Neuroprotective effects of bioactive compounds (resveratrol and genistein) in various mouse models

by

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Abstract

Obesity is a complex disease which has adverse effects on various organ including liver, heart and brain. It was shown that obesity is a significant risk factor for developing type 2 diabetes, heart disease and neurodegenerative disease such as Alzheimer's disease (AD). The relationship between obesity, type 2 diabetes and cognitive impairment is important, considering the increased aging population in whom cognitive dysfunction will carry huge individual, financial and societal burdens. Bioactive compounds such as carotenoids, isoflavones, and polyphenols have received increased attention due to their antioxidant, anti-inflammatory, anti-aging, and anti-cancer properties. These effects make them promising candidates for the prevention and treatment of obesity, type 2 diabetes and AD. This study is mainly focusing on resveratrol and genistein. Promoting regular exercise is also an effective non-pharmacological approach that prevent the progression of metabolic and neurodegenerative diseases. Therefore, the first objective of this study is to examine the neuroprotective effects of chronic resveratrol supplementation with exercise training in the 3xTg-AD mouse model. Our observations suggest that resveratrol ameliorated neuroinflammation, decreased accumulation of amyloid beta (Aβ) oligomers, improved levels of neurotrophic factors and synaptic markers, inhibited apoptosis, autophagy and ubiquitination in the brain of the 3xTg-AD mouse. Exercise improved few markers related to neuroprotection, but when combined with resveratrol treatment, the benefits achieved were as effective as resveratrol treatment alone.

Secondly, we would like to investigate the beneficial effects of genistein on diabetes-induced brain damage. Leptin deficient (ob/ob) mice and high-fat/high-sugar (HFHS) diet-induced diabetic mice were chosen. Our results suggest that genistein improved brain insulin signaling, increase neurotrophic support and alleviated AD-related pathology in the brain of ob/ob mice. In the brain HFHS diet-fed mice, genistein supplementation coupled with exercise training

decreased the accumulation of Aβ, improved the expression of neurotrophic factor as well as ameliorated apoptosis. Moreover, it was suggested that the neuroprotective effects of genistein are associated with its capability to bind the estrogen receptor. Further cell culture study was conducted, and the results demonstrate that the anti-apoptotic actions of genistein were selectively mediated by estrogen receptor in PC12 cells following high glucose and palmitate exposure.

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Chapter 1: Introduction

Obesity is a complex disease in which excess fat has accumulated in the body. Nowadays, it has an epidemic expansion rate in the world. Obesity is a leading risk factor for early death, approximately 8% of worldwide deaths were attributed to obesity in 2017 [1]. Obesity has adverse effects on general health and has shown to increase the risk of type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), hypertension, arthritis, and cancer. Recently, the association between obesity and damaged brain function has received increasing attention. Mid-life obesity has an immediate adverse impact on cognitive performance, such as verbal learning, working memory, and decision-making [2][3]. Increasing age accompanying the negative consequences of obesity (such as T2DM, chronic inflammation) are likely to increase the risk of cognitive impairment and dementia in later life [4]. Alzheimer's disease (AD) is the most common form of dementia. It was reported that obesity and T2DM are risk factors for AD [5]. Several overlapping neurodegenerative mechanisms were observed in these disorders, including oxidative stress, inflammation, mitochondria dysfunction, and impaired insulin signaling [6].

Bioactive compounds are extra-nutritional constituents in foods, which have specific biological effects. They have been intensively studied to evaluate their impact on several diseases. Due to the safety, low price, and pharmacological effects, they become interesting candidates to help manage obesity, T2DM, and some neurodegenerative diseases [6][7]. Our research is mainly focused on resveratrol and genistein. Resveratrol is a polyphenolic compound mainly found in grape skins and red wines. It was shown to improve mitochondrial function, maintain metal homeostasis and exhibit anti-inflammatory, antioxidant, and anti-aging properties [8]. Resveratrol was reported to diminish lipid accumulation as well as decrease body weight in obese animals [9][10]. Moreover, several studies demonstrate that in diabetic rats, resveratrol

has an impact on insulin secretion and plasma insulin concentration. And it can reduce hyperglycemia [11][12][13]. The neuroprotective effects of resveratrol have shown in both preclinical and clinical studies [14]. In humans, evidence from randomized clinical trials indicates that resveratrol is able to improve perceived performances and some cognitive tests and improve cerebral blood flow and cerebrospinal fluid level [15]. Genistein is an isoflavone mostly found in soy products. It shows antioxidant, anti-inflammatory, and anti-apoptosis qualities. Also, genistein was known as a phytoestrogen since it has a similar structure to estrogen and can bind the estrogen receptor. Several preclinical evidence suggests that genistein may be a novel intervention therapy for obesity and T2DM through inhibiting leptin synthesis and secretion, decreasing lipogenesis, and protecting b cell function [16][17][18]. Moreover, genistein may play a role in preventing and treating neurodegenerative diseases, such as AD and Parkinson's disease, through multiple neuroprotective pathways [19][20].

The purpose of this study was to examine the neuroprotective effects of bioactive compounds (resveratrol and genistein) in the AD mouse model or diabetic mouse model and to examine the diabetic-induced brain damage.

Chapter 2: Literature review

2.1 Obesity and cognitive function

The prevalence of obesity has been increased at an alarming rate worldwide over the past 50 years, reaching a pandemic level. About 13% of the world's adult population were obese in 2016. And it was projected that the numbers of obese adults in 2030 are 573 million [21]. Obesity has shown to enhance the risk of both mortality and somatic morbidity. A relationship between obesity and cognitive dysfunction is receiving increasing attention. It was reported that a higher body mass index (BMI) was associated with cognitive decline in healthy, middle-aged men and women, as evidence by the scores of word-list learning test [22]. A further study showed poorer memory performance also exists in young and middle-aged adults with higher BMI than lower BMI controls [2]. Moreover, several studies have demonstrated that the decision-making performance is changed in obese subjects. For example, morbidly obese participants had significantly worse performance than comparison participants in the Iowa Gambling Task, and 69% of obese participants showed clinically impaired decision-making ability [3].

Besides, the association between midlife obesity and the risk of dementia in later life has been reported. Xu et al. found that obesity at midlife was associated with dementia with an odds ratio of 3.88 when compared with normal BMI individuals [4]. A cohort projections model was built to examine the impact of midlife BMI on dementia in the Australian population. Interestingly, this study predicted that the dementia cases among older Australians (aged 65-69 years) would be lower by 10% in 2050 if the prevalence of midlife obesity were decreased to 20% throughout 2015-2025 [23]. However, a recent retrospective cohort study contradicted the hypothesis that midlife obesity could increase the risk of dementia in later life [24]. The reason for these conflicting reports needs further investigation.

2.2 Type 2 Diabetes and cognitive decline

Obesity is a well-established risk factor for T2DM. T2DM is characterized by hyperglycemia that results from relative insulin deficiency due to β cell dysfunction and insulin resistance in target organs. Approximately 6.28% of the world's population were affected by T2DM in 2017, and the burden of T2DM is rising at an extremely rapid rate [25]. People living longer with T2DM may develop new complications such as diabetic retinopathy, nephropathy, CVD. Cognitive dysfunction is one of these emerging complications. Evidence from longitudinal and cross-sectional data strongly supports that T2DM is associated with cognitive decline and dementia. The risk of dementia is approximately 1.5-2.5 higher in T2DM patients when compared with the general population [26][27][28]. T2DM has associated with worse performance in several cognitive domains, including attention, verbal fluency and verbal memory, processing speed and executive function [29][30][31]. The etiology of cognitive dysfunction in people with T2DM is multifactorial. Early-onset of T2DM, poor glycemic control with the presence of the cerebral microvascular disease may introduce early cognitive deficits. During acute hyperglycemia, it was shown that cognitive functions, including working memory, speed of information processing, attention, were impaired in people with T2DM. Also, the mood state has deteriorated in these people [32]. Chronic hyperglycemia is associated with microvascular changes in the retina, kidney and nerves. Ding et al. found that diabetic retinopathy was related to estimated lifetime cognitive impairment in the elderly with T2DM [33]. Moreover, T2DM is independently associated with an increased risk of CVD. The presence of CVD at midlife significantly increases the risk of dementia at late-life. Therefore, preserving the cerebral microvasculature and decreasing the risk of developing micro- and macrovascular disease could be the target for preventing cognitive dysfunction in the diabetic population. Besides, it has demonstrated that people with T2DM have dysregulated hypothalamic-pituitary-adrenal (HPA) axis, leading to increased blood levels of cortisol [34].

Emerging evidence suggests that the HPA axis dysregulation might contribute to cognitive decline and mood disturbances in people with T2DM. For example, impaired negative feedback of the HPA axis, as indicated by higher cortisol levels, was associated with deteriorated general cognitive ability and poorer performance on working memory, processing speed, mental flexibility, and nonverbal memory [35][36]. Furthermore, inflammatory mediators and increased viscosity might also play a role in the development of cognitive decline in people with T2DM.

2.3 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia and it is a detrimental neurodegenerative disorder. AD patients gradually lose their memory and the ability to carry out daily life activities. They will also exhibit hostile behavior and language deficits. In 2019, around 5.8 million Americans of all ages were living with AD [37]. It is suggested that the number of AD patients will increase rapidly in the coming years since it is predicted that the population of Americans age 65 and older will increase from 55 million in 2019 and to 88 million by 2050 [37]. Six drugs have been approved by the US. Food and Drug Administration for the treatment of AD. However, these drugs could only temporarily improve symptoms, and none of these pharmacological treatments could prevent or stop the damage and/or destruction of neurons that cause AD symptoms [37]. Great efforts are currently underway by the researchers to find new strategies that may display safe and reliable efficacy. The two main neuropathological hallmarks of AD are extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). The main constituent of the NTFs is the microtubule-related protein Tau. Various kinases abnormally phosphorylate the Tau within the NTFs. The senile plaques are characterized by the presence of a central core accumulation of amyloid beta (Aβ) peptide [39]. The Aβ peptide is formed by the sequential cleavage of amyloid beta precursor protein (APP). APP is a transmembrane protein involved in neurite growth, axonal transport,

and neuronal development [40]. Two alternative pathways exist for APP proteolysis: one of them leads to the generation of the Aβ peptide and the other does not. The amyloidogenic pathway generates Aβ through the following process: APP is firstly recognized by β-secretase, which generates soluble β-APP fragments (sAPPβ) and C-terminal fragment β (CTFβ, C99). Next, C99 is cleaved by γ-secretase and produces APP intracellular domain (AICD) and Aβ. The non-amyloidogenic pathway is an innate way to prevent the production of Aβ. The process begins when APP is cleaved by α-secretase and produces soluble α-APP fragments (sAPPα) and C-terminal fragment α (CTFα, C83). C83 is further cleaved by γ-secretase, producing AICD and non-toxic P3 fragments [41]. Under normal physiological conditions, 90% or more of APP is cleaved by α-secretase and the remaining APP is cleaved by β-secretase. Hence, Aβ is a minor product [42].

2.4 Obesity, type 2 diabetes and Alzheimer's disease

Recent studies indicate that obesity and diabetes increase the risk of AD. A population-based Rotterdam study first reported the association between T2DM and AD: older adults with T2DM had doubled risk for AD [43]. Later, a nationwide population-based study identified that patients newly diagnosed with diabetes experienced a higher risk of AD than non-diabetic control during 11 years of follow-up [44]. Besides, several epidemiological studies show that people with a higher BMI at midlife have an increased risk of AD later in life [45]. High BMI was also associated with reduced brain volume in temporal, frontal, parietal, and occipital lobes of AD patients [46]. A meta-analyses study reviewed longitudinal epidemiological studies of BMI, diabetes, insulin and glucose levels on risk for AD. They concluded that obesity and diabetes significantly and independently enhance the risk for AD [47]. Animal studies have further supported the link between obesity, T2DM and AD. To investigate the interaction between these diseases, Daisuke et al. crossed APP23 transgenic mice (a well-established AD mice model) with two types of diabetic mice (NSY mice and ob/ob mice), then analyzed the

metabolic and brain pathology of mice [48]. The results indicated that these diabetic AD mice have exacerbated cognitive dysfunction and accelerated diabetic phenotype compared to the non-crossed mice. The relationship between Aβ and insulin resistance has been explored in AD model Tg2576 mice. Researchers found that the increased Aβ level in the brain of high-fat diet-fed Tg2576 mice led to increased food intake and abnormal feeding behavior, then resulting in obesity and insulin resistance in these mice. Insulin resistance may in turn worsen Aβ formation and AD progression [49]. The following are possible mechanisms that help to explain the proposed link between T2DM and AD: (1) Impaired insulin signaling leads to increased glycogen synthase kinase 3β (GSK3β) activation, which is one of the kinases that responsible for Tau protein phosphorylation. Enhanced GSK3β activity could upregulate the level of hyperphosphorylated Tau, increase the formation of NFTs, and improve the production of Aβ [50][51]. (2) Insulin-degrading enzyme (IDE) could degrade both insulin and Aβ. Under the condition of hyperinsulinemia, there is a competition between Aβ and insulin for IDE, which eventually decreases Aβ degradation [7]. (3) Chronic inflammation, oxidative stress and protein misfolding are some of the pathways in the brain of diabetic subjects that similar to early events in AD [6].

2.5 Resveratrol

2.5.1 Source and metabolism

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) was first isolated from the roots of the white hellebore in 1939 [52]. The major dietary sources of resveratrol include wines, grapes, soy, peanuts and peanut products. It can be found in the trans or cis configurations, or the glucosylated form [53]. Analyze shows that grapes and peanuts contain mainly trans-resveratrol glucoside while red wines are the primary source of the aglycones cis- and trans-resveratrol [53]. Resveratrol can be perceived by the intestine enterocyte and undergoes sulfation and glucuronidation. Conjugated resveratrol exits the cell via specific transporters on

the apical membrane or the basolateral membrane. A small faction of resveratrol could escape the enterocyte as the free form. Then resveratrol and conjugated metabolites move towards the large intestine, where they can be metabolized by the gut microbiota to generate dihydroresveratrol, 3,4'-dihydroxy-trans-stilbene and lunularin. From the gut, resveratrol and metabolites may be absorbed by the liver or other tissues for further metabolism or excretion. Conjugated resveratrol and metabolites can undergo enterohepatic circulation, they may leave the liver and be reabsorbed in the intestine; after hydrolysis in the intestine, resveratrol and metabolites enter the portal circulation to reach the liver again. From the liver, resveratrol and metabolites could enter the systemic circulation and be absorbed by peripheral tissues, such as adipose tissue [52].

2.5.2 Bioavailability of resveratrol

Resveratrol has a high absorption rate due to its lipophilic characteristics. However, the rapid and extensive metabolism in the intestine/liver leads to low bioavailability of resveratrol and trace amounts of free resveratrol in the systemic circulation. Walle et al. examined the absorption, metabolism and bioavailability of 25mg resveratrol after oral or i.v. doses in six human volunteers, the results showed that about 75% administrated resveratrol was absorbed, resulting in peak plasma levels of resveratrol plus metabolites of 400-500 ng/ml (about 2 μM). However, less than 5ng/ml of nonmetabolized resveratrol was detected in plasma [54]. In addition, a single 500 mg oral dose of resveratrol leads to 70-80 ng/ml plasma levels of free resveratrol in 15 healthy volunteers [55]. Despite the low bioavailability of resveratrol, animal studies indicate the efficacy of resveratrol, which may be explained by the pharmacological activities of its metabolites. Besides, the enterohepatic recirculation of its metabolites, followed by the deconjugation and reabsorption, contributing to the efficiency of resveratrol administration [56]. Finally, *in vivo* effects may be explained by converting conjugated resveratrol again to free resveratrol in target tissues such as the liver [57].

2.5.3 Pharmacological effect

The notion that resveratrol may have beneficial effects on human health arose from the epidemiological data and "French paradox." The "French paradox" is an observation that the French population has a low risk of cardiovascular diseases despite a high intake of saturated fat. And these protective effects may attribute to relatively high wine consumption [58]. In later studies, resveratrol was designated as a substance that partly contributing to such beneficial effects [59][60]. More biological properties of resveratrol have been extensively studied, including antioxidant, anti-inflammatory, antiallergenic, antitumor and antiaging properties. In human mesenchymal stem cell lines, resveratrol promotes spontaneous osteogenesis but prevents adipogenesis, suggesting its role in bone regeneration [61]. In cultured prostate cancer cells, resveratrol negatively modulates cell growth by affecting mitogenesis and inducing apoptosis, suggesting its antitumor effects [62]. Dysregulated vascular smooth muscle cell (VSMC) proliferation and exacerbated platelet aggregation is important risk factors for atherosclerosis. Several studies demonstrated that resveratrol suppresses platelet aggregation and diminishes VSMC proliferation [63][64], which contributes to the vasculoprotective effects of resveratrol. Moreover, resveratrol exhibits antioxidant properties, suggesting the therapeutic potential of resveratrol in the treatment of diabetes, cancer and neurodegenerative disease.

2.5.4 Resveratrol, obesity and T2DM

Obesity is characterized by excessive accumulation of triacylglycerols (TAG) in adipose tissue. Resveratrol exhibits anti-obesity action by targeting the adipose tissue. Adipocytes are differentiated from preadipocyte, and CCAAT-enhancer-binding protein (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory element-binding protein 1c (SREBP-1c) play an essential role in this differentiation process [65]. Several studies suggest that resveratrol can inhibit adipogenesis by reducing the expression of C/EBPα, PPARγ and SREBP-1c [66][67]. The apoptotic effect of resveratrol in adipocytes has

been demonstrated in cell studies. The range of 20-400 μM resveratrol increases pre-adipocytes or adipocytes apoptosis [68][69]. Besides, fatty acid uptake from circulating TAG-rich lipoproteins and *de novo* lipogenesis are two crucial metabolic pathways contributing to the fat accumulation in adipose tissue. Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are two rate-limiting enzymes in *de novo* lipogenesis. The enzyme lipoprotein lipase (LPL) hydrolyzes lipoprotein TAG into free fatty acids and monoacylglycerol molecules. It has been reported that the expression and activity of FAS and/or the activation of ACC was inhibited by resveratrol [70][71]. With regard to LPL, it was observed that resveratrol reduced both gene expression and activity of LPL [72]. Moreover, resveratrol increases fatty acid consumption in the liver and skeletal muscle by improving mitochondriogenesis, reducing available adipose tissue fatty acid stored as TAG [73]. As a consequence, resveratrol induces the reduction in body fat by regulating these metabolic pathways.

Cell and animal studies have provided strong evidence for resveratrol as a component of management for T2DM. The blood glucose-lowering effect of resveratrol has been implicated in diabetic animals. For example, in streptozotocin (STZ)–induced diabetic rats, resveratrol administration (given orally or injected intraperitoneally) was able to dramatically reduce blood glucose [13][74][75]. Both insulin-dependent and insulin-independent effects seem to contribute to the glucose-lowering effect of resveratrol. Resveratrol treatment was shown to increase the expression and translocation of glucose transporter GLUT4, enhancing intracellular glucose transport [76]. Moreover, Palsamy et al. reported that 30 days of oral treatment of resveratrol restore the activities of key enzymes of the glycolytic pathway in the liver and kidney of diabetic rats. Further, resveratrol treatment increased the hepatic glycogen stores, suggesting the potential of resveratrol to decrease blood glucose levels in diabetic rats [77]. Besides, the influence of resveratrol on insulin secretion and blood insulin level is another crucial aspect of resveratrol's action. In high-fat diet or high cholesterol-fructose diet-fed mice,

resveratrol supplementation reversed hyperinsulinemia [78]. Resveratrol was reported to reduce glucose-induced insulin secretion in pancreatic islets, suggesting an insulin-suppressive effect [12]. Numerous data indicate that chronic over-secretion of insulin leads to β cell failure, whereas temporary rest of β cell may delay the onset of T2DM [79]. Therefore, the inhibitory effect of resveratrol on insulin secretion may play a role in the prevention and treatment of T2DM.

2.5.5 Resveratrol and AD

It has been suggested that dietary interventions such as caloric restriction (CR) could extend the lifespan and possibly prevent some neurodegenerative diseases, including AD [80]. However, it is hard for humans to commit themselves to CR over a long time. Therefore, researchers have focused on finding alternative compounds called caloric restriction mimetics (CRMs) that mimic the functional and metabolic effects of CR without the necessity to reduce food intake [80]. Resveratrol has shown to be an important CRM due to its actions on Sirtuin 1 (SIRT1). SIRT1 is a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, governing several central metabolic pathways such as circadian rhythm, energy metabolism, and aging [81]. With regard to AD, the overexpression of SIRT1 has been shown to (1) decrease the production of Aβ by upregulating α-secretase, (2) protect against Aβ-induced neurotoxicity through inhibiting NF-κB pathway or by inducing autophagy, (3) enhance the clearance of Aβ by astrocytes and delay the accumulation of amyloid deposits, (4) ameliorate NTF pathology by inhibiting the phosphorylation and aggregation of Tau protein [82][83][84][85]. Resveratrol is a potent agonist of SIRT1. Through the activation of SIRT1, resveratrol could protect against oxidative stress, decrease Aβ deposition and toxicity, attenuate neuroinflammation, reduce cognitive impairment in AD cell or animal models [64][87][88]. In addition to SIRT1-dependent pathways, resveratrol could target AD pathology through other mechanisms. The excess accumulation of metals in the brain could exacerbate Aβ-induced

oxidative stress. Also, a high concentration of copper, iron and zinc are able to bind to Aβ, promoting Aβ aggregation and accelerating cerebral oxidative stress [89]. In human neuroblastoma cells, resveratrol reduced Aβ-Zinc and Aβ-Iron complexes-induced toxicity and upregulated antioxidant enzyme levels [90]. In pyramidal neurons, resveratrol was shown to attenuate Aβ-induced excitotoxicity by restoring the activity of two voltage-gated potassium channels. Moreover, the Advanced glycation end products and their receptors (RAGEs) on the blood-brain barrier (BBB) are important for the transportation of Aβ peptide to the brain. Resveratrol supplementation was shown to preserve BBB integrity via decreasing RAGEs expression and inhibiting the degradation of junction proteins [91]. Overall, resveratrol could be a promising compound for the prevention and treatment of AD due to its diverse pharmacological properties.

2.6 Genistein

2.6.1 Source and metabolism of genistein

Leguminous plant foods contain genistein. Soy-based foods, such as soy milk, soy flour, soy protein isolate, and tempeh, are the best-known sources of genistein. The raw mature seeds of soybeans contain an average of 80.99 mg/100g of genistein [92]. The second most important source of genistein is legumes including lupin, broad beans, and garbanzo beans (chickpeas). The legumes contain 0.2 to 0.6 mg/100g of genistein. The content of genistein in fruits, vegetables, nuts is quite variable, ranging from 0.03mg/100g to 0.2mg/100g [93]. Soy-based infant formula has been commercially available since 1960s, it results in significantly higher plasma concentration of isoflavones in infants than endogenous estrogen concentration in infants. Moreover, there is no significant difference in health outcomes such as height, weight, the menstrual and reproductive history between infants exposure to cow milk formula and soy-based formula [94]. In infants consuming soy-based formula (expose to 4-7mg/kg/day of total genistein), circulating levels of genistein are approximately 1-5μM. In contrast, adults

consuming a moderate to a large amount of soy-based foods (expose to ~1mg/kg/day of total genistein) resulted in circulating levels of 0.5μM of total genistein [95].

