy, oxidative stress, inflammation, and cholinesterases in CVD, providing valuable insights for future research.

Keywords: homocysteine; oxidative stress; Nrf2; inflammation; cholinesterase; heart

Abbreviations

AchE - acetylcholinesterase

BuchE - Butyrylcholinesterase

CAT - catalase

CVD - cardiovascular diseases

DCFH - Dihydrodichlorofuorescein

GPx – glutathione peroxidase

Gr - glutathione reductase

GSH - Glutathione

Hcy – homocysteine

HHcy - hyperhomocysteine

NADPH - nicotinamide adenine dinucleotide phosphate

NFκB - nuclear factor kappa B

Nrf2 - nuclear factor erythroid 2-related factor 2

ROS – reactive oxygen species

SOD - superoxide dismutase

TBARS - Thiobarbituric Acid Reactive Substances

TNF-α - Tumour Necrosis Factor alpha

TNFRSF1 – tumor necrosis factor receptor superfamily, member 1A

1.Introduction

Homocysteine (Hcy) is a non-proteinogenic sulfur amino acid product of the conversion of methionine, an essential amino acid obtained through the diet[1]. The biological control of Hcy plasma levels occur by the regulation of two metabolic pathways: remethylation to methionine or transsulfuration forming cystathione [2]. Both processes are dependent of vitamins from the B complex and enzymes metabolic homeostasis, meaning that an impair on the functioning of these pathways, whether due to a genetic mutation in enzymes or deficient nutrition of vitamins, cofactors in these pathways, can lead to an increase in Hcy levels [3,4].

Elevated plasma Hcy levels are defined as hyperhomocysteinemia (HHcy). While normal Hcy levels are defined between 4 and 15 uL/mol, values between 16 and 30 uL/mol are categorized into a mild HHcy condition, and may still be characterized as moderate (30-100 uL/mol) and severe (>100 uL/mol) [5]. Mild HHcy is a condition associated as a risk factor for multiple pathological processes and cardiovascular diseases, such as heart failure [6,7].

When intracellular Hcy accumulates, this amino acid tends to be exported, in order to avoid a possible cytotoxic effect. However, as a consequence, this action exposes the endothelium to large amounts of Hcy [8]. Hcy transport to the endothelium is then mediated by multiple system amino acid transporters that are components of the solute transporter superfamily [9]. Nonetheless, as the cholinergic system is associated with oxide nitric acid (vascular tone regulator) [10] as well as heart rate maintance[11], and mild HHcy is associated with alterations to the AchE activity [12], an impaired enzyme could contribute for an increased risk of cardiovascular diseases (CVD) through the cholinergic system.

Experimental studies conducted by our group have demonstrated a relationship between Hcy and deleterious effects on heart through an increase of reactive oxygen species

(ROS) [13], as well as a decrease in butyrylcholinesterase (BuchE)[14]. Furthermore, animals treated chronically with mild HHcy showed a decrease in the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the heart [15].

In parallel, as an important regulator of oxidative stress response, a decrease in nuclear factor erythroid 2-related factor 2 (Nrf2)[16] could determine further cardiovascular events in mild HHcy being associated with differential response in aging[17]. Knowledge of the mechanisms behind the action of enzymatic biomarkers in Mild HHcy, as well as non-enzymatics such as glutathione (GSH), derive mostly, however, from infant or young adult animal models on the heart [13], serum [14] and brain [12]. Tissue damage, in parallel, is a signaling step towards the beginning of the inflammatory process, with nuclear factor kappa B p65 (NFκB) being a mediator of this process [18] that may present an increase in its expression with elevated Hcy levels [19]. Consequentially, NFκB can lead, as a promoter, to an increase in pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukine 6 (IL-6) and interleukine 10 (IL-10).

Nevertheless, mild elevated levels of Hcy have been shown to be related to aging [20], so that, of the general population, with a prevalence of 5 to 7% for the condition, the most affected is the elderly population [21]. Furthermore, with an increased age, mild HHcy may be related to reduced enzymatic function, decreased vitamin intake, such as B12 and B6, as well as the use of drugs, such as tobacco, and a sedentary lifestyle [22,23]. Then, as age relates to increased risk of elevated concentration of Hcy over time, it becomes important to adress the characteristic effect of HHcy in biological models towards this age gap. In this regard, rats from the Wistar colony constitute a great model for replicating these phenomena, given the similarity between structures and stages of the development [24].

Since Hcy can represent a risk factor to the cardiovascular system, in the present study we explored the potential toxic effects of mild HHcy in the heart and serum of aged rats. We

determined oxidative stress, inflammatory and cholinesterases parameters and hypotesize that impairments related to elevated levels of Hcy could be enhanced through advanced age by biochemical and molecular mechanisms compromised in such groups, adressing potential links between age and mild HHcy.

2. Materials and Methods

2.1 Animals and Compliance with Ethical Standards

Forty-eight male Wistar rats (365 days old) were acquired from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences at the Universidade Federal do Rio Grande do Sul, in Porto Alegre, Brazil. Animals were maintained in a colony room (12:12 h light/dark cycle) with controlled temperature (22±1 °C) and water and 20% (w/w) protein commercial chow ad libitum. All procedures followed the Arouca Law (Brazilian Federal Law no 11.794/2008), the National Institutes of Health guide for the care and use of laboratory animals (NIH publication 8023/1996), and the ARRIVE and UFRGS Ethics Committee guidelines. The experimental protocol was approved by the Ethics Committee of the UFRGS (#36,991). The reagents and inputs used in the assays were purchased from Sigma-Aldrich (San Luis, Missouri, USA) and Merck Millipore (Burlington, Massachusetts, USA).

