LUIZ CARLOS MAIA LADEIRA

EFEITOS DA INFUSÃO DE *Camellia sinensis* (L.) Kuntze SOBRE PARÂMETROS MORFOFISIOLÓGICOS CARDÍACOS E RENAIS DE RATOS WISTAR COM DIABETES TIPO I

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

Orientador: Izabel Regina dos Santos Costa Maldonado

Coorientadores: Eliziária Cardoso dos Santos
Mariana Machado Neves
Marcio Roberto Silva

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Izabel Regina dos Santos Costa Maldonado
Orientadora

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"Ora, tendo a intenção de empregar toda a minha vida na pesquisa de uma ciência tão necessária, e havendo encontrado um caminho que se me afigura tal que se deve infalivelmente encontrá-la, se o seguirmos, exceto se disso sejamos impossibilitados, ou pela breve duração da vida, ou pela falta de experiências, julguei que não havia melhor remédio contra esses dois impedimentos a não ser comunicar com fidelidade ao público o pouco que já tivesse descoberto, e convidar os bons espíritos a empregarem todas as forças para ir além, contribuindo, cada qual de acordo com sua inclinação e sua capacidade, para as experiências que seria necessário realizar, e comunicando ao público todas as coisas que aprendesse, para que os últimos começassem onde os precedentes houvessem acabado, e assim, somando as vidas e os trabalhos de muitos, fôssemos, todos juntos, muito mais longe do que poderia ir cada um em particular."

(René Descartes, 1637)

RESUMO

LADEIRA, Luiz Carlos Maia, D.Sc., Universidade Federal de Viçosa, junho de 2021. **Efeitos da infusão de *Camellia sinensis* (L.) Kuntze sobre parâmetros morfofisiológicos cardíacos e renais de ratos Wistar com diabetes tipo 1.** Orientadora: Izabel Regina dos Santos Costa Maldonado. Coorientadores: Eliziária Cardoso dos Santos, Mariana Machado Neves e Marcio Roberto Silva.

Introdução: O diabetes tipo 1 é um grupo heterogêneo de distúrbios metabólicos que se desenvolve principalmente na infância e adolescência. A hiperglicemia decorrente do diabetes gera estresse metabólico sistêmico favorecendo o desenvolvimento de várias comorbidades, dentre elas a nefropatia diabética (ND) e a cardiomiopatia diabética (CD). Ambas as doenças são prevalentes em pacientes com diabetes e se não tratadas ou prevenidas podem evoluir para falências dos órgãos e morte do paciente. O chá verde é tradicionalmente utilizado como tratamento do diabetes e seus efeitos foram relacionados à capacidade hipoglicemiante, reduzindo a sobrecarga glicêmica e o dano oxidativo nos tecidos. Entretanto, estes resultados ainda são controversos e nem sempre o chá exerce uma ação hipoglicemiante, podendo levar a efeitos positivos por outras vias. **Objetivo:** Avaliar como os efeitos da infusão de chá verde (*Camellia sinensis* L. Kuntze) afetam parâmetros morfológicos, bioquímicos e funcionais dos rins e do coração frente a um estado hiperglicêmico grave gerado pelo diabetes tipo 1 experimental em animais jovens. **Metodologia:** Utilizamos neste trabalho um total de 18 ratos Wistar, machos e jovens. Tratamos seis ratos com diabetes tipo 1 induzido por estreptozotocina, com 100 mg/kg de chá verde, diariamente, por 42 dias. Além disso, um grupo controle saudável (n=6) e um diabético (n=6) também compuseram o experimento. A infusão foi preparada com o objetivo de reproduzir a forma consumida normalmente por humanos e os animais foram mantidos em condições controladas de temperatura (22 ± 2 ºC) e luminosidade (12/12h), e receberam alimento e água *ad libitum*. Todos os procedimentos deste experimento foram aprovados pelo CEUA/UFV (protocolo nº 53/2018). No Artigo 1 (Capítulo 2), foram avaliados marcadores sorológicos da função renal e marcadores teciduais de estresse oxidativo, homeostasia iônica e função de transportadores de íons, alterações morfológicas glomerulares e tubulares, bem como o dano ao DNA em células renais. Ainda, utilizamos a ferramenta de *network pharmacology* para explorar as vias de sinalização relacionadas aos resultados encontrados *in vivo*. No Artigo 2 (Capítulo 3) foram avaliados os marcadores séricos e teciduais para função cardíaca e estresse oxidativo. Além disso, analisamos por microscopia de campo claro, as alterações morfológicas e os danos ao DNA. Ainda, avaliamos também as alterações

teciduais e ultraestruturais mitocondriais em fragmentos do ventrículo esquerdo por microscopia eletrônica de varredura. **Resultados:** Nossos resultados revelaram que uma dose diária de 100 mg/kg de tratamento com infusão de chá verde por 42 dias evitou danos renais cardíacos desencadeados pela hiperglicemia em ratos jovens com diabetes tipo 1 de início precoce, mesmo sem conseguir controlar a hiperglicemia grave nos animais. Os dados relativos às análises renais (Capítulo 2) revelaram que os componentes do chá verde interagem em vias de sinalização que regulam o metabolismo energético, incluindo a síntese e degradação da glicose e do glicogênio, além da reabsorção de glicose pelos rins, manejo da hipóxia e morte celular por apoptose. Tais interações levaram à redução do acúmulo de glicogênio no órgão e proteção do DNA ao dano oxidativo. Além disso, o chá verde foi capaz de prevenir danos morfológicos nos glomérulos, sugerindo um efeito protetor ao órgão e a preservação de sua função. No coração (Capítulo 3), apesar da falta de efeito direto sobre as atividades das enzimas antioxidantes, o chá verde preveniu a fibrose cardíaca e a hipertrofia dos cardiomiócitos, mantendo a distância de difusão dos vasos sanguíneos e a área de secção transversal das fibras em níveis semelhantes aos encontrados nos animais saudáveis. Além disso, a quantidade de células marcadas com iodeto de propídio foram mais baixas no grupo tratado com chá verde do que nos animais com diabetes não tratado, indicando um efeito protetor do chá verde contra danos ao DNA. Ainda, menores taxas de infiltração de mastócitos foram encontradas nos animais tratados com chá verde quando comparados ao controle diabético. Da mesma forma, menores taxas de mastócitos ativados também foram encontradas no grupo tratado com chá verde quando comparado ao controle diabético. Adicionalmente, foram encontradas alterações morfológicas nas mitocôndrias dos animais diabéticos, com maiores frequências de fusão mitocondrial que no grupo controle, e que foram prevenidas pelo tratamento com chá verde. Esses resultados positivos refletiram nos níveis mais baixos de creatina quinase (CK-MB) e lactato desidrogenase (LDH), sugerindo uma melhor função cardíaca no grupo tratado com chá verde, independentemente de quaisquer melhorias nos valores de glicose no sangue. **Conclusões:** A ingestão da infusão de chá verde é capaz de prevenir a remodelação dos tecidos do coração e dos rins, neutralizando as alterações induzidas pelo diabetes, prevenindo fibrose no miocárdio e pericárdio e a nefrose glicogênica nos rins, a remodelação vascular no miocárdio e infiltração e ativação de mastócitos no coração, e o desenvolvimento de alterações patológicas nos glomérulos. Além disso, o chá verde foi capaz de prevenir danos ao DNA dos cardiomiócitos e nas células renais, e controlar a dinâmica morfológica da mitocôndria, que ocorre como uma adaptação metabólica ao diabetes. Esses resultados benéficos, considerados

em conjunto, refletem-se no potencial efeito protetor da infusão de chá verde frente às comorbidades decorrentes do diabetes envolvidas neste estudo.

Palavras-chave: Cardiomiopatia diabética. Chá verde. Diabetes tipo 1. Fitoterapia. Nefropatia diabética.

ABSTRACT

LADEIRA, Luiz Carlos Maia, D.Sc., Universidade Federal de Viçosa, June, 2021. **Effects of *Camellia sinensis* (L.) Kuntze infusion on cardiac and renal morphophysiological parameters of Wistar rats with type 1 diabetes.** Adviser: Izabel Regina dos Santos Costa Maldonado. Co-advisers: Eliziária Cardoso dos Santos, Mariana Machado Neves and Marcio Roberto Silva.

Introduction: Type 1 diabetes is a heterogeneous group of metabolic disorders that develop mainly in childhood and adolescence. Hyperglycemia resulting from diabetes generates systemic metabolic stress, favoring the development of several comorbidities, including diabetic nephropathy (DN) and diabetic cardiomyopathy (DC). Both diseases are prevalent in patients with diabetes and if left untreated or prevented they can progress to organ failure and patient death. Green tea is traditionally used as a treatment for diabetes and its effects were related to its hypoglycemic capacity, reducing glycemic overload and oxidative damage to tissues. However, these results are still controversial and tea does not always exert a hypoglycemic action, which can lead to positive effects in other ways. **Objective:** To evaluate how the effects of green tea infusion (*Camellia sinensis* L. Kuntze) affect morphological, biochemical and functional parameters of the kidneys and heart in a severe hyperglycemic state generated by experimental type 1 diabetes in young animals. **Methodology:** In this work we used a total of 18 male and young Wistar rats. We treated six streptozotocin-induced type 1 diabetes rats with 100 mg/kg of green tea daily for 42 days. In addition, a healthy control group (n=6) and a diabetic group (n=6) also composed the experiment. The infusion was prepared with the objective of reproducing the form normally consumed by humans and the animals were kept under controlled conditions of temperature (22 ± 2 ºC) and light (12/12h), and received food and water *ad libitum*. All procedures of this experiment were approved by CEUA/UFV (protocol no. 53/2018). In the Article 1 (Chapter 2), serological markers of renal function and tissue markers of oxidative stress, ionic homeostasis and ion transporter function, glomerular and tubular morphological changes, as well as DNA damage in renal cells were evaluated. Furthermore, we used the network pharmacology tool to explore the signaling pathways related to the results found in vivo. In the Article 2 (Chapter 3), serum and tissue markers for cardiac function and oxidative stress were evaluated. In addition, we analyzed by brightfield microscopy, morphological changes and DNA damage. Furthermore, we also evaluated mitochondrial tissue and ultrastructural changes in left ventricular fragments by scanning

electron microscopy. **Results:** Our results revealed that a daily dose of 100 mg/kg of green tea infusion treatment for 42 days prevented cardiac renal damage triggered by hyperglycemia in young rats with early-onset type 1 diabetes, even without being able to control severe hyperglycemia in animals. The data related to kidney analysis (Chapter 2) revealed that the components of green tea interact in signaling pathways that regulate energy metabolism, including the synthesis and degradation of glucose and glycogen, in addition to glucose reabsorption by the kidneys, management of hypoxia and cell death by apoptosis. Such interactions led to a reduction in the accumulation of glycogen in the organ and protection of DNA from oxidative damage. Furthermore, green tea was able to prevent morphological damage to the glomeruli, suggesting a protective effect on the organ and the preservation of its function. In the heart (Chapter 3), despite the lack of direct effect on the activities of antioxidant enzymes, green tea prevented cardiac fibrosis and cardiomyocyte hypertrophy, maintaining the diffusion distance of blood vessels and the cross-sectional area of fibers in levels similar to those found in healthy animals. Furthermore, the amounts of cells labeled with propidium iodide were lower in the group treated with green tea than in animals with untreated diabetes, indicating a protective effect of green tea against DNA damage. Also, lower rates of mast cell infiltration were found in animals treated with green tea when compared to diabetic control. Likewise, lower rates of activated mast cells were also found in the group treated with green tea when compared to the diabetic control. Additionally, morphological alterations were found in the mitochondria of diabetic animals, with higher frequencies of mitochondrial fusion than in the control group, which were prevented by treatment with green tea. These positive results reflected lower creatine kinase (CK-MB) and lactate dehydrogenase (LDH) levels, suggesting better cardiac function in the green tea-treated group, regardless of any improvements in blood glucose values. **Conclusions:** The ingestion of green tea infusion is able to prevent the remodeling of heart and kidney tissues, neutralizing the changes induced by diabetes, preventing fibrosis in the myocardium and pericardium and glycogenic nephrosis in the kidney, vascular remodeling in the myocardium and infiltration and activation of mast cells in the heart, and the development of pathological changes in the glomeruli. Furthermore, green tea was able to prevent damage to the DNA of cardiomyocytes and renal cells, and to control the morphological dynamics of the mitochondria, which occur as a metabolic adaptation to diabetes. These beneficial results, taken together, are reflected in the potential protective effect of green tea infusion against comorbidities resulting from diabetes involved in this study.

Keywords: Diabetic cardiomyopathy. Diabetic nephropathy. Green Tea. Phytotherapy. Type 1 diabetes.

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LISTA DE ABREVIAÇÕES

67LR – 67kDa laminin receptor

ABTS – 2,2'–Azinobis–[3–ethylbenzthiazoline–6–sulfonic acid]

ACCORD – Action to Control Cardiovascular Risk in Diabetes

AGE/RAGE – Advanced glycated end–products and its receptor

AKT – Proteína kinase B

AMPK – 5'–AMP–activated protein kinase

AO – Acridine Orange

APC – APC Regulator of WNT Signaling Pathway

ATP – Adenosina trifosfato

BAX – BCL2 Associated X

BCL2 – BCL2 Apoptosis Regulator

BID – BH3 Interacting Domain Death Agonist

BW – Body weight

C – Carbon

Ca – Calcium

Ca^{2+} – Calcium ion

CaCl – Calcium chloride

CASP3 – Caspase 3

CASP8 – Caspase 8

CASP9 – Caspase 9

CAT – Catalase

CAV1 – Caveolin 1

CCL2 – C–C Motif Chemokine Ligand 2

CDK2 – Cyclin Dependent Kinase 2

CDKN1A – Cyclin Dependent Kinase Inhibitor 1

CEUA – Comissão de Ética no Uso de Animais

CIAPIN1 – Cytokine Induced Apoptosis Inhibitor 1

c–kit – Proto–oncogene c–kit

CK–MB – Creatine kinase

Cl – Chlorine

CONCEA – Conselho Nacional de Controle de Experimentação Animal

CPI – Compound–protein interactome

CTNNB1 – Catenin Beta 1

Ctrl – Control group

Cu – Copper

DAPI – 4',6'–diamino–2–fenil–indol

DB02077 – L–N(omega)–nitroarginine–(4R)–amino–L–proline amide (NOS3)

DB08019, DB08018 and NOS3– Nitric Oxide Synthase 3

DC – Diabetic cardiomyopathy

DKG – Diacylglycerol kinase

DM – Diabetes mellitus

DM1 – Diabetes mellitus tipo 1

DM2 – Diabetes mellitus tipo 2

DMSO – Dimethyl sulfoxide

DN – Diabetic nephropathy

DNA – Deoxyribonucleic acid

EGCG – Epigallocatechin gallate

eNOS – Endothelial nitric oxide synthase

EROs – Espécies reativas de oxigênio

Fe – Iron

FGF – Fibroblast growth factor

FGFR – Fibroblast growth factor receptor

FOS – Fos Proto–Oncogene

FRAP – Ferric reducing antioxidant power

GLUT1 – Glucose transporter 1

GLUT4 – Glucose transporter 4

GSK3β – Glycogen synthase kinase–3 β

GST – Glutathione S–transferase

GTI – Green tea infusion

H&E – Hematoxylin and Eosin

H_2O_2 – Hydrogen peroxide

H6PD – Hexose–6–Phosphate Dehydrogenase/Glucose 1–Dehydrogenase

HIF1A – Hypoxia Inducible Factor 1 Subunit Alpha

HMG1 – High–mobility group box 1

HPLC – High performance liquid chromatography

HSP90AA1 – Heat Shock Protein 90 Alpha Family Class A Member 1

i.p. – Intraperitoneal

IL6 – Interleukin 6

IL8 – Interleukin 8

JUN – Jun Proto–Oncogene

K – Potassium

K^+ – Potassium ion

KCl – Potassium chloride

KW – kidney weight

LDH – Lactate dehydrogenase

LV – Left ventricle

MAP2K1 – Mitogen–Activated Protein Kinase Kinase 1

MAPK1 – Mitogen–Activated Protein Kinase 1

MAPK3 – Mitogen–Activated Protein Kinase 3

MAPK8 – Mitogen–Activated Protein Kinase 8

MAPKAPK5 – MAPK Activated Protein Kinase 5

Mg – Magnesium

MgCl – Magnesium chloride

MLH1 – MutL Homolog 1

Mn – Manganese

mTOR – Mammalian target of rapamycin

MTRR – 5–Methyltetrahydrofolate–Homocysteine Methyltransferase Reductase

Na – Sodium

Na^+ – Sodium ion

NaCl – Sodium chloride

NADPH – Reduced nicotinamide adenine dinucleotide phosphate

NaOH – Sodium hydroxide

NDOR1 – NADPH–dependent diflavin reductase

NFκB – Nuclear factor κ B

NO_2^-/NO_3^- – Nitrate and nitrite

NOS1 – Nitric Oxide Synthase 1

NOS2 – Nitric Oxide Synthase 2

NR1H4 – Nuclear Receptor Subfamily 1 Group H Member 4

O – Oxygen

O_2 – Oxygen

O_2^- – Superoxide

OW – Organ weight

PARP1 – Poly (ADP–Ribose) Polymerase 1

PDGF – Platelet–derived growth factor

PDK – Pyruvate dehydrogenase kinase

PGD – Phosphogluconate Dehydrogenase

PGLS – 6–Phosphogluconolactonase

PI – Propidium iodide

PI3K – Phosphoinositide 3–kinase

PIN1 – Peptidylprolyl Cis/Trans Isomerase, NIMA–Interacting 1

PKC–β – Protein kinase C beta

POR – Cytochrome P450 Oxidoreductase

PPI – Protein–Protein Interactome

ROS – Reactive oxygen species

RPIA – Ribose 5–Phosphate Isomerase A

RSI – Renal somatic index

SCF – Stem cell factor

SD – Standard deviation

Se – Selenium

SEI – Secondary electrons

SEM – Scanning electron microscope

SGLT1 – Sodium–dependent glucose transporter 1

SGLT2 – Sodium–dependent glucose transporter 2

SOD – Superoxide dismutase

SOES – Specific Organ Expression Score

STAT3 – Signal transducer and activator of transcription 3

STZ – Streptozotocin

TCA – Trichloroacetic acid

TCF7L2 – Transcription Factor 7 Like 2

TE – Trolox equivalent

TGF–β – Transforming growth factor–beta

TLR4 – Toll–like receptor 4

TP53 – Tumor protein 53

TRIF – TIR domain–containing adaptor–inducing Interferon– β

TYW1 – TRNA–YW Synthesizing Protein 1 Homolog

UBC – Ubiquitin C

UFV – Universidade Federal de Viçosa

VE – Ventrículo esquerdo

Zn – Zinc

LISTA DE SÍMBOLOS

% – Porcentagem

® – Marca registrada

µm – Micrômetro

µm² – Micrômetro quadrado

cm – Centímetros

dL – Decilitro

g – Grama

h – Horas

Kg – Quilograma

Kv – Quilovolt

L – Litro

M – Mol

mg – Miligrama

mL – Mililitro

mm – Milímetro

mm² – Milímetro quadrado

mm^3 – Milímetro cúbico

ºC – Graus Célsius

pH – Potencial hidrogeniônico

rpm – Rotações por minuto

β – Beta

κ – Kappa

SUMÁRIO

Capítulo 1

Introdução (Histórico da doença)

A primeira descrição de uma doença que seria nomeada "diabetes" data de 1550 a.C. e foi encontrada em papiros egípcios descobertos pelo alemão George Ebers em 1862 d.C. Por mais de três milênios os papiros de Ebers guardaram os registros das características observadas pelos egípcios: poliúria (aumento na produção de urina), polidipsia (aumento no consumo de água) e perda de peso, quadro em que, naqueles tempos, levava à morte pela doença inevitavelmente (VIGGIANO, 2009).

Muitos séculos se passaram até a palavra "diabete" ser usada para descrever tal patologia, sendo no século II da era moderna (d.C.) que Arataeus da Cappadocia[1], notável médico grego, usou o termo "*διαβήτης"* ou "*diabeinein*" – que significa "fluir por um sifão" – para nomear uma "doença terrível, que se desenvolve durante um longo período de tempo", como descrito pelo próprio médico (TEKINER, 2015). Arataeus acreditava que a poliúria dos indivíduos com diabetes acontecia pelo "derretimento de suas carnes e membros em urina" e que era causada por outras doenças em outros órgãos, como a bexiga e os rins (TEKINER, 2015). Já naquele tempo, o médico grego observou que o desenvolvimento da doença era de

[1] Arataeus da Cappadocia - Αρεταίος ο Καππαδόκης – (séc. I – II d.C.) um dos grandes médicos da antiguidade greco-romana após Hipócrates. Seu pensamento era moldado pela escola pneumática – que acreditava que a saúde depende do balanço harmônico entre os elementos básicos (calor, frio, úmido e seco) e a *pneuma* (elemento espírito). É autor do tratado de medicina "Das causas, sintomas e cura das doenças", obra de grande importância na história da medicina que descreve em detalhes variadas doenças (TEKINER, 2015).

natureza crônica e, que ao estar completamente estabelecida, os danos causados levariam o paciente a uma vida sofrida e curta.

Dois tipos distintos de diabetes foram descritos pelos médicos indianos Sushruta e Charaka, já no tempo comum (400 – 500 d. C.), que hoje são conhecidos como diabetes mellitus tipo 1 e diabetes mellitus tipo 2 (AHMED, 2002). O termo *mellitus* foi usado pela primeira vez em 1675, pelo médico inglês Thomas Willis, para diferenciar o tipo de diabetes onde a urina do paciente era adocicada (*mellitus* vem de mel) do tipo onde ela não possui o gosto doce (diabetes *insipidus*, onde *insipidus* é o termo latino para insípido, sem sabor) (VECCHIO et al., 2018). Tal sabor adocicado na urina provém da excreção anormal de glicose pelos rins, descoberto em 1776 por Matthew Dobson (VECCHIO et al., 2018).

No século seguinte o jovem pesquisador Paul Langerhans[2] identifica, em 1869, estruturas pancreáticas conhecidas hoje como "Ilhotas de Langerhans" (ou ilhotas pancreáticas), onde se localizam as células responsáveis pela produção de insulina, também chamadas de células beta (β). Porém, naquele tempo, o jovem pesquisador ainda não sabia da importância que seu achado teria na medicina moderna. Langerhans achava que a estrutura se tratava de linfonodos presentes no pâncreas. O termo Ilhota de Langerhans foi criado pelo histologista francês Gustave-Édouard Laguesse, e se popularizou posteriormente (JOLLES, 2002). Foi somente a partir de 1909, com o trabalho de Jean de Mayer, e 1910 com Sir Edward Albert Sharpey-Schafer, que de forma independente[3], nomearam uma molécula hipotética secretada pelas ilhotas de Langerhans, responsável por reduzir os sintomas do diabetes

[2] Paul Langerhans descreveu as ilhotas pancreáticas aos seus 22 anos de idade, durante seu doutorado no Berlin Pathological Institute.

[3] O crédito da criação do nome pode variar dependendo da fonte. Alguns autores afirmam ter sido de Meyer o primeiro, outros Sharpey-Schafer. É importante, entretanto, levar em conta que no início do século XX, antes da invenção da internet, a comunicação científica levava mais tempo e o trabalho independente dos pesquisadores leva alguns autores a creditarem a criação a ambos (VECCHIO et al., 2018).

(VECCHIO et al., 2018). A *Insulina* é então nomeada assim por ser produto da ilhota (ilhota vem do latim: *insula*, ou ilha).

A Figura 01 mostra detalhes do pâncreas com destaque para a ilhota pancreática, estrutura onde se localizam as células β.

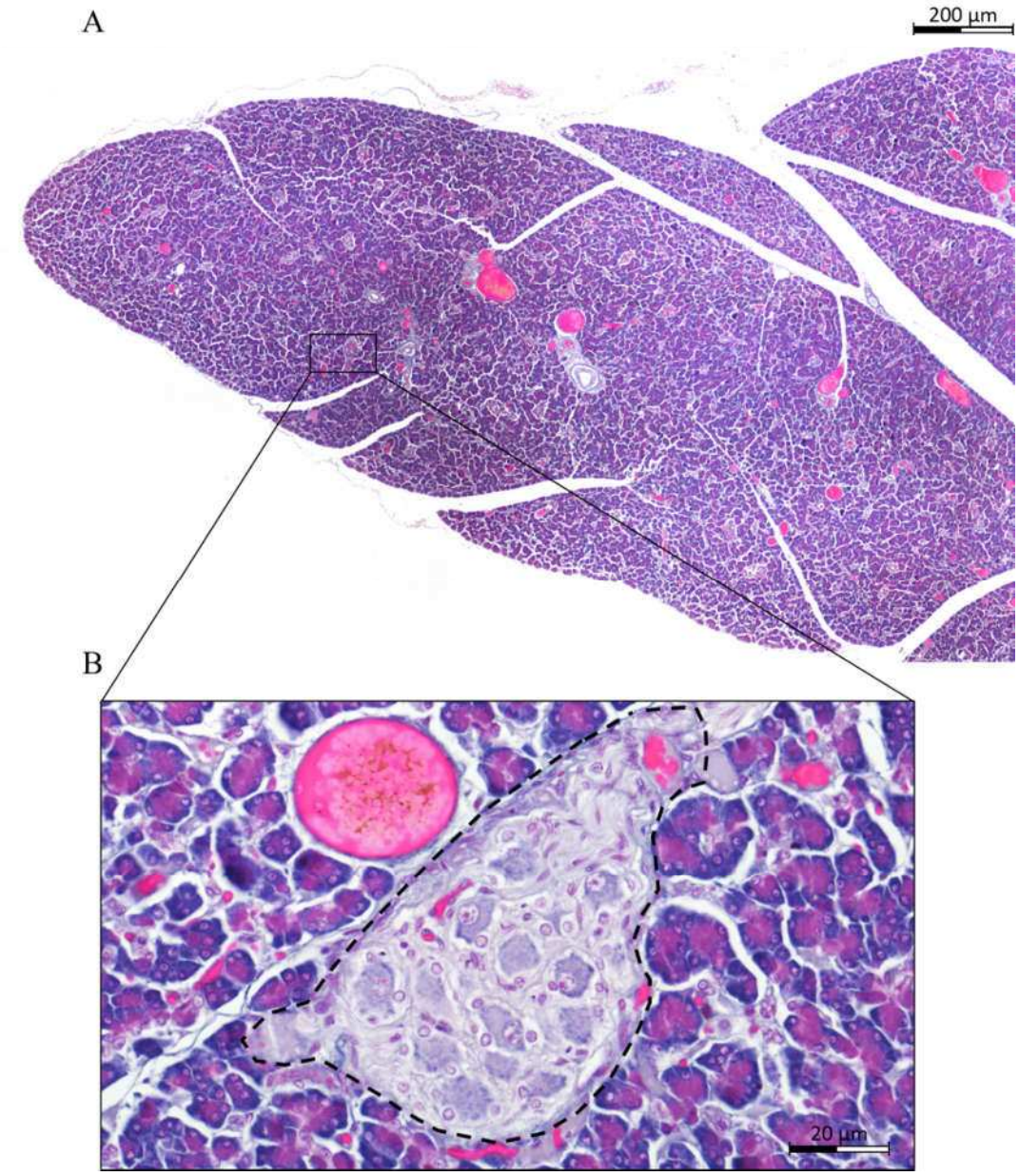

Figura 01. Pâncreas e ilhota pancreática. Fotografia produzida a partir de lâmina do acervo do Departamento de Biologia Geral da Universidade Federal de Viçosa, corada com H.C. Floxina. **A** – Pâncreas. **B** – Detalhe do pâncreas em região de ilhota pancreática (região delimitada pelo pontilhado), rodeada pelos ácinos, unidades secretoras do pâncreas exócrino. Na ilhota, as

células volumosas com citoplasma em azul correspondem às células beta, produtoras de insulina. As mais clarinhas são células alfa, secretoras de glucagon. As estruturas cujo lúmen aparece rosado são vasos sanguíneos. Fotografia do acervo pessoal do autor.

Ainda em 1910, o médico estadunidense Joseph Pratt relaciona o pâncreas ao diabetes (PRATT, 1910) e afirma que o pâncreas tem uma função importante no metabolismo da glicose, possivelmente por meio de "secreções internas" (endócrinas), que ainda não haviam sido provadas mas que eram altamente prováveis dadas as evidências à época. Pratt foi o primeiro a fazer essa suposição, porém outros pesquisadores já haviam feito diversas descobertas em relação aos extratos pancreáticos de várias naturezas e sobre as funções pancreáticas utilizando órgãos caninos e humanos. Dez anos após Pratt, Moses Barron, em 1920, relaciona as Ilhotas Pancreáticas ao diabetes no trabalho intitulado "*Relation of the Islets of Langerhans to Diabetes with special reference to cases of pancreatic lithiasis*" (VECCHIO et al., 2018).

O trabalho de Barron foi determinante para o que se sucederia até a descoberta da insulina. No mesmo ano (1920) o médico canadense Frederick Grant Banting, trabalhando no laboratório liderado por John James Richard MacLeod na Universidade de Toronto, conseguiu isolar o extrato das ilhotas pancreáticas. Banting teve ajuda do estudante Charles Best na execução dos experimentos para extração do extrato de pâncreas de cães. Com este extrato, pela primeira vez, Banting e Best conseguiram controlar a hiperglicemia decorrente do diabetes em animais. Com ajuda do bioquímico James Collip na purificação do extrato, foi possível alcançar sucesso nos testes em humanos (VECCHIO et al., 2018). Após as publicações, a empresa farmacêutica Eli Lilly and Company, em parceria com os pesquisadores, lançam a insulina comercialmente no mercado em 1923, revolucionando o tratamento do diabetes. Fredrick Grant Banting e John James Richard Macleod receberam o Nobel de Fisiologia e Medicina de 1923 e dividiram o prêmio com os colegas Charles Best e James Collip, que participaram da descoberta.

Outras descobertas e outros pesquisadores foram de grande importância na história do diabetes, e são apresentados mais detalhadamente nos trabalhos de Vecchio e colegas (2018) e Ahmed (2002). Ainda, a matéria publicada na revista Pesquisa Fapesp intitulada "A descoberta da Insulina", em homenagem aos 100 anos de sua história, conta detalhes da participação brasileira no processo de industrialização e escalonamento da produção da insulina humana recombinante (FIORAVANTI, 2021), que possibilitou um imenso avanço na produção e distribuição do hormônio no mundo todo.

O diabetes e as comorbidades cardíaca e renal

O diabetes *mellitus* (DM) afeta, segundo as estimativas mais recentes, cerca de 9,3% da população mundial (463 milhões de pessoas), podendo chegar a 700,2 milhões de pessoas com a doença em 2045 (INTERNATIONAL DIABETES FEDERATION, 2019). O Brasil era o quarto país com maior incidência de DM em adultos no mundo, com 14,3 milhões de casos estimados em 2017 (SOCIEDADE BRASILEIRA DE DIABETES, 2017). Nos dados de 2019, o Brasil é o quinto colocado, com 16,8 milhões de pessoas diagnosticadas com a condição, atrás da China, Índia, Estados Unidos da América e Paquistão. Considerando os casos em crianças e adolescentes, o Brasil é o terceiro no mundo. (INTERNATIONAL DIABETES FEDERATION, 2019; SOCIEDADE BRASILEIRA DE DIABETES, 2019). Ainda, a projeção é que este número chegue a 26 milhões de indivíduos com diabetes somente no Brasil.

A definição utilizada pela Sociedade Brasileira de Diabetes é de que a doença "consiste em um distúrbio metabólico caracterizado por hiperglicemia persistente, decorrente de deficiência na produção de insulina ou na sua ação, ou em ambos os mecanismos" (SOCIEDADE BRASILEIRA DE DIABETES, 2019).

A maioria dos casos de diabetes se divide em dois tipos: diabetes *mellitus* tipo 1 (DM1) e tipo 2 (DM2). Ainda, figuram entre os casos de DM o diabetes gestacional e outros tipos específicos de diabetes. A condição originária da produção insuficiente ou ausência completa da produção e secreção de insulina pelo pâncreas, o que comumente exige a reposição sintética do hormônio, é categorizada como DM1. Ainda, o DM1 pode ser subdividido em DM tipo 1 A, quando a causa da deficiência insulínica está associada à destruição autoimune das células β pancreáticas, e DM tipo 1 B, quando essa deficiência é de natureza idiopática (AMERICAN DIABETES ASSOCIATION, 2021; SOCIEDADE BRASILEIRA DE DIABETES, 2019).