Isoflavones are primarily found in soy-based foods in two chemical forms. Genistin is a glycoside form of genistein that conjugated with sugar molecule, and aglycones are the unconjugated form of genistein. The main dietary source of genistein is the biologically active compound genistin [96]. After ingestion, intestinal β-glucosidases hydrolyze genistin, resulting in the release of the sugar molecule from the genistin and leaving the isoflavones aglycones: genistein [96]. The aglycone form genistein is either rapidly absorbed intact or metabolized by intestinal microflora. Metabolites dihydrogenistein and dihydrodaidzein can also be effectively absorbed by passive diffusion [97]. Besides, the pharmacokinetic studies showed gender-related differences in absorption: 56% of total genistein (both parent compound and metabolites) were absorbed in male rats and 100% in female rats [98]. After entering the portal vein, the remaining aglycone form genistein can be further metabolized by liver, kidney and heart. A recent study found that genistein-7-glucuronide-4'-sulfate (G-7G-4'S) and genistein-4',7-diglucuronide (G-4',7-diG) are the major metabolites in human plasma [99].

2.6.2 Bioavailability of genistein

Bioavailability indicates the amount of genistein that becomes available for tissue distribution where physiological effects are exerted. The pharmacokinetic studies indicated that aglycone form genistein had poor oral bioavailability but total genistein (both parent compound and metabolites) had decent bioavailability in rodents [100][98]. The plasma level of total genistein is usually 10 fold more than the value of aglycone form genistein, represented by much higher area under the curve (AUC) in plasma. The higher bioavailability of total genistein suggested that genistein has high absorption in gastrointestinal tract. However, the extensive metabolism may cause the low oral bioavailability for aglycone form genistein [101].The gender difference was reported in pharmacokinetics and bioavailability of genistein. A two-fold higher oral bioavailability of both aglycone form genistein and total genistein was observed in female than

in male rats [102]. Moreover, the concentrations of genistein were significantly higher in liver and reproductive organs in female than in male rats. The type of food matrix or form could also influence the pharmacokinetics and bioavailability of genistein. A solid matrix of soy foods yields a slower absorption rate and a lower peak plasma concentrations than a liquid matrix, such as soy milk [103]. Furthermore, the aglycone form genistein in fermented food such as tempeh could be absorbed more rapidly than the conjugated form genistein.

2.6.3 Chemical structure of genistein

Understanding the chemical structure of genistein is important for understanding its biological activity. Genistein was originally isolated from the "dyer's broom" plant, *Genista tinctorial*. It was chemically synthesized by Baker and Robinson in 1928 [104]. Genistein has a diphenol structure. The chemical structure is a 4,5,7-trihydroxyisoflavone. Genistein has a similar structure and functionality to 17β-estradiol due to the presence of hydroxyl groups on carbons 4 and 7. The hydroxyl groups establish an important contact with the estrogen receptor (ER). For example, the carbon 4 of the phenol binds to Glu305 and Arg307 on the ER, the carbon 7 of the phenol binds to His 475 on the ER [104]. Genistein could bind to both isoforms of ER (ERα and ERβ) [105], and it has been reported to possess more than a 7-fold higher binding affinity for ERβ than ERα. Therefore, genistein may avoid unwanted ERα agonist side effects, such as cancer promotion [106].

2.6.4 Pharmacological effect

Genistein has a wide range of potential beneficial health effects. Genistein could ameliorate post-menopausal ailments, prevent cardiovascular disease, breast and prostate cancers, and protect neuron functions [107]. Inhibition of tumor cell growth is one of the most recognized protective roles of genistein. Disrupted expression and/or function of cell cycle regulators, such as cyclin-dependent kinase, is essential for cancer development and progression. Genistein could inhibit tumor cell advancement through regulating these cell-cycle arrest-associated

molecules [108]. In addition, genistein may indirectly influence the growth rate of tumor cells by interfering with insulin-like growth factor 1 (IGF-1) that stimulates proliferation [109]. Moreover, genistein is capable of inhibiting transforming growth factor-β (TGF-β) signaling, decreasing tyrosine kinase activity [110][111], inducing apoptosis of tumor cells by altering regulatory genes such Akt and nuclear factor kappa B (NFκB) [112]. It could also induce the expression of antioxidant enzymes [113] and reverse promoter hypermethylation of some tumor suppressor genes [109]. All these properties contribute to the role of genistein on the prevention of cancers.

In addition, genistein displays a wide range of benefits on metabolic diseases such as obesity, type 2 diabetes mellitus, and atherogenesis. For example, genistein increased insulin secretion by reducing intracellular calcium concentration in mice pancreatic islets [114]. Furthermore, genistein has the ability to promote insulin receptor sensitivity. Cortisol is a steroidal hormone that increases the blood sugar levels through improving carbohydrate, fat, and protein metabolism as well as gluconeogenesis. In adrenocortical cell lines, genistein treatment suppressed the cAMP-stimulated cortisol synthesis [115]. Moreover, genistein is involved in modulation of cellular and humoral immunity at multiple levels, such as the inhibition of the activation of NFκB, the reduction of inflammatory cytokines level [114], which indicate that genistein has anti-inflammatory functions.

Serval studies have shown that soy isoflavones help to catalyze possible memory recovery, learning ability improvement, and neuroprotection effects [116][117], because genistein is able to cross the blood-brain barrier [118]. Due to its estrogenic property, genistein has been observed to upregulate the level of nerve growth factor and choline acetyltransferase in the hippocampus of rats [119]. The antioxidant properties of genistein protect the brain from oxidative stress and suppress Aβ-induced toxicity. In addition, genistein has been shown to improve synapse development, the interaction between transcription factor with neurotrophic

genes, and cognitive function [120][121]. Overall, these observations suggest that genistein could serve as a key chemical component in preventing or treating some of the neurodegenerative diseases.

2.6.5 Genistein and obesity and T2M

Genistein has been suggested as a promising therapeutic agent for obesity and T2DM. Experiment data indicate that the beneficial effects of genistein on obesity and T2DM may attribute to its action on leptin secretion, adipocyte differentiation, obesity-related inflammation and oxidative stress, and its protective effects of β cells [122][123][124][125]. Leptin is an important adipocyte-secreted hormone with a key role in regulating energy expenditure and energy intake, by controlling the appetite and metabolism [126]. Normally, an increase in leptin concentration leads to a decreased appetite and a reduced body weight. However, common obese individuals exhibit leptin resistance: despite the elevated leptin concentration, individuals do not respond to this upregulation by decreasing food intake and losing weight [127]. Experiment data suggests that genistein could inhibit leptin synthesis and secretion, and ameliorate the hyperleptinemia state [128][16]. In addition, similar to resveratrol, genistein is able to suppress adipocyte differentiation and adipogenesis through down-regulation of C/EBPα, PPARγ and SREBP-1c [122][123]. Moreover, it was showed that genistein reduced ATP levels in adipocytes by attenuating the activity of mitochondria [129]. Since most cellular processes require energy, insufficient ATP may result in diminished fatty acid synthesis, decrease leptin secretion and decreased lipogenesis. Genistein was indeed demonstrated as exhibiting antilipogenic effect, partially via the depletion of ATP [17].

An adequate β cell number and insulin secretion are important for preventing the onset and development of T2DM. Several cell studies and animal studies have shown that genistein could protect β cell and improve insulin secretion. In mouse pancreatic islets, genistein acutely enhanced glucose-stimulated insulin secretion through activating the cAMP/protein kinase A

(PKA) signaling cascade [18]. In human pancreatic β cells, genistein significantly reversed high glucose-induced cell proliferation inhibition and apoptosis through the ER-dependent pathway [130]. Furthermore, genistein was shown to stimulate β cell proliferation by enhancing the expression of cyclin D1, a key cell-cycle regulator important for β-cell growth. Consistent with the results of the cell study, animal study showed that genistein is able to preserve β cell mass, downregulating hyperglycemia, reducing glucose tolerance and insulin levels in diabetic mice [124] Taken together these results suggest the protective effect of genistein on β cell.

Obesity and T2DM are characterized by chronic inflammation with permanently enhanced oxidative stress. It has been suggested that the elevated free fatty acid levels could improve the production of reactive oxygen species in obesity and diabetes subjects. Also, visceral fat accumulation may contribute to the pro-oxidative stress and the pro-inflammatory associated with obesity [131]. Thus, compounds possess antioxidant and anti-inflammatory properties could be the promising candidate for the treatment of obesity and diabetes. Numerous studies have pinpointed genistein has beneficial on obesity and diabetes by ameliorating oxidative stress and inflammation. It has been suggested that genistein acts as an antioxidant by improving the expression of antioxidant enzymes such as catalase and manganese superoxide dismutase (MnSOD) and chelating metals [125][132]. In STZ-induced diabetic mice, genistein administration reverted reactive oxygen species overproduction, restored reduced glutathione/oxidized glutathione (GSH/GSSG) ratio, and improved the antioxidant enzyme activities [125]. In addition to oxidative stress, genistein has been indicated to ameliorate the inflammatory state in obesity or diabetes subjects. For instance, it mitigated the expression of proinflammatory cytokines and decreased the release of TNF-α from activated microglia in the retina of STZ-injected rats, thus offering an anti-inflammatory effect [133]. Collectively, these preclinical evidence suggest that genistein may represent a novel intervention therapy to

modulate the pathological pathways among obesity and diabetics.

2.6.6 Genistein and AD

2.6.6.1 Genistein and Aβ metabolism

Aβ peptide plays an essential role in the degeneration of neurons and synaptic gaps in the brain regions implicated in learning and memory in AD. The Aβ peptide is formed by the sequential cleavage of amyloid β precursor protein (APP). APP is a transmembrane protein that is involved in neurite growth, axonal transport, and neuronal development [40]. There are two alternate pathways exist for APP proteolysis: one of them leads to generation of the Aβ peptide and the other does not. The amyloidogenic pathway generates Aβ through the following process: APP is firstly recognized by β-secretase which generates soluble β-APP fragments (sAPPβ) and C-terminal fragment β (CTFβ, C99). Next, C99 is cleaved by γ-secretase and produces APP intracellular domain (AICD) and Aβ. The non-amyloidogenic pathway is an innate way to prevent the production of Aβ. The process begins when APP is cleaved by α-secretase and produces soluble α-APP fragments (sAPPα) and C-terminal fragment α (CTFα, C83). C83 is further cleaved by γ-secretase, producing AICD and non-toxic P3 fragments [41]. Under normal physiological conditions, 90% or more of APP is cleaved by α-secretase and the remaining APP is cleaved by β-secretase, hence, Aβ is a minor product [42]. Efficient degradation or clearance of Aβ is critical to maintain low levels Aβ and to avoid Aβ-induced toxicity. In normal subjects, Aβ could be efficiently cleared or degraded by enzymes such as insulin-degrading enzyme (IDE), neprilysin (NEP), angiotensin converting enzyme (ACE), endothelin-converting enzyme (ECE), and matrix metalloproteinases [134]. However, pathological conditions or aged subjects exhibit a decreased metabolic ability to degrade Aβ and an increased risk for Aβ accumulation. Aβ40 (containing 40 amino acid residues) and Aβ42 (containing 42 amino acid residues) are primary components of the accumulated Aβ [135]. Increased levels of Aβ42 or elevated ratio of Aβ42 to Aβ40 promotes Aβ oligomerization,

amyloid fibril formation, and senile plaque formation. Senile plaques are a neuropathological hallmark of AD. Excessive accumulation of Aβ causes neurotoxicity, neuronal cell death, and neurodegeneration. Therefore, regulation of Aβ metabolism could be a useful treatment target for AD.

Protein kinase C (PKC) is a phospholipid dependent serine/threonine kinase. Isoenzymes of PKC, such as PKCα and PKCε, have been shown to regulate Aβ production and clearance. For example, it has been demonstrated that the overexpression or activation of PKCε improves the activity of ECE type 1 [136] and thereby promoting the degradation of Aβ. PKCα decreases Aβ production by upregulating α-secretase activity [137]. Therefore, molecules or drugs which activate PKC can reduce Aβ and hold an important role in the treatment of AD. In rat hippocampal neurons, these positive effects were observed after genistein treatment. 2 hours pre-treatment with genistein (0.375 μg/mL) significantly increased the α-secretase activity via activation of the PKC signaling pathway. The activation further inhibited the formation of Aβ and Aβ-induced neurotoxicity [138].

γ-secretase complex has been suggested to be a therapeutic target for AD treatment [139]. Presenilin is catalytic member of the γ-secretase complex. Ubiquitin-1 has been demonstrated as an important factor in regulating presenilin biogenesis and promoting presenilin accumulation [140][141]. However, Naoko et al. reported that genistein (50μM) likely downregulated presenilin by inhibiting the ubiquitin-1 expression in lymphoid cells [142]. Therefore, genistein may serve as an inhibitor of γ-secretase and thereby downregulating Aβ generation. The β-site of the APP cleaving enzyme 1 (BACE1) is a major β-secretase. Yown et al. revealed that genistein (50, 100 μM) exerted a notable BACE1 inhibition effect through interacting with amino acid residues in BACE1 [143]. In female AD mice with genetic deficiency of aromatase (these mice brain contained non-detectable levels of estrogen), genistein treatment (26mg/day) significantly reduced brain amyloid plaque formation by

downregulating BACE1 protein expression [144]. In contrast, in AD mice with an ovariectomy (mice brain still contained certain levels of estrogen), genistein did not inhibit amyloid plaque formation. These results suggest that the effect of genistein on AD pathologies may depend on endogenous brain estrogen levels in aged females.

Additionally, genistein treatment is involved in Aβ clearance. It was reported that the treatment of genistein improved NEP2 protease activity, a protease that has found to induce Aβ degradation [144]. The study conducted by Bonet et al. revealed that the treatment of an AD mouse model with genistein (0.022 mg/kg/day) resulted in decreased number of Aβ and amyloid plaques [145]. The primary reason was demonstrated by further cell culture study that genistein could bind to peroxisome proliferator-activated receptor gamma (PPAR-γ) moiety of the retinoid X receptor (RXR/ PPAR-γ) dimeric receptor. Moreover, binding of genistein promoted the PPAR-γ mediated release of Apolipoprotein E (ApoE) which contributed to Aβ clearance. In addition, a recent study found that autophagy-dependent mechanisms are involved in genistein-mediated Aβ clearance [146]. Autophagy is a natural regulated clearance system for cellular waste, including unnecessary or dysfunctional components. Autophagy plays an essential role in Aβ metabolism. In addition to Aβ degradation, it influences extracellular delivery of Aβ [147]. In the streptozotocin (STZ)-induced sporadic AD rat model, genistein (150mg/kg body weight/day) resulted in degradation of Aβ in the rat brain. The cell culture study revealed that these effects are stimulated by genistein-regulated autophagy [46]. Overall, genistein could reduce Aβ through upregulation of non-amyloidogenic process, downregulation of amyloidogenic process, and improvement of Aβ clearance (see Figure 1).

2.6.6.2 Genistein and Aβ-induced oxidative stress

Due to the high utilization of oxygen, the brain is vulnerable to oxidative damage stress. Oxidative damage has been shown to play an essential role in the pathogenesis of AD. The brains contains several curial metal ions such as Cu, Fe and Mn. The ions play the role in

metalloproteins of structural component or catalytic center, and they are needed to regulate the neural activity in the synapses. Moreover, these metal ions have been implicated in the production or deterioration of oxidative damage [148]. The metal ion homeostasis is disrupted in AD [149] and high content of Cu and Zn can increase the binding with Aβ. The increased binding caused the formation of metal-induced aggregates that further transform into amyloid fibrils [150]. High concentration of metal ions were found trapped into amyloid plaques in AD brains [151]. The Aβ-metal ions complex can catalyze the production of reactive oxygen species (ROS), leading to oxidative damage on both Aβ itself and surrounding molecules such as proteins, lipids, nucleic acids [148]. In response to redox imbalance, the brain is endowed with an antioxidant defense system, which consists of antioxidant enzymes (e.g. redox glutathione (GSH)) and non-enzymatic molecules. An imbalance between oxidant agents and antioxidant capacity plays an important role in the development of AD.

Genistein exhibits antioxidant properties, therefore, it may be useful in the treatment or prevention of AD. It was reported that Aβ-treated PC12 cells had significantly higher ROS levels, lower GSH levels and GSH/oxidative glutathione (GSSG) ratio compared to control group. Moreover, these cells exhibited decreased nuclear factor erythroid 2 – related factor 2 (Nrf2), heme – oxygenase – 1 (HO-1) as well as decreased cell viability. All these parameters were restored by genistein pre-treatment (25μm, 50μm, 100μm) [152]. Nrf2 is a transcription factor, in response to ROS, it binds to the promoter region of antioxidant genes such as HO-1 and glutathione regulatory enzymes to regulate gene expression [153]. Overall, genistein protected PC12 cells from Aβ-induced oxidative damage through activating Nrf2/HO-1 signaling pathway. The cerebrovascular endothelial cells exhibited the same oxidative damage including increased ROS, impaired redox balance and decreased Nrf2 after Aβ-treatment. However, genistein pre-treatment (100μm) ameliorated these effects [154]. Furthermore, phosphatidylinositol 3-kinase (PI3K) is reported to control Nrf2 activation. To examine how

genistein up-regulates the Nrf2, another group of cells was treated with PI3K inhibitor and results demonstrated that the effects of genistein on Nrf2 were attenuated. Therefore, genistein might activate Nrf2 signaling pathway via up-regulation of PI3K protein expression to protect cerebrovascular cells from Aβ-induced oxidative damage [154]. In addition, in Aβ-injected AD rats, a one-time genistein treatment (oral gavage, 10mg/kg body weight) attenuated the increased level of malondialdehyde (lipid peroxidation marker) caused by Aβ injection, but did not improve the level of antioxidant enzyme, superoxide dismutase (SOD) activity [155]. A previous review article suggested that the antioxidant effect of genistein is not based on chemical structure and suggested a possible signaling pathway of the antioxidant effect of genistein [106]. The binding of genistein with the estrogen receptor activates a signaling pathway that leading to phosphorylation of mitogen-activated protein kinase kinase (MEK) which causes the activation of mitogen-activated protein kinase (MAPK) and subsequent phosphorylation of IKβ kinase. IKβ kinase further causes translocation of NFκB to the nucleus to upregulate the expression of antioxidant enzymes manganese superoxide dismutase (MnSOD) and glutathione peroxidase. These enzymes are then directed to mitochondrial to protect against oxidative stress.

2.6.6.3 Genistein and Aβ-induced mitochondria dysfunction

Mitochondria are the major intracellular source of ROS because of the unavoidable electron leakage from the election transport chain [156]. Mitochondria dysfunction has been reported in AD, including reduced enzyme metabolism, alternated key enzymes in oxidative phosphorylation, calcium dyshomeostasis, and damaged mitochondria DNA (mtDNA) [156]. As aforementioned, Aβ could induce oxidative damage in neuronal cells or the brain of rats. Ma et al. found that Aβ could also increase the level of ROS in mitochondria of C6 cells and the mitochondria antioxidant enzymes MnSOD and GSH were decreased after Aβ treatment [157]. The excessive production of ROS in mitochondria may attack mtDNA and cause harmful

mutations [158]. mtRNA mutations could decrease ATP production and increase ROS production. In addition, mutation of mtDNA is related to impaired respiratory chain activity and disrupted mitochondrial energy metabolism, leading to a range of neurodegenerative disease including AD [159]. In C6 cells, Ma et al. found that Aβ can cause mtDNA oxidative damage as evidence by increased level of 8-hydroxydeoxyguanosine (8-OHdG), a marker for oxidative DNA damages. However, genistein pre-treatment (50μM) not only restored mitochondria ROS and GSH levels, but inhibited mtDNA oxidative damage caused by Aβ in C6 cells. In addition, Ding et al. [160] and Ma et al. [152] found that Aβ inhibited mitochondria membrane potential in primary cerebral neuron and PC12 cells, respectively, while genistein pre-treatment reversed mitochondria membrane potential in both experiments. In conclusion, these data suggest that mitochondria may be a promising new target of genistein for the treatment of AD.

2.6.6.4 Genistein and Aβ-induced apoptosis

Apoptosis is a form of programmed cell death and involves the action of catabolic enzymes such as proteases and nucleases [161]. Changes in nuclear morphology and chromatin biochemistry are the main characteristic features of apoptosis. It has been suggested that mitochondria play an essential role in apoptosis [162]. Increased mitochondria membrane permeabilization may promote the release of mitochondria apoptosis-inducing factor and cytochrome C. In the cytosol, cytochrome C mediates the activation of caspase-9 and caspase-3, leading to apoptotic cell death [163]. Bcl-2 family proteins display either anti-apoptotic or pro-apoptotic effects. For example, Bax, Bak and Bim are pro-apoptotic proteins and Bcl-2, Bcl-w are anti-apoptotic proteins. Through controlling cytochrome C release, Bcl-2 family proteins regulate apoptosis [162]. Several studies have shown that apoptotic cascades are upregulated in AD and can be activated further under the exposure of Aβ. The cascade upregulations were demonstrated through decreased mitochondria membrane potential,

increased ROS production and caspase activation, and dysregulation of calcium homeostasis. For example, after treated with Aβ, hippocampal neuronal cells exhibited increased apoptosis, characterized by reduced cell viability, and neuronal DNA condensation and fragmentation [164]. However, genistein treatment significantly blocked several Aβ-induced apoptotic signals, including ROS production, caspase-3 activation, DNA fragmentation and increased intracellular calcium level. Moreover, researchers also found that genistein protected neurons from Aβ-induced damage via an ER-mediated pathway at the nanomolar (100nM) level and it protected neurons from Aβ-induced damage via its antioxidant properties at the micromolar (40μM) level [164]. A study performed by You et al. investigated new molecular mechanisms underlying effect of genistein on Aβ-induced apoptosis. Incubation with Aβ significantly increased PC12 cell apoptosis and genistein pre-treatment decreased the apoptosis rate as compared with the control group. Further studies have shown that genistein attenuated cell apoptosis through inhibition of Aβ-induced Jun-N-terminal kinase (JNK) and reduction of JNK-dependent decreases in Bcl-w and increases in Bim. Bcl-2 family proteins further reduced cytochrome C release from the mitochondria, inhibited caspase-3 activity, and attenuated apoptotic cell death [165]. In addition, genistein treatment (10, 30, 90mg/kg) decreased the upregulated apoptosis, expression levels of caspase-3, Bax and cytochrome C, and reduced neuronal loss in the hippocampus of AD rats [166].