2.2 Chronic Treatments

Male Wistar rats at 365 days of age were treated for a period of 30 days (365 to 395 days of life), twice a day, with an interval of 12 h between injections. Animals treated with homocysteine, 30 DL-HCY (Sigma Aldrich®, catalog no. H4628) (4.055 mg/kg of body weight), were administered subcutaneously (sc), towards plasma concentration levels would be similar to those found in patients with hyperhomocysteinemia, as described by Scherer et al (2011), with control group receiving saline (sodium chloride solution) (Fig. 1).

Rats were decapitated 12 h after the last Hcy injection. Then, heart and serum were used for respective analyses, with a sample size determined and delineated in corresponding methods and figures caption. For biochemical analyses, we used 8-7 animals per group and a 7-6 animals per group for western blot and gene expression analysis.

2.3 Oxidative Stress Parameters Assays

2.3.1 Tissue Preparation

After euthanasia by decapitation, the heart was removed and homogenized 1:40 (w/v) in 20mM sodium phosphate buffer (pH 7.4) with 140 mM KCl. The homogenates were centrifuged (800 x g, 10 min at 4°C). The supernatants were separated and stored (at -70 °C) until use to determine the parameters of the oxidative stress and antioxidant enzimes. Serum was obtained by centrifugation (3000 rpm, 10 min at 4°C) of whole blood collected immediately after euthanasia, and diluted 1:5 in 20mM sodium phosphate buffer (pH 7.4) with 140 mM KCl for oxidative stress parameters determination.

2.3 2 2′,7′-Dihydrodichlorofuorescein (DCFH) Oxidation

ROS was measured according to the LeBel method [26]. In this method DCFH-diacetate is cleaved by cellular esterases, and, in the process, the DCFH formed is oxidized to 2′,7′-DCF by ROS present in the sample supernatants. The supernatants were incubated with a DCFH-diacetate solution (at 37 °C for 30 min), and the resulting fluorescence was measured at 488 nm excitation and 525 nm emission. DCF content was determined by a standard DCF calibration curve, and the DCF formed was expressed as nanomole of DCF per mg of protein.

2.3.3 Thiobarbituric Acid Reactive Substances (TBARS)

Lipid redox status was evaluated by Ohkawa method [27]. In this method, the lipoperoxidation product reactive to thiobarbituric acid, the malondialdehyde (MDA), is measured. Thiobarbituric acid reactive substances (TBARS) formation was measured by incubation of supernatants with 1.15% KCl, 20% trichloroacetic acid, and 0.8% thiobarbituric acid and heated in a boiling water bath (60 min). The concentration of TBARS in the samples was determined from a calibration curve with 1,1,3,3-tetramethoxypropane as standard.

TBARS level was measured in a spectrophotometer at 535 nm, and the results are expressed as nanomole of MDA per mg protein.

2.3.4 Sulfhydryl Content

To assess oxidative changes in proteins, sulfhydryl content of the samples was measured as previously described by Aksenov and Markesbery [28]. In the reactional medium, were added the sample supernatant, saline phosphate bufer (pH 7.4) with 1 mM EDTA, 5,5-dithiobis-2-nitrobenzoic acid 0.01 mol/L in ethanol, and 0.2 M potassium phosphate solution (pH 8.0). After 30 min of incubation at room temperature in the dark, the yellow color formed was measured at 412 nm on a spectrophotometer. The results are expressed as nanomoles of TNB per mg of protein.

2.3.5 Nitrite levels

Nitrite levels were determined according to Green method [29]. Samples were incubated for 10 min at room temperature with Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1- Naphthyl) ethylenediamine dihydrochloride). Sodium nitrite calibration curve was used as the standard, and absorbance was measured at 543 nm. The results were expressed as micromole of nitrite per milligram of protein.

2.3.6 GSH Levels

GSH levels were measured as determined by Browne and Armstrong [30]. First, samples were deproteinized by mixing with metaphosphoric acid and centrifugated (1000 x g, 10 min, 4 °C). After, equal volumes of sample and o-phthaldialdehyde (1 mg/mL in methanol) were incubated at room temperature for 15 min. Fluorescence was measured using excitation of 350 nm and emission of 420 nm. Calibration curve was performed with standard GSH

(0.001–0.1 mM), and GSH concentrations were calculated as nanomoles of GSH per milligram of protein.

2.3.7 SOD Activity

SOD activity was determined according to Marklund method [31]. In this method, pyrogallol was used and, according to its autooxidation, SOD activity was indirectly determined. The inhibition of pyrogallol autoxidation was measured at 420 nm. A calibration curve was performed using purified SOD as a standard, and the results were expressed as units per mg of protein.

2.3.8 CAT Activity

CAT activity was measured as previously described by Aebi [32]. Sample supernatants were mixed with 0.1% Triton X-100, preincubated for 15 min at room temperature, and added 10 mM potassium phosphate buffer (pH 7.0) with 20 mM H2O2. The degradation of H2O2 was monitored at 240 nm for 3-4 min. A CAT unit is defined as 1 mM H2O2 consumed per minute at 37 °C. The activity was expressed in units per mg of protein.

2.3.9 GPx Activity

GPx activity was measured according to Wendel (1981) [33] with some modifications[13] using tert-butyl hydroperoxide as a substrate. The reactional medium contained 0.1 mmol/L nicotinamide adenine dinucleotide phosphate (NADPH), 2mmol/L glutathione, 0.15 U/mL glutathione reductase (GR), 0.4 mmol/L azide and 0.5 mmol/L tert-butyl hydroperoxide. NADPH disappearance was monitored at 340 nm for 3-4 minutes. One GPx unit is defined as 1 μmol of NADPH consumed per minute, and represented as GPx units per milligram of protein.