Por outro lado, a doença desenvolvida a partir da redução progressiva da produção de insulina combinada à resistência sistêmica a sua ação é categorizada como DM2, que pode ser tratada com a reposição da insulina em casos mais graves ou outros agentes hipoglicemiantes, como a metformina, em casos menos graves. Além dos agentes hipoglicemiantes, a terapia nutricional e a fitoterapia são grandes aliadas no tratamento do diabetes e prevenção do agravamento das patologias decorrentes dele (AMERICAN DIABETES ASSOCIATION, 2021; SOCIEDADE BRASILEIRA DE DIABETES, 2019).

O DM1 está associado a várias complicações sistêmicas, geralmente causadas por distúrbios micro e macrovasculares que resultam em retinopatia, doença das artérias coronarianas, além de comprometimento do sistema arterial periférico (SOCIEDADE BRASILEIRA DE DIABETES, 2019). Além disso, também ocorrem danos hepáticos e no sistema reprodutor (BAOTHMAN et al., 2016; SOUZA et al., 2018, 2019), comprometimento renal (RASCH, 1980; SERTORIO et al., 2019) e danos cardíacos, favorecendo o aparecimento da nefropatia diabética (HERMAN-EDELSTEIN; DOI, 2016) e da cardiomiopatia diabética (BOUERI et al., 2015; DA SILVA et al., 2013; RITCHIE; DALE ABEL, 2020).

A nefropatia diabética

A nefropatia diabética atinge entre 25% e 35% dos pacientes, independente do tipo de diabetes *mellitus* (HERMAN-EDELSTEIN; DOI, 2016), e é responsável por cerca de 45% dos casos de falência renal nesta população (SU et al., 2020).

Seus sintomas iniciais são o aumento na taxa de filtração glomerular, caracterizando a poliúria, e alterações morfológicas glomerulares e tubulares, que se refletem no aumento da excreção de proteínas, principalmente albumina. Além disso alterações glomerulares típicas como a expansão mesangial, espessamento da cápsula glomerular (de Bowman), e alargamento do espaço urinário contribuem para o comprometimento da função renal. Nos túbulos é comum encontrar alterações como a cariomegalia, fusão exacerbada de mitocôndrias e vacuolização das células (GILBERT, 2017; HERMAN-EDELSTEIN; DOI, 2016).

Uma das características mais frequentes e iniciais neste processo patológico é o acúmulo de glicogênio nos túbulos proximais (GILBERT, 2017; HARAGUCHI et al., 2020). A glicose presente no filtrado glomerular é normalmente reabsorvida quase que completamente nos túbulos proximais pelos transportadores de glicose dependentes de sódio 2 (SGLT2) e em menor quantidade pelos mesmos transportadores do tipo 1 (SGLT1), e só é detectada na urina quando essa capacidade de reabsorção é extrapolada (BAILEY, 2011; VALLON; THOMSON, 2017). No diabetes, por motivos de maior demanda de adenosina trifosfato (ATP) para as funções vitais celulares e para a manutenção da reabsorção aumentada de glicose, a expressão do SGLT2, também, aumentada, eleva a disponibilidade de glicose no meio intracelular (HERMAN-EDELSTEIN; DOI, 2016). Ainda, as células do túbulo proximal tem uma grande capacidade gliconeogênica, e se utilizam basicamente de substratos como o lactato, glutamina e glicerol para isso, porém este processo é reforçado no diabetes (EID et al., 2006). Desta forma, o acúmulo de glicogênio no órgão é facilitado pela soma destes processos: reabsorção e

gliconeogênese aumentadas (HERMAN-EDELSTEIN; DOI, 2016; MATHER; POLLOCK, 2011). Tal acúmulo, se não tratado, pode progredir para lesões pré-neoplásicas e posteriormente câncer renal (RIBBACK et al., 2015).

Em nível macroscópico, os rins sofrem hipertrofia e hiperplasia como um mecanismo compensatório aos danos causados pela hiperglicemia, de forma a preservar a função glomerular (HERMAN-EDELSTEIN; DOI, 2016). Entretanto, quando a glicemia elevada se instala ainda em idade jovem, os danos podem ser extensos o suficiente para inibir o mecanismo de compensação do órgão, resultando em aumento diminuto ou ausente do peso e tamanho dos rins (ARATAKI, 1926).

Os mecanismos moleculares e as alterações morfológicas causadas pela alteração destes mecanismos serão descritos e explorados no Capítulo 2.

A cardiomiopatia diabética

No músculo cardíaco o DM predispõe a complicações funcionais, teciduais e metabólicas. Em nível celular há desregulação da homeostase de cálcio (Ca^{2+}), sódio (Na^{+}) e potássio (K^{+}) com prejuízo no funcionamento das bombas destes íons (Ca^{2+}ATPase e Na-K-ATPase); disfunção mitocondrial e aumento na produção de espécies reativas de oxigênio (EROs) e nitrogênio; desregulação do ciclo celular; apoptose e autofagia; inflamação; e hipertrofia celular, dentre outras alterações (BABU; SABITHA; SHYAMALADEVI, 2006a; BATTIPROLU et al., 2013; CHEN et al., 2017; DA SILVA et al., 2016, 2015; HUANG et al., 2017; LIAO et al., 2016; OKOSHI et al., 2007; OU et al., 2010; VARGA et al., 2015).

A doença impacta no processo de remodelamento patológico do ventrículo esquerdo (VE), como a redução na densidade capilar, aumento na quantidade de colágeno total e fibras

reticulares, necrose e vacuolização dos cardiomiócitos, além de estimular o aumento da quantidade de glicogênio citoplasmático e glicoproteínas na matriz extracelular (BABU et al., 2007; DA SILVA et al., 2013, 2016; LEVELT et al., 2018; OKOSHI et al., 2007; ZHI; PRINS; MARWICK, 2004).

Estas alterações celulares e teciduais levam disfunção dos cardiomiócitos, causando redução na fração de ejeção do ventrículo e na frequência cardíaca (DA SILVA et al., 2015; HUANG et al., 2017; OU et al., 2010). Quando não tratadas, tais alterações podem evoluir para falência cardíaca e morte súbita (DA SILVA et al., 2015), fazendo com que a disfunção cardíaca (CHEN et al., 2017; YE et al., 2004), seja responsável por cerca de 80% das mortes decorrentes do diabetes (BABU; SABITHA; SHYAMALADEVI, 2006a).

Um marco da disfunção cardíaca amplamente aceito na comunidade médica e científica é a disfunção diastólica do ventrículo esquerdo, que é um dos primeiros sinais da cardiomiopatia diabética. Geralmente ela é detectada antes da disfunção sistólica do ventrículo esquerdo (BOYER et al., 2004; RITCHIE; DALE ABEL, 2020). Tais disfunções tem sido atribuídas às alterações morfológicas cardíacas, já em estágios avançados da doença (WOOD; PIRAN; LIU, 2011). No modelo experimental de diabetes induzido por estreptozotocina, 42 dias são suficientes para o agravamento da doença e o aparecimento das disfunções sistólica e diastólica, já sendo possível detectar alterações morfológicas no coração (GERBER; ARONOW; MATLIB, 2006). Estes danos funcionais cardíacos geralmente são silenciosos no DM e frenquentemente só são detectados nas fases mais avançadas da doença (RITCHIE; DALE ABEL, 2020), tornando ainda mais difícil o manejo apropriado e agravando ainda mais a condição.

A reposição de insulina exógena (insulinoterapia) é a principal forma de controle da glicemia e danos associados a ela em indivíduos com DM tipo 1 e DM tipo 2 grave

(SOCIEDADE BRASILEIRA DE DIABETES, 2019). Entretanto, o estudo conduzido pelo grupo de estudos ACCORD (*Action to Control Cardiovascular Risk in Diabetes*), por 9 anos, descreveu que o controle glicêmico intensivo não apresenta efeitos quanto a redução nas chances de falência cardíaca, e ainda, aumenta a chance de morte por falência cardíaca em pacientes com DM2 (ACCORD STUDY GROUP, 2016). O tratamento convencional para a falência cardíaca é o mesmo para pacientes com ou sem diagnóstico de DM e tratamentos que consideram mecanismos específicos (i.e., *endotypes*) para a falência cardíaca associada ao diabetes ainda não estão disponíveis (RITCHIE; DALE ABEL, 2020).

No Capítulo 3 são exploradas as alterações morfológicas e os mecanismos moleculares que levam a tais alterações no coração dos animais diabéticos.

O diabetes tipo 1

Apesar da maior frequência do DM2 dentre os indivíduos com diabetes (aproximadamente 90% a 95% dos casos), o DM1 é uma das doenças mais prevalentes na infância e adolescência (NOVATO; GROSSI, 2011). Nesta população jovem com diabetes, a prevalência do DM1 chega a 90% dos casos (SOCIEDADE BRASILEIRA DE DIABETES, 2019). Tal fato é agravante da condição, em que a exposição precoce à hiperglicemia antecipa os danos e o desenvolvimento de comorbidades associadas em uma população jovem. Quando estes pacientes não tem acesso à insulina, sua expectativa de vida é drasticamente reduzida. Além disso a educação no manejo do diabetes é um determinante no sucesso da insulinoterapia (SOCIEDADE BRASILEIRA DE DIABETES, 2019).

O tratamento do jovem com diabetes tem suas peculiaridades inerentes à esta fase da vida, visto que fatores sociais e familiares influenciarão grandemente no sucesso do controle

metabólico (DA CRUZ; COLLET; NÓBREGA, 2018). Mudanças na sensibilidade à insulina relacionadas à maturidade sexual e ao crescimento, bem como a capacidade de iniciar o autocuidado devem ser monitoradas para poder balizar os ajustes nas dosagens da insulina. Entretanto, o tratamento do diabetes vai além da insulinoterapia. A terapêutica do DM1, historicamente, segue a tríade composta por insulina, alimentação e atividade física. e educação, monitorização e orientação para os pacientes e seus familiares. Porém, com os avanços na terapia os fatores psicossociais devem ser considerados e a monitoração e educação no diabetes passam a ser essenciais no tratamento (SOCIEDADE BRASILEIRA DE DIABETES, 2019). Dentre os cuidados relacionados à alimentação, o acompanhamento nutricional e a prescrição de fitoterápicos auxilia em várias frentes, como o aumento da sensibilidade à insulina, controle da glicemia e prevenção e redução dos danos associados às comorbidades.

Estratégias Terapêuticas

A insulinoterapia é a principal forma de controle da glicemia em indivíduos com DM1 (SOCIEDADE BRASILEIRA DE DIABETES, 2019) podendo ser uma estratégia eficiente no controle e tratamento das alterações metabólicas e funcionais nos indivíduos que possuem diabetes, porém com algumas inconsistências. Em estudos com ratos Wistar foi demonstrado que a insulina foi capaz de controlar algumas alterações funcionais cardíacas, com melhora na frequência cardíaca e tempo de relaxamento miocárdio em animais experimentais tanto em fêmeas (FEIN et al., 1981) quanto em machos (DA SILVA et al., 2015). A insulina ainda mostrou-se eficiente em modular algumas vias de neutralização de EROs reduzindo a oxidação de proteínas, além de favorecer o aumento da captação de Ca^{2+} nos cardiomiócitos com consequente melhoria da função cardíaca (DA SILVA et al., 2015). Por outro lado, não foi capaz de reverter completamente outras alterações metabólicas (i.e. níveis de superoxido

dismutase, glutationa, produção de peróxido de hidrogênio [H_2O_2]) e funcionais (fração de ejeção e encurtamento fracionário) dos corações de animais com diabetes (DA SILVA et al., 2015). Em outro estudo a insulina foi capaz de reverter algumas alterações mitocondriais no coração, normalizando a homeostase de íons, reduzindo o estresse oxidativo e aumentando a eficiência da fosforilação oxidativa, além de normalizar os níveis glicêmicos no indivíduo diabético (MOREIRA et al., 2006). Porém, mesmo com a insulinoretapia, o risco de danos cardíacos ainda é elevado no paciente diabético (HÖLSCHER; BODE; BUGGER, 2016), e o controle metabólico baseado na insulinoterapia está associado à maiores taxas de falência cardíaca e morte, como revelado pelo estudo ACCORD (ACCORD STUDY GROUP, 2016).

A combinação de insulinoterapia com outros tratamentos essenciais, como o exercício físico, e tratamentos complementares, como o uso de fitoterápicos, tem demonstrado resultados ainda melhores no manejo dos sintomas e danos causados pelo DM (DA SILVA et al., 2015; LE DOUAIRON LAHAYE et al., 2011, 2012; WU et al., 2004).

O chá verde (*Camellia sinensis* (L.) Kuntze (Theaceae)), é uma bebida popularmente utilizada como medicamento tradicional, na forma de infusão, para vários propósitos incluindo a hiperglicemia e o controle do peso corporal (BARKAOUI et al., 2017; CHOPADE et al., 2008; FALLAH HUSEINI et al., 2006; MENG et al., 2019; RACHID et al., 2012; SEA-TAN; GROVE; LAMBERT, 2011). Ainda, é sabido que ele possui diversos efeitos positivos no manejo do diabetes (MENG et al., 2019; MOHABBULLA MOHIB et al., 2016).

Um estudo recente demonstrou os mecanismos de reconhecimento e iniciação da sinalização, até então desconhecidos, da epigalocatequina gallato (EGCG), principal componente ativo do chá verde, com foco especialmente nos podócitos (HAYASHI et al., 2020). Tal mecanismo se dá através da ativação do receptor de laminina de 67kDa (67LR) pelo EGCG, levando à mecanismos de preservação da morfologia e função da filtração gomerular,

regulada principalmente pelos podócitos, sugerindo uma melhora na nefropatia diabética. Tal mecanismo de ativação de sinalização intracelular também poderia explicar os efeitos positivos encontrados em outros órgãos, como o coração que também expressa o receptor 67LR.

Os efeitos positivos do chá verde na nefropatia diabética eram creditados à capacidade hipoglicemiante do chá (RENNO et al., 2008; YOKOZAWA; NOH; PARK, 2012), que levaria à menor sobrecarga glicêmica no órgão e consequentemente à menor dano oxidativo e glicação de proteínas. Entretanto, em humanos estes efeitos ainda são controversos. O primeiro estudo clínico controlado duplo-cego tratando pacientes com diabetes (100% DM2) com polifenóis proveniente do chá verde descreve uma redução na morte dos podócitos por apoptose e uma melhora considerável na função renal, com consequente redução da microalbuminúria (BORGES et al., 2016). Outro estudo clínico controlado duplo-cego mais recente, com 70,3% dos pacientes sendo DM1, falhou em alcançar controle glicêmico ou em melhorar a função renal após tratamento com o chá verde (VAZ et al., 2018). Entretanto, as catequinas do chá verde conseguem inibir a gliconeogênese (COLLINS et al., 2007), reduzindo o acúmulo de glicogênio e o desenvolvimento da nefrose glicogênica. Ainda, a EGCG pode ativar a proteína kinase B (AKT) aumentando a sinalização de vias de sobrevivência celular, preservando a morfologia do néfron e a função renal (HAYASHI et al., 2020). Estes efeitos podem contribuir para a prevenção do desenvolvimento da nefropatia diabética no paciente jovem (HARAGUCHI et al., 2020), por mecanismos que independem do controle glicêmico.

No coração do indivíduo com diabetes, o chá verde e seus polifenóis estão associados à redução do dano oxidativo, inflamação, fibrose e morte celular (OTHMAN et al., 2017). Entretanto, no estudo de Othman e colaboradores utilizando o modelo de DM2 induzido por estreptozotocina e nicotinamida, o chá verde conseguiu alcançar o efeito de controle glicêmico, reduzindo os níveis de glicose sanguínea nos animais tratados a níveis comparáveis aos dos

animais não diabéticos. Outros estudos também descrevem efeitos positivos relacionados à prevenção e ao tratamento da cardiomiopatia diabética, porém com algumas inconsistências que podem levar à conclusões precipitadas quanto a estes resultados. Na maioria dos estudos, efeitos positivos como a melhora da pressão arterial, redução da hipertrofia cardíaca e da morte celular, manutenção do perfil lipídico e da homeostase iônica no órgão e a prevenção de dano oxidativo que poderia levar à falência do órgão foram alcançados em animais com DM1 induzido experimentalmente após idade adulta, onde os efeitos metabólicos da hiperglicemia não são tão graves quanto quando induzidos em animais jovens (BABU et al., 2007; BABU; SABITHA; SHYAMALADEVI, 2006b; FIORINO et al., 2012; SAMARGHANDIAN; AZIMI-NEZHAD; FARKHONDEH, 2017).

Desta forma, este trabalho foi proposto para investigar os efeitos do tratamento com chá verde no diabetes tipo 1 induzido por estreptozotocina em animais jovens, de forma a avaliar o potencial preventivo da bebida especificamente no desenvolvimento da nefropatia diabética e da cardiomiopatia diabética.

Hipótese

Durante o desenvolvimento do projeto percebemos que na maioria dos trabalhos o tratamento com o chá verde era capaz de controlar a glicemia dos animais, mesmo que de forma parcial, e a isso era creditado os outros efeitos positivos encontrados. Desta forma, nossa principal hipótese era de que *a infusão de chá verde é eficiente em atenuar os danos renais e cardíacos causados pelo diabetes tipo 1, ainda, através do controle glicêmico.* Do mesmo modo, a hipótese nula automaticamente seria de que a infusão de chá verde não é eficiente em atenuar os danos causados pela hiperglicemia. O leitor encontrará nos próximos capítulos um cenário que não previmos: o tratamento não foi capaz de controlar a hiperglicemia, nem mesmo de forma parcial, porém foi capaz de prevenir muitos dos danos causados pelo diabetes induzido, tanto nos rins como no coração dos animais.

Objetivos

Investigar os efeitos do tratamento com chá verde (*Camellia sinensis* L. Kuntze) no diabetes tipo 1 induzido por estreptozotocina em animais jovens, de forma a avaliar o potencial preventivo da bebida especificamente no desenvolvimento da nefropatia diabética e da cardiomiopatia diabética.

Metodologia geral

Considerações éticas

O estudo foi conduzido em ratos (*Rattus novergicus*) e todos os procedimentos experimentais foram realizados em consonância com os padrões determinados pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA). O estudo foi submetido à avaliação da Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa (CEUA-UFV), e aprovado, tendo como registro o número de protocolo 53/2018. O certificado de autorização do CEUA-UFV pode ser encontrado no Anexo I.

Modelo animal

Foram utilizados 18 ratos da linhagem Wistar, machos, com 30 dias de idade, provenientes do Biotério Central da Universidade Federal de Viçosa. Os animais foram distribuídos aleatoriamente em alojamentos plásticos (41x34x16cm), com grade de aço, sendo dois animais por gaiola, em ambiente de temperatura (22 ± 2 ºC) e luz controladas, em ciclo claro-escuro de 12 h, tendo acesso a alimento (dieta padrão para roedores) e água *ad libitum*.

Preparo da infusão de chá verde

Foram adquiridos e homogeneizados o conteúdo de 5 embalagens de lotes diferentes de chá verde (*Camellia sinensis*) puro da marca comercial Leão® - Food and Beverages (Coca-Cola Company®). Os lotes foram misturados (1:1) e a infusão foi preparada misturando-se as folhas com água destilada aquecida (1:40 w/v, 80 °C) (PERVA-UZUNALIĆ et al., 2006). A mistura permaneceu em infusão por 20 minutos sob agitação em um agitador magnético. Após, a infusão foi filtrada em filtro de papel com poros de 0,45 µm, congelada a -80 °C e liofilizada.

As amostras liofilizadas foram armazenadas e foram dosadas e ressuspendidas em água destilada no momento de uso. A avaliação da quantidade total de fenóis e de EGCG, e a capacidade antioxidante total do extrato está detalhada na metodologia dos próximos capítulos.

Desenho experimental

Após sete dias de aclimatação no biotério, os grupos foram designados a seus respectivos propósitos (tratamento e controles) por sorteio. Um grupo (n = 6) foi designado como controle saudável. O diabetes tipo 1 experimental foi induzido nos outros 12 ratos dos 2 grupos restantes. Todos os animais ficaram em jejum de 12 h e após isso, o diabetes foi induzido pela injeção intraperitoneal (i.p.) de uma dose única de estreptozotocina (STZ) (Sigma Chemical Co., St, Louis, MO, USA) na dosagem de 60 mg/Kg de peso corporal, diluída em solução tampão citrato de sódio 0,01M, pH 4,5 (DA SILVA et al., 2016). O grupo controle recebeu a injeção de apenas tampão, no mesmo volume, pela mesma rota (DA SILVA et al., 2016), de forma a reproduzir o estresse da injeção. Após dois dias da indução, foi feito novo jejum de 12 h e coletamos amostras de sangue da veia caudal para medir a glicemia usando um glicosímetro (Accu-Chek® Performa, Roche LTDA). Todos os animais que foram induzidos ao diabetes apresentaram a glicemia de jejum acima de 250 mg/dL e foram incluídos no estudo (OTHMAN et al., 2017). Os ratos hiperglicêmicos integraram os grupos diabéticos (n = 6, cada). Desta forma, o experimento consistiu em três grupos experimentais: controle saudável (n = 6); controle diabético (n = 6); diabéticos tratados com infusão de chá verde (n = 6), que recebeu uma dosagem de 100 mg de extrato liofilizado de chá verde por Kg de peso corporal, ressuspendidos num volume de 0,6 mL de água destilada. Os grupos controle receberam apenas a água, no volume de 0,6 mL. Todos os tratamentos foram administrados via gavagem, diariamente, por 42 dias.

Após o período experimental, os animais foram eutanasiados por aprofundamento em anestesia (tiopental sódico, 60 mg/Kg i.p.) seguido de punção cardíaca e exsanguinação (RASHEED et al., 2018).

Referências

ACCORD STUDY GROUP. Nine-Year Effects of 3.7 Years of Intensive Glycemic Control on Cardiovascular Outcomes. **Diabetes Care**, v. 39, n. 5, p. 701–708, maio 2016.

AHMED, A. M. History of diabetes mellitus. **Saudi Medical Journal**, v. 23, n. 4, p. 373–378, 2002.

AMERICAN DIABETES ASSOCIATION. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2021. **Diabetes Care**, v. 44, n. Supplement 1, p. S15–S33, 9 jan. 2021.

ARATAKI, M. On the postnatal growth of the kidney, with special reference to the number and size of the glomeruli (albino rat). **American Journal of Anatomy**, v. 36, n. 3, p. 399–436, 1926.

BABU, P. V. A. et al. Green tea attenuates diabetes induced Maillard-type fluorescence and collagen cross-linking in the heart of streptozotocin diabetic rats. **Pharmacological Research**, v. 55, n. 5, p. 433–440, maio 2007.

BABU, P. V. A.; SABITHA, K. E.; SHYAMALADEVI, C. S. Green tea impedes dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca2+-ATPase and Na+/K+-ATPase activity in the heart of streptozotocin-diabetic rats. **Chemico-Biological Interactions**, v. 162, n. 2, p. 157–164, ago. 2006a.

BABU, P. V. A.; SABITHA, K. E.; SHYAMALADEVI, C. S. Therapeutic effect of green tea extract on oxidative stress in aorta and heart of streptozotocin diabetic rats. **Chemico-Biological Interactions**, v. 162, n. 2, p. 114–120, ago. 2006b.

BAILEY, C. J. Renal glucose reabsorption inhibitors to treat diabetes. **Trends in Pharmacological Sciences**, v. 32, n. 2, p. 63–71, 2011.

BAOTHMAN, O. A. et al. The role of Gut Microbiota in the development of obesity and Diabetes. **Lipids in Health and Disease**, v. 15, n. 1, 2016.

BARKAOUI, M. et al. Ethnobotanical survey of medicinal plants used in the traditional treatment of diabetes in Chtouka Ait Baha and Tiznit (Western Anti-Atlas), Morocco. **Journal of Ethnopharmacology**, v. 198, n. June 2016, p. 338–350, 2017.

BATTIPROLU, P. K. et al. Diabetic cardiomyopathy and metabolic remodeling of the heart. **Life Sciences**, v. 92, n. 11, p. 609–615, 2013.

BORGES, C. M. et al. The use of green tea polyphenols for treating residual albuminuria in diabetic nephropathy: A double-blind randomised clinical trial. **Scientific Reports**, v. 6, n. June, p. 1–9, 2016.

BOUERI, B. F. DA C. et al. Body composition in male rats subjected to early weaning and treated with diet containing flour or flaxseed oil after 21 days until 60 days. **Journal of Developmental Origins of Health and Disease**, v. 6, n. 6, p. 553–557, 17 dez. 2015.

BOYER, J. K. et al. Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. **American Journal of Cardiology**, v. 93, n. 7, p. 870–875, 2004.

CHEN, T.-S. et al. Green tea epigallocatechin gallate enhances cardiac function restoration

through survival signaling expression in diabetes mellitus rats with autologous adipose tissue-derived stem cells. **Journal of Applied Physiology**, v. 123, n. 5, p. 1081–1091, nov. 2017.

CHOPADE, V. V et al. Green tea (Camellia sinensis): chemistry , traditional , medicinal uses and its pharmacological activities- a review. **Pharmacognosy Reviews**, v. 2, n. 3, p. 157–162, 2008.

COLLINS, Q. F. et al. Epigallocatechin-3-gallate (EGCG), A Green Tea Polyphenol, Suppresses Hepatic Gluconeogenesis through 5′-AMP-activated Protein Kinase. **Journal of Biological Chemistry**, v. 282, n. 41, p. 30143–30149, 12 out. 2007.

DA CRUZ, D. S. M.; COLLET, N.; NÓBREGA, V. M. Qualidade de vida relacionada à saúde de adolescentes com dm1- revisão integrativa. **Ciencia e Saude Coletiva**, v. 23, n. 3, p. 973–989, 2018.

DA SILVA, E. et al. Ventricular remodeling in growing rats with experimental diabetes: The impact of swimming training. **Pathology Research and Practice**, v. 209, n. 10, p. 618–626, 2013.

DA SILVA, E. et al. Swimming training attenuates the morphological reorganization of the myocardium and local inflammation in the left ventricle of growing rats with untreated experimental diabetes. **Pathology - Research and Practice**, v. 212, n. 4, p. 325–334, abr. 2016.

DA SILVA, M. F. et al. Attenuation of Ca 2+ homeostasis, oxidative stress, and mitochondrial dysfunctions in diabetic rat heart: insulin therapy or aerobic exercise? **Journal of Applied Physiology**, v. 119, n. 2, p. 148–156, 15 jul. 2015.

EID, A. et al. Intrinsic gluconeogenesis is enhanced in renal proximal tubules of Zucker diabetic fatty rats. **Journal of the American Society of Nephrology**, v. 17, n. 2, p. 398–405, 2006.

FALLAH HUSEINI, H. et al. Review of anti-diabetic medicinal plant used in traditional medicine. **Journal of Medicinal Plants**, v. 5, n. SUPPL. 2, p. 1–8, 2006.

FEIN, F. S. et al. Reversibility of diabetic cardiomyopathy with insulin in rats. **Circulation Research**, v. 49, n. 6, p. 1251–1261, 1981.

FIORAVANTI, C. A descoberta da insulina. **Pesquisa Fapesp**, abr. 2021. Disponível em: <https://revistapesquisa.fapesp.br/a-descoberta-da-insulina/>

FIORINO, P. et al. The effects of green tea consumption on cardiometabolic alterations induced by experimental diabetes. **Experimental Diabetes Research**, v. 2012, 2012.

GERBER, L. K.; ARONOW, B. J.; MATLIB, M. A. Activation of a novel long-chain free fatty acid generation and export system in mitochondria of diabetic rat hearts. **American Journal of Physiology - Cell Physiology**, v. 291, n. 6, p. 1198–1207, 2006.

GILBERT, R. E. Proximal tubulopathy: Prime mover and key therapeutic target in diabetic kidney disease. **Diabetes**, v. 66, n. 4, p. 791–800, 2017.

HARAGUCHI, R. et al. New Insights into the Pathogenesis of Diabetic Nephropathy: Proximal Renal Tubules Are Primary Target of Oxidative Stress in Diabetic Kidney. **Acta Histochemica Et Cytochemica**, v. 53, n. 2, p. 21–31, 2020.

HAYASHI, D. et al. The mechanisms of ameliorating effect of a green tea polyphenol on diabetic nephropathy based on diacylglycerol kinase α. **Scientific Reports**, v. 10, n. 1, p. 1–12, 2020.

HERMAN-EDELSTEIN, M.; DOI, S. Q. **Pathophysiology of diabetic nephropathy**. Elsevier Inc., 2016.

HÖLSCHER, M. E.; BODE, C.; BUGGER, H. Diabetic cardiomyopathy: Does the type of diabetes matter? **International Journal of Molecular Sciences**, v. 17, n. 12, 2016.

HUANG, P. C. et al. Cellular apoptosis and cardiac dysfunction in STZ-induced diabetic rats attenuated by anthocyanins via activation of IGFI-R/PI3K/Akt survival signaling. **Environmental Toxicology**, v. 32, n. 12, p. 2471–2480, 2017.

INTERNATIONAL DIABETES FEDERATION. **IDF Diabetes Atlas**. 9th edn ed. Brussels, Belgium. 2019.

JOLLES, S. Paul Langerhans. **Journal of Clinical Pathology**, v. 55, n. 4, p. 243–243, 1 abr. 2002.

LE DOUAIRON LAHAYE, S. et al. Effects of exercise training combined with insulin treatment on cardiac NOS1 signaling pathways in type 1 diabetic rats. **Molecular and Cellular Biochemistry**, v. 347, n. 1–2, p. 53–62, 2011.

LE DOUAIRON LAHAYE, S. et al. Combined insulin treatment and intense exercise training improved basal cardiac function and Ca 2+ -cycling proteins expression in type 1 diabetic rats . **Applied Physiology, Nutrition, and Metabolism**, v. 37, n. 1, p. 53–62, 2012.

LEVELT, E. et al. Diabetic cardiomyopathy: pathophysiology and potential metabolic interventions state of the art review. **European Journal of Endocrinology**, v. 178, n. 4, p. R127–R139, abr. 2018.

LIAO, H.-E. et al. Deep Sea Minerals Prolong Life Span of Streptozotocin-Induced Diabetic Rats by Compensatory Augmentation of the IGF-I-Survival Signaling and Inhibition of Apoptosis. **Environmental toxicology**, v. 31, p. 769–781, 2016.

MATHER, A.; POLLOCK, C. Glucose handling by the kidney. **Kidney International**, v. 79, n. 120, p. S1–S6, mar. 2011.

MENG, J.-M. et al. Effects and Mechanisms of Tea for the Prevention and Management of Diabetes Mellitus and Diabetic Complications: An Updated Review. **Antioxidants**, v. 8, n. 6, p. 170, 10 jun. 2019.

MOHABBULLA MOHIB, M. et al. Protective role of green tea on diabetic nephropathy - A review. **Cogent Biology**, v. 2, n. 1, 18 out. 2016.

MOREIRA, P. et al. Insulin Attenuates Diabetes-Related Mitochondrial Alterations: A Comparative Study. **Medicinal Chemistry**, v. 2, n. 3, p. 299–308, 2006.

NOVATO, T. DE S.; GROSSI, S. A. A. Fatores associados á qualidade de vida de jovens com diabetes mellitus do tipo 1. **Revista da Escola de Enfermagem**, v. 45, n. 3, p. 770–776, 2011.

OKOSHI, K. et al. Miocardiopatia diabética. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 51, n. 2, p. 160–167, 2007.

OTHMAN, A. I. et al. Epigallocatechin-3-gallate protects against diabetic cardiomyopathy through modulating the cardiometabolic risk factors, oxidative stress, inflammation, cell death and fibrosis in streptozotocin-nicotinamide-induced diabetic rats. **Biomedicine and Pharmacotherapy**, v. 94, p. 362–373, 2017.

OU, H. C. et al. Cardiac contractile dysfunction and apoptosis in streptozotocin-induced

diabetic rats are ameliorated by garlic oil supplementation. **Journal of Agricultural and Food Chemistry**, v. 58, n. 19, p. 10347–10355, 2010.

PERVA-UZUNALIĆ, A. et al. Extraction of active ingredients from green tea (Camellia sinensis): Extraction efficiency of major catechins and caffeine. **Food Chemistry**, v. 96, n. 4, p. 597–605, jun. 2006.

PRATT, J. H. THE RELATION OF THE PANCREAS TO DIABETES. **JAMA: The Journal of the American Medical Association**, v. 55, n. 25, p. 2112, 17 dez. 1910.