2.6.6.5 Genistein and Tau metabolism in AD

Tau proteins play an important role in the central nervous systems by promoting microtubule assembly, which stabilizes and affects the dynamics of microtubules in neurons [167]. Tau has more than 30 phosphorylation sites. When it is abnormally hyperphosphorylated, Tau destabilizes microtubules and aggregates into intracellular neurofibrillary tangle (NTF), a key hallmarks of AD. NTFs could block the neuron's transport system, interrupting the synaptic communication between neurons. Tau phosphorylation is primarily determined by the balance

between Tau protein kinases and phosphatases. Tau protein kinases include glycogen synthase kinase-3 (GSK-3), cyclin-dependent protein kinase-5 (CDK-5), MAPK, and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) [168]. It has been reported that in SH-SY5Y cells, Aβ inhibited Akt activation. Reduced Akt activation decreases the inhibitory phosphorylation of GSK-3β and activates it which in turn promotes Tau hyperphosphorylation and cell death. However, genistein treatment (1nM, 10nM) was able to reverse Tau hyperphosphorylation by preventing Akt inactivation [169]. In addition, it was reported that in Aβ-induced PC12 cells, administration of genistein (25, 50, 100 μmol/L) reduced level of phosphorylated Tau through downregulation of CaMKIV signalling pathway [170]. These results were further verified in the AD rat model [171]. Deregulation of protein phosphatase 2A (PP2A) has been linked to has been linked to Tau hyperphosphorylation, synaptic deficits, and amyloid- genesis [172]. It was reported that the upregulation of cancerous inhibitor of PP2A (CIP2A) may contribute to phosphorylation of Tau and overproduction of Aβ by inhibiting PP2A in AD brain [72]. Interestingly, genistein (30mM) ameliorated Tau hyperphosphorylation and inhibited Aβ production through reducing the inhibitory effect of CIP2A on PP2A in HEK293-T cells [173].

2.6.6.6 Genistein improves cognitive decline in AD

Importantly, several *in vivo* studies have been shown genistein could ameliorate learning and memory deficit in the AD animal model. It was reported that Aβ-injected adult Wistar rats exhibited impaired short-term spatial recognition memory in the Y-maze task, decreased recall and retention ability in the passive avoidance test, and performed more errors in the armed radial maze test. However, all these parameters were significantly improved by genistein pre-treatment (10mg/kg). Furthermore, these positive actions were further prevented by use of an ER antagonist, indicating that genistein ameliorates Aβ-induced learning and memory deficits through estrogenic pathways [155]. In addition, a recent study found that impaired behavior of rats with induced AD was corrected by genistein (150mg/kg/day) as determined in behavioral

testing, including Morris water maze test, open field test, elevated plus-maze test, and locomotor measurements in an actometer [146]. No clinical trial has been undertaken to test the influence of genistein specifically, although clinical trials have examined the effect of soy isoflavone supplementation that contains genistein. In one study, 65 elderly men and women with AD were treated with 100mg/day soy isoflavones. However, 6 months of treatment did not benefit cognition among those subjects [174]. The study utilized a glycoside form of isoflavones rather than an aglycones form, which may have influenced the bioavailability. Small sample size as well as regional and racial homogeneity of the sample were weakness of this study. Overall, more clinical studies are warranted.

2.6.6.7 Genistein and inflammation in AD

Neuroinflammation has been implicated in neurodegenerative disease including AD. The glial cells such as microglia and astrocytes were suggested to contribute to the inflammatory process in AD [175]. Microglia is the resident phagocytes of the central nervous system. Under pathological triggers such as neuronal death, microglia could migrate to the site of injury and initiate an innate immune response [175]. In AD, the binding of Aβ oligomers and fibrils with microglia via receptors including toll-like receptors (TLR2, TLR4, TLR6), could activate microglia and induce production of proinflammatory cytokines from microglia. Next to activated microglia, increased reactive astrogliosis enhances hypertrophic reactive astrocytes accumulation around senile plaque [176]. Astrocytes could release cytotoxic molecules like cytokines, nitric oxide, and interleukin, thereby exacerbating the inflammation response, and inducing neuronal degeneration.

Valles et al. demonstrated that Aβ can cause inflammation not only in neuronal cells, but in astrocytes in primary culture, as evidenced by increased inflammatory mediators, such as interleukin 1β (IL-1β), cyclooxygenase 2 (COX-2), tumor necrosis factor α (TNF-α), and inducible nitric oxide synthase (iNOS) in astrocytes in primary culture [177]. All these effects

were prevented by genistein pre-treatment (0.5mM). Therefore, genistein may play a role in delaying the onset and the progression of AD involving chronically activated astrocytes. Moreover, genistein can act as an agonist to PPARγ and induce PPARγ expression in these astrocytes. Activation of PPARγ has been shown to suppress inflammation in AD model. Therefore, some of the anti-inflammatory effects of genistein in astrocytes may be regulated by PPARγ suppressing inflammatory response caused by Aβ. In addition, it was revealed that Aβ induced inflammation in BV-2 microglia cells through TLRs signaling pathway. Pre-incubation of genistein can regulate TLRs and then inhibit this inflammatory response [178]. Zhao et al. obtained results that were consistent with other studies indicating that genistein could reduce the levels of iNOS, COX-2 and IL-6 caused by Aβ treatment in C6 glial cells [117]. Moreover, since NFκB signalling pathway has been suggested to be regulated by genistein [179], Zhao et al. then investigated whether genistein alleviated Aβ-induced inflammation through mediating the NFκB pathway. Their results indicated that genistein likely improves lysis of NFκB P65 subunit, thereby inhibiting the translocation of NFκB to the nucleus and preventing Aβ-induced inflammation [179]. A further study demonstrated that the reduction of NFκB is associated with the downregulation of expression of TLR4 [157]. Overall, it is likely that genistein could prevent the Aβ-induced neuroinflammation through TLR4/ NFκB pathway in glial cells.

Figures and figure legends

Figure 1

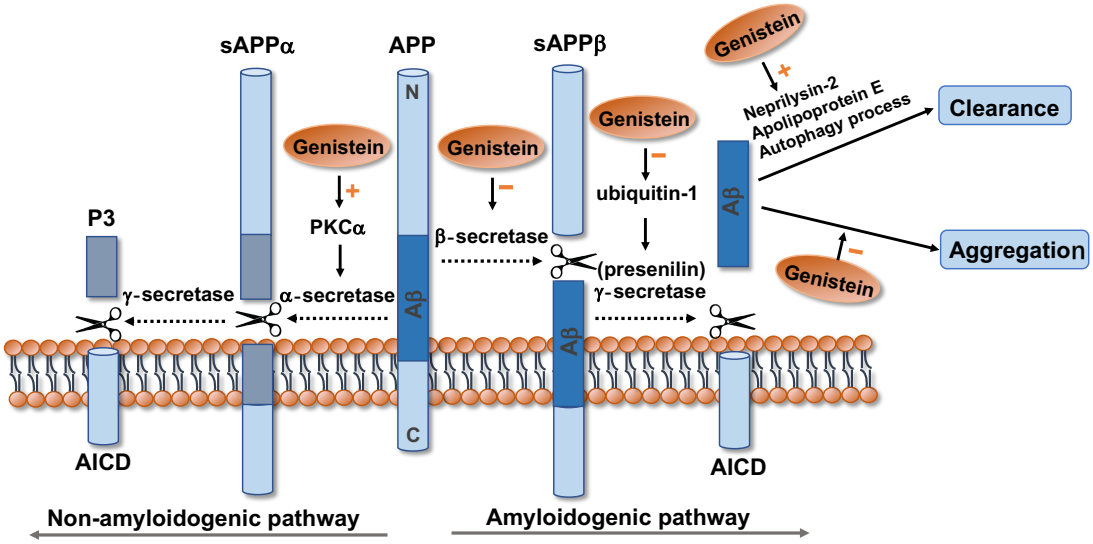


Fig.1 The effect of genistein on Aβ metabolism. Genistein could regulate the level of Aβ through upregulating non-amyloidogenic process, downregulating amyloidogenic process, and improving Aβ clearance. (Aβ: amyloid beta; AICD: APP intracellular domain; APP: amyloid β precursor protein; sAPPα: soluble α-APP fragments; sAPPβ: soluble β-APP fragments; PKCα: protein kinase Cα.)

Chapter 3: Neuroprotective effects of chronic resveratrol treatment and exercise training in the 3xTg-AD mouse model of Alzheimer's disease

3.1 Abstract

To date, there is no cure or effective treatment for Alzheimer's disease (AD), a chronic neurodegenerative condition that affects memory, language, and behavior. AD is characterized by neuroinflammation, accumulation of brain amyloid-beta (Aβ) oligomers and neurofibrillary tangles, increased neuronal apoptosis, and loss of synaptic function. Promoting regular exercise and a diet containing polyphenols are effective non-pharmacological approaches that prevent the progression of neurodegenerative diseases. In this study, we measured various conformational toxic species of Aβ and markers of inflammation, apoptosis, endolysosomal degradation, and neuroprotection after 5 months of exercise training (ET), resveratrol (Resv) treatment, or combination treatment in the 3xTg-AD mouse model of AD. Our main results indicate that Resv decreased neuroinflammation and accumulation of Aβ oligomers, increased levels of neurotrophins, synaptic markers, silent information regulator, and decreased markers of apoptosis, autophagy, endolysosomal degradation and ubiquitination in the brains of 3xTg-AD mice. ET improved some markers related to neuroprotection, but when combined with Resv treatment, the benefits achieved were as effective as Resv treatment alone. Our results show that the neuroprotective effects of Resv, ET or Resv and ET are associated with reduced toxicity of Aβ oligomers, suppression of neuronal autophagy, decreased apoptosis, and upregulation of key growth-related proteins.

3.2 Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia and patients with this neurodegenerative disease exhibit cognitive impairment, memory loss, and behavioral

manifestations [180]. According to recent statistics by the World Alzheimer's Report, nearly 47 million people worldwide are living with dementia and the number of new cases is expected to exceed 130 million by 2050 [181]. AD affects one in seven Americans aged 65 years and the prevalence of this disease will undoubtedly increase with the prolonged life expectancy.

Although the causes of this chronic disease are not fully understood, abnormal accumulation of Aβ into plaques and hyperphosphorylated tau, leading to neurofibrillary tangle (NFT) formation have been identified as the two major hallmarks of AD. The cognitive decline, memory loss, and dementia in AD are also associated with neuroinflammation, oxidative stress, mitochondrial dysfunction, apoptosis, and misfolded proteins [182][183][167]. Additionally, there is currently a substantial number of investigations aimed at understanding the role of folding of aggregated Aβ oligomers, which form prior to the development of plaques [184][185][186]. It has been well-established that aggregated Aβ, particularly in oligomer conformation, contributes to mislocalization and hyperphosphorylation of tau [187][188][189][190][191]. Recently, it has been observed that intraneuronal Aβ accumulates in early events of AD, which can be detected with M78 antibodies but not by regular Aβ antibodies, including 4G8 and 6E10 [192].

Despite the medical and economic significance of AD with a growing aging population, current drug treatment remains inadequate. Exercise is a safe and effective non-pharmacological approach known to delay the progression of neurodegenerative diseases. Exercise training (ET) performed on a regular basis improves the quality of life and reduces the incidence of cognitive defects and the progression of AD [193][194][195]. In the 3xTg mouse model of AD, ET affords neuroprotection by reducing Aβ content [196][197], inflammation and apoptosis in the hippocampus [198][199][200][201] and by improving mitochondrial function and neurogenesis [199]. Similarly, the consumption of natural products containing polyphenols has gained interest as a non-pharmacological approach for the treatment and prevention of AD.

Resveratrol (3,5,4-trihydroxy-trans-stilbene, Resv) is a polyphenolic phytoalexin and the main ingredient found in wine, grape seeds, and nuts [8]. Evidence indicates that Resv attenuates learning impairment and delays the onset of neurodegeneration in transgenic murine models of AD [202][203]. In addition, extracts of polyphenolic grape seed attenuate cognitive deficits in Tg256 mice and reduce Aβ oligomer content in the brain [204]. A significant reduction in the number of activated microglia and decreased inflammation in APP/PS1 mice following Resv treatment has been reported [205]. Resv also selectively remodels soluble oligomers and other structures of Aβ into alternative species that are non-toxic [206] and is known to induce the expression of brain-derived neurotrophic factor (BDNF), indicating a beneficial effect on neurotrophin synthesis [207]. In pregnant rats, Resv differentially activates promoters of the BDNF gene [208] and increases the expression of BDNF in the primary culture of neurons and glial cells [209], suggesting a potential role in synaptic plasticity and memory formation [210]. Taken together, the results of these studies clearly highlight the beneficial effects of ET and Resv and their potential use in the treatment of AD.

The effects of Resv treatment in combination with chronic ET, both of which can afford neuroprotection have not been well characterized in the 3xTg mouse model of AD. Recent reports have demonstrated that combined Resv and ET treatment have the advantage of improving both cardiovascular function and fracture resistance in the 3xTg mouse [211][212], suggesting that benefits of such treatment may also occur in other tissues, including the brains of these mice. Therefore, considering the benefits of both ET and the consumption of Resv in the prevention of neurodegenerative diseases, the purpose of this study was to examine the role of ET and dietary supplementation with Resv on different conformational toxic species of Aβ, Aβ-induced apoptosis, inflammation, neuroprotection, and regulation of the endolysosomal pathway in the 3xTg mouse. Since previous studies have indicated that ET alone is protective against neurodegeneration [196][197][198][199][200][201], we aimed to determine whether

the combined treatment of ET with Resv supplementation would provide added protection against the development of AD-induced pathology.

3.3 Materials and methods

3.3.1 Mouse model of AD and treatment protocols

The animal protocols described in this study were approved by the Midwestern University Institutional Animal Care and Use Committee and adhered to the guidelines in the National Institute of Health's Guide for the Care and Use of Laboratory Animals (Publ. No. 85–23, 1986). Male triple transgenic mice (3xTg-AD, Jackson Laboratories, Bar Harbor, ME, USA), aged 8 weeks, were used in this study. This mouse model of AD, which harbors three mutant genes (Aβ precursor protein, presenilin-1, tau) was selected because of its similarity to the human condition of familial AD [213][214]. Aged-matched, non-transgenic wild type (B6129SS2/J) mice were used as controls. Male mice were assigned to five groups (n = 8/group): (1) wild type control (WT), (2) AD control (3xTg-AD), (3) 3xTg-AD exercise training (3xTg-AD + ET), (4) 3xTg-AD resveratrol-treated) and (5) 3xTg-AD resveratrol-treated and exercise (3xTg-AD + Resv + ET).

Mice were trained using an electrical treadmill (Exer 3/6, Columbus Instruments, Columbus OH, USA) using an incremental training protocol. Mice were first acclimated to daily 10-min exercise sessions at 10m/min for a period of two weeks. After this period of acclimation, training consisted of the following as described earlier [211]: week 1, 20 min at 10m/min; week 2, 30 min at 12m/min. At week 3, the duration was increased to 45 min and intensity to 15 m/minute corresponding to ~80% maximal oxygen-carrying capacity based on treadmill belt speed [215].

Resv (synthetic, 99% Lallilab Inc. Durham, NC, USA) was incorporated into food pellets (Dyets Inc. Bethlehem, PA, USA, Dyet #102270, modified AIN-93G purified rodent diet with 4 g/kg resveratrol). This diet was selected based on earlier studies showing cardioprotective,

anti-inflammatory, and neuroprotective effects without any negative effects [216][217]. Mice in the WT, AD-3xTg, and 3xTg-AD + ET groups received the same purified diet without the addition of Resv. Mice were housed two per cage and maintained in a room with a 12:12 h light–dark cycle and given food and water ad libitum. Mice were treated with Resv, ET, or both for a period of 5 months. Following treatment, mice were euthanized by CO2 gas followed by cervical dislocation. Brain tissue was harvested, quickly frozen in liquid nitrogen and stored at −80 °C.

3.3.2 Western blot analysis

Analysis was performed using soluble fractions isolated from brains using a method described previously [218]. Briefly, brain tissue was homogenized in Triton lysis buffer containing the following: 50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, aprotinin and protease inhibitor cocktail. Homogenates were then centrifuged at 14,000 rpm for 1 hr at 4 °C. The supernatants were collected and the concentration of protein in lysates was measured using standard techniques (Pierce 660-nm protein assay reagent, Thermo Scientific, Rockford, IL, USA). Protein (20 μg) from each fraction was resolved by SDS–PAGE and transferred onto polyvinylidene difluoride membranes. Antibodies against BACE-1, A11, tau, MC-1, PARP, GFAP, BDNF, NGF, synaptophysin, PSD-95, caspase (3, 7, 9), Adam10, and SIRT1 were purchased from Abcam, Cambridge, MA, USA. Antibodies against pGSK3β, α-synuclein, KF-κB, LC3-1, p62, and AMPK were obtained from Cell Signaling Technology, Danvers, MA). Antibodies against IRS-1, LAMP8, and Ub1 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. GAPDH was used as an internal control (Abcam, Cambridge, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St-Louis, MO, USA). The immunoreactive bands were visualized with an enhanced chemiluminescence reagent. Aβ conformation antibodies A11 and M78 were a generous gift from Dr. Charles Glabe of UC Irvine. Western blot images were quantified using image ISO lite software

3.3.3 Statistical analysis

Quantification of Western blot data was performed by using Li Core Image J software and data were analyzed using Graph pad prism. A one-way analysis of variance followed by the Tukey–Kramer post hoc test was used to determine differences in group means. All values are reported as mean ± SEM and significance set at $p < 0.05$.

3.4 Results

Mice in the ET groups (3xTg-AD and 3xTg-AD + Resv groups) ran without reluctance and completed the exercise protocol. In addition, no signs of poor health or distress were observed in mice treated with Resv. This was confirmed by the observation that there were no differences in food intake expressed in g/kg/day (WT, 0.114 ± 0.001; 3xTg-AD, 0.126 ± 0.008; 3xTg-AD + ET, 0.120 ± 0.009; 3xTg-AD + Resv, 0.117 ± 0.007; 3xTg-AD + Resv + ET, 0.136 ± 0.007; $F = 1.8738$, $p = 0.1544$) and body weight between groups recorded at the end of the treatment protocol (range 35 ± 2 to 39 ± 2 g, NS) [211]. Based on the composition of the diet, Resv intake was estimated at 0.481 g/kg/24-h and 0.557 g/kg/24-h in 3xTg-AD mice + Resv and 3xTg-AD + Resv + ET mice, respectively.

Figure 2 illustrates the protein expression profile in brain tissue of 7-month-old control 3xTg-AD mice compared to brain tissue obtained from age-matched WT controls. Compared to control mice, brain tissue from mice in the 3xTg-AD group exhibited significantly higher levels of proteins linked to neuroinflammation, toxic species of Aβ, apoptosis, autophagy and ubiquitination. However, protein levels of neurotrophins, synaptic markers and SIRT1 were reduced in the brains of 3xTg-AD mice compared to WT mice.

3.4.1 Resv or Resv with ET reduces neuroinflammation in 3xTg-AD mice

Inflammation is an important contributor to the pathogenesis of AD. Activation of microglia, astrocytes, and increases in expression levels of inflammatory markers are observed in the brain of AD [36]. As shown in Figure 3, the brains from 3xTg-AD mice exhibited a significant

increase in inflammation expressed as NF-ƙB, GFAP, and PARP. There was no effect of ET on the levels of inflammatory markers. However, treatment with Resv or in combination with ET resulted in a significant decrease in inflammation compared to 3xTg-AD mice.

3.4.2 Resv or Resv with ET attenuates the accumulation of toxic conformational species of Aβ

The 3xTg-AD mouse develops age-related progressive neuropathology, including plaques and NFT. At 6 months of age, extracellular Aβ deposits in the frontal cortex are evident. In older mice, as expected, Aβ deposits are increased in this area of the brain [214]. Western blot analysis of conformational antibodies to detect structural aggregates of Aβ was performed in the brain of 3xTg-AD mice. Protein expression levels of Aβ detected by 4G8, BACE 1, A11, and M78 antibodies are illustrated in Figure 4a. As expected, protein expression of 4G8 and BACE 1 was increased in the brain of 3xTg-AD mice. Treatment with Resv or ET was associated with a reduction in 25 kDa molecular weight of Aβ detected by 4G8 and 65 kDa protein detected by BACE-1 antibodies compared to 3xTg-AD mice (Figure 4a). Combination treatment was also effective in reducing 4G8 and BACE 1 protein levels compared to brains from 3xTg-AD mice. M78 stains neuronal nuclei, whereas at a later stage of AD, M78 immunoreactivity localizes with a subset of amyloid plaques that are distinct and shows no immunoreactivity with Aβ or APP antibodies [192]. Protein levels of Aβ oligomers and intracellular Aβ fibrils recognized by A11 and M78 antibody, respectively, were elevated in the brain of 3xTg-AD mice compared to WT mice. ET, Resv, and combination treatment were beneficial in reducing protein levels of different conformation of Aβ recognized by the A11 and M78 antibodies (Figure 4a). Toxic oligomeric species Aβ*56 detected by A11 antibody was significantly reduced by the treatment of Resv. In addition, higher molecular weight fibrils above 200 Kd detected by M78 antibody was significantly reduced with the treatment of Resv. The brains of untreated 3xTg-AD mice or treated with Resv or in combination with ET showed significantly increased total tau content compared to WT mice. The oligomeric species of tau

protein detected by MC1 antibodies in 3xTg-AD mice treated with Resv or in combination with ET were significantly decreased (Figure 4b). Although total tau protein levels were increased in transgenic mice, we did not observe any hyperphosphorylated tau due to their relatively young age, although tau pathology is evident in older 3xTg-AD mice [213][214].

Abnormal expression of α-synuclein protein occurs spontaneously in the brain and has been associated with oxidative stress, impaired proteasome function and mitochondrial abnormalities. Oxidative stress can induce aggregation of α-synuclein protein into amyloid-like fibrils [219][220]. Western blot data demonstrated a significant increase in α-synuclein protein expression in brains of 3xTg-AD mice compared to WT mice (Figure 4b). A significant reduction in the accumulation of α-synuclein protein in 3xTg-AD mice detected by α-synuclein antibodies was observed following Resv treatment when compared to 3xTg-AD mice. ET had no effect on protein expression of α-synuclein, but in combination with Resv, protein expression of α-synuclein was significantly decreased. Taken together, our results suggest that Resv can reduce the formation of toxic species of Aβ and tau and prevent the accumulation of misfolded proteins in brains of 3xTg-AD mice. The effects of ET are not as robust as those exhibited with Resv. However, combination treatment appears to be as effective as Resv treatment alone.

