PHARMACOLOGICAL ACTIONS OF DANGGUI BUXUE TANG ON OXYGEN-GLUCOSE DEPRIVATION AND REOXYGENATION INSULTED MOUSE BRAIN ENDOTHELIAL CELLS AND OVARIECTOMIZED SPRAGUE DAWLEY RATS

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CERTIFICATE OF ORIGINALITY

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Abstract

Menopause marks the permanent end of menstruation, resulting from decreased ovarian follicle activity and hormonal changes. The notable drop in estrogen level is a key risk factor for vascular dementia in menopausal women, which is characterized by cerebral hypoperfusion and disruption of the blood-brain barrier. While hormone replacement therapy remains the main treatment for menopausal symptoms, its long-term use is associated with increased risks of chronic diseases, limiting its clinical use in managing menopausal symptoms. Hence, there is an urgent need for safe and effective alternative therapies.

Danggui Buxue Tang (DBT), a traditional Chinese herbal formula made from *Astragali Radix* (Huangqi) and *Angelicae Sinensis Radix* (Danggui), is known for its estrogenic and osteogenic properties and is widely used among menopausal women in Asia. Despite its widespread use, the effects of DBT on cerebral endothelial cells and cognitive function in ovariectomized rats are not yet fully understood. We hypothesized that DBT may help prevent menopause-related cognitive decline via upregulation of estrogen receptor (GPR30) and activating the Nrf2/HO-1 signalling pathway.

The effects of DBT on the proliferation and migration of bEnd.3 cells were assessed using MTT and wound scratch assays, respectively. The expression of tight junction proteins were examined by western blotting, and ROS levels were evaluated using DCFDA staining. Our result showed that DBT (0.01-10 mg/mL) increased proliferation and migration of bEnd.3 cells in a concentration-dependent manner. Cell viability was markedly reduced by

60% following oxygen-glucose deprivation/reperfusion (OGD/R). DBT (0.01 – 3 mg/mL) suppressed the OGD/R-induced cell death in a concentration-dependent manner. Additionally, reductions in ZO-1 and Claudin-5 protein expression caused by OGD/R were normalized by DBT (0.1–3 mg/mL). Significant reductions in ROS levels were also observed when cells were treated with 0.3–3 mg/mL DBT.

In *in vivo* study, ovariectomized Sprague Dawley rats demonstrated the effects of 8-week DBT administration on cognitive function and brain histology. Immunohistochemical analysis revealed that DBT treatment (3 g/mg/day) attenuated OVX-induced astrocyte reactivity, as evidenced by reduced GFAP expression and normalized astrocyte morphology in the hippocampus. While DBT showed limited effectiveness in restoring vascular density, it partially preserved vessel morphology as indicated by CD31 immunostaining. Besides, cognitive impairment was established in ovariectomized Sprague Dawley rats as shown by the results of the Morris water maze, novel object recognition and rotarod test. Following the 8-week feeding of DBT to ovariectomized rats, the cognitive deficits moderately improved.

In conclusion, this study demonstrated, for the first time, that DBT as a classical Chinese formula can protect brain endothelial cells from OGD/R insult and improve cognitive functions in cognitively impaired ovariectomized rats. These findings support that DBT could be an alternative therapy for the management of menopause-associated vascular dementia.

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1 Introduction

1.1 Menopause and Postmenopausal Syndromes

1.1.1 Causes and Pathophysiology

Menopause, which is the permanent cessation of menses, is characterized by the decline of ovarian follicular activity and hormone fluctuation, for instance, estradiol, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and progesterone. (Burger et al., 2002). Majority of women experience spontaneous menopause between their 40s - 50s, with an average age of 51-year-old (Greendale et al., 1999). Prior to the onset of amenorrhea, women first experience perimenopause, during which hormonal levels begin to oscillate. This phase is marked by elevated FSH and LH levels resulting from a reduction in the number of mature follicles produced by the ovaries, while estrogen levels fluctuate and gradually decline. Besides, some women undergo induced menopause because of oophorectomy, which leads to a rapid and significant decrease in hormone levels within a single day, contrasting sharply with the gradual hormonal changes seen in spontaneous menopause. Among these symptoms, cognitive impairment and emotional disturbances are particularly noteworthy; many women report difficulties with memory, attention, and mood regulation during this transition. These cognitive and emotional challenges are believed to be closely linked to the substantial decline in estrogen levels that occurs during menopause (Albert & Newhouse, 2019; Russell et al., 2019).