RACHID, A. et al. Ethnopharmacological survey of medicinal plants used in the traditional treatment of diabetes mellitus in the North Western and South Western Algeria. **Journal of Medicinal Plants Research**, v. 6, n. 10, p. 2041–2050, 2012.

RASCH, R. Prevention of diabetic glomerulopathy in streptozotocin diabetic rats by insulin treatment - Albumin excretion. **Diabetologia**, v. 18, n. 5, p. 413–416, 1980.

RASHEED, N. O. A. A. et al. Paradoxical cardiotoxicity of intraperitoneally-injected epigallocatechin gallate preparation in diabetic mice. **Scientific Reports**, v. 8, n. 1, p. 7880, 18 dez. 2018.

RENNO, W. M. et al. Effect of greean tea on kidney tubules of diabetic rats. **British Journal of Nutrition**, v. 100, n. 03, p. 652–659, 6 set. 2008.

RIBBACK, S. et al. PI3K/AKT/mTOR pathway plays a major pathogenetic role in glycogen accumulation and tumor development in renal distal tubules of rats and men. **Oncotarget**, v. 6, n. 15, p. 13036–13048, 2015.

RITCHIE, R. H.; DALE ABEL, E. Basic Mechanisms of Diabetic Heart Disease. **Circulation Research**, p. 1501–1525, 2020.

SAMARGHANDIAN, S.; AZIMI-NEZHAD, M.; FARKHONDEH, T. Catechin Treatment Ameliorates Diabetes and Its Complications in Streptozotocin-Induced Diabetic Rats. **Dose-Response**, v. 15, n. 1, p. 155932581769115, 6 mar. 2017.

SEA-TAN, S.; GROVE, K. A.; LAMBERT, J. D. Weight control and prevention of metabolic syndrome by green tea. **Pharmacological Research**, v. 64, n. 2, p. 146–154, ago. 2011.

SERTORIO, M. N. et al. Arsenic exposure intensifies glycogen nephrosis in diabetic rats. **Environmental Science and Pollution Research**, v. 26, n. 12, p. 12459–12469, 7 abr. 2019.

SOCIEDADE BRASILEIRA DE DIABETES. **Diretrizes - Sociedade Brasileira de Diabetes 2017-2018**. São Paulo: Clannad, 2017.

SOCIEDADE BRASILEIRA DE DIABETES. **Diretrizes Sociedade Brasileira de Diabetes 2019-2020**. São Paulo: Clannad, 2019.

SOUZA, A. C. F. et al. Arsenic aggravates oxidative stress causing hepatic alterations and inflammation in diabetic rats. **Life Sciences**, v. 209, n. 9, p. 472–480, set. 2018.

SOUZA, A. C. F. et al. Combined effects of arsenic exposure and diabetes on male reproductive functions. **Andrology**, v. 7, n. 5, p. 730–740, 2019.

SU, J. et al. Mechanism of progression of diabetic kidney disease mediated by podocyte mitochondrial injury. **Molecular Biology Reports**, n. 0123456789, 12 set. 2020.

TEKINER, H. Aretaeus of Cappadocia and his treatises on diseases. **Turkish Neurosurgery**,

v. 25, n. 3, p. 508–512, 2015.

VALLON, V.; THOMSON, S. C. Targeting renal glucose reabsorption to treat hyperglycaemia: the pleiotropic effects of SGLT2 inhibition. **Diabetologia**, v. 60, n. 2, p. 215–225, 2017.

VARGA, Z. V. et al. Interplay of oxidative, nitrosative/nitrative stress, inflammation, cell death and autophagy in diabetic cardiomyopathy. **Biochimica et Biophysica Acta - Molecular Basis of Disease**, v. 1852, n. 2, p. 232–242, 2015.

VAZ, S. R. et al. Effects of green tea extract on oxidative stress and renal function in diabetic individuals: A randomized, double-blinded, controlled trial. **Journal of Functional Foods**, v. 46, n. April, p. 195–201, 2018.

VECCHIO, I. et al. The Discovery of Insulin: An Important Milestone in the History of Medicine. **Frontiers in Endocrinology**, v. 9, n. October, p. 1–8, 23 out. 2018.

VIGGIANO, C. E. Diabetes Mellitus. In: CUPPARI, L. (Ed.). . **Nutrição nas doenças crônicas não-transmissíveis**. 1ª ed. Barueri, SP: Manole, 2009. p. 143–189.

WOOD, P.; PIRAN, S.; LIU, P. P. Diastolic heart failure: Progress, treatment challenges, and prevention. **Canadian Journal of Cardiology**, v. 27, n. 3, p. 302–310, 2011.

WU, L.-Y. et al. Effect of Green Tea Supplementation on Insulin Sensitivity in Sprague−Dawley Rats. **Journal of Agricultural and Food Chemistry**, v. 52, n. 3, p. 643–648, 2004.

YE, G. et al. Catalase Protects Cardiomyocyte Function in Models of Type 1 and Type 2 Diabetes. **Diabetes**, v. 53, p. 1336–1343, 2004.

YOKOZAWA, T.; NOH, J. S.; PARK, C. H. Green tea polyphenols for the protection against renal damage caused by oxidative stress. **Evidence-based Complementary and Alternative Medicine**, v. 2012, 2012.

ZHI, Y. F.; PRINS, J. B.; MARWICK, T. H. Diabetic cardiomyopathy: Evidence, mechanisms, and therapeutic implications. **Endocrine Reviews**, v. 25, n. 4, p. 543–567, 2004.

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Green tea infusion prevents diabetic nephropathy aggravation in recent-onset type 1 diabetes regardless of glycemic control

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Green tea infusion prevents diabetic nephropathy aggravation in recent-onset type 1 diabetes regardless of glycemic control

Running title: Tea and diabetic nephropathy

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Abstract

Ethnopharmacological relevance: Green tea, traditionally used as antidiabetic medicine, affects positively the diabetic nephropathy and it was assumed that these beneficial effects were due to the tea's hypoglycemiant capacity, reducing the glycemic overload and, consequently, the advanced glycation end products rate and oxidative damage. However, these results are still controversial because tea is not always able to exert a hypoglycemic action, as shown by previous studies.

Aim: Investigate if green tea infusion can generate positive outcomes for the kidney independently of glycemic control, using a model of severe type 1 diabetes.

Material and methods: We treated streptozotocin type 1 diabetic young rats with 100 mg/Kg of green tea, daily, for 42 days, and evaluated the serum and tissue markers for stress and function, also, we analyzed the ion dynamics in the organ and the morphological alterations promoted by diabetes and green tea treatment. Besides, we analyzed, by an in silico approach, the interactions of the green tea main catechins with the proteins expressed in the kidney.

Results: Our findings reveals that the components of green tea can interact with proteins participating in cell signaling pathways that regulate energy metabolism, including glucose and glycogen synthesis, glucose reabsorption, hypoxia management, and cell death by apoptosis. Such interaction leads to reduced accumulation of glycogen in the organ, as well as protects DNA. These results also reflect in a preserved glomerulus morphology, with improvement in pathological features, and suggesting a prevention of kidney function impairment.

Conclusion: Our results show that such benefits are achieved regardless of the blood glucose status, and are not dependent on the reduction of hyperglycemia.

Keywords

Diabetic nephropathy; type 1 diabetes; recent-onset diabetes; diabetic kidney disease; green tea.

1. Introduction

Diabetic nephropathy (DN) affects 25% - 35% of type 1 and 2 diabetic patients (Herman-Edelstein and Doi, 2016), and account for about 45% of the patients with end-stage renal disease (Su et al., 2020). It progresses from the increase in the glomerular filtration rate to the total failure of the kidneys, passing through alterations that indicate damage to the renal glomeruli and tubules, albuminuria, mesangial expansion, fibrosis, and vascular damage (Gilbert, 2017; Herman-Edelstein and Doi, 2016). In addition, glycogenic accumulation in the proximal tubules is a common feature in DN, and one of the earliest signals of metabolic impairment in the organ (Gilbert, 2017; Haraguchi et al., 2020). Such damage can progress in renal cells to pre-neoplastic lesions which, if left untreated, may progress to renal cancer (Ribback et al., 2015).

Green tea (*Camellia sinensis* (L.) Kuntze (Theaceae)), popularly used as a traditional medicine, in the form of infusion, for many porpoises including hyperglycaemia (Barkaoui et al., 2017; Chopade et al., 2008; Fallah Huseini et al., 2006; Rachid et al., 2012), is known to exert positive effects in diabetes management (Meng et al., 2019; Mohabbulla Mohib et al., 2016). Recent studies have shed light on the mechanisms that tea catechins affect positively the DN, with special focus on the podocyte (Hayashi et al., 2020), through the activation of the 67kDa laminin receptor (67LR) by the epigallocatechin gallate (EGCG), the main polyphenol in green tea. Such interaction results in the preservation of podocyte morphology and the glomerular filtration function, suggesting an improvement in DN. However, tubular alterations, with glycogen accumulation, and aberrant activation of the advanced glycated end-products and its receptor (AGE/RAGE system), affecting the cellular renovation and survival, are seem to be the primary cause of proximal tubular function disruptment (Haraguchi et al., 2020). This, in turn, can affect glomerular function by the proximal tubule/glomerulus feedback system, leading to glomerular damage and contributing to the progression of DN.

It was assumed that the beneficial effects of the green tea on proximal tubules were due to the tea's hypoglycemiant capacity (Renno et al., 2008; Yokozawa et al., 2012), reducing the glycemic overload and consequently AGE rate and oxidative damage. However, tea effects in diabetic human subjects are still controversial. The first double-blind controlled trial treating diabetic patients (being 100% type 2 diabetic) with green tea polyphenols describes a reduction in podocyte apoptosis and an improvement of kidney function by reducing microalbuminuria (Borges et al., 2016). Another double-blind controlled trial conducted with diabetic adult patients (being 70.3% type 1 diabetic) fail to achieve glycemic control or improve renal function after green tea consumption (Vaz et al., 2018). On the other hand, tea catechins can inhibit gluconeogenesis by activating the 5'AMP-activated protein kinase (AMPK) (Collins et al., 2007), possibly reducing glycogenic nephrosis. Also, EGCG can activate the protein kinase B (AKT) pathway enhancing cell survival and preserving nephron morphology (Hayashi et al., 2020). These effects may contribute to the prevention of DN development in recent-onset diabetes (Haraguchi et al., 2020).

In a previous study, our group demonstrated that the infusion of green tea was not able to prevent hyperglycemia in animals with experimental type 1 diabetes induced by streptozotocin (STZ) in young male Wistar rats (Ladeira et al., 2020a). Therefore, in the same model, we tested the hypothesis that the beneficial effects of tea in DN go beyond glycemic control. In this way, we investigated the effects of green tea infusion treatment on diabetic kidney disease in recent-onset type 1 diabetic young rats. Also, we used bioinformatics tools to explore tea catechin interaction in signaling pathways in the kidney.

2. Materials and methods

2.1. Animals and ethics

Eighteen male Wistar rats (30 days old; 82.52 ± 10.83g) were housed, two per cage, in polypropylene cages with autoclaved sawdust as cage bed, under controlled conditions of temperature (22 ± 2 ºC) and light-dark cycles (12/12h), and received food (Presence Alimentos, Paulínea, SP, Brazil) and water *ad libitum*. The use of animals in the research was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa (CEUA/UFV – protocol number 53/2018).

2.2. Green tea infusion preparation and analysis

Green tea (*Camellia sinensis*) leaves were obtained from Leão® - Food and Beverages (Coca-Cola Company®, lot LO159), and prepared as infusion, to mimic the way it is normally consumed by humans. The infusion was prepared mixing the leaves with warm distilled water (1:40 w/v, 80 °C) (Perva-Uzunalić et al., 2006). The mixture remained infused for 20 minutes on a magnetic stirrer. Then, it was filtered through a 0.45 µm porous filter, frozen at -80 °C and lyophilized. The lyophilized samples were resuspended in distilled water at the moment of use. The treatment and placebo (water) were administered by gavage.

The chromatographic profile, or fingerprint, was determined as described by Kim-Park et al. (2016), with some modifications. High-performance liquid chromatography (HPLC) (Prominence LC-20A, Shimadzu, Kyoto, Japan), equipped with Diode Arrangement Detector (DAD), LC-20AD pump, SPD-M20A detector, CTO-20A oven and LabSolutions software, was used to determine the EGCG content using a maximal absorption peaks at 272nm. It was used a Vydac C18 (4.6 x 250 mm) column, at 30 °C, with a 5µL injection volume. The mobile phase was composed of water and 2.0% acetic acid (1:1). The infusion lyophilized powder was

suspended in methanol before analysis. The mobile phase flow rate was 1.0 mL/min and the run time was 15 min. The retention time of the main component, EGCG, was 4.5 min and the total amount of it was calculated using a standard curve ($r^2 = 0.9967$) developed under the same conditions using an EGCG chemical standard (≥ 98.0%, Sigma Aldrich Inc. - CAS Number 989-51-5. St. Louis, MO, USA). The EGCG content was shown to be 19.38% of the total GTI content. The fingerprint is presented in the Figure 1.

Also, we determined the total phenolic content and antioxidant capacity as previously described (Ladeira et al., 2020a). GTI presented a total amount of phenolic components of 3.88 ± 2.49 mg gallic acid equivalent (GAE)/g GTI. The extract presented an antioxidant capacity of 3.26 ± 0.06 µMol Trolox equivalent (TE)/g GTI in the 2,2'-Azinobis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) assay and 46.38 ± 4.10 µMol FeSO4/g GTI in the ferric reducing antioxidant power (FRAP) assay.

2.3. Experimental design, euthanasia, and tissue collection

Twelve rats were randomly selected to integrate the diabetics groups. After 12h fasting, diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) (Sigma Chemical Co., St, Louis, MO, USA) at a dosage of 60 mg/kg of body weight (BW) diluted in 0.01 M sodium citrate buffer, pH 4.5. The healthy control group (n=6) received the buffer alone (i.p.) to simulate the injection stress. Fasting blood glucose levels were measured after 2 days using a glucometer (Accu-Chek® Performa, Roche LTDA. Jaguaré, SP, Brazil) in blood samples collected at the tail vein. All STZ-injected animals presented the fasting glycemia levels higher than 250 mg/dL and were included in the study. The diabetic rats were divided into two groups (n=6, each). Therefore, the experiment consisted of three groups: the healthy control group (Ctrl, n=6), which received water as a placebo; the diabetic control group (STZ, n=6), which also received water; and the diabetic group treated with the green tea infusion

(STZ+GTI, n=6), that received the GTI (100 mg/kg body weight). All treatments were administered by gavage, daily, for 42 days. The dosage was equivalent to 7 cups (200mL) of tea, prepared according to the manufacturer instructions, mimicking a feasible human consumption dosage, considering survey data from the Asian population (Mineharu et al., 2011).

We monitored body weight and water consumption using a precision scale (BEL M503, e=0.001g, Piracicaba, SP, Brazil), and 12h fasting blood glucose using test strips and a glucometer (Accu-Chek® Performa, Roche LTDA. Jaguaré, SP, Brazil) in blood samples from the tail vein.

After the experimental period, the animals were euthanized by deep anesthesia (sodium thiopental, 60 mg/kg i.p.) followed by cardiac puncture and exsanguination. The kidneys were removed and weighed. One kidney (right) of each animal was frozen in liquid nitrogen and stored at -80 °C for enzymatic analysis, the other one (left) was immersed in Karnovsky fixative solution for 24h for histopathological analyses. The renal somatic index (RSI) was calculated using the ratio between the kidney weight (KW) and BW, where RSI = KW/BW × 100 (Sertorio et al., 2019).

2.4. Renal function markers

Blood samples collected by cardiac puncture at the euthanasia were centrifuged at 4600 rpm for 15 min at 4 ºC and the serum was separated. Then we performed the analysis for quantification of urea and creatinine in the serum using biochemical kits (Bioclin Laboratories, Belo Horizonte, MG, Brazil) at the BS-200 equipment (Bioclin Laboratories, Belo Horizonte, MG, Brazil) following the manufacturer's instructions.

2.5. Antioxidant enzyme and nitric oxide analysis

The antioxidant enzyme analysis was performed with the supernatant obtained from 100 mg of frozen kidney tissue homogenized in ice-cold phosphate buffer (pH 7.0) and centrifuged at 12000 rpm for 10 minutes at 4 ºC. The activity of the superoxide dismutase enzyme (SOD) was assessed by the pyrogallol method based on the ability of this enzyme to catalyze the reaction of the superoxide (O^{-2}) and hydrogen peroxide (H_2O_2) (Dieterich et al., 2000). The glutathione S-transferase (GST) activity was measured according to the method of Habig et al. (1974), and calculated from the rate of NADPH oxidation. The activity of catalase (CAT) was determined by measuring the kinetics of hydrogen peroxide (H_2O_2) decomposition as described by Aebi (1984). The nitric oxide (NO_2^- and NO_3^-) levels were quantified by the Griess method (Ricart-Jané et al., 2002). The values of enzyme activities were normalized by the total protein content, determined with the Folin–Ciocalteu method according to Lowry et al. (1994).

2.6. Determination of Ca^{2+}, Na^+/K^+, Mg^{2+}, and total ATPase activities

The ATPase activity was determined following the procedure described by Al-Numair et al (2015). Briefly, 100mg of kidney fragments were homogenized in Tris-HCl buffer (0.1M, pH 7.4) and centrifuged at 12000 rpm for 10 min at 5ºC. The supernatant was used for the determination of the ATPase activity using NaCl, KCl, MgCl, and CaCl solutions at 0.1M. ATP solution (0.1M) was used as a substrate to generate free phosphate by the ATPases. The reaction was stopped with a cold solution of 10% TCA. Then, we centrifuged at 6000 rpm for 10 min and the supernatant was used to determine the inorganic phosphorus content by the Fiske and Subbarow method (Fiske and Subbarow, 1925). The ATPase activities were expressed as µMol of inorganic phosphorus/min mg of protein.

2.7. Chemical elements analysis

The proportion of chemical elements in the renal cortex was assessed per area in fragments of frozen kidney, as described before (Ladeira et al., 2020b). We measured the proportion of sodium (Na), magnesium (Mg), chlorine (Cl), potassium (K), and calcium (Ca). Fragments were dried at 60 °C for 96h, coated with carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK), and analyzed in a scanning electron microscope (JEOL, JSM-6010LA) with a Silicon Drift type X-ray detector system. The analysis was performed in an area of 50 μm², using an accelerating voltage of 20 kV and a working distance of 10 mm. The results were expressed as a mean value of the proportions between the elements present in the samples.

2.8. Histopathological, stereological analysis, and assessment of DNA damage

The fragments fixed in Karnovsky solution were then dehydrated in a crescent ethanol series and embedded in Historesin® (Leica, Nussloch, Germany). A rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany) was used to cut the material into histological sections of 3 μm thickness, then, the section was mounted in glass slides and reacted with periodic acid and Schiff reagent (PAS), and counterstained with hematoxylin for histopathological and stereological evaluation. The analysis was carried as described before by Sertorio *et al* (2019). Also, slices stained with Toluidine Blue – Sodium borate 1% were used to analyze qualitatively the glomeruli morphopathological features. We analyzed 40 glomeruli, randomly photographed, per experimental animal.

DNA damage was evaluated in sections of the kidney cortex stained with acridine orange (AO; green) and propidium iodide (PI; red) (Bernas et al., 2005; Suzuki et al., 1997). This fluorescent stain allows to evaluate the DNA damage, as damaged DNA presents red color, marked with PI, and integral DNA is marked in green by the AO (Dias et al., 2019). Digital

images were captured using a photomicroscope (Olympus AX 70 TRF, Tokyo, Japan) and analyzed with Image-Pro Plus® 4.5 (Media Cybernetics, Silver Spring, MD) software according to Lima *et al* (2018).

2.9. Statistical analysis

All the results were submitted to the Shapiro-Wilk test to check normality. The data expressed as percentages were transformed by angular transformation before the analysis. Results were expressed as mean ± standard deviation (mean ± SD) and analyzed using unpaired *t*-test when the variances are equal (by *F* test) and unpaired *t*-test with Welch's correction for data with unequal variances (Ctrl vs STZ; STZ vs STZ+GTI). The non-parametric data were compared with the Mann-Whitney test. The correlation analysis was carried out following Pearson's correlation method, as the analyzed data were normally distributed. Statistical significance was established at $P \leq 0.05$.

2.10. In silico pathway exploration

After the in vivo experiment, we explored, through an in silico approach, the interactions of green tea catechins with proteins, in search of possible signaling pathways involved in the generation of the observed effects. For this, we built and analyzed a network of interactions based on information from the STRING and STITCH databases (Szklarczyk et al., 2017, 2016).

A chemo-biology interactome network was built to elucidate the interactions between the tea compounds (catechins) and proteins expressed in the kidneys related to the positive effects founded in the in vivo experiment with diabetic rats. A prospective evaluation of compound-protein interactions (CPI) was done with the STITCH v.5.0 database (http://stitch.embl.de/) (Szklarczyk et al., 2016). The CPI settings were done according to (de

Godoi et al., 2020). Briefly, the network downloaded from the database was limited to no more than 50 interactions, medium confidence score (0.400), network depth equal to 1, and the following methods of predictions were activated: experiments, databases, co-expression, and predictions. The search was set to retrieve results for seven green tea catechins (Catechin, Catechin gallate, Epicatechin, Epicatechin Gallate, Epigallocatechin Gallate, Gallocatechin, and Gallocatechin Gallate), using the *Homo sapiens* species. All the catechins were imputed individually in the search, however, only four (Catechin, Epicatechin, Epicatechin Gallate, and Epigallocatechin Gallate) retrieve results of interactions, generating four small CPI subnetworks (data not showed), that were used in the posterior analysis.

The four catechin-proteins network analysis was performed using Cytoscape v.3.8.0 (Shannon, 2003). The four subnetworks were merged using the merge tool with the union function of the software. Then, we "STRINGfy" the resultant network, through the STRING v.1.5.1 (Szklarczyk et al., 2017) to enable the protein interaction functions analysis. After that, we performed the Molecular Complex Detection analysis to detect clusters (i.e. densely connected regions) that may suggest functional protein complexes, with the MCODE v.1.6.1 app (Bader and Hogue, 2003). To that, the app was set up as described before (de Godoi et al., 2020). An MCODE score was calculated for each cluster. Additionally, the Reactome Pathways (Jassal et al., 2020) related to diabetic nephropathy pathogenesis were selected.

To identify proteins that could be considered as a key regulator of essential biological processes to the network da network, we performed a centrality analysis, using the CentiScaPe v.2.2 app (Scardoni et al., 2009) for Cytoscape. This app identifies the node (i.e. protein) that has a central position in the network by measuring the "betweenness" and "degree" of the node. Nodes with high betweenness and degree levels are named "bottlenecks" and are more probable to connect different clusters in the network (Yu et al., 2007).

The functional information about all the network proteins, as the tissue-specific expression score and the cellular location score, was accessed by the ClueGO v.2.5.7 and CluePedia v.1.5.7 apps (Bindea et al., 2013, 2009). The Specific Organ Expression Score (SOES) was accessed in this analysis and a filter to protein expression was used to apply the SOES to the PPI (Protein-Protein Interactome). Protein functions were accessed in the Human Gene database - GeneCards (http://www.genecards.org/) (Rebhan et al., 1998) and compared with the functions related to their effects in diabetic nephropathy, described in the scientific literature.

3. Results

3.1. Experimental results

Diabetic animals showed classical signs of polydipsia (Table 1) and polyuria observed during the experiment (noted in the cage bed). The initial body weight was maintained throughout the experimental period in the animals of the two diabetic groups, indicating a stagnation in the body weight gain, and a commitment of the body development by hyperglycemia, when compared to the healthy control group. Both diabetic groups remain severely hyperglycemic, and green tea infusion did not reduce blood glucose levels in the treated group.

The kidney weight was reduced in the diabetic groups when compared with the Ctrl group ($P < 0.0001$) and it was reflected in the kidney somatic index ($P < 0.0001$). In addition, this result may be related to the body development impairment due to hyperglycemia, as showed by bodyweight reduced values. These data are presented in Table 1.

The serological analysis revealed that diabetes increased the serum levels of urea and the GTI did not act modifying this parameter (Figure 2, A). In the same way, creatinine levels

were also higher in the diabetic groups than the healthy control without effect by GTI treatment (Figure 2, B).

The GTI was capable of inducing a higher activity of GST enzyme (Figure 3, C), and nitric oxide levels were increased in both diabetics groups, without any effect of GTI treatment (Figure 3, D). The activity of SOD and CAT in the kidney were not impacted by diabetes or GTI treatment in the kidney.

Figure 4 shows the measurements of microelements and ions that participate in the filtration and reabsorption dynamics in the kidney. Despite diabetes have not affected any of the elements analyzed (Figure 4, A – F), green tea infusion altered Mg and Cl amounts compared to the STZ group (Figure 4, B and D). Although all altered values (Mg and Cl) remain between the Ctrl normal reported values, the relationship between all these elements were impaired by diabetes (Figure 4, G), and GTI was not able to restore the homeostatic environment of ion dynamics. Additionally, we detected a reduced activity of the Na^+/K^+ ATPase pump in the diabetic group (Figure 4, H). The Ca^{2+} and Mg^{2+} ATPases, as the total ATPase activity were not affected.

Histopathological analysis revealed a reduced glomerular volume in the diabetic groups (Figure 5, C), despite no differences in the glomeruli number per area (mm^2) (Figure 5, B). Sections of the healthy control group did not show any pathological feature, and the measurements are compatible with the described ones for the species. However, the diabetic groups presented an abnormal accumulation of glycogen in the tubules, known as glycogen nephrosis. The volume of glycogen accumulation in the diabetic group was increased compared with the Ctrl group (Figure 5, D), however the GTI treatment was able to prevent the glycogen granules accumulation in the diabetic animals (Figure 5, D).

Diabetes led to a reduced proportion of AO-positive cells in the renal cortex (Figure 6, A), indicating a reduced proportion of cells without DNA damage. A direct consequence of that is the increased proportion of IP-positive cells, shown in Figure 6, B. On the other hand, GTI was able to counteract these effects, improving the proportion of AO-positive cells (Figure 6, A), and reducing the proportion of the IP-positive cells (Figure 6, B).

Glomerular morphological analysis reveals diabetic glomerulus surrounded by flattened epithelial cells, with pathological alterations that were less frequent in the group treated with GTI. Diffuse mesangial expansion was more frequent, present in almost every glomeruli in the STZ group. Bowman's capsule lesions were more frequent in the untreated diabetic than in the STZ+GTI group. Nodular mesangial expansion was not observed in any group. Moderate dilation in the lumen of the proximal tubule was more frequent in the STZ group. Also in the STZ group, the basal region of the proximal tubular cells presented the accumulation of aggregated stained granules, more densely than in the healthy group, possible mitochondria aggregation (Itagaki et al., 1995). Furthermore, karyocytomegaly was frequently observed in the STZ group and less frequency in the STZ+GTI group, as so as cytoplasmatic microvesicles, possibly lipid droplets, in the proximal tubule cells (Figure 7).

3.2. Virtual analysis

The STRING network is presented in Figure 8, A, and highlights the two main functional clusters (Cluster 1 and Cluster 2). The Reactome Pathway analysis for each cluster is summarized in Table 2. The centrality analysis showed that protein kinase B 1 (AKT1) is the protein classified as the "bottleneck" in the network and has the capacity to integrate the functional pathways that participate in the catechins effects in the kidney (Figure 8, B). The implications of AKT1 in green tea induced signaling in diabetic nephropathy are discussed below. All proteins in the PPI network are expressed in the normal kidney in different degrees.

4. Discussion

Our results showed that green tea infusion treatment was able to prevent glycogen accumulation in the renal tubules, reduce the DNA damage caused by the hyperglycemic state in renal cortex cells, and act preventing the aggravation of glomerular morphological alterations, independently of any hyperglycemia reduction. These outcomes confirm that green tea positive effects in diabetic nephrosis are broader than glycemic regulation related effects. Although our study has not shown a strong improvement in organ function, DNA preservation is determinant in cell survival and proper function, and glomerular morphological integrity is elemental to the filtration process. Such results, together with the *in silico* considerations, may indicate key points in the signaling pathways to improve diabetic nephropathy treatment, as coadjuvant, and prevention, with an herbal medicine, widely distributed and highly accepted around the world.

In adult animals, the weight of the kidney is increased by the damage caused by hyperglycemia. Such injuries lead to hypertrophy and compensatory hyperplasia in the tubules, in order to preserve the glomerular filtration function, thus increasing the kidney's weight (Herman-Edelstein and Doi, 2016). However, our animals were induced to diabetes at a younger age, so that they had not passed the full development process of the body and organs, including the kidneys, that would still go through a period of growth, with subsequent weight gain (Arataki, 1926). The damage caused by hyperglycemia at this stage of life seems to have been severe enough to delay the progression of the organ's normal growth, stagnating the weight gain together with the entire body development of the animal, as described in other experimental conditions with young animals (da Silva et al., 2016; Haraguchi et al., 2020; Silva et al., 2009). Besides, such damage may have extended to prevent green tea's positive effects on kidney function markers found in other studies with adult animals (Hayashi et al., 2020; Renno et al., 2008). Our data suggest that diabetes, when rises early, impairs the development of the kidney,

as well as the glomerulus, reflected in the low volume of the glomerulus and appearance of pathological features (e.g. mesangial expansion, karyocytomegaly, and glomerular basal membrane alterations) in the diabetic animals compared to healthy control. Although we have not observed statistical difference, the size of glomerulus was observed to have a higher mean and a lower variance (SD) in the group treated with green tea compared with the diabetic one, approaching the characteristics that describe the control group. Such data are in line with the protective effects on glomerular morphology exercised by EGCG, the main catechin found in green tea (Yoon et al., 2014).

It is known that catechins in green tea have a hypoglycemic and preventive effect on high glucose levels (Fu et al., 2017), and it was assumed that the beneficial effects of green tea in diabetic nephropathy, especially concerning the tubular glycogen nephrosis, were due to this hypoglycemiant capacity (Renno et al., 2008). However, green tea treatment, or its isolated catechin administration, can generate positive outcomes without the achievement of proper glycemic control (Hayashi et al., 2020), confirming that tea's effect on diabetes goes beyond improving glucose-related harms.

The glycogen accumulation in renal tubules, as presented in our study, is a hallmark of experimental diabetic nephropathy induced by STZ or Alloxan in experimental models (Kang et al., 2005). In normal conditions, glucose is reabsorbed almost completely in the proximal tubule by sodium-dependent glucose transporter 2 (SGLT2) and, in lower levels, by sodium-dependent glucose transporter 1 (SGLT1), and appears in the urine when the absorptive capacity is extrapolated (Bailey, 2011; Vallon and Thomson, 2017). Additionally, proximal tubule cells have a greater capacity to perform gluconeogenesis from lactate, glutamine, and glycerol, and this is an upregulated process in diabetes (Eid et al., 2006). The glycogen accumulated in the tubule may result from the sum of factors including abnormally increased absorption, and increased gluconeogenesis (Herman-Edelstein and Doi, 2016; Mather and Pollock, 2011).

Green tea catechins are shown to act as SGLT1 inhibitors *in vitro* (Kobayashi et al., 2000), suggesting that tea treatment forces the glucose reabsorption process in the kidney to be done by SGLT2 alone. At the time, there is no evidence suggesting an inhibitory effect of catechins on SGLT2. However, EGCG was shown to inhibit glucose production via gluconeogenesis in cells by activating the AMPK (Collins et al., 2007). Also, EGCG suppresses gluconeogenic gene expression (e.g. glucose-6-phosphatase and phosphoenolpyruvate carboxykinase) via the phosphoinositide 3-kinase (PI3K) pathway (Waltner-Law et al., 2002). Such a mechanism in kidney cells could lead to a reduced glucose overload and the improvement of glycogen accumulation in proximal tubules and may explain the positive outcomes of GTI treatment in our study.

