

Diabetes-Induced Tyrosine Nitration Impairs Glutamine Synthetase Activity and Exacerbates Neurotoxicity in a Rat Model

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Abstract

Diabetes-induced oxidative and nitrative stress have been well-documented in experimental and clinical samples. Tyrosine nitration is a post-translational protein modification that can modulate protein function. Glutamine synthetase (GS), an enzyme in astroglia and Müller cells is involved in detoxification of glutamate to glutamine and, hence protect neurons from accumulation of glutamate and neuronal cell death. We propose to test the hypothesis that diabetes-induced tyrosine nitration of GS impairs its activity ensuing glutamate accumulation and retinal neurodegeneration. Diabetes was induced by a single STZ injection and one group received epicatechin (100 mg/Kg/po) or vehicle every other day. After 8 weeks, retinal cell death, GS tyrosine nitration and GS activity were determined. Diabetes-induced significant increases in tyrosine-nitration of GS in the diabetic retina compared to controls. These effects coincided with significant decrease in GS activity in the diabetic retina. Treatment with the nitration inhibitor, epicatechin significantly reduced nitration of GS and improved its activity. Retinal neuronal death was indicated by numerous TUNEL-labeled cells in diabetic rats, but not in epicatechin-treated diabetic rats compared with controls. Together, these results suggest that diabetes-induced neurodegeneration could be attributed at least in part due to tyrosine nitration- impaired GS activity. Tyrosine nitration inhibitors are potential add-on therapy to glycemic control that can provide neuroprotection in patients with diabetes.

Keywords

Diabetes, Müller cells, Nitrotyrosine, Glutamine synthetase, Neurotoxicity

Introduction

Diabetes-induced oxidative and nitrative stress have been well-documented in experimental models and clinical samples [1-3]. Multiple molecular pathways have been identified including, the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase, and uncoupled nitric oxide synthases (reviewed in [4]). Retina is a susceptible target for oxidative and nitrative stress [5] as demonstrated by large body of work showing positive correlation of nitrotyrosine with accelerated retinal endothelial cell death, breakdown of the blood-retinal barrier (BRB), and accelerated neuronal cell death in experimental models of diabetes [6-9]. These studies suggest a key role of peroxynitrite affecting different retinal neurons and capillaries. However, the causal role of diabetes-induced tyrosine nitration in mediating glial injury and dysfunction has not been elucidated.

Müller cells, a specialized and the primary macroglial cells in the retina, perform wide-array of functions to maintain retinal homeostasis (reviewed in [10]). Under physiological condition, Müller cells recycle neurotransmitters and prevent glutamate toxicity by transporting glutamate and metabolizing glutamate by glutamine synthetase (GS) to glutamine that is released back to neurons for re-synthesis of glutamate and gamma-aminobutyric acid (GABA). Under stress condition including diabetes, these functions can be impaired resulting in glutamate accumulation, as reported in the vitreous of diabetic patients [11] and in the retina of diabetic animals



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[12-15]. Prior studies demonstrated that GS is a susceptible target for tyrosine nitration, which subsequently can impair the enzyme activity [16,17]. Diabetes-associated tyrosine nitration has been shown to play critical role in interrupting survival signal and accelerating cell death in both neurons and capillaries [3,7,18,19], however, the specific impact of tyrosine nitration on glial function has not been fully elucidated. Together, these observations prompted us to study the role of diabetes-induced tyrosine nitration in mediating glial injury and GS dysfunction.

Materials and Methods

Experimental animals and retina isolation

Eight-week-old male Sprague-Dawley rats (≥ 200 g) were obtained from Charles River and made diabetic by a single tail-vein injection of streptozotocin (STZ, Sigma, 65 mg/kg body weight in 0.1 M citrate-buffered saline, pH 4.5). All animal procedures were performed in accordance with Association for Research in Vision and Ophthalmology (ARVO) statement for use of animals in ophthalmic and vision research, and Charlie Norwood VA Medical Center Animal Care and Use Committee (ACORP # 16-01-088). Diabetes was confirmed by detection of glucose in the urine of and blood of injected animals > 250 mg/dL. A group of diabetic rats received oral gavages of 100 mg/kg of epicatechin in PBS every other day for 8-weeks. STZ-injected animals had significant increases of blood glucose level (486 ± 32 mg/dL) compared to controls (130 ± 8 mg/dL). Treatment of diabetic rats with epicatechin did not alter blood glucose level (446 ± 30 mg/dL) compared to untreated-diabetic rats. After 8-weeks of diabetes, eyes were enucleated, retinas were dissected immediately for further analyses.

Glutamine Synthetase (GS) activity

GS enzyme activity was assayed as described previously [20]. Briefly, retinas were homogenized on ice in a solution containing 100 mM imidazole-HCl buffer, pH 7.0, 5 mM EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate. After centrifugation ($10,000 \times g$ for 30 min at 4 °C), the supernatant was subjected to the assay. The assay mixture contained 30 mM imidazole, 100 mM sodium glutamine, 8.5 mM adenosine 5-triphosphate, 1 mM phosphoenolpyruvate, 60 mM magnesium chloride, 20 mM potassium chloride, 45 mM ammonium chloride, 0.25 mM β -nicotinamide adenine dinucleotide, 28 units pyruvate kinase, and 40 units L-lactic dehydrogenase in a final volume of 3 ml. The reaction was initiated by addition of 100 μ l retina lysate, and the decrease in absorbance at 340 nm was measured at 37 °C with a plate reader (BioTek Synergy2, Winooski, VT, USA). Protein concentration of retina samples was determined by the Bradford method using Bio-Rad assay solution. GS activity is expressed as μ mol of l-glutamine produced per min per g of tissue protein [μ mole/(min \times g)].

Immunoprecipitation and western blot analysis of GS

Individual rat retinas were homogenized in radioimmunoprecipitation assay buffer as described previously [7]. For GS tyrosine nitration, 100 μ g protein was incubated with anti-GS antibody and protein A/G agarose (Santa Cruz, TX, USA) and mixed overnight at 4 °C. The precipitated proteins were recovered by centrifugation for 5 min at 700 g and washed two-times in 50 mM Tris (pH 7.5) containing 0.1% (w/v) SDS and 150 mM NaCl. Immunoprecipitates were analyzed by SDS-PAGE, and blotted with anti-nitrotyrosine monoclonal antibody or anti-GS for equal loading. Antibody for nitrotyrosine was purchased from Millipore (Millipore-Sigma, Burlington, MA, USA) and polyclonal anti-glutamine synthetase was purchased from (Santa Cruz, TX, USA). The primary antibody was detected using a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (EMD, La Jolla, CA, USA) and enhanced chemiluminescence. The films were scanned, and the band intensity was quantified using ImageJ densitometry software version, and expressed as optical density (OD).

Terminal dUTP nick end-labeling (TUNEL) analysis

Eyes were mounted in OCT and sections (10 μ m) were collected and stored at -80 °C. TUNEL was performed in these frozen sections using the Apop TAG *in situ* cell



death detection kit (TUNEL-FITC; Chemicon International) as described previously [3]. Retina sections were counterstained with propidium iodide (PI) and cover-slipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). Micrographs were captured at 20X by fluorescent microscope (AxioObserver.Z1; Zeiss, Jena, Germany).

Immunostaining of Glial activation using GFAP

The distribution of GFAP in frozen eye sections was analyzed using immunolocalization techniques as described previously [3]. Retinal sections were fixed with 4% paraformaldehyde then reacted with a mouse anti-GFAP (Cell signaling) antibody followed by anti-mouse antibody (Molecular Probes). Images were captured using at 20X by fluorescent microscope (AxioObserver.Z1; Zeiss, Jena, Germany).

Oxidized and reduced glutathione

Total glutathione including GSH and oxidized glutathione (GSSG) were measured according to manufacturer's protocol (Northwest Life Science, Vancouver, WA) as described previously [18]. For total glutathione, retinas were lysed in phosphate buffer (100 mM potassium phosphate and 1 mM EDTA) and were mixed with an equal amount of 10 mM 5,5-dithiobis (2-nitrobenzoic acid) in the presence of glutathione reductase and NADPH producing a measurable color. The yellow color was measured at a wavelength of 412 nm. To detect GSSG, samples were treated with 10 mM 2-vinylpyridine (Sigma-Aldrich) in ethanol to sequester all the reduced GSH then measured using the same protocol of the glutathione. GSH was calculated as the difference between total glutathione and GSSG. The ratio between the oxidized glutathione (GSSG) and reduced-form GSH was calculated for controls and diabetic rats.

Data analysis

Results are expressed as mean \pm SE, and the data was processed for statistical analysis. One-way ANOVA was used to assess significant differences among groups by GraphPad Software Version.6 (San Diego, CA). Significance was defined at probability $p < 0.05$.

Results

Diabetes triggered glial Müller cell injury and reduced antioxidant defense

Glial activation as indicated by glial fibrillary acidic protein (GFAP) is a common response to stress condition. Therefore, we assessed glial injury in response to the diabetic insult by immunolocalization of GFAP. As shown in [Figure 1A](#), diabetic rat retinas demonstrated a substantial increase in the intensity of GFAP immuno-reactivity in the filaments of Müller cells that extended from the ganglion cell layer (GCL) and inner nuclear layer (INL) into the outer nuclear layer (ONL) of retina as compared with controls. As shown in [Figure 1B](#), analysis of the ratio of oxidised glutathione (GSSG)/free glutathione (GSH) was used to measure the retinal antioxidant defense. The results showed that diabetes significantly decreased the antioxidant defense as indicated by a twofold increase in GSSG/GSH ratio compared with controls.

Diabetes induced GS tyrosine nitration and impairs its activity

Diabetes-induced peroxynitrite formation and its subsequent alteration of protein function via tyrosine nitration are well-documented [21]. Recent studies demonstrated that glutamine synthetase (GS) is a susceptible target for tyrosine nitration [16]. Therefore, we evaluated the specific tyrosine nitration levels of the GS and to what extent it can alter its activity in diabetic rat retinas. As shown in [Figure 2](#), diabetes significantly caused remarkable tyrosine nitration (2-fold) of GS that was significantly reduced by treatment with epicatechin. We next evaluated the effects of tyrosine nitration on GS activity. Indeed, diabetes-induced GS tyrosine nitration was positively correlated with significant inhibition (40%) of GS activity ([Figure 3](#)). Treatment with epicatechin partially and significantly restored GS activity. These results suggest a causal role of tyrosine nitration in impairing the function of GS which can lead to accumulation of glutamate and possibly cause neurotoxicity.



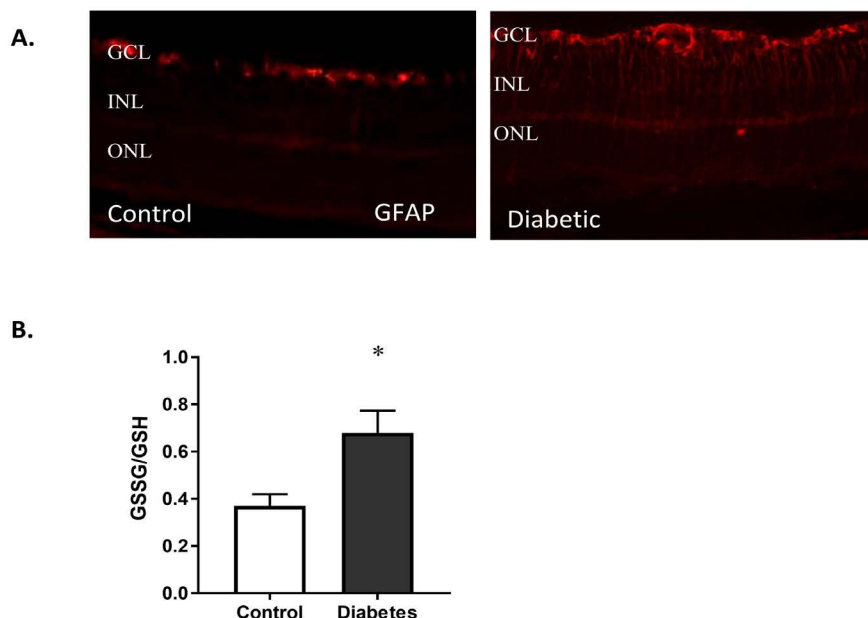


Figure 1: Diabetes triggers Müller cells activation and reduced antioxidant defense.

A) Representative Images of glial fibrillary acidic protein (GFAP) showing abundant immunofluorescence at the end-feet of the Müller cells and the radial processes stained intensely throughout both the inner and the outer retina in the diabetic retinas compared to normal controls. (GCL, ganglion cell layer, INL, inner nuclear layer and ONL, outer nuclear layer). Similar results were obtained from 4-additional animals/group; B) Statistical analysis of the ratio between oxidized glutathione (GSSG) and reduced glutathione (GSH) from retinas from various groups. Diabetes markedly increased GSSG/GSH ratio compared to controls ($n = 4$ retinas/group, * $P < 0.05$, vs. control).

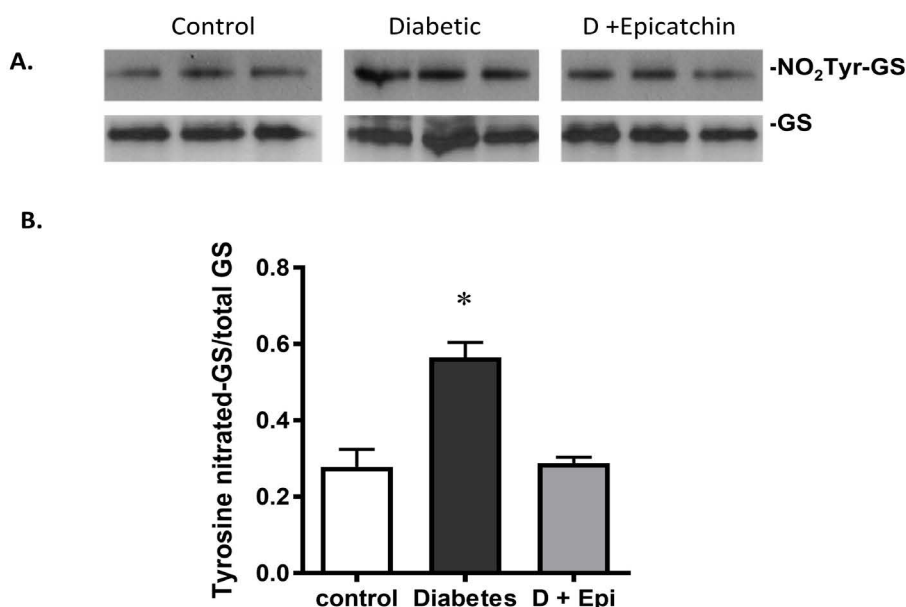


Figure 2: Diabetes causes GS tyrosine nitration.

A) Representative images from retinal homogenate immune-precipitated with anti-glutamine synthetase (GS) and Western blot analysis using anti-nitrotyrosine antibody from controls, diabetic and epicatechin-treated diabetic rats; B) Statistical analysis show that diabetes significantly increased tyrosine nitration of GS in the diabetic retina compared with normal controls. Treatment of diabetic rats with epicatechin (100 mg/Kg/QOD) significantly reduced tyrosine nitration of GS. ($n = 4-6$ retinas/group, * $P < 0.05$, vs. control).

Diabetes accelerated neuronal death

We next evaluated neuronal death after 8-weeks of diabetes. The results demonstrated that diabetic rat retina showed significant increases in TUNEL-positive cells mainly in retinal ganglion cells (GCL), inner retinal layers (INL) and outer retinal layers (ONL) compared to controls (Figure 4). Treatment of diabetic animals with epicatechin significantly reduced the number of TUNEL-positive cells.



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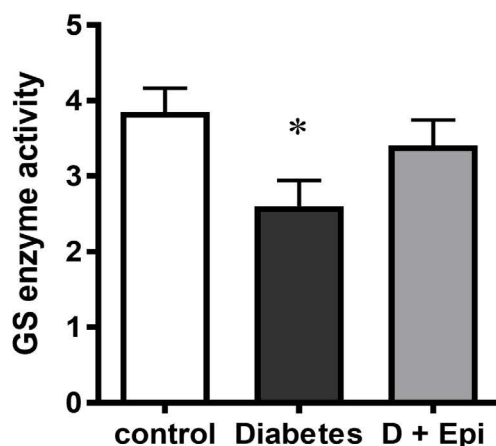


Figure 3: Diabetes impaired GS activity.

Glutamine synthetase activity measured by the ability of the sample to decrease the absorbance measured at 340 nm at 37 °C (see methods) and the results demonstrated significant inhibition of GS activity in diabetic rat retina compared to controls. Treatment of diabetic rats with epicatechin (100 mg/Kg/QOD) significantly improved GS activity compared to untreated diabetic rats. (*n* = 4-5 retinas/group, **P* < 0.05, vs. control).

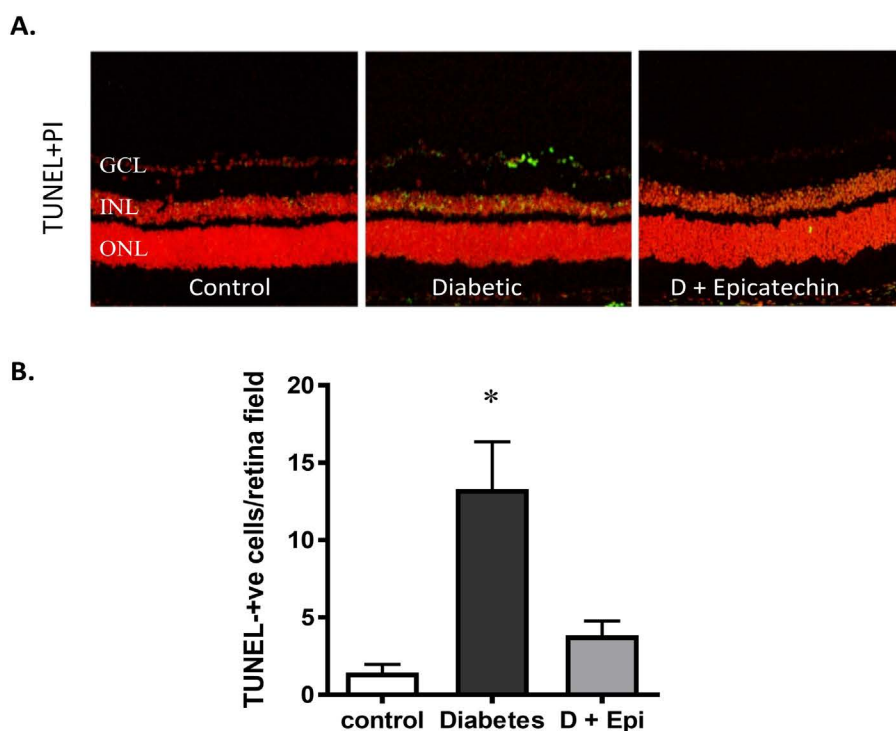


Figure 4: Diabetes causes retinal neurodegeneration.

A) Representative images from the control and diabetic rat retinas showing TUNEL-positive cells (green) in different retinal layers. TUNEL-positive cells were distributed mainly in the ganglion cell layer (GCL), inner nuclear layer (INL) and to less extent in the outer nuclear layer (ONL); B) Statistical analysis of TUNEL-positive nuclei from various groups showing that diabetes triggered retinal cell death compared to controls and epicatechin-treated diabetic rats. At least 4 fields/mid-peripheral retina were counted for each retina from one animal. (*n* = 4-5 retinas/group, **P* < 0.05, vs. control).

Discussion

The imbalance between excessive generation of free radicals and the antioxidants mechanisms is a hallmark in the pathogenesis of diabetic retinopathy (reviewed in [4]). Over the past decade, research focused on identifying molecular pathways by which hyperglycemia triggers formation of free radicals and oxidative stress. We and others have identified several pathways including the mitochondrial electron transport chain [22], uncoupling of nitric oxide synthase, activation of polyol pathway [6,23,24] and NADPH oxidases [25,26]. In addition to upregulation of oxidative stress sources, the antioxidant defense system is also compromised in diabetes. The



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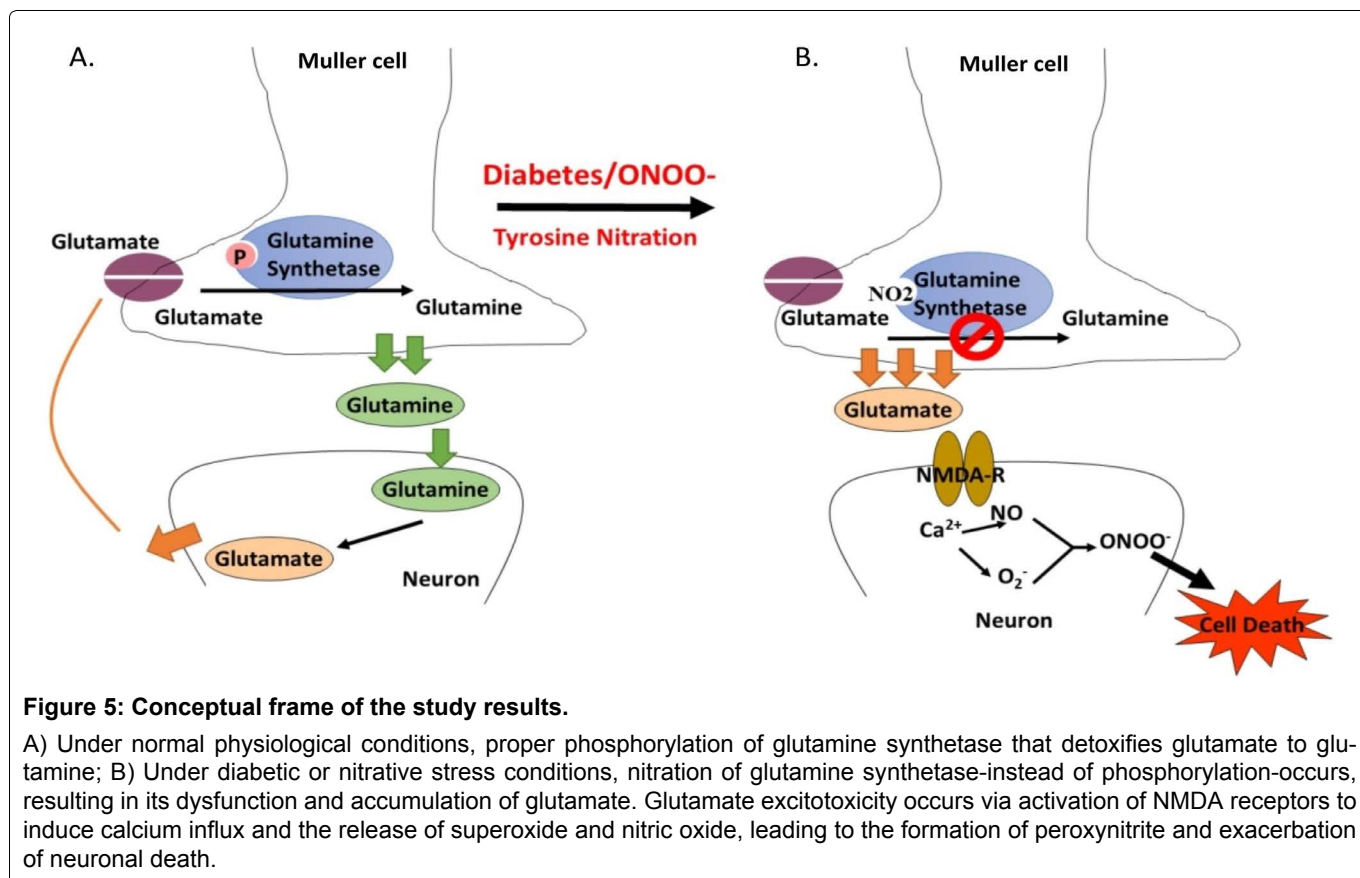


Figure 5: Conceptual frame of the study results.

A) Under normal physiological conditions, proper phosphorylation of glutamine synthetase that detoxifies glutamate to glutamine; B) Under diabetic or nitrative stress conditions, nitration of glutamine synthetase-instead of phosphorylation-occurs, resulting in its dysfunction and accumulation of glutamate. Glutamate excitotoxicity occurs via activation of NMDA receptors to induce calcium influx and the release of superoxide and nitric oxide, leading to the formation of peroxynitrite and exacerbation of neuronal death.

levels of the glutathione (GSH), the main intracellular antioxidant become markedly reduced, and the enzymes responsible for glutathione redox cycle (glutathione peroxidase and glutathione reductase) are compromised [27,28]. In support, our results showed that retinas from 8-weeks of STZ-diabetes showed marked increase in the ratio of oxidized glutathione to the reduced glutathione (Figure 1B).

Diabetes-induced retinal oxidative and nitrative stress have been well documented in patients and animals and have been positively correlated with neuronal cell death [3,29-33]. However, the effects of diabetes-induced oxidative and nitrative stress on macro-glial activation and how this can affect neuronal function are not fully elucidated. The results showed 8-weeks of diabetes induced prominent glial Müller cell activation compared to controls. Although alterations in glutamate transporter activity during diabetes remain controversial, impairment in GS activity was previously reported [34-36]. Interestingly, GS has been reported to be a susceptible target for tyrosine nitration that might reduce its activity [16,17]. Therefore, we investigated GS activity and GS nitration in diabetic rat retinas. Our results showed 2-fold increase in GS tyrosine nitration that was associated with significant reduction (34%) of GS activity in diabetic retina compared to controls. These results concurred with previous reports showing that GS activity is regulated by tyrosine nitration [37,38]. Our results lend further support to previous reports showing that diabetes can alter glial function, impair GS activity and decrease its expression [20,34,39]. Previous studies documented the adverse effects of diabetes on impairing the function of Müller cells in transporting glutamate by glutamate transporter or metabolizing glutamate by glutamine synthetase [12-14,40].

Treatment of diabetic animals with the nitration inhibitor, epicatechin significantly reduced tyrosine nitration of GS and restored GS enzyme activity compared to untreated diabetic rats. Epicatechin, a polyphenolic flavonoid is one of several green tea constituents including epigallocatechin gallate, epigallocatechin, epicatechin gallate and epicatechin. The limited bioavailability of green tea extracts usually restricts their use as effective therapeutics. We used a repeated dosing of epicatechin by oral gavage (100 mg/kg/day), as it has proven successful in improving epicatechin bioavailability to cross blood-brain barrier [41]. Our results lend further support to prior



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work showing that neuroprotective effects of epicatechin in diabetic animals [20,42] and in models of cardiovascular and neuropsychological health [43].

Significant increases in tyrosine nitration have been demonstrated in plasma from diabetic patients [1], retinas from diabetic patients [3]. Tyrosine nitration and subsequent loss of function have been documented in models of diabetes [7,21,44-46]. Here, we show the impact of GS tyrosine nitration on impairing the enzyme activity and hence glutamate accumulation, as reported in the vitreous of diabetic patients [11] and in the retina of diabetic animals [13,14,30]. As depicted in Figure 5, glutamate excitotoxicity occurs via activation of NMDA receptors to induce calcium influx and the release of superoxide and nitric oxide, leading to the formation of peroxynitrite and neuronal death [47]. Therefore, we next evaluated neuronal cell death in the diabetic animals. Indeed our results showed significant increases in TUNEL-positive cells that were immune-localized in retinal ganglion cells and inner retinal layers in diabetic retina compared to controls. These findings suggest a loop where diabetes-induced oxidative and nitrative stress alter the function of Müller by impairing GS activity leading to glutamate neurotoxicity and sustaining neuronal death. Finally, tyrosine nitration inhibitors such as epicatechin can be potential add-on therapy to anti-diabetic agents that can restore neuroglial healthy function and provide neuroprotection in patients with diabetes.

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