3.4.3 Resv or Resv with ET increases protein expression of neurotrophins and synaptic markers in 3xTg-AD mice

In addition to the presence of plaque and NFT pathology, low levels of neurotrophins and synaptic markers contribute to the pathogenesis of AD. The effects of treatment on the protein expression of neuronal and synaptic markers are illustrated in Figure 5. Our results show that protein levels of BDNF, NGF, synaptophysin, and PSD-95 detected by respective antibodies were significantly reduced in the brain of 3xTg-AD mice compared to WT mice. ET had no effect on the protein expression of neuronal and synaptic markers. However, treatment with Resv increased protein expression of NGF and synaptophysin. Protein levels of BDNF, NGF,

synaptophysin and PSD-95 were significantly increased following combined ET and Resv treatment compared to 3xTg-AD mice. Therefore, our results indicate that either Resv treatment alone or with ET exerts beneficial effects on the expression of neurotrophins and synaptic markers.

3.4.4 Resv or Resv with ET reduces apoptosis and decreases protein expression of autophagy and accumulation of ubiquitinated proteins

Aβ and hyperphosphorylated tau lead to apoptosis and autophagy, which ultimately causes cognitive dysfunction in AD. The effects of treatment on the expression of markers of apoptosis are shown in Figure 6. The protein content of caspase-3, caspase-7, caspase-9, and adam-10 was increased in the brain from 3xTg-AD mice compared to WT mice. Treatment with Resv resulted in a significant decrease in apoptosis when compared to 3xTg-AD mice (detected by antibodies specific for activated caspases), while ET had no effect. However, combined treatment was just as effective as Resv on protein expression of these markers of apoptosis.

Protein expression levels of autophagy detected by antibodies for LC3-1 and antibodies for the endolysosomal proteins cathepsin B, cathepsin D, and LAMP2 are illustrated in Figure 7. Brain from 3xTg-AD mice expressed significantly higher protein expression levels of cathepsin B and D, LAMP2, and LC3-1 compared to WT mice. ET significantly reduced protein levels of cathepsin B, LAMP2, and LC3-1 when compared to the brains of 3xTg-AD mice. Treatment with Resv and combination treatment were both associated with a greater reduction in the expression as detected by these antibodies.

Protein turnover by autophagy and the ubiquitin–proteasome system is mediated, in part, by p62 [221][222]. The role of this multifunctional protein is supported by the observation that p62 knockout mice exhibit an accumulation of NFT associated with defects in synaptic function [223]. The effects of treatment on p62 protein expression are shown in Figure 7. Compared to WT mice, the brains of 3xTg-AD mice demonstrated a significant reduction in p62. This reduction was reversed in mice treated with Resv and with combined treatment.

The effects of treatment on protein ubiquitination were examined. As shown in Figure 8, a significant increase in the protein expression of ubiquitinated proteins using Ub1 was observed in brains of 3xTg-AD mice compared to WT mice. A similar increase in protein expression of ubiquitinated protein Ub1 was observed in brains of 3xTg-AD mice after ET compared to WT mice. However, the level of ubiquitinated proteins was normalized with Resv and with combination treatment. Taken together, our results indicate that treating 3xTg-AD mice with Resv or in combination with ET may prevent apoptosis and autophagy and reduce the accumulation of misfolded and ubiquitinated proteins.

3.4.5 Resv or Resv with ET increases the expression level of SIRT1

The effects of Resv and ET on SIRT1 protein expression are illustrated in Figure 9. Western blot analysis revealed decreased protein expression of SIRT1 in 3xTg-AD mice compared to WT mice. The expression of SIRT1 also remained low in the brain of mice after ET. However, following treatment with Resv or in combination with ET, SIRT1 protein expression was significantly increased compared to 3xTg-AD mice. No differences in the expression of AMPK between groups were observed. A summary of the effects of treatment on the expression of proteins or markers of concern is illustrated in Figure 10. Changes in protein expression are compared to brains of mice from the control 3xTg-AD group. Of the 27 proteins examined, 8 were improved with ET, 23 with Resv, and 25 proteins of interest were beneficially affected by combined ET and Resv treatment. ET did not improve the expression of neuroinflammatory, synaptic, and apoptotic markers. However, some markers of autophagy were improved and toxic species of Aβ were reduced with ET. Resv treatment or in combination with ET afforded the greatest benefits on the expression of proteins.

3.5 Discussion

It is well-established that exercise and healthful eating are protective against the development and progression of various age-related and neurodegenerative diseases. Performing aerobic

exercise on a regular basis and the consumption of a diet rich in polyphenols are not only associated with fewer side effects and better adherence but also improve the quality of life in patients with progressive neurodegenerative diseases. These non-pharmacological strategies are known to preserve the blood–brain barrier (BBB), improve CNS immunity, reduce hippocampal atrophy, and improve blood flow and cognitive function, and functional ability [81][224][225]. In this study, we examined the effects of chronic Resv treatment and ET in the form of treadmill running on common markers of AD-induced pathology in the brain. We used the 3xTg-AD mouse model because of its close representation of the human condition. To reflect the early stages in the development of AD in the absence of significant tau pathology [214], treatment was initiated in 2-month-old mice and continued for a period of 5 months. Our analysis of brain tissue from 3xTg-AD mice revealed, as expected, severe pathologic disturbances that are not only consistent with earlier studies using this model, but also reminiscent of the human condition of AD. Brains from 3xTg-AD mice displayed neuroinflammation, accumulation of toxic species of Aβ, increased apoptosis and autophagy, and decreased expression of synaptic markers. In terms of the effects of treatment, our data show that: (1) ET decreased the accumulation of Aβ oligomers and markers of autophagy, (2) Resv reduced markers related to inflammation, toxic species of Aβ, apoptosis, autophagy, ubiquitination and endolysosomal degradation, (3) Resv increased the expression of neurotrophins and SIRT1, and (4) combined ET and Resv was as effective and beneficial as Resv treatment alone except for an increase in synaptophysin and PSD95 synthesis. Our results support the relationship between regular exercise and polyphenol consumption and neuroprotection in the 3xTg-Ad mice [196].

The deficit in cognitive function and behavioral changes in patients with AD is associated with an inflammatory response involving microglia, astrocytes, and macrophages [226]. Aggregation of Aβ leads to the activation of microglia and causes the release of

proinflammatory mediators including cytokines, free radicals, which in turn increase Aβ production [205][227][228]. Previous studies suggested that the formation of Aβ annular protofibrils (APFs) in astrocytes is linked to the pathogenesis of AD and prevention of these APF formations could be a relevant target for the prevention of Aβ toxicity in AD [229]. Resv has been shown to exert anti-inflammatory properties by reducing the production of proinflammatory markers and suppressing the activation of microglia and astrocytes [230]. Our findings support and extend previous studies on the benefits afforded by Resv against the progression of AD. However, ET alone was not accompanied by a reduction in the expression of neuroinflammatory markers in brain tissue. This is in contrast to previous studies that have demonstrated protection with ET following forced treadmill exercise [199], voluntary wheel running exercise [200] and loaded resistance training [198] in the 3xTg-AD mouse. Decreases in protein expression of TNF-α, IL-6, and GFAP in the hippocampus, cortex, and hypothalamus were seen under these training conditions, resulting in an improvement in cognitive function [198][199]. In addition, treadmill running suppressed the accumulation of neuroinflammatory markers in the hippocampus of 3xTg-AD mice fed an obesogenic diet consisting of high fat [201], which is associated with increased risk of AD and known to hasten the development of AD-related neuropathology [231][232].

The reasons for these discrepancies in protection against the increase in neuroinflammatory markers with treadmill running are not known but may relate to differences in the age of mice at the start of the exercise program as well as the intensity of exercise. Where benefits were reported with treadmill exercise, protection against neuroinflammation was achieved at a lower exercise intensity [199], which likely produced a lower stress response. Higher exercise intensity elicits specific negative adaptations, including adrenal hypertrophy, increased corticosterone secretion, and suppressed antigen-specific IgM production [233]. An increased stress response is also known to contribute to the development of age-related

neurodegenerative diseases and AD [234][235]. Despite the lack of effect of ET on inflammatory markers in our study, most reports support the premise that ET, regardless of the paradigm, exerts an important role in suppressing neuroinflammation in 3xTg-AD mice.

In addition to the accumulation of Aβ into plaques and hyperphosphorylated tau leading to NFT, the formation of aggregated Aβ prior to the formation of plaque has been recognized as major hallmarks of AD [184][185][186]. Similarly, accumulation of tau oligomers in the synaptic space leads to synaptic dysfunction, amnesia, and neurodegeneration [236]. Emerging evidence also suggests the involvement of disrupted synaptic vesicle cycle function, which is a site of both Aβ production and toxicity, in the pathology of AD [237]. In addition, the ectopic release of glutamate near axonal swellings surrounding amyloid plaques can lead to increased glutamatergic activity [238]. Aggregated Aβ oligomer conformation and its contribution to mislocalization and hyperphosphorylation of tau have been well established [187][188][189]. Recently, it has been observed that interneuronal Aβ accumulates in the early development of AD and is detected by M78Ab, but not with regular Aβ antibodies such as 4G8 and 6E10 [192]. Studies suggest that Resv has a potential role in the treatment of hippocampal neurons against Aβ-induced toxicity and neurotoxicity induced by oxidative stress [239][240]. In rats with AD, this effect appears to be mediated by the ability of Resv to cross the BBB [241][242] and increase the expression of antioxidant mechanisms [243][244][245] and reduce the production of inflammatory markers in the brain. Resv may have beneficial effects by inhibiting the extension and destabilizing the polymerization of Aβ peptides and inhibiting Aβ cytotoxicity [246]. Soluble oligomers are toxic and distinct from monomers or fibril aggregates identified in AD brains [247]. During the early progression of AD pathogenesis, Aβ, APP or APP-CTFs (carboxy terminal of amyloid precursor protein) accumulate and aggregate intracellularly (usually in the perinuclear compartment) into a conformation that is recognized by M78Ab and colocalizes with Aβ and APP-CTF [192]. At intermediate stages of plaque pathology, M78

stains neuronal nuclei, whereas at a later stage M78 immunoreactivity localizes with a subset of amyloid plaques that are distinct and show no or less immunoreactivity with Aβ or APP antibodies [192]. This suggests that neuritic plaques arise from degenerative neurons with intracellular immunoreactivity. Our data show a significant reduction in M78, A11, and BACE1 expression in brains of 3xTg-AD mice following Resv treatment. This is consistent with the decreases in expression levels of tau and phosphorylated tau recently reported with Resv [248]. Further, we found that ET also alleviated the AD-induced increases in M78, A11, and BACE1 expression. Although we are the first to document the beneficial effects of ET on toxic species of Aβ detected by these antibodies, other studies nonetheless support the role of exercise in alleviating Aβ pathology. Five weeks of treadmill running prevented the increase in Aβ42/40 ratio from occurring in the cerebral cortex of 7-month-old 3xTg-AD mice [249]. Voluntary wheel running also ameliorated Aβ levels in brains of 3xTg-AD mice although it appears that this effect is dependent on the age of mice at the onset of exercise training [196]. Volitional running initiated in 4-month-old 3xTg-AD mice for a period of 4 weeks was associated with a reduction in soluble Aβ40 levels in the hippocampus whereas no reduction was observed with training initiated in 7-month-old mice after 6 months of training. Recent studies have also supported these beneficial effects of treadmill or wheel running initiated in young mice on AD-related pathology, including decreases in Aβ 1–40 and 1–42 in the hippocampus and cerebral cortex after 20 weeks of treadmill running initiated in 4-month-old 3xTg-AD mice fed a high-fat diet [201] and after 12 weeks of treadmill running in 3 month-old 3xTg-AD mice [199]. In addition to the benefits afforded by aerobic exercise training, resistance training attenuates the amyloid burden in the 3xTg-AD mouse regardless of the age of mice at the onset of exercise [197][198]. It is evident from these studies that exercise, regardless of the form, slows the accumulation of Aβ species. ET combined with Resv affords greater protection than ET alone. In addition to the role of Aβ, the pathogenesis of AD involves the hyperphosphorylation of tau.

Evidence suggests that tau also undergoes differential structural changes to the oligomeric state before the formation of tau fibrils [236][250][251]. Tau oligomers are neurotoxic and have been isolated at early stages and prior to the onset of clinical symptoms in AD patients [252][253]. Our results indicate that Resv treatment reduced tau oligomers using MC1 Ab when compared to 3xTg-AD mice, indicating neuroprotection from the accumulation of abnormal proteins. In addition, the effects of ET on Aβ accumulation and tau oligomer expression are in line with early studies indicating a lowering effect in the brains of 3xTg-AD mice.

The involvement of neurotrophic and synaptic markers in the pathogenesis of AD has been previously described [254]. Studies have shown that plasma levels of BNDF and gene and protein expression of BDNF in the hippocampus and cortex are reduced in AD brains [255][256][257][258][259]. BDNF acts as an important mediator of synaptic plasticity and memory formation [210]. NGF plays a role in aging and age-related neurodegenerative diseases because of the presence of abnormalities in trophic signaling [260][261]. In addition, lower levels of PSD95 were detected in AD because of its role as a major scaffolding protein of the dendritic spines and in trafficking of glutamate receptors, ion channels, and adhesion molecules [262][263][264][265][266]. Synaptic plasticity is a cellular substrate for learning and memory and synaptic loss may contribute to impaired synaptic plasticity in AD [267][268][269]. Levels of BDNF in serum and brain tissue are increased with ET [270] preventing loss of synaptic stability and function and cell death. In our study, we found that ET alone had no beneficial effect on the expression of BDNF, synaptophysin, and PSD95 in brains of 3xTg-AD mice. In contrast, other studies have reported the benefits of ET on these proteins with both treadmill running and resistance training [199][201]. Those studies also report increases in the levels of synaptotagmin-1 and synaptobrevin-1 following ET. However, it should be emphasized that the increase in these markers was observed in specific areas of the brain, including the hippocampus and cerebral cortex, while our results were obtained from

crude whole brain homogenates. This could explain why no significant changes were detected after 5 months of ET in our study whereas localized increases in the hippocampus were seen just after 12 weeks of training. That is, our current findings do not suggest a lack of effect of ET on the expression of the markers but rather caution the interpretation of results based on regions of the brain analyzed. On the other hand, we found that 3xTg-AD mice treated with Resv alone or combined with ET showed an improvement in the expression of several markers compared to control 3xTg-AD mice (Figure 10). Treatment with Resv improved levels of NGF and synaptophysin while Resv in combination with ET provided additional benefits by also increasing the expression of BDNF and PSD95. The observation that the reduction in protein levels of these neurotrophins was prevented suggests that these interventions offer a robust neuroprotective role.

The accumulation of Aβ may cause both neuronal and synapse loss from apoptosis and the role of resveratrol in reducing apoptotic markers has been reported [207]. Our data demonstrate that 3xTg-AD mice treated with Resv show significant reductions in the expression levels of apoptotic markers. Further, dysregulation in the neuronal endocytic pathway occurs prior to the accumulation of Aβ and tau is considered a seminal event in the pathogenesis of AD [271]. The endosomal–lysosomal system plays an important role since this system is involved in APP processing, uptake of Aβ and its accumulation. Studies have shown an association between impaired autophagy induction and the increase in autophagy suppressing molecules with AD [272][273][274]. Autophagosome/lysosome and the UPS are the two major proteolytic systems in the brain. Endolysosomes play a critical role in amyloidogenic processing of APP [275][276] and Aβ acts a substrate for autophagy [277]. Our results show that 3xTg-AD mice treated with Resv show a significant reduction in the expression levels of endolysosomal and autophagic markers. Defects in Ub-mediated protein degradation and increased levels of Ub-conjugated proteins may also explain the increased formation of NFT [278]. The role of p62,

which contains ubiquitin-binding protein and is involved in autophagy [279][280], should not be ruled out as reports have demonstrated that the levels of NFT are increased in the brains of p62 knockout mice [223]. For degradation, p62 binds to NFT, which accumulates after phosphorylation of tau. Sequestration of p62 in NFT limits its cellular function by creating a reduced pool of p62 in the cytosol [281]. Our results show that treating 3xTg-AD mice with Resv leads to a significant increase in p62 protein levels. Resv alone or in the presence of ET significantly reduced the expression levels of ubiquitinated proteins, suggesting improved degradation and disposal of proteins.

Figures and figure legends

Figure 2

Neuro-inflammation	Toxic species of Aβ	Neurotrophins & synaptic markers	Markers of apoptosis	Markers of autophagy	Ubiquitination & metabolism
NF-κB (1.9↑) GFAP (12.2↑) PARP (6.8↑)	4G8 (10.6↑) GSK3β (0.4↑) BACE (5.9↑) MC1 (2.1↑) A11 (17.9↑) α-synuclein (7.3↑) M78 (16.3↑) Total tau (3.6↑)	BDNF (1.7↓) NGF (9.5↓) Synaptophysin (0.5↓) PSD95 (1.4↓)	caspase-3 (3.6↑) caspase-7 (5.9↑) caspase-9 (23.8↑) ADAM-10 (5.1↑)	cathepsin B (3.5↑) cathepsin D (2.5↑) Lamp 2 (4.6↑) LC3-1 (6.4↑) P62 (1.3↓)	Ub1 (1.9↑) Sirt1 (4.6↓)

Fig.2 Relative changes in the expression of proteins of interest in brain tissue of 3xTg-AD mice. Changes shown as increased (↑) or decreased (↓) in protein expression in brain of 7-month-old 3xTg-AD mice compared to brain from age-matched WT mice. Numbers in parentheses represent the fold change compared to WT mice. Based on the Western blot data, proteins relating to neuroinflammation, toxic species of Aβ, apoptosis, autophagy and ubiquitination were increased whereas protein levels of neurotrophins, synaptic markers and SIRT1 were reduced in the 3xTg-AD mouse brain compared to age-matched WT mice.

Figure 3

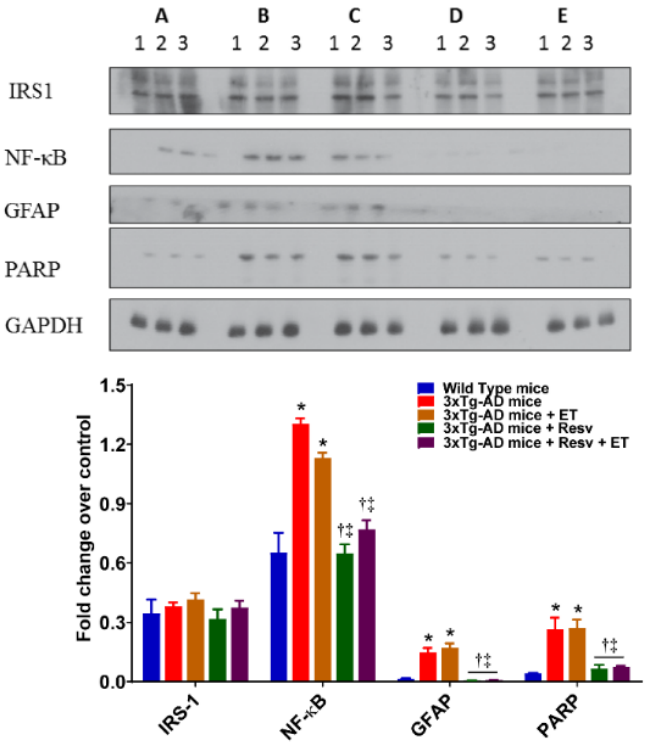
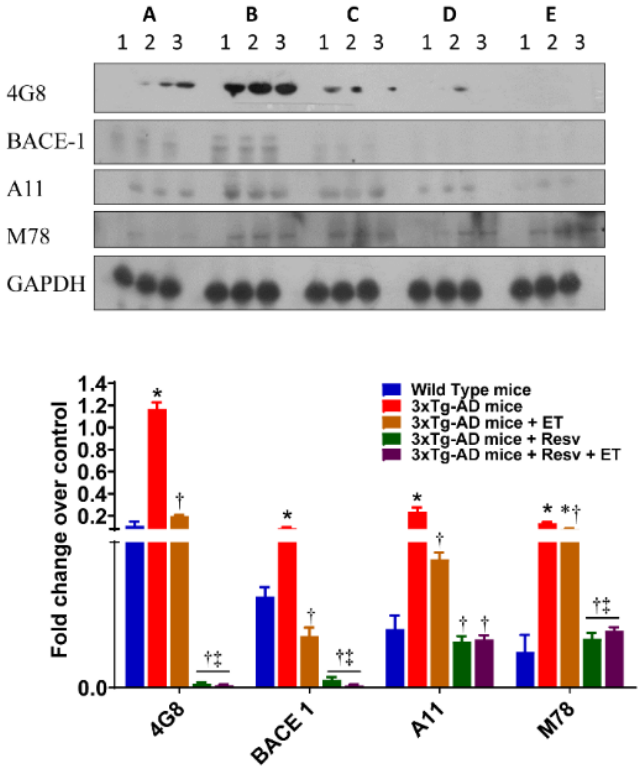


Fig.3 The effects of Resv and exercise training (ET) markers of neuroinflammation. Treating 3xTg-AD mice with Resv or Resv with ET decreases the expression of key inflammatory markers. Corresponding densitometry measurements: A, wild-type (WT) mice; B, 3xTg-AD mice; C, 3xTg-AD mice + ET; D, 3xTg-AD mice + Resv; E, 3xTg-AD mice + Resv + ET. NF-κB, nuclear factor-kappa B; GFAP, glial fibrillary acidic protein; PARP, poly (ADP-ribose) polymerase; IRS-1, insulin-receptor substrate. AD, Alzheimer's disease; ET, exercise training; Resv, resveratrol. Data are presented as mean ± SEM for 3 mice per group. *, compared to WT mice, $p < 0.001$; †, compared to 3xTg-AD mice, $p < 0.001$; ‡, compared to 3xTg-AD mice + ET, $p < 0.05$ for NF-κB.