Furthermore, diabetes can increase the expression of SGLT2 and sodium-hydrogen antiporter 3 (NHE3), in response to the higher demand for adenosine triphosphate (ATP) to maintain the glucose reabsorption flow (Herman-Edelstein and Doi, 2016). The great capacity of tubular cells to perform gluconeogenesis, a process that consumes a lot of ATP, further increases the demand for the molecule (Gilbert, 2017). Such increased demand for energy therefore enhances the oxygen (O_2) demand creating a hypoxic environment in the tubular cells (Herman-Edelstein and Doi, 2016). However, the blood supply of O_2 in this case is severely affected by the endothelial damage caused by glucose, leading to loss and obstruction of capillaries, worsen the oxygen supply (Herman-Edelstein and Doi, 2016). In this way, a deeper hypoxic environment is generated, favorable to the activation of apoptosis via the Caspase pathway, and the fibrosis development in the organ by stimulating the Transforming growth factor-beta (TGF-β) pathway. In turn, the progression of fibrosis further worsens hypoxia, aggravating cell death in the organ (Gilbert, 2017). This mechanism is also accompanied by increased expression of stem cell factor (SCF) and proto-oncogene c-kit (c-kit) (Yin et al., 2018). In contrast, ellagic acid, a derivative polyphenol found in green tea (Yang and Tomás-

Barberán, 2019), is shown to inhibit tyrosinase activity (Yoshimura et al., 2005) inhibiting the SCT-Kit pathway and alleviating the damages caused by hypoxia.

In this same line, green tea extract can inhibit the fibroblast growth factor receptor (FGFR) signaling by reducing the expression of fibroblast growth factor (FGF) (Sartippour et al., 2002), and EGCG impedes the signaling pathway of the platelet-derived growth factor (PDGF), other profibrotic factors (Park et al., 2006).

Hypoxia can aggravate diabetic kidney disease by upregulating the expression of Toll-Like Receptor 4 (TLR4) ligands in diabetes, as fibronectin (Zhang et al., 2018) and high-mobility group box 1 (HMGB1) (Feng et al., 2020). The activation of the TLR4 signal mediated by the TIR-domain-containing adaptor-inducing Interferon-β (TRIF) culminate in the activation of the nuclear factor κ B (NF-κB) that lead to inflammation and fibrosis in the kidney (Feng et al., 2020). However, EGCG was shown to inhibit the TLR pathway activation *in vitro* (Youn et al., 2006) and to reduce the NFκB expression (Yamabe et al., 2006) suggesting that tea may act through this mechanism to promote anti-inflammatory and antifibrotic protection in the kidney.

Our results showed that green tea was able to reduce the binding of propidium iodide to DNA and enhance GST activity, suggesting an improvement in DNA integrity or that there was some reduction in the damage caused by hyperglycemia or oxidizing agents. A previous study showed that EGCG can inhibit apoptosis induced by oxidative stress (Itoh et al., 2005) preserving renal cells in an *in vitro* model. Also, green tea polyphenols can contribute to reduce apoptosis levels in diabetic nephropathy by blocking the glycogen synthase kinase-3 β (GSK3β) interaction with the tumor protein 53 (TP53), reducing Caspase 3 activity in podocytes leading to higher cell survival rates (Borges et al., 2016; Peixoto et al., 2015). A review study by Mohabbulla Mohib et al. (2016) summarizes other possible mechanisms that green tea protects

the nuclear envelope and the genome, including the stabilization of the DNA strand and also the reduction of NF-κB expression culminating in the already discussed positive outcomes.

The homeostatic maintenance of the ions inside the cell may influence the antioxidant enzyme activities (Soetan et al., 2010). Our results show that green tea was not able to reverse the dysregulation in the relationship between the ions in the kidneys, which actively participate in the functioning of antioxidant enzymes. Also, oxidative stress may be responsible to inhibit Na^+/K^+ ATPase activity by oxidation of thiol groups in the pumps (Al-Numair et al., 2015), and despite the increased GST activity shown in our study, Na^+/K^+ ATPase function was not recovered.

The PI3K/AKT/mammalian target of rapamycin (mTOR) pathway is linked to metabolic regulation in diabetic nephropathy and also in the development of human kidney cancer. This signal cascade is upregulated in diabetes and is closely related to glycogen tubular accumulation (Ribback et al., 2015). EGCG is shown to inhibit both PI3K and mTOR, by competitively binding in the ATP-binding sites in these proteins (Van Aller et al., 2011). Additionally, mTOR inhibition can restore the autophagy mechanism, reduced by mTOR overexpression in diabetes, and contribute to cellular renovation in the kidney. Also, the PI3K/AKT/mTOR pathway is related to *de novo* lipogenesis in the kidney (Ribback et al., 2015), which can lead to lipid accumulation, as in line with the microvesicles showed in Figure 6 D. Green tea treated animals didn't present this cytoplasmic microvesicles.

Our *in silico* results show that protein kinase B (AKT) is the central protein in the catechin mediated effects in the kidney. EGCG can activate the diacylglycerol kinase (DGK) pathway, promoting the inactivation of protein kinase C beta (PKC-β) and improving the condition of diabetic nephropathy (Hayashi et al., 2020). Such a process is initiated the interaction of EGCG with the 67-kDa laminin receptor (67LR), which is known as an EGCG

receptor (Tachibana et al., 2004), and is also capable of activating the AKT in the kidney (Kumazoe et al., 2020). Hayashi et al (2015) showed that EGCG activates DGK-α via 67LR binding. In a recent study (Hayashi et al., 2020), the authors proposed that this mechanism occurs by activating 67LR receptors in the cell membrane, which, when activated, promotes the translocation of the DGK to the membrane, through the formation of 67LR-DGK-α and α3-β1 integrin's complex, promoting greater focal adhesion of podocyte foot process in the glomerular basement membrane, ensuring cell adhesion, in addition to inhibiting α and β PKC (Hayashi, 2020), preserving glomerular morphology. This mechanism may be responsible for the positive effects concerning glomerular preservation by green tea ingestion. Other catechins present in green tea composition may exert effect by AKT pathway activation by a different receptor, as they do not bind with the 67LR (Tachibana et al., 2004), however the primary membrane receptor for them are still unknown.

5. Conclusion

The components of green tea can interact with proteins participating in cell signaling pathways that regulate energy metabolism, including glucose and glycogen synthesis, glucose reabsorption, hypoxia management, and cell death by apoptosis. Such interaction leads to reduced accumulation of glycogen in the kidney's cells of the proximal tubules in diabetes, as well as to reduce DNA damage. These results also reflect in a preserved glomerulus morphology, with improvement in pathological features, and suggesting a prevention of kidney function impairment. Our results show that such benefits are achieved regardless of the blood glucose status, and are not dependent on the reduction of hyperglycemia to be achieved.

Abbreviations

67LR - 67kDa laminin receptor; ABTS - 2,2'-Azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]; NOS1 - Nitric Oxide Synthase 1; AGE/RAGE - advanced glycated end-products and its receptor; AKT - protein kinase B; AMPK - 5'-AMP-activated protein kinase; AO – acridine Orange; APC - APC Regulator Of WNT Signaling Pathway; ATP - adenosine triphosphate; BAX - BCL2 Associated X; BCL2 – BCL2 Apoptosis Regulator; BID - BH3 Interacting Domain Death Agonist; BW – body weight; Ca – calcium; CaCl – calcium chloride; CASP3 – Caspase 3; CASP8 – Caspase 8; CASP9 – Caspase 9; CAT – catalase; CAV1 - Caveolin 1; CCL2 - C-C Motif Chemokine Ligand 2; CDK2 - Cyclin Dependent Kinase 2; CDKN1A - Cyclin Dependent Kinase Inhibitor 1; CIAPIN1 - Cytokine Induced Apoptosis Inhibitor 1; c-kit – proto-oncogene c-kit; Cl – chlorine; CPI – compound-protein interactions; CTNNB1 - Catenin Beta 1; Ctrl – control group; DB02077 - L-N(omega)-nitroarginine-(4R)-amino-L-proline amide (NOS3); DB08019, DB08018 and NOS3- Nitric Oxide Synthase 3; DKG - diacylglycerol kinase; DN – Diabetic nephropathy; EGCG - epigallocatechin gallate; FGF – fibroblast growth fator; FGFR – fibroblast growth factor receptor; FOS - Fos Proto-Oncogene; FRAP – ferric reducing antioxidant power; GSK3β - glycogen synthase kinase-3 β; GST – glutathione S-transferase; GTI – Green tea infusion; H_2O_2 – hydrogen peroxide; H6PD - Hexose-6-Phosphate Dehydrogenase/Glucose 1-Dehydrogenase; HIF1A - Hypoxia Inducible Factor 1 Subunit Alpha; HMG1 – high-mobility group box 1; HSP90AA1 - Heat Shock Protein 90 Alpha Family Class A Member 1; i.p. – intraperitoneal; IL6 – Interleukin 6; IL8 – Interleukin 8; JUN - Jun Proto-Oncogene; K – potassium; KCl – potassium chloride; KW – kidney weight; MAP2K1 - Mitogen-Activated Protein Kinase Kinase 1; MAPK1 - Mitogen-Activated Protein Kinase 1; MAPK3 - Mitogen-Activated Protein Kinase 3; MAPK8 - Mitogen-Activated Protein Kinase 8; MAPKAPK5 - MAPK Activated Protein Kinase 5; Mg – magnesium; MgCl – magnesium chloride; MLH1 - MutL Homolog 1; mTOR – mammalian target of rapamycin; MTRR - 5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase; Na – sodium; NaCl – sodium chloride; NADPH – reduced nicotinamide adenine dinucleotide phosphate; NDOR1 - NADPH-dependent diflavin reductase; NFκB - nuclear factor κ B; NO_2^-/NO_3^- - nitric oxide; NOS2 - Nitric Oxide Synthase 2; NR1H4 - Nuclear Receptor Subfamily 1 Group H Member 4 ; O_2 – oxygen; O^-_2 – superoxide; PARP1 - Poly(ADP-Ribose) Polymerase 1; PDGF – platelet-derived growth fator; PGD - Phosphogluconate Dehydrogenase; PGLS - 6-Phosphogluconolactonase; PI – propidium iodide; PI3K – phosphoinositide 3-kinase; PIN1 - Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1; PKC-β – protein kinase C beta; POR - Cytochrome P450 Oxidoreductase; PPI – Protein-Protein Interactome; RPIA - Ribose 5-Phosphate Isomerase A; RSI – renal somatic index; SCF – stem cell fator; SD – standard deviation; SGLT1 – sodium-dependent glucose transporter 1; SGLT2 – sodium-dependent glucose transporter 2; SOD – superoxide dismutase; SOES – Specific Organ Expression Score; STAT3 - Signal transducer and activator of transcription 3; STZ – streptozotocin; TCA - Trichloroacetic acid; TCF7L2 - Transcription Factor 7 Like 2; TE – Trolox equivalente; TGF-β – transforming growth factor-beta; TLR4 – toll-like receptor 4; TP53 – tumor protein 53; TRIF – TIR domain-containing adaptor-inducing Interferon- β; TYW1 - TRNA-YW Synthesizing Protein 1 Homolog; UBC - Ubiquitin C.

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References

Aebi, H., 1984. [13] Catalase in vitro, in: Methods in Enzymology, Methods in Enzymology. Elsevier, pp. 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Al-Numair, K.S., Veeramani, C., Alsaif, M.A., Chandramohan, G., 2015. Influence of kaempferol, a flavonoid compound, on membrane-bound ATPases in streptozotocin-induced diabetic rats. Pharm. Biol. 53, 1372–1378. https://doi.org/10.3109/13880209.2014.982301

Arataki, M., 1926. On the postnatal growth of the kidney, with special reference to the number and size of the glomeruli (albino rat). Am. J. Anat. 36, 399–436. https://doi.org/10.1002/aja.1000360302

Bader, G.D., Hogue, C.W.V., 2003. An automated method for finding molecular complexes in large protein interaction networks. BMC Bioinformatics 4, 1–27. https://doi.org/10.1186/1471-2105-4-2

Bailey, C.J., 2011. Renal glucose reabsorption inhibitors to treat diabetes. Trends Pharmacol. Sci. 32, 63–71. https://doi.org/10.1016/j.tips.2010.11.011

Barkaoui, M., Katiri, A., Boubaker, H., Msanda, F., 2017. Ethnobotanical survey of medicinal plants used in the traditional treatment of diabetes in Chtouka Ait Baha and Tiznit (Western Anti-Atlas), Morocco. J. Ethnopharmacol. 198, 338–350. https://doi.org/10.1016/j.jep.2017.01.023

Bernas, T., Asem, E.K., Robinson, J.P., Cook, P.R., Dobrucki, J.W., 2005. Confocal Fluorescence Imaging of Photosensitised DNA Denaturation in Cell Nuclei. Photochem. Photobiol. 33342, 960–969. https://doi.org/10.1562/2004-11-11-ra-369

Bindea, G., Galon, J., Mlecnik, B., 2013. CluePedia Cytoscape plugin: Pathway insights using integrated experimental and in silico data. Bioinformatics 29, 661–663. https://doi.org/10.1093/bioinformatics/btt019

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.H., Pagès, F., Trajanoski, Z., Galon, J., 2009. ClueGO: A Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25, 1091–1093. https://doi.org/10.1093/bioinformatics/btp101

Borges, C.M., Papadimitriou, A., Duarte, D.A., Lopes De Faria, J.M., Lopes De Faria, J.B., 2016. The use of green tea polyphenols for treating residual albuminuria in diabetic nephropathy: A double-blind randomised clinical trial. Sci. Rep. 6, 1–9. https://doi.org/10.1038/srep28282

Chopade, V. V, Phatak, A.A., Upaganlawar, A.B., Tankar, A.A., 2008. Green tea (Camellia sinensis): chemistry , traditional , medicinal uses and its pharmacological activities- a review. Pharmacogn. Rev. 2, 157–162.

Collins, Q.F., Liu, H.-Y.Y., Pi, J., Liu, Z., Quon, M.J., Cao, W., 2007. Epigallocatechin-3-gallate (EGCG), A Green Tea Polyphenol, Suppresses Hepatic Gluconeogenesis through 5′-AMP-activated Protein Kinase. J. Biol. Chem. 282, 30143–30149. https://doi.org/10.1074/jbc.M702390200

da Silva, E., Natali, A.J., da Silva, M.F., de Jesus Gomes, G., da Cunha, D.N.Q., Toledo, M.M., Drummond, F.R., Ramos, R.M.S., dos Santos, E.C., Novaes, R.D., de Oliveira, L.L.,

Maldonado, I.R. dos S.C., 2016. Swimming training attenuates the morphological reorganization of the myocardium and local inflammation in the left ventricle of growing rats with untreated experimental diabetes. Pathol. - Res. Pract. 212, 325–334. https://doi.org/10.1016/j.prp.2016.02.005

de Godoi, R.S., Almerão, M.P., da Silva, F.R., 2020. In silico evaluation of the antidiabetic activity of natural compounds from Hovenia dulcis Thunberg. J. Herb. Med. 100349. https://doi.org/10.1016/j.hermed.2020.100349

Dias, F.C.R., Martins, A.L.P., de Melo, F.C.S.A., Cupertino, M. do C., Gomes, M. de L.M., de Oliveira, J.M., Damasceno, E.M., Silva, J., Otoni, W.C., da Matta, S.L.P., 2019. Hydroalcoholic extract of Pfaffia glomerata alters the organization of the seminiferous tubules by modulating the oxidative state and the microstructural reorganization of the mice testes. J. Ethnopharmacol. 233, 179–189. https://doi.org/10.1016/j.jep.2018.12.047

Dieterich, S., Bieligk, U., Beulich, K., Hasenfuss, G., Prestle, J., 2000. Gene Expression of Antioxidative Enzymes in the Human Heart : Increased Expression of Catalase in the End-Stage Failing Heart. Circulation 101, 33–39. https://doi.org/10.1161/01.CIR.101.1.33

Eid, A., Bodin, S., Ferrier, B., Delage, H., Boghossian, M., Martin, M., Baverel, G., Conjard, A., 2006. Intrinsic gluconeogenesis is enhanced in renal proximal tubules of Zucker diabetic fatty rats. J. Am. Soc. Nephrol. 17, 398–405. https://doi.org/10.1681/ASN.2005070742

Fallah Huseini, H., Fakhrzadeh, H., Larijani, B., Shikh Samani, A.H., 2006. Review of anti-diabetic medicinal plant used in traditional medicine. J. Med. Plants 5, 1–8.

Feng, Q., Liu, D., Lu, Y., Liu, Z., 2020. The Interplay of Renin-Angiotensin System and Toll-Like Receptor 4 in the Inflammation of Diabetic Nephropathy. J. Immunol. Res. 2020, 1–11. https://doi.org/10.1155/2020/6193407

Fiske, C.C.H., Subbarow, Y.Y., 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375–400.

Fu, Q.-Y., Li, Q.-S., Lin, X.-M., Qiao, R.-Y., Yang, R., Li, X.-M., Dong, Z.-B., Xiang, L.-P., Zheng, X.-Q., Lu, J.-L., Yuan, C.-B., Ye, J.-H., Liang, Y.-R., 2017. Antidiabetic Effects of Tea. Molecules 22, 849. https://doi.org/10.3390/molecules22050849

Gilbert, R.E., 2017. Proximal tubulopathy: Prime mover and key therapeutic target in diabetic kidney disease. Diabetes 66, 791–800. https://doi.org/10.2337/db16-0796

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-Transferases: The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 25, 7130–7139.

Haraguchi, R., Kohara, Y., Matsubayashi, K., Kitazawa, R., Kitazawa, S., 2020. New Insights into the Pathogenesis of Diabetic Nephropathy: Proximal Renal Tubules Are Primary Target of Oxidative Stress in Diabetic Kidney. Acta Histochem. Cytochem. 53, 21–31. https://doi.org/10.1267/ahc.20008

Hayashi, D., Ueda, S., Yamanoue, M., Saito, N., Ashida, H., Shirai, Y., 2015. Epigallocatechin-3-gallate activates diacylglycerol kinase alpha via a 67 kDa laminin receptor: A possibility of galloylated catechins as functional food to prevent and/or improve diabetic renal dysfunctions. J. Funct. Foods 15, 561–569. https://doi.org/10.1016/j.jff.2015.04.005

Hayashi, D., Wang, L., Ueda, S., Yamanoue, M., Ashida, H., Shirai, Y., 2020. The mechanisms

of ameliorating effect of a green tea polyphenol on diabetic nephropathy based on diacylglycerol kinase α. Sci. Rep. 10, 1–12. https://doi.org/10.1038/s41598-020-68716-6

Herman-Edelstein, M., Doi, S.Q., 2016. Pathophysiology of diabetic nephropathy, Proteinuria: Basic Mechanisms, Pathophysiology and Clinical Relevance. Elsevier Inc. https://doi.org/10.1007/978-3-319-43359-2_4

Itagaki, S. ichi, Nishida, E., Lee, M.J., Doi, K., 1995. Histopathology of subacute renal lesions in mice induced by streptozotocin. Exp. Toxicol. Pathol. 47, 485–491. https://doi.org/10.1016/S0940-2993(11)80332-5

Itoh, Y., Yasui, T., Okada, A., Tozawa, K., Hayashi, Y., Kohri, K., 2005. Examination of the anti-oxidative effect in renal tubular cells and apoptosis by oxidative stress. Urol. Res. 33, 261–266. https://doi.org/10.1007/s00240-005-0465-7

Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., Sidiropoulos, K., Cook, J., Gillespie, M., Haw, R., Loney, F., May, B., Milacic, M., Rothfels, K., Sevilla, C., Shamovsky, V., Shorser, S., Varusai, T., Weiser, J., Wu, G., Stein, L., Hermjakob, H., D'Eustachio, P., 2020. The reactome pathway knowledgebase. Nucleic Acids Res. 48, D498–D503. https://doi.org/10.1093/nar/gkz1031

Kang, J., Dai, X.-S., Yu, T.-B., Wen, B., Yang, Z.-W., 2005. Glycogen accumulation in renal tubules, a key morphological change in the diabetic rat kidney. Acta Diabetol. 42, 110–116. https://doi.org/10.1007/s00592-005-0188-9

Kim-Park, W.K., Allam, E.S., Palasuk, J., Kowolik, M., Park, K.K., Windsor, L.J., 2016. Green tea catechin inhibits the activity and neutrophil release of Matrix Metalloproteinase-9. J. Tradit. Complement. Med. 6, 343–346. https://doi.org/10.1016/j.jtcme.2015.02.002

Kobayashi, Y., Suzuki, M., Satsu, H., Arai, S., Hara, Y., Suzuki, K., Miyamoto, Y., Shimizu, M., 2000. Green Tea Polyphenols Inhibit the Sodium-Dependent Glucose Transporter of Intestinal Epithelial Cells by a Competitive Mechanism. J. Agric. Food Chem. 48, 5618–5623. https://doi.org/10.1021/jf0006832

Kumazoe, M., Fujimura, Y., Tachibana, H., 2020. 67-kDa Laminin Receptor Mediates the Beneficial Effects of Green Tea Polyphenol EGCG. Curr. Pharmacol. Reports. https://doi.org/10.1007/s40495-020-00228-3

Ladeira, L.C.M., dos Santos, E.C., Mendes, B.F., Gutierrez, E.A., Santos, C.F.F., de Souza, F.B., Machado-Neves, M., Maldonado, I.R. dos S.C., 2020a. Green tea infusion aggravates nutritional status of the juvenile untreated STZ-induced type 1 diabetic rat. bioRxiv 35. https://doi.org/10.1101/2020.01.13.904896

Ladeira, L.C.M., dos Santos, E.C., Valente, G.E., da Silva, J., Santos, T.A., dos Santos Costa Maldonado, I.R., 2020b. Could biological tissue preservation methods change chemical elements proportion measured by energy dispersive X-ray spectroscopy? Biol. Trace Elem. Res. 196, 168–172. https://doi.org/10.1007/s12011-019-01909-x

Lima, G.D. de A., Sertorio, M.N., Souza, A.C.F., Menezes, T.P., Mouro, V.G.S., Gonçalves, N.M., Oliveira, J.M. de, Henry, M., Machado-Neves, M., 2018. Fertility in male rats: Disentangling adverse effects of arsenic compounds. Reprod. Toxicol. 78, 130–140. https://doi.org/10.1016/j.reprotox.2018.04.015

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randal, R.J., 1994. Protein Measurement with the Folin Phenol Reagent. Anal. Biochem. 217, 220–230. https://doi.org/10.1016/0304-

3894(92)87011-4

Mather, A., Pollock, C., 2011. Glucose handling by the kidney. Kidney Int. 79, S1–S6. https://doi.org/10.1038/ki.2010.509

Meng, J.-M., Cao, S.-Y., Wei, X.-L., Gan, R.-Y., Wang, Y.-F., Cai, S.-X., Xu, X.-Y., Zhang, P.-Z., Li, H.-B., 2019. Effects and Mechanisms of Tea for the Prevention and Management of Diabetes Mellitus and Diabetic Complications: An Updated Review. Antioxidants 8, 170. https://doi.org/10.3390/antiox8060170

Mineharu, Y., Koizumi, A., Wada, Y., Iso, H., Watanabe, Y., Date, C., Yamamoto, A., Kikuchi, S., Inaba, Y., Toyoshima, H., Kondo, T., Tamakoshi, A., 2011. Coffee, green tea, black tea and oolong tea consumption and risk of mortality from cardiovascular disease in Japanese men and women. J. Epidemiol. Community Health 65, 230–240. https://doi.org/10.1136/jech.2009.097311

Mohabbulla Mohib, M., Fazla Rabby, S.M., Paran, T.Z., Mehedee Hasan, M., Ahmed, I., Hasan, N., Abu Taher Sagor, M., Mohiuddin, S., 2016. Protective role of green tea on diabetic nephropathy - A review. Cogent Biol. 2. https://doi.org/10.1080/23312025.2016.1248166

Park, J.S., Kim, M.H., Chang, H.J., Kim, K.M., Kim, S.M., Shin, B.A., Ahn, B.W., Jung, Y.D., 2006. Epigallocatechin-3-gallate inhibits the PDGF-induced VEGF expression in human vascular smooth muscle cells via blocking PDGF receptor and Erk-1/2. Int. J. Oncol. 29, 1247–1252. https://doi.org/10.3892/ijo.29.5.1247

Peixoto, E.B., Papadimitriou, A., Teixeira, D.A.T., Montemurro, C., Duarte, D.A., Silva, K.C., Joazeiro, P.P., Lopes de Faria, J.M., Lopes de Faria, J.B., 2015. Reduced LRP6 expression and increase in the interaction of GSK3β with p53 contribute to podocyte apoptosis in diabetes mellitus and are prevented by green tea. J. Nutr. Biochem. 26, 416–430. https://doi.org/10.1016/j.jnutbio.2014.11.012

Perva-Uzunalić, A., Škerget, M., Knez, Ž., Weinreich, B., Otto, F., Grüner, S., 2006. Extraction of active ingredients from green tea (Camellia sinensis): Extraction efficiency of major catechins and caffeine. Food Chem. 96, 597–605. https://doi.org/10.1016/j.foodchem.2005.03.015

Rachid, A., Rabah, D., Farid, L., Zohra, S.F., Houcine, B., 2012. Ethnopharmacological survey of medicinal plants used in the traditional treatment of diabetes mellitus in the North Western and South Western Algeria. J. Med. Plants Res. 6, 2041–2050. https://doi.org/10.5897/JMPR11.1796

Rebhan, M., Chalifa-Caspi, V., Prilusky, J., Lancet, D., 1998. GeneCards: A novel functional genomics compendium with automated data mining and query reformulation support. Bioinformatics 14, 656–664. https://doi.org/10.1093/bioinformatics/14.8.656

Renno, W.M., Abdeen, S., Alkhalaf, M., Asfar, S., 2008. Effect of greean tea on kidney tubules of diabetic rats. Br. J. Nutr. 100, 652–659. https://doi.org/10.1017/S0007114508911533

Ribback, S., Cigliano, A., Kroeger, N., Pilo, M.G., Terracciano, L., Burchardt, M., Bannasch, P., Calvisi, D.F., Dombrowski, F., 2015. PI3K/AKT/mTOR pathway plays a major pathogenetic role in glycogen accumulation and tumor development in renal distal tubules of rats and men. Oncotarget 6, 13036–13048. https://doi.org/10.18632/oncotarget.3675

Ricart-Jané, D., Llobera, M., López-Tejero, M.D., 2002. Anticoagulants and other preanalytical

factors interfere in plasma nitrate/nitrite quantification by the Griess method. Nitric Oxide - Biol. Chem. 6, 178–185. https://doi.org/10.1006/niox.2001.0392

Sartippour, M.R., Heber, D., Zhang, L., Beatty, P., Elashoff, D., Elashoff, R., Go, V.L., Brooks, M.N., 2002. Inhibition of fibroblast growth factors by green tea. Int. J. Oncol. 21, 487–491. https://doi.org/10.3892/ijo.21.3.487

Scardoni, G., Petterlini, M., Laudanna, C., 2009. Analyzing biological network parameters with CentiScaPe. Bioinformatics 25, 2857–2859. https://doi.org/10.1093/bioinformatics/btp517

Sertorio, M.N., Souza, A.C.F., Bastos, D.S.S., Santos, F.C., Ervilha, L.O.G., Fernandes, K.M., de Oliveira, L.L., Machado-Neves, M., 2019. Arsenic exposure intensifies glycogen nephrosis in diabetic rats. Environ. Sci. Pollut. Res. 26, 12459–12469. https://doi.org/10.1007/s11356-019-04597-1

Shannon, P., 2003. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303

Silva, M.J., Brodt, M.D., Lynch, M.A., McKenzie, J.A., Tanouye, K.M., Nyman, J.S., Wang, X., 2009. Type 1 Diabetes in Young Rats Leads to Progressive Trabecular Bone Loss, Cessation of Cortical Bone Growth, and Diminished Whole Bone Strength and Fatigue Life. J. Bone Miner. Res. 24, 1618–1627. https://doi.org/10.1359/jbmr.090316

Soetan, K.O., Olaiya, C.O., Oyewole, O.E., 2010. The importance of mineral elements for humans , domestic animals and plants : A review. African J. Food Sci. 4, 200–222.

Su, J., Ye, D., Gao, C., Huang, Q., Gui, D., 2020. Mechanism of progression of diabetic kidney disease mediated by podocyte mitochondrial injury. Mol. Biol. Rep. https://doi.org/10.1007/s11033-020-05749-0

Suzuki, T., Fujikura, K., Higashiyama, T., Takata, K., 1997. DNA staining for fluorescence and laser confocal microscopy. J. Histochem. Cytochem. 45, 49–53. https://doi.org/10.1177/002215549704500107

Szklarczyk, D., Morris, J.H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N.T., Roth, A., Bork, P., Jensen, L.J., Von Mering, C., 2017. The STRING database in 2017: Quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res. 45, D362–D368. https://doi.org/10.1093/nar/gkw937

Szklarczyk, D., Santos, A., Von Mering, C., Jensen, L.J., Bork, P., Kuhn, M., 2016. STITCH 5: Augmenting protein-chemical interaction networks with tissue and affinity data. Nucleic Acids Res. 44, D380–D384. https://doi.org/10.1093/nar/gkv1277

Tachibana, H., Koga, K., Fujimura, Y., Yamada, K., 2004. A receptor for green tea polyphenol EGCG. Nat. Struct. Mol. Biol. 11, 380–381. https://doi.org/10.1038/nsmb743

Vallon, V., Thomson, S.C., 2017. Targeting renal glucose reabsorption to treat hyperglycaemia: the pleiotropic effects of SGLT2 inhibition. Diabetologia 60, 215–225. https://doi.org/10.1007/s00125-016-4157-3

Van Aller, G.S., Carson, J.D., Tang, W., Peng, H., Zhao, L., Copeland, R.A., Tummino, P.J., Luo, L., 2011. Epigallocatechin gallate (EGCG), a major component of green tea, is a dual phosphoinositide-3-kinase/mTOR inhibitor. Biochem. Biophys. Res. Commun. 406, 194–199. https://doi.org/10.1016/j.bbrc.2011.02.010

Vaz, S.R., de Amorim, L.M.N., de Nascimento, P.V.F., Veloso, V.S.P., Nogueira, M.S., Castro, I.A., Mota, J.F., Botelho, P.B., 2018. Effects of green tea extract on oxidative stress and renal function in diabetic individuals: A randomized, double-blinded, controlled trial. J. Funct. Foods 46, 195–201. https://doi.org/10.1016/j.jff.2018.04.059

Waltner-Law, M.E., Wang, X.L., Law, B.K., Hall, R.K., Nawano, M., Granner, D.K., 2002. Epigallocatechin Gallate, a Constituent of Green Tea, Represses Hepatic Glucose Production. J. Biol. Chem. 277, 34933–34940. https://doi.org/10.1074/jbc.M204672200

Yamabe, N., Yokozawa, T., Oya, T., Kim, M., 2006. Therapeutic Potential of (-)-Epigallocatechin 3- O -Gallate on Renal Damage in Diabetic Nephropathy Model Rats. J. Pharmacol. Exp. Ther. 319, 228–236. https://doi.org/10.1124/jpet.106.107029

Yang, X., Tomás-Barberán, F.A., 2019. Tea Is a Significant Dietary Source of Ellagitannins and Ellagic Acid. J. Agric. Food Chem. 67, 5394–5404. https://doi.org/10.1021/acs.jafc.8b05010

Yin, D.D., Luo, J.H., Zhao, Z.Y., Liao, Y.J., Li, Y., 2018. Tranilast prevents renal interstitial fibrosis by blocking mast cell infiltration in a rat model of diabetic kidney disease. Mol. Med. Rep. 17, 7356–7364. https://doi.org/10.3892/mmr.2018.8776

Yokozawa, T., Noh, J.S., Park, C.H., 2012. Green tea polyphenols for the protection against renal damage caused by oxidative stress. Evidence-based Complement. Altern. Med. 2012. https://doi.org/10.1155/2012/845917

Yoon, S.P., Maeng, Y.H., Hong, R., Lee, B.R., Kim, C.G., Kim, H.L., Chung, J.H., Shin, B.C., 2014. Protective effects of epigallocatechin gallate (EGCG) on streptozotocin-induced diabetic nephropathy in mice. Acta Histochem. 116, 1210–1215. https://doi.org/10.1016/j.acthis.2014.07.003

Yoshimura, M., Watanabe, Y., Kasai, K., Yamakoshi, J., Koga, T., 2005. Inhibitory effect of an ellagic acid-rich pomegranate extract on tyrosinase activity and ultraviolet-induced pigmentation. Biosci. Biotechnol. Biochem. 69, 2368–2373. https://doi.org/10.1271/bbb.69.2368

Youn, H.S., Lee, J.Y., Saitoh, S.I., Miyake, K., Kang, K.W., Choi, Y.J., Hwang, D.H., 2006. Suppression of MyD88- and TRIF-dependent signaling pathways of toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea. Biochem. Pharmacol. 72, 850–859. https://doi.org/10.1016/j.bcp.2006.06.021

Yu, H., Kim, P.M., Sprecher, E., Trifonov, V., Gerstein, M., 2007. The importance of bottlenecks in protein networks: Correlation with gene essentiality and expression dynamics. PLoS Comput. Biol. 3, 713–720. https://doi.org/10.1371/journal.pcbi.0030059

Zhang, X., Guo, K., Xia, F., Zhao, X., Huang, Z., Niu, J., 2018. FGF23 C-tail improves diabetic nephropathy by attenuating renal fibrosis and inflammation. BMC Biotechnol. 18, 1–9. https://doi.org/10.1186/s12896-018-0449-7

Tables

Table 1. Blood Glucose, biometric parameters and water consumption of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group.