The vascular contributions to cognitive decline are equally significant. Menopause-associated estrogen depletion exacerbates oxidative stress, a key driver of vascular

dementia (VaD). In ischemic brains, reactive oxygen species (ROS) and reactive nitrogen species (RNS) overwhelm antioxidant defenses, damaging cerebral vasculature and neurons (Shi et al., 2012). Estrogen normally upregulates antioxidant enzymes like superoxide dismutase (SOD) and catalase, but its decline during menopause leaves the brain vulnerable to ROS-mediated endothelial dysfunction, blood-brain barrier (BBB) leakage, and neuroinflammation (Bellanti et al., 2013; Xiao et al., 2018). Chronic hypoperfusion from microvascular injury, which is common in postmenopausal women, further accelerates white matter damage and neuronal apoptosis which are hallmarks of VaD (Rajeev et al., 2023).

1.1.2 Current Treatment for Menopausal Women

To alleviate distressing symptoms in menopausal women, hormone replacement therapy (HRT) is often used by taking either estrogen alone or combined with progesterone orally, transdermally, or intravaginally. It was approved by the FDA for treating hot flashes and osteoporosis in the 80s and it started to be a popular therapy to conserve femininity. After the approval, numerous studies have been conducted to investigate the benefits of HRT. It was suggested that HRT was useful in preventing chronic disease, for instance, coronary artery disease and stroke. It could also prolong women's life expectancy (Grady et al., 1992). However, debate on the safety of HRT has continued for decades since the Women's Health Initiative (WHI) reported that chances of breast cancer, coronary heart disease, and stroke increased in post-menopausal women undertaking HRT. Some trials discovered that estrogen can no longer exert neuroprotection in aged women with pre-existing coronary heart disease, showing that estrogen might not reverse damaged endothelium (Rossouw,

2002). Furthermore, there are conflicts that HRT might be responsible for the higher risk of Alzheimer's disease in women. A case-control study comprised 230,580 women conducted in Finland from 2005 to 2011 revealed that the use of estrogen and progesterone had a positive relationship with the pathogenesis of Alzheimer's disease (Irntiaz et al., 2017).

Emerging evidence underscores the importance of timing in HRT initiation for cognitive health, as encapsulated in the critical window hypothesis. Observational studies suggest that initiating HRT during perimenopause or early postmenopause (within 5–10 years of menopause onset) may confer neuroprotective benefits. For instance, women starting HRT closer to menopause exhibited better cognitive performance and reduced AD risk, likely due to estrogen’s role in preserving synaptic plasticity, neurogenesis, and cerebral blood flow (Mills et al., 2023). While neuroimaging data linked early postmenopausal HT to enhanced prefrontal cortex activity during cognitive tasks, counteracting age-related declines (Girard et al., 2017).

Conversely, initiating HRT later in life (≥10 years postmenopause or after age 65) is associated with increased dementia risk. A landmark study found late-life HRT initiation raised dementia risk by 48%, attributed to exacerbation of vascular pathology and neuroinflammation in aging brains (Whitmer et al., 2011). Randomized trials, such as the WHI, reinforced these risks, showing that conjugated equine estrogens combined with medroxyprogesterone acetate increased dementia incidence in women aged over 65 (Shumaker et al., 2003). These findings align with preclinical models where early estrogen

replacement rescued synaptic deficits, while late administration failed to reverse established neurodegeneration.

The critical window theory posits that estrogen's neuroprotective effects depend on initiating therapy before irreversible vascular or neuronal damage occurs. Early HRT may mitigate oxidative stress, enhance antioxidant defenses, and maintain BBB integrity. Beyond this window, delayed HRT initiation fails to replicate these protective effects, potentially exacerbating neurodegeneration and cognitive decline. Thus, aligning therapeutic strategies with this temporal framework could optimize neuroprotection while minimizing risks, offering a targeted approach to reducing dementia incidence in aging women.

1.2 Vascular Dementia

1.2.1 Causes and Risk Factors

VaD is mainly caused by decreased blood flow to the brain, which deprives neurons of vital nutrients, resulting in cell death and shrinkage of brain tissue (Venkat et al., 2015). This blood supply impairment can be caused by a variety of vascular problems, such as strokes or multiple mini strokes that cause multi-infarct dementia. Other cardiovascular diseases such as heart disease, hypertension, hyperlipidemia, and diabetes, as well as atherosclerosis, could be risk factors for VaD (Morgan & Mc Auley, 2024). A common feature across these risk factors is the elevated production of ROS, which is believed to be one of the main contributors to the development of VaD. ROS are highly reactive molecules that contain oxygen and are naturally produced as byproducts of cellular metabolism, especially during mitochondrial respiration. Common types of ROS include

superoxide anion (O_2^-), hydrogen peroxide, and hydroxyl radical. Oxidative stress occurs when there is an imbalance of oxidants and antioxidants in the body. Normally, the body has its defence system comprising enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase, along with non-enzymatic antioxidants like vitamins C and E (Cervellati & Bergamini, 2016). These antioxidants work together to neutralize ROS and protect cells from oxidative damage. However, when oxidative stress persists due to increased ROS production or diminished antioxidant capacity, it can lead to significant cellular injury. Studies have shown that estrogen could protect cells from oxidative stress by the modification of antioxidant enzymes (Nathan & Chaudhuri, 1998; Nilsen, 2008). For instance, the intracellular ROS production was reduced by the upregulation of manganese SOD and extracellular SOD in vascular smooth muscle cells pretreated with 1 μmol/L of estrogen before exposing to 1 μmol/L Angiotensin II (Strehlow et al., 2003). When the estrogen level decrease during menopause, the protective effect will be diminished and that could explain why VaD has higher prevalence in menopausal women than man.

1.2.2 Blood-Brain Barrier Dysfunction in VaD

In addition to these factors, changes in the blood-brain barrier (BBB) play a major role in the pathophysiology of VaD. The BBB, which serves as a protective barrier between the bloodstream and the brain, is essential for maintaining the brain's microenvironment. However, in VaD, BBB breakdown is a common consequence of the underlying vascular damage and oxidative stress, leading to increased permeability which allows harmful substances to infiltrate the brain.

Several key cell types contribute to the structure and function of the BBB, each plays a critical role in maintaining its integrity. Firstly, endothelial cells, which form the inner lining of blood vessels, are the primary structural components of the BBB. These cells are tightly connected by tight junctions that control the passage of substances between the bloodstream and the brain, ensuring selective permeability (Yazdani et al., 2019). In VaD, endothelial dysfunction is a hallmark of BBB breakdown, often characterized by alterations in tight junctions, which are induced by pro-inflammatory cytokines and other molecular signals (Wang et al., 2018). Concurrently, the reduced blood flow associated with ischemic events and chronic hypoperfusion in VaD exacerbates this endothelial damage (Yu et al., 2022). The resulting oxygen and nutrient deprivation triggers a cascade of detrimental effects, including endothelial cell apoptosis and increased BBB permeability. The BBB disruption further compromises cerebral perfusion and accelerates the progression of VaD pathology (Rajeev et al., 2022).

Surrounding the endothelial cells are pericytes, contractile cells that wrap around the capillaries and microvessels of the brain (Armulik et al., 2011). Pericytes are crucial for stabilizing the endothelial cell layer and regulating the blood-brain flow within the capillary network. They also play a role in the development of tight junctions and contribute to the structural integrity of the BBB. Pericytes are involved in the regulation of blood flow through the capillaries by contracting and relaxing, thus controlling the microcirculation and supporting the overall function of the BBB (Bell et al., 2023). Loss of pericytes or their dysfunction has been linked to BBB breakdown in various neurovascular diseases, including VaD.

Thirdly, astrocytes are the star-shaped glial cells in the brain that are important for BBB functions. Astrocytic end-feet surround the blood vessels, and they are involved in signalling to the endothelial cells, helping to regulate the permeability of the BBB (Heithoff et al., 2021). However, in response to injury, oxidative stress, or chronic inflammation, astrocytes can become activated and release pro-inflammatory cytokines like interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) (Cai et al., 2010). In the context of VaD, reactive astrocytes can worsen BBB breakdown, promote neuronal cell death, and contribute to the cognitive decline seen in the disease.

Finally, microglia that serve as the brain's first line of defence against injury or infection are the resident immune cells of the central nervous system. These cells are constantly monitoring the brain for any signs of damage or pathogens. In the case of neurovascular diseases like VaD, microglia can become activated by the presence of inflammatory cytokines, oxidative stress, or other insults (Qin et al., 2023). While their role is essential in protecting the brain from harm, chronic activation of microglia can contribute to the breakdown of the BBB by releasing pro-inflammatory cytokines and other mediators that disrupt the endothelial cell layer (Sweeney et al., 2018). This activation further exacerbates neuroinflammation and neuronal damage, creating a vicious cycle of BBB disruption and neurodegeneration.

By controlling the flow of chemicals between the bloodstream and brain tissue, the BBB is essential to preserve the microenvironment of the brain. Due to the increased permeability caused by BBB malfunction in VaD, dangerous chemicals can enter the brain and worsen

neuronal damage. The integrity of the BBB can be compromised by various insults linked to VaD, including acute ischemia, chronic hypoperfusion, and hypertension, which contribute to inflammation and leakage into the brain (Hussain et al., 2021; Ueno et al., 2016). The disruption of the BBB not only promotes neuroinflammation but also interferes with the mechanisms of waste clearance and nutrition transport that are vital to the health of neurons.

The interplay of these cells forms a dynamic neurovascular unit that preserves the brain's delicate microenvironment by regulating molecular transport into the brain. In conditions like VaD, dysfunction or injury to any of these cells can lead to a compromised BBB, allowing neurotoxic molecules, immune cells, and other damaging agents to infiltrate the brain and contribute to cognitive decline.

1.3 Estrogen and Estrogen Receptors

1.3.1 Role of Estrogen in Neuroprotection and Emotional Health

Estrogen is mainly produced by the ovaries in women and is found to be neuroprotective in various studies. Numerous animal studies have demonstrated that males and females can respond to brain injury differently (Ma et al., 2019; Rubin & Lipton, 2019). The relationship between sex differences and brain injury has been demonstrated in several *in vivo* studies. For instance, it has been shown that female mice resisted middle cerebral artery occlusion (MCAO)-induced injury with smaller infarct size when compared to the males (Park et al., 2006). The sex-specific effects noted in these studies are likely attributed to inherent hormonal differences, especially the elevated and cyclical estrogen levels in

females and the influence of the estrous cycle, which contribute to estrogen receptor-driven changes in the female hippocampus (Waters et al., 2009). In addition to promoting the development of new brain cells and enhancing blood flow, estrogen is also known to safeguard the brain from oxidative stress and inflammation. Studies have shown that estrogen may have neuroprotective effects in ischemic stroke. For example, administration of estrogen before or after a stroke decreased the infarct size and improved neurological outcomes in the rodent model of ischemic stroke (Dubal & Wise, 2002; Suzuki et al., 2009). Estrogen is also believed to be protective against the blood-brain barrier, which is made up of tight junction proteins between endothelial cells (Kuruca et al., 2017). The depletion of estrogen in menopausal women seems to result in a higher prevalence of stroke, which progressively leads to VaD (Gannon et al., 2023). All these demonstrate that estrogen has neurotrophic effect, which explains the frequent occurrence of VaD among menopausal women.

The decline in estrogen levels during menopause is closely associated with an increased risk of emotional disturbances, particularly depression, which in turn may contribute to cognitive deficit (Greendale et al., 2020). This relationship is supported by a large-scale retrospective cohort study conducted in South Korea, which followed over 1.6 million women aged 40 to 60 years who were free of dementia at baseline (Yoo et al., 2024). The study found that women diagnosed with depression prior to the onset of dementia had a significantly higher risk of developing young-onset dementia, including both Alzheimer's disease and VaD. Specifically, the adjusted hazard ratio for VaD was 2.03 in premenopausal women and 1.85 in postmenopausal women with depression, compared to

their non-depressed counterparts. Furthermore, the study identified that reproductive factors, such as early menopause (before age 40), further amplified the risk of young-onset dementia, suggesting that prolonged estrogen deficiency may exacerbate vulnerability to neurodegenerative processes. Huang et al. (2015) established menopause depression SD rat model by ovariectomy in combination with chronic unpredictable mild stress to increase levels of stress hormones such as corticotropin releasing hormone, adrenocorticotropic hormone and cortisol. It was also reported that ovariectomized Wistar rats had elevated immobility time and reduced swimming time in forced swimming test, indicative of depressive-like behaviour (Fedotova, 2012). Collectively, these data underscore the vital role of estrogen in regulating mood and emotional stability during menopause. Moreover, the co-occurrence of depression and estrogen deficiency may synergistically exacerbate cerebrovascular dysfunction, thereby increasing the vulnerability of menopausal women to VaD. This triad highlights the importance of addressing both emotional and vascular health in the management of menopausal women to mitigate the risk of cognitive decline.

Estrogen exerts a significant influence on cognitive function and emotional well-being through its interaction with specific receptors and the activation of diverse signalling pathways. Estrogen binds to both traditional estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) and the novel receptor GPR30, causing genomic and non-genomic effects (Acconcia & Kumar, 2006; Bjornstrom & Sjoberg, 2005). In the genomic estrogen pathway, estrogen enters the nucleus after binding to the intracellular ERα and ERβ to form the estrogen-receptor dimer complex. The complex functions as a transcription factor that controls gene transcription in the nucleus by binding to estrogen response sites or activator

protein-1 and specificity protein-1 on the estrogen-responsive gene promoters. The regulation of autophagy, proliferation, apoptosis, survival, differentiation, and vasodilation is ultimately governed by the resulting gene products. Due to the intracellular position, activation usually takes hours or longer, which results in a gradual effect. On the other hand, by binding to membrane-bound ERα, ERβ, and GPR30, estrogen also mediates the non-genomic signalling pathway, which happens instantly. This pathway quickly activates nuclear transcription factors by controlling the opening of ion channels or the activation of related enzymes like mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and Ca^{2+} mobilization (Prossnitz & Maggiolini, 2009; Pupo et al., 2016).

1.3.2 GPR30: A Novel Estrogen Receptor in Brain Function

The seven-transmembrane G protein-coupled receptor GPR30, located in the endoplasmic reticulum, is an important mediator of the effects of estrogen. It was first categorized as an orphan receptor and was later found to be a particular receptor for estrogen, exhibiting a high affinity and special signalling properties that set it apart from the traditional estrogen receptors (Prossnitz & Barton, 2009). It is found in different tissues, including the central nervous system, adipose tissue, vascular tissue, and reproductive systems, suggesting its involvement in a variety of physiological processes. While traditional estrogen receptors have been extensively studied regarding their roles in cognition, GPR30's contributions are gaining attention. Studies have demonstrated that selective deletion of GPR30 in astrocytes, rather than neurons, resulted in impaired learning and memory in female mice (X. Wang et al., 2023). Specifically, the study indicated that GPR30 deletion increased the transcription of deleterious A1 astrocytes and decreased A2 astrocytes through the

modulation of PJA1 expression and Serpina3n. Astrocytes are star-shaped glial cells that play essential roles in supporting neurons, maintaining the blood-brain barrier, and regulating the brain's microenvironment. Recent research has shown that astrocytes can adopt different reactive states in response to injury or disease, notably the neurotoxic A1 phenotype and the neuroprotective A2 phenotype (Ding et al., 2021). A1 astrocytes, induced by pro-inflammatory signals, contribute to neuronal damage and synaptic loss, whereas A2 astrocytes, stimulated by anti-inflammatory cues, promote neuronal survival and tissue repair (Ding et al., 2021). The imbalance of A1/A2 leads to destroyed synapses and reduced neurotrophic factors, contributing to neurodegeneration and cognitive decline (Ding et al., 2021). Additionally, GPR30 has been implicated in enhancing spatial memory and social cognition (Hadjimarkou & Vasudevan, 2018). Activation of this receptor has been shown to induce rapid estrogenic responses that improve hippocampal memory by involving the regulation of synaptic plasticity (Briz et al., 2015; Waters et al., 2015). Research also indicates that GPR30 mediates these effects through various signalling pathways, including the PI3K/Akt pathway, which is known to support neurogenesis and protect neurons from excitotoxicity (Peng et al., 2024).

In addition to its influence on neuropsychological function, GPR30 is believed to take part in regulating mood disturbances but it is still controversial. Kang et. al. (2024) investigated the effect of paeonol in alleviating neuropsychiatric symptoms in OVX ICR mice by performing various behavioural tests. The administration of paeonol effectively improved depression and cognitive impairments by restoring GPR30 expression in the prefrontal cortex and hippocampus of OVX mice and the results were comparable to those achieved with estrogen administration. Yet, another finding suggested that the activation of GPR30

by G1 agonist could lead to anxiogenic effect (Kastenberger et al., 2012). These conflicting results highlight the complexities of GPR30's role in mood regulation, underscoring the need for further research to clarify its mechanisms and implications in emotional health.

1.3.3 Sex Differences in Neuroprotection

Notably, sex differences have been increasingly recognized as significant factors influencing the outcomes and mechanisms of cerebrovascular disease. One of the critical aspects is the role of GPR30. Broughton et al. (2013) examined the expression of GPR30 in the brains of C57Bl/6J mice and human by immunohistochemistry, western blot and quantitative real-time PCR. The receptor expression was found to be increased in the peri-infarct regions of the brain in male mice after stroke, while OVX female mice showed either no change or a decrease in GPR30 expression. Most importantly, GPR30 is expressed in human brain. In contrast to control males, GPR30 expression was shown to be higher in the post-stroke brain of male patients, whereas immunoreactivity in female patients is not significantly different (Broughton et al., 2013). Apart from expression, another study demonstrated the differing effects of GPR30 activation on stroke outcomes in males and females (Broughton et al., 2014). It was shown that G1 (a selective GPR30 agonist) activated GRP30 leading to increased infarct volume and neurological deficits, as well as increased apoptosis in neurons in male mice with focal cerebral ischemia. These harmful effects were mitigated by G15 