2.4 Cholinesterases Parameters Assays

2.4.1 Tissue Preparation

For AchE activity, heart samples were homogenized 1:40 (w/v) in 0.1 mM potassium phosphate buffer (pH 7.5) and centrifuged (800×g for 10 min at 4 °C). Serum was diluted 1:5 in 0.1 mM potassium phosphate buffer (pH 7.5) for BuchE activity assay.

2.4.2 AchE activity

AChE activity was determined according to the method of Ellman (1961)[34] with some modifications [13]. Acetylthiocholine (0.8 mM) hydrolysis rates were measured in the reactional medium by adding heart supernatant with 30 mM phosphate bufer (pH 7.5) and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid—DTNB) at 25 °C. After, the reaction mixture was incubated for 3 min and lastly acetylthiocholine (0.8 mM) was added. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 3-4 min, measured at intervals of 30 s.

2.4.3 BuchE activity

BuChE activity was determined by the method of Ellman et al. (1961)[34] with some modifications [13].To determinate the hydrolysis rate, 15 microliters of diluted serum were added to the reaction mixture containing 100 mM potassium phosphate buffer pH 7.5 and 1.0 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and preincubated for 3 min. After, acetylthiocholine (0.8 mM) was added and the hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 3-4 min (intervals of 30 s) at 25 C.

2.5 RNA extraction and RT qPCR

Total RNA was isolated from wistar rat heart using TRIzol Reagent. Afterwards extracted RNA (1 μg) was then submitted to cDNA synthesis by High Capacity cDNA Reverse Transcription Kit. Quantitative PCR determination of the messenger RNAs (mRNAs) encoding interleukin-6 (Rn01410330_m1), interleukin-10 (Rn00563409_m1), NFκB1 (Rn01399572_m1), TNFRSF1 (Rn01492348_m1) and β-actin (Rn00667869_m1) were performed using the TaqMan realtime RT-PCR system with inventory primers and probes purchased from Applied Biosystems (Thermo Fisher Scientifc), as referred for each gene. Target mRNA levels were normalized to β-actin levels. Results were analyzed employing the$2^{-\Delta\Delta Ct}$ method [35].

2.6 Western Blot Analysis

2.6.1 Cellular Fractionation for Cytosolic and Nuclear Protein Abundance

To obtain the cytosolic and nuclear protein abundance of Nrf2, NFκB p65 and p-NFκB p65, samples were fractioned as described by (Ferreira et al. 2018). Heart tissue was homogenized in phosphate buffered saline (PBS) and centrifugated (4000 rpm, 3min, 4°C). Then, after centrifuge, supernatants were discarded and pellets were resuspended in 700 μL hypotonic lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT), 5 mM NaF, and 1 mM sodium orthovanadate plus protease inhibitor cocktail. Samples homogenate were then lysed with 18 μL 10% IGEPAL and incubated 5 min at - 4°C. After, homogenates were centrifuged (at 14,000×g, 30 s, 4 °C), and supernatants containing the cytosolic fractions were stored (at −80 °C). The nuclear pellets were resuspended in 450 μL ice-cold hypertonic extraction buffer containing 10 mM HEPES (pH 7.9), 0.40 mM NaCl, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF, 1 mM DTT, 5 mM NaF, 1 mM sodium orthovanadate, 0.25 mM

EDTA, 25% glycerol plus protease inhibitor cocktail. Lastly, after 40 min of intermittent mixing at - 4°C, extracts were centrifuged (at 14,000×g, 10 min, 4 °C), and supernatants containing nuclear fractions were stored (at −80 °C). Then, samples were dissolved 1:1 in Laemmli bufer 2× and boiled for 5 min.

2.6.2 Western Blot Analysis of Cytosolic and Nuclear Nrf2, NFκB p65 and p-NFκB p65 Fractions

Western blot of cytosolic and nuclear fractions was performed as described by (Ferreira et al. 2018). Total protein of supernatant fractions was separated by 10% SDS–PAGE (40 μg/lane of total protein) and transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to nitrocellulose membranes (1 h at 15 V) in a transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-bufered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5% bovine serum albumin; MTBS). After incubation, the membrane was washed twice for 5 min with TBS plus 0.05% Tween 20 (TTBS). The membranes were then incubated for overnight (4°C) with the appropriate primary antibody against Nrf2 (#550014740, Sigma-Aldrich; diluted 1:1000), p- NFκB (#3033, Cell Signaling Technology; diluted 1:1000), NFκB p65 (#B1609, Santa Cruz Biotechnology; diluted 1:500) and GAPDH (G8795, Sigma-Aldrich; diluted 1:1000); The blot was then washed twice for 5 min with TTBS and incubated for 2 h in antibody solution containing specific peroxidase-conjugated anti-mouse IgG (#7076, Cell Signaling Technology; diluted 1:1000) or anti-rabbit IgG (#7074, Cell Signaling Technology; diluted 1:1000). The blot was washed twice again for 5 min with TTBS and twice for 5 min with TBS. Then, the blot was revealed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences).

2.6.3 Protein Determination

Sample protein levels were determined by the method of Lowry et al. (1951)[36] or Bradford (1976)[37], using bovine serum albumin as standard.

2.6 Statistical Analysis

Sample size was determined by calculation performed using the Minitab VS.17 program (Minitab Inc, State College, PA, USA) with an allowed α error of up to 0.05, with 2 levels and an estimated power of 92%. All analyses were performed using the GraphPad Prism 8.0 software on a compatible computer. Data were evaluated for normality by the Shapiro–Wilk test and outliers removed using ROUT test (Q = 10%). Statistical analyses were performed using Student´s t-test, expressed as mean ± SEM and considered statistically significant when $p<0.05$.

3. Results

Towards assessing effects of the chronic mild HHcy in the heart and serum, firstly pro-oxidative parameters were tested. In both heart and serum an increased formed DCF was observed [Fig.2A, $t = 6.382$, $df = 13$, $p<0.0001$; Fig2.B, $t = 2.822$, $df = 14$, $p=0.0136$]. Other pro-oxidants measures evaluated including nitrites, sulfhydryl and TBARS were not altered significantly on the heart or serum in this analysis ($p> 0.05$).

Sequentially, anti-oxidant enzymes SOD, CAT and GPx as well as GSH content were investigated on the heart (Fig. 3), with a decrease in the SOD [$t = 3.041$, $df = 14$, $p=0.0088$], CAT [$t= 5.391$, $df =13$, $p=0.0001$] and GPx [$t = 3.757$, $df = 13$, $p=0.0024$] activity as well as a decrease in the GSH content [$t = 4.096$, $df = 12$, $p=0.0015$] and an increase in SOD/CAT ratio [$t = 3.447$, $df = 12$, $p=0.0048$]. To investigate whether the changes observed where related to the protein levels of Nrf2, an immunocontent analysis was conducted and a decrease (Fig. 4) in both cytosolic [$t= 2.297$, $df = 10$, $p=0.0445$] and nuclear [$t= 4.119$, $df = 10$, $p=0.0021$] fraction was observed.

To further address through which parameters the chronic mild HHcy impacts the cardiovascular system, the NFκB pathway was targeted and the immunocontent of both NFκB p65 and p- NFκB p65 were evaluated in the heart (Fig. 5), with an increase (Fig 5A) in the cytosolic fraction of NFκB p65 [$t= 2.335$, $df = 10$, $p=0.0417$] being detected in this analysis. No further statistically significant differences were observed ($p>0.05$).

Next, to undertake a view of the expression levels of genes that encode for parameters related to the inflammatory profile and NFκB pathway [38], IL-6, IL-10, TNFRSF1 and NFκB1 were evaluated and an increase in both IL-6 [$t = 2.353$, $df = 13$, $p=0.0350$] and IL-10 [$t = 2.496$, $df = 12$, $p=0.0281$] was observed, with no significant alterations to the TNFRSF1 and NFκB1 expression levels detected ($p>0.05$).

Lastly, as AchE and BuchE activity can be related to physiological cardiovascular function [39], both colinesterases were evaluated, AchE at the heart and BuchE in the serum. At present analysis either enzymes activities were found decreased relative to control [Fig.7A, t = 1,13= 2.266, df = 13, p=0.0411; Fig.7B t = 2.700, df = 12, p=0.0193].

4. Discussion

Mild HHcy induces alterations in the cardiovascular system leading to CVD and increased cardiovascular mortality [40]. Thus, the present study investigated the chronic effects of mild Hcy administration subcutaneously, on the heart and serum of aged Wistar rats. To better understand the toxic effects of mild HHcy, which can be enhanced by aging, characteristics in response to oxidative stress, inflammatory profile, and cholinesterase activities were investigated. We believe, HHcy is thought to induce several cellular dysfunctions by diminished anti-oxidant response [15], increased ROS concentration [41], altered AchE [12] and BuchE activity [42], and to present an effect on the NFκB p65 protein by increased activation [43], which leads to an up-regulation of pro-inflammatory cytokines.

Hcy auto-oxidation to form itself is one major source of ROS formation in mild HHcy [44]. ROS are unstable molecules, that, in biological systems, are carefully maintained in a redox homeostasis [45]. The oxidative stress alterations in biological systems are potential sources of damage to all macromolecules [46], such as DNA, proteins and lipids, contributing to atherosclerosis and cardiovascular events [47]. The most relevant ROS and associated with a deleterious effect are the superoxide anion radical, hydroxyl radical, nitric oxide, hydrogen peroxide, peroxynitrite and hypochlorous acid [48,49]. Any alterations to the presence of those molecules can cause an adaptative modulation of anti-oxidant defense systems and corresponding detoxification. Mitochondria complexes present a decreased activity when associated with high levels of Hcy [9,45], and as mitochondria represent primary sources of ROS

formation, results found could be attributed to those factors. Nonetheless, up to 10% of Hcy produced by the cell cannot be metabolized, and that quantity is transported to the endothelium [50], where effects of this exposure are contributors to oxidative stress [51] and atherosclerotic complications [52], yet its underlying mechanisms are poorly understood.

SOD is an enzyme that catalyzes the dismutation of superoxide anion into oxygen and hydrogen peroxide [53]. The product of the reaction can be catalyzed by the CAT activity, an enzyme found in most organisms, and decompose hydrogen peroxide into water and oxygen [54]. Both enzymes are related to the anti-oxidant defenses, and a ratio between them is an important measurement of cell detoxification systems [55]. When their activity is impaired, a corresponding relation could be traced to their ratio, where an increased SOD/CAT proportion, as present in our study (Fig.3), may indicate that their products and substracts are not being properly detoxified, and consequentially, ROS molecules may interact for extended periods with cellular components [56].

Likewise, another relevant enzyme related to the decomposition of H2O2 is the GPx. Through its reaction, GSH and hydrogen peroxide are converted into GSSG and water, being an important cardiovascular protector towards limiting lipid peroxidation [57]. To reduce GSSG in GSH, cell utilizes GR, an enzyme that requires for this reaction NADPH, consequently, a decrease in GSH content, as observed in this present study (Fig 3.), may disrupt other pathways dependent of NADPH [58]. Furthermore, homeostatic levels of GSH are associated with Hcy recycling back to Met and maintaining the homocysteine pathway's integrity [59], with a decrease in its content related to multiple factors such as low B complex vitamin intake [60]. These findings could indicate a magnifying effect of aging on mild HHcy, as significances observed on antioxidant enzymes impaired, as well as ROS increase, where only matched by studies in our group with infants in Hcy concentrations of 100 uL/mol or higher, classified as homocystinuria [13].

Nrf2 characterizes as a protein important to the oxidative stress response mechanism and aging [17]. Its function as a transcription factor regulates the genetic expression of other proteins related to detoxifying and corresponding elimination of ROS from biological systems [58]. In the present study, both cytosolic and nuclear fractions of Nrf2 were observed reduced, which may impair the transcription of the anti-oxidant enzymes SOD, CAT and GPx [61] as, when Nrf2 translocates to the nucleus, it binds to the antioxidant response element, a cis-acting regulatory element found in the promoter region of those genes, rendering a critical step in the cellular response to oxidative stress and the activation of the genes necessary to counteract the damaging effects of ROS [62].

The detrimental effects of chronic mild HHcy to the oxidative stress parameters we also observed an increase in the cytosolic fraction of NFκB p65 protein, which corresponds to an inflammatory profile. The NFκB p65 protein is a transcription factor located in the cytosol and bound to IkB in an inactive status [63]. Phosphorylation of the protein, p-NFκB, induces release from the inhibitory proteins, activation and translocation to the nucleus, where it binds to specific promoter regions of genes involved in several cellular processes such as inflammation and immune response [38]. An increased cytosolic fraction of NFκB p65 could indicate a pro-inflammatory state that contributes to cardiovascular events by endothelial dysfunction [63]. In endothelial cells, Nf-Kβ p65 activation can lead to increased expression of adhesion molecules and pro-inflammatory cytokines, which contribute to vascular inflammation and atherosclerosis [64].

Cytokines constitute a group of small proteins important to cell signaling, regulating inflammation. Our study evaluated the expression of genes that code for cytokines IL-6, IL-10 and the corresponding receptor of TNF-α, TNFRSF1, as well as of NFκB1, with an increase in both IL-6 and IL-10 observed on the heart. Those results indicate that, as elevated IL-6 levels can serve as a marker of ongoing inflammation in the cardiovascular system [64], an

increase in IL-10 on the heart may represent a counter-regulatory response to the inflammation caused by Hcy [65], as well as an attempt to regulate the damage caused by inflammation to the heart tissue. Such interaction could suggest that an imbalance in inflammatory response may contribute to the progression of cardiovascular disease in the chronic mild HHcy [66].

As cholinesterases regulate acetylcoline (Ach) presence and signaling through hydrolysis of the molecule, a potential effect of Hcy on the cardiovascular system could be derived from prolonged Ach signaling and influence on heart rate and blood pressure regulation [67]. In our study, both AchE, evaluated in the heart, and BuchE, in the serum, activities were found decreased (Fig 8). AchE reduced activity, may indirectly point a potential enzymatic role in hypertension and CVD [39], as well as an impaired BuchE activity, which, has been associated with increased cardiovascular events such as heart attacks and strokes, as well as a dysregulated control of the vascular tone [68].

Some limitations must be taken into account in this study. First, only whole heart homogenized and serum parameters were evaluated, however with aging, morphological and functional changes are observed in other tissues, such as liver, kidneys, and central nervous system, exceptionally as multiple studies link mild HHcy and increased risk of alzheimer disease [69], and should be adressed. In addition, an evaluation of the immunocontent for the anti-oxidant enzymes and cholinesterases was not done, yet should provide a better understanding of the mechanisms by which their activity may be impaired. Finally, other protocols could prove useful to determine the best window to evaluate changes related to increased Hcy plasma levels, in the cardiovascular system, such as studies in slices and cell culture.

In conclusion, the number of changes elated can demonstrate the impact of mild concentrations of Hcy on the cardiovascular system when subcutaneously administered on

aged wistar rats. An important and magnifying factor to those oxidative stress, inflammatory and cholinergic features may come from the age associated to the animal model [70], and positively translate to humans, as increased age represent a greater risk to mild HHcy [71]. Characteristics such as an overall decreased enzymatic function, an impaired cellular response to stressor mechanisms and DNA methylation levels may elude most findings in our study and link with cohort developments in humans for mild HHcy and correlated pathologies.

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Availability of data and material

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

Code availability

Not applicable.

Authors' contributions

G.R.K.P and A.T.S.W. designed the studies. G. R. K. P., A. S. R, O. V. R. J contributed to the data collection; G. R. K. P. and A.T.S.W. revised critically, analyzed and interpreted the work. All authors have read, revised, and approved the final version of this manuscript.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Artwork and Tables

Fig. 1:

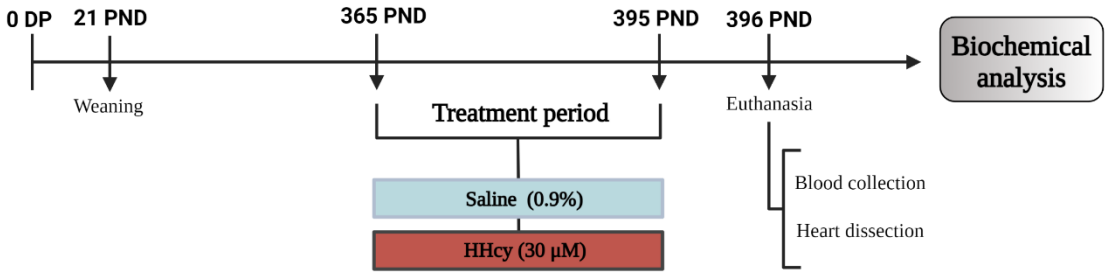


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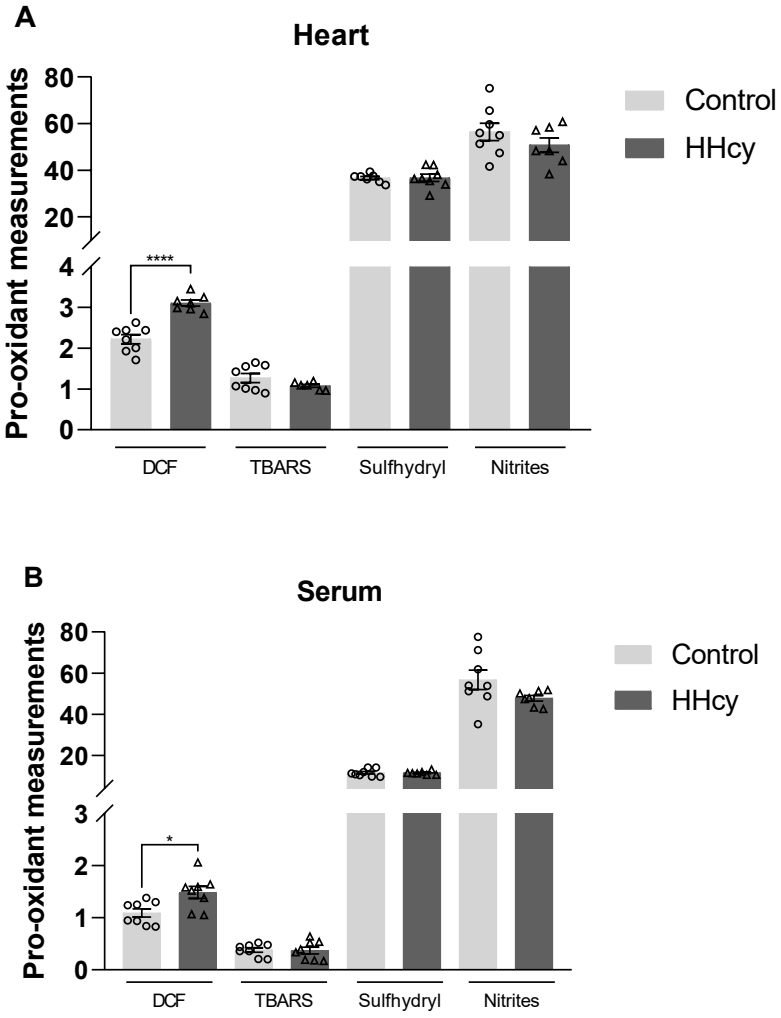


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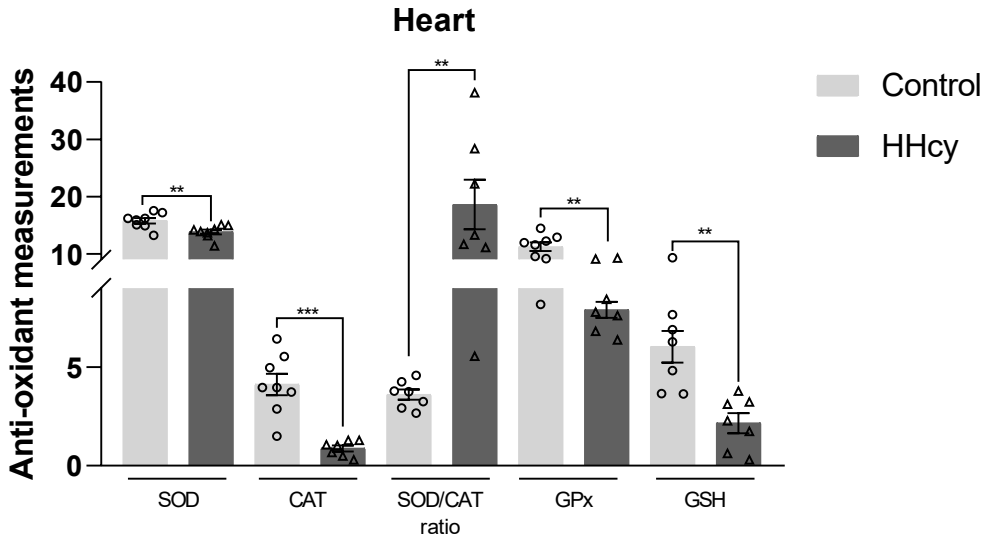

Heart
Anti-oxidant measurements
40
30
20
10
5
0
**
***
**
**
**
Control
HHcy
SOD
CAT
SOD/CAT ratio
GPx
GSH

Fig 4:

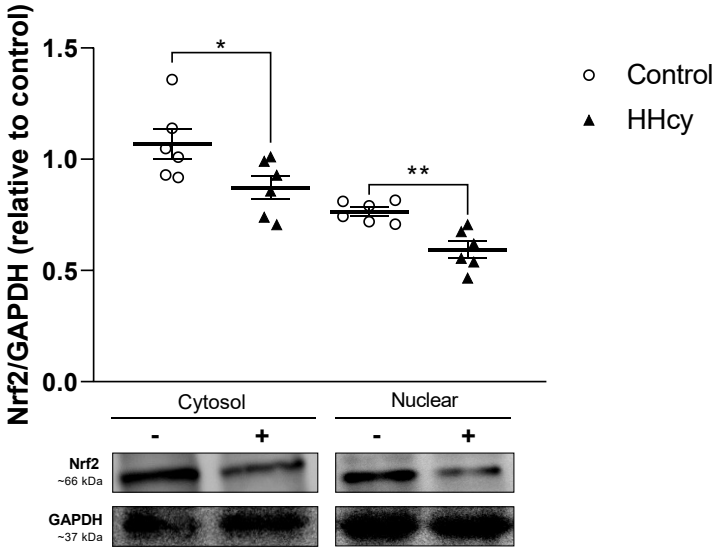
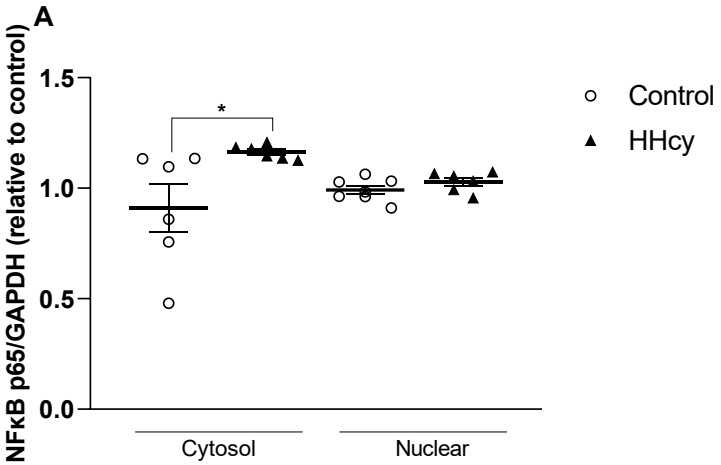
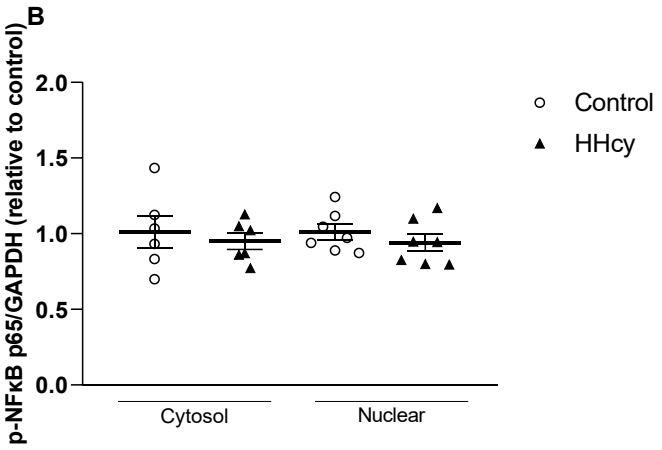

Nrf2/GAPDH (relative to control)
1.5
1.0
0.5
0.0
*
**
Control
HHcy
Cytosol
Nuclear
-
+
-
+
Nrf2
~66 kDa
GAPDH
~37 kDa

Fig 5:


A
NFκB p65/GAPDH (relative to control)
1.5
1.0
0.5
0.0
*
Control
HHcy
Cytosol
Nuclear


B
p-NFκB p65/GAPDH (relative to control)
2.0
1.5
1.0
0.5
0.0
Control
HHcy
Cytosol
Nuclear

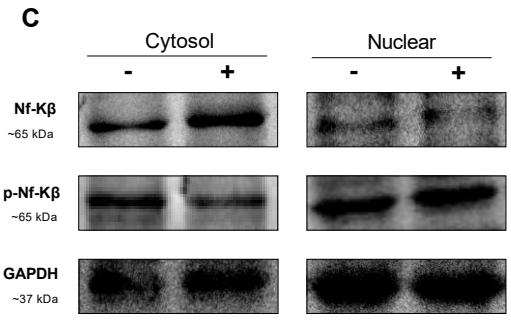

C
Cytosol
Nuclear
-
+
-
+
Nf-Kβ
~65 kDa
p-Nf-Kβ
~65 kDa
GAPDH
~37 kDa

Fig 6:

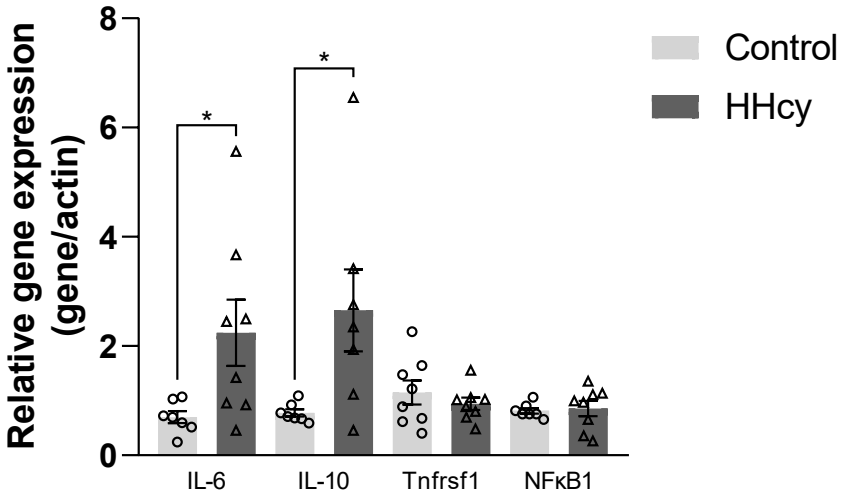
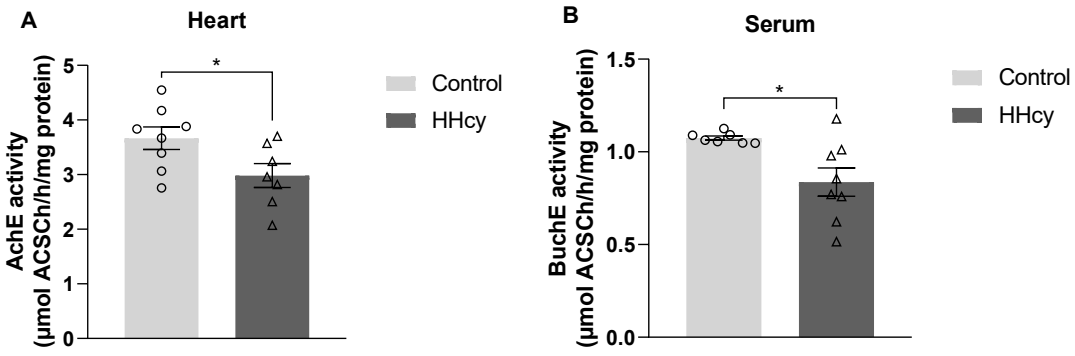

Relative gene expression
(gene/actin)
8
6
4
2
0
*
*
IL-6
IL-10
Tnfrsf1
NFκB1
Control
HHcy

Fig 7:


A
Heart
AchE activity
(µmol ACSCh/h/mg protein)
5
4
3
2
1
0
*
Control
HHcy
B
Serum
BuchE activity
(µmol ACSCh/h/mg protein)
1.5
1.0
0.5
0.0
*
Control
HHcy

Artwork and table captions

Fig. 1. Experimental timeline of biochemical and biomolecular assays

Fig. 2. Effects of Chronic mild HHcy on Heart (A) and serum (B) pro-oxidant measurements: DCF formed, TBARS, nitrite levels and sulfhydryl content. DCF and nitrites levels were expressed as μmol per mg of protein. TBARS level was expressed as nmol of MDA per of mg protein and sulfhydryl content was expressed as nmol of TNB per mg of protein.The data are expressed as mean ±S.E.M. of n= 7-8 per group with Student t-test performed. ****$p<0.0001$ and *$p<0.05$ from control group.

Fig. 3. Effects of Chronic mild HHcy on Heart anti-oxidant parameters: SOD, CAT and GPx activity, as well as GSH content. All anti-oxidant enzymes were expressed as units per mg of protein. GSH content was expressed as nmol per of mg protein. The data are expressed as mean ±S.E.M. of n= 7-8 per group with Student t-test performed. ***$p=0.0001$ and **$p<0.01$ from control group.

Fig. 4. Efect of chronic mild HHcy on Nrf2 protein content. The data are expressed as mean ±S.E.M. of n= 6 per group with Student t-test performed. *$p<0.05$ and **$p<0.01$ from control group.

Fig. 5. Efects of chronic mild HHcy on NFκB p65 and p-NFκB 65 protein content: NFκB p65 protein content (A), p-NFκB protein content (B) and band images (C). The data are expressed as mean ±S.E.M. of n= 6-7 per group with Student t-test performed. *$p<0.05$ from control group.

Fig. 6. Efects of chronic mild HHcy in the quantitative analysis of RT-PCR: Expression of IL-6, IL-10, Tnfrsf1, and NFκB1 gene, in the heart of rats. The data are expressed as mean ±S.E.M. of n= 7-8 per group with Student t-test performed. *$p<0.05$ from control group.

Fig. 7. Effects of Chronic mild HHcy on heart AchE (A) and serum BuchE (B) activity. Enzymes activity were expressed as μmol ACSCh/h/mg protein. The data are expressed as mean ±S.E.M. of n= 7-8 per group with Student t-test performed. *$p<0.05$ from control group.

3 CONCLUSÕES

A HHcy leve crônica, em ratos Wistar envelhecidos, causou danos a diferentes parâmetros bioquímicos no coração e soro, como seguem:

- Aumento das espécies reativas de oxigênio e diminuição das enzimas antioxidantes SOD, CAT e GPx;
- Redução na fração citosólica e nuclear do conteúdo de Nrf2 no coração
- Redução na atividade enzimática das colinesterases AchE (coração) e BuchE (soro)
- Aumento na fração citosólica de NFκB p65 no coração
- Um aumento na expressão das interleucinas IL-6 e IL-10 no coração

Dessa forma, destaca-se que, a HHcy leve caracteriza-se como fator de risco relevante a eventos cardiovasculares em destaque para uma faixa etária avançada, a qual pode, através desses dados, compreender um papel signficante de ampliar uma resposta ao estresse oxidativo, perfil inflamatório e colinesterases.

4 PERSPECTIVAS

Objetiva-se, como perspectiva aos resultados desse trabalho:

- Avaliar esses mesmos parâmetros também em fêmeas, conforme a influência dos hormônios sexuais podem vir a afetar diferencialmente o sistema cardiovascular;
- buscar avaliar outras estruturas do sistema periférico, como rins e fígado, importantes a depuração em sistemas biológicos;
- Realizar análises histológicas destes tecidos, coração, fígado, rins, afim de aprofundar influências funcionais decorrentes de possíveis alterações bioquímicas e biomoleculares;
- Destacar estudos em fatias e culturas celulares, objetivando, dessa forma, avaliação de demais parâmetros singulares.

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In addition to original research articles, the Journal publishes articles categorized as **Reviews, Mini-Reviews, Biolological Perspectives,** and **Commentaries**. Authors who wish to publish a Review or Mini-Review should send their curriculum vitae along with an outline of the proposed article for prior approval by the Editor-in-Chief. **Reviews** have a maximum of 6,000 words, 100 references, and 4 tables or figures (one of which is mechanistic); **Mini-Reviews** have a maximum of 4,000 words, 50 references, and 2 tables or figures (one of which is mechanistic). **Biological Perspectives** are submitted by invitation only and provide streamlined, mechanistic insight into the current state of research in the discussed topic. **Commentaries** are submitted by invitation only and relate to a concurrently published manuscript. All invited papers are subject to the same review process as unsolicited articles.

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[Revised: January 2012]