	Ctrl	**STZ**	**STZ+GTI**
Blood Glucose (mg/dL)	85.38 ± 7.53	475.00 ± 33.14*	542.80 ± 42.20#
Initial body weight (g)	84.26 ± 14.97	81.27 ± 9.46	81.75 ± 7.57
Final body weight (g)	288.10 ± 44.16	93.08 ± 23.42*	99.75 ± 13.04
Body weight gain (g)	203.80 ± 30.81	11.82 ± 21.87*	18.00 ± 16.18
Kidney weight (g)	0.98 ± 0.07	0.78 ± 0.18#	0.86 ± 0.08
Renal somatic index (%)	0.34 ± 0.05	0.84 ± 0.13*	0.87 ± 0.10
Initial water consumption (mL/day)	44.45 ± 10.02	44.48 ± 9.87	41.78 ± 11.16
Final water consumption (mL/day)	39.25 ± 6.13	118.8 ± 17.45*	139.8 ± 10.26#

Mean ± SD. Data were compared by Student *t*-test (Ctrl vs STZ; STZ vs STZ+GTI) considering statistical differences when $P \leq 0.05$. Asterisk (*) indicates difference with $P < 0.0001$, and the hash (#) indicates different means with $P < 0.05$. (n = 6 animals/group).

Table 2. Reactome pathways identified for each cluster with specific interest to the diabetic nephropathy pathological state, identified by the comparison of the CPI network with the Reactome Pathway database with the corresponding adjusted *P*-values.

Cluster	Proteins	Reactome Pathway	Adjusted *P*-value
Cluster 1	NR1H4, CCL2, IL8, IL6, MAPKAPK5, STAT3, PIN1, PARP1, CASP8, MTOR, MAPK3, MAPK8, MLH1, CASP3, CASP9, GSK3B, HIF1A, BID, MAPK1, MAP2K1, BAX, BCL2, FOS, JUN, APC, AKT1, CDKN1A, CDK2, TP53, CTNNB1, TCF7L2	Apoptosis	1.76×10^{-7}
		Signaling by SCF-KIT	1.06×10^{-6}
		Signaling by FGFR in disease	4.35×10^{-6}
		Signaling by PDGF	6.00×10^{-6}
		TRIF-mediated TLR3/TLR4 signaling	6.00×10^{-6}
		AKT phosphorylates targets in the cytosol	1.15×10^{-5}
Cluster 2	DB02077, RPIA, POR, H6PD, PGLS, PGD, AKT1, TYW1, MTRR, NOS2, DB08019, DB08018, AC1NDS4X, NOS1, CAV1, NDOR1, CIAPIN1, HSP90AA1, UBC, NOS3	eNOS activation	1.05×10^{-6}
		Pentose phosphate pathway	1.30×10^{-5}
		AKT phosphorylates targets in the cytosol	3.70×10^{-5}
		Metabolism of carbohydrates	1.53×10^{-4}
		Cellular response to hypoxia	1.50×10^{-3}
		PI3K/AKT/mTOR activation	6.29×10^{-3}

Figures

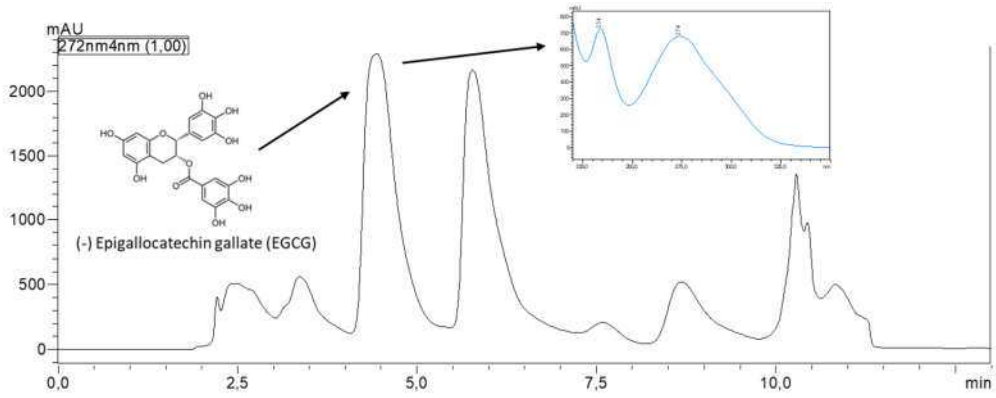

Figure 1. Chromatogram of the green tea infusion (*Camellia sinensis*). In detail: peak of the major compound (Epigallocatechin gallate).

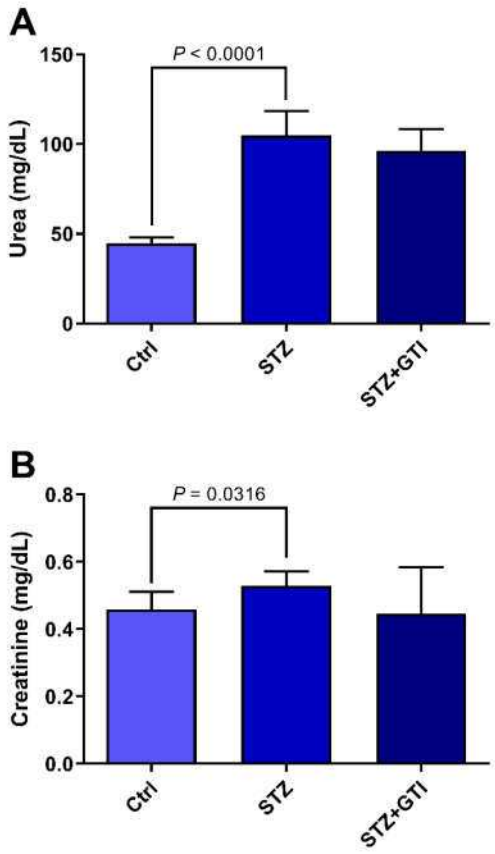

Figure 2. Renal function markers of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Urea (mg/dL). **B** – Creatinine (mg/dL). Mean ± SD. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student *t*-test (Ctrl vs STZ; STZ vs STZ+GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

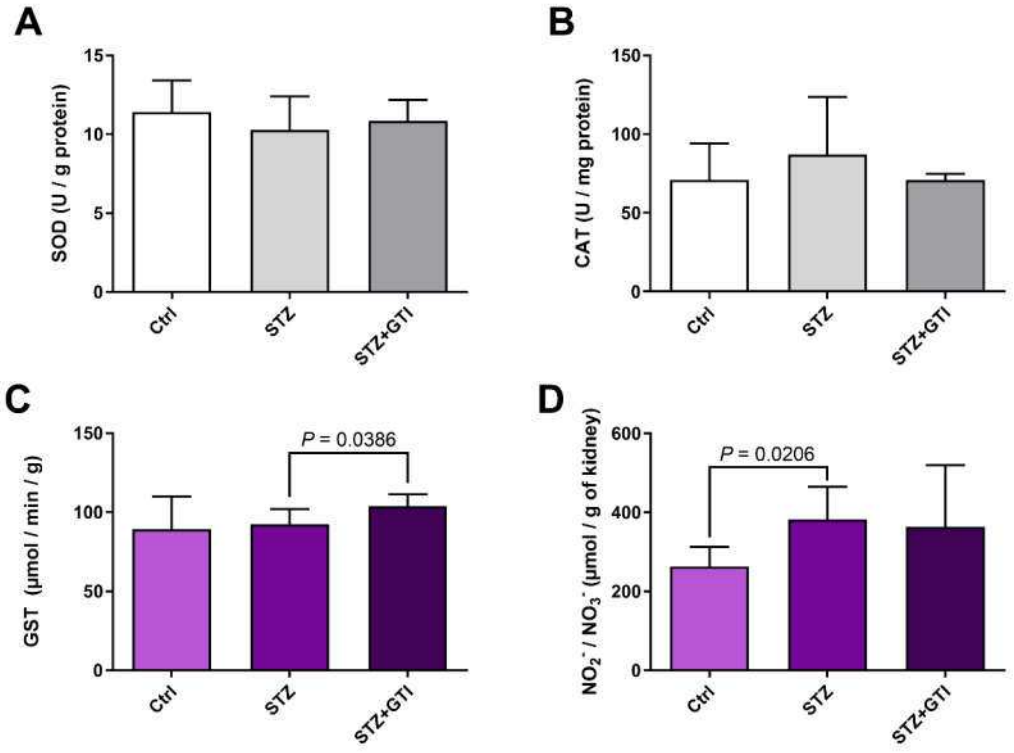

Figure 3. Antioxidant enzymes and nitric oxide levels of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Superoxide dismutase. **B** – Catalase. **C** – Glutathione S-Transferase. **D** – Nitric oxide. Mean ± SD. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student *t*-test (Ctrl vs STZ; STZ vs STZ+GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

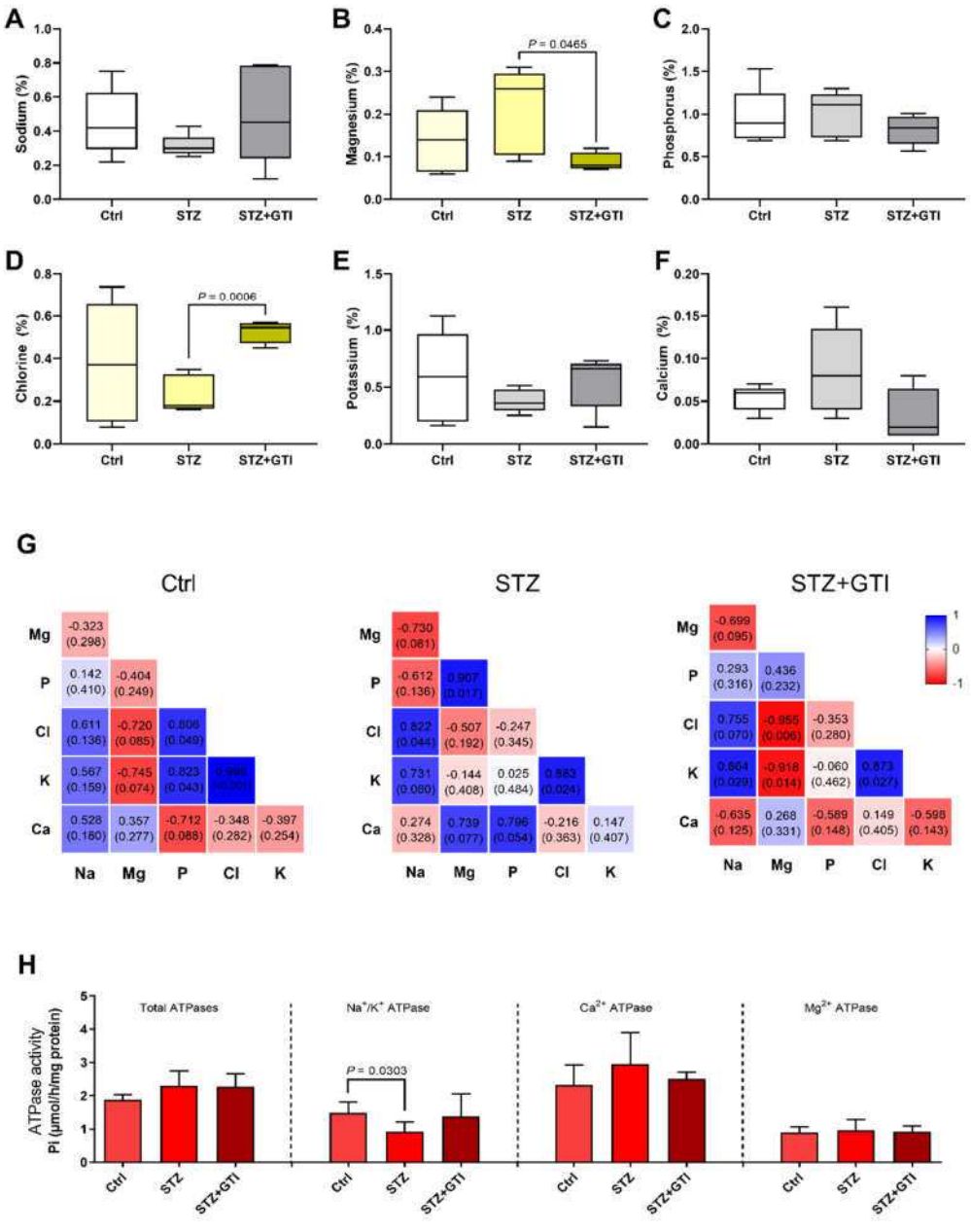

Figure 4. Microelement proportions and its correlations, and ATPase activity in the kidney of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Sodium (%). **B** – Magnesium (%). **C** – Phosphorus (%). **D** – Chlorine (%). **E** – Potassium (%). **F** – Calcium (%). **G** – Elemental correlations. **H** - Na^+/K^+, Ca^{2+}, Mg^{2+} and total ATPase activity. Mean ± SD. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student *t*-test (Ctrl vs STZ; STZ vs STZ+GTI)

considering statistical differences when $P \leq 0.05$. The correlations were calculated by Pearson's method and the r^2 is shown in the upper number of each graph cell, the bottom number in each graph cell corresponds to the *P*-value of each correlation. (n = 6 animals/group).

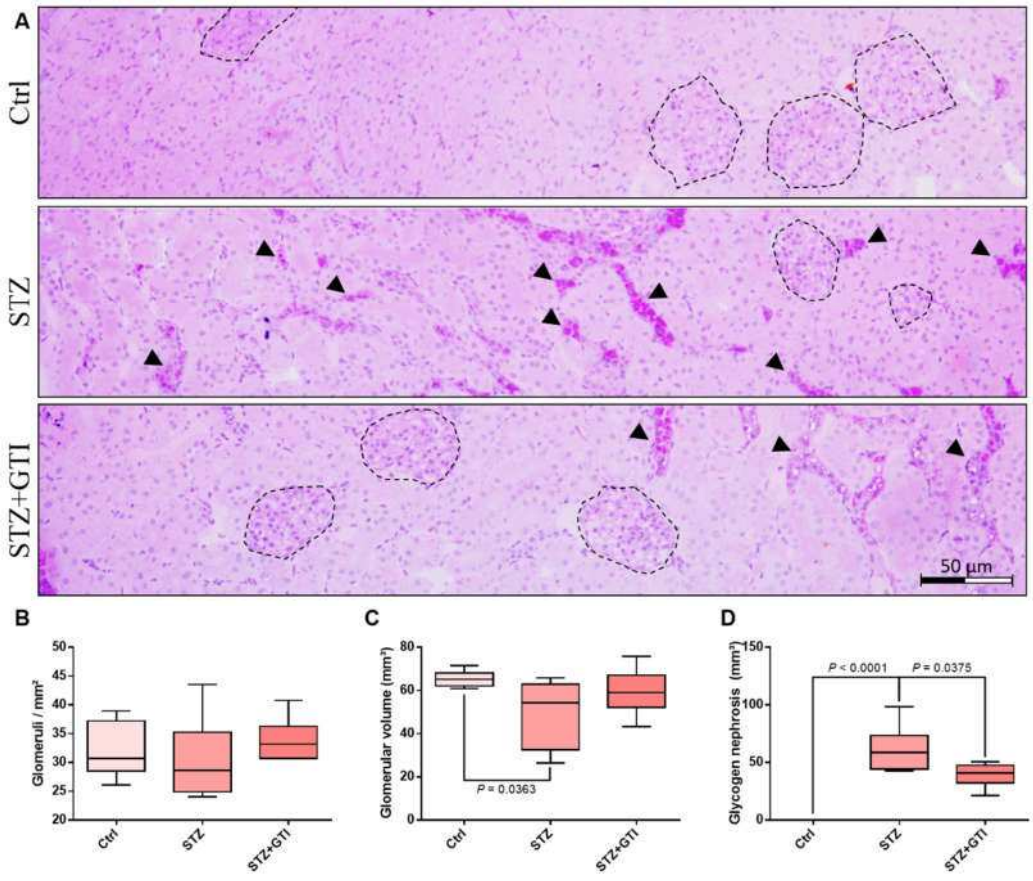

Figure 5. Representative PAS stained photomicrographs, histopathological and stereological parameters of the kidney´s cortex of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Kidney's cortex photomicrography. The glomeruli are delimited by the dotted line. The glycogen nephrosis areas are indicated by the arrowheads. The scale bar is indicated in the figure. **B** – Glomeruli / mm². **C** – Total glomerular volume (mm³). **D** – Glycogen nephrosis volume (mm³). The box represents the interquartile interval with the median indicated (horizontal line), and the whiskers represent the superior and inferior quartiles. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student *t*-test (Ctrl vs STZ; STZ vs STZ+GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

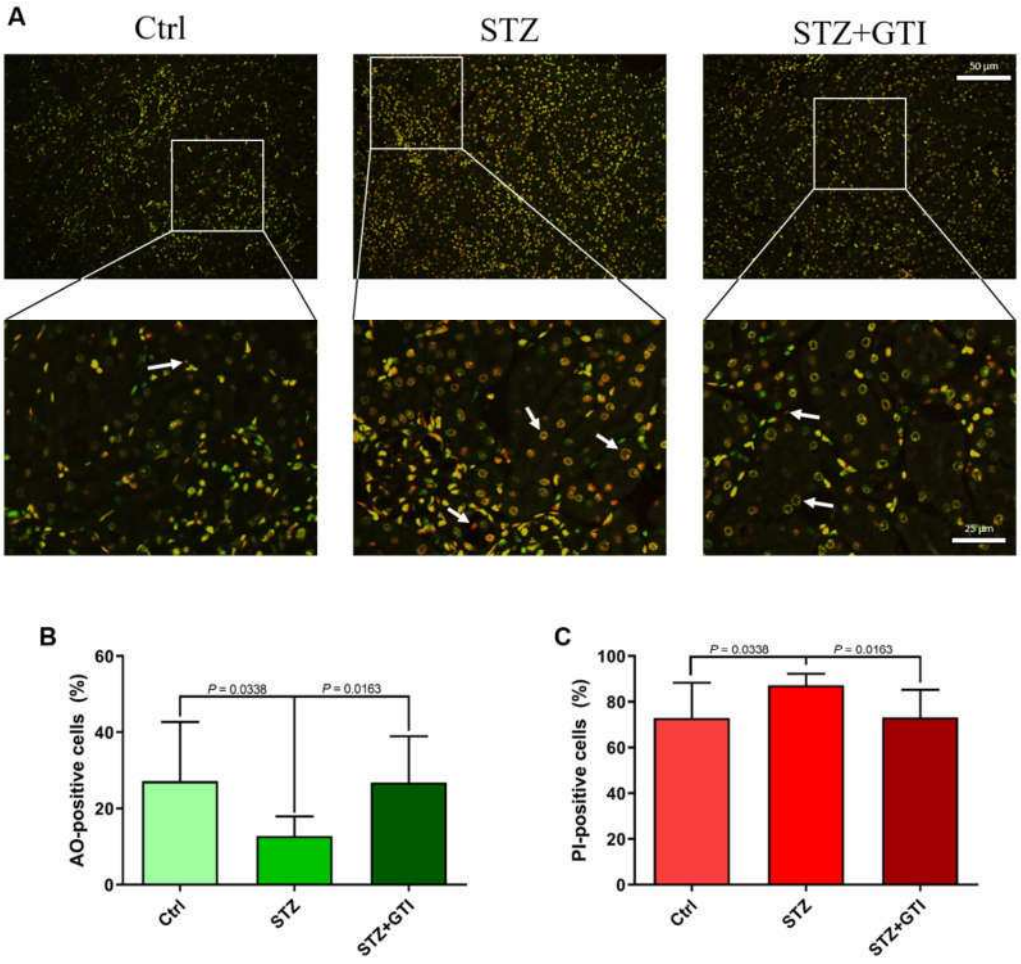

Figure 6. Representative acridine orange (AO) and propidium iodide (IP) stained photomicrographs of the kidney´s cortex of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Kidney's cortex photomicrography. Green nuclei – AO-positive; Yellow to reddish nuclei – IP-positive; Arrows indicate PI-positive nuclei. Scales bars are indicated in the figure. **B** – AO-positive cells (%). **C** – IP-positive cells (%). Mean ± SD. The statistical differences are indicated with lines with the *P*-value above them. Data were compared by Student *t*-test (Ctrl vs STZ; STZ vs STZ+GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

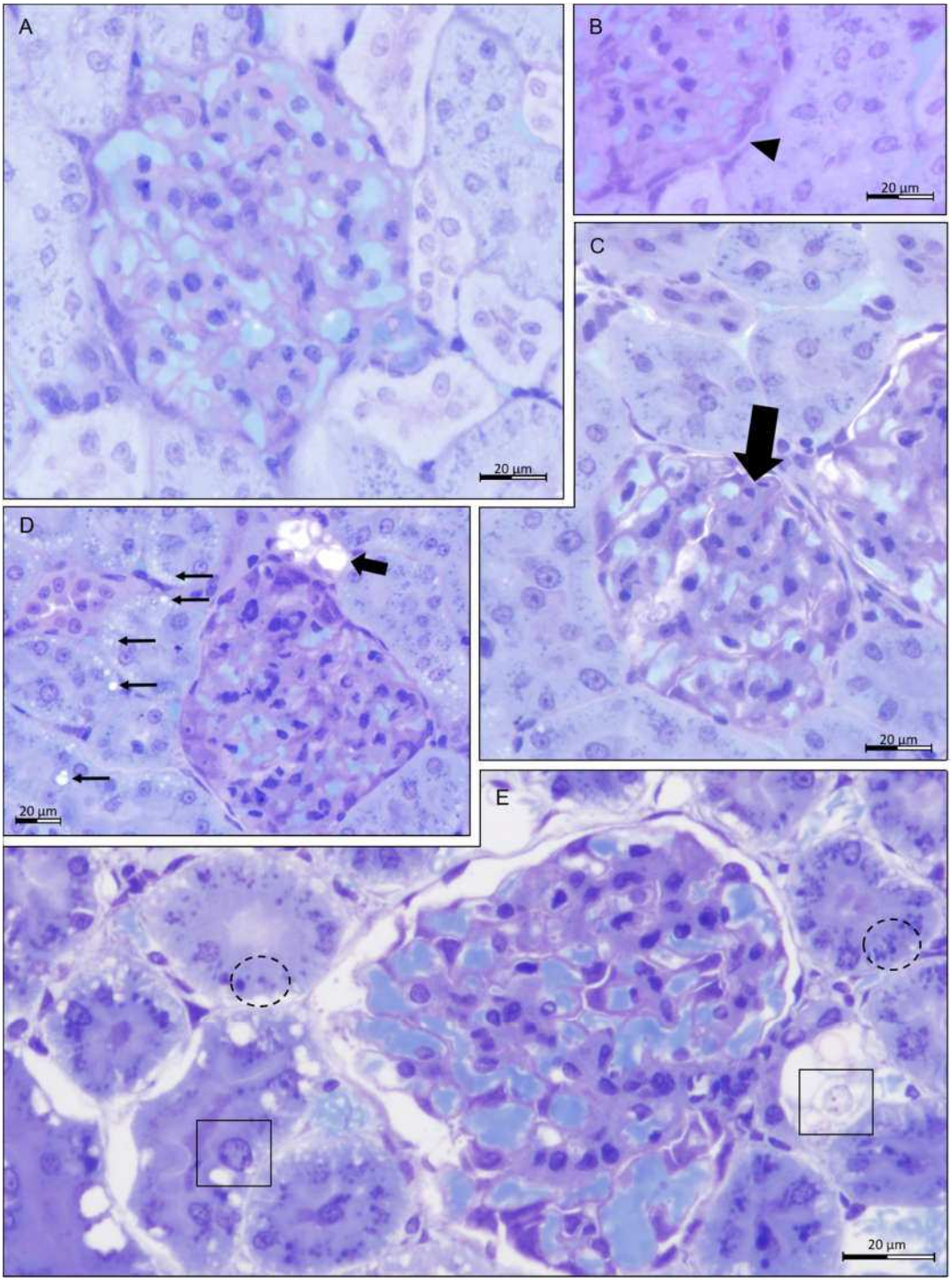

Figure 7. Representative photomicrographs of the glomerulus, stained with Toluidine Blue – Sodium borate 1%, of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – A normal glomeruli of an animal from the healthy control group. **B** – Diabetic glomeruli. Arrowhead indicates a thickening in the glomeruli basal membrane. **C** –

Diabetic glomeruli. Arrow indicates a region of diffuse mesangial expansion. **D** – Diabetic glomeruli. Thick arrow indicates a remarkable vacuolization in the macula densa region. Thin arrows indicate cytoplasmic microvesicles in the proximal tubule cells. **E** – Diabetic glomeruli. Squares indicate karyocytomegaly in the proximal tubule. Dotted circles indicate basal regions in the tubular cells with accumulation of stained granules, possible mitochondria aggregation. The glomeruli present a dilated Bowman's space.

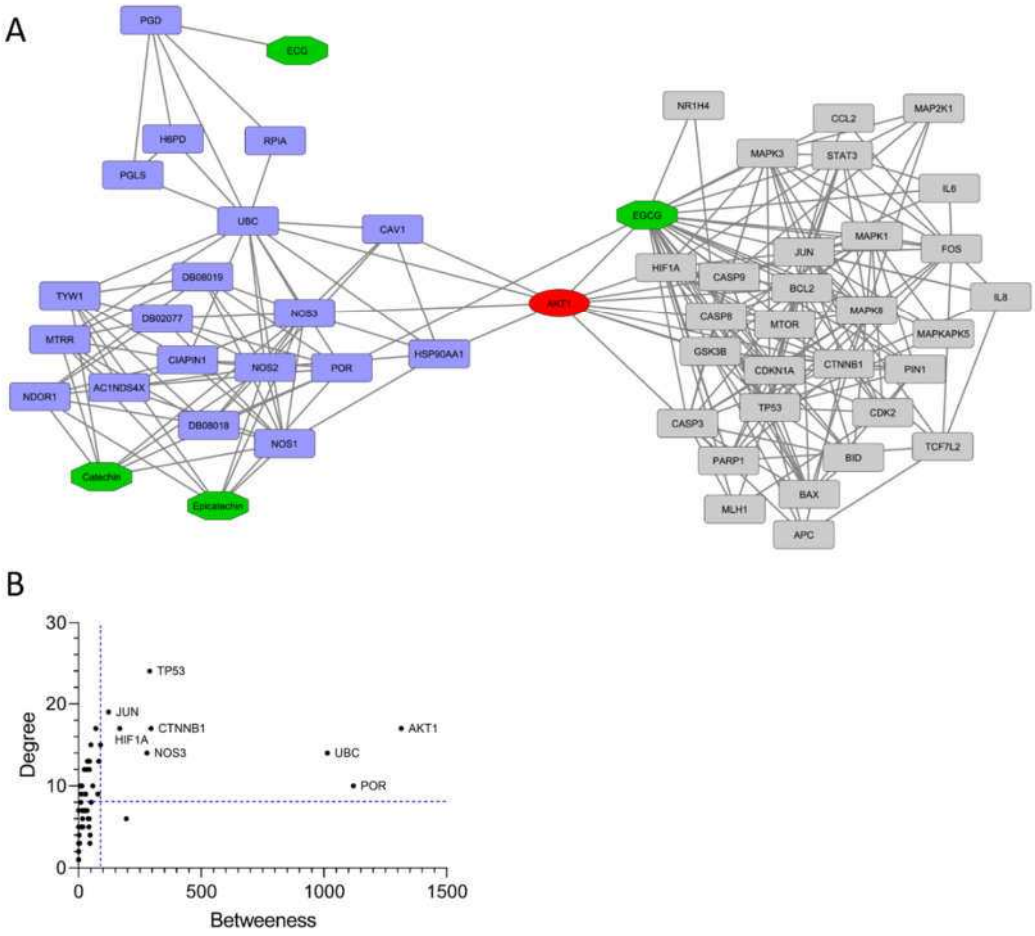

Figure 8. *In silico* exploration of catechins effects in the kidney. **A** – Compound-Protein Interactome network, highlighting tea catechins (green nodes), bottleneck protein (red node), cluster 1 (grey nodes), and cluster 2 (light blue nodes). **B** – Centrality analysis for the CPI network, the blue lines represent the threshold of the parameter.

Capítulo 3

Green tea protects against diabetic cardiomyopathy-induced morphophysiological damage in recent-onset severe type 1 diabetes

Abstract

Diabetic cardiomyopathy (DC) is a comorbidity resulting from diabetes, which develops from the stress generated by hyperglycemia, causing morphophysiological damage to the heart, which may progress to heart failure and death. Although there is no specific treatment for this type of heart failure, it is known that antioxidant substances can help relieving the symptoms. Green tea is traditionally used as a treatment for diabetes and its effects have been related to its hypoglycemic capacity, reducing oxidative damage. We investigated whether the infusion of green tea could prevent the development of morphophysiological changes in the heart caused by diabetes. We treated six young male Wistar rats, with type 1 diabetes induced by streptozotocin, with 100 mg/kg of green tea, daily, for 42 days. In addition, a healthy control group (n=6) and a diabetic group (n=6) also integrated the experiment. The infusion was prepared with the objective of reproducing the usual consumption by humans and the animals were kept under controlled conditions of temperature (22 ± 2 ºC) and light cycle (12/12h), and received food and water *ad libitum*. Serum and tissue markers for cardiac function and oxidative stress were evaluated. In addition, we analyzed morphological changes and DNA damage by bright field microscopy. Furthermore, we also evaluated the cardiac tissue and ultrastructural changes in mitochondria in the left ventricular fragments by scanning electron microscopy. Our results revealed that a daily dose of 100 mg/kg of green tea infusion treatment for 42 days prevented cardiac damage triggered by hyperglycemia in young rats with early-onset type 1 diabetes, even without being able to control the severe hyperglycemia in the animals. The green tea infusion was able to prevent the remodeling of the heart, attenuating the changes induced by diabetes, preventing fibrosis in the myocardium and pericardium, vascular remodeling in the myocardium and infiltration and activation of mast cells in the heart. Furthermore, it prevented damage to the cardiomyocytes DNA and control the morphological dynamics of the

mitochondria, which occur as a metabolic adaptation to diabetes. These beneficial results, taken together, are reflected in a positive profile of cardiac function markers.

Keywords

Diabetic cardiomyopathy; Diabetic heart disease; Green tea; Recent-onset diabetes; Type 1 diabetes.

1. Introduction

Diabetes Mellitus (DM) affects 9.3% (463 million) of the global population, and the projected numbers reach up to 700.2 million people with the disease in 2045 (INTERNATIONAL DIABETES FEDERATION, 2019). In the heart, DM predisposes to morphologic, metabolic, and functional complications that might lead to heart failure and evolve to the patient's death. At the cellular level, impaired processes include dysregulation of ion homeostasis with impaired functioning of these ion pumps (Ca^{2+} ATPase and Na-K-ATPase), intense generation of reactive oxygen species (ROS), switch of energy substrate to lipids, cardiomyocyte hypertrophy, mitochondrial dysfunction, and cell death (BUGGER; ABEL, 2014; CHEN et al., 2017; DA SILVA et al., 2016, 2015; LIAO et al., 2016; OU et al., 2010; RITCHIE; DALE ABEL, 2020).

DM may also impacts the process of pathological remodeling of the left ventricle (LV), with a reduction in capillary density, reduced coronary microvascular perfusion, inflammation, vacuolization of cardiomyocytes, necrosis, and fibrosis, in addition to stimulating an increase in the amount of cytoplasmic glycogen and glycoproteins in the extracellular matrix (BABU et al., 2007; DA SILVA et al., 2013, 2016; RITCHIE; DALE ABEL, 2020). These alterations may lead to cardiomyocyte dysfunction, causing a reduction in ejection fraction of the LV and heart rate (DA SILVA et al., 2015; HUANG et al., 2017). When left untreated, such changes can progress to heart failure and sudden death (DA SILVA et al., 2015; RITCHIE; DALE ABEL, 2020). Cardiac dysfunction may result from both types of DM, DM type 1 and type 2 (CHEN et al., 2017; YE et al., 2004) and accounts for about 80% of deaths of diabetic patients (BABU; SABITHA; SHYAMALADEVI, 2006a).

