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Modulation of Alzheimer-like Pathology in Type 2 Diabetic Rats via Grape Seed or Ginseng Extracts

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Abstract:
This study was aimed to investigate the potential role of grape seed extract (GSE) or ginseng extract (GE) against some brain cortical indices related to Alzheimer's disease (AD). AD developed after 6 months of type 2 diabetes mellitus (T2DM) course. T2DM was induced by high fat diet and streptozotocin injection (i.p 45 mg/kg b.w.). Rats were randomized into control, T2DM, T2DM+GSE, T2DM+GE. Administration of extracts (100 mg/kg b.w. each) was started just after diagnosis of T2DM, and continued for 6 consecutive months. Treatment with GSE or GE provided significant modulation of type 2 diabetes metabolic disturbances, manifested by the attenuated serum levels of glucose, total cholesterol, triglycerides and blood HbA1C. Both extracts also opposed type 2 diabetic central neuropathy as seen in decreasing tissue elevation of amyloid precursor protein, amyloid-β1-42, phospho-tau, TNF-α and MDA. Nevertheless, the extracts increased both the ACh and GSH brain cortical contents. In conclusion, the present data added further evidence that T2DM induced by high fat diet and streptozotocin could be used to study AD-like pathology. Aside from administration of GSE or GE for prolonged time might be successful as dietary supplement in ameliorating brain cortex biochemical changes related to AD associated with T2DM.

Keywords: *Type 2 diabetes mellitus, Alzheimer's disease, grape seed extract, ginseng extract*

1. Introduction

Type 2 diabetes mellitus (T2DM) is a disease of older age, although over the last decades its incidence has increased among young adults and even adolescents due to high fat-containing diet and sedentary life style. T2DM patients have a 30-65% increased risk for developing Alzheimer's disease (AD) (Ahtiluoto *et al.*, 2010). The localization of insulin receptors in the hippocampus and medial temporal cortex in rats is consistent with the evidence that insulin influences memory, an important aspect that links AD with T2DM and a subtle effect of T2DM on whole brain cortical atrophy was observed (Brundel *et al.*, 2010).

Prasad *et al.* (2014) reported that hyperglycemia has been linked to altered blood brain barrier (BBB) transport functions (e.g., glucose, insulin, choline, amino acids, etc.), integrity (tight junction disruption), and oxidative stress in the CNS microcapillaries. On a cellular basis, inhibition of insulin/IGF signaling contributes to AD-type neurodegeneration by activation of kinases that phosphorylate tau protein; increasing expression of amyloid precursor protein (APP) and accumulation of amyloid β_{1-42}($A\beta_{1-42}$); elevating levels of oxidative stress that damage proteins, RNA, DNA, and lipids; and activation of pro-inflammatory cascades (De la Monte, 2012).

Different hypotheses can be involved in AD; the first is cholinergic hypothesis, in which there is a decrease in the neurotransmitter acetylcholine (ACh) (Narahashi *et al.*, 2003). The second is amyloid hypothesis, in which the APP is broken to give short proteins named amyloid-β, $A\beta_{1-42}$ especially is aggregated in neurons (Härd and Lendel, 2012). The third is tau hypothesis, in which the microtubule-associated protein tau abnormalities initiate the disease cascade, when hyperphosphorylated (Fratiglioni and Qiu, 2009). The brain is believed to be vulnerable to oxidative damage, an earlier event associated with neurodegeneration (Migliore *et al.*, 2005).

Grape seed extract (GSE) is an industrial derivative from grape seeds that have great concentrations of vitamin E, polyphenols as flavonoids, linoleic acid, oligoproanthocyanidins (also known as procyanidins) and polymers of catechins (Kijima *et al.*, 2006).

Phenolics in the seeds represent 60-70% of total phenolics in the grape, which attributes to the powerful antioxidant capacity of GSE and also found to inhibit enzymes that lead to inflammatory actions (Shi *et al.*, 2003).

Panax ginseng C.A. Meyer (P. ginseng), commonly known as 'Korean ginseng', is one of the most popular adaptogens among medicinal plants with a long history of traditional use (Kennedy and Scholey, 2003). It is composed of a series of saponins known as ginsenosides which have anti-oxidative properties apart from its immunomodulatory, antihyperlipidemic and neuroprotective activities (Mannaa *et al.*, 2006). Long term administration of ginseng root extract has also been reported to ameliorate hyperglycemia and improve insulin sensitivity (Lee *et al.*, 2009). Panax ginseng standardized extract (G115) was used in the present study, as it is standardized to its ginsenosides content, the most widely studied in the scientific literature, and readily available as an over-the-counter supplement (Kampen *et al.*, 2003).

The objective of the current study was to expand the knowledge about the link between T2DM and AD and to spot light on the possible beneficial effect of GSE and GE against AD-like pathology as a result of T2DM.

2. Materials and Methods

2.1. Drugs and Chemicals

Grape seed extract (Arab Co. for Pharmaceuticals & Medicinal Plants, Mepaco, Egypt) and ginseng extract G115 (Egyptian International Pharmaceutical Industry Company, EIPICO, Egypt, under license of Pharmaton SA Lugano-Bioggio/Switzerland) were used in the present study. The two extracts were standardized using high performance liquid chromatography (HPLC) and gas chromatography (GC) respectively. The extracts were freshly dissolved in distilled water and given orally using oral gavage. Streptozotocin (STZ) was purchased from Sigma–Aldrich Co. (St Louis, MO USA). All other chemicals were of highest analytical grade.

2.2. Animals

Adult male Wistar rats, weighing 160-180 g, were housed in standard polypropylene cages (four rats per cage), and were kept on a light/dark cycle of equal duration, under constant environmental conditions. Rats were fed commercially available rat normal pellet diet (NPD) and water *ad libitum* for one week prior to the dietary manipulation for acclimatization. All the procedures in this study were in accordance with the guidelines for the care and use of laboratory animals as adopted by the Ethics Committee of the Faculty of Pharmacy, Cairo University [(Permit Number: BC (120)].

2.3. Development of Type 2 Diabetic Rats

Sixty rats were fed high fat diet (HFD) according to Srinivasan *et al.* (2005), and ten rats were fed NPD *ad libitum* for the initial period of 2 weeks. Then HFD-fed rats were fasted overnight and injected i.p. with low dose of streptozotocin (STZ) in citrate buffer (pH 4.4) as a vehicle at a dose 45 mg/kg b.w. the following morning (Sawant *et al.*, 2004), while the respective control rats were injected citrate buffer only.

Blood samples were collected and centrifuged to separate clear sera for biochemical estimations of fasting serum glucose, insulin, triglycerides (TG) and total cholesterol (TC) 7 days after STZ injection. Rats with fasting serum glucose of ≥200 mg/dl, normal or hyper-insulinemia, hypertriglyceridemia and hypercholesterolemia were considered type 2 diabetic. The rats were allowed to continue on their respective diets until the end of the study.

Type 2 diabetic rats were randomly allocated into three groups (n=10 -15). One group served as diabetic untreated group and received nothing, the second group received GSE orally (100 mg/kg b.w.) (El-Awdan *et al.*, 2013), and the third received GE orally (100 mg/kg b.w.) (Sawiress, 2011). Drug treatment was started just after diagnosis of T2DM and continued daily for 6 consecutive months. Ten rats fed NPD served as non-diabetic control group. After 6 months, rats were sacrificed by decapitation and blood samples were collected for separation of sera. Whole blood was collected in EDTA tubes for estimation of glycated hemoglobin (HbA_{1C}). Brain was removed instantly after decapitation and rinsed with cold normal saline. The brain cortex was dissected out and quickly stored below 80°C for further determination of biochemical parameters.

2.4. Determination of Biochemical Parameters in Blood

Serum glucose, TC and TG were estimated by enzymatic methods using colorimetric kits (Spinreact, Spain) according to the method of Trinder, 1969, Fossati and Prencipe, 1982 and Meiattini *et al.*, 1978 respectively. Furthermore, serum insulin level was determined by enzyme-linked immunosorbent assay (ELISA) using a diagnostic kit (Glory Science, USA).HbA_{1C} was determined in the whole blood using a column chromatography kit (Teco diagnostics, USA) (Trivelli *et al.*, 1971).

2.5. Determination of Biochemical Parameters in Rat Brain Cortex

A portion of the brain cortex was homogenized in phosphate buffer saline (PBS) (pH 7.4) using grinder and then centrifuged for 20 minutes at the speed 3000 rpm. This supernatant was used for estimation of ACh and $A\beta_{1-42}$ levels by ELISA using diagnostic kits (Glory Science, USA) for each, and tumor necrosis factor-α (TNF-α) by ELISA using diagnostic kit (Labs Biotechnology inc., Canada). Another portion of the brain cortex was homogenized in ice-cold saline (20% w/v) and used to measure lipid peroxidation product, malondialdehyde (MDA) and glutathione (GSH) colorimetrically according to the methods of Mihara and Uchiyama, 1978 and Ellman, 1959 respectively.

APP gene expression in brain cortex was determined using quantitative real time polymerase chain reaction (RT-PCR). About 30mg of brain cortex was homogenized in 175 µl lyses buffer for RNA extraction. Total RNA was isolated from homogenate using RNeasy Purification Reagent (Qiagen, Valencia, CA). Purity (A_{260}/A_{280} ratio) and concentration of RNA were obtained using spectrophotometry (Gene Quant 1300, Uppsala, Sweden). First-strand cDNA was synthesized from 4 µg of total RNA using an Oligo(dT)12-18 primer and Superscript™ II RNase Reverse Transcriptase, supplied by SuperScript Choice System (Life Technologies, Breda, Netherlands). This mixture was incubated at 42°C for 1 hour. Real-time PCR (RT-PCR) amplification was carried out using 10 µL amplification mixtures containing SYBR I Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems). PCR reactions consisting of 95°C for 10 minutes (1 cycle), 94°C for 15 seconds, and 60°C for 1 minute (40 cycles) were done. Data were analyzed with the ABI Prism sequence detection system software and quantified using software from PE Biosystems (Foster City, CA, USA). The target gene was normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which was not changed by any of the treatments. The sequences of PCR primer pairs used for the APP gene and GAPDH gene are shown in table 1. Relative expression of studied gene was calculated using the comparative cycle threshold method. All values were normalized to GAPDH (Livak and Schmittgen, 2001).

Primer	Gene Bank Accession Number	Primer Sequence
APP	X07648	F: 5'-GGA TGC GGA GTT CGG ACA TG-3' R: 5'-GTT CTG CAT CTG CTC AAA G-3'
GAPDH	XM_005253678.1	F: 5'-ACCACAGTCCATGCCATCAC-3' R: 5'-TCCACCACCATGTTGCTGTA-3'

Table 1: The oligprimers sequence of APP and GAPDH

Phosphorylated Tau protein in the brain cortex was determined using western blotting. About 300 mg of brain cerebral cortex was added to 150 µl of RIPA buffer (Sigma) and homogenized. Protein concentration was determined with the BCA Protein Assay kit (Thermoscientific). The isolated proteins were separated according to their molecular weight by SDS-PAGE with 12% polyacrylamide gels. To make proteins available for antibody detection, proteins were transferred onto a nitrocellulose membrane (Whatman) by western blotting. The membrane was then incubated overnight with primary antibody against phospho-tau: Anti- phospho-Tau antibody (Sigma). On the next day, β-actin monoclonal antibody (Sigma) was added. The secondary antibody linked to HRP reporter enzyme (Dianova) was incubated together with the membrane. Membrane was then incubated in chemiluminescent substrate which emitted light when exposed to HRP bound to the secondary antibody. The western blots shown were then quantified by detecting densitrometric intensity of each band with the Chemidoc XRS (Biorad). All values were normalized to the amount of β-actin staining.

2.6. Statistical Analysis

Data were expressed as means ± standard error mean (SEM). Statistical significance was taken as $P < 0.05$ for all experiments, using either unpaired t-test or one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. All statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA).

3. Results

Serum glucose, insulin, total cholesterol and triglycerides one week after STZ injection

Animals received HFD and injected with low dose STZ showed an obvious increase in serum glucose, TC and TG with normal insulin level compared to control group (table 2). These animals were considered as a model of T2DM and were used for the rest of the experiment.

	Normal group (n = 10)	**Diabetic group (n = 30)**
Glucose (mg/dl)	92.74±2.66	289.8±13.16 [a]
Insulin (mIU/L)	5.22±0.24	5.7±0.13
Total Cholesterol (mg/dl)	64.63±3	87.23±2 [a]
Triglycerides (mg/dl)	80.1±3.7	178.6±6.84 [a]

Table 2: Serum glucose, insulin, total cholesterol and triglycerides one week after STZ
Values are expressed as mean ± SEM in each group
[a] significant difference from non-diabetic control at $p < 0.05$

3.1. Effect of Administration of Either GSE or GE to Diabetic Rats for 6 Months on Blood Glucose, Hba_{1c}, TC and TG

As illustrated in table 3,T2DM aggravated the blood glucose, HbA_{1C},TC and TG levels by 244.15, 164.96, 133.99 and 181.68 % compared to normal control group.While, treatment with GSE or GE for 6 months succeeded to maintain these parameters in normalized levels.

	Normal Control		Diabetic	
		Control	GSE	GE
Glucose (mg/dl)	86.38±2.63	210.9±17.92 [a]	80.47±1.6 [b]	87.83±5.2 [b]
HbA_{1C} (%)	4.91±0.22	8.1±0.46 [a]	6.22±0.3 [a,b]	6.31±0.32 [a,b]
Total Cholesterol (mg/dl)	80.15±3.05	107.4±5.7 [a]	90.66±4.12 [b]	77.28±4.51 [b]
Triglycerides (mg/dl)	88.67±4.02	161.1±10.84 [a]	91.04±4.71 [b]	98.38±8.49 [b]

Table 3: Levels of glucose, total cholesterol, triglycerides and HbA_{1C} after oral administration of GSE or GE (100mg/kg each) for 6 months
Values are expressed as mean ± SEM in each group. (n=8)
[a] significant difference from non-diabetic control at $p < 0.05$
[b] significant difference from diabetic control at $p < 0.05$

3.2. Effect of Administration of GSE and GE to Diabetic Rats for 6 Months on Brain Cortical TNF-A, GSH, MDA and Ach

Table 4 shows the highly magnified inflammatory cytokine TNF-α in brain cortex of diabetic rats by a 699.06 % of the normal control group. This redundancy was abolished by treatment with either GSE or GE, although the effect of GSE was more potent. Regarding the potent natural antioxidant GSH, its brain cortical level was significantly reduced after 6 months diabetic course reaching 38.34 % of normal (table 4).No doubt that GSE or GE administration succeeded to maintain GSH levels, and again the effect of GSE was more obvious. T2DM insult has boosted the level of MDA by 467.25 % as compared to the normal control group, effect that was reverted by the two tested extracts, but was clearer with GSE (Table 4).Table 4 also depicted the marked depression in brain cortical ACh in diabetic rats (reaching 74.09 % of normal control group). Treatment with GSE or GE fenced against that depression. Surprisingly, GE had yet non-significant but apparently a more potent effect than GSE, where it enhanced ACh level reaching 108.3 % of normal group, compared to only 100.06 % effect of GSE.

	Normal Control		Diabetic	
		Control	GSE	GE
TNF-α(ng per g tissue)	1.07±0.1	7.48±0.6 [a]	2.43±0.47 [b]	3.25±0.53 [a,b]
GSH (mg per g tissue)	0.266±0.02	0.102±0.01 [a]	0.219±0.01 [b]	0.202±0.01 [a,b]
MDA(nmol per g tissue)	6.84±0.62	31.96±2.59 [a]	12.68±1.5 [b]	21.01±1.83 [a,b]
ACh (pg per g tissue)	66.94	49.6 [a]	66.98 [b]	72.65 [b]

Table 4: Brain cortical TNF-α, GSH, MDA and ACh after oral administration of GSE or GE (100mg/kg each) for 6 months
Values are expressed as mean ± SEM in each group. (n= 8)
[a] significant difference from normal at $p < 0.05$
[b] significant difference from diabetic at $p < 0.05$

3.3. Effect of Administration of Either GSE or GE to Diabetic Rats for 6 Months on Brain Cortical Gene Expression of APP and Level of $A\beta_{1-42}$

Table 5 illustrates the boosting effect of T2DM on APP gene expression and $A\beta_{1-42}$content in brain cortex after 6 months. This effect was hindered by GSE and GE administration, although parameters didn't reach normal levels, but were significantly different from diabetic control group. It is worth mentioning that GE showed significantly lower APP expression than GSE, where in group administered GE, APP expression reached only 347 % of normal, while GSE reached 508 % of normal.

3.4. Effect of Administration of Either GSE or GE to Diabetic Rats for 6 Months on Brain Cortical Protein Levels of P-Tau

Western blotting analysis revealed that cortical phosphorylation of tau protein was significantly reinforced by T2DM by 511.11 % when compared to normal healthy control group (table 5 and fig.1) in cerebral cortex. Treatment with either GSE or GE significantly reduced phosphorylated tau when compared to diabetic control group.

	Normal Control		Diabetic	
		Control	GSE	GE
APP (relative to GAPDH protein)	1	10.81±0.49 [a]	5.08±0.46 [a,b]	3.47±0.38 [a,b]
$A\beta_{1-42}$ (nmol per g tissue)	2.77±0.05	3.63±0.04 [a]	3.05±0.06 [a,b]	3.16±0.03 [a,b]
p-tau (relative to β-actin)	0.18±0.01	0.92±0.02 [a]	0.29±0.04 [ab]	0.33±0.02 [ab]

Table 5: Brain cortical APP gene expression and $A\beta_{1-42}$ level after oral administration of GSE or GE (100mg/kg each) for 6 months
Values are expressed as mean ± SEM in each group. (n= 8)
[a] significant difference from non-diabetic control at $p < 0.05$
[b] significant difference from diabetic control at $p < 0.05$

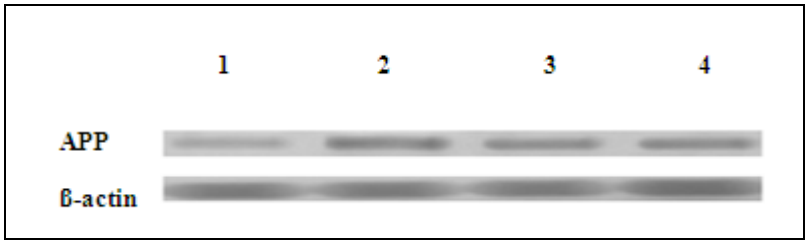

Figure 1: Western blotting of brain cortical p-tau and control gene (β-actin) in all rats (n= 8/group). Lanes 1, 2, 3 and 4 are representative for normal control, diabetic control, GSE-treated and GE-treated respectively.

4. Discussion

This study may introduce a new approach in establishing a model of T2DM-induced AD and using grape seed extract and ginseng extract on guarding against this complication.

In the current study, a model of T2DM was represented, since rats showing hyperglycemia with normal/high insulin level, hypercholesterolemia and hypertriglyceridemia were selected. This model was established by Srinivasan *et al.* (2005), giving a model similar to late stage type 2 diabetic patients. Consumption of HFD for the initial period of 2 weeks was expected to give a rise to insulin resistance - a major predisposing factor for T2DM (Sawant *et al.*, 2004). It was also proved that if a low dose STZ was used after HFD in rats, the pancreatic β-cell mass would be modestly impaired without completely impairment in glucose tolerance (Srinivasan *et al.*, 2005).

Our data revealed that T2DM for 6 months can lead to AD-like brain pathology, as it was evidenced that apoptotic neurons were upregulated in AD (Sharma *et al.*, 2012). AD in the present study was indicated in type 2 diabetic group by the manifested deficiency in brain cortical ACh and GSH, in addition to boosting APP mRNA, as well as levels of $A\beta_{1-42}$, p-tau, TNF-α and MDA.

Impaired brain insulin signaling in AD due to metabolic stress and neuroinflammation is a similar mechanism that accounts for peripheral insulin resistance in T2DM (De Felice *et al.*, 2014). In type 2 diabetes, abnormal TNF-α signaling leads to activation of kinase c-Jun N terminal kinase (JNK), which through a certain cascade results in insulin resistance, an action similar to that of amyloid beta oligomers in the brain (Bomfim *et al.*, 2012). Defective insulin signaling in the brain may also enhance the amyloidogenic processing of APP (De Felice *et al.*, 2014). Disinhibition of glycogen synthase kinase-3 (GSK-3), the prime enzyme responsible for tau phosphorylation, due to insulin deficiency may be a cause behind increased p-tau (Doble and Woodget, 2007). Ghribi *et al.* (2006) has also assumed that increased Aβ may trigger phosphorylation of tau by activation of extracellular signal-regulated protein kinase (ERK).

Hypercholesterolemia arose in the current study correlates positively to neurodegenerative diseases (Charradi *et al.*, 2012). Its exact mechanism in AD brains is still unclear, as cholesterol is unable to pass the BBB. However, it was indicated that hypercholesterolemia resulted in decreasing the number of cholinergic neurons and ACh levels in the rat brain cortex and also elevated p-tau (Ullrich *et al.*, 2010). Consistent with our results, hypercholesterolemia significantly upregulated APP expression almost by formation of caveolae and lipid rafts (Li *et al.*, 2007).

The diminished ACh in diabetic rats may be also due to reduced BBB transport of choline (ACh substrate) (Mooradian, 1987). Increased acetylcholinesterase (AChE)-which is responsible for Ach degradation-and decreased choline acetyltransferase (ChAT)-which is responsible for its synthesis-were observed by Kumar *et al.* (2011) in cerebral cortex of diabetic rats; may be another illustration for ACh shortage.

Di Bona *et al.* (2010) proved that TNF-α was significantly increased in cerebral cortex of AD rats, as found in our study, via activation of glia that damage neurons (Umegaki, 2014). Binding of TNF-α to its receptors was found to stimulate several intracellular signaling pathways linked to AD, as the activation of proteinkinase C, JNK, p38 mitogen activated protein kinase (p38/MAPK), PI3 kinase, ERK, as well as activation of caspase-1 and -3 (Sharma *et al.*, 2012). Concerning oxidative stress in cerebral cortex in diabetic control rats, it was revealed by elevated MDA and diminished GSH levels, where Migliore *et al.* (2005) mentioned that these findings are present in AD.

Our results revealed that oral administration of GSE or GE for 6 consecutive months improved type 2 diabetic cases. Effect of GSE may be partly due to enhancing insulin secretion and act as insulin sensitizer (El-Awdan *et al.*, 2013). GSE procyanidins were reported to exhibit insulinomimetic properties by stimulating glucose uptake in insulin sensitive cell lines (Pinent *et al.*, 2004). Studies showed that GSE strongly inhibited both pancreatic α-amylase and intestinal α-glucosidase activity, two key glucosidases required for starch digestion in humans (Yilmazer-Musa *et al.*, 2012). Anti-hyperlipidemic effect of GSE can be attributed to enhancing bile acid synthesis and excretion (Sethupathy *et al.*, 2002). GSE flavonoids raised cortical GSH in diabetic rats, may be via induction of γ-glutamyl cysteine synthetase expression, rate-limiting enzyme for GSH synthesis (Balu *et al.*, 2006). Furthermore, GSE elevated ACh probably via decreasing AChE activity (Ullrich *et al.*, 2010). GSE significantly decreased $A\beta_{1-42}$ deposition, as was observed by Wang *et al.* (2009).

Consistent with our results found in animals administered GE in comparison to control, it was reported that GE succeeded in reduction of plasma glucose in type 2 diabetic rodents (Kennedy and Scholey, 2003), which found that beneficial effect of GE might be via insulin sparing, increased glucose transport and slowing food digestion rate. Ginsenoside Rg1 (active constituent in GE) induces neuroprotection through ameliorating amyloid pathology and modulating APP process (Fang *et al.*, 2012). Possible ginseng-mediated neuroprotective mechanisms are that ginseng increases nitric oxide synthesis in cerebral tissue, in addition to protection against lipid

peroxidation (Kennedy and Scholey, 2003). Wang *et al.* (2011) suggested that ginsenoside Rb1 is effective in preventing Aβ induced loss of brain functions. In-vitro experiment showed that ginsenoside Rb1 could reduce the tau phosphorylation in cultured cortical and hippocampal neurons isolated from rat models of AD (Cheng *et al*., 2005).

Gathering our results together, it is very clear that using GSE and GE plays an important role in guarding against AD as a type 2 diabetic central neuropathy.

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