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Green tea infusion prevents diabetic nephropathy's 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 hypoglycemic 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.

58 1. Introduction

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

68 Green tea (*Camellia sinensis* (L.) Kuntze (Theaceae)), popularly used as a traditional
69 medicine, in the form of infusion, for many porpoises including hyperglycaemia (Barkaoui et al.,
70 2017; Chopade et al., 2008; Fallah Huseini et al., 2006; Rachid et al., 2012), is known to exert
71 positive effects in diabetes management (Meng et al., 2019; Mohabbulla Mohib et al., 2016).
72 Recent studies have shed light on the mechanisms that tea catechins affect positively the DN, with
73 special focus on the podocyte (Hayashi et al., 2020), through the activation of the 67kDa laminin
74 receptor (67LR) by the epigallocatechin gallate (EGCG), the main polyphenol in green tea. Such
75 interaction results in the preservation of podocyte morphology and the glomerular filtration
76 function, suggesting an improvement in DN. However, tubular alterations, with glycogen
77 accumulation, and aberrant activation of the advanced glycated end-products and its receptor
78 (AGE/RAGE system), affecting the cellular renovation and survival, are seem to be the primary
79 cause of proximal tubular function disruptment (Haraguchi et al., 2020). This, in turn, can affect
80 glomerular function by the proximal tubule/glomerulus feedback system, leading to glomerular
81 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 hypoglycemic 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, we 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; $82.52 \pm 10.83\text{g}$) were housed, two per cage, in polypropylene cages with autoclaved sawdust as cage bed, under controlled conditions of temperature ($22 \pm 2\text{ }^{\circ}\text{C}$) and light-dark cycles (12/12h), and received food (Presença 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, $80\text{ }^{\circ}\text{C}$) (Perva-Uzunalić et al., 2006). The mixture remained infused for 20 minutes on a magnetic stirrer. Then, it was filtered through a $0.45\text{ }\mu\text{m}$ porous filter, frozen at $-80\text{ }^{\circ}\text{C}$ and lyophilized. The lyophilized samples were resuspended in distilled water at the moment of use.

The chromatographic profile, or fingerprint, was determined as described by Kim-Park et al. (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 $30\text{ }^{\circ}\text{C}$, with a $5\mu\text{L}$ 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 ($\geq 98.0\%$, 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 μ Mol Trolox equivalent (TE)/g GTI in the 2,2'-Azinobis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) assay and 46.38 ± 4.10 μ Mol 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 $RSI = KW/BW \times 100$ (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 °C 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 °C. 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 ($O_2^{\cdot -}$) 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 5°C. 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 μm^2 , 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 and 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).

222

223 2.9. Statistical analysis

224 All the results were submitted to the Shapiro-Wilk test to check normality. The data expressed
225 as percentages were transformed by angular transformation before the analysis. Results were
226 expressed as mean $\pm$ standard deviation (mean $\pm$ SD) and analyzed using unpaired *t*-test when the
227 variances are equal (by *F* test) and unpaired *t*-test with Welch's correction for data with unequal
228 variances (Ctrl vs STZ; STZ vs STZ+GTI). The non-parametric data were compared with the
229 Mann-Whitney test. The correlation analysis was carried out following Pearson's correlation
230 method, as the analyzed data were normally distributed. Statistical significance was established at *P*
231 ≤ 0.05 .

232

233 2.10. In silico pathway exploration

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

238 A chemo-biology interactome network was built to elucidate the interactions between the tea
239 compounds (catechins) and proteins expressed in the kidneys related to the positive effects founded
240 in the in vivo experiment with diabetic rats. A prospective evaluation of compound-protein
241 interactions (CPI) was done with the STITCH v.5.0 database (<http://stitch.embl.de/>) (Szklarczyk et
242 al., 2016). The CPI settings were done according to (de Godoi et al., 2020). Briefly, the network
243 downloaded from the database was limited to no more than 50 interactions, medium confidence
244 score (0.400), network depth equal to 1, and the following methods of predictions were activated:
245 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, 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 (O₂) demand creating a hypoxic environment in the tubular cells (Herman-Edelstein and Doi, 2016). However, the blood supply of O₂ 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- κ B) 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 NF κ B 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 (GSK3 β) 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- κ B 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 α 3- β 1 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; CaCl₂ - 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 factor; FGFR - fibroblast growth factor receptor; FOS - Fos Proto-Oncogene; FRAP - ferric reducing antioxidant power; GSK3 β - glycogen synthase kinase-3 β ; GST - glutathione S-transferase; GTI - Green tea infusion; H₂O₂ - 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; MgCl₂ - 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; NF κ B - nuclear factor κ B; NO₂⁻/NO₃⁻ - nitric oxide; NOS2 - Nitric Oxide Synthase 2; NRIH4 - Nuclear Receptor Subfamily 1 Group H Member 4 ; O₂ - oxygen; O₂⁻ - superoxide; PARP1 - Poly(ADP-Ribose) Polymerase 1; PDGF - platelet-derived growth factor; 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 factor; 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.

Disclosures

The authors have no conflicts of interest to disclose.

Authors' contributions

LCML planned and carried the experiment, performed the analysis and interpreted the results, wrote the original draft, reviewed and final edited the manuscript. ECS participate in the

conceptualization of the experiment, interpretation of results and writing of the manuscript. TAS, JS, GDAL, performed the analysis, interpreted results, reviewed and approved the final manuscript. MMN participate in the conceptualization of the experiment, provided resources, reviewed and approved the final manuscript. RCS and MBF provided resources, participate in the interpretation of results, reviewed and approved the final manuscript. IRSCM participate in the conceptualization of the experiment, interpretation of results, supervision and administration of the project, reviewed and approved the final manuscript.

Availability of data and material

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

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Ethics statement

The use of animals in this study was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa (CEUA/UFV – protocol number 53/2018).

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790 **Figure captions**

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

793

794 **Figure 2.** Renal function markers of male Wistar diabetic rats treated or not with green tea infusion
 795 and a healthy control group. **A** – Urea (mg/dL). **B** – Creatinine (mg/dL). Mean $\pm$ SD. The statistical
 796 differences are indicated with lines with the *P*-value above or below them. Data were compared by
 797 Student *t*-test (Ctrl vs STZ; STZ vs STZ+GTI) considering statistical differences when $P \leq 0.05$. (n
 798 = 6 animals/group).

799

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

805

806 **Figure 4.** Microelement proportions and its correlations, and ATPase activity in the kidney of male
 807 Wistar diabetic rats treated or not with green tea infusion and a healthy control group. **A** – Sodium
 808 (%). **B** – Magnesium (%). **C** – Phosphorus (%). **D** – Chlorine (%). **E** – Potassium (%). **F** – Calcium
 809 (%). **G** – Elemental correlations. **H** - Na^+/K^+ , Ca^{2+} , Mg^{2+} and total ATPase activity. Mean $\pm$ 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 $\pm$ 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.

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

Figure 1

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