Figure 4

(a)


A B C D E
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
4G8
BACE-1
A11
M78
GAPDH
Fold change over control
Wild Type mice
3xTg-AD mice
3xTg-AD mice + ET
3xTg-AD mice + Resv
3xTg-AD mice + Resv + ET
4G8
BACE 1
A11
M78

(b)

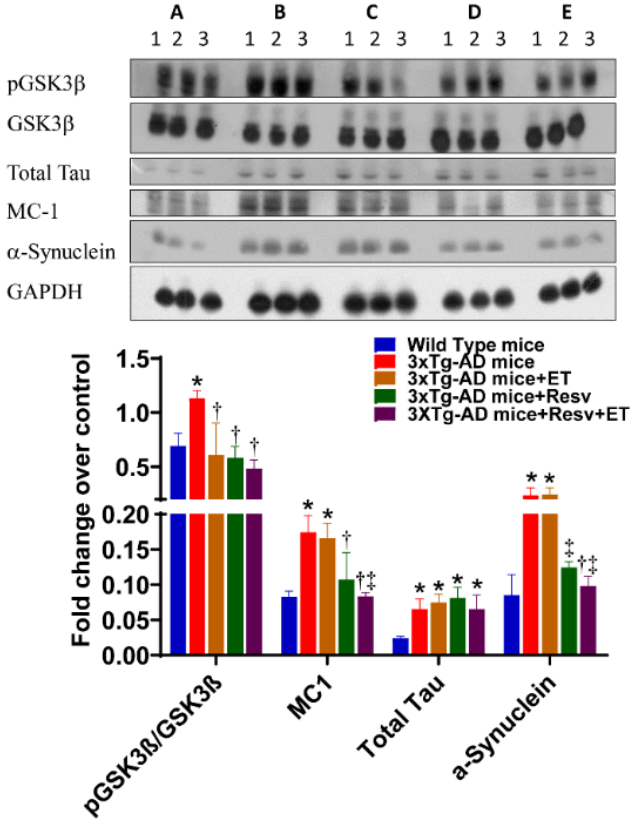

A B C D E
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
pGSK3β
GSK3β
Total Tau
MC-1
α-Synuclein
GAPDH
Fold change over control
Wild Type mice
3xTg-AD mice
3xTg-AD mice+ET
3xTg-AD mice+Resv
3XTg-AD mice+Resv+ET
pGSK3ß/GSK3ß
MC1
Total Tau
a-Synuclein

Fig.4 The effects of Resv and ET on conformational toxic species of Aβ. (a) Treating 3xTg-AD mice with Resv or Resv with ET reduces extracellular and intracellular Aβ accumulation in brain. Aβ content, oligomers of Aβ and intracellular Aβ detected by A11 and M78 antibody. (b) Protein content of pGSK3-β was reduced in 3xTg-AD mice treated with Resv, ET, and Resv with ET. Tau oligomers in brain detected by using the MC1 antibody were also reduced with treatment with Resv or Resv with ET but with no change in total tau. Misfolded protein expression levels using α-synuclein antibody were reduced in 3xTg-AD mice treated with Resv or Resv with ET. Corresponding densitometry measurements: A, WT mice; B, 3xTg-AD mice; C, 3xTg-AD mice + ET; D, 3xTg-AD mice Resv; E, 3xTg-AD mice + Resv + ET. BACE1, beta-secretase enzyme 1; pGSK3β, glycogen synthase kinase beta (*p* = phosphorylated). AD, Alzheimer's disease; ET, exercise training; Resv, resveratrol. Data are presented as mean ± SEM for 3 mice per group. *, compared to WT mice, $p < 0.01$; †, compared to 3xTg-AD mice, $p < 0.01$; ‡, compared to 3xTg-AD mice + ET, $p < 0.01$.

Figure 5

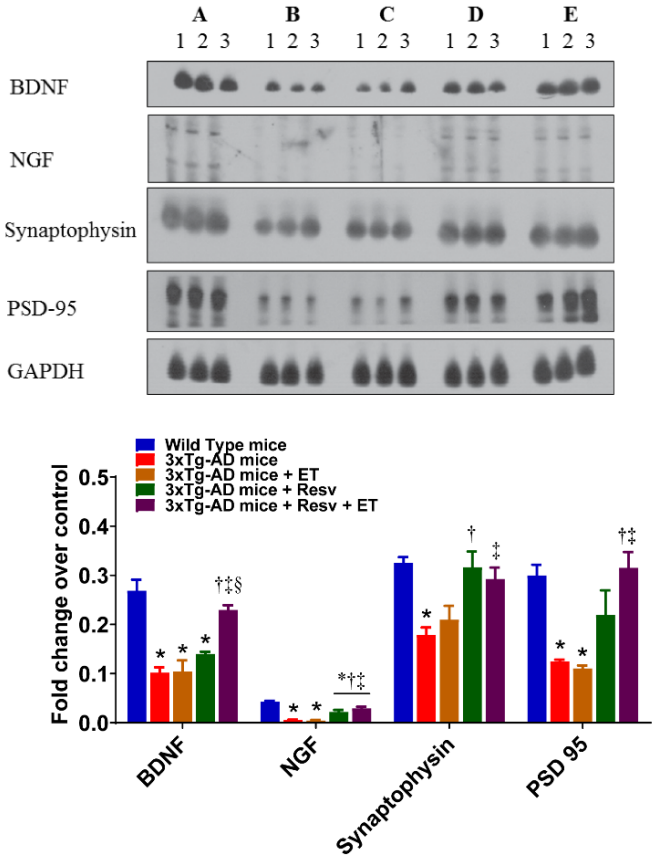


Fig.5 The effects of Resv and ET on the expression of neurotrophins and synaptic markers. Treatment with Resv or Resv with ET increased the expression of neurotrophins and synaptic markers. Representative immunoblots show significant increases in expression of the neurotrophins BDNF, NGF and synaptic markers synaptophysin and PSD-95 in 3xTg-AD mice treated with Resv or Resv with ET. Corresponding densitometry measurements: A, WT mice; B, 3xTg-AD mice; C, 3xTg-AD mice + ET; D, 3xTg-AD mice + Resv; E, 3xTg-AD mice + Resv + ET. BDNF, brain-derived neurotropic factor; NGF, nerve growth factor; PSD95, postsynaptic density 95. AD, Alzheimer's disease; ET, exercise training; Resv, resveratrol. Data are presented as mean ± SEM for 3 mice per group. *, compared to WT mice, $p < 0.05$; †, compared to 3xTg-AD mice, $p < 0.05$; ‡, compared to 3xTg-AD mice + ET, $p < 0.05$; §, compared to 3xTg-AD mice + Resv.

Figure 6

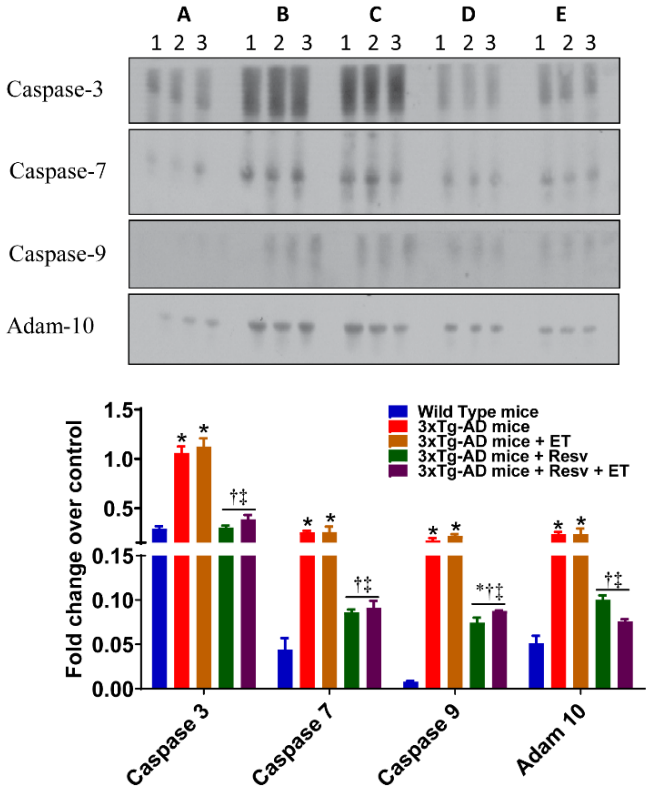


Fig.6 The effects of Resv and ET on the expression of markers of apoptosis. Treatment with Resv or Resv with ET decreased the expression of markers of apoptosis. Representative immunoblots show significant decreases in the expression of caspase 3, 7, 9, and Adam 10. Corresponding densitometry measurements: A, WT mice; B, 3xTg-AD mice; C, 3xTg-AD mice + ET; D, 3xTg-AD mice + Resv; E, 3xTg-AD mice + Resv + ET. Adam 10, disintegrin and metallopeptidase domain-containing protein 10. AD, Alzheimer's disease; ET, exercise training; Resv, resveratrol. Data are presented as mean ± SEM for 3 mice per group. *, compared to WT mice, $p < 0.05$; †, compared to 3xTg-AD mice, $p < 0.05$; ‡, compared to 3xTg-AD mice + ET, $p < 0.05$.

Figure 7

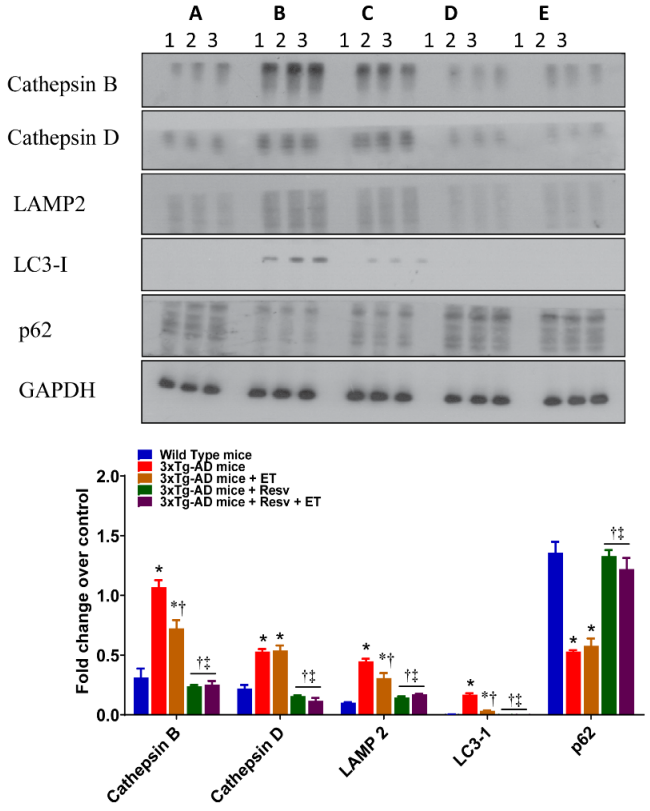


Fig.7 The effects of Resv and ET on the expression of markers of autophagy. Treatment with Resv or Resv with ET decreased protein expression of key autophagy markers. Autophagy was expressed using LC3-1 and Cathepsin B, Cathepsin D, and LAMP2 were measured as endolysosomal markers. Representative immunoblots show significant reductions in the expression of all markers with ET, Resv, and Resv with ET. The significant decrease in the expression p62 in brains of 3xTg-AD mice was prevented with Resv or Resv with ET. Corresponding densitometry measurements: A, WT mice; B, 3xTg-AD mice; C, 3xTg-AD mice+ET; D, 3xTg-AD mice + Resv; E, 3xTg-AD mice + Resv + ET. LAMP2, lysosomal-associated membrane protein; LC3-1, microtubule-associated protein 1 light chain 3 beta; p62, sequestosome 1/ubiquitin-binding protein. AD, Alzheimer's disease; ET, exercise training; Resv, resveratrol. Data are presented as mean ± SEM for 3 mice per group. *, compared to WT mice, $p < 0.05$; †, compared to 3xTg-AD mice, $p < 0.05$; ‡, compared to 3xTg-AD mice + ET, $p < 0.05$.

Figure 8

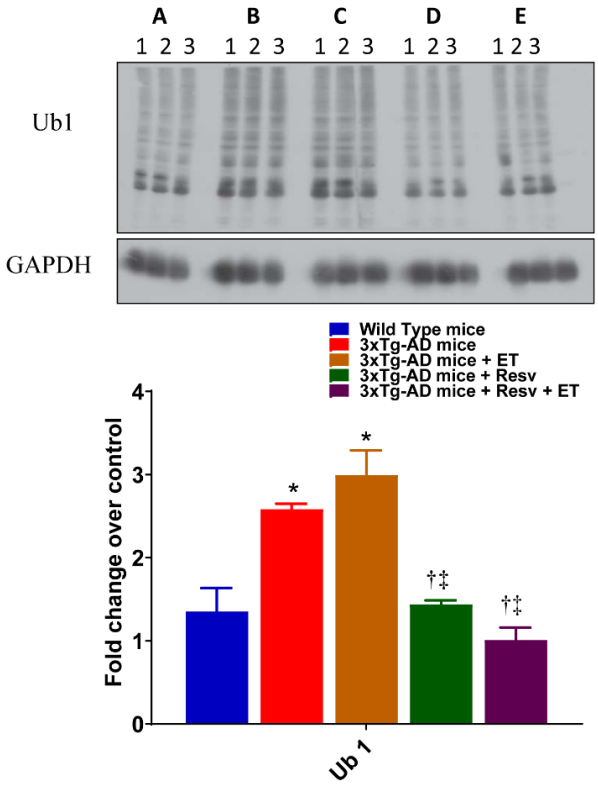


Fig.8 The effects of Resv and ET on protein ubiquitination. Treatment with Resv or Resv with ET decreased protein ubiquitination, expressed as Ub1. Corresponding densitometry measurements: A, WT mice; B, 3xTg-AD mice; C, 3xTg-AD mice + ET; D, 3xTg-AD mice + Resv; E, 3xTg-AD mice + Resv + ET. AD, Alzheimer's disease; ET, exercise training; Resv, resveratrol. Data presented as mean ± SD for 3 mice per group. *, compared to WT mice, $p < 0.05$; †, compared to 3xTg-AD mice, $p < 0.05$; ‡, compared to 3xTg-AD mice + ET, $p < 0.05$.

Figure 9

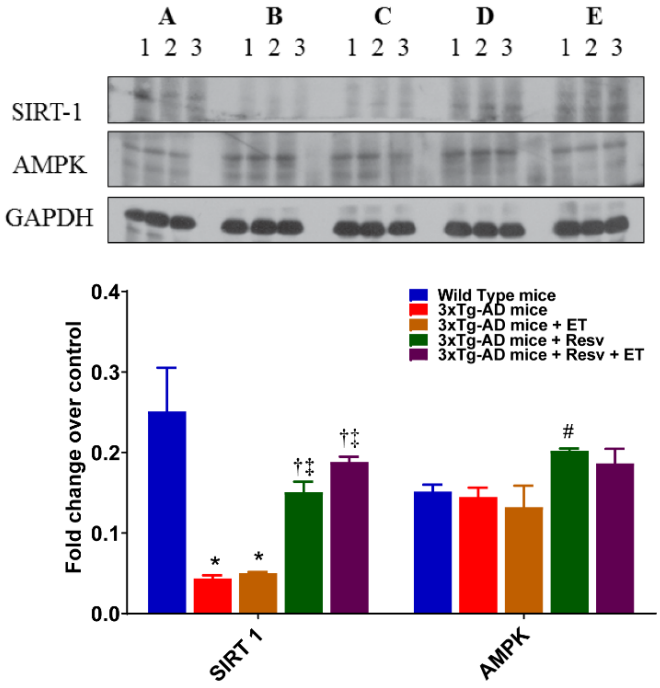


Fig.9 The effects of Resv and ET on protein expression of SIRT1 and AMPK. Representative immunoblots show a significant decrease in the expression level of SIRT1 in 3xTg-AD mice. Treatment with Resv or Resv with ET increased the expression SIRT1. Expression levels of AMPK were not significantly different between groups. Corresponding densitometry measurements: A, WT mice; B, 3xTg-AD mice; C, 3xTg-AD mice + ET; D, 3xTg-AD mice + Resv; E, 3xTg-AD mice + Resv + ET. SIRT1, silent information regulator 1; AMPK, AMP-activated protein kinase. AD, Alzheimer's disease; ET, exercise training; Resv, resveratrol. Data presented as mean ± SEM for 3 mice per group. *, compared to WT mice, $p < 0.05$; †, compared to 3xTg-AD mice, $p < 0.05$; ‡, compared to 3xTg-AD mice + ET, $p < 0.05$; #, $p = 0.0578$ compared to WT mice.

Figure 10

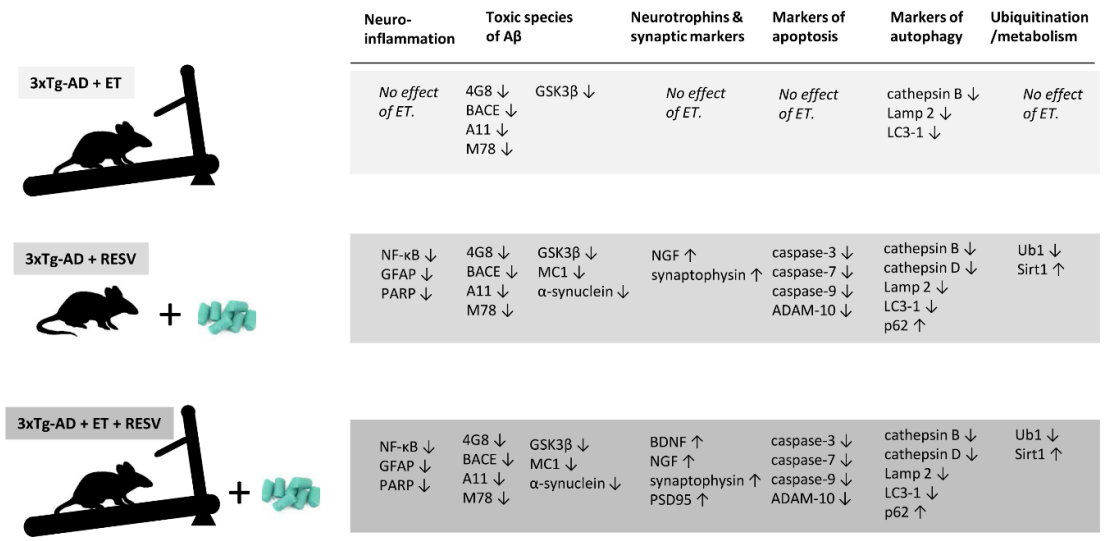


Fig.10 Summary of the effects of Resv and ET on proteins of interest. Changes are expressed as increased (↑) or decreased (↓) based on the Western blot data compared to brain tissue obtained from 3xTg-AD mice serving as controls (Figure 2). The greatest protection was afforded by treatment with Resv alone or by Resv combined with ET.

Chapter 4: Beneficial effect of genistein on diabetes-induced brain damage in the ob/ob mouse model

4.1 Abstract

Diabetes mellitus (DM)-induced brain damage is characterized by cellular, molecular and functional changes. The mechanisms include oxidative stress, neuroinflammation, reduction of neurotrophic factors, insulin resistance, excessive amyloid beta (Aβ) deposition and Tau phosphorylation. Both antidiabetic and neuroprotective effects of the phytoestrogen genistein have been reported. However, the beneficial effect of genistein in brain of the ob/ob mouse model of severe obesity and diabetes remains to be determined. In this study, female ob/ob mice and lean control mice were fed with either a standard diet or a diet containing genistein (600mg/kg) for a period of 4 weeks. Body weight was monitored weekly. Blood was collected for the measurement of glucose, insulin and common cytokines. Mice brains were isolated for western immunoblotting analyses. Treatment with genistein reduced weight gain of ob/ob mice and decreased hyperglycemia compared to ob/ob mice fed the standard diet. The main findings show that genistein treatment increased insulin sensitivity and the expression levels of the neurotrophic factors nerve growth factor (NGF) and brain-derived neurotrophic factors (BDNF). In these mice, genistein also reduced Aβ deposition and the level of hyper-phosphorylated Tau protein. The results of our study indicate beneficial effects of genistein in the diabetic mice brain, including improving brain insulin signaling, increasing neurotrophic support, and alleviating Alzheimer's disease-related pathology.

4.2 Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia. Type 1 DM (T1DM) is caused by impairment in insulin production from the pancreas. In contrast, type 2 DM (T2DM) is the result of peripheral insulin resistance in which the tissues fail to respond to

insulin effectively. DM is associated with various complications, including retinopathy, nephropathy, and neuropathy [282]. Several experimental and clinical studies have demonstrated brain damage in patients with DM. These individuals exhibit both cellular and functional changes, such as progressive cognitive deterioration and neurodegeneration [283][284][26]. Systematic reviews and meta-analysis studies have reported that patients with T2DM have a 50-100% higher risk of developing dementia compared to the non-diabetic population [26]. Results from longitudinal studies have shown that middle-aged to older adults with T2DM performed worse in memory, information-processing speed, attention tasks, and in overall cognitive function compare to non-diabetic controls over a follow-up of 3-6 years [285][286][287]. In addition, T2DM patients are at increased risk of developing Alzheimer's disease (AD) [288]. AD is the most common form of dementia, which is characterized by the accumulation of extracellular amyloid beta (Aβ) peptide formed senile plaques and intracellular hyperphosphorylated Tau-formed neurofibrillary tangles [218]. The possible mechanisms of diabetes-induced brain damage include oxidative stress, neuroinflammation, neuronal apoptosis, reduction of neurotrophic factors, insulin receptors downregulation, insulin resistance, excessive Aβ deposition and Tau phosphorylation, mitochondria dysfunction [282][289].

The consumption of plant-based foods rich in isoflavones is known to provide health benefits [290]. Several studies have demonstrated beneficial effects of isoflavones, especially genistein, which is the most abundant isoflavones found in soy [291]. Due to its similar structure with endogenous estrogen 17β-estradiol and to its affinity to the estrogen receptor, genistein displays estrogenic properties [292]. Evidence from human and animal studies indicates that genistein could be a potential preventative and therapeutic treatment for DM [124][293]. In experimental models of T1DM and T2DM, genistein treatment significantly decreased blood glucose, improved glucose tolerance and insulin sensitivity, possibly by modulating pancreatic

beta cell function and alleviating inflammation and oxidative stress [292][294]. Few studies, however, have reported the protective effects of genistein in the diabetic brain [295][125]. especially in T2DM. Also, whether genistein exhibits beneficial effects within the brain of the genetic ob/ob model of severe obesity and insulin resistance is not well understood. The ob/ob mouse is an animal model of peripheral neuropathy of type 2 diabetes and obesity. Ob/ob mice possess a recessive mutation in the leptin, which is a hormone that helps to regulate energy expenditure and food intake. Mutation in the leptin gene results in hyperphagia, hyperglycemia, and subsequent development of insulin resistance, obesity and diabetes [296]. Therefore, in this study, we used the naturally occurring soy isoflavone genistein to determine whether an improvement in insulin signaling is observing in the ob/ob mouse brain. We also examined the impacts of genistein on neurotrophic factors, Aβ deposition, and Tau metabolism. We hypothesize that genistein exerts beneficial effects by alleviating these neurodegenerative factors in this model of obesity and diabetes.

4.3 Material and methods

4.3.1Animals

The study utilized female ob/ob mice (B6.Cg-Lep^{ob}/J) and lean control mice (C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at five weeks of age. Ob/ob mice display severe obesity and insulin resistance as a result of a spontaneous mutation of the leptin receptor gene. Although this mutation rarely causes diabetes [297], a feature of this model of leptin deficiency is the hyperphagia exhibited by mice and the gradual onset of diabetes, producing a phenotype similar to human patients with T2DM. Female mice were selected since AD is more prevalent in females [298]. As a phytoestrogen that mimics estrogen, genistein may have more protective effects in the brain of female mice. All mice were housed in an animal facility maintained at a room temperature of 22°C with a 12-hour light/dark cycle, they consumed food and water ad libitum. Body weight was measured weekly and the general health

was monitored weekly during the study. This study was approved by the Institutional Animal Care and Use Committee at Midwestern University, and carefully followed the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

4.3.2 Study design

After one week of acclimatization, mice were divided into four groups (n=8-13). (1) lean control mice fed a standard diet (SD), (2) lean mice fed a genistein diet, (3) ob/ob mice fed a SD, (4) ob/ob mice fed a genistein diet. The formulated genistein diet was prepared in powder form (Dyets Inc., Bethlehem, PA, USA) and contained 600 mg genistein/kg diet. The concentration of 600 mg/kg was chosen because it is effective in maintain a significant improvement in plasma levels of genistein [299]. Moreover, our previous studies have showed that 600 mg/kg could induce significant physiological effects with four weeks of treatment in mouse model [300][301][302]. After four weeks of treatment, mice were sacrificed with CO_2 asphyxiation, followed by bilateral pneumothorax. Brains were isolated and immediately snap-frozen in liquid nitrogen and stored at -80°C for western immunoblotting analyses.

4.3.3 Plasma and cytokine assays

Cardiac blood was collected at the time of euthanasia and plasma maintained at -80^{0}C until use. Plasma was utilized for the measurement of glucose using a colorimetric assay kit (Wako Chemicals USA, Richmond, VA, USA) and insulin (Alpco Research and Clinical Immunoassays, Salem, NH, USA) assayed according to manufacture specifications. In a separate series of age-matched female ob/ob treated with genistein, plasma was collected and assayed using for common cytokines using the Millipore Milliplex assay (Burlington, MA).

4.3.4 Antibodies

Antibodies against pAkt (Ser-473), pIR (Tyr-1150/1151) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against IR, Tau, PHF-6 (Tau phospho Thr 231), CDK-5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Beta-actin, PSD-95 antibodies were obtained from Abcam (Cambridge, MA). 4G8 antibody was purchased from

Covance (Princeton, NJ). Antibodies against pIRS-1(Ser307) and IRS-1 were purchased from Fisher Scientific (Hampton, NH). PHF-1 (Tau phospho Ser 396/Ser404) antibody was obtained from Alzforum. Antibody OC was obtained from Millipore (Burlington, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

4.3.5 Western blot analysis

Whole mouse brains were homogenized in Triton lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/mL leupeptin, aprotinin and protease inhibitor cocktail). Samples were centrifuged at 12,000 rpm for 20 min at 4°C, and supernatants were collected. Protein concentrations of the brain lysates were measured with the Pierce 660-nm protein assay reagent (Thermo Scientific, Rockford, IL). The samples were separated on 8%-15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and then transferred onto polyvinylidene difluoride membranes. Immunoblots were blocked for one hour in Tris-buffered saline Tween-20 (TBST) containing 5% dry milk. The blots were incubated with appropriate primary antibodies in TBST at 4°C overnight. After washing, the blots were then incubated with secondary antibodies for two hours at room temperature. The immunoreactive bands were visualized with Enhanced Chemiluminescence reagent [219].

4.3.6 Statistics

The two-way analysis of variance (GraphPad Prism), followed by Tukey's test was used to determine significant differences between different group means. All values are reported as mean ± SEM. $P<0.05$ were considered to be statistically significant.

4.4 Results

4.4.1 Genistein reduces weight gain and plasma glucose of ob/ob mice

At the beginning of treatment and at the time of sacrifice, ob/ob mice had higher body mass than lean mice. Ob/ob mice fed 600 mg genistein/kg diet for 4 weeks gained ~44% less weight compared to those SD-fed ob/ob mice, while no significant change of weight between SD-fed

and genistein-fed lean control mice (Figure 11). At the completion of the 4-week treatment, genistein-fed ob/ob mice showed a significant reduction (17%) in plasma glucose levels when compared with SD-fed ob/ob mice (Table 1). Typical for the ob/ob model of obesity and diabetes, serum insulin levels were significantly elevated in ob/ob mice compared to lean control mice. However, genistein diet had no significant effect on insulin level for ob/ob mice. Interestingly, genistein diet significantly elevated serum insulin levels in lean control mice. This result is consistent with previous studies that genistein could improve pancreatic beta-cell function and stimulate insulin secretion [291][18]. Cytokines measured in plasma of mice demonstrated the effects of obesity and diabetes. Plasma levels of LIX, IL-1a, CXCL1, IL-5, IL-6, and IL-13 were significantly increased in ob/ob mice compared to control mice. Treating ob/ob mice with genistein resulted in a significant reduction in plasma levels of LIX, IL-1a, and IL-13. All other cytokines measured using the Milliplex assay kit revealed either no effects of the obese diabetic state or treatment (Table 2).

4.4.2 Genistein mitigates brain insulin resistance in ob/ob mice

T2DM mice not only exhibit systemic insulin resistance (IR) but also brain IR. To evaluate the insulin sensitivity in the brain of ob/ob mice and the effects of genistein, we analyzed the activation of insulin receptor and its downstream pathway in mice brain. As shown in Figure 12, the levels of insulin receptor phosphorylation at Tyr-1150/1151 in SD-fed ob/ob mice were lower than SD-fed control mice (Figure 12A). Also, there was a significant increase in insulin receptor substrate-1 (IRS-1) phosphorylation at Ser307 in SD-fed ob/ob mice as compared to control lean mice (Figure 12B), which suggests the inactivation of IRS-1. Moreover, the activation or phosphorylation of Akt was inhibited in SD-fed ob/ob mice (Figure 12C). These data indicate the increased insulin resistance in the brain of SD-fed ob/ob mice. However, genistein diet rescued the inactivation of the insulin receptor, IRS-1, and Akt in ob/ob mice, suggesting that short term treatment with genistein improves brain insulin sensitivity in the ob/ob model of diabetic obesity. There were no significant differences in these markers of

insulin sensitivity between SD-fed control mice and the genistein-fed control mice.

4.4.3 Genistein improves the level of neurotrophic factors in the brain of ob/ob mice

Neurotrophic factors are polypeptides known to regulate the proliferation, survival, differentiation, and migration of cells in the nervous system. Nerve growth factor (NGF) and brain-derived neurotrophic factors (BDNF) are the two main neurotrophic factors. Evidence indicates the role of neurotrophic factors in the pathogenesis of several neuronal and non-neuronal diseases, including AD and DM [303]. As shown in Figure 3, SD-fed ob/ob mice exhibited diminished levels of NGF (Figure 13A) and BDNF (Figure 13B) compared to control lean mice. However, expression levels of NGF and BDNF were dramatically elevated by supplementation with genistein in the diet. In addition, it has been reported that the compromised synaptic function could lead to various neurodegenerative disorders [304]. In order to evaluate synaptic function in T2DM mice and the effect of genistein, the protein content of postsynaptic density protein (PSD95) was measured. As shown in Figure 13C, our results reveal a significant difference in PSD95 levels between SD-fed ob/ob mice and control lean mice. Genistein supplementation improved PSD95 levels in the brain of ob/ob mice, although this was not statistically different.

4.4.4 Genistein attenuates the accumulation of Aβ and the phosphorylation of Tau in the brain of ob/ob mice

Previous studies have demonstrated that diabetes-associated brain damage has similar pathology with AD. In addition to the decrease in neurotrophic and synaptic factors, we hypothesize that Aβ deposition is observed in ob/ob mice brain, and genistein is involved in the process of regulating Aβ deposition. Here, the antibody 4G8 was used to detect Aβ peptide and the antibody OC to recognize amyloid fibrils. As shown in Figure 14A, when compared with SD-fed lean mice, SD-fed ob/ob mice had significantly increased Aβ deposition, while treatment with genistein diet mitigated these changes. This benefit of genistein was also observed in OC-detected content (Figure 14B). In addition, we assessed the neurofibrillary

tangle, through detecting the level of hyper-phosphorylated Tau protein. Antibody PHF-1 recognizes Tau protein phosphorylated at serine residues 396 and 404. The antibody PHF-6 recognizes Tau protein when phosphorylated at Thr231. As illustrated in Figure 15, the brain from ob/ob mice possessed significantly higher level of hyper-phosphorylated Tau than control mice. However, supplementation with genistein effectively reduced the phosphorylation level of Tau protein in those ob/ob mice. Furthermore, numerous serine/threonine kinases could phosphorylate Tau, such as cyclin dependent kinase 5 (CDK5). We observed enhanced expression of CDK5 in the brain of ob/ob mice compared to the control mice while treatment with genistein reduced levels of CDK5.

4.5 Discussion

In recent years, considerable attention has focused on diabetes pathology and the development of therapeutic or preventive strategies for the treatment of DM and its complications. Natural bioactive compounds attracted extensive interest due to abundant availability and few side effects [6]. As an isoflavone found in a variety of plants, genistein exhibits anti-diabetic effects in both *in vivo* and *in vitro* studies due to its anti-antioxidant and estrogenic properties [291]. Further, genistein treatment is reported to ameliorate diabetic peripheral neuropathy, inhibit oxidative stress and proinflammatory cytokine production, and restore NGF levels in brain of STZ-diabetic mice [125]. In the HFD-fed *ApoE* knockout mice model, genistein supplementation not only reduced neuroinflammation and oxidative stress-mediated insulin resistance, but decreased the level of Aβ peptide and hyperphosphorylated Tau protein, two major hallmarks of AD pathology [295]. Our results are consistent with these earlier reports, and we extend these observations by demonstrating beneficial effects on the brain afforded by genistein in the obese diabetic ob/ob mice.

Insulin receptors are expressed in the peripheral systems as well as in central nervous systems. Similar to systemic IR, brain IR is defined as reduced sensitivity in brain cells to the action of

insulin [305]. Evidence from experimental animal models suggests that systemic IR is linked with brain IR and both are observed in animal models of T2DM. Indeed, both high-fat diet (HFD)-induced diabetic mice and genetic diabetic mice (ob/ob and db/db mice) exhibit systemic and brain IR and abnormalities such as altered synaptic integrity and cognitive behaviors [306][307]. The insulin receptor is a heterotetrameric transmembrane protein made up of two extracellular α-subunits and two transmembrane β-subunits [308]. In the brain, binding of insulin to insulin receptors results in rapid autophosphorylation of the insulin receptor, which initiates a signal transduction cascade leading to tyrosine phosphorylation and activation of IRS-1. IRS proteins then activate phosphatidylinositol-3 kinase (PI3 kinases), followed by activation of downstream signaling cascade involving Akt and glycogen synthase kinase 3 (GSK3) [218]. Further, IRS-1 protein degradation could result from the serine phosphorylation of IRS-1. Our results demonstrate that ob/ob mice represent increased central IR, as evidenced by decreased insulin receptor phosphorylation, increased serine phosphorylation or inactivation of IRS-1 and inhibited Akt activation. However, feeding ob/ob mice with genistein diet improved insulin sensitivity. These results are consistent with previously published studies that genistein affords protection against central IR. In the cerebral cortex of aged female rats, acute treatment with genistein (40mg/kg body weight) reversed the deterioration in the interactions between PI3K and tyrosine phosphorylation of IRS-1, as well as stimulated Akt activation [309]. Also, in HFD-fed *ApoE* knockout mice model, genistein supplementation (500mg/kg diet) was reported to lessen central IR by decreasing phosphorylated IRS-1 levels [295].

Neurotrophic factors have multiple functions in the nervous system. In addition to their role in neuronal survival and growth, neurotrophic factors are capable of inducing synaptic plasticity, modulating the formation of long-term memories and maintaining neuron functions [310]. Evidence for a role of NGF and BDNF in the pathogenesis of DM is supported by the

observation that serum BDNF levels are lower in T2DM patients than non-diabetic subjects [311]. Also, Passaro et al. found that plasma BDNF levels are lower in patients with both T2DM and dementia than in non-diabetic patients with dementia [312]. In the early stage of STZ-induced diabetes in mice, gene expression of BDNF and NGF is lower compared to non-diabetic mice [313]. Furthermore, increasing evidence demonstrated a link between BDNF and steroid hormones such as estradiol. Lauren et al. reported that 17β-estradiol regulates hippocampal function by its impact on BDNF expression [314]. Genistein has been suggested to improve spatial learning and memory by activating BDNF signaling [315]. In the present study, our results show that genistein prevents a decrease in BDNF expression from occurring in the brain of ob/ob female mice. This effect is consistent with previous findings that estrogen plays a crucial role in BDNF expression and may improve the DM state. Furthermore, diminished levels of NGF were shown in the serum of both STZ-induced diabetic mice and diabetic patients [316]. Tosaki et al. indicated that high glucose condition induced the reduction of NGF secretion in Schwann cells, which could contribute to the disturbed axonal regeneration and may result in the development of diabetic neuropathy [317]. Interestingly, repeated treatment with genistein improves NGF production in the sciatic nerve of STZ-induced diabetic mice, showing a neurotrophic activity [125]. The results of our study further substantiate the effect of genistein on NGF normalization in the ob/ob genetic model of diabetes and obesity. Synaptic dysfunction contributes impairment of brain function and decline in learning and memory ability that occurs in the early stage of AD [318]. In the present study, the synaptic protein PSD-95 was used to evaluate the synaptic function. Our data show that the expression of PSD-95 was significantly decreased in the brain of ob/ob female mice. 4-weeks treatment with genistein diet improved the level of PSD-95 by ~25% in ob/ob mice, although this was not significant. We hypothesize that increasing the duration of the treatment period beyond 4-weeks could result in significant changes in PSD-95 in the brain of those ob/ob mice.

Previous studies have demonstrated that the pathology of diabetes-associated cognitive decline is similar to AD. The hallmark of AD, consisting of increased levels of aggregated Aβ protein, was observed in db/db mice and HFD-induced diabetic mice [26][307]. Aβ peptides are formed from amyloid precursor protein through sequential proteolytic cleavage by two enzymes, β- and α-secretases. Aβ could oligomerize into amyloid fibrils, and excessive oligomerization creates senile plaques in neural tissues. Our results indicate that brain from ob/ob female mice exhibits both significantly enhanced Aβ and amyloid fibrils levels when compared to lean control mice. However, genistein supplementation significantly reduced Aβ deposition in these female ob/ob mice. The inhibitory effect of genistein on Aβ aggregation has been proposed in a previous study: in an AD mouse model, treatment with genistein resulted in the rapid clearance of Aβ and the improvement of cognitive function [319]. Genistein supplementation also alleviated Aβ deposition in HFD-fed *Apo E* knockout mice [295]. Furthermore, several studies have reported a reduction in Aβ-induced neuronal apoptosis, astrogliosis, inflammation, and neurotoxicity following genistein treatment [164][320][321][322]. In contrast, a recent study reported that genistein caused an accumulation in Aβ in human neuroblastoma cells [323]. The reasons for these contrasting effects are not known, but may relate to the bioavailability of genistein in the plasma [324]. Overall, the long-term impact of treatment with genistein cannot be overlooked, further studies are needed to determine the effective concentration of genistein associated with neuroprotection.

Neurofibrillary tangles consist of hyperphosphorylated Tau protein. Under normal conditions, Tau modulates the stability of microtubes. In AD, Tau proteins undergo dynamic, site-specific phosphorylation by various kinases, such as CDK5, GSK3β [325]. Inappropriate phosphorylation leads to dysfunction of Tau, decreased cell viability, and pathogenesis of the neurological disease [325]. In this study, 4-weeks genistein diet significantly reduced the level of hyperphosphorylated Tau in ob/ob mice. The inhibition of CDK5 might partially contribute

to this result. Previous studies indicate that genistein could modulate Tau hyperphosphorylation through regulating calcium/calmodulin-dependent protein kinase IV or GSK3β in AD mice or *Apo E* knockout mice [295][171]. The autophagy-dependent mechanism may also be responsible for regulating the concentrations of hyperphosphorylated Tau by genistein in AD mice [146]. In addition, a recent study demonstrated that the hyperphosphorylation of Tau in the brain of ob/ob mice might relate to hypothermia and the decreased thyroid hormone levels [326]. Interestingly, treatment of ob/ob mice with genistein elevated thyroid hormone levels and induced a thermogenic change in these mice [302], suggesting that genistein might be a novel therapy in the elimination of hyper-phosphorylated Tau.

The diminished level of Aβ observed in the brain of genistein-fed ob/ob mice may be explained by improved insulin signaling. Brain IR has been suggested to accelerate AD pathology, including processing or aggregation of Aβ and hyperphosphorylation Tau. Diet-induced IR is suggested to promote Aβ generation in the brain of AD mice by enhancing γ- secretases activities and inhibiting insulin degrading enzyme (IDE) activities [327]. The IR has also been implicated in Tau aggregation. The downstream signaling effect of PKB/Akt, GSK-3β functions as a kinase responsible for the phosphorylation of Tau [328]. Also, administration of insulin could suppress protein phosphatase 2 activity, which is the primary Tau phosphatase contributes to Tau de-phosphorylation [329]. Overall, brain IR may increase the tendency for Tau aggregation by improving Tau phosphorylation and inhibiting Tau de-phosphorylation.

In addition to its role in AD pathology, IR in brain has a direct effect on synaptic function and cognition. For instance, the downregulation of the hypothalamic insulin receptor in mice elicited a decreased expression of BDNF as well as an impaired long-term potentiation and spatial learning [330]. The role of insulin as a synaptic modulator promoting neuroplasticity in the developing and adult brain has been proposed. Moreover, IR in T2DM impairs hippocampal synaptic plasticity [331][332]. Our data indicate that ob/ob mice exhibit central IR

accompanied with decreased synaptic plasticity. However, genistein supplementation improved brain insulin sensitivity and increased levels of the synaptic marker, PSD-95, by nearly 25%. Therefore, it is reasonable to hypothesize that genistein could ameliorate synaptic plasticity in the brain of diabetic mice by mitigating insulin resistance.

4.6 Conclusion

In summary, considering the high risk of deterioration in brain function induced by DM, the identification of preventive strategies and complementary therapies has been an important research goal. The results of this study indicate that genistein could improve the obesity and diabetes-related neuronal damage in the brain of ob/ob mice. The beneficial effects of genistein are associated with increasing insulin sensitivity and expression of neurotrophic factors as well as inhibiting Tau protein phosphorylation and Aβ deposition. As emerging evidence indicates that the putative mechanisms of genistein include estrogen receptor (ER) regulation [333], it is likely that genistein impacts survival and cell growth, synaptic plasticity, and cognitive function through the ER-mediated pathways [333][334]. Therefore, further studies are needed to explore the role of ER-mediated or non-ER mediated mechanisms in neuroprotective effects of genistein in the DM animal model. Also, the present study only used female mice. The male mice model needed to be included in future studies to examine whether the effect of genistein is sex-dependent.

Tables

Table 1 Effect of genistein on blood glucose concentrations and body weight gain of ob/ob mice

Parameter	lean SD	lean G	ob/ob SD	ob/ob G
Glucose (mg/dL)	241±8	261±10	467±17*	389±40$^{\#}$
Insulin (pg/ml)	1285±345	10670±449†	8408±1249$^{\$}$	6858±209
Weight gain (g)	1.96±0.33	1.40±0.47	12.29±0.80*	6.89±0.70$^{\&}$

Values are expressed as mean ± SEM for 13 mice in each group. *$p < 0.001$ compared to lean mice fed the SD and G diet. $^{\#}p < 0.05$ compared to ob/ob mice fed the SD. $^{\&}p < 0.001$ compared to ob/ob mice fed the SD. $^{\$}$ $p < 0.001$ compared to lean mice fed the SD. $^{\dagger}p < 0.001$ compared to lean mice fed the SD. SD, standard diet; G, genistein.

Table 2 Effect of genistein on the levels of plasma cytokines of ob/ob mice

Cytokine(pg/ml)	lean SD	ob/ob SD	ob/ob G
LIX	3176±1265 (6)	8344±1209 (7)*	3721±974 (8)$^{\#}$
IL-1a	91.7±30.1 (6)	273.5±72.3 (6)*	92.8±14.7 (7) $^{\#}$
KC (CXCL1)	36.7±8.1 (6)	83.8±11.5 (7)*	71.5±7.6 (8)
MCP-1	23.1±4.9 (6)	46.4±7.7 (7)*	36.8±5.9 (8)
IL-2	2.9±0.3 (6)	2.8±0.5 (7)	3.2±0.4 (8)
IL-4	0.1±0.01 (6)	0.2± 0.1(5)	0.1± 0.01 (6)
IL-5	6.7±0.9 (6)	2.8±0.4 (6)*	2.9±0.4 (8)
IL-6	0.8±0.2 (4)	2.5±0.6 (5)*	4.7±2.7 (3)
IL-7	6.2±2.7 (6)	29.9±20.2 (7)	3.9±1.8 (5)
IL-10	2.9±0.3 (6)	2.7±0.8 (6)	1.7±0.2 (7)
IL-12 (p70)	2.6±0.2 (5)	14.9±6.8 (6)	1.5±0.2 (6)
IL-13	50.3±4.7 (6)	36.8±2.7 (7)*	26.6±3.3 (8) $^{\#}$
IL-17	11.7±1.0 (6)	8.8±1.2 (7)	10.3±2.1 (8)
IL-1B	3.3±0.6 (6)	2.4±0.2 (3)	3.8±1.8 (3)
GM-CSF	7.9±0.9 (6)	8.4±1.4 (6)	7.3±0.6 (6)
MIP-2	39.0±2.9 (6)	39.9±1.8 (7)	36.7±2.1 (8)
TNFα	3.5±0.3 (6)	3.2±0.5 (6)	2.7±0.5 (8)

Values are expressed as mean ± SEM for 3-8 mice per group. Numbers in parentheses indicate the sample size for each cytokine assayed per group. *$p < 0.05$ compared to lean mice fed the SD. $^{\#}p < 0.05$ compared to ob/ob mice fed the SD. SD, standard diet; G, genistein. LIX, lipopolysaccharide-induced CXC chemokine; IL, interleukin; KC CXCL1, C-X-C motif chemokine-1; MCP, monocyte chemoattractant protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; TNFα, tumor necrosis factor alpha.

Figures and figure legends

Figure 11

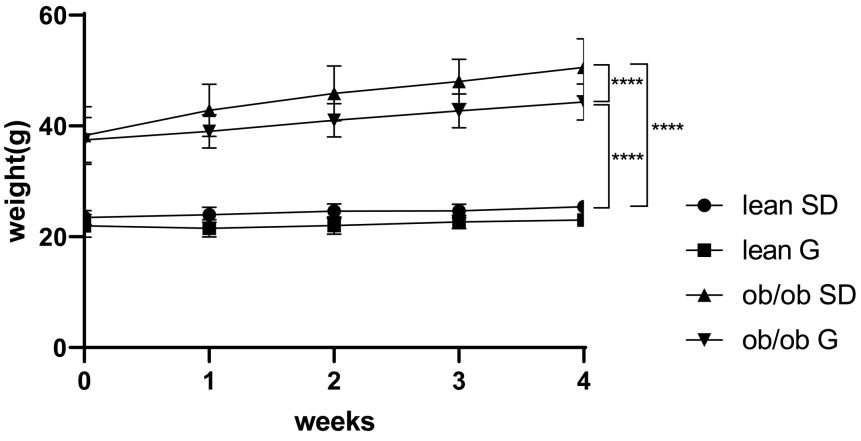


Fig.11 Weight change of mice during 4 weeks treatment.

Lean mice and ob/ob mice were fed with a standard diet or genistein diet for 4 weeks. Body weight was monitored weekly. SD, standard diet; G, genistein. ****$p \leq 0.0001$.

Figure 12

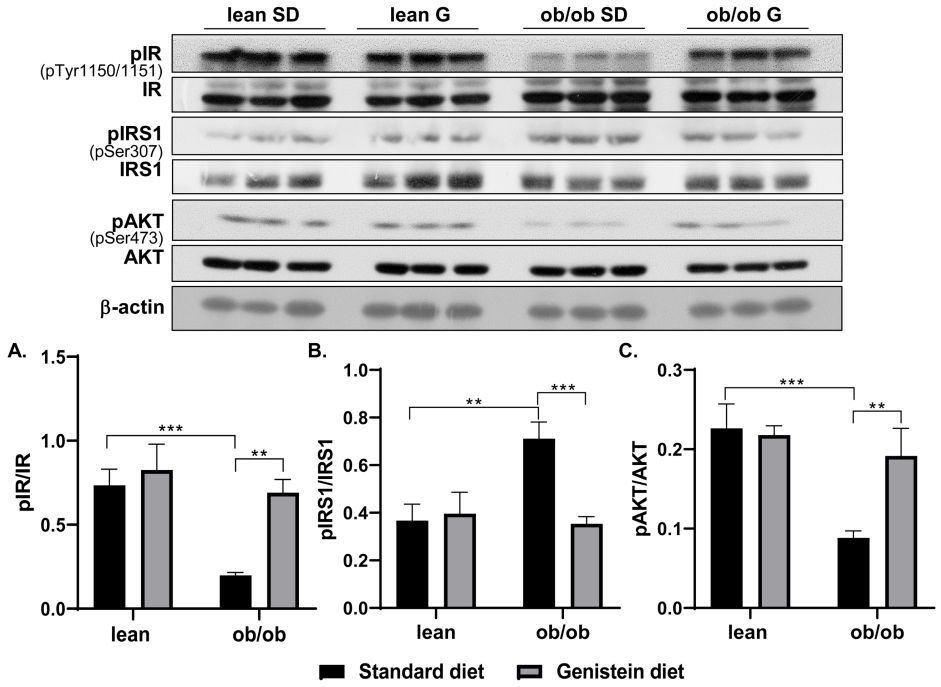


Fig.12 Effect of genistein on central insulin signaling in brain of ob/ob mice.

(**A**) Representative blot images with corresponding densitometry measurements of the insulin receptor. IR, insulin receptor; pIR, IR phosphorylation at Tyr-1150/1151; (**B**) Representative blot images with the corresponding densitometry measurements of insulin receptor substrate-1. IRS-1, insulin receptor substrate-1; pIRS-1, IRS-1 phosphorylation at Ser307; (**C**) Representative blot images with the corresponding densitometry measurements of Akt. pAkt, Akt phosphorylation at Ser473. Data are presented as mean ± SEM for 3 mice per group. ** $p \leq 0.01$, *** $p \leq 0.001$. SD, standard diet; G, genistein.

Figure 13

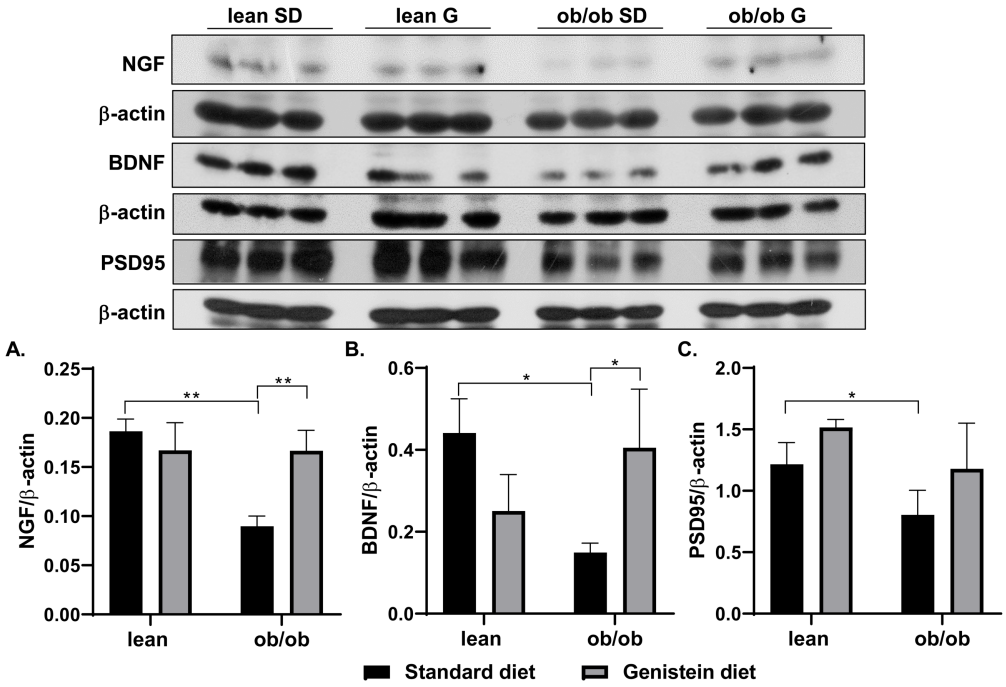


Fig.13 Effect of genistein on the levels of neurotrophins and synaptic marker in brain of ob/ob mice.

(**A**) Representative blot images with the corresponding densitometry measurement of nerve growth factor (NGF). (**B**) Representative blot images with the corresponding densitometry measurements of brain-derived neurotropic factor (BDNF). (**C**) Representative blot images with the corresponding densitometry measurement of postsynaptic density 95 (PSD95). Data are presented as mean ± SEM for 3 mice per group. *$p \leq 0.05$, **$p \leq 0.01$. SD, standard diet; G, genistein.

Figure 14

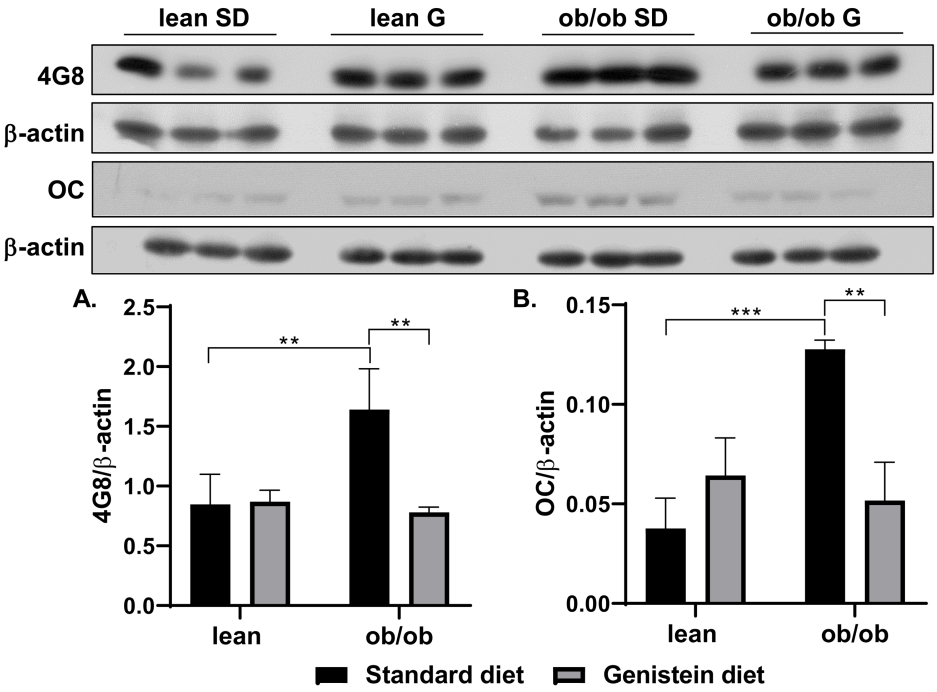


Fig.14 Effect of genistein on Aβ deposition in the brain of ob/ob mice. Representative blot images of Aβ deposition markers and the corresponding densitometry measurements. OC, amyloid fibrils. Data are presented as mean ± SEM for 3 mice per group. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. SD, standard diet; G, genistein.

Figure 15

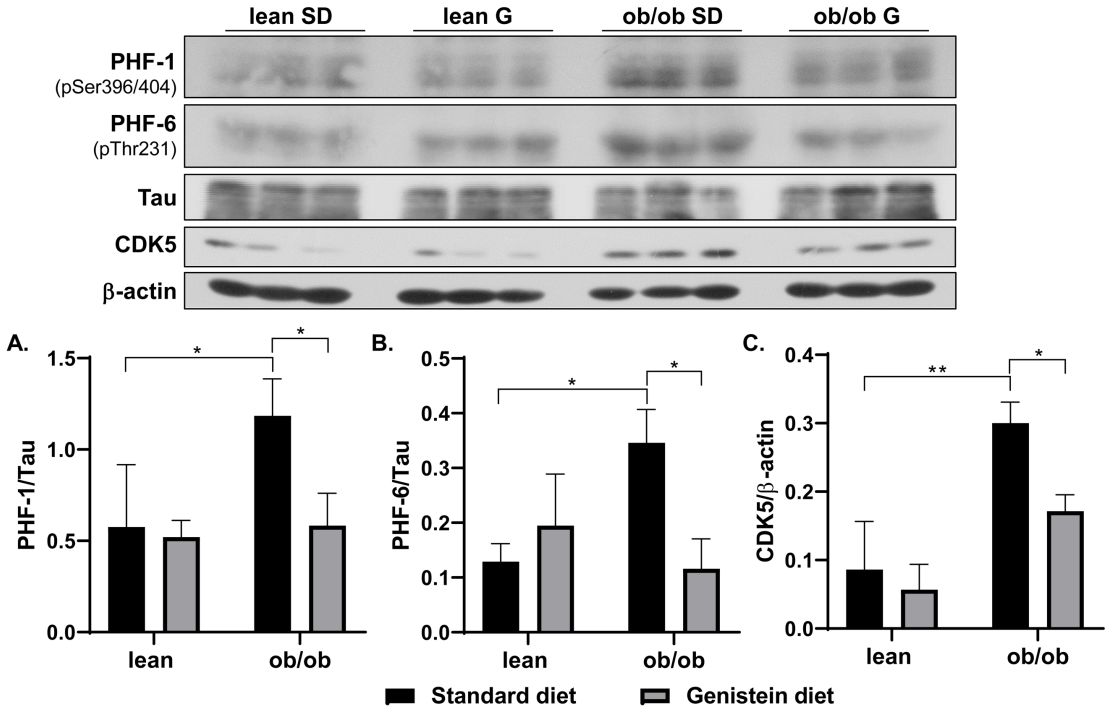


Fig.15 Effect of genistein on the level of hyper-phosphorylated Tau in the brain of ob/ob mice. Representative blot images of neurofibrillary tangle formation markers, kinases and the corresponding densitometry measurements. PHF-1, Tau phosphorylation at Ser396/Ser404; PHF-6, Tau phosphorylation at Thr231. CDK5, cyclin dependent kinase 5. Data are presented as mean ± SEM for 3 mice per group. *$p \leq 0.05$, **$p \leq 0.01$. SD, standard diet; G, genistein.

Chapter 5. Beneficial effects of exercise and/or genistein treatment on high-fat, high-sugar diet-induced brain damage in C57BL/6 mice

5.1 Abstract

Western diet, defined as high dietary intake of sugars, saturated fats and low intake of fiber, represents an increasing health risk contributing to the occurrence of brain damage and neurodegenerative disease. A diet containing genistein accompanied by regular exercise has been associated with improved brain function. In this study, we examined the beneficial effects of genistein supplementation and exercise training on the brain of high-fat, high-sugar (HFHS) diet-fed mice. Our results indicate that genistein decreased the accumulation of amyloid beta protein (Aβ), improved the expression of brain-derived neurotrophic factor, and ameliorated apoptosis in the brain of HFHS diet-fed mice. Exercise improved some neuroprotection-related markers, and when combined with genistein supplementation, the benefits obtained were as useful as genistein treatment alone. Moreover, there was evidence suggesting that the neuroprotective effects of genistein are associated with its capability to bind to the estrogen receptor (ER). We used high glucose (HG) and palmitate (PA) to induce apoptosis in PC12 cells, and our results show that genistein ameliorated HG and PA-induced apoptosis via ER-mediated mechanism.

5.2 Introduction

Over the past few decades, there has been a profound change in the food environment. Advances in technology introduced increased affordability and availability of fast food, refined grains and sugars [335]. As a result, the dietary composition has been shifted from diets high in fiber and complex carbohydrate, to diets high in sugars, refined grains, high fat foods: a dietary pattern that is termed as “the Western diet” [336]. Increased proportion of fat and sugar in the diet has been accompanied by adverse effects on general health, increase risk of obesity,

type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) [337][338]. Moreover, emerging evidence has been suggested the damaging effect of the Western diet on the brain [339][340]. Both human epidemiological and animal studies have been shown that intake of high-fat, high-sugar diet (HFHS) is associated with declined cognitive function, worse performance on cognitive tests and increased risk of neurodegenerative disease [339][340][341].

Increasing the intake of bioactive compounds in habitual diets has been suggested as a potential dietetic treatment to improve brain health. Among the different bioactive compounds, genistein has exhibited the ability to protect the brain from oxidative stress, improve synapse development and cognitive function [120][121]. Genistein belongs to the class of soy isoflavones and is found in soy-based foods such as soy flour and soy milk. Evidence from animal and human studies support the notion that genistein may offer protection against a broad range of human conditions, including menopausal symptoms, breast cancer, T2DM, CVD, and neurodegenerative disease such as AD [104][342][19]. Exercise is a non-pharmacologic therapy and affordable alternative for different pathological conditions and for the maintenance of general health. Also, exercise training has been shown to the ability to improve memory and cognitive processes and maintain brain health [343]. Given the beneficial effects of genistein and exercise, we aimed to examine the neuroprotective effects of genistein and exercise training in the HFHS diet-induced obesity mouse model.

Moreover, genistein processes a structure that is similar to estrogen. Therefore, genistein can bind the estrogen receptor (ER) [19]. There is evidence suggesting that genistein exerts anti-apoptotic actions via ER-mediated mechanisms, which may attribute to its neuroprotective function [344][345]. In this study, we exposed the PC12 cells to saturated fatty acid palmitate (PA) and high glucose (HG), to determine their effect on the cell viability and apoptotic process. We also investigated whether treatment with genistein could attenuate these effects in PC12

cell cultures. Further, we explored whether genistein protects against HG and PA-induced neural toxicity involves ER-mediated downregulation of apoptotic signaling.

5.3 Methods

5.3.1 Animals

Fifty female mice of the strain C57BL6 (Jax Labs, ME, USA), aged 6 weeks, were used in the study. Mice were assigned to five groups (n=10/group): (1) control, (2) HFHS diet, (3) HFHS diet + exercise, (4) HFHS diet + genistein, (5) HFHS diet + exercise + genistein. HFHS diet consisted of 60% fat (32.3g/kg of corn oil and 316.6g/kg of lard), 20% protein and 20% carbohydrate (Dyets Inc. Bethlehem, PA, USA) and 42 g/L sugar dissolved in drinking water (55% fructose/45% sucrose). This diet is known to induce significant insulin resistance and visceral obesity in the C57BL/6 mouse [218]. Control mice were fed with a standard diet that contained 20.3 g protein, 66 g carbohydrate, and 5 g fat. Exercise training consisted of low-intensity treadmill running for 30 min/day, 5 days/week. Genistein treatment was incorporated into food pellet at 600 mg genistein/kg HFSD diet (Dyets Inc., PA, USA). This genistein dose is sufficient to produce significant increases in plasma levels of free genistein [299]. Mice were maintained in a room with a light/dark period of 12-h and at a temperature of 22 °C. After 12 weeks treatment, mice were sacrificed, and brain tissue was frozen in liquid nitrogen for Western blotting analyses. The animal protocols described in this study were approved by the Institutional Animal Care and Use Committee at Midwestern University. Guidelines in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals were followed.

5.3.2 Cell culture

Undifferentiated PC12 cells (ATCC® CRL-1721™) were maintained in RPMI-1640 Medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS), penicillin/streptomycin (50 units/50 µg of each per mL) at 37°C with 95% air–5% CO_2. Culture

medium was replaced every 2–3 days. PC12 cells were differentiated by exposure to 50 ng/mL mouse nerve growth factor (Alomone Laboratories, Israel) for 5-7 days in RPMI-1640 Medium supplemented with 1% FBS, penicillin/streptomycin (described hereafter as differentiation medium).

5.3.3 Cell treatment

In order to measure the toxic effects of glucose and saturated fatty acid, cells were exposed to either 13.5mg/ml glucose, 0.3mM PA bound to 0.15mM fatty acid-free bovine serum albumin (BSA) or to 13.5mg/ml glucose in the presence of PA. In order to prevent toxic effects of high levels of glucose and fatty acid, in some experiments, cells were pretreated with genistein (2, 5, 10, 20μM) alone or in combination with estrogen receptor antagonists tamoxifen (5μM) for 2h in differentiation medium, followed by HG and PA treatment.

5.3.4 Preparation of BSA-bound fatty acids

PA (Santa Cruz 215881) was conjugated to fatty acid-free BSA (Sigma A8806). Following previously described methods [346], PA was dissolved in 100% ethanol at 70°C to make a 0.3 mM stock solution. Fatty acid-free BSA was dissolved in differentiation medium to make a 0.15mM solution and sterilized by filtering through a 0.2 μM filter. Then dilute dissolved PA into fatty acid-free BSA to generate a complex of PA: BSA at 2:1 molar ratio. For control, the cells were treated with differentiation medium containing 150 μM fatty acid-free BSA and 0.1% ethanol.

5.3.5 Determination of cell proliferation

The proliferation of PC12 cells was assessed by MTT assay. PC 12 cells were seeded in 96-well plates at the initial density of 3000 cells per well, and then differentiated for 5-7 days before the treatment. After the treatment for 24 h, discarded treatment medium and added 50 μL of serum-free medium and 50 μL of MTT Reagent into each well, followed by incubation for 3 h. Then the supernatant was discarded and 150 μL of MTT Solvent was added into each

well. The absorbance at 590nm was determined by a microplate reader. Cell proliferation rate was calculated from the absorbance value.

5.3.6 Western blot analysis

Triton lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/mL leupeptin, aprotinin and protease inhibitor cocktail) was used to homogenize whole brain samples. Cells were harvested in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin. Protein concentrations of the brain and cell lysates were measured by the Pierce 660-nm protein assay reagent (Thermo Scientific, Rockford, IL). The samples were run on 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels. The proteins were transferred onto polyvinylidene difluoride membranes. Transferred membranes were blocked for one hour in Tris-buffered saline Tween-20 (TBST) containing 5% milk. The blots were then incubated with various primary antibodies in TBST at 4°C overnight. After washing, the blots were incubated with secondary antibodies for two hours at room temperature. Enhanced Chemiluminescence reagents were used to visualize the immunoreactive bands.

5.3.7 Statistical analysis

Quantification of Western blot data was performed by Image J software and data were analyzed using Graph pad prism. A one-way analysis of variance followed by the Tukey's test was used to determine differences in group means. All values are reported as mean ± SEM. $P<0.05$ was considered to be statistically significant.

5.4 Results

Mice metabolic data were previously published [347]. Briefly, mice fed an HFHS diet for 12 weeks had significantly greater body mass, plasma glucose, insulin and IL-6 than control mice

fed a standard diet (p<0.05). HFHS diet-fed mice treated with genistein, either alone or combined with exercise, had significantly decreased body mass and plasma glucose levels in comparison to HFHS controls.

5.4.1 Genistein or genistein with exercise reduces the accumulation of Aβ and hyperphosphorylated tau in HFHS diet-fed mice

Evidence suggests that increasing intake of saturated fat and sugar in the diet may increase the risk of AD [348]. Amyloid plaques and neurofibrillary tangles (NFT) are two main pathological conditions of AD. Amyloid plaques are extracellular accumulation of the Aβ, and the NFT are intracellular deposit of the hyperphosphorylated tau protein. In order to examine how genistein affects HFHS diet-induced changes in AD neuropathology, we measured the levels of Aβ and hyperphosphorylated tau in different groups. Protein expression levels of Aβ detected by 4G8 and expression levels of hyperphosphorylated tau detected by PHF antibody are illustrated in Figure 16 and Figure 17 respectively. As expected, HFHS diet significantly increased the level of Aβ protein and hyperphosphorylated tau protein. Treatment with genistein or exercise was associated with a reduction of Aβ compared to HFHS-fed mice. Although genistein or exercise alone did not significantly change the level of hyperphosphorylated tau, combination treatment was effective in reducing both 4G8 and PHF levels compared to brains from HFHS diet treatment group.

A Disintegrin And Metalloprotease 10 (ADAM10) is an α-secretase that responsible for the non-amyloidogenic pathway of amyloid precursor protein (APP). We observed decreased expression of ADAM10 in the brain of HFHS diet-treated mice compared to the control mice while treatment with genistein or exercise improved levels of ADAM10. Cyclin dependent kinase 5 (CDK5) is a key kinase involved in the phosphorylation of tau protein. We found a significant increase in the level of CDK5 in the HFHS group, while combining exercise and genistein treatment returned CDK5 level to that of control mice.

5.4.2 Genistein and/or exercise increases the expression of BDNF in HFHS diet-fed mice

Brain-derived neurotrophic factor (BDNF), a key molecule involved in synapse function and neurite growth, is emerging as a crucial player in synaptic plasticity, learning and memory [210]. Our results show that the protein level of BDNF in the brain of mice fed a HFHS diet were reduced compared to mice fed a control diet. Treatment with genistein alone or exercise alone resulted in a significant increase in BDNF level compared to HFHS diet-treated mice. Combination treatment was associated with a greater improvement in the expression of BDNF.

5.4.3 Genistein or genistein with exercise decreases apoptosis in HFHS diet-fed mice

Moreover, we evaluated if the protective potential of genistein and exercise involves the regulation of the apoptotic process in HFHS diet-treated mice. The two main pathways of apoptosis are intrinsic (mitochondria) and extrinsic (death ligand) pathways. Cytochrome c plays a key role in the intrinsic pathway and can trigger the activation cascade of caspases [349]. Our results indicate that HFHS diet led to an increase in cytochrome c levels while genistein and exercise combination treatment returned cytochrome c level to that of control. Caspase-3 is an executioner caspase in apoptosis due to its role in regulating the destruction of cellular structures or degradation of cytoskeletal proteins [350]. Caspase-3 is activated by intrinsic (mitochondria) or/and extrinsic (death ligand) pathways. We found that the expression of activated caspase-3 was significantly increased in brain of mice fed a HFHS diet (figure 19). However, the addition of genistein to HFHS diet or exercise, or combination treatment reversed the caspase-3 level to normal.

5.4.4 Incubation of PC12 cells with high glucose and palmitate results in loss of viability

Differentiated PC12 cells were treated either with HG, PA or co-treated with HG and PA for 24 h. Subsequently, the effect of HG and PA on PC12 cell viability was determined using an MTT assay. As shown in Figure 20A, PC12 cell viability was significantly decreased by the presence of HG and PA. The combined treatment of HG and PA, which significantly decreased cell viability to 40% ($p<0.01$) was selected for subsequent experiments. The effects of different

concentrations of genistein on cell viability were also investigated. Genistein induced no cytotoxic effects at concentrations 2, 5, 10, 50 μM (Fig. 20B), although concentrations at 100 μM significantly decreased cell viability compared with the control (p<0.05).

5.4.5 Genistein attenuates HG and PA-induced loss of cell viability

In a separated experimental group, to examine the effect of genistein on HG and PA-induced cytotoxicity, PC12 cells were pretreated with 2μM or 5μM genistein, then incubated in HG and PA medium. It was demonstrated that the viability of PC12 cells pre-treated with genistein improved significantly by compared with cells that underwent treatment with HG and PA alone (p<0.05). We further examined the cell viability when the cells were pretreated with genistein and an ER antagonist (tamoxifen) to determine whether the genistein-induced proliferative effect in HG and PA-containing medium was associated with the activation of ER. As shown in Figure 21, the cell viability of the group pre-treated with 5μM genistein and tamoxifen was significantly lower than the group pre-treated with genistein alone. These results indicated that genistein may exert its proliferative effect in HG and PA-containing medium, partially through the ER-mediated pathway.

5.4.6 Genistein protects against HG and PA-induced apoptosis in PC12 cells via ER-mediated mechanism

Control, HG+PA, HG+PA+genistein (HG+PA+G), HG+PA+genistein+tamoxifen (HG+PA+G+T), four group cells were harvested into lysis buffer for western blotting analysis. The expression of caspase-3 and Bcl-2 were examined. Figure 22 shows significant caspase-3 activation following 24 hours of HG and PA exposure (p<0.01). Preincubation with 5 μM of genistein prevented the increase in caspase-3 activation. The level of expression of caspase-3 in genistein pretreatment groups was not significantly different from that of the control group. Moreover, as shown in Figure 22A, the protein levels of caspase-3 in HG+PA+G+T group were significantly higher than HG+PA+G treated cultures (p<0.05) and were similar to that in HG+PA-only treated cultures. Results of the Bcl-2 western blotting presented in Figure 22B

demonstrate that genistein added to the cell culture medium significantly (p<0.05) improved the downregulation of Bcl-2 expression, an effect shown to occur within the HG+PA exposure in PC12 cell cultures. However, the addition of tamoxifen significantly attenuated genistein effects on Bcl-2 expression. These results indicate that genistein inhibits the HG+PA-induced caspase-3 activation and Bcl-2 downregulation via an ER-mediated mechanism, as the addition of tamoxifen returned caspase-3 and Bcl-2 levels to that of HG+PA- only treated cultures.

5.5 Discussion

An unhealthy diet such as the "Western diet" is one of the greatest factors contributing to the risk of obesity. The link between obesity and cognitive function has been well established. The negative consequences of obesity (and T2DM) are associated with impaired cognitive performance, cognitive decline and increased incidence of dementia [351]. Exercise is recommended as a non-pharmacological strategy to help reduce obesity, improve cardiovascular health, and delay the effects of pathological neurodegeneration on the brain [343]. In addition, the natural bioactive compound, genistein has been studied as a promising therapy for the treatment of obesity or diabetes-induced cognitive dysfunction. Our previous study indicated the neuroprotective effect of genistein in the genetically obese (ob/ob) mouse model [352], however, the role of genistein in diet-induced changes in brain function is not well studied. In this study, mice were fed a HFHS diet and the effects of genistein supplementation and exercise training were examined.

Firstly, we found that the HFHS diet is related to the increased level of Aβ protein and the exacerbation in tau phosphorylation, which are hallmarks of AD in the brain. These results are consistent with previous studies [353][348]. The HFHS diet in this study is sufficient to induce visceral obesity, hyperglycemia and insulin resistance in the C57BL/6 mice [347]. Increasing evidence has shown that insulin resistance is associated with AD pathology. Insulin could

regulate Aβ level through modulating the balance between Aβ production and degradation. Also, the defect in insulin signaling pathway may promote the phosphorylation of tau protein through activating the glycogen synthase kinase 3 (GSK-3) [354]. In addition, impaired insulin signaling leads to the impairment or dysfunction of mitochondrial, resulting in increased production of reactive oxygen species and neuropathology in the brain [355]. Our results demonstrate that genistein or exercise treatment ameliorated HFHS diet-induced increased Aβ level. The reduction effect of genistein on Aβ level has been reported in the AD mouse model and the ob/ob mouse model [352][319]. However, to the best of our knowledge, this study is the first to show that genistein reduces the accumulation of Aβ in a diet-induced obese mouse model.

APP is a transmembrane protein found in most tissues. In pathological conditions, APP is mainly cleaved by β- and γ-secretase, resulting in the production of Aβ peptides. In physiological conditions, APP is mainly processed by ADAM10, releasing a soluble fragment (sAPPα) in a non-amyloidogenic pathway [356]. Activating the non-amyloidogenic pathway by potentiating ADAM10 activity could significantly decrease Aβ release. Also, product sAPPα has neuroprotective properties [356]. Therefore, a drug able to improve ADAM10 activity can be a potential therapy for AD to reduce Aβ generation and favor neurogenesis. We observed a decreased ADAM10 protein level in HFHS diet-treated mice compared to control mice, although it was not significant. However, genistein or exercise treatment significantly improved the protein level of ADAM10, which may contribute to the deduction of Aβ level in brain of mice fed a HFHS diet. We also observed increased hyperphosphorylated tau and CDK5 levels in HFHS diet-treated group. A combination of genistein and exercise training was able to ameliorate the increased CDK5 levels, which may contribute to the reduction of hyperphosphorylated tau in the mice brain.

BDNF plays an important role in synaptic plasticity, neuronal growth and animal behavior. An

animal's energy status can affect levels of BDNF protein expression. Previous studies have shown that a high-fat, refined sugar diet reduces levels of BDNF, which was associated with a deficiency in learning performance [357][358]. Our study suggests that mice maintained on the HFHS diet for 12 weeks showed a decrease in levels of BDNF protein, this is consistent with previous studies. Some interventions, such as exercise and an enriched environment can improve BDNF expression [359]. Vaynman et al. reported that exercise utilizes BDNF action to improve learning and memory, enhance cognitive function in rats [360]. In our study, we also observed enhanced BDNF level in the HFHS+Exercise treatment group compared to the HFHS group. Moreover, the improvement effect of genistein on BDNF expression have been reported in the ovariectomized rat model [315][361]. However, our results are novel as they provide the first evidence that genistein ameliorates the effect of a HFHS diet on BDNF expression.

Genistein is a phytoestrogen since it has a similar structure to the female hormone estrogen. Genistein can bind ER and previous findings suggest that genistein exerts its effect through modulating estrogenic pathways. For example, genistein enhanced the acetylcholinesterase activity of PC12 cells by binding to the ER [362]. Genistein also exerted anti-apoptotic actions by activating the ER in primary neuronal cells following glutamate exposure [344]. In our diet-induced obesity mouse model, we found that genistein ameliorates HFHS diet-induced apoptotic processes in the brain of mice. We then performed a cell culture study to examine if the ER is involved in the action of genistein.

The rat pheochromocytoma cell line PC12 was used in our study, which is widely accepted as an *in vitro* model for neurotoxicity research [363]. PC12 cells were exposed to the high concentration of glucose (3 times higher than the control medium) and PA in culture medium. PA is the predominant saturated fatty acid in the high-fat diet, which is also associated with accumulation of PA in the hypothalamus [364]. We observed that either HG, PA or a

combination of HG and PA treatment led to significantly decrease in PC12 cell viability. Alleviation of HG+PA-induced PC12 cell death was observed when genistein was used at doses 2 and 5 μM. However, the addition of ER antagonist tamoxifen had a robust effect against genistein actions on neuronal cell death.

Moreover, in our cell model, genistein also exhibited anti-apoptotic property. We observed that HG+PA exposure significantly decreased the expression level of anti-apoptotic protein Bcl-2, which is involved in the regulation of mitochondrial membrane permeability and the release of apoptogenic substances. Pre-treatment of genistein markedly increased the expression of Bcl-2 in HG+P-exposed cultures. This is in accordance with previous findings of Sonee et al, who indicated that the neuroprotective effect of genistein in cortical cells was attributed to the regulation of the Bcl-2 [365]. In addition, HG+PA exposure triggered the activation of the executioner of apoptotic cascades, caspase-3, and significantly increased the expression of caspase-3. Our results show that genistein also prevents the upregulation of caspase-3 in HG+PA-exposed cultures. Moreover, we observed that the addition of the ER antagonist tamoxifen reversed genistein downregulation of caspase 3 activity and upregulation of Bcl-2 expression with HG+P exposure, suggesting that these effects of genistein were ER-dependent. Collectively, our results demonstrate that genistein act as ER agonist in PC12 cells and activate estrogenic neural protective mechanisms. Since there are different subtypes of ER, further studies sought to determine if ER-mediated effects of genistein in PC12 cells were specific to a particular ER-subtype, ER-subtype specific antagonist could be used in cell culture.

5.6 Summary

In conclusion, our study demonstrates the possibility of a HFHS diet contributing to the neuropathology, including upregulation of Aβ and hyperphosphorylated tau proteins, downregulation of BDNF expression and activation of apoptotic cascades. Genistein supplementation and exercise training ameliorated the HFHS-related biochemical disturbances.

Moreover, our cell model demonstrates that the anti-apoptotic actions of genistein were selectively mediated by ER in PC12 cells following high-glucose and palmitate exposure.

Figures and figure legends

Figure 16

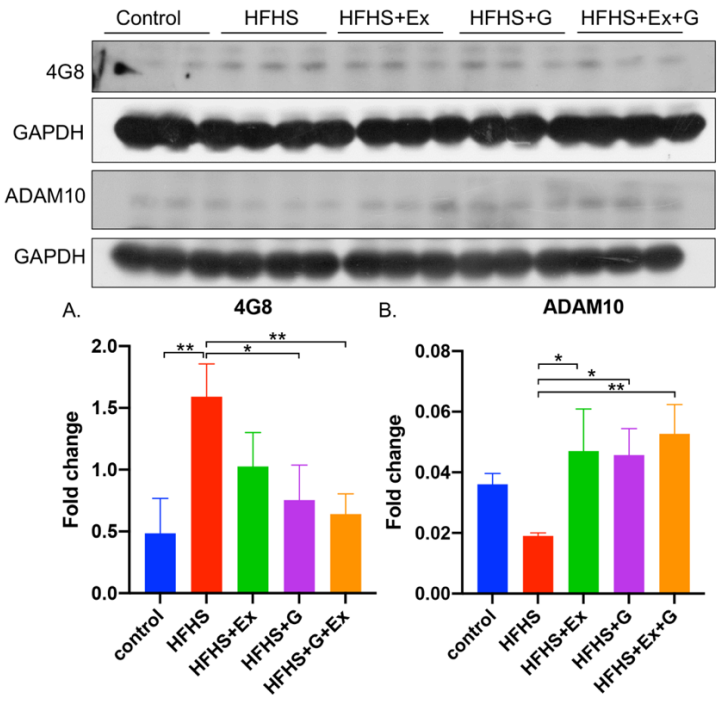


Fig.16 Effect of genistein or/and exercise on Aβ deposition in mice brain. (A) Representative blot images with the corresponding densitometry measurement of 4G8. (B) Representative blot images with the corresponding densitometry measurement of ADAM10. HFHS: high-fat, high-sugar diet; HFHS+Ex: HFHS+exercise; HFHS+G: HFHS+genistein; HFHS+G+Ex: HFHS+genistein+exercise. Data are presented as mean ± SEM for 3 mice per group. *$p \leq 0.05$, **$p \leq 0.01$.

Figure 17

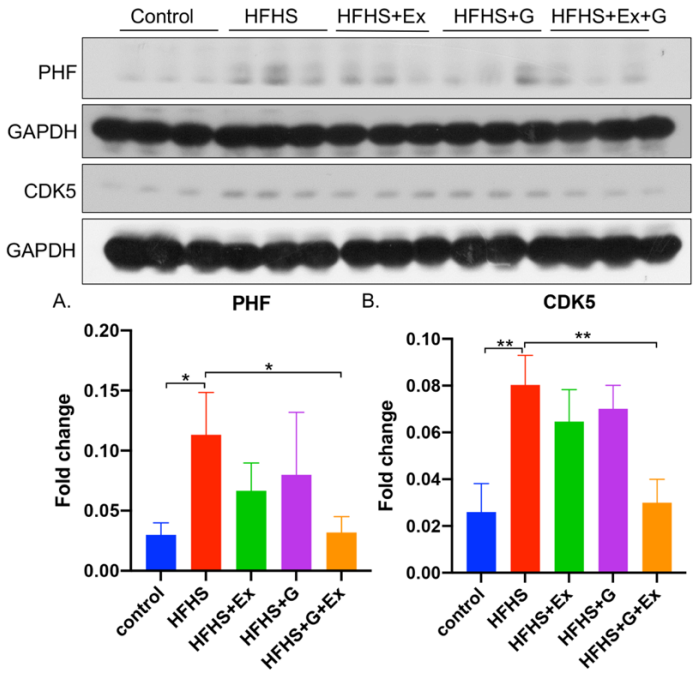


Fig.17 Effect of genistein or/and exercise on phosphorylation of tau in mice brain. (A) Representative blot images with the corresponding densitometry measurement of PHF. (B) Representative blot images with the corresponding densitometry measurement of CDK5. HFHS: high-fat, high-sugar diet; HFHS+Ex: HFHS+exercise; HFHS+G: HFHS+genistein; HFHS+G+Ex: HFHS+genistein+exercise. Data are presented as mean ± SEM for 3 mice per group. *$p \leq 0.05$, **$p \leq 0.01$.

Figure 18

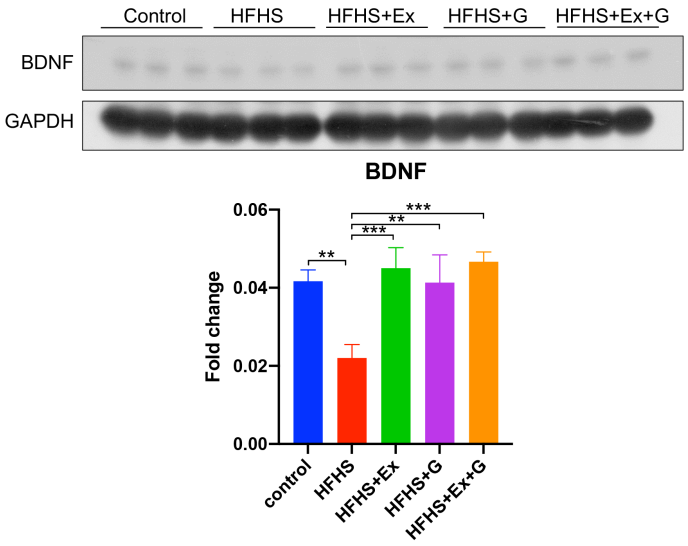


Fig.18 Effect of genistein or/and exercise on BDNF expression in mice brain. HFHS: high-fat, high-sugar diet; HFHS+Ex: HFHS+exercise; HFHS+G: HFHS+genistein; HFHS+G+Ex: HFHS+genistein+exercise. Data are presented as mean ± SEM for 3 mice per group. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Figure 19

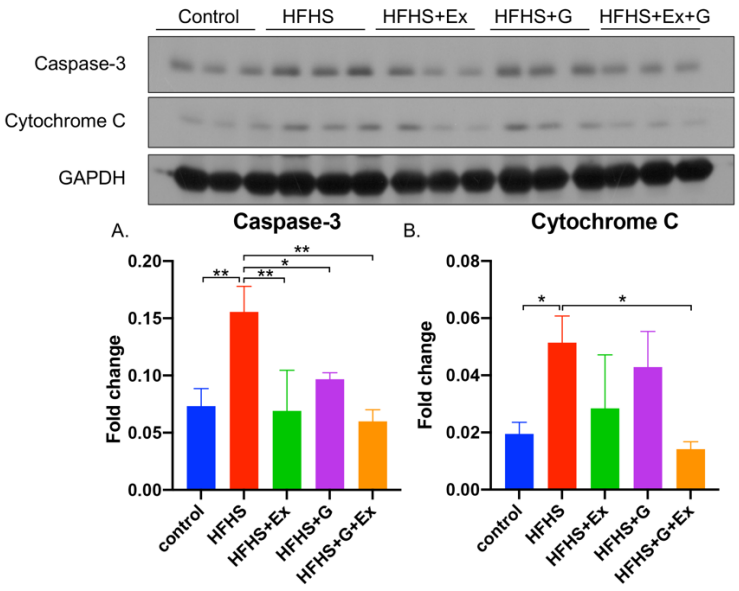


Fig.19 Effect of genistein or/and exercise on apoptosis in mice brain. (A) Representative blot images with the corresponding densitometry measurement of Caspase-3. (B) Representative blot images with the corresponding densitometry measurement of Cytochrome C. HFHS: high-fat, high-sugar diet; HFHS+Ex: HFHS+exercise; HFHS+G: HFHS+genistein; HFHS+G+Ex: HFHS+genistein+exercise. Data are presented as mean ± SEM for 3 mice per group. *$p \leq 0.05$, **$p \leq 0.01$.

Figure 20

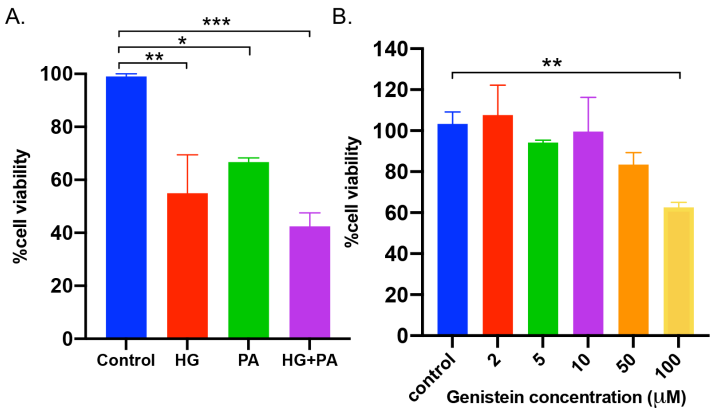


Fig.20 (A) Effect of high glucose or/and palmitate on PC12 cell viability. (B) Effect of genistein on PC12 cell viability. HG: high glucose; PA: palmitate; HG+PA: high glucose+palmitate. Data are presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 21

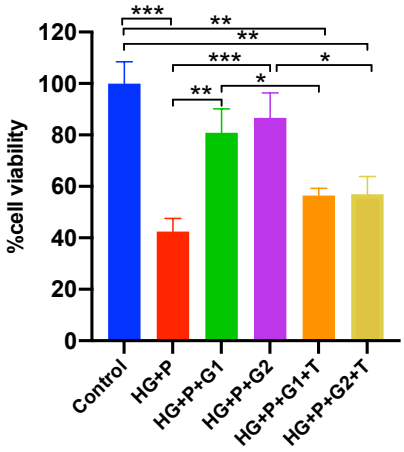


Fig.21 Effect of genistein alone or with tamoxifen on cell viability in high glucose and palmitate-treated PC12 cells. HG+P: high glucose+palmitate; HG+P+G1: high glucose+palmitate+2μM genistein; HG+P+G2: high glucose+palmitate+5μM genistein; HG+P+G1+T: high glucose+palmitate+2μM genistein+tamoxifen; HG+P+G2+T: high glucose+palmitate+5μM genistein+tamoxifen. Data are presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 22

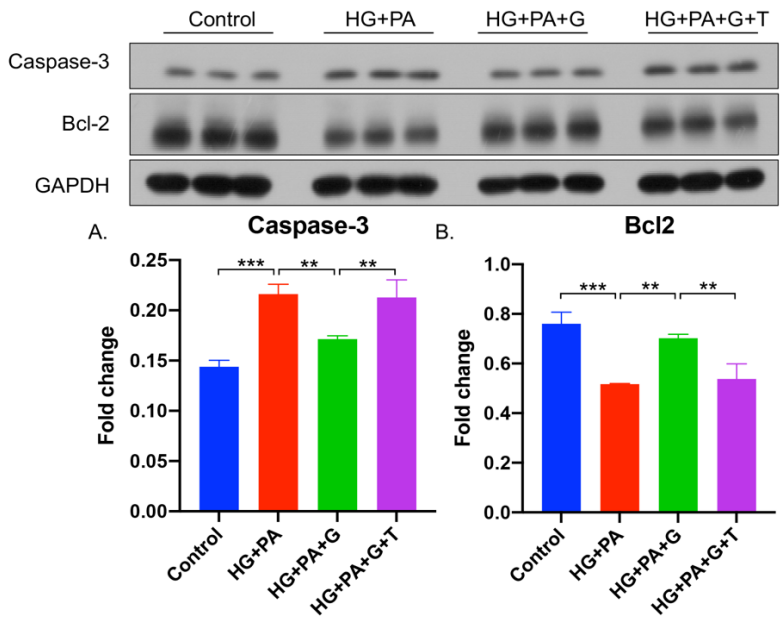


Fig.22 Effect of genistein alone or with tamoxifen on high glucose and palmitate-induced apoptosis in PC12 cells. (A) Representative blot images with the corresponding densitometry measurement of Caspase-3. (B) Representative blot images with the corresponding densitometry measurement of Bcl-2. HG+PA: high glucose+palmitate; HG+PA+G: high glucose+palmitate+5μM genistein; HG+PA+G+T: high glucose+palmitate+5μM genistein+tamoxifen. Data are presented as mean ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Chapter 6: Summary and conclusion

Bioactive compounds are being studied to evaluate their effects on general health and various diseases. In the first part of this study, the role of chronic resveratrol supplementation in AD mouse was identified. Aerobic exercise, as a non-pharmacological strategy to prevent AD, was also examined in this study. Our results indicate that consuming a diet rich in resveratrol and performing regular exercise was associated with decreased toxicity of Aβ oligomers, suppressed neuronal autophagy, reduced apoptosis, and upregulated key growth-related proteins in the brain of 3xTg-AD mice. Secondly, we investigated the brain damage in diabetic mice and explored the protective role of genistein in the brain of mice. Both genetically obese (ob/ob) and high-fat/high-sugar (HFHS) diet-induced diabetic mice were examined. We found the impaired insulin signaling, decreased neurotrophic support, increased apoptosis, and AD-related pathology in the brain of ob/ob or HFHS diet-fed mice. However, genistein supplementation ameliorated or reversed these negative consequences in mice brains. Furthermore, the results of cell culture study demonstrate that the anti-apoptotic actions of genistein were selectively mediated by estrogen receptor in high glucose and palmitate-treated PC12 cells. Taken together, our results emphasized the neuroprotective effects of bioactive compounds resveratrol and genistein. We also verified the association between type 2 diabetes and deteriorated brain function, and highlighted key biological pathway changes through which the genistein supplementation may protect against diabetes-induced brain damage.

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