A landmark of cardiac dysfunction widely accepted in the medical and scientific community is the diastolic dysfunction of the left ventricle, one of the first signs of diabetic

cardiomyopathy (DC) (RITCHIE; DALE ABEL, 2020). It is usually detected before left ventricular systolic dysfunction (BOYER et al., 2004; RITCHIE; DALE ABEL, 2020). Such dysfunctions have been attributed to cardiac morphological changes, already at the advanced stages of the disease (WOOD; PIRAN; LIU, 2011). In the experimental model of diabetes induced by streptozotocin (STZ) in rats, 42 days are sufficient for the worsening of the disease and the appearance of systolic and diastolic dysfunctions, and this period usually allows the detection of morphological changes in the heart (GERBER; ARONOW; MATLIB, 2006). Cardiac functional damage is usually silent in DM patients and is often only detected in the more advanced stages of the disease (RITCHIE; DALE ABEL, 2020), making appropriate management even more complex and further aggravating the original condition.

In general, insulin therapy is the primary hyperglycemic damage control strategy in type 1 and severe type 2 DM patients (SOCIEDADE BRASILEIRA DE DIABETES, 2017). However, a long-term research conducted by the ACCORD study group found that intensive glycemic control has no effect on reducing the chances of heart failure and also increases the cardiac-related deaths in patients with type 2 DM (ACCORD STUDY GROUP, 2016). Whether the patient has a DM diagnosis or not, conventional treatment for heart failure is currently the same in both cases, since specific treatment for heart failure associated with diabetes mellitus is not yet available (RITCHIE; DALE ABEL, 2020).

Among the therapeutics of cardiac disfunction, those that can modulate lipid metabolism might be great allies for DM patients (RITCHIE et al., 2017). *Camellia sinensis* (L.) Kuntze (Theaceae) teas are quite popular in traditional medicine and are consumed worldwide for different purposes. Green tea is the most trendy of them, and its therapeutic potential is frequently linked to effects related to metabolic, glycemic, and weight control (BARKAOUI et

al., 2017; CHOPADE et al., 2008; FALLAH HUSEINI et al., 2006; MENG et al., 2019; RACHID et al., 2012; SEA-TAN; GROVE; LAMBERT, 2011).

Epigallocatechin gallate (EGCG), the main polyphenol in green tea, can protect the heart of adult Wistar rats with experimental type 2 diabetes induced by STZ and nicotinamide by reducing oxidative stress, inflammation, fibrosis, and cell death (OTHMAN et al., 2017). However, in Othman's study, the used EGCG doses made possible a glycemic reduction to levels near the healthy control (< 100 mg/dL). Other studies also associated positive effects of green tea intake to improvements on glycemic control (BABU et al., 2007; BABU; SABITHA; SHYAMALADEVI, 2006b; FIORINO et al., 2012; SAMARGHANDIAN; AZIMI-NEZHAD; FARKHONDEH, 2017), although evidence in young developing animals is scarce.

In a previous study, we demonstrated that the green tea infusion could counteract kidney damage progression, even though green tea was ineffective in preventing hyperglycemia in pre-clinical type 1 diabetes models (LADEIRA et al., 2021). Here we aimed to investigate the effects of green tea infusion treatment on diabetic cardiomyopathy induced in recent-onset experimental type 1 diabetic young rats to further evaluate whether green tea may also prevent heart damage under uncontrolled hyperglycemia.

2. Materials and methods

2.1. Green tea infusion preparation and analysis

Green tea (*Camellia sinensis*) infusion was prepared and analyzed as previously described before (LADEIRA et al., 2021). Briefly, leaves were obtained from Leão® - Food and Beverages (Coca-Cola Company®). The infusion was prepared mixing the leaves with warm distilled water (1:40 w/v, 80 °C) (PERVA-UZUNALIĆ et al., 2006). The mixture remained

infused for 20 minutes on a magnetic stirrer. Then, it was filtered through a 0.45 µm porous filter, frozen at -80 °C, and lyophilized. The lyophilized samples were resuspended in distilled water at the moment of use.

We determined the total phenolic and EGCG content and antioxidant capacity as previously described (LADEIRA et al., 2020a). The HPLC fingerprint and the total phenolic content and antioxidant capacity results are summarized in Figure 1.

2.2. Animals and treatments

Eighteen male Wistar rats (30-days-old; weighting 82.52 ± 10.83g) were housed under controlled conditions of temperature (22 ± 2 ºC) and light/dark cycles (12/12h). They received food (Presence Alimentos, Paulínea, SP, Brazil) and water *ad libitum*. The use of animals in the research was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa (CEUA/UFV – protocol number 53/2018).

The animals were randomly assigned to three groups, and after 12h fasting, diabetes was induced in 12 animals (2 groups) by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) (Sigma Chemical Co., St, Louis, MO, USA) at a dosage of 60 mg/kg of body weight (BW) diluted in 0.01 M sodium citrate buffer, pH 4.5 (DA SILVA et al., 2016). The healthy control group (n=6) received the buffer alone (i.p.) to simulate the injection stress (LAHAYE et al., 2010). Fasting blood glucose levels were measured after two days using a glucometer (Accu-Chek® Performa, Roche LTDA. Jaguaré, SP, Brazil) in blood samples collected at the tail vein. All animals presented fasting glycemia levels higher than 250 mg/dL and were included in the study. The experiment consisted of three groups: the healthy control group (Healthy Ctrl, n=6), which received water as placebo; the diabetic control group (Diabetic, n = 6), that also received water; and the diabetic group treated with the green tea infusion (Diabetic

+ GTI, n = 6), that was treated with green tea infusion (GTI) (100 mg/kg body weight). All treatments (GTI and water) were administered by gavage, daily, for 42 days.

We monitored the body weight using a precision scale (BEL M503, e = 0.001g, Piracicaba, SP, Brazil), and 12h fasting blood glucose in blood samples from the tail vein.

On the 43rd day, the animals were euthanized by deep anesthesia (sodium thiopental, 60 mg/kg i.p.) followed by cardiac puncture and exsanguination. The hearts were removed, weighed and their volume was determined using a submersion method (SCHERLE, 1970). The left ventricles (LV) were dissected, weighed and their volume was determined, then they were divided into three fragments. One fragment was frozen in liquid nitrogen and stored at -80 °C for enzymatic and chemical elements analysis. The second was immersed in Karnovsky fixative solution for 24h for histopathological analyses (KARNOVSKY, 1965). The third was immersed in Glutaraldehyde 4% solution for electron microscopy analysis. The relative weight of the heart and LV were calculated using the ratio between the organ weight (OW) and body weight (BW), where Relative Weight = OW/BW × 100 (SERTORIO et al., 2019).

2.3. Serum biochemical analysis

Blood samples collected by cardiac puncture following the euthanasia were centrifuged at 4600 rpm for 15 min at 4 ºC, and the serum was separated. Then we performed the analysis for quantification of creatine kinase (CK-MB) and lactate dehydrogenase (LDH) in the serum using biochemical kits (Bioclin Laboratories, Belo Horizonte, MG, Brazil) at the BS-200 equipment (Bioclin Laboratories, Belo Horizonte, MG, Brazil) following the manufacturer's instructions.

2.4. Anti-oxidant capacity

The oxidative and nitrosative stress markers analysis was performed with the supernatant obtained from the following: 100 mg of frozen LV tissue was homogenized in ice-cold phosphate buffer (pH 7.0) and centrifuged at 12000 rpm for 10 minutes at 4 ºC. We quantified the activity of the superoxide dismutase (SOD) (DIETERICH et al., 2000), glutathione-S-transferase (GST) (HABIG; PABST; JAKOBY, 1974), and catalase (CAT) (AEBI, 1984). The nitric oxide (NO_2^-/NO_3^-) was quantified by the Griess method (RICART-JANÉ; LLOBERA; LÓPEZ-TEJERO, 2002). The total antioxidant capacity by ferric reducing antioxidant power (FRAP) as described before by Benzie and Strain (1996). The values of enzyme activities were normalized by the total protein content, determined with the Folin–Ciocalteu method according to Lowry et al. (1994).

2.5. Chemical elements analysis

The proportion of chemical elements in the LV was assessed per area in fragments of the frozen LV, as described before (LADEIRA et al., 2020b). We measured the proportion of calcium (Ca), sodium (Na), magnesium (Mg), manganese (Mn), potassium (K), iron (Fe), copper (Cu), zinc (Zn), and selenium (Se). Fragments were dried at 60 °C for 96h, mounted in a stub, and analyzed in a scanning electron microscope (JEOL, JSM-6010LA) with a Silicon Drift type X-ray detector system. The analysis was performed under a low vacuum in an area of 250 μm^2, using an accelerating voltage of 20 kV and a working distance of 10 mm. Data were normalized using the carbon (C) and oxygen (O) measurements. The results were expressed as a mean value of the proportions between the elements present in the samples.

2.6. Histopathological, stereological analysis, and assessment of DNA damage

The fragments fixed in Karnovsky solution were divided following the *orientator* method to obtain uniform and isotropic random sections, necessary for the stereological study (MANDARIM-DE-LACERDA, 2003). Then, they were dehydrated in a crescent ethanol series and embedded in Historesin® (Leica, Nussloch, Germany). A rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany) was used to cut the material into histological sections of 3μm thickness, then the section was mounted in glass slides (MISHIMA et al., 2021).

The sections were stained with Hematoxylin and Eosin (H&E) for histopathological and stereological evaluation (NOVAES et al., 2018). In summary, we quantified the volume density occupied by cardiomyocytes (Vv [cmy] %), interstitium (Vv [int] %), and blood vessels (Vv [bvs] %), the length density of the cardiomyocytes (Lv [cmy]) and blood vessels (Lv [bvs]), the mean diffusion distance from capillary to tissue (r [bvs] μm), and the mean cross-sectional area of cardiomyocytes (a [cmy] μm^2). Additionally, we calculated the blood vessels and interstitium relative volumes to the cardiomyocyte volume: Vv [bvs] / Vv [cmy] and Vv [int] / Vv [cmy]. All the stereological methods and equations used in this work were previously described (NOVAES et al., 2018).

We also stained sections with Toluidine Blue – Sodium borate 1% for mast cell quantification and classification and stratified into activated and inactivated mast cells based on the morphology of the cell, being degranulating cells considered activated ones (YIN et al., 2018). In addition, DAPI (4',6'-diamino-2-fenil-indol) was used to count cell number in the cardiac tissue sections (NOVAES et al., 2018).

DNA damage was evaluated in sections of the LV stained with acridine orange (AO; green) and propidium iodide (PI; red) (BERNAS et al., 2005; SUZUKI et al., 1997). This fluorescent stain allows to evaluate DNA damage, as damaged DNA presents red color, marked

with PI, and integral DNA is marked in green by the AO, also, in the superposed images, the yellow marked nuclei was considered as in the initial damage process (LIMA et al., 2018). Digital images were captured using a photomicroscope (Olympus AX 70 TRF, Tokyo, Japan) and analyzed with Image-Pro Plus® 4.5 (Media Cybernetics, Silver Spring, MD).

2.7. Qualitative analysis of the extracellular matrix

Left ventricle glutaraldehyde fixed fragments were submitted to the NaOH decellularization maceration process for the isolation of the fibrillary collagen matrix (ROSSI; ABREU; SANTORO, 1998). The fragments were immersed in a 10% NaOH (w/w) solution for 7 days at room temperature. Then they were rinsed in distilled water until they became transparent. After, they were immersed in 1% tannic acid solution for 4 hours and rinsed in distilled water overnight, rinsed again, and post-fixed in a 1% osmium tetroxide solution for 2 hours. Subsequently, the fragments were dehydrated in a crescent series of ethanol (70% to Absolute ethanol), submitted to critical point drying (CPD 030, Baltec, Witten, North Rhine-Westphalia, Germany), coated with powdered gold, and observed under a scanning electron microscope (SEM) (JEOL, JSM-6010LA), with an accelerating voltage of 5 kV and a minimum working distance of 10 mm (STEPHENSON et al., 2016). The extracellular matrix of the left ventricle fragments was analyzed qualitatively.

2.8. Qualitative analysis of the left ventricle fragments

Glutaraldehyde fixed fragments were dissected and prepared for cryofractured scanning electron microscopy imaging as described before (CURY et al., 2013). Briefly, the fragments were rinsed in distilled water and immersed in a crescent series of dimethyl sulfoxide (DMSO)

solution (12.5%, 25%, and 50%). The fragments were frozen in liquid nitrogen and fractured with a frozen razor. Then, they were immersed in the 50% DMSO solution for 30 min, rinsed in distilled water, post-fixed with a 2% osmium tetroxide solution for 2h (4º C), and immersed in a 2% tannic acid solution for 1h at room temperature. Subsequently, the fragments were dehydrated in a crescent series of ethanol and followed the same final steps of the extracellular matrix imaging preparation described in item 2.7. The images were composed by the detection of secondary electrons (SEI).

2.9. Statistical analysis

The study design and statistical analysis were inspired by previous studies (CHOO, 2003; TANG et al., 2013; ZHANG et al., 2021). All animals were evaluated. All the results were submitted to the Shapiro-Wilk test to check normality. The data expressed as percentages were transformed by angular transformation before the analysis. Results were expressed as mean ± standard deviation (mean ± SD) and analyzed using unpaired *t*-test when the variances are equal (by *F* test) and unpaired *t*-test with Welch's correction for data with unequal variances (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI). The non-parametric data were compared with the Mann-Whitney test. Statistical significance was established at $P \leq 0.05$.

3. Results

After diabetes induction and subsequent hyperglycemia confirmation two days later, both diabetic groups maintained high blood glucose levels, which remained above 400 mg/dL, compared with the healthy control group (glucose < 100 mg/dL). Both diabetic groups remained severely hyperglycemic, and notably, green tea infusion did not reduce blood glucose levels in the treated group. The body weight was reduced in the two diabetic groups, indicating a

commitment of the body development by hyperglycemia compared to the Healthy Ctrl group (Table 1).

The heart weight and volume were reduced in the diabetic groups compared to the Healthy Ctrl group. However, it was not reflected in the relative heart weight or volume, indicating that the organ's growth was proportional to the animal's body growth. The LV, on the other hand, was affected in both parameters, absolute and relative weight and absolute volume. The absolute weight and volume were reduced in the diabetic groups, and the relative weight was increased (Table 1), indicating that the LV could have suffered hypertrophy in these groups.

The serum biochemical analysis revealed that diabetes increased CK-MB and LDH levels, and the oral administration of green tea infusion was able to prevent the rise of these two heart function markers levels in the GTI treated group (Figure 2).

Hyperglycemia reduced the NO_2/NO_3 and the total antioxidant capacity (FRAP) in both diabetic groups (Figure 3); however, differences in the antioxidant enzyme activities were not detected (i. e. CAT, SOD, and GST).

Elemental mapping of the LV fragments showed a homogenous distribution of the chemical elements that participate in the cardiomyocyte function and contraction in all three groups (Figure 4, A). Diabetes induced a reduction in the sodium proportion and a rise in the magnesium proportion in the tissue (Figure 4, B and C). GTI improved sodium proportion, reducing its levels in the Diabetic + GTI group; however, no effect was found for Mg proportion.

Diabetes led to a reduced proportion of AO-positive cells in the LV (Figure 5, D), indicating a reduced proportion of cells without DNA damage. A direct consequence of that is the increased proportion of IP-positive cells, showed in Figure 4, F. On the other hand, GTI was able to counteract these effects, reducing the proportion of the IP-positive cells (Figure 5,

F), even without statistical differences in the AO-positive cell counting, possibly due to the variance in the initial damaged DNA %, indicated in Figure 5 (E). No effect was observed in the nuclei classified as initial damaged DNA labeled. Figure 5 (A to C) shows representative AO-IP labeled histological LV slices with green, yellow, and red fluorescence.

Animals from both diabetic groups exhibited increased cell count, which could indicate an inflammatory process (Figure 6, G). Diabetes led to increased counting of the total, inactive, and activated mast cells, and green tea infusion was able to induce a reduction or prevent the rise in these three parameters (Figure 6, H, I and J), even exerting no effect on total cell counting (Figure 6, G).

Stereological analysis showed that diabetes induced a deep myocardial remodeling compared with healthy control animals (Figure 7). Animals in the Diabetic group presented a higher Vv [int] % and Vv [int] / Vv [cmy] compared with Healthy Ctrl (Figure 7, F and I), indicating an expansion of the interstitial component of the tissue. Also, diabetes induced a reduction on vascular parameters Vv [bvs] % and Vv [bvs] / Vv [cmy] in the diabetic animals without treatment (Figure 7, G and H). Simultaneously, green tea infusion prevented this remodeling, leading to values numerically near the Healthy group, on all these parameters in the GTI treated animals.

Besides, diabetes induced a marked vacuolization in the cardiomyocytes in the LV of animals in the Diabetic group without GTI treatment (Figure 7, C'), not observed in the Heathy Ctrl nor the Diabetic + GTI groups.

Similar to the Vv [cmy] %, the relative cardiomyocyte length (LV [cmy]) was not affected (Figure 8, A); however, diabetes led to a reduced blood vessel relative length, prevented by green tea treatment (LV [bvs], Figure 8, B). Furthermore, the cardiomyocyte cross-sectional

area and the diffusion distance of blood vessels and capillaries were increased by diabetes, changes also prevented by green tea treatment (Figure 8, C and D).

Qualitative analysis of the LV extracellular matrix revealed a well-organized thin collagen fibers matrix in the myocardium and pericardium layers in the Healthy Ctrl group animals (Figure 9, A and B). Untreated diabetic animals presented thick and densely compacted collagen fibers in the myocardium compatible with tissue fibrosis (Figure 9, C and C'). Moreover, the pericardium fibers also appear more densely organized than the Healthy Ctrl group (Figure 9, D). On the other hand, green tea treated diabetic group LV collagen fiber matrix resembles the healthy animal's extracellular matrix, with the fibers loosely organized in the regions surrounding the cardiomyocytes and without the presence of compaction in the myocardium and pericardium (Figure 9, E, F, and G).

Scanning electron microscopic qualitative analysis of the cryofractured myocardium revealed a well-vascularized tissue in the Healthy control animals, without the presence of leucocytes (Figure 10, A) and typical cardiomyocyte internal organization, with a well-delimited sarcomere unit by t-tubules structures along the Z-line and multiple individual mitochondria (Figure 10, B and C). In diabetic group animals, leucocytes were more frequent, and an extracellular matrix with bundles of collagen fibers was also present (Figure 10, D). Additionally, leucocytes were frequent in the SEM images of the diabetic group (Figure 10, E). Myofibrils and sarcomeres were well-delimited; however, mitochondria appeared to fuse, forming structures similar to bunches of grapes (Figure 10, F). Green tea treated diabetic group animal's myocardium, and cardiomyocytes structures resembled the Healthy Ctrl group ones (Figure 10, G and H); however, despite the well-defined mitochondria structures, fusion points between them were still present (Figure 10, H). Figure 11 summarizes the main results of this study.

4. Discussion

Our results revealed that a daily dose of 100 mg/kg of green tea infusion treatment for 42 days prevented heart damage triggered by hyperglycemia in young early-onset type 1 diabetic rats. Despite the lack of a direct effect on the activities of antioxidant enzymes, green tea prevented cardiac fibrosis and cardiomyocyte hypertrophy, maintaining the diffusion distance of the blood vessels and the cross-sectional area of the fibers at similar levels to those found in healthy animals. Besides, our results indicate a protective effect of green tea against DNA damage. These positive results reflected in the lower levels of CK-MB and LDH levels, suggesting a better cardiac function in the Diabetic + GTI treated group, regardless of any improvements on blood glucose values.

Diabetes modulates the energy metabolism in the heart to shift towards lipid oxidation instead of the typical turnover of various substrates, including glucose, ketone bodies, and amino acids, occurring under normal conditions (BERTRAND et al., 2020). This intricated systemic metabolic control is already reviewed by many studies (BAYEVA; SAWICKI; ARDEHALI, 2013; LEVELT et al., 2018; RITCHIE et al., 2017; RITCHIE; DALE ABEL, 2020), and the main reason for this alternating substrate preference seems to be associated to the fact that the primary glucose transporter in cardiomyocytes in the adult heart (GLUT4) is insulin-dependent. The lack of this hormone in type 1 diabetes leads to insufficient signaling to induce GLUT4 translocation to the cellular membrane (RITCHIE; DALE ABEL, 2020). However, cardiac-specific GLUT4 knockout mice were able to express GLUT1 in cardiac tissue and maintain basal glucose uptake as an adaptation to the diabetic condition (ABEL et al., 1999). This mechanism may compensate for the lack of the main glucose transporter to allow the maintenance of basal glucose uptake. However, GLUT1 does not transport glucose as efficiently as GLUT4, so the metabolism seems to be modulated towards a preference for fatty acids oxidation (BERTRAND et al., 2020). Also, beta oxidation induces a simultaneous

activation of the pyruvate dehydrogenase kinase (PDK) and inhibition of pyruvate dehydrogenase, which reduces glucose oxidation in order to improve beta oxidation (RITCHIE et al., 2017). Accordingly, glucose uptake and use is fairly reduced, both in experimental models of diabetes (RITCHIE; DALE ABEL, 2020; SOWTON; GRIFFIN; MURRAY, 2019) and in humans (COOK et al., 2010; NIELSEN et al., 2018; SOWTON; GRIFFIN; MURRAY, 2019). Yet, lipid accumulation in cardiomyocytes, like the evidence found in non-treated diabetic animals in this study, might indicate lipotoxic damage (RITCHIE et al., 2017; SOWTON; GRIFFIN; MURRAY, 2019).

As already discussed in our previous work (LADEIRA et al., 2021), PI3K/AKT/mTOR pathway is related to *de novo* lipogenesis, which can lead to lipid accumulation and aggravation of lipotoxicity, which is in line with the microvesicles in diabetic animals, showed in Figure 7 C'. Like the results described for the kidney in our previous work (LADEIRA et al., 2021), GTI treated animals did not present these findings, possibly by the inhibition of both PI3K and mTOR, by EGCG through competitively binding in the ATP-binding sites of these proteins (VAN ALLER et al., 2011).

With low levels of intracellular glucose, muscles depend almost exclusively on fatty acids oxidation (BAYEVA; SAWICKI; ARDEHALI, 2013; BERTRAND et al., 2020; SOWTON; GRIFFIN; MURRAY, 2019), a process that requires more oxygen than carbohydrate metabolism (PERONNET; MASSICOTTE, 1991). Nevertheless, the blood O_2 supply is highly affected by the endothelial damage (including capillary loss and obstruction) caused by hyperglycemia (HERMAN-EDELSTEIN; DOI, 2016). Accordingly, diabetic patients often show reduced coronary blood volume and blood flow (HANSEN et al., 2002; MOHAMMED et al., 2015), which worsens the oxygen delivery to the organ (RITCHIE; DALE ABEL, 2020). Once installed, a hypoxic environment favors the Caspase pathway activation (HO et al., 2006) and the development of cardiac fibrosis through TGF-β pathway stimulation (WANG et al.,

2016). In fact, fibrosis, induced through the increased O_2 and nutrients diffusion distance and decreased capillary density, is frequently observed in pre-clinical studies of diabetes in juvenile animals (DA SILVA et al., 2013, 2016). Yet, coronary reduction in the human heart, leading to reduced O_2 delivery, was already linked to fibrosis development (MOHAMMED et al., 2015). This whole mechanism worsens as it feeds back.

Green tea extract has shown to modulate the extracellular matrix architecture through inhibition of the fibroblast growth factor receptor (FGFR) signaling pathway by reducing the expression of fibroblast growth factor (FGF) and blocking the signaling of the platelet-derived growth factor (PDGF), another profibrotic factor, as shown in a study using EGCG (PARK et al., 2006; SARTIPPOUR et al., 2002). These mechanisms may be involved in the fibrosis reduction, with collagen extracellular matrix modulation and diffusion distance restauration on the heart. Yet, fibrosis reduction can help mitigate the damage caused by hypoxia, permitting a better diffusion of oxygen and improving metabolism efficiency.

Fibrosis and cardiomyocyte hypertrophy, found in our untreated diabetic animals, can be induced by TGF-β in many cardiac conditions, including diabetes (WENZEL et al., 2010; YUE et al., 2017). However, this factor is also associated with the up-regulation of the 67-kDa laminin receptor (67LR) expression in the left ventricle cardiomyocytes (WENZEL et al., 2010). 67LR was identified to be the primary receptor for EGCG (TACHIBANA et al., 2004) and is capable of triggering the protein kinase B (Akt) pathway (KUMAZOE; FUJIMURA; TACHIBANA, 2020), leading to the activation of the diacylglycerol kinase (DGK) pathway, inactivating the protein kinase C beta (PKC-β) (HAYASHI et al., 2020). These effects combined might result in improvements in the ongoing systemic vascular disfunction (ISHII et al., 1996), counteracting one of the major causes associated with cardiac damage progression. This compensatory mechanism particularly benefits the green tea mode of action since TGF-β

can elevate the expression of the receptor of the tea's main active compound in the damaged tissue, providing a better EGCG action.

Circulating fatty acids and TGF-β also play a role in the activation of Toll-Like Receptor 4 (TLR4), which culminates in the activation of the nuclear factor κ B (NFκB) that worsens fibrosis and leads to cardiac inflammation (FRATI et al., 2017). This mechanism is consistent with the intensification of cardiac cell number and mast cells in our study, that could indicate an inflammatory process. Interestingly, EGCG was shown to inhibit the TLR pathway activation *in vitro* (YOUN et al., 2006) and reduce the NFκB expression (YAMABE et al., 2006), suggesting that green tea may act through this mechanism to promote anti-inflammatory and antifibrotic protection. Although we have not found a reduction in total cell count promoted by the green tea treatment, mast cells were less frequent and less activated in the Diabetic + GTI group. Whole green tea preparation, like its infusion, was shown to reduce mast cell activation *in vitro* and *in vivo* (BALAJI et al., 2014; INOUE; SUZUKI; RA, 2010). In addition, a study with isolated EGCG describes its capacity in reducing mast cell activation, controlling its degranulation (LI; CHAI; SONG, 2005). Other green tea catechins (e.g., epicatechin) also have an effect on inhibiting mast cell activity, although not as efficiently EGCG (INOUE; SUZUKI; RA, 2010). Mast cell infiltration and activation are regulated by many types of proteins, expressed in humans and rodents (GILFILLAN; TKACZYK, 2006), and the reduced degranulation might be related to the phosphorylation inhibition of signaling factor such as AKT and NF-κB (LI et al., 2021).

Our results revealed that propidium iodide bonded to cardiomyocytes DNA was reduced in the green tea treated group, presenting similar levels of that found in the healthy animals. Despite that, antioxidant enzyme activities were not impacted by diabetes nor green tea infusion. Also, NO_3^-/NO_4^- levels were found to be reduced in both diabetic groups, which is consistent with diabetic tissue damage, such as fibrosis and microvascular injuries (JOSHI et

al., 2013), as hyperglycemia reduces the endothelial nitric oxide synthase (eNOS) enzyme activity. However, none of these results alone explain the preservation of DNA damage in the heart. We hypothesize that such protection might be related to the antioxidant capacity of green tea itself and the possible lipotoxic prevention promoted by green tea, although underlying mechanisms need a deeper investigation. A previous study showed that EGCG could inhibit apoptosis induced by oxidative stress (ITOH et al., 2005), preserving renal cells in an *in vitro* model. In addition, whole green tea extract was shown to prevent apoptosis and improve the endogenous antioxidant system in adult diabetic animals (OTHMAN et al., 2017). Green tea can also reduce Caspase 3 activity in a diabetic nephropathy model, leading to reduced DNA damage levels and higher cell survival rates (PEIXOTO et al., 2015). Although this mechanism has been described in kidney cells, cardiomyocytes express the complete protein apparatus that would enable, in the heart, the same protection previously reported.

Ion balance is directly involved in the antioxidant enzyme activities (SOETAN; OLAIYA; OYEWOLE, 2010). In our study, however, the main involved ions did not show different levels than those found in healthy animals. Sodium and magnesium, on the other hand, were affected by diabetes. These ions are involved in several cellular functions (HOLROYDE et al., 1980; PFEIFFER et al., 2014). Diabetes reduces the exchange capacity of the sodium ATPase pump, resulting in a deficient transport of the ion to the cell (BABU; SABITHA; SHYAMALADEVI, 2006a). Magnesium also participates in ATP metabolism (SULLIVAN et al., 1971). Green tea treatment modulated these two ions positively, bringing them to levels similar to those found in the control group, contributing to prevent damage in the tissue.

In pre-clinical studies, CK-MB and LDH have the same pattern of variation of Troponin T (OTHMAN et al., 2017), a specific marker of cardiac damage, and may indicate the global status of heart health. A study with type 2 diabetic adult rats treated with a single dose of EGCG (2.0 mg/kg) found that this catechin protects against the progression of diabetic cardiomyopathy

by oxidative stress modulation and morphological damage protection, reflecting on the cardiac function (OTHMAN et al., 2017). However, the use of green tea catechins in adult diabetic animals can also control the rise of glycemic levels, as seen in many studies (BABU et al., 2007; OTHMAN et al., 2017; RENNO et al., 2008), being this a significant source of tissue protection.

5. Conclusion

The ingestion of green tea infusion is capable of preventing some tissue remodeling in the heart, counteracting changes induced by diabetes, preventing fibrosis in the myocardium and pericardium, and infiltration and activation of mast cells in the heart. Moreover, green tea was able to prevent damage to cardiomyocytes' DNA and control mitochondria morphological dynamics, which occur as a metabolic adaptation to diabetes. These beneficial outcomes might contribute to an overall improvement of the cardiac function when green tea is consumed.

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References

ABEL, E. D. et al. Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. **Journal of Clinical Investigation**, v. 104, n. 12, p. 1703–1714, 1999.

ACCORD STUDY GROUP. Nine-Year Effects of 3.7 Years of Intensive Glycemic Control on Cardiovascular Outcomes. **Diabetes Care**, v. 39, n. 5, p. 701–708, maio 2016.

AEBI, H. [13] Catalase in vitro. In: **Methods in Enzymology**. Methods in Enzymology. [s.l.] Elsevier, 1984. v. 105p. 121–126.

BABU, P. V. A. et al. Green tea attenuates diabetes induced Maillard-type fluorescence and collagen cross-linking in the heart of streptozotocin diabetic rats. **Pharmacological Research**, v. 55, n. 5, p. 433–440, maio 2007.

BABU, P. V. A.; SABITHA, K. E.; SHYAMALADEVI, C. S. Green tea impedes dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca2+-ATPase and Na+/K+-ATPase activity in the heart of streptozotocin-diabetic rats. **Chemico-Biological Interactions**, v. 162, n. 2, p. 157–164, ago. 2006a.

BABU, P. V. A.; SABITHA, K. E.; SHYAMALADEVI, C. S. Therapeutic effect of green tea extract on oxidative stress in aorta and heart of streptozotocin diabetic rats. **Chemico-Biological Interactions**, v. 162, n. 2, p. 114–120, ago. 2006b.

BALAJI, G. et al. Mast cell stabilizing and anti-anaphylactic activity of aqueous extract of green tea (Camellia sinensis). **International Journal of Veterinary Science and Medicine**, v. 2, n. 1, p. 89–94, 2014.

BARKAOUI, M. et al. Ethnobotanical survey of medicinal plants used in the traditional treatment of diabetes in Chtouka Ait Baha and Tiznit (Western Anti-Atlas), Morocco. **Journal of Ethnopharmacology**, v. 198, n. June 2016, p. 338–350, 2017.

BAYEVA, M.; SAWICKI, K. T.; ARDEHALI, H. Taking diabetes to heart--deregulation of myocardial lipid metabolism in diabetic cardiomyopathy. **Journal of the American Heart Association**, v. 2, n. 6, p. 1–17, 2013.

BENZIE, I. F. F.; STRAIN, J. J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. **Analytical Biochemistry**, v. 239, n. 1, p. 70–76, jul. 1996.

BERNAS, T. et al. Confocal Fluorescence Imaging of Photosensitised DNA Denaturation in Cell Nuclei. **Photochemistry and Photobiology**, v. 33342, p. 960–969, 2005.

BERTRAND, L. et al. Glucose transporters in cardiovascular system in health and disease.

Pflugers Archiv European Journal of Physiology, v. 472, n. 9, p. 1385–1399, 2020.

BOYER, J. K. et al. Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. **American Journal of Cardiology**, v. 93, n. 7, p. 870–875, 2004.

BUGGER, H.; ABEL, E. D. Molecular mechanisms of diabetic cardiomyopathy. **Diabetologia**, v. 57, n. 4, p. 660–671, 2014.

