UNIVERSIDADE FEDERAL DO PARÁ
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E BIOQUÍMICA

MANOEL BENEDITO SOUSA CANTÃO

N-ACETILCISTEÍNA ATENUA ATIVIDADE DE MMP-2 E PREVINE ESTRESSE OXIDATIVO NA AORTA DE CAMUNDONGOS QUE RECEBERAM ADMINISTRAÇÃO SISTÊMICA DE MMP-2

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RESUMO

O aumento da atividade da metaloproteinase de matriz-2 (MMP-2) está implicado nas alterações bioquímicas na disfunção vascular. Evidências demonstraram que a MMP-2 ativa vias pró-oxidantes, o que pode levar à disfunção endotelial e aumento da vasoconstrição. Dessa forma, os compostos antioxidantes poderiam prevenir danos vasculares ao endotélio e à musculatura lisa vascular. A N-acetilcisteína (NAC) é um antioxidante que funciona como eliminador de radicais livres e precursor da síntese de glutationa reduzida e pode modular o estado redox. Portanto, este estudo levanta a hipótese de que o aumento sistêmico da MMP-2 promove estresse oxidativo no vaso e que o tratamento com o antioxidante N-acetilcisteína pode prevenir o desequilíbrio redox. Assim, este estudo teve como objetivo avaliar o efeito da NAC no estresse oxidativo vascular induzido pelo aumento sistêmico da MMP-2 em camundongos adultos. O estudo foi aprovado pelo CONCEA-UFPA nº. 3610090519. A MMP-2 foi obtida através de expressão bacteriana e purificação em coluna cromatográfica. Camundongos C57BL/6 adultos com 8 semanas de idade (n=32) foram divididos em quatro grupos (n=8): solução salina (solução salina i.p.); MMP-2 (1,2 µg/g animal i.p); NAC (40 mg/kg animal via oral) e NAC 40 mg/kg i.p + MMP-2 (1,2 µg/g animal i.p). O tratamento com NAC e a administração de MMP-2 ocorreram concomitantemente durante 4 semanas. Após esse período, os animais foram eutanasiados e a aorta torácica foi coletada para análise bioquímica. A expressão e atividade da MMP-2 foram determinadas por zimografia in situ e imunofluorescência para MMP-2. Os níveis de ROS foram determinados pelo ensaio DHE. Os níveis dos indicadores de estresse oxidativo e das enzimas de defesa antioxidante foram determinados por testes bioquímicos para avaliação dos níveis de nitrito, TBARS, SOD, Catalase e glutationa total. Também medimos a expressão de Nitrotirosina, Nrf2 e iNOS por imunofluorescência. Para as análises estatísticas foi utilizado o programa GraphPad Prism ® 9.0 (GraphPad Software, San Diego, CA, EUA), sendo realizados o teste ANOVA a dois critérios e o pós-teste de Tukey. Os resultados foram expressos como média ± erro padrão da média (EPM), considerando $p<0,05$ estatisticamente significativo. O tratamento com NAC reduziu significativamente a atividade gelatinolítica e a expressão de MMP-2 in situ na aorta, prevenindo o desequilíbrio redox ao reduzir os níveis vasculares de ERO, modulando o sistema antioxidante, reduzindo os níveis de nitrito e iNOS e mantendo a expressão significativa de eNOS.

Nossos resultados sugerem que o NAC previne o desequilíbrio redox induzido pelo aumento sistêmico da MMP-2.

Palavras-chave: Desbalanço redox, Aorta, MMP-2, Antioxidante, N-acetilcisteína.

ABSTRACT

Increased matrix metalloproteinase-2 (MMP-2) activity is implicated in the biochemical changes in vascular dysfunction. Evidence demonstrated that MMP-2 activates pro-oxidant pathways, which can lead to endothelial dysfunction and increased vasoconstriction. This way, antioxidant compounds could prevent vascular damage to the endothelium and vascular smooth muscles. N-acetylcysteine (NAC) is an antioxidant that functions as a free radical scavenger and precursor to synthesizing reduced glutathione and can modulate the redox state. Therefore, this study hypothesizes that the systemic increase in MMP-2 promotes oxidative stress in the vessel and that treatment with the antioxidant N-acetylcysteine can prevent redox imbalance. Thus, this study aimed to evaluate the effect of NAC on vascular oxidative stress induced by systemic increase in MMP-2 in adult mice. The study was approved by CONCEA-UFPA nº. 3610090519. MMP-2 was obtained through bacterial expression and purification using a chromatographic column. Adult C57BL/6 mice aged 8 weeks (n=32) were divided into four groups (n=8): saline (i.p. saline); MMP-2 (1,2 µg/g animal i.p); NAC (40 mg/kg animal p.o.) and NAC 40 mg/kg i.p + MMP-2 (1.2 µg/g animal i.p). NAC treatment and MMP-2 administration occurred concomitantly for 4 weeks. After this period, the animals were euthanized, and the thoracic aorta was collected for biochemical analysis. MMP-2 expression and activity were determined by in situ zymography and immunofluorescence for MMP-2. ROS levels were determined by DHE assay. The levels of oxidative stress indicators and antioxidant defense enzymes were determined by biochemical tests to assess the levels of nitrite, TBARS, SOD, Catalase and total glutathione. We also measured the expression of Nitrotyrosine, Nrf2, eNOS and iNOS by immunofluorescence. The GraphPad Prism ® 9.0 program (GraphPad Software, San Diego, CA, USA) was used for statistical analyses, and the two-way ANOVA test and Tukey post-test were performed. The results were expressed as the mean ± standard error of the mean (SEM), considering $p<0.05$ statistically significant. Treatment with NAC significantly reduced gelatinolytic activity and MMP-2 expression in situ in the aorta, preventing redox imbalance by reducing vascular ROS levels, modulating the antioxidant system, reducing nitrite and iNOS levels, and maintaining significant eNOS expression. Our results suggest that NAC prevents redox imbalance induced by systemic increase in MMP-2.

Keywords: Redox imbalance, Aorta, MMP-2, Antioxidant, N-acetylcysteine

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CD36	Agrupamento de diferenciação 36
cDNA	DNA complementar
CXCL5	Ligante de quimiocina 5
CXCL6	Ligante de quimiocina 6
CXCL8	Ligante de quimiocina 8
DHE	Dihidroetídio
ECM	Matriz extracelular
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio de imunoabsorção enzimática
eNOS	Óxido nítrico endotelial
EphB2	Receptor 2 de efrina tipo B
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSH	Glutationa oxidada
HF/IC	Insuficiência cardíaca
ICAM	Molécula de adesão intracelular – 1
ICFEp	Insuficiência cardíaca com a fração de ejeção preservada
IL-1β	Interleucina – 1 beta
IL-8	Interleucina – 8
iNOS	Óxido nítrico induzível
MDA	Malondialdeído
MMP	Metaloproteinase de matriz
MMP-2	Metaloproteinase de matriz – 2
MMP-9	Metaloproteinase de matriz – 9
NAC	N-acetilcisteína
NO	Óxido nítrico
Nrf2	Fator nuclear eritróide 2 relacionado ao fator 2
NT-Pro-BNP	N-terminal do pró-hormônio do peptídeo natriurético do tipo B

PBS	Solução salina tamponada com fosfato
PDGFR	Receptor do fator de crescimento derivado de plaquetas
RNS	Espécies reativas de nitrogênio
RONS	Espécies reativas de oxigênio e nitrogênio
ROS	Espécies reativas de oxigênio
RT-PCR	Reação em cadeia da polimerase com transcrição reversa
SDS-PAGE	Eletroforese em gel de poliacrilamida dodecil sulfato de sódio
SOD	Superóxido dismutase
TGFβ	Fator de crescimento transformador beta
TIMP-1	Inibidor tecidual de metaloproteinase – 1
TIMPs	Inibidores teciduais de metaloproteinase
TNF-α	Fator de necrose tumoral alfa
VCAM	Molécula de adesão celular vascular
VE	Ventrículo esquerdo
VSMC	Célula muscular lisa vascular

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1. VISÃO INTEGRADORA DO PROBLEMA

Os antioxidantes são compostos capazes de neutralizar radicais livres por meio de doação de equivalentes redutores para reduzir a sobrecarga e os níveis de substratos envolvidos na geração espécies reativas de oxigênio e nitrogênio (RONS). O estresse oxidativo é o produto do desbalanço redox causado pelo aumento de radicais livres e diminuição das respostas antioxidantes (BIELLI et al., 2015; KATHY K. GRIENDLING; MASUKO USHIO-FUKAI, 1998). A disfunção vascular está diretamente ligada ao estresse oxidativo (OBRADOVIC et al., 2020). A geração de EROs na vasculatura tanto pela redução do oxigênio quanto pela atividade desequilibrada dos sistemas enzimáticos como a NADPH oxidase e a xantina oxidase levam a interação de EROs com o óxido nítrico (NO) vascular (PARAVICINI; TOUYZ, 2008; VIRDIS; DURANTI; TADDEI, 2011). A diminuição da biodisponibilidade do NO pela formação do peroxinitrito através da reação com o ânion superóxido leva a disfunção vascular e endotelial ocasionando também dano lipídico oxidativo e nitrosilação de proteínas (HSIEH et al., 2014). O NO é produzido por uma família de enzimas chamadas óxido nítrico sintases (NOS). As NOS vasculares envolvem a óxido nítrico sintase endotelial (eNOS/NOS3) e a induzível (iNOS/NOS2) (FÖRSTERMANN; SESSA, 2012). Durante o desenvolvimento da disfunção vascular, a iNOS é regulada positivamente por meio das respostas as EROS e aos agentes inflamatórios agravando o status disfuncional da vasculatura (GUNNETT et al., 2005).

Evidências já demonstraram que o estresse oxidativo está envolvido no comprometimento da saúde vascular e no aparecimento de doenças vasculares (AL GHOULEH et al., 2011; TANIYAMA; GRIENDLING, 2003). Patologias como a hipertensão, aterosclerose, aneurisma e dissecção da aorta torácica e doença vascular periférica são as principais causas de morbimortalidade no mundo acometendo mais de 1,28 bilhões de indivíduos no mundo e gastos globais somando cerca de US$ 51.2 bilhões por ano (TSAO et al., 2023; XIE et al., 2016). Foi demonstrado que nas doenças vasculares, como hipertensão e aneurisma da aorta, os níveis circulantes de MMP-2 estão aumentados nestes pacientes (CIONE et al., 2020; DEROSA et al., 2006; GOODALL et al., 2001). Consistentemente já foi demonstrado que a MMP-2 especificamente participa da gênese, progressão e agravamento das doenças vasculares (BARBOUR et al., 2006; EUGSTER et al., 2005).

A MMP-2 é uma gelatinase dentro da classe das metaloproteinases da matriz extracelular (MMPs) capaz de degradar substratos da matriz extracelular, como colágeno, elastina, fibronectina e laminina e substratos não matriciais (CUI; HU; KHALIL, 2017; NAGASE; VISSE; MURPHY, 2006). Estudos sugerem que o aumento da atividade da MMP-2 contribui para o remodelamento hipertrófico e eutrófico arterial, bem como a hipertrofia e hiperplasia das CMLV na hipertensão (CASTRO et al., 2008, 2009, 2012; GUIMARAES et al., 2011). A MMP-2 pode contribuir para o aumento do tônus vascular pela clivagem proteolítica de peptídeos vasoativos como o peptídeo do gene relacionado a calcitonina e adrenomedulina, como também a porção extracelular do receptor β2 adrenérgico, diminuindo a sinalização vasodilatadora (FERNANDEZ-PATRON et al., 2000; FERNANDEZ-PATRON; RADOMSKI; DAVIDGE, 1999; RODRIGUES et al., 2010).

A atividade da MMP-2 é regulada positivamente pelo estresse oxidativo. Em níveis pós traducionais a MMP-2 é ativada por peroxinitrito sem a clivagem do seu propeptídeo, interrompendo sua latência. De fato, concentrações elevadas de peroxinitrito induz a s-glutatiolação do resíduo de cisteína presente no sítio catalítico da MMP-2 (OKAMOTO et al., 2001; VIAPPIANI et al., 2009). Prado et al., mostrou que a MMP-2 não só é ativada por EROs como também ativa vias pró-oxidantes que prejudicam o estado redox vascular. A MMP-2 pode clivar o fator de crescimento epidermal ligado a heparina ativando uma cascata de sinalização a jusante ativando a NADPH oxidase e consequentemente aumentando as EROs vasculares e a contratilidade vascular (PRADO et al., 2018). Barhoumi et al., demonstrou que a MMP-2 especificamente é necessária para o estresse oxidativo vascular via Ang-II, levando à disfunção endotelial, remodelação, estresse oxidativo e inflamação vascular (BARHOUMI et al., 2017). Desta forma, a utilização de compostos antioxidantes seriam opções viáveis para contribuir para a redução desse mecanismo de feedback entre MMP-2 e EROs, já que o aumento na produção de EROs levaram a várias alterações na atividade de inúmeras proteínas alvo, como a glutationilação da MMP-2 e no desacoplamento da eNOS e expressão de iNOS na disfunção vascular (SUGAMURA; KEANEY, 2011; XIA et al., 2006).

Nesse sentido, a N-acetilcisteína (NAC) um fármaco utilizado na prática clínica desde 1960 como agente mucolítico, tem sido investigada como agente antioxidante pelo seu efeito como precursor da glutationa reduzida (RAGUE, 2021). A glutationa

(GSH) é um tripeptídeo que é sintetizado e mantido em altas concentrações nas células. Para isso, nessa etapa sintética, a NAC penetra na membrana celular por meio da hidrólise em cisteína, conjugando-se com o l-glutamato e l-glicina formando a glutationa reduzida (GSH) (SAMUNI et al., 2013). Além da GSH, existe a glutationa oxidada (GSSH), que é subproduto de reações enzimáticas envolvendo a GSH. A GSSH é reciclada em GSH pela enzima glutationa redutase (GR) ou exportada para fora da célula para manter o equilíbrio redox intracelular entre GSH e GSSG. A GSH é capaz de oferecer equivalentes redutores para as EROs por meio do seu grupo sufidrila livre para apoiar a atividade da glutationa peroxidase, além de reagir com o ânion superóxido, atuando em conjunto com as superóxidos dismutases (SODs) (ATES; ABRAHAM; ERCAL, 2008; RUSHWORTH; MEGSON, 2014). Mudanças no estado redox intracelular têm sido implicados nas doenças vasculares. Estudos mostraram que na doença vascular periférica os níveis de GSSH estão aumentados em comparação aos de GSH, devido ao desbalanço redox principalmente(PRASAI et al., 2018). A síntese prejudicada da glutationa, por exemplo, foi implicada a progressão da aterosclerose pelo estresse oxidativo (BARAJAS-ESPINOSA et al., 2014; CHEN et al., 2010; HUA CAI AND DAVID G. HARRISON, 2000).

Em vista disso, o objetivo deste estudo foi avaliar os efeitos do antioxidante N-acetilcisteína sobre as alterações vasculares induzidas pelo aumento sistêmico de MMP-2 por 4 semanas. Isso foi feito por meio da avaliação da morfometria da parede vascular e dos conteúdos da MEC, quantificação da expressão e atividade da MMP-2, níveis de EROs e peroxidação lipídica, avaliação da atividade e níveis das enzimas sistema antioxidante endógeno e exógeno, bem como a avaliação da biodisponibilidade de NO na aorta que receberam administração sistêmica de MMP-2 e o tratamento antioxidante.

Levando em consideração que o aumento da síntese e atividade da MMP-2 levam a disfunção vascular e endotelial pela redução de agentes vasoativos e mudanças no estado oxidativo dos vasos sanguíneos, e que a NAC atue como um agente antioxidante, neste trabalho investigamos tanto o potencial da NAC de atenuar a atividade da MMP-2 como o de atuar como antioxidante sobre o desbalanço redox ocasionado pelo aumento sistêmico de MMP-2. Este trabalho apresenta resultados promissores sobre a atuação da NAC sobre a expressão e atividade de MMP-2,

estresse oxidativo e biodisponibilidade de óxido de nítrico na aorta de ratos que receberam injeção sistêmica de MMP-2 por quatro semanas de tratamento.

2. ARTIGO: O papel da metaloproteinase de matriz – 9 (MMP-9) na remodelação e disfunção cardíaca e como possível biomarcador sanguíneo na insuficiência cardíaca.

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The role of matrix metalloproteinase-9 in cardiac remodeling and dysfunction and as a possible blood biomarker in heart failure

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Systolic and diastolic dysfunction

ABSTRACT

Heart failure (HF) is the leading cause of morbidity and mortality in cardiovascular diseases, being responsible for many hospitalizations annually. HF is considered a public health problem with significant economic and social impact, which makes searches essential for strategies that improve the ability to predict and diagnose HF. In this way, biomarkers can help in risk stratification for a more personalized approach to patients with HF. Preclinical and clinical evidence shows the participation of matrix metalloproteinase 9 (MMP-9) in the HF process. In this review, we will demonstrate the critical role that MMP-9 plays in cardiac remodeling and dysfunction. We will also show its importance as a blood biomarker in acute and chronic HF patients.

1. Introduction

Heart failure (HF) occurs due to structural, conduction and functional changes in the heart. Originating from an initial harmful event, which can be insidious, such as diabetes, atherosclerosis, hypertension, obesity, and Chagas disease, or abrupt, as in acute myocardial infarction [1].

HF affects approximately 64 million people worldwide. The prevalence of people living with HF varies geographically, with higher rates in Central Europe, the United States, the Middle East, and North Africa and lower rates in Southeast Asia. The incidence of HF is higher in the African American population compared to the white population [2,3]. The Framingham study, a cohort study developed with the support of Boston University, showed that the prevalence of men with HF increases with age, and the same correlation was found for women [4]. The aging of the population and the increase in life expectancy lead to an increase in the prevalence of HF. Despite improvements in the management of people living with HF through therapeutic innovations, hospitalization and death rates remain unacceptably high. Early detection of susceptible individuals is imperative since the symptoms present are non-specific in the early stages of the disease [2,5,6].

The progression of HF is associated with heart remodeling as a result of maladaptive pathophysiological processes in the neurohumoral pathways, which will influence the survival processes of cardiomyocytes and fibroblasts, changes in fibroblast phenotypes and changes in components of the extracellular matrix (MEC) [7–11].

The cleavage and maintenance of ECM content is carried out by matrix metalloproteinases (MMPs), zinc-dependent endopeptidases, which play an essential role in many pathophysiological processes in cardiovascular diseases. The activity of MMPs is controlled at the transcriptional level and after the protein translation, mainly by the Tissue Inhibitors of Metalloproteinases (TIMPs) [12,13].

Among the MMPs, MMP-9 is most investigated in HF due to its participation in regulating cardiac remodeling through the direct degradation of ECM proteins, activating pro-fibrotic pathways, and activating cytokines and chemokines [14]. Studies in patients with HF, which investigated the increase in circulating MMP-9 activity and myocardial remodeling, showed a positive correlation between these variables. MMP-9 levels can serve as a marker to stratify the risk of poor prognosis, as well as serve as an indicator of the efficiency of established therapy and implementation of specific therapeutic interventions [15,16].

This review will address the classification, expressing cells, transcription process, translation, activation, proteolytic targets, and the

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effect of increasing MMP-9 on HF. We will discuss the role of MMP-9 in structural, conduction and functional changes in the heart in preclinical and clinical studies. We will present a possible future direction for using MMP-9 as a biomarker and its translational potential as an indicator in therapies for patients with HF.

2. Structure, regulation and activation of MMP-9

2.1. Classification and structure of MMP-9

The first descriptions of MMPs occurred in the 1960s with the identification of a protease with collagenolytic capacity in the tail of tadpoles [17]. Years later, an MMP nomenclature committee was created to standardize the terminology and classify this family of proteases [18]. Therefore, for a protease to be considered MMP, it must have sequence homology with MMP-1, have proteolytic activity coordinated by zinc in the catalytic site, degrade at least one component of the extracellular matrix (ECM) [19,20], be inhibited by ethylenediaminetetraacetic acid (EDTA) [21], phenanthroline[22] or tissue inhibitors of MMPs (TIMPs) [23] and be activated by proteases [24] and mercurial compounds [25].

Until now, 28 MMPs were identified and cloned, and 24 were expressed in humans [26]. They are subdivided into groups according to their structure and main cleavage component. In general, the structures of MMPs are formed by a signal peptide, propeptide, and catalytic site containing a zinc atom and a hemopexin domain [27]. In the group of gelatinases, which are enzymes specialized in degrading denatured collagen (gelatin), we have MMP-9, which differs from other MMPs in that it has glycosylation domains in the pro-domain and three fibronectin domains in the catalytic site [19].(Fig. 1) The catalytic activity of MMP-9 is controlled by alpha-2-macroglobulin and tissue inhibitors (TIMP) 1–4, but mainly by TIMP-1 [28].

2.2. Cells that express MMP-9

The first description of MMP-9 occurred in 1974 with the purification of a protease with gelatinolytic activity in human leukocytes [29]. Later, Hibbs et al., 1987 demonstrated that neutrophils could secrete MMP-9 under Phorbol 12-myristate 13-acetate (PMA) stimulation. Later, MMP-9 was also characterized as a product of alveolar macrophage secretion [30], and peripheral blood monocytes can secrete MMP-9 and TIMP-1 [31]. Triebel et al. demonstrated that 10 % of MMP-9 secreted from polymorphonuclear leukocytes is conjugated with TIMP-1. Studies carried out in the late 1980s identified MMP-9 in fibroblasts, epithelial cells, and tumor cells and, through cloning techniques, the gene and the complete protein structure of the 92 kDa glycosylated proenzyme, which is secreted in a non-covalent with TIMP-1 [25,32,33].MMP-9 can also be secreted by cardiomyocytes when stimulated by inflammatory cytokines and reactive oxygen species (ROS) [34].

2.3. MMP-9 transcription and translation process

In humans, the MMP-9 gene is present on chromosome 20. The MMP-9 promoter is in a 2Kb 5′ flanking region containing binding sites for AP-1, AP-2, PEA3, GC, TIE, KRE, RCE, TATA, NF-κβ, Sp1, TIE, RUNX2, Ets-1 and Cmyc [35–40]. Transcription is stimulated by inflammatory cytokines such as TNF-α, IL-1α, IL-β and IL-6 [41–44]. After transcription, the mRNA is translated into a protein of 707 amino acids. The signal peptide in the protein structure is responsible for translocating the translated protein from the endoplasmic reticulum and secretion into the extracellular environment of MMP-9 in the form of zymogen (Pro-MMP-9). In this configuration, the MMP-9 catalytic site is unavailable for substrate binding, as the zinc in the catalytic site is linked to a cysteine present in the propeptide by a sulfhydryl bond. In humans, pro-MMP-9 is 92 kDa, which differs from the size found in mice, which is 105 kDa (due to 23 extra amino acids) [45,46]. Regarding MMP-9 isoforms, five have been described: 1) Pro-MMP-9 (92 kDa), 2) MMP-9 (82 kDa), 3) MMP-9 – Lipocalin (125 kDa), an isoform found in human neutrophils due to a covalent bond between cysteine present at position 87 and lipocalin, which protects it from proteolytic degradation; 4) MMP-9 heterodimer linked to TIMP-1 (220 kDa); 5) MMP-9 of 65 kDa [47–53].

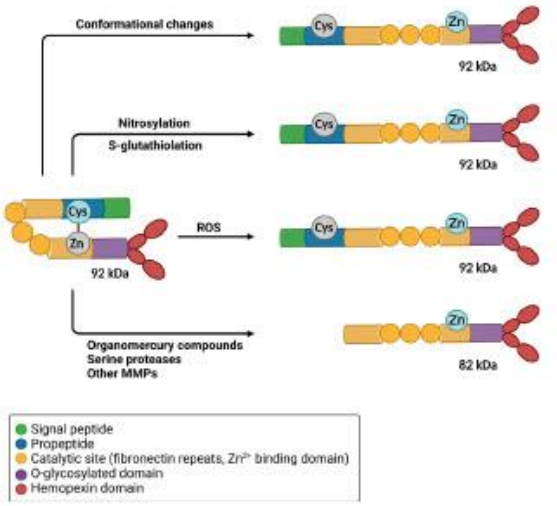


Fig. 1. MMP-9 structure, activation and isoforms. MMP-9 has in its structure a signal peptide (Pre), a propeptide (Pro) containing cysteine and glycosylation domains, a catalytic domain (having a zinc ion and three fibronectin repeats that confer affinity to gelatin), and a hemopexin domain (HP). The inactive isoform of MMP-9 has a molecular size of 92 kDa (pro-MMP-9). Inactivity is guaranteed by a cysteine residue in the propeptide domain, which binds to Zn^{2+} in the catalytic domain, preventing substrate binding and proteolysis. MMP-9 can also be activated by S-glutathionylation, Nitrosylation, and ROS that react with the sulfhydryl bond and expose the catalytic site, allowing interaction with substrates. Conformational changes of pro-MMP-9 may allow proteolytic activity even with the presence of the inhibitory propeptide. Other forms of activation of pro-MMP-9 include proteolytic activation by serine proteases and other MMPs that remove the inhibitory propeptide, generating an 82 kDa MMP-9 with proteolytic activity. Organomercurial compounds can also interact with the sulfhydryl bond, leading to proteolytic activation of MMP-9, which removes the propeptide by autolysis, generating an 82 kDa MMP-9. In addition, removal of the hemopexin-like C- terminal of MMP-9 giving the 65 kDa isoform of MMP-9.

2.4. MMP-9 activation process

The MMP-9 activation process can occur through other proteases, such as serine and MMPs [29,50], as well as physical agents, organomercurial compounds [24,25] and reactive species [24]. In the process of proteolytic activation, the propeptide is removed, leaving the catalytic site exposed for binding to substrates and reducing the molecular weight of MMP-9–82 kDa. Upon activation by organomercurial compounds, 73 amino acids are removed from the NH2 terminal region of the enzyme, producing an active form. During the activation process by ROS, the sulfhydryl bond between the propeptide and the catalytic site is broken, making it active. However, the molecular weight of 92 kDa remains [54]. Interestingly, conformational changes in the three-dimensional structure of MMP-9 (without amino acid loss) can lead to catalytic exposure and proteolytic activity of MMP-9 [55] (Fig. 1).

2.5. Proteolytic targets of MMP-9

MMP-9 can act in tissue remodeling and modulate organ function through proteolysis of ECM components and signaling proteins that can influence, for example, inflammatory processes. In the ECM, MMP-9 is capable of degrading denatured collagen (gelatin), native collagen types I, IV, V, VII, X, XI, elastin, fibronectin, laminin, aggrecan, binding protein, vitronectin, actin, tubulin, gelsolin, moesin, ezrin, subunits of the Arp2/3 complex, filamin B and stathmin [30,47,56–59]. Among the non-ECM components, MMP-9 is capable of cleaving IL-1β, IL-8, TGF-β, CD36, Osteopontin, Citrate Synthase, myelin basic protein, ICAM-1, occludins, serpins, protein1/CAP1, EphB2, integrin beta 2/CD18, alpha b crystallin [60–69].

3. Roles of MMP-9 in heart failure

A series of pathological conditions result in structural and functional changes in the heart, generating the inability to contract and relax adequately, impairing blood pumping, and requiring a high filling pressure at rest or during exertion to meet tissue metabolic needs, leading to the clinical syndrome of HF [60,70]. Within the multiplicity of factors that trigger HF, there are several pathways of neurohormonal activation and remodeling proteins, the understanding of which is necessary to search for better treatment and diagnosis strategies for

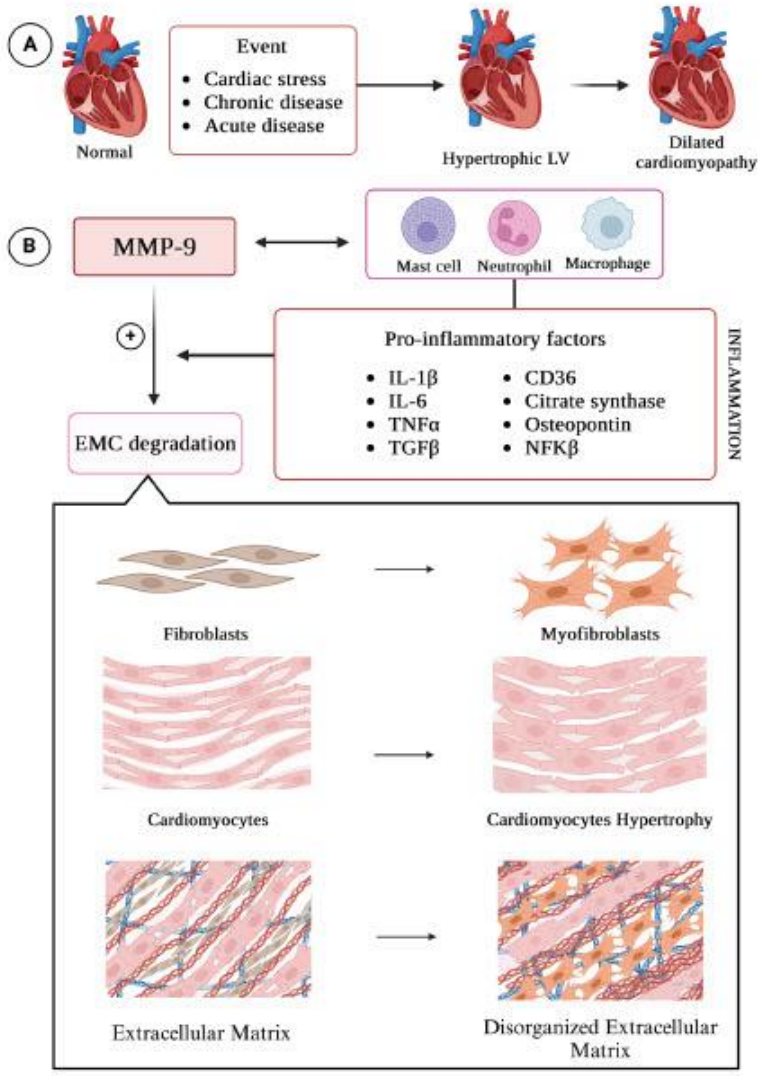


Fig. 2. Cardiac remodeling process induced by MMP-9 in heart failure. Different cardiac events (hypertension, diabetes, and myocardial infarction) induce changes in the cardiac phenotype, initiating the adaptive remodeling process that leads to ventricular hypertrophy. However, this adaptive process fails to maintain adequate ventricular function, resulting in ventricular dilation. During the remodeling process, there is an intense inflammatory response triggered by the migration of neutrophils, macrophages and mast cells that release MMP-9 during degranulation and by the increase in gene expression with the release and modulation of inflammatory factors (IL-1β, IL-6, TNF-α, TGF-β, CD36, citrate synthase, osteopontin, NF-κβ) that alter the inflammatory response and tissue regeneration. Established cardiac dysfunction leads to cardiomyocyte hypertrophy to normalize cardiac contractility. In addition, MMP-9, through proteolysis, alters the components of the extracellular matrix, causing intense collagen deposition and disorganization of the extracellular matrix. Scar tissue results from the activation of fibroblasts into myofibroblasts by the action of TGF-β.

patients with HF [71–74].

In this context, MMP-9 stands out due to its proteolytic capacity on several substrates, including structural components of the ECM, cytokines, chemokines, growth factors and adhesion molecules, which directly contribute to remodeling in HF through multiple mechanisms [75–77]. (Fig. 2)

Myocardial infarction is the leading cause of HF, and among the events after a cardiac ischemic injury, remodeling occurs as part of the healing response. Two overlapping processes occur inflammation and tissue repair [78,79]. Initially, there is an intense release of inflammatory mediators, which activate the complement system via Toll-like, which induces the production of reactive species, activating MMP-9 [80,81]. The second is characterized by the release of cytokines that promote fibrosis and proliferation of fibroblasts, resulting in scar tissue formation [82–85].

The inflammatory cytokines Il-1β, TNF-α and NFkβ released in cardiac tissue lead to increased gene expression MMP-9 in cardiac fibroblasts via direct stimulation of transcription factors and ROS, increasing the process of collagen deposition in the cardiac matrix [86,87]. In an animal study of cardiomyopathy induced by TNF-α and NF-κβ, it was demonstrated that MMP-9 is a critical protease in the dysfunction remodeling process, as the deletion of NF-κβ was shown to improve cardiac remodeling and function via decreasing levels of MMP-9 [88].

Increased IL-6 in cardiac tissue also regulates the inflammatory process in heart failure [78,89], modulating the ventricular remodeling process [89], mainly by modulating surface receptors and adhesion proteins on neutrophils [90]. It is known that Il-6 can induce the expression of MMP-9 in different cells [44,91]. However, the importance of this has not yet been evaluated in heart failure.

During ischemic injury, MMP-9 is secreted by activated neutrophils, and in later stages of injury, it is secreted by macrophages, lymphocytes, and fibroblasts [86,92,93]. Neutrophils present an essential difference when compared to other cell types. Mature neutrophils produce MMP-9 associated with lipocalin, forming a complex (NGAL-MMP-9) stored in granules, ready to be released quickly under a given stimulus [94,95]. The presence of MMP-9 in cardiac tissue can also lead to the cleavage of pro-IL-1β into IL-1β and is capable of truncating IL-8 into a form with more significant activity, leading to the release of more MMP- 9 by the neutrophil, providing an essential positive feedback loop for neutrophil activation and chemotaxis [96–98].

A study that evaluated the time of MMP-9 expression after ischemic injury demonstrated that the up-regulation processes of the MMP-9 gene increase from the 3rd day onwards, leading to a transcription peak on the 7th day after the infarction. Furthermore, it has been demonstrated that MMP-9 is expressed mainly in the infarct's border region, produced more by macrophages and lymphocytes attracted to the lesion [99].

Macrophages attracted to the injury are essential for the phagocytosis of cardiomyocytes undergoing apoptosis during myocardial injury [100]. However, they modulate an intense inflammatory response and signal fibroblasts to produce collagen [101,102], negatively affecting ventricular function [101]. Ventricular remodeling and function worsen with increased secretion of MMP-9 by macrophages, as demonstrated in transgenic animals that overexpress MMP-9 in macrophages [92]. However, interestingly, these same transgenic animals that overexpress MMP-9 in macrophages when subjected to ischemic injury showed improved ventricular function, decreased inflammatory response in the infarcted area, and reduced fibrotic response within five days after myocardial infarction, effects that were related to a rearrangement of ECM proteins [103].

MMP-9 also modulates the inflammatory process during remodeling through the proteolysis of CD36, a multifunctional plasma membrane protein that influences inflammation and apoptosis [104]. Thus, MMP-9 decreases macrophage phagocytosis and prolongs neutrophil inflammation during myocardial infarction, leading to neutrophil persistence and activation in the injured myocardium [98]. Furthermore, MMP-9 contributes to the inflammatory process through the cleavage of several inflammatory mediators, such as chemokines CXCL5, CXCL6, and CXCL8, activator protein-1 and specificity protein-1, participating in tissue restoration, through the formation of scar tissue, with abundant collagen content [105]. In addition, MMP-9 can cleave pro-TGF-β and release its soluble form, increasing collagen deposition during myocardial injury [106].

Clinical studies have confirmed the findings in vitro and in vivo animal models of cardiac dysfunction, showing that MMP-9 is a marker of cardiac remodeling and injury [12,14,15,107]. Confirming the preclinical and clinical findings, studies of gene deletion of MMP-9 showed benefits in improving ventricular function [108,109], improving remodeling and fibrosis in the infarcted area [109], in addition to more remarkable survival from infarction compared to conventional treatments [110]. Although studies show elevated levels of MMP-9 during cardiac injury and that MMP-9 knockout animals showed beneficial results for cardiac remodeling and function, the mechanisms involved were still unclear. Thus, seeking to elucidate the mechanisms involved in HF by MMP-9, Rouet-Benzineb *et al.* investigated *in vitro* the direct proteolytic action of MMP-9 on components of the cardiac contractile machinery (actin, tropomyosin, troponin T and light and heavy chain myosin), MMP-9 was able to cleave only heavy chain myosin. In the hearts of patients with dilated cardiomyopathy, MMP-9 was identified within cardiomyocytes in the tubular system with a striated structure, being associated with fragments of the heavy chain myosin, suggesting the proteolytic action of MMP-9 on this component of the contractile machinery [111]. MMP-9 could also cleave *in vitro* actinin and actin, cytoskeletal proteins contributing to ventricular function [112,113]. Recently, it was demonstrated that molar concentrations of MMP-9 can lead to the cleavage of TIMP-1–4, which alters their inhibitory effect on other MMPs, which affects the tissue MMP-TIMPs balance [114]. However, the influence of TIMPs cleavage by MMP-9 has not yet been evaluated on cardiac function.

The studies above showed the participation of MMP-9 in pathological processes that lead to HF. However, Zhu *et al.* recently showed direct evidence of the involvement of MMP-9 in ventricular remodeling and dysfunction through the administration of recombinant MMP-9. Even without pathological stimulus, these animals showed cardiomyocyte hypertrophy, cytoplasmic lysis, decreased collagen deposition, diastolic dysfunction, and increased blood pressure [76].

4. MMP-9 as a biomarker in HF

HF annually causes thousands of deaths and hospitalizations globally [2,115], a situation that highlights the relevance of primary prevention [116]. However, for preventive measures to exist, it is necessary to screen for predisposing conditions in individuals that pose a future risk of developing HF [117,118]. People who develop coronary disease, for example, commonly have none or just one risk factor among the traditional risk factors already established and are, therefore, less likely to be targets of preventive therapies. Thus, there is a growing need to investigate new risk factors that may have predictive value in HF [119]. In this context, increased activity and expression of MMP-9 may function as a risk factor, as this protease has been associated with pathological processes that culminate in the development of HF [120]. Using it as a biomarker can be a valuable tool as an indicator of pathological processes or even in therapeutic intervention [121–123].

MMP-9, as a circulating biomarker, can be informative of biological pathways that precede the disease and function as a biomarker in already established HF [124]. The criteria for evaluating new biomarkers have been postulated by several groups, emphasizing that a biomarker must be easy to measure and add new information on the pathophysiology of a disease, in addition to cost-effectiveness, safety and replication of the predictive value in different cohort studies [125]. Several clinical studies have demonstrated the predictive value of MMP-9 in the genesis and pathophysiology of cardiovascular diseases, as well as HF [121,122,126]

Prospective case-control studies that investigated the expression of MMP-9 in 30 patients with rheumatic valvular heart disease and its value in predicting atrial fibrillation (AF), concluded that the expression of MMP-9 is elevated in patients with AF and that MMP-9 possibly influences collagen metabolism, promoting myocardial fibrosis and cardiac remodeling in these patients, leading to the emergence and maintenance of AF. In this study, the enzyme-linked immunosorbent assay was used to measure the level of MMP-9 in serum, and the distribution of the protein in myocardial tissues was evaluated by immunohistochemistry [121].

Another case-control study that evaluated the association between polymorphisms in the MMP-9 gene and coronary artery disease (CAD) in the Han Chinese population concluded that the single nucleotide polymorphism of rs3918242 in the MMP-9 gene is associated with CAD and Elevated serum MMP-9 levels are also associated with CAD in the Han Chinese population. Polymerase chain reaction-based restriction fragment length polymorphism was used to determine the rs3918242 and rs17576 genotypes in the MMP-9 gene, and total serum MMP-9 levels were measured using enzyme-linked immunosorbent assay in the groups [126].

A meta-analysis that evaluated case-control studies comparing circulating concentrations of MMP-9 and abdominal aortic aneurysm (AAA) demonstrated that, in the pooled analysis, circulating concentrations of MMP-9 were significantly higher in the AAA group than in the control group in random effect models (SMD, 0.70; 95 % CI, 0.23–1.17; P = 0.004), with significant heterogeneity of study results (P<0.00001) [127].

The Framingham Study, a cohort study with 699 participants, with a mean age of 57 years, free of heart failure and previous myocardial infarction, investigated the cross-sectional relationship between plasma MMP-9 and vascular risk factors (heart failure and acute coronary syndrome) and echocardiographic measurements of the left ventricle (LV). In multivariable models, increased heart rate (95 % CI) and antihypertensive treatment (95 % CI) were the main clinical correlates linked to detectable plasma MMP-9. In multivariable-adjusted models, detectable plasma MMP-9 was associated with increased LV internal dimensions (LVDD) 95 % CI, increased LV wall thickness (WTLV) 95 % CI, and greater LV mass in men (P = 0.06). These observations indicate that plasma MMP-9 level may be a marker for cardiac extracellular matrix degradation, a process involved in LV remodeling [122].

Case-control study of patients with idiopathic dilated cardiomyopathy (DCM) that evaluated the relative activity of MMP-9, TIMP-1 and TIMP-2 observed an intense increase of 50 % in activity and 422 % in expression, P<0.05 of MMP-9 and increase 500 % in expression, P<0.05 of TIMP-1 and TIMP- 2 in DCM patients, from heart biopsy. Gel zymography of the substrate was performed to quantify the activity of MMPs, and to measure the expression of MMP-9, TIMP-1 and TIMP-2, a western blotting assay was performed. The authors suggest that selective inhibition of MMP species in the LV myocardium may provide a new therapeutic target in patients with DCM [123].

A study carried out in patients without heart failure (ejection fraction > 55 %) and patients with congestive heart failure (ejection fraction < 25 %) from the Vesnarinone Trial clinical study (VEST) demonstrated that plasma levels of MMP-9 increased by 25 ± 6 in patients without heart failure to 48 ± 11 in patients with nonischemic heart failure and 100 ± 29 in patients with ischemic heart failure (15 ng/mL, P < 0.05). Interestingly, MMP-9 levels in patients with congestive heart failure remained elevated during the 48 hours of evaluation. The MMP-9/TIMP-1 ratio increased 3-fold, whereas the MMP-9/TIMP-2 ratio increased 16-fold in CHF patients (P < 0.05). Enzyme-linked immunosorbent assay kits measured plasma levels of MMP-9, TIMP-1 and TIMP-2. Thus, the authors conclude that serial measurements of plasma MMP/TIMP levels may have diagnostic/prognostic significance in patients with CHF [12].

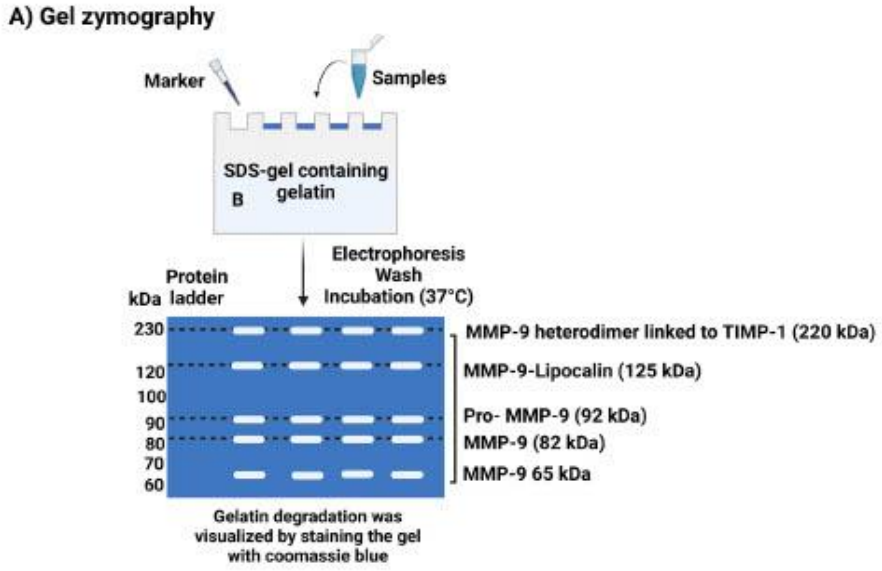
In a multicenter, double-blind, randomized and controlled study in patients with stage II to IV heart failure (NYHA-FC), it was found at the beginning of the study that plasma MMP-9 correlated positively with end-systolic volume (ESV) (Spearman correlation coefficient ρ = 0.17, P = 0.02) and negatively with left ventricular ejection fraction LVEF (ρ = −0.18, P = 0.01). Temporal changes in the MMP-9 level were inversely correlated with changes in LVEF (ρ = −0.16, P = 0.04). In a multivariable analysis adjusted for clinical characteristics and treatment, a minor proportional change in MMP-9 level after 43 weeks (below versus above the median) predicted a concomitant improvement in LVEF (odds ratio = 2.35, 95 % CI 1.24–4.46; P < 0.0001). Elevated plasma MMP-9 levels correlated with lower LVEF and higher FSV, while increasing MMP-9 levels are associated with a concomitant deterioration in LV function. These findings suggest that monitoring plasma markers of myocardial matrix remodeling may provide important prognostic information regarding ongoing adverse LV remodeling in patients with HF [124].

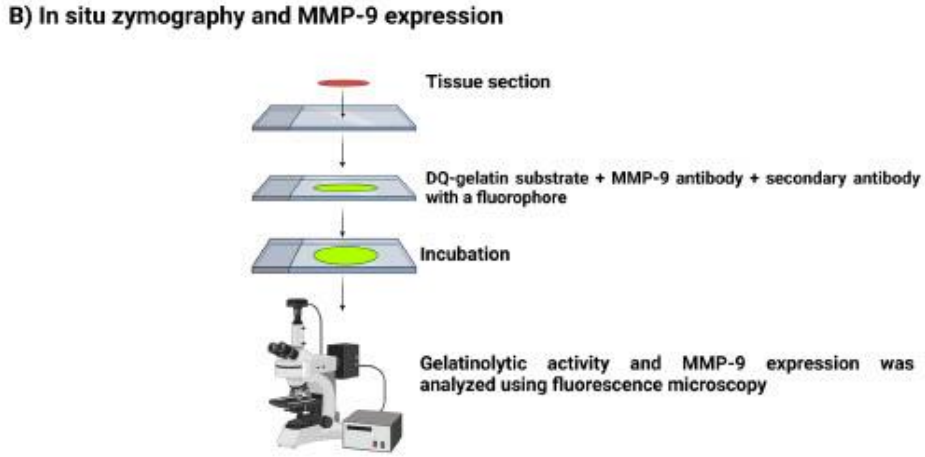
Patients without heart disease (control, n = 241), with left ventricular hypertrophy without heart failure (LVH, n = 61), and with heart failure (DHF, n = 61) were recruited from locally sponsored health fairs, response to multimedia stories, medical referral, and echocardiographic studies. Control patients had MMP-9 levels of 95.0 ± 3.8 ng/mL. LVH (126.7 ± 7.2 ng/mL) and DHF (123.8 ± 8.6) patients showed increased MMP-9. Various unadjusted and adjusted modeling and cross-validation interactions were performed for clinical variables. For LVH: The best fitting model was achieved using 5 biomarkers consisting of NT-proBNP, MMP-7, MMP-9, TIMP-1 and PIIINP. The sensitivity and specificity of this multi-biomarker plasma profile exceeded that of any single biomarker (including NT-proBNP). They exceeded any currently available screening algorithm (e.g., clinical examination and ECG) for LVH. They concluded that plasma biomarkers, such as MMPs, which reflect changes in collagen homeostasis, combined in a multimarker panel have discriminative value in the identification of LVH and DHF [107]. On the other hand, a cross-sectional study to compare the predictive values of NTproBNP, sST2 and MMPs was carried out in 113 patients with heart failure with different ejection fractions admitted to the Fujian Provincial Hospital from December 2016 to March 2018. In the evaluation of the diagnostic value Of the four biomarkers for three types of heart failure, the AUC for the diagnosis of HFpEF and HFrEF by plasma NT-proBNP is higher than that of plasma sST2, MMP-2 and MMP-9, suggesting that the diagnostic predictive value of NT-proBNP Plasma proBNP for HFpEF and HFrEF is better than that of other markers [128].

Multifactorial diseases such as HF, resulting from different etiologies, can make it challenging to choose an isolated biomarker [129]. However, the data presented in the studies mentioned above reinforces the potential of MMP-9 as a biomarker in prognosis and diagnosis and as a tool for new therapeutic targets. MMP-9 was measured using simple and easy-to-use methods such as zymography and ELISA or more robust methods such as ELISA, RT-PCR and Western blotting (Fig. 3). Currently, the gold standard biomarker used in HF is the measurement of the natriuretic peptides NT-Pro-BNP and BNP [130].However, the sensitivity and specificity of plasma multimarker profiles, together with MMP-9, may have more consistent discriminative value in patients with IC [107].The plasma elevation of MMP-9 activity and expression may function as a risk factor for the development of HF [120].

5. Techniques used to measure activity, levels and expression of MMP-9

MMP-9 is a protease that can be in its inactive or activated form. Therefore, it is interesting to consider its activity in addition to evaluating changes in MMP-9 levels in pathological conditions. Therefore, sometimes there are no changes in levels, but the activity is altered, leading to increased degradation of substrates. In addition, sometimes the levels are increased, but the activity is not due to the increase in tissue inhibitors such as TIMP-1. Therefore, performing a gel zymogram or fluorimetric assay together with an ELISA assay generates results that are better used to study the participation of MMP-9 in a pathological process. Below, we briefly describe tests that can be performed to





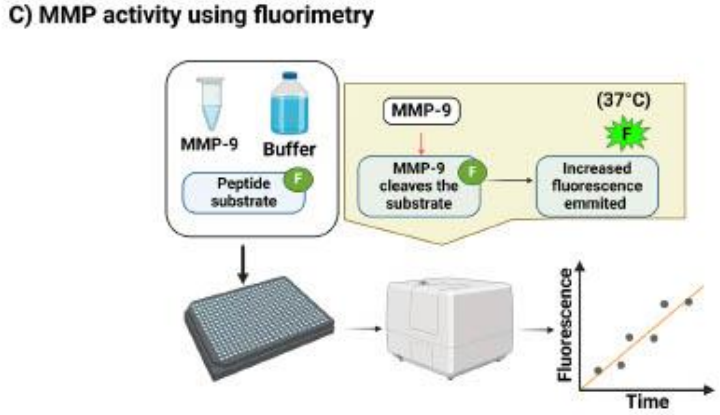


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Fig. 3. Techniques for assessing MMP-9 activity and levels. A. zymography was the first technique to be introduced to evaluate MMP-9 activity and can be used in samples of serum, plasma, tissues, biological fluids and cell culture. It's a widely used and susceptible technique. B. In situ zymography was introduced to evaluate the activity of active MMP-9 directly in the tissue; it has the advantage of being performed together with immunohistochemistry, leading to the evaluation of activity and expression concomitantly. C. Fluorescent substrates can evaluate MMP-9 activity in serum, plasma, tissue, biological fluids, and cell culture samples. Compared to gel zymography, it is a faster assay in which it is possible to obtain the result in 1–2 hours.

evaluate the activity, levels and expression of MMP-9. Some recommendations must be adopted before carrying out the tests. For example, MMP-9 is a protease susceptible to self-cleavage, and care must be taken when refrigerating it. Generally, keep samples at −20°C for a few months, but storage time can be increased when refrigerated at −80°C.

5.1. Techniques for evaluate MMP-9 activity (Fig. 3)

5.1.1. Gel zymography

Gel zymography was the first technique created to evaluate the enzymatic activity of MMP-9. In this assay, samples of body fluids or tissue extracts are applied to SDS-PAGE gel copolymerized with gelatin [131,132]. During sample collection, enzyme stability can be maintained using MMP protease inhibitors (usually phenanthroline) plus serine protease, cysteine protease and phosphatase inhibitors. During the electrophoresis run, the SDS is removed, and the sample is refolded. The inhibitors are washed out, and therefore, we can visualize activity. The great advantage of this gel is its sensitivity, which makes it possible to visualize enzymatic activity in the picogram range of protein and distinguish the different forms and molecular weights of MMP-9. The disadvantage of this technique is that the MMP-TIMP connection is broken during the race, and when using triton x-100, all MMPs are activated (even though they are not active in the tissue). In general, MMP-9 activity can be visualized in zymograms within 24 h due to the gel incubation time of 18 h in Tris $CaCl_2$ [133,134]

5.1.2. In situ zymography

In situ zymography is a method developed to correct the disadvantage found in the gel zymography assay, as this methodology allows the measurement of only active MMPs in the tissue (not bound to TIMPs, for example). This assay was first developed using colorimetric substrates but was soon replaced by fluorescent, more sensitive substrates. The enzymatic activity of MMP is quickly observed under a fluorescence microscope (in hours) [135,136]. An advantage of this test is the performance of double labeling using antibodies, making it possible to mark MMP-9 and cellular components to identify where the increase in enzymatic activity occurs (intracellular or extracellular) [137]. [1] *In situ* zymography can detect enzymes in biopsy samples, preferably unfixed. The test's specificity can be increased using controls during the assay, such as sections incubated with MMP inhibitors (phenanthroline) [138, 139].

5.1.3. Fluorimetric MMP-9 assay

This technique uses quenched substrates that, after undergoing proteolysis, emit fluorescence and can be used to determine the increased activity of MMP-9 in any samples of body fluid and tissue extracts [140]. It is a sensitive and rapid technique, which can become specific through the use of particular substrates for MMP-9 or by performing the enzymatic assay in the presence of specific MMP-9 inhibitors to prove that proteolysis is due to the presence of MMP-9 in the fabric. Quantification of MMP-9 enzymatic activity is determined by measuring the emitted fluorescence intensity, which is observed within one or two hours [141,142].

5.2. Techniques for evaluate MMP-9 expression and levels (Fig. 4)

5.2.1. RT-PCR for MMP-9

Reverse transcription followed by polymerase chain reaction (RT-PCR) is a susceptible technique used to determine gene expression in blood samples or tissue extracts [143]. In this assay, RNA is extracted from the sample using reverse transcriptase to obtain cDNA used for the PCR reaction using specific primers for MMP-9. The number of copies is obtained by measuring the fluorescence intensity emitted, normalized by housekeeping genes, the response being evaluated within 24 hours [144].

5.2.2. Western blotting

Western blotting is another powerful, susceptible technique used to evaluate the protein expression of any isoform of MMP-9. Western blotting can be utilized on all samples, whether body fluids or tissue extracts [145]. It is usually a confirmatory tool for confirming results obtained by immunohistochemistry and ELISA, as it is possible to visualize the specification and sensitivity of the antibody [146].

5.2.3. ELISA

It is a powerful technique used to measure the levels of any MMP-9 isoform by using monoclonal or polyclonal antibodies. 125 The commercial ELISA kits available for measuring MMP-9 from the companies Abcam, R&D system, ThermoFisher and Elabscience can detect MMP-9 in general in the range between 0.1 and 17 ng/mL. The kits have high specificity for MMP-9, do not react with analogs, and have a sensitivity that varies from 0.01 to 0.1 ng/mL, depending on the kit. They can detect MMP-9 in different body fluids (serum, plasma, urine), tissue and cell culture extracts and culture supernatants. The time to view the result varies between 1:30 and 3 hours [147,148].

6. MMP-9 and its translational potential in therapies for patients with HF

Preclinical and clinical studies demonstrate that MMP-9 has a dual effect on HF, which can be beneficial or harmful, depending on the time course and cellular source [143–145]. Current research seeks to understand the role of MMP-9 in the pathogenesis and pathophysiology of HF to search for appropriate therapeutic strategies. Inhibition of MMP-9 has been used in the treatment of HF; primarily, non-selective inhibitors, such as doxycycline, showed promising results in animal models; however, in clinical studies, the results were not satisfactory [146–149]. Recently, a selective inhibitor constructed by the Fields laboratory, a triple helix peptide incorporating a binding sequence to the fibronectin II insertion, reduces collagen cleavage by MMP-9, showing promising results [150].

Monitoring MMP-9, its tissue inhibitors and the cleavage products of MMP-9 substrates can be helpful in the early diagnosis of HF [151], and in monitoring patients with HF. The McDonald laboratory demonstrated an association between high plasma levels of MMP-9 and an increased risk of developing HF in asymptomatic hypertensive patients. This study suggests that inflammation and fibrosis profiles may vary during HF and that the assessment of MMP-9 and TIMP-1 allows early detection of HF [152].

In a cohort study in patients with coronary artery disease (CAD), with stable or unstable angina, plasma concentrations of MMP-9 were identified as a marker of risk of future mortality from cardiovascular disease and myocardial infarction, in addition, the MMP-9/R279Q polymorphism has been linked to future CV events in patients with stable angina [153]. Another study that evaluated heart attack patients with ST-segment elevation suggested that high levels of MMP-9 over time are a strong predictor of cardiovascular mortality and heart failure [154]. A recent study investigated the predictive value of plasma MMP-9

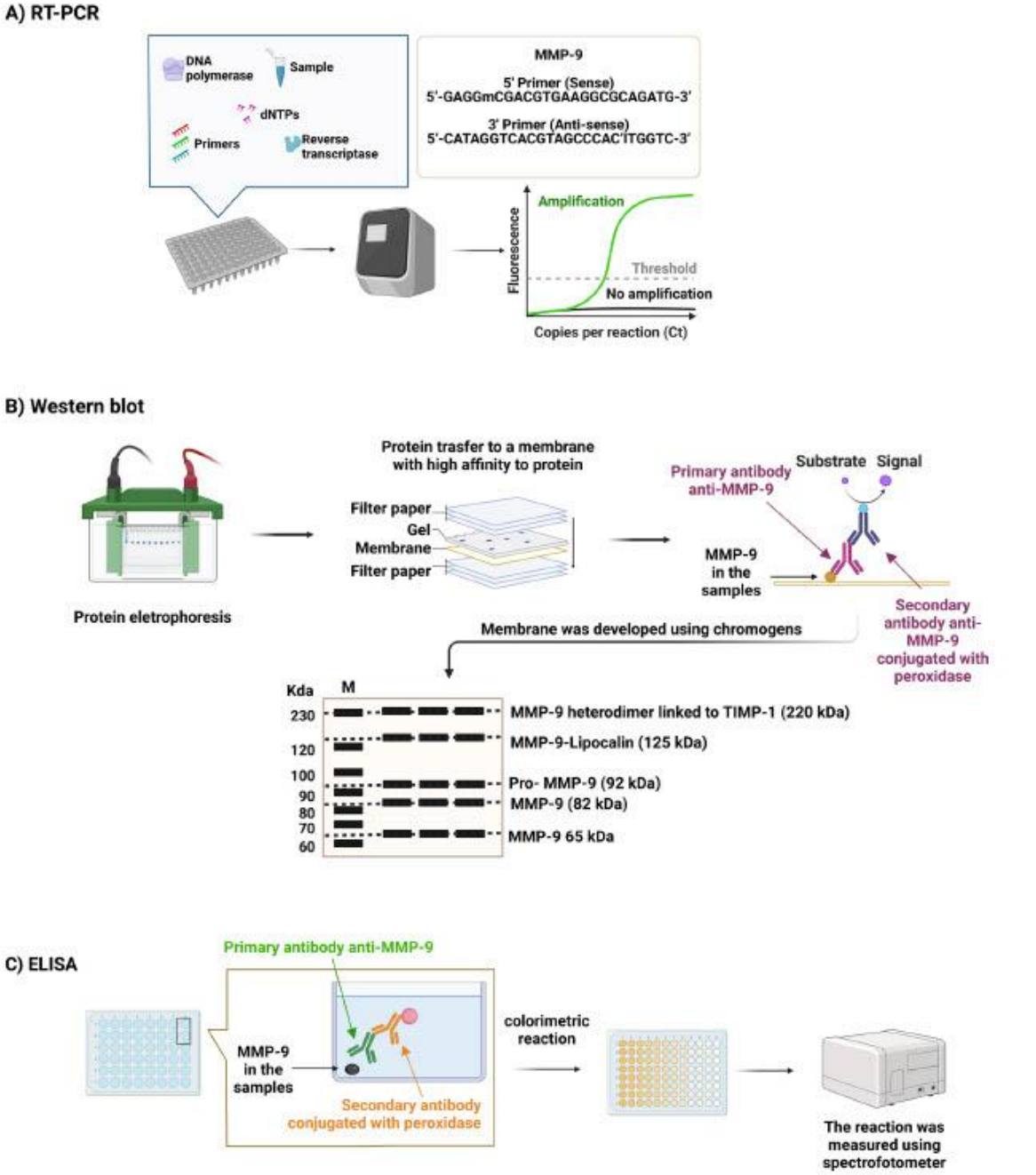

Fig. 4. Techniques for assessing MMP-9 expression and levels. A. RT-PCR is a highly sensitive technique for evaluating gene expression in blood and tissue samples. B. Western blotting is a highly sensitive technique that also uses a specific antibody for MMP-9 to determine the expression of this protease in serum, plasma, tissues, biological fluids, and cell culture samples. C. ELISA is a powerful technique that uses specific antibodies against MMP-9 to assess levels in different biological fluids and tissues.

concentrations associated with echocardiographic parameters in patients with systolic HF. The authors concluded that MMP-9 combined with diastolic dysfunction variables contribute to risk stratification in patients with systolic heart failure [155]. In patients with heart failure with preserved ejection fraction, MMP-9 proteolysis products such as active TNF-α, active IL-1β, and active TGF-β may be biomarkers associated with MMP-9 activity [156].

The most used contemporary cardiovascular biomarkers are natriuretic peptides, galectin-3 and ST2 in the diagnosis and prognosis of cardiac failure [157].Troponins I (cTnI) and T (cTnT) are currently the

main biomarkers of myocardial injury. They are components of the myocardial contractile apparatus and are expressed almost exclusively by the heart. Increases in cTnI always result from the heart, while situations of injury to the skeletal muscle can lead to an increase in cTnT. There are highly sensitive cTn assays available for routine clinical examinations, and an increase in cTn above the 99th percentile is required for diagnosing myocardial injury [158].

A cohort study of 395 outpatients with systolic heart failure (BSI) who participated in the Penn Heart Failure Study evaluated MMP-9 as a remodeling biomarker and predictor of outcomes and compared its performance with BNP. In their results, MMP-9 had no significant correlation with the left ventricular end-diastolic dimension index (LVEDDI) or ejection fraction (EF), while BNP showed highly significant correlations LVEDDI and EF: ($P < 0.0001$). In multivariate linear regression models, MMP-9 again showed no significant associations with LVEDDI and EF, while BNP showed independent solid associations with LVEDDI ($P<0.001$) and EF ($P = 0.002$). Kaplan-Meier showed no difference in hospitalization-free survival by baseline tertile of MMP-9 ($P = 0.7$), whereas a higher tertile of BNP predicted worse survival ($P < 0.0001$). The authors concluded that MMP-9, compared to BNP, is a poor biomarker of remodeling and progression in BSI patients from clinical practice [159].

Although MMP-9 is not a protease exclusively expressed by the heart, it presents changes in its levels in other diseases, such as cancer, inflammatory and neurodegenerative diseases [160–163]. Its systemic elevation is closely related to cardiovascular remodeling [76]. Pathological processes that generate primary or secondary aggression in the heart also trigger an increase in the expression and activity of MMP-9, promoting tissue remodeling [164].Pathways that lead to fibrosis in cardiac tissue are also activated, generating changes in the electrical impulse and function of the heart. When the cardiac injury occurs, the elevation of MMP-9 influences the inflammatory process [165,166]. It triggers inflammatory cells and is secreted by them, interfering with tissue repair. Elevated MMP-9 activity also activates apoptotic pathways that compromise cardiomyocyte survival [167].

An ideal cardiac biomarker in HF should appear early in the circulation, allowing early disease detection [156]. Another important characteristic is that it presents an increase in its levels related to the prognosis and that its measurement is easily carried out with good sensitivity and analytical precision. In this review, we show that MMP-9 presents these characteristics and, associated with biomarkers already used, can contribute to managing patients with HF [168,169]. However, an evaluation with more robust cohort studies is necessary for analytical considerations and clinical relevance of MMP-9 as a possible biomarker in HF. As mentioned previously, it derives from multiple processes that can contribute to its temporal and spatial variation.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare no conflict of interest.

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3. ARTIGO: N-acetilcisteína atenua atividade de MMP-2 e previne estresse oxidativo na aorta de camundongos que receberam administração sistêmica de MMP-2.

N-acetylcysteine attenuates MMP-2 activity and prevents oxidative stress in the aorta of mice that received systemic administration of MMP-2

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Abstract: Increased matrix metalloproteinase-2 (MMP-2) activity is implicated in the biochemical changes in vascular dysfunction. MMP-2 activates pro-oxidant pathways, which can lead to endothelial dysfunction and increased vasoconstriction. This way, antioxidant compounds could prevent damage to the endothelium and vascular smooth muscle cells. N-acetylcysteine (NAC) is an antioxidant that functions as a free radical scavenger and precursor to synthesizing reduced glutathione and can modulate the redox state. Therefore, this study hypothesizes that the systemic increase in MMP-2 promotes oxidative stress in the vessel and that treatment with the antioxidant N-acetylcysteine can prevent redox imbalance. Thus, this study aimed to evaluate the effect of NAC on vascular oxidative stress induced by systemic increase in MMP-2 in adult mice. MMP-2 was obtained through bacterial expression and purification using a chromatographic column. 8 weeks old male C57BL/6 mice (n=32) were divided into four groups (n=8 each): saline (i.p. saline); MMP-2 (1,2 µg/g animal i.p); NAC (40 mg/kg animal p.o.) and NAC 40 mg/kg i.p + MMP-2 (1.2 µg/g animal i.p). NAC treatment and

MMP-2 administration occurred concomitantly for 4 weeks. After this period, the animals were euthanized, and the thoracic aorta was collected for biochemical analysis. MMP-2 expression and activity were determined by in situ zymography and immunofluorescence for MMP-2. ROS levels were determined by DHE assay. The levels of oxidative stress indicators and antioxidant defense enzymes were determined by biochemical tests to assess the levels of nitrite, TBARS, SOD, Catalase and total glutathione. We also measured the expression of Nitrotyrosine, Nrf2, eNOS and iNOS by immunofluorescence. The GraphPad Prism ® 9.0 program (GraphPad Software, San Diego, CA, USA) was used for statistical analyses, and the two-way ANOVA test and Tukey post-test were performed. The results were expressed as the mean ± standard error of the mean (SEM), considering $p<0.05$ statistically significant. Treatment with NAC significantly reduced gelatinolytic activity and MMP-2 expression in situ in the aorta, preventing redox imbalance by reducing vascular ROS levels, modulating the antioxidant system, reducing nitrite and iNOS levels, and maintaining significant eNOS expression. Our results suggest that NAC prevents redox imbalance induced by systemic increase in MMP-2.

Keywords: Redox imbalance, Aorta, MMP-2, Antioxidant, N- acetylcysteine

1. Introduction

Cardiovascular diseases affect around 1.28 billion individuals. [1,2]. Retirados os custos com DCV a pedido do Prof. Tiago. Cardiovascular diseases, for example, such as hypertension, atherosclerosis, thoracic aortic dissection and aneurysm, aortic stenosis, stroke and peripheral vascular diseases, are significant global public health problems [3]. Vascular remodeling is one of the causes of dysfunction and collagen content and elastin fragmentation contribute to increased matrix metalloproteinase activity [4].

Matrix metalloproteinases (MMPs), a group of endopeptidases that degrade components of the extracellular matrix (ECM), have been implicated in the intracellular and extracellular mechanisms that lead to cardiovascular diseases (CVDs) [5]. MMP-2, a gelatinase that belongs to the MMP family, has received attention due to its direct role in remodeling and modulating the vessel's redox state [6-8].

Mechanical stress in VSMC increases MMP-2 levels and activity that it increase reactive oxygen species (ROS) and platelet-derived growth fator receptor (PDGFR) activation [13]. Angiotensin II increases the MMP-2 activity, which results in a degradation of collagen and elastin, inducing migration and rearrangement of VSMCs from the aorta [9]

Oxidative stress also modulates MMP-2 activity. Increased ROS in the vasculature due to mechanical stress has been associated with upregulation of MMP-2 RNAm [10-11]. Peroxynitrite induces s-glutathiolation activating MMP-2 [12,13]. MMP-2 transactivates heparin-binding epidermal growth factor (HB-EGF), activating the EGF receptor (EGFR) and increasing ROS concentrations. [14]. Increased ROS production can lead to changes such as eNOS uncoupling and increased iNOS expression in vascular dysfunction [15,16].

Substances that present antioxidant activity and have their use approved in clinical practice can function as adjuvant therapy for CVD [17, 18, 19]. Using drugs that already have a known mechanism of action and possible targets and repositioning them to validate a new treatment helps to reduce costs and accelerate their use in clinical practice [20, 21]. Therefore, N-acetylcysteine (NAC), a drug used since 1960 as a mucolytic agent, has been investigated as an antioxidant agent due to its effect as a precursor of reduced glutathione [22]. NAC penetrates the cell membrane and conjugates with l-glutamate and l-glycine to form reduced glutathione (GSH) [23]. GSH is capable of offering reducing equivalents for ROS through its free sulfhydryl group to support the activity of glutathione peroxidase, in addition to reacting

with superoxide anion, acting together with superoxide dismutases (SODs) [24–26]. In addition to its antioxidant effects, NAC can directly inhibit MMP-2. [27,28].

Thus, considering that increased synthesis and activity of MMP-2 leads to vascular dysfunction due to changes in the oxidative state of blood vessels and that NAC acts as an antioxidant agent, we hypothesize that increasing MMP-2 in the vessel promotes redox imbalance and that NAC can prevent such changes. Therefore, this study aimed to evaluate the effect of NAC on MMP-2 activity and redox balance in the aorta of mice that received systemic administration of MMP-2.

2. Materials and methods

2.1. Animals

All animal experiments were carried out by the principles described in the Guide for the Production, Maintenance or Use of Animals for Teaching or Scientific Research Activities, according to the National Council for the Control of Animal Experimentation (CONCEA). The experimental procedures were conducted by a protocol approved by the Ethics Committee on the Use of Animals of the Federal University of Pará (CEUA-UFPA, Nº 5223300323). 8 weeks-old male C57BL/6 mice from Evandro Chagas Animal Facility Institute were used. The animals were housed in the Animal House of the Oxidative Stress Research Laboratory in cages and maintained under controlled temperature conditions (25 ± 1ºC) in an alternating 12-hour light/dark cycle, with water and food provided *ad libitum.*

2.2. Design

We performed a *time course* of MMP-2 administration for one, two and four weeks to evaluate the effects of MMP-2 injection on vascular remodeling and redox status. The animals were divided into Control (0.9% saline solution ip) and MMP-2 (1.2 µg/g of animal/ ip). With the results obtained in the first protocol, we carried out a second one using the antioxidant NAC. In this, the animals were divided into 4 experimental groups: Control (0.9% saline solution ip); NAC (40 mg/kg/ vo); MMP-2 (1.2 µg/g animal/ip); MMP-2 (1.2 µg/g of animal/ ip) + NAC (40 mg/kg/ vo). The MMP-2 used in this study was obtained by expressing and purifying the pET5rhMMP-2 clone as previously described [29], and NAC was obtained from Sigma (A7250). MMP-2 administration and co-treatment with NAC were carried out for 4 weeks. The dose of NAC was selected through previous studies [30]. The dose for daily injection of rhMMP-2 protein was based on studies in patients with arterial hypertension and thoracic aortic aneurysm. These studies showed increased MMP-2 plasma levels to two and five times above the concentration found in control patients [31–34]. Then, based on the doses described above, a mouse weighing 25 g received an amount of 3.7 µg, which, divided by the mouse's plasma volume, approximately 1.250 µL, would give the expected three-fold increase in plasma MMP-2, levels that are compatible with the concentrations reported in the plasma of patients in these studies. At the end of the experimental protocol, the animals were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) and euthanized by exsanguination to collect the aorta.

2.3. MMP-2 gelatinolytic activity (*in situ zymography*) and MMP-2 expression in the aorta

Aortas were frozen in cryoprotectant fluid (Tissue Tek) and cut in a cryostat into 5 µm sections. First, these sections were incubated with fluorescent gelatin substrate (DQ- gelatin, Invitrogen) for 1h in a dark, humid chamber at 37ºC. They were then washed in PBS and fixed with 2% formalin. To colocalize the gelatinolytic activity increase with MMP-2, the sections were incubated with anti-MMP-2 antibody (Abcam, 1:300) overnight at 4ºC. The following day,

the sections were washed with PBS and incubated with anti-rabbit IgG secondary antibody conjugated to Alexa 594 (Abcam, 1:500) in a dark, humid chamber for 1 h at room temperature. Finally, the sections were incubated with a fluorescence preservation solution containing DAPI (Sigma, F6057). Images were acquired using a ZEISS fluorescence microscope. *In situ,* gelatinolytic activity and MMP-2 expression were quantified using the Image J program, as described in a previous study [35].

2.4. ROS levels

Vascular reactive oxygen species (ROS) production was determined in situ in aortic sections by dihydroethidium, following the method previously described by [35]. Aortic segments (5 µm) were incubated in a light-protected and humidified chamber (37°C, 30 min) with 10 -5 µM dihydroethidium solution (DHE). Fluorescence was detected with a 585–590-nm long-pass filter under a microscope (Company - Zeeis) with a 40 objective lens coupled to a digital camera. Fluorescent images were recorded and analyzed by measuring the mean optical density of the fluorescence by Image J (NHI). Fluorescence in each image was evaluated at least in 30 locations and normalized by the area.

2.5. Morphometric Analysis of the Vascular Wall

Thoracic aortas were fixed in 4% formalin, dehydrated, and treated with 70%, 90%, 95% alcohol and 100% absolute alcohol (3X) for 2h. Samples were then placed in equal parts alcohol and xylene overnight and clarified in xylene with changes every two hours. Three exchanges were performed and subsequently embedded in paraffin. Then, hematoxylin-eosin, picrosirius and orcein stains were performed to analyze morphometric indices: cross-sectional area, media layer thickness, lumen diameter and collagen and elastin content. All images were obtained using a 10x magnification objective under an optical microscope. The measurement will be performed using the National's 64-bit Image J.

2.6. Lipid peroxidation

Aortas were macerated on dry ice and resuspended in PBS. Then, the aortic extract was homogenized in a thiobarbituric acid solution (1% v/v) and heated at 100ºC for 1h. The pink reaction product was quantified in a spectrophotometer (SpectraMax M5, Molecular Devices) at a wavelength of 535 nm. The results obtained were compared with a standard MDA curve. Values were expressed as nmol/mg of protein.

2.7. Nitrotyrosine Levels

Aortas were frozen in cryoprotectant liquid and cut as described above 5 µm thick sections were fixed in 4% formalin for 10 min. and incubated with PBS blocking solution containing BSA (1%), glycine (0.3 mol/L) and Tween (0.1% v/v) for 60 min., at 37ºC, to block nonspecific bonds and permeabilize the fabric. Then, the sections were incubated with an Anti-Nitrotyrosine antibody (Sigma, 1:500) overnight at 4ºC. The following day, the sections were washed in PBS and incubated for 60 min at room temperature with anti-rabbit IgG secondary antibody conjugated to Alexa 488 (Abcam, 1:500). Slides were mounted with Fluoroshield solution containing DAPI (F6057, Sigma) and images were acquired using a ZEISS fluorescence microscope. The images were quantified using the Image J program, as described in a previous study [35].

2.8. Evaluation of antioxidant system.

To evaluate the role of the antioxidant system in the redox imbalance induced by MMP-2, the aortas were macerated in dry ice and resuspended in PBS. The levels of reduced and oxidized glutathione were initially obtained by measuring total glutathione levels. In this way,

the aortic extract was treated with glutathione reductase. Next, the sample was reacted with 5,5′-dithiobis (2-nitrobenzoic acid), which has a yellow color. The reaction product was read after 5 minutes in a spectrophotometer at a wavelength of 412 nm. The results were expressed using the reduced glutathione curve and expressed in µ mol/ mg of protein. The assay was performed similarly without using glutathione reductase to calculate reduced glutathione levels. Oxidized glutathione was calculated using the total glutathione – reduced glutathione ratio. For the total glutathione assay, the Cayman Chemical Company Glutathione Assay Kit (N° 703002) was used.

SOD activity, in turn, was calculated by reacting the aortic extract with hypoxanthine and NBT and the reaction product was read in a spectrophotometer at a wavelength of 415 nm. Results were expressed as units of SOD/mg protein, calculated using a standard curve for SOD. CAT activity, in turn, was measured using an aortic extract that reacted with hydrogen peroxide as a product of the formaldehyde reaction. The purple reaction product was measured in a spectrophotometer at 540 nm. CAT activity was measured using a formaldehyde curve. For the SOD and catalase activity assays, the Cayman Chemical Company Kits (N° 706002, N° 707002, respectively) were used.

2.9. Expression of Nuclear Factor Erythroid 2-related Factor 2 (Nrf2)

5 µm of aorta were fixed in 2% formalin for 10 min. and incubated with PBS blocking solution containing BSA (1% v/v), glycine (0.3 mol/L) and Tween (0.1% v/v) for 60 min., at 37ºC. After blocking, the sections were incubated with anti-Nrf2 primary antibody (R&D Systems, 1:500) overnight at 4ºC. The following day, the sections were washed in PBS and incubated with Rhodamine-conjugated anti-rat IgG secondary antibody (Sigma, 1:500) for 1h at room temperature. Slides were mounted with Fluoroshield solution containing DAPI (F6057, Sigma), and images were acquired using a ZEISS fluorescence microscope. The images were quantified using the Image J, as described in a previous study [35].

2.10. Nitrite levels and iNOS expression

The aortic extract was incubated with Griess reagent to measure nitrite levels. The pink reaction product was read in a spectrophotometer at 550 nm (BIO-RAD Model 450 Microplate Reader). Nitrite levels were calculated using a sodium nitrite standard curve. The results were expressed in µmol/mg of protein. To assay nitrite levels, the Cayman Chemical Company Nitrate/Nitrite colorimetric assay kit (N° 780001) was used. To evaluate iNOS expression, 5-µm-thick sections were fixed in 4% formalin for 10 min. and incubated with PBS blocking solution containing BSA (1% v/v), glycine (0,3 mol/L) and Tween (0.1% v/v) for 60 min., at 37ºC, to block nonspecific bonds and permeabilize the fabric. Afterward, the sections were incubated with the Anti-iNOS antibody (Abcam, 1:200) overnight at 4ºC. The following day, sections were washed in PBS and incubated for 60 min at room temperature with secondary antibody. Rhodamine-conjugated anti-rat IgG (Sigma, 1:500), respectively. The slides were mounted with Fluoroshield solution containing DAPI (F6057, Sigma), and images were acquired under a ZEISS fluorescence microscope using a 40x magnification objective. The images were quantified using the Image J, as described in a previous study. [35].

2.11. Statistical analysis

Prism ® 9.0 program (GraphPad Software, San Diego, CA, USA) was used. The normality of the data was assessed using the Shapiro-Wilk normality test, and the data were considered normal. Two-way ANOVA test and Tukey post-test were performed. The results were expressed as the mean ± standard error of the mean (SEM). $p<0.05$ was considerad statistically significant.

3. Results

3.1. Time-course of systemic MMP-2 administration promoted oxidative stress without generating remodeling in the aorta.

The MMP-2 group showed higher levels of lipid peroxidation, nitrite, GSH and increased SOD activity only in the fourth week of MMP-2 administration when compared to the vehicle control group ($p>0.05$, **Figure 1. E-H**). Catalase enzyme activity increased significantly from the second to fourth week in the MMP-2 group compared to the vehicle control group $p<0.05$, **Figure 1. I)**. Regarding vascular remodeling, the MMP-2 group maintained measurements of cross-sectional area (CSA), collagen and elastin content in the aorta similar to those in the vehicle control group ($p>0.05$, **Figure 1. A-D**).

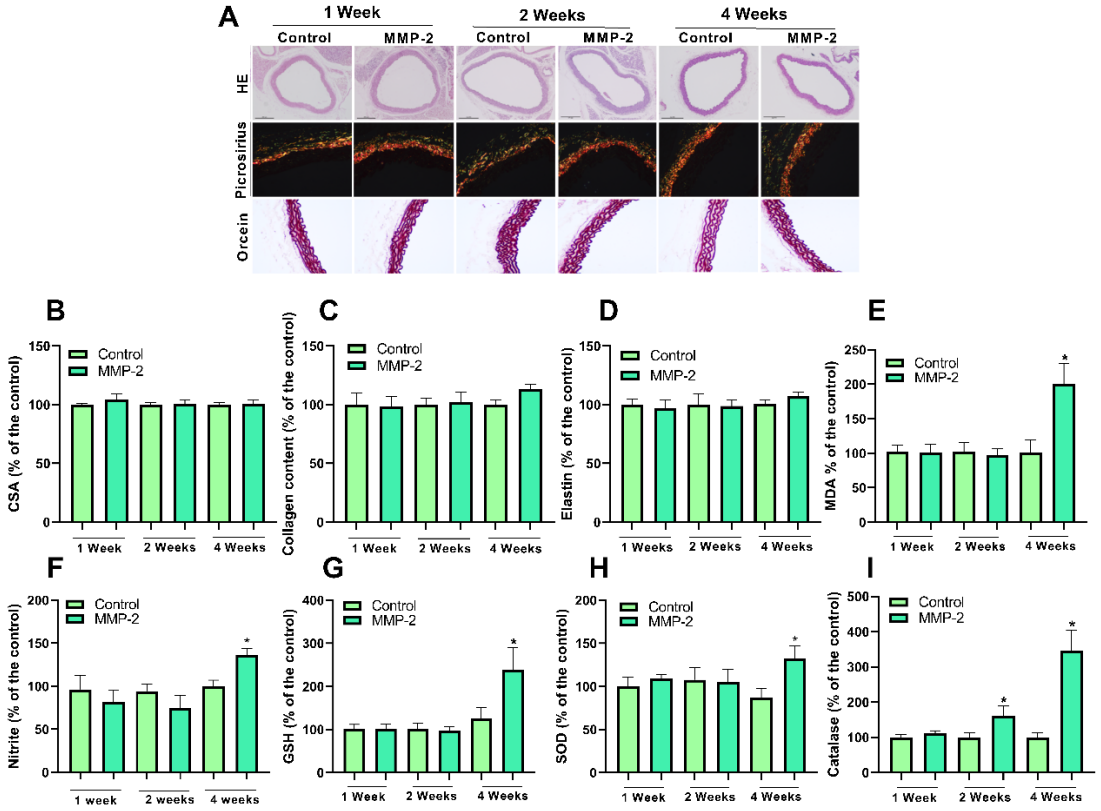

Figure 1. Time-course of systemic MMP-2 administration promoted oxidative stress without generating remodeling in the aorta. **A.** Representative panel of optical microscope photomicrographs of aorta samples stained with hematoxylin and eosin (HE), picrosirius (collagen), and orcein (elastin) – Scale: 250 µm. **B.** Graphical representation of the measurement of the medial cross-sectional area of the thoracic aorta (CSA). **C.** Graphical representation of the quantification of aortic collagen content. **D.** Graphical representation of the elastin surface area values of the aortic medium. **E.** Quantification of lipid peroxidation in the aorta. **F.** Quantification of nitrite in the aorta**. G** Quantification of GSH levels in the aorta. **H.** Quantification of SOD activity. **I.** Quantification of catalase activity in the aorta. Data are represented as a percentage (P%); * $p<0.05$ *vs.* control, two-way ANOVA (n=5/group).

3.2. NAC reduces gelatinolytic activity and MMP-2 expression *in situ* in the aorta.

In situ, gelatinolytic activity was found in the aortas of animals that received systemic administration of MMP-2 compared to the vehicle group (p<0.05**, Figure 2. A-B**). The MMP-2 group also showed a greater presence of this protease in the aortas, as demonstrated by immunofluorescence for MMP-2 (p<0.05, **Figure 2. A-C**). On the other hand, the MMP-2 + NAC group showed lower gelatinolytic activity and MMP-2 expression than the MMP-2 group (p<0.05, **Figure 2. A-C**). There was no difference in gelatinolytic activity and MMP-2 expression between the vehicle control and NAC groups (p>0.05, **Figure 2. A-C**).

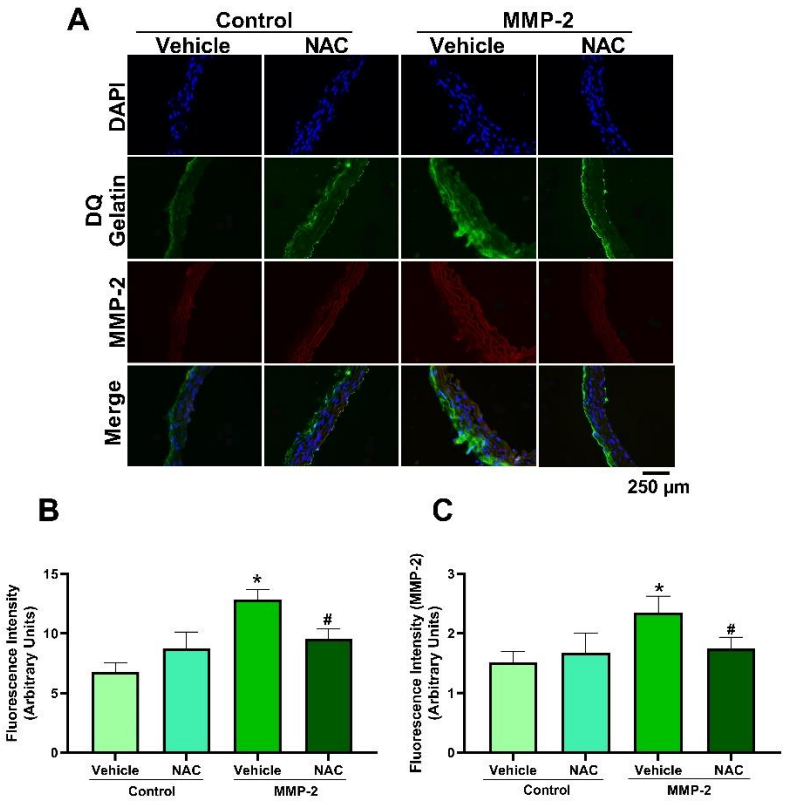

Figure 2. NAC reduces *in situ* gelatinolytic activity and MMP-2 expression in the aorta. **A**. Representative photomicrographs from the fluorescence microscope illustrating nuclear labeling with DAPI, DQ-gelatin (*in situ* zymography), MMP-2 (immunofluorescence for MMP-2) and the superimposition of the 3 images (merge). **B**. Graphical representation of *in situ* zymography quantification expressed as green fluorescence intensity. **C**. Graphical representation of the quantification of the fluorescence intensity of the immunostaining for MMP-2. Data are represented by mean ± SEM; * p<0.05 *vs* control, # p<0.05 *vs* MMP-2, two-way ANOVA (n=5/group).

3.3. NAC prevents MMP-2-induced redox imbalance in the aorta by modulating the antioxidant system.

Higher levels of ROS were found in the aortas of animals that received systemic administration of MMP-2 compared to the control group (p<0.05, **Figure 3. A-B**). The MMP-2 group showed an increase in lipid peroxidation (p< 0.05. **Figure 3. C**) of Nrf2 expression, SOD and catalase activity. It altered the GSSH: GSH ratio compared to the control group (p<0.05. **Figure 3. D-I**). Treatment with the antioxidant NAC significantly reduced the levels of ROS, lipid peroxidation, Nrf2 expression, SOD and catalase activity compared and the GSSH: GSH ratio when compared to the MMP-2 group (p<0.05, **Figure 3. A-I**). There was no difference in ROS content between the control and NAC groups (p>0.05, **Figure 3. A-B**). The NAC group

showed lower lipid peroxidation, increased GSH, Nrf2 expression and more significant catalase activity than the control group (p<0.05, **Figure 3. C-I**).

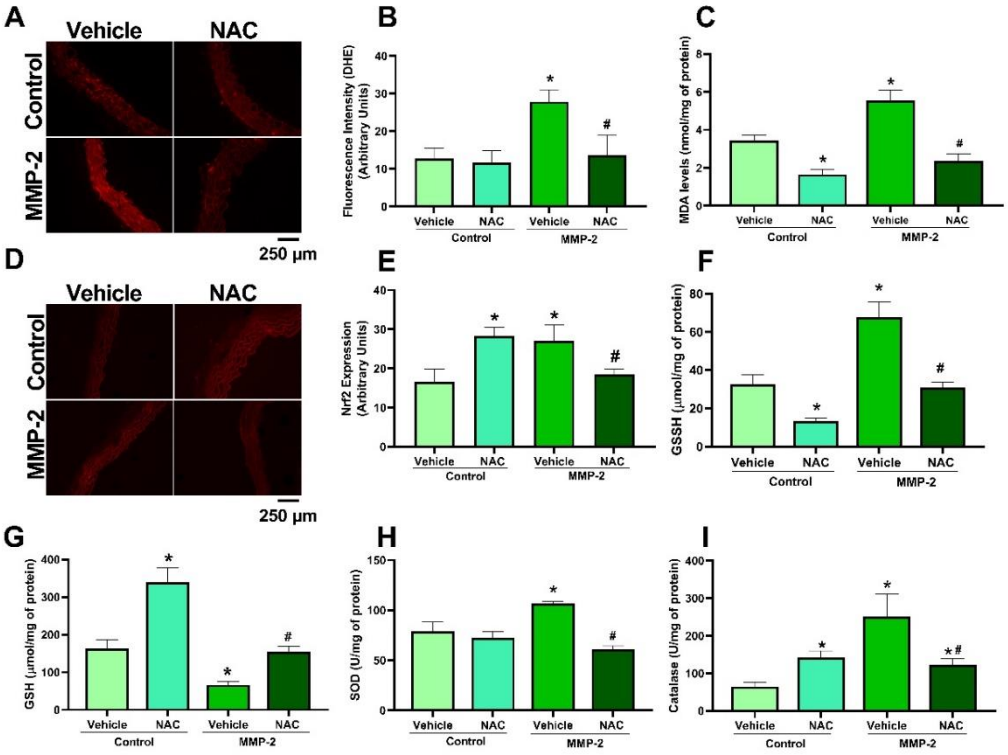

Figure 3. NAC prevents MMP-2-induced redox imbalance in the aorta by modulating the antioxidant system. **A**. Representative confocal microscope photomicrographs illustrating nuclear labeling with the DHE probe representing labeled ROS levels. **B.** Graphical representation of the quantification of nuclear red fluorescence intensity labeled with the DHE probe. **C**. Quantification of lipid peroxidation in the aorta. **D.** Representative photomicrographs from the fluorescence microscope illustrating Nrf2 labeling (immunofluorescence for Nrf2). **E**. Graphic quantification of the red fluorescence intensity of the immunostaining for Nrf2. **F.** Quantification of GSSH levels in the aorta. **G.** Quantification of reduced GSH levels in the aorta. **H.** Quantification of SOD activity. **I.** Quantification of catalase activity in the aorta. Data are represented by mean ± SEM; * p<0.05 *vs* control, # p<0.05 *vs* MMP-2, two-way ANOVA (n=5/group).

3.4. NAC prevented nitrosylation by modulating nitrite levels and the iNOS enzyme.

Higher expression of iNOS, nitrite levels and protein nitrosylation were found in the aortas of animals that received systemic administration of MMP-2 compared to the control group (p<0.05. **Figure 4. A-E**). Treatment with NAC significantly reduced nitrite levels, iNOS expression and protein nitrosylation compared to the MMP-2 group (p<0.05. **Figure 4. A-E**). The NAC control group showed lower iNOS expression than the vehicle control group (p<0.05, **Figure 4. B**).

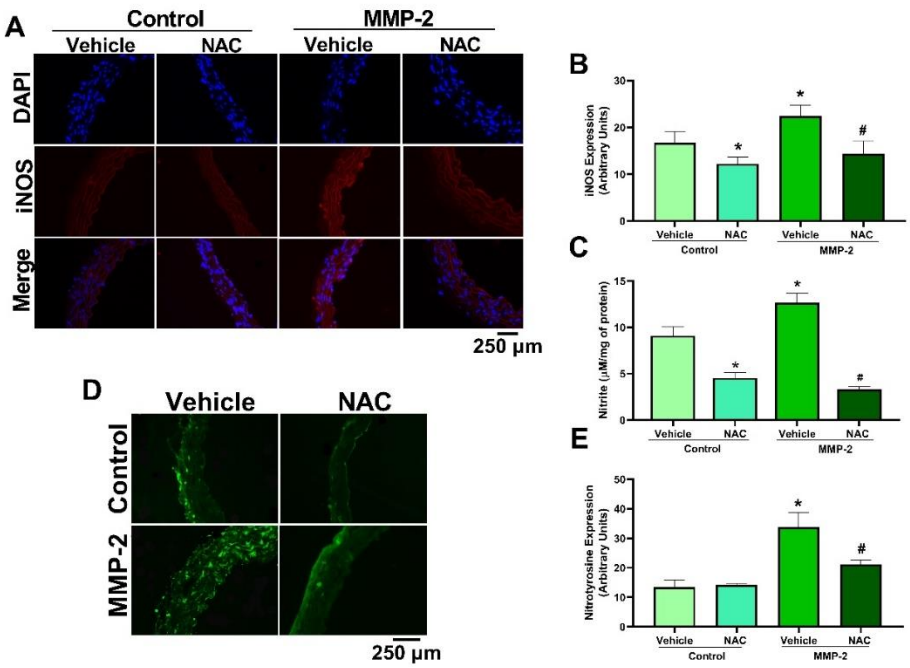

Figure 4. NAC prevents vascular dysfunction by reducing nitrite levels and expression of iNOS and eNOS. **A.** Representative photomicrographs from the confocal microscope representing nuclear labeling with DAPI and iNOS and overlapping of the three images (merge). **B.** Quantification of fluorescence intensity for iNOS expression. **C**. Quantification of nitrite levels in the aorta. **D.** Representative photomicrographs from the fluorescence microscope representing the labeling for Nitrotyrosine. **E.** Quantification of fluorescence intensity for immunostaining for Nitrotyrosine. Data are represented by mean ± SEM; * p<0.05 vs control, # p<0.05 vs MMP-2, two-way ANOVA (n=5/group).

4. Discussion

The main finding of this study was that the antioxidant NAC is capable of preventing oxidative changes in the aorta of mice induced by the increase in the activity and expression of MMP-2 in the vessel, resulting from the systemic increase in MMP-2. We believe this study can help elucidate the pathophysiological events that occur in the vessel after increased MMP-2 activity, even in the absence of an underlying disease. The role of MMP-2 in altering the oxidative state of the aorta as a primary event is demonstrated by the sequence of modifications called vascular remodeling.

In this study, systemic administration of MMP-2 for four weeks led to increased gelatinolytic activity and redox imbalance in the aorta. These results are in line with the study by Prado et al., who demonstrated that MMP-2 alone is capable of activating pro-oxidant pathways dependent on EGFR activation and that the increase in MMP-2 proteolytic activity correlates with increased reactive species [14]. MMP2 mediates the effects of Ang II, leading to endothelial dysfunction, vascular remodeling, oxidative stress, and inflammation in a model of Ang II-induced hypertension. Barhoumi et al. (2017) demonstrated that Ang II increases the expression of MMP-2 in immune system cells that infiltrate the aorta and perivascular fat. [36]. In our study, the systemic increase in MMP-2 induced by intraperitoneal administrations increased the presence of MMP-2 in the aortic tissue, suggesting that the increase in MMP-2 derived from other compartments of the organism can lead to oxidative imbalances in the aorta. This is an exciting result, as in periodontal disease, cancer and inflammatory diseases, there is an increase in MMP-2 secretion, which can lead to vascular changes [37].

The main objective of this study was to evaluate whether NAC prevented oxidative changes induced by MMP-2. Notably, NAC prevented redox imbalance and decreased the activity and expression of MMP-2, consequently decreasing the activation of pro-oxidant pathways. The inhibitory activity of NAC on MMP-2 was also observed in previous studies [27,28], which linked the inhibitory activity of NAC to its ability to interact directly with MMP-2.

Our results demonstrated that the NAC prevented redox imbalance through modulation of the antioxidant system. NAC is a thiol, precursor of GSH, leading to increased synthesis of reduced glutathione, which maintains this tripeptide at positive levels in intracellular stores [38]. GSH, in turn, has antioxidant capacity due to the presence of a free sulfhydryl group that serves as a donor source of electrons for free radicals, therefore functioning as a reducing equivalent [39]. GSH exists in the cell in its reduced and oxidized form (GSSH), which are responsible for maintaining the intracellular redox state through the accumulation of disulfides that provide a reducing environment within cells [10,40]. Oxidative stress leads to an imbalance in the glutathione redox pair, favoring the accumulation of GSSH and decreasing GSH levels, reducing antioxidant capacity [41]Consistent with our results, the MMP-2 group altered the GSSH/GSH ratio, and NAC normalized it in animals receiving MMP-2.

The MMP-2 group also showed increased SOD and catalase activity, possibly related to the body's failed attempt to prevent oxidative stress. On the other hand, treatment with NAC in animals that received MMP-2 normalized SOD and catalase activity. We believe these results are related to the decrease in the bioavailability of oxidizing molecules due to the increase in GSH levels. Other authors also found similar results [42–45].

Among the transcriptional factors of antioxidant defense, Nrf2 is the main redox state-sensitive factor that regulates the gene expression of many enzymes of the antioxidant system [46]. Under conditions of oxidative stress, Nrf2 activates antioxidant response elements of several genes, initiating the antioxidant response [47]. Nrf2 knockout mice presented secondary complications aggravated by increased lipid peroxidation and decreased GSH/GSSH ratio after SAH [48]. It was previously demonstrated that increased MMP-2 activity correlates with increased Nrf2 levels, possibly related to this protease's ability to induce oxidative stress [49]. In our results, the MMP-2 group also showed increased expression of Nrf2, which is in accordance with the increased SOD and catalase activity found in this group. Likewise, the MMP-2 + NAC group showed a decrease in Nrf2 levels and SOD and catalase activity.

One of the main sources of ROS in vascular dysfunction processes occurs through increased expression of iNOS [50], which is positively regulated through responses to ROS [51]. Different from eNOS and nNOS, the NO generated by iNOS is mainly generated in peroxynitrite due to the chemical interaction of NO and O^{2-} [16]. The presence of iNOS and nitrotyrosine in the vessels are strong indicators that high levels of peroxynitrite were formed [52]. Our results showed increased expression of iNOS, nitrite and nitrotyrosine in the MMP-2 group, which were prevented by treatment with NAC. These results are in line with previous studies that showed that iNOS leads to increased oxidative and nitrosative stress in the vasculature [53–55].

Among MMPs, MMP-2 is considered one of the main proteases involved in aortic vascular remodeling. This was demonstrated in time-course experiments that evaluated vascular hypertrophy and fibrosis in the aorta of animals with two-kidney-one-clip hypertension [56]. Non-selective inhibitors of MMPs (doxycycline) and antioxidants have also been shown to decrease remodeling by modulating MMP-2 activity and expression [57–59]. In line with these studies, MMP-2 knockout animals attenuated vascular hypertrophy and fibrosis in angiotensin II-induced hypertension [36]. In our study, administration of MMP-2 did not generate aortic

hypertrophy or alter the deposition of extracellular matrix components, suggesting that the oxidative changes found precede the morphological changes induced by MMP-2.

Evidence reinforces oxidative stress as a critical factor in the worsening of vascular diseases, mainly biochemical, structural and functional changes [60–62]. Many of them are responsible for increased contractility, remodeling and vascular dysfunction, characteristics of vascular diseases [9]. Therefore, some limitations of our study must be considered in this aspect. First, we did not investigate the endothelial and vascular function of the aortas. Furthermore, we believe in the hypothesis that oxidative changes precede morphological changes; therefore, a longer period of MMP-2 administration can lead to vascular remodeling. Future work being developed by our group seeks to resolve these limitations.

In conclusion, our results suggest that NAC has potential MMP-2 inhibitory and antioxidant activity, preventing oxidative changes induced by the vascular increase in MMP-2. Therefore, NAC may be a viable therapeutic alternative for approaches related to maintaining vascular health.

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3. CONCLUSÕES INTEGRADORAS

As doenças vasculares têm impacto significativo sobre a qualidade de população mundial como uma das principais causa de morbimortalidade e hospitalizações por pessoa. Apesar de os mecanismos fisiopatológicos envolvidos na disfunção vascular estejam bem esclarecidos, mecanismos como a modulação da MMP-2 e o desbalanço redox precisam de investigação mais detalhada tendo vista seu papel crucial na gênese de um processo que leva ao remodelamento vascular e consequentemente a alterações mais generalizadas.

A avaliação de fármacos reposicionados para atuar sobre mecanismos inibitórios e regulatórios na patologia vascular permite uma abordagem adicional para uma terapia em potencial. Neste sentido, o cotratamento com a NAC proposto em nosso estudo para atenuar a atividade da MMP-2 e reduzir o estresse oxidativo na aorta foi considerado promissor, devido seus efeitos significativos sobre a redução significativa dos marcadores de estresse oxidativo e aumento da capacidade antioxidante, bem como seus efeitos positivos sobre a biodisponibilidade de NO. Contudo, estudos adicionais devem ser realizados a fim de avaliar outras possíveis vias e marcadores importantes envolvidos no processo de disfunção vascular.

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