CHEN, T.-S. et al. Green tea epigallocatechin gallate enhances cardiac function restoration through survival signaling expression in diabetes mellitus rats with autologous adipose tissue-derived stem cells. **Journal of Applied Physiology**, v. 123, n. 5, p. 1081–1091, nov. 2017.

CHOO, J. J. Green tea reduces body fat accretion caused by high-fat diet in rats through -adrenoceptor activation of thermogenesis in brown adipose tissue. **The Journal of Nutritional Biochemistry**, v. 11, p. 671–676, 2003.

CHOPADE, V. V et al. Green tea (Camellia sinensis): chemistry , traditional , medicinal uses and its pharmacological activities- a review. **Pharmacognosy Reviews**, v. 2, n. 3, p. 157–162, 2008.

COOK, S. A. et al. Abnormal myocardial insulin signalling in type 2 diabetes and left-ventricular dysfunction. **European Heart Journal**, v. 31, n. 1, p. 100–111, 2010.

CURY, D. P. et al. Morphometric, quantitative, and three-dimensional analysis of the heart muscle fibers of old rats: Transmission electron microscopy and high-resolution scanning electron microscopy methods. **Microscopy Research and Technique**, v. 76, n. 2, p. 184–195, 2013.

DA SILVA, E. et al. Ventricular remodeling in growing rats with experimental diabetes: The impact of swimming training. **Pathology Research and Practice**, v. 209, n. 10, p. 618–626, 2013.

DA SILVA, E. et al. Swimming training attenuates the morphological reorganization of the myocardium and local inflammation in the left ventricle of growing rats with untreated experimental diabetes. **Pathology - Research and Practice**, v. 212, n. 4, p. 325–334, abr. 2016.

DA SILVA, M. F. et al. Attenuation of Ca 2+ homeostasis, oxidative stress, and mitochondrial dysfunctions in diabetic rat heart: insulin therapy or aerobic exercise? **Journal of Applied Physiology**, v. 119, n. 2, p. 148–156, 15 jul. 2015.

DIETERICH, S. et al. Gene Expression of Antioxidative Enzymes in the Human Heart : Increased Expression of Catalase in the End-Stage Failing Heart. **Circulation**, v. 101, n. 1, p. 33–39, 2000.

FALLAH HUSEINI, H. et al. Review of anti-diabetic medicinal plant used in traditional medicine. **Journal of Medicinal Plants**, v. 5, n. SUPPL. 2, p. 1–8, 2006.

FIORINO, P. et al. The effects of green tea consumption on cardiometabolic alterations induced by experimental diabetes. **Experimental Diabetes Research**, v. 2012, 2012.

FRATI, G. et al. An overview of the inflammatory signalling mechanisms in the myocardium underlying the development of diabetic cardiomyopathy. **Cardiovascular Research**, v. 113, n. 4, p. 378–388, 15 mar. 2017.

GERBER, L. K.; ARONOW, B. J.; MATLIB, M. A. Activation of a novel long-chain free fatty

acid generation and export system in mitochondria of diabetic rat hearts. **American Journal of Physiology - Cell Physiology**, v. 291, n. 6, p. 1198–1207, 2006.

GILFILLAN, A. M.; TKACZYK, C. Integrated signalling pathways for mast-cell activation. **Nature Reviews Immunology**, v. 6, n. 3, p. 218–230, 10 mar. 2006.

HABIG, W. H.; PABST, M. J.; JAKOBY, W. B. Glutathione S-Transferases: The first enzymatic step in mercapturic acid formation. **The Journal of Biological Chemistry**, v. 25, n. 22, p. 7130–7139, 1974.

HANSEN, A. et al. C-peptide exerts beneficial effects on myocardial blood flow and function in patients with type 1 diabetes. **Diabetes**, v. 51, n. 10, p. 3077–3082, 2002.

HAYASHI, D. et al. The mechanisms of ameliorating effect of a green tea polyphenol on diabetic nephropathy based on diacylglycerol kinase α. **Scientific Reports**, v. 10, n. 1, p. 1–12, 2020.

HERMAN-EDELSTEIN, M.; DOI, S. Q. **Pathophysiology of diabetic nephropathy**. [s.l.] Elsevier Inc., 2016.

HO, F. Y. et al. The critical role of caspases activation in hypoxia/reoxygenation induced apoptosis. **Biochemical and Biophysical Research Communications**, v. 345, n. 3, p. 1131–1137, 2006.

HOLROYDE, M. J. et al. The calcium and magnesium binding sites on cardiac troponin and their role in the regulation of myofibrillar adenosine triphosphatase. **Journal of Biological Chemistry**, v. 255, n. 24, p. 11688–11693, 1980.

HUANG, P. C. et al. Cellular apoptosis and cardiac dysfunction in STZ-induced diabetic rats attenuated by anthocyanins via activation of IGFI-R/PI3K/Akt survival signaling. **Environmental Toxicology**, v. 32, n. 12, p. 2471–2480, 2017.

INOUE, T.; SUZUKI, Y.; RA, C. Epigallocatechin-3-gallate inhibits mast cell degranulation, leukotriene C4 secretion, and calcium influx via mitochondrial calcium dysfunction. **Free Radical Biology and Medicine**, v. 49, n. 4, p. 632–640, 2010.

INTERNATIONAL DIABETES FEDERATION. **IDF Diabetes Atlas**. 9th edn ed. Brussels, Belgium: [s.n.].

ISHII, H. et al. Amelioration of Vascular Dysfunctions in Diabetic Rats by an Oral PKC beta Inhibitor. **Science**, v. 272, n. 5262, p. 728–731, 3 maio 1996.

ITOH, Y. et al. Examination of the anti-oxidative effect in renal tubular cells and apoptosis by oxidative stress. **Urological Research**, v. 33, n. 4, p. 261–266, 2005.

JOSHI, M. S. et al. Functional relevance of genetic variations of endothelial nitric oxide synthase and vascular endothelial growth factor in diabetic coronary microvessel dysfunction. **Clinical and Experimental Pharmacology and Physiology**, v. 40, n. 4, p. 253–261, 2013.

KARNOVSKY, M. J. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. **J. Cell Biol.**, v. 27, n. NOVEMBER 1964, p. 137–138, 1965.

KUMAZOE, M.; FUJIMURA, Y.; TACHIBANA, H. 67-kDa Laminin Receptor Mediates the Beneficial Effects of Green Tea Polyphenol EGCG. **Current Pharmacology Reports**, 24 jul. 2020.

LADEIRA, L. C. M. et al. Green tea infusion aggravates nutritional status of the juvenile

untreated STZ-induced type 1 diabetic rat. **bioRxiv**, p. 35, 1 jan. 2020a.

LADEIRA, L. C. M. et al. Could biological tissue preservation methods change chemical elements proportion measured by energy dispersive X-ray spectroscopy? **Biological Trace Element Research**, v. 196, n. 1, p. 168–172, 25 jul. 2020b.

LADEIRA, L. C. M. et al. Green tea infusion prevents diabetic nephropathy aggravation in recent-onset type 1 diabetes regardless of glycemic control. **Journal of Ethnopharmacology**, v. 274, n. January, p. 114032, jun. 2021.

LAHAYE, S. L. D. et al. Intense exercise training induces adaptation in expression and responsiveness of cardiac β-adrenoceptors in diabetic rats. **Cardiovascular Diabetology**, v. 9, n. 1, p. 72, 2010.

LEVELT, E. et al. Diabetic cardiomyopathy: pathophysiology and potential metabolic interventions state of the art review. **European Journal of Endocrinology**, v. 178, n. 4, p. R127–R139, abr. 2018.

LI, G. Z.; CHAI, O. H.; SONG, C. H. Inhibitory effects of epigallocatechin gallate on compound 48/80-inducedmast cell activation and passive cutaneous anaphylaxis. **Experimental & Molecular Medicine**, v. 37, n. 4, p. 290–296, 1 ago. 2005.

LI, Q.-S. et al. The anti-allergic potential of tea: a review of its components, mechanisms and risks. **Food & Function**, v. 12, n. 1, p. 57–69, 2021.

LIAO, H.-E. et al. Deep Sea Minerals Prolong Life Span of Streptozotocin-Induced Diabetic Rats by Compensatory Augmentation of the IGF-I-Survival Signaling and Inhibition of Apoptosis. **Environmental toxicology**, v. 31, p. 769–781, 2016.

LIMA, G. D. DE A. et al. Fertility in male rats: Disentangling adverse effects of arsenic compounds. **Reproductive Toxicology**, v. 78, p. 130–140, 2018.

LOWRY, O. H. et al. Protein Measurement with the Folin Phenol Reagent. **Analytical Biochemistry**, v. 217, n. 2, p. 220–230, 1994.

MANDARIM-DE-LACERDA, C. A. Stereological tools in biomedical research. **Anais da Academia Brasileira de Ciencias**, v. 75, n. 4, p. 469–486, 2003.

MENG, J.-M. et al. Effects and Mechanisms of Tea for the Prevention and Management of Diabetes Mellitus and Diabetic Complications: An Updated Review. **Antioxidants**, v. 8, n. 6, p. 170, 10 jun. 2019.

MISHIMA, M. D. V. et al. Cardioprotective action of chia (Salvia hispanica L.) in ovariectomized rats fed a high fat diet. **Food & Function**, p. 0–41, 2021.

MOHAMMED, S. F. et al. Coronary Microvascular Rarefaction and Myocardial Fibrosis in Heart Failure With Preserved Ejection Fraction. **Circulation**, v. 131, n. 6, p. 550–559, 10 fev. 2015.

NIELSEN, R. et al. Heart failure patients with prediabetes and newly diagnosed diabetes display abnormalities in myocardial metabolism. **Journal of Nuclear Cardiology**, v. 25, n. 1, p. 169–176, 2018.

NOVAES, R. D. et al. Aluminum: A potentially toxic metal with dose-dependent effects on cardiac bioaccumulation, mineral distribution, DNA oxidation and microstructural remodeling. **Environmental Pollution**, v. 242, n. July, p. 814–826, nov. 2018.

OTHMAN, A. I. et al. Epigallocatechin-3-gallate protects against diabetic cardiomyopathy through modulating the cardiometabolic risk factors, oxidative stress, inflammation, cell death and fibrosis in streptozotocin-nicotinamide-induced diabetic rats. **Biomedicine and Pharmacotherapy**, v. 94, p. 362–373, 2017.

OU, H. C. et al. Cardiac contractile dysfunction and apoptosis in streptozotocin-induced diabetic rats are ameliorated by garlic oil supplementation. **Journal of Agricultural and Food Chemistry**, v. 58, n. 19, p. 10347–10355, 2010.

PARK, J. S. et al. Epigallocatechin-3-gallate inhibits the PDGF-induced VEGF expression in human vascular smooth muscle cells via blocking PDGF receptor and Erk-1/2. **International Journal of Oncology**, v. 29, n. 5, p. 1247–1252, 2006.

PEIXOTO, E. B. et al. Reduced LRP6 expression and increase in the interaction of GSK3β with p53 contribute to podocyte apoptosis in diabetes mellitus and are prevented by green tea. **Journal of Nutritional Biochemistry**, v. 26, n. 4, p. 416–430, 2015.

PERONNET, F.; MASSICOTTE, D. Table of nonprotein respiratory quotient: an update. **Canadian journal of sport sciences**, v. 16, p. 23–29, 1991.

PERVA-UZUNALIĆ, A. et al. Extraction of active ingredients from green tea (Camellia sinensis): Extraction efficiency of major catechins and caffeine. **Food Chemistry**, v. 96, n. 4, p. 597–605, jun. 2006.

PFEIFFER, E. R. et al. Biomechanics of Cardiac Electromechanical Coupling and Mechanoelectric Feedback. **Journal of Biomechanical Engineering**, v. 136, n. 2, p. 021007, 5 fev. 2014.

RACHID, A. et al. Ethnopharmacological survey of medicinal plants used in the traditional treatment of diabetes mellitus in the North Western and South Western Algeria. **Journal of Medicinal Plants Research**, v. 6, n. 10, p. 2041–2050, 2012.

RENNO, W. M. et al. Effect of greean tea on kidney tubules of diabetic rats. **British Journal of Nutrition**, v. 100, n. 03, p. 652–659, 6 set. 2008.

RICART-JANÉ, D.; LLOBERA, M.; LÓPEZ-TEJERO, M. D. Anticoagulants and other preanalytical factors interfere in plasma nitrate/nitrite quantification by the Griess method. **Nitric Oxide - Biology and Chemistry**, v. 6, n. 2, p. 178–185, 2002.

RITCHIE, R. H. et al. Lipid metabolism and its implications for type 1 diabetes-associated cardiomyopathy. **Journal of Molecular Endocrinology**, v. 58, n. 4, p. R225–R240, 2017.

RITCHIE, R. H.; DALE ABEL, E. Basic Mechanisms of Diabetic Heart Disease. **Circulation Research**, p. 1501–1525, 2020.

ROSSI, M. A.; ABREU, M. A.; SANTORO, L. B. Connective tissue skeleton of the human heart: A demonstration by cell- maceration scanning electron microscope method. **Circulation**, v. 97, n. 9, p. 934–935, 1998.

SAMARGHANDIAN, S.; AZIMI-NEZHAD, M.; FARKHONDEH, T. Catechin Treatment Ameliorates Diabetes and Its Complications in Streptozotocin-Induced Diabetic Rats. **Dose-Response**, v. 15, n. 1, p. 155932581769115, 6 mar. 2017.

SARTIPPOUR, M. R. et al. Inhibition of fibroblast growth factors by green tea. **International journal of oncology**, v. 21, n. 3, p. 487–491, 2002.

SCHERLE, W. A simple method for volumetry of organs in quantitative stereology. **Mikroskopie**, v. 26, n. 1, p. 57–60, 1970.

SEA-TAN, S.; GROVE, K. A.; LAMBERT, J. D. Weight control and prevention of metabolic syndrome by green tea. **Pharmacological Research**, v. 64, n. 2, p. 146–154, ago. 2011.

SERTORIO, M. N. et al. Arsenic exposure intensifies glycogen nephrosis in diabetic rats. **Environmental Science and Pollution Research**, v. 26, n. 12, p. 12459–12469, 7 abr. 2019.

SOCIEDADE BRASILEIRA DE DIABETES. **Diretrizes - Sociedade Brasileira de Diabetes 2017-2018**.

SOETAN, K. O.; OLAIYA, C. O.; OYEWOLE, O. E. The importance of mineral elements for humans , domestic animals and plants : A review. **African Journal of Food Science**, v. 4, n. May, p. 200–222, 2010.

SOWTON, A. P.; GRIFFIN, J. L.; MURRAY, A. J. Metabolic profiling of the diabetic heart: Toward a richer picture. **Frontiers in Physiology**, v. 10, n. MAY, p. 1–16, 2019.

STEPHENSON, M. K. et al. Scanning electron microscopy of maceraated tissue to visualize the extracellular matrix. **Journal of Visualized Experiments**, v. 2016, n. 112, p. 1–8, 2016.

SULLIVAN, J. M.; ALPERS, J. B. In vitro regulation of rat heart 5'-nucleotidase by adenine nucleotides and magnesium. **Journal of Biological Chemistry**, v. 246, n. 9, p. 3057–3063, 1971.

SUZUKI, T. et al. DNA staining for fluorescence and laser confocal microscopy. **Journal of Histochemistry and Cytochemistry**, v. 45, n. 1, p. 49–53, 1997.

TACHIBANA, H. et al. A receptor for green tea polyphenol EGCG. **Nature Structural and Molecular Biology**, v. 11, n. 4, p. 380–381, 2004.

TANG, W. et al. Anti-diabetic activity of chemically profiled green tea and black tea extracts in a type 2 diabetes mice model via different mechanisms. **Journal of Functional Foods**, v. 5, n. 4, p. 1784–1793, out. 2013.

VAN ALLER, G. S. et al. Epigallocatechin gallate (EGCG), a major component of green tea, is a dual phosphoinositide-3-kinase/mTOR inhibitor. **Biochemical and Biophysical Research Communications**, v. 406, n. 2, p. 194–199, 2011.

WANG, J.-H. et al. Hypoxia-stimulated cardiac fibroblast production of IL-6 promotes myocardial fibrosis via the TGF-β1 signaling pathway. **Laboratory Investigation**, v. 96, n. 8, p. 839–852, 27 ago. 2016.

WENZEL, S. et al. TGF-β1 improves cardiac performance via up-regulation of laminin receptor 37/67 in adult ventricular cardiomyocytes. **Basic Research in Cardiology**, v. 105, n. 5, p. 621–629, 2010.

WOOD, P.; PIRAN, S.; LIU, P. P. Diastolic heart failure: Progress, treatment challenges, and prevention. **Canadian Journal of Cardiology**, v. 27, n. 3, p. 302–310, 2011.

YAMABE, N. et al. Therapeutic Potential of (-)-Epigallocatechin 3- O -Gallate on Renal Damage in Diabetic Nephropathy Model Rats. **Journal of Pharmacology and Experimental Therapeutics**, v. 319, n. 1, p. 228–236, out. 2006.

YE, G. et al. Catalase Protects Cardiomyocyte Function in Models of Type 1 and Type 2 Diabetes. **Diabetes**, v. 53, p. 1336–1343, 2004.

YIN, D. D. et al. Tranilast prevents renal interstitial fibrosis by blocking mast cell infiltration in a rat model of diabetic kidney disease. **Molecular Medicine Reports**, v. 17, n. 5, p. 7356–7364, 2018.

YOUN, H. S. et al. Suppression of MyD88- and TRIF-dependent signaling pathways of toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea. **Biochemical Pharmacology**, v. 72, n. 7, p. 850–859, 2006.

YUE, Y. et al. Transforming growth factor beta (TGF-β) mediates cardiac fibrosis and induces diabetic cardiomyopathy. **Diabetes Research and Clinical Practice**, v. 133, p. 124–130, 2017.

ZHANG, X. et al. Forskolin Protected against Streptozotocin-Induced Diabetic Cardiomyopathy via Inhibition of Oxidative Stress and Cardiac Fibrosis in Mice. **BioMed Research International**, v. 2021, p. 1–8, 2021.

Tables

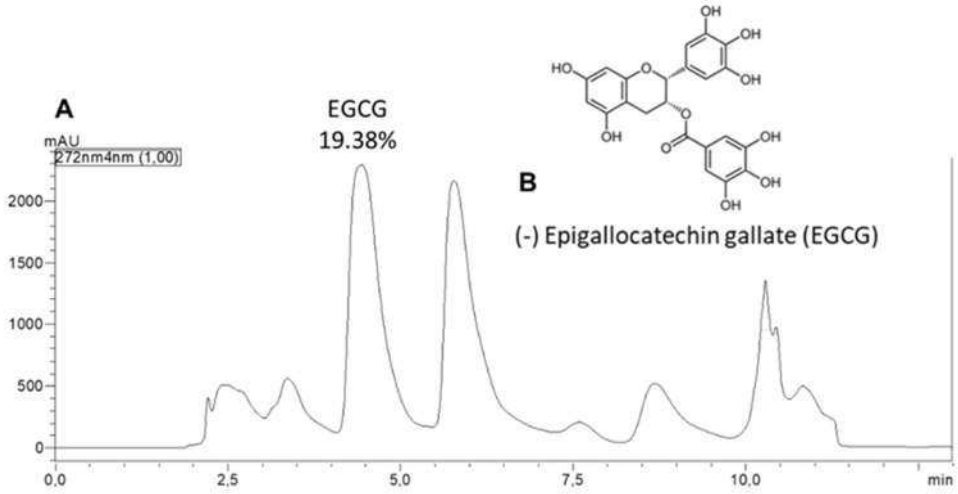
Table 1.

Blood Glucose, biometric parameters, and water consumption of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group.

	Healthy Ctrl	Diabetic	Diabetic + GTI
Blood glucose (mg/dL)	85.38 ± 7.53	475.00 ± 33.14*	542.80 ± 42.20#
Initial body weight (g)	84.26 ± 14.97	81.27 ± 9.46	81.75 ± 7.57
Final body weight (g)	288.10 ± 44.16	93.08 ± 23.42*	99.75 ± 13.04
Body weight gain (g)	203.80 ± 30.81	11.82 ± 21.87*	18.00 ± 16.18
Heart weight (g)	1.76 ± 0.11	0.57 ± 0.14*	0.62 ± 0.13
Heart relative weight (%)	0.65 ± 0.05	0.62 ± 0.06	0.64 ± 0.11
Heart volume (mm^3)	1.35 ± 0.23	0.34 ± 0.05*	0.45 ± 0.14
Heart relative volume (%)	0.49 ± 0.08	0.42 ± 0.06	0.46 ± 0.13
LV weight (g)	0.67 ± 0.08	0.29 ± 0.06*	0.30 ± 0.03
LV relative weight (%)	0.24 ± 0.02	0.31 ± 0.02*	0.31 ± 0.01
LV volume (mm^3)	0.52 ± 0.14	0.17 ± 0.02*	0.21 ± 0.04
LV relative volume (%)	0.19 ± 0.04	0.21 ± 0.01	0.22 ± 0.03

Mean ± SD. Data were compared by Student *t*-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. One asterisk (*) indicates a difference with $P < 0.001$, and the hash (#) indicates different means with $P < 0.05$. (n = 6 animals/group). LV = left ventricle.

Figures



C

Analysis	Green Tea Infusion
Total phenolic content	3.88 ± 2.49 mg GAE/g GTI
Total antioxidant capacity (ABTS)	3.26 ± 0.06 µMol TE/g GTI
Total antioxidant capacity (FRAP)	46.38 ± 4.1 µMol $FeSO_4$/g GTI
EGCG (% w/w)	19.38 %

Figure 1. Chromatogram of the green tea infusion (*Camellia sinensis*). **A –** HPLC fingerprint of the green tea infusion. **B -** Molecular representation of epigallocatechin-3-gallate (EGCG). **C** – Total phenolic and EGCG content and total antioxidant capacity of green tea infusion samples.

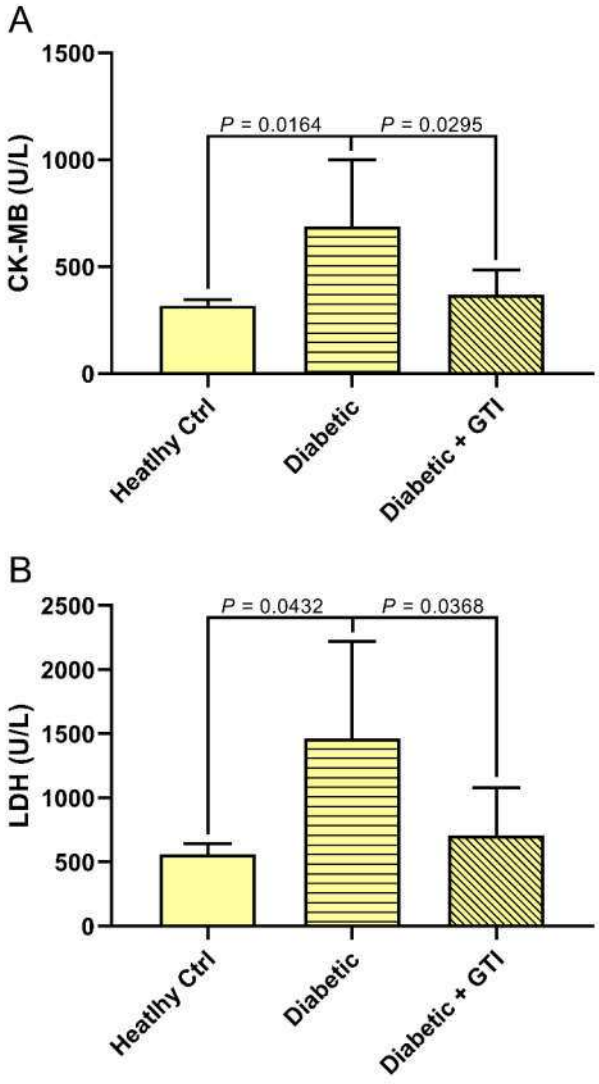


Figure 2. Cardiac function markers of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Creatine Kinase - CK-MB (U/L). **B** – Lactate Dehydrogenase - LDH (U/L). Mean ± SD. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student t-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

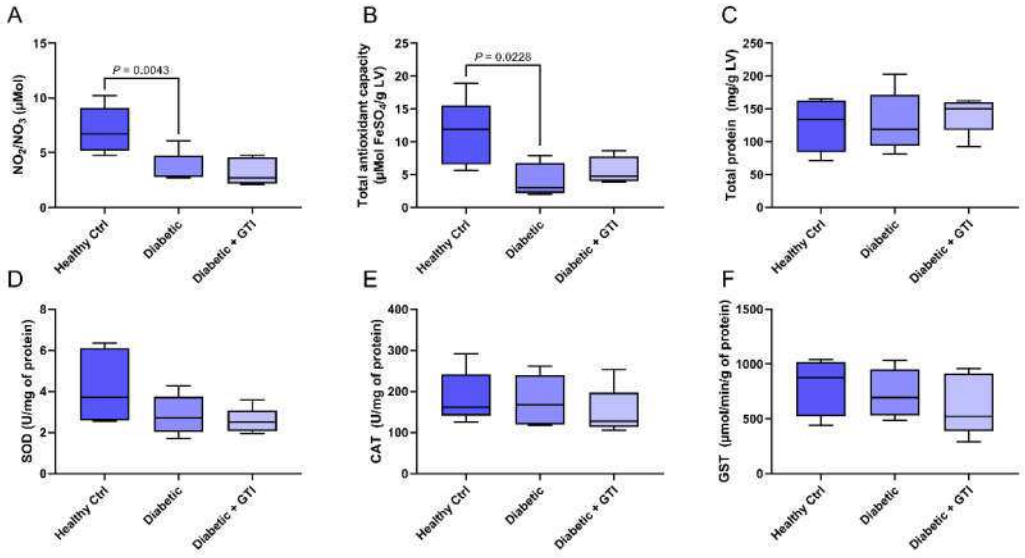


Figure 3. Antioxidant enzymes, total antioxidant capacity, protein and nitric oxide levels of the heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – NO_2/NO_3. **B** – Total antioxidant capacity (FRAP). **C** – Protein levels. **D** - Superoxide dismutase. **E** – Catalase. **F** – Glutathione S-Transferase. The box represents the interquartile interval with the mean indicated (horizontal line), and the whiskers represent the superior and inferior quartiles. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student t-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

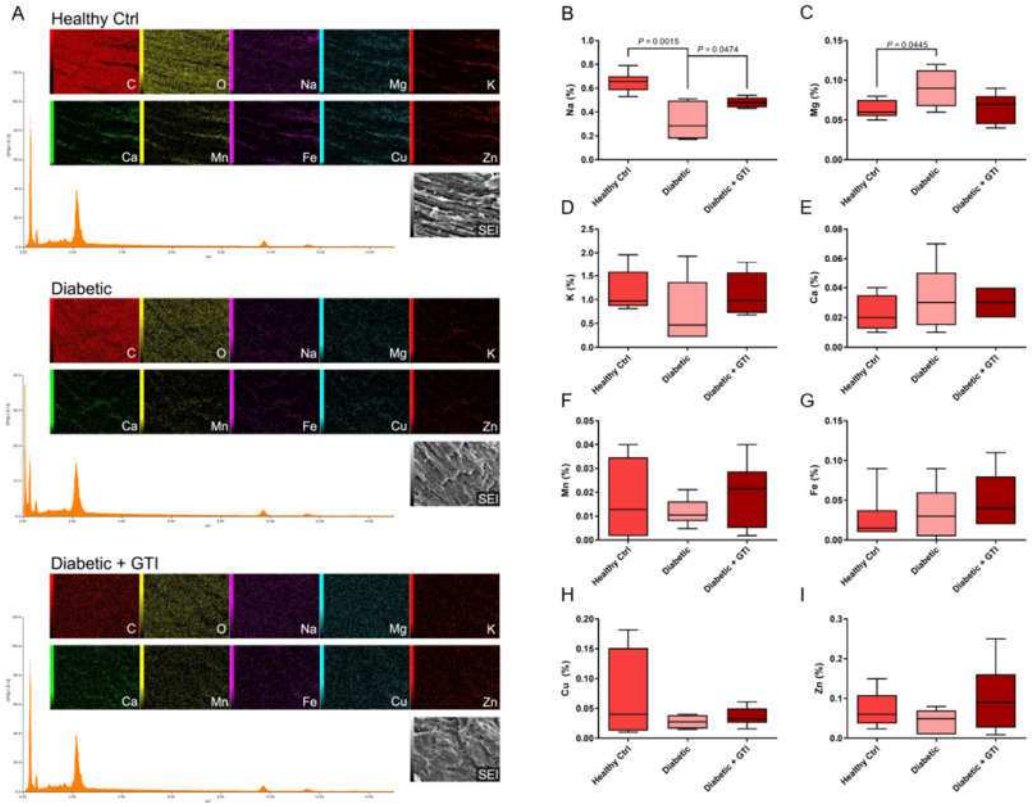


Figure 4. Microelement mapping and proportions in the heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Elemental mapping. **B** – Sodium (%). **C** – Magnesium (%). **D** – Potassium (%). **E** – Calcium (%). **F** – Manganese (%). **G** – Iron (%). **H** – Copper (%). **I** – Zinc (%). The box represents the interquartile interval with the mean indicated (horizontal line), and the whiskers represent the superior and inferior quartiles. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student t-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

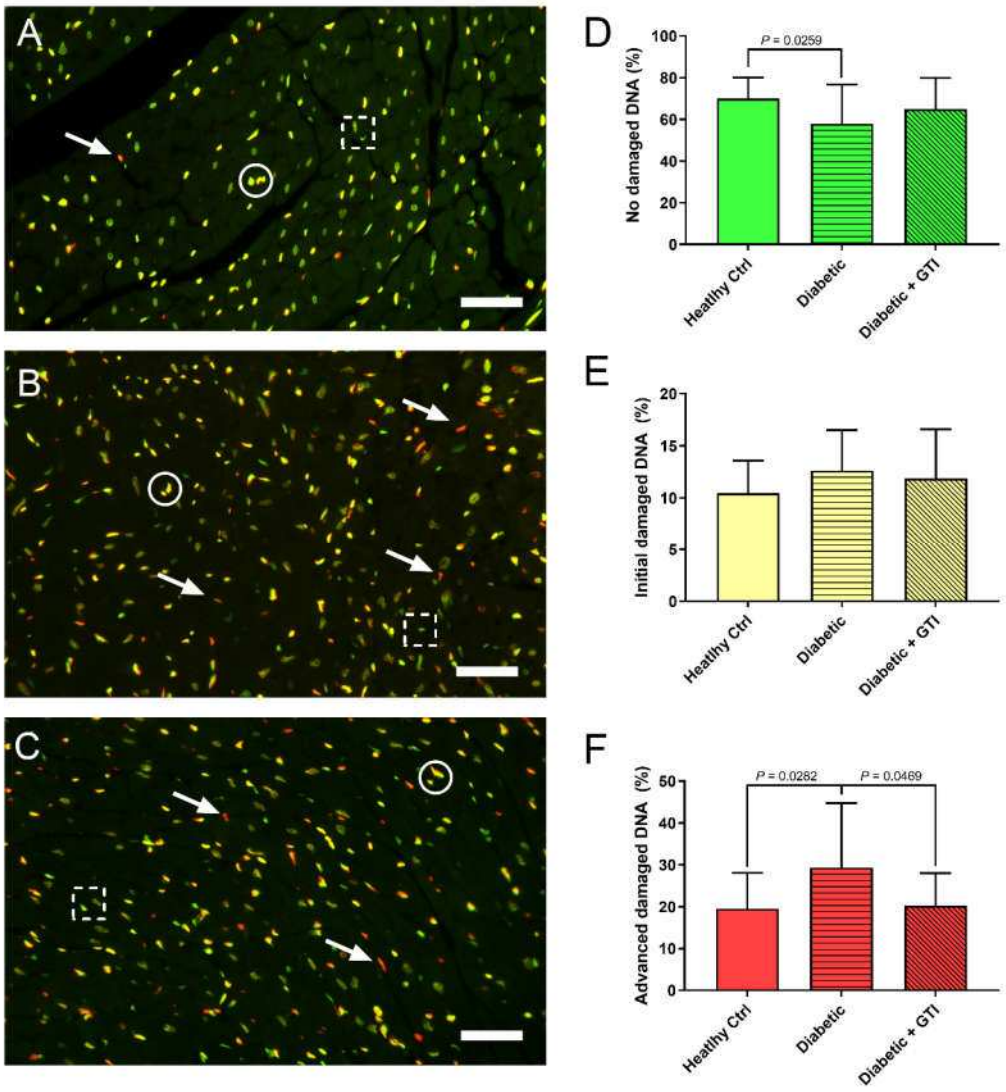


Figure 5. Representative acridine orange (AO) and propidium iodide (IP) stained photomicrographs of the heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Healthy Ctrl group - left ventricle photomicrography. **B** – Diabetic group - left ventricle photomicrography. **C** – Diabetic + GTI group - left ventricle photomicrography. Green nuclei – AO-positive; Yellow to reddish nuclei – IP-positive; Dotted squares delimitate AO-positive labeled nuclei; circles delimitate AO and IP labeled nuclei; arrows indicate PI-positive nuclei. **D** - AO-positive cells – no damaged DNA (%). **E** – AO/IP-positive cells – initial damaged DNA (%). **F** - IP-positive cells – damaged

DNA (%). Scale bars = 250 μm. Mean ± SD. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student t-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

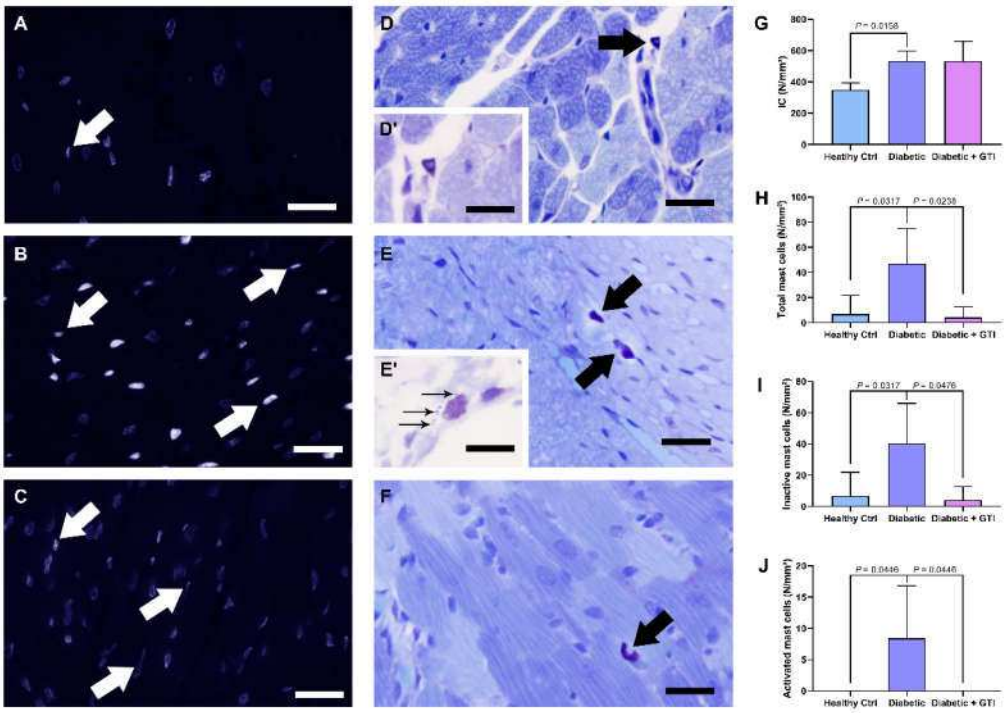


Figure 6. Total cell count and mast cell infiltration and activation on the heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Healthy Ctrl group – DAPI labeled left ventricle photomicrography. **B** – Diabetic group - DAPI labeled left ventricle photomicrography. **C** – Diabetic + GTI group - DAPI labeled left ventricle photomicrography. White arrows indicate non-cardiomyocyte cell nuclei. **D** – Heathy Ctrl group – Toluidine Blue stained left ventricle photomicrography. **D' –** Inactive mast cell. **E** – Diabetic group - Toluidine Blue stained left ventricle photomicrography. **E'** – Activated mast cell. **F** – Diabetic + GTI group - Toluidine Blue stained left ventricle photomicrography**.** Thick black arrows indicate mast cells and thin black arrow indicates mast cell granules. **G** – Infiltrated cells (N/mm²). **H** – Total mast cell (N/mm²). **I** – Inactive mast cell (N/mm²). **I** – Activated mast cell (N/mm²). Scale bars: A, B and C = 100 µm; D, E and F = 150 µm; D' and E' = 50 µm. Mean ± SD. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student t-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

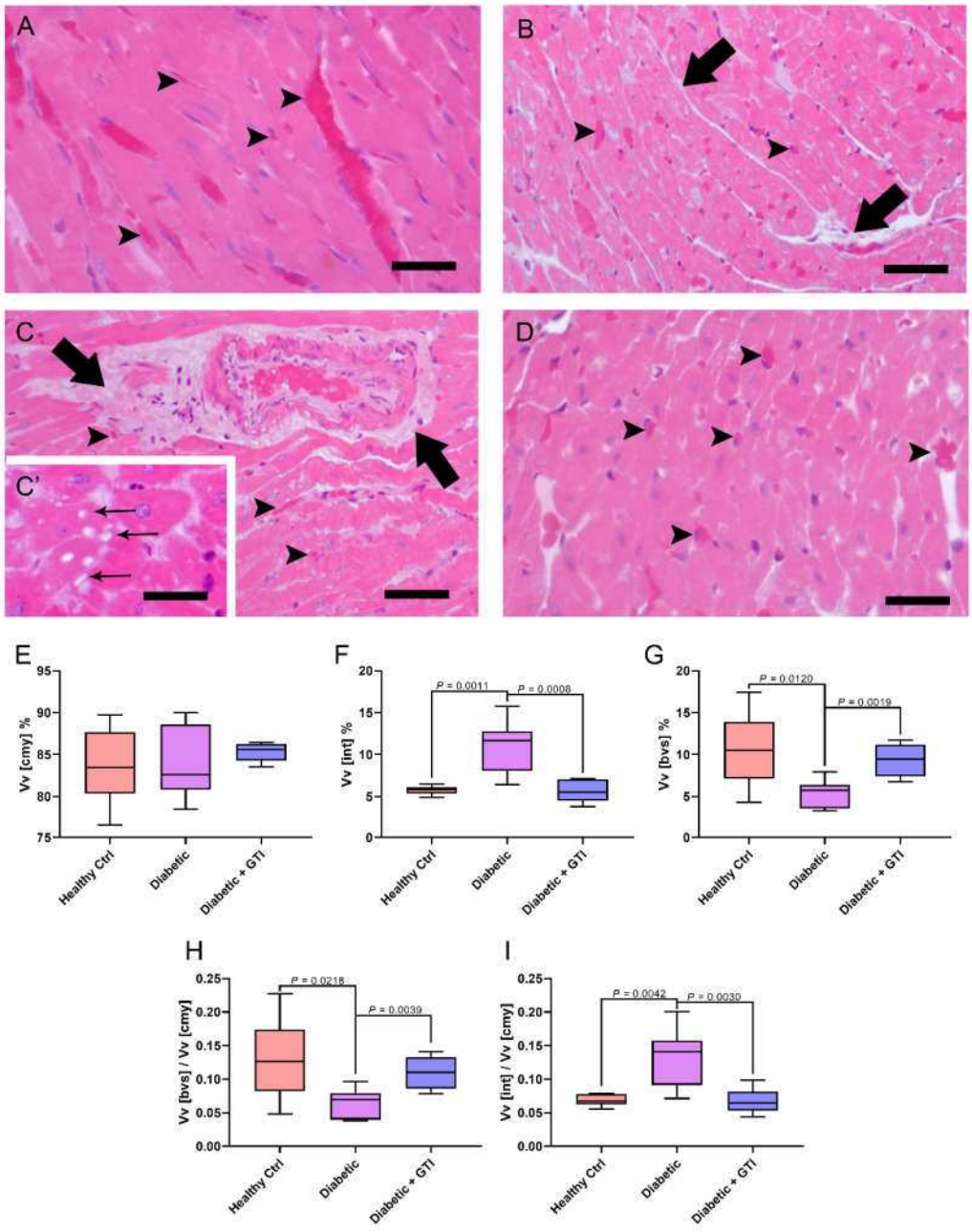


Figure 7. Volume density of morphological features of the heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Healthy Ctrl group - left ventricle photomicrography. **B** – Diabetic group - left ventricle photomicrography. **C** – Diabetic group – perivascular fibrosis highlight. **C'** – Diabetic group – unspecific vacuolization. **D** – Diabetic + GTI group - left ventricle photomicrography. Arrowheads

indicate blood vessels, thick arrows indicate connective tissue in the interstitium, and thin arrow indicates vacuoles. **E** – Cardiomyocyte volume density (Vv [cmy] %). **F** – Interstitium volume density (Vv [Int] %). **G** – Blood vessels volume density (Vv [bvs] %). **H** - Vv [bvs] / Vv [cmy]. **I** – and Vv [int] / Vv [cmy]. Scale bars: A and C' = 50 μm; B, C and D = 150 μm. The box represents the interquartile interval with the mean indicated (horizontal line), and the whiskers represent the superior and inferior quartiles. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student t-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

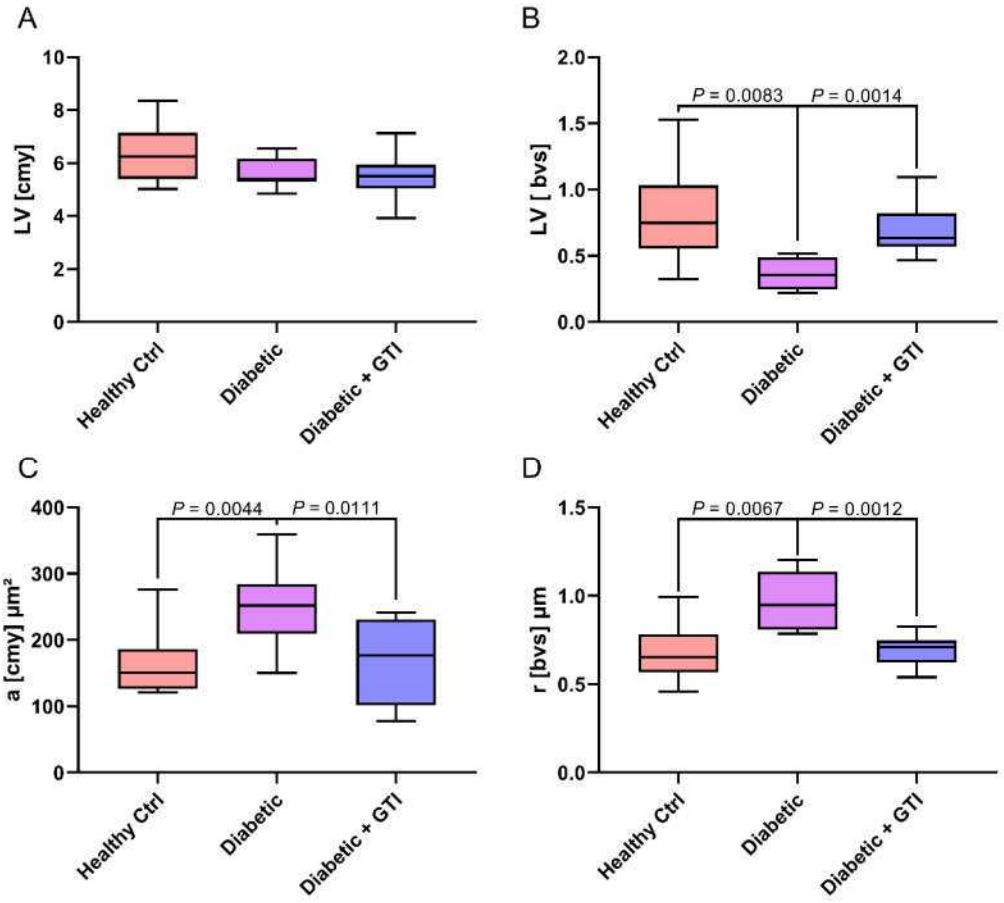


Figure 8. Histomorphological features of the heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Length density of cardiomyocytes (Lv [cmy]). **B** – Length density of blood vessels (Lv [bvs]). **C** – Cross-sectional area of cardiomyocytes (a [cmy] µm²). **D** – Diffusion distance from capillary to tissue (r [bvs] µm). The box represents the interquartile interval with the mean indicated (horizontal line), and the whiskers represent the superior and inferior quartiles. The statistical differences are indicated with lines with the *P*-value above or below them. Data were compared by Student t-test (Healthy Ctrl vs. Diabetic; Diabetic vs. Diabetic + GTI) considering statistical differences when $P \leq 0.05$. (n = 6 animals/group).

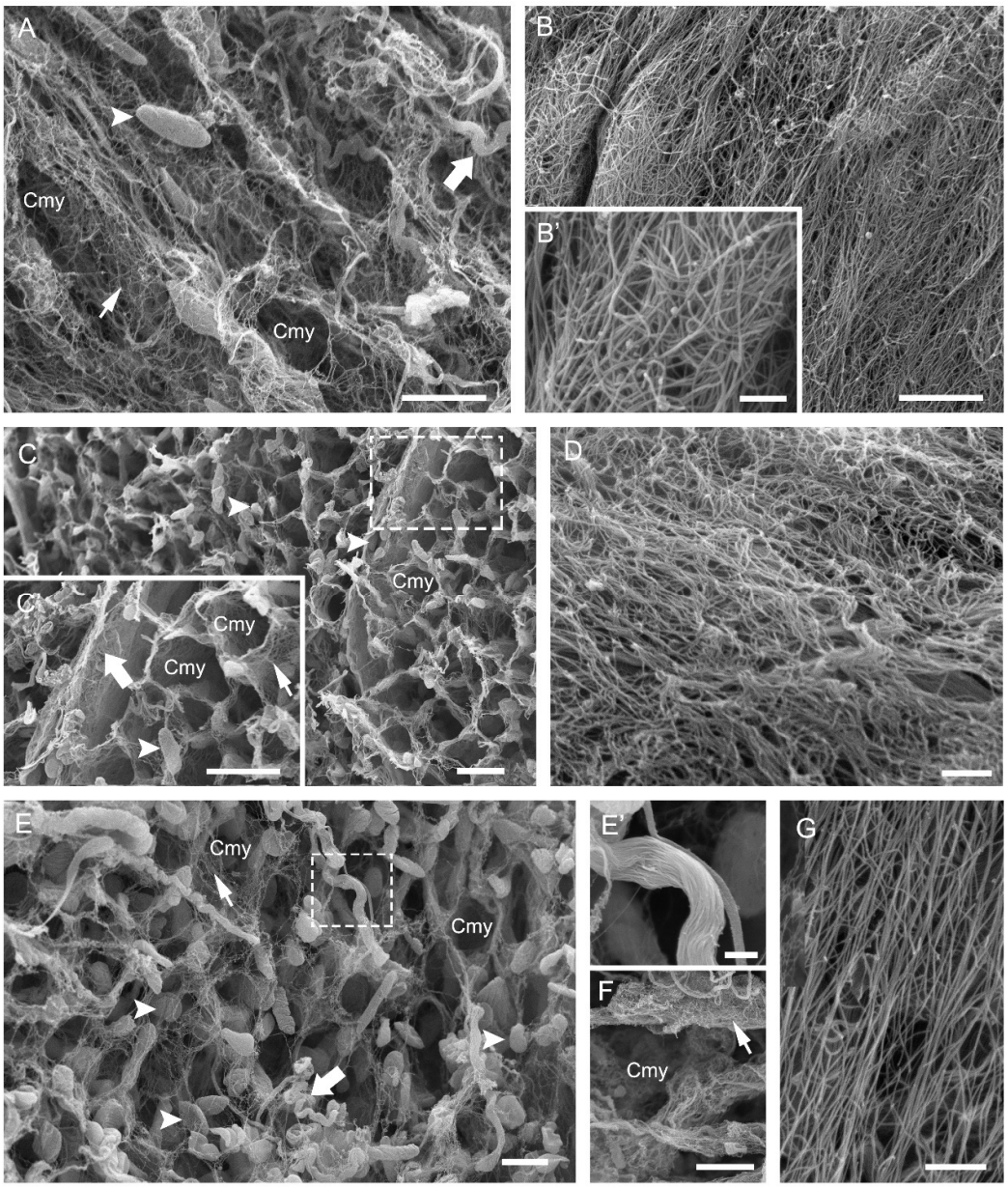


Figure 9. Representative scanning electron micrographs of the collagen matrix in the heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Healthy Ctrl group - myocardium extracellular matrix. **B** – Heathy Ctrl group - pericardium collagen scaffold. **B'** – Heathy Ctrl group - pericardium collagen fibers highlight. **C** – Diabetic group – myocardium extracellular matrix. **C'** – Diabetic group – myocardium extracellular matrix highlight. **D** – Diabetic group - pericardium collagen scaffold. **E** – Diabetic

+ GTI group - myocardium extracellular matrix. **E'** – Diabetic + GTI group – type 1 collagen bundle highlight. **F** – Diabetic + GTI group – collagen fibrin net highlight. **G** – Diabetic + GTI group – pericardium collagen scaffold. Cmy indicates space occupied by cardiomyocytes in the collagenic matrix; thick arrows indicate thick collagen bundle; thin arrows indicate thin collagens nets; arrowheads indicate erythrocytes. Scale bars: A = 10 µm; B = 5 µm; B' = 1 µm; C and C' = 20 µm; D = 2 µm; E = 10 µm; F = 2 µm; G = 20 µm; H = 2 µm.

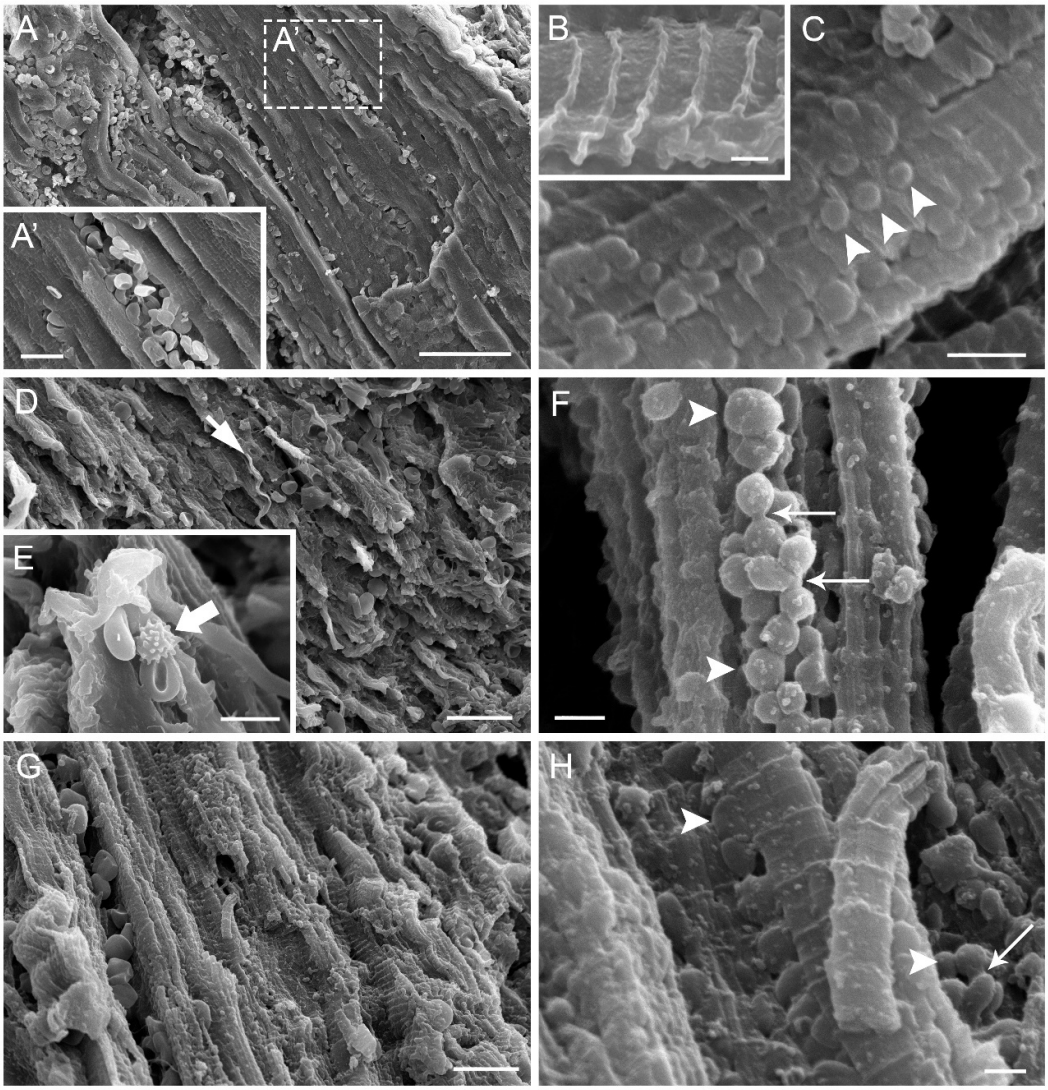


Figure 10. Representative scanning electron micrographs of the cryofractured heart's left ventricle of male Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Healthy Ctrl group - myocardium. **A'** – Heathy Ctrl group – myocardium vascularization highlight. **B** – Heathy Ctrl group – myofibril details. **C** – Heathy Ctrl group – myofibrils, with mitochondria organization. **D** – Diabetic group - myocardium. **E** – Diabetic group – leucocyte and erythrocytes. **F** – Diabetic group – mitochondria organization, with highlight to fusion point between them. **G** – Diabetic + GTI group – myocardium. **H** – Diabetic + GTI group – mitochondria organization, with highlight to fusion point between them.

Arrowheads indicate mitochondria; small arrow indicates collagen bundles; thick arrow indicates leucocyte; thin arrow indicates fusion points in the mitochondria. Scale bars: A = 50 µm; A' = 10 µm; B = 1 µm; C = 2 µm; D = 20 µm; E = 5 µm; F = 1 µm; G = 10 µm; H = 1 µm.

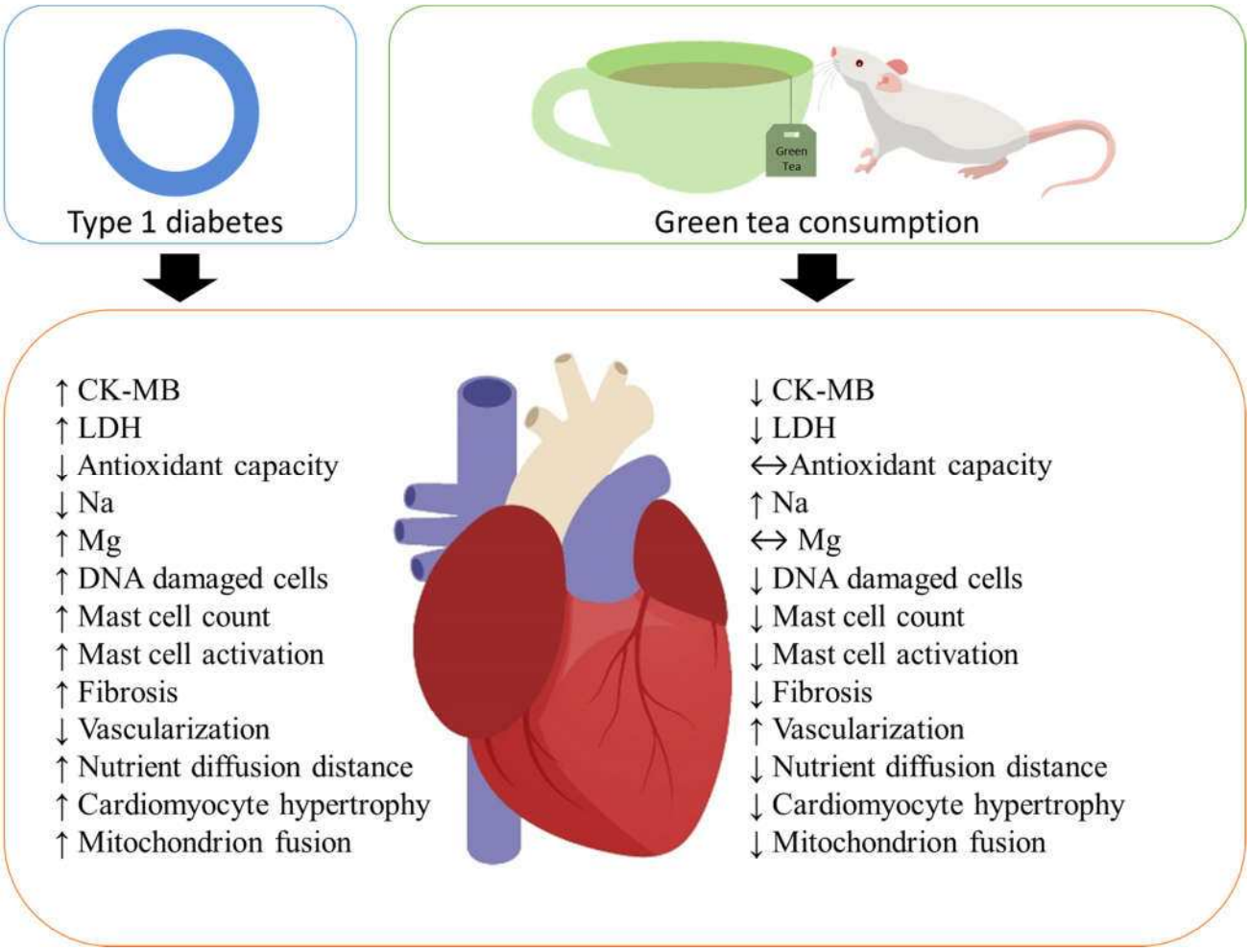


Figure 11. Effects of untreated type 1 diabetes and green tea treated type 1 diabetes on the heart's left ventricle of male Wistar diabetic rats. Figure created with elements by Freepik.com.

Conclusões gerais

Os resultados desta tese indicam que a ingestão da infusão de chá verde na dose estudada é capaz de prevenir alguns aspectos da remodelação dos tecidos do coração e dos rins, prevenindo o agravamento das alterações induzidas pelo diabetes tipo 1 nestes órgãos durante o aparecimento e desenvolvimento da doença ainda na juventude. Além disso, o tratamento foi capaz de prevenir o acúmulo de glicogênio nos túbulos renais, reduzir o dano no DNA das células renais e prevenir o agravamento de alterações morfológicas glomerulares.

No coração, o chá verde foi capaz de prevenir em vários aspectos o avanço dos danos teciduais causados pelo diabetes. O chá preveniu a fibrose cardíaca, a hipertrofia dos cardiomiócitos, o aumento da distância de difusão dos vasos sanguíneos, a infiltração e degranulação dos mastócitos e anomalias morfológicas mitocondriais, sendo possível encontrar valores iguais aos encontrados em animais saudáveis. Além disso, protegeu o DNA dos cardiomiócitos. Tudo isso refletiu nos níveis dos marcadores de função cardíaca, indicando uma melhora significativa na função do órgão, também independente do controle glicêmico.

Tudo isso independentemente de controle glicêmico, visto que nosso tratamento não afetou a glicemia. Tais fatos confirmam que os efeitos do chá verde nas doenças relacionadas ao diabetes são independentes do controle glicêmico. Tais resultados, considerados em conjunto, refletem-se em um efeito protetor da infusão de chá verde frente ao desenvolvimento da nefropatia e da cardiomiopatia decorrentes do diabetes.

Considerações finais

Além dos capítulos que compõem o texto desta tese, o doutorado me abriu diversas oportunidades de trabalho em diferentes equipes de pesquisa e treinamento no ensino e extensão. Junto a colegas do Programa de Pós-graduação em Biologia Celular e Estrutural, em especial a Drª. Nadja Biondine Marriel, criamos o primeiro Curso de Verão em Biologia Celular e Estrutural com o intuito de promover o programa e abrir a oportunidade para outros profissionais conhecerem nosso curso e o trabalho desenvolvido por nós. Em duas edições trouxemos alunos de diversos estados do país para Viçosa – MG, para uma semana de intenso treinamento e troca de conhecimento na área. Alguns destes profissionais hoje são estudantes de mestrado e doutorado do programa.

Criamos também os cursos Lúdicos de Biologia Celular e de Histologia, onde ministramos aulas para mais 300 alunos de diversos cursos de graduação das grandes áreas das ciências biológicas e da saúde, ciências agrárias e ciências exatas, durante 2 anos. Nosso curso tinha o objetivo de proporcionar um espaço de aprendizado dessas disciplinas utilizando-se de metodologias ativas no ensino. Além disso, foi um grande espaço de formação docente para os estudantes de pós-graduação envolvidos. Um relato detalhado desta experiência pode ser encontrado no artigo publicado por parte da equipe (https://doi.org/10.21284/elo.v10i.12290)

Durante dois anos, integrei a Comissão Coordenadora do Programa de Pós-Graduação como representante discente. Tive a oportunidade de participar de processos administrativos internos ao programa e contribuir representando as demandas dos meus colegas junto à comissão coordenadora.

Além disso, e de outras experiências únicas proporcionadas pelo doutorado, como resultado do trabalho desta tese e do trabalho desenvolvido enquanto pesquisador colaborador em outros grupos de pesquisa, pude integrar equipes que desenvolveram trabalhos diversos sobre alimentos funcionais, fitoterapia, toxicologia e ensino em biologia. Os produtos destas colaborações deixo listados abaixo:

[1] Ladeira LCM, dos Santos EC, Valente GE, da Silva J, Santos TA, dos Santos Costa Maldonado IR. Could biological tissue preservation methods change chemical elements proportion measured by energy dispersive X-ray spectroscopy? **Biol Trace Elem Res** 2020;196:168–72. https://doi.org/10.1007/s12011-019-01909-x.

[2] Ladeira LCM, dos Santos EC, Mendes BF, Gutierrez EA, Santos CFF, de Souza FB, et al. Green tea infusion aggravates nutritional status of the juvenile untreated STZ-induced type 1 diabetic rat. **BioRxiv** 2020:35. https://doi.org/10.1101/2020.01.13.904896.

[3] Mouro VGS, Ladeira LCM, Lozi AA, de Medeiros TS, Silva MR, de Oliveira EL, et al. Different Routes of Administration Lead to Different Oxidative Damage and Tissue Disorganization Levels on the Subacute Cadmium Toxicity in the Liver. **Biol Trace Elem Res** 2021. https://doi.org/10.1007/s12011-020-02570-5.

[4] Mishima MDV, Ladeira LCM, da Silva BP, Toledo RCL, de Oliveira TV, Costa NMB, et al. Cardioprotective action of chia (Salvia hispanica L.) in ovariectomized rats fed a high fat diet. **Food Funct** 2021:0–41. https://doi.org/10.1039/D0FO03206A.

[5] Ladeira LCM, dos Santos EC, Santos TA, da Silva J, Lima GD de A, Machado-Neves M, et al. Green tea infusion prevents diabetic nephropathy aggravation in recent-onset type 1 diabetes regardless of glycemic control. **J Ethnopharmacol** 2021;274:114032. https://doi.org/10.1016/j.jep.2021.114032.

[6] de Souza FB, Novaes RD, Santos CFF, de Deus FA, Santos FC, Ladeira LCM, et al. High-fat diet and caffeine interact to modulate bone microstructure and biomechanics in mice. **Life Sci** 2021;276:119450. https://doi.org/10.1016/j.lfs.2021.119450.

[7] Guimarães-Ervilha LO, Ladeira LCM, Carvalho RPR, Bento IP da S, Bastos DSS, Souza ACF, et al. Green Tea Infusion Ameliorates Histological Damages in Testis and Epididymis of Diabetic Rats. **Microsc Microanal** 2021:1–13. https://doi.org/10.1017/S1431927621012071.

[8] Marriel NB, Ladeira LCM, Araújo R dos S, Silva J da, Martins ALP, Tavares MG. O lúdico no ensino de biologia celular: possibilidades no ensino superior. **Rev ELO – Diálogos Em Extensão** 2021;10:1–11. https://doi.org/10.21284/elo.v10i.12290.

Anexo I

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 53/2018, intitulado **"Efeitos da infusão de chá verde (*Camellia sinensis*) combinada com insulinoterapia sobre parâmetros cardíacos e bioquímicos de ratos wistar diabéticos"**, coordenado pela professora Izabel Regina dos Santos Costa Maldonado do Departamento de Biologia Geral, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 21/09/2018, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 53/2018, named **"Effects of green tea infusion (*Camellia sinensis*) combined with insulin therapy on cardiac and biochemical parameters of diabetic wistar rats"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on September 21, 2018 valid for 12 months.

Atima Clemente Alves Zuanon

Profª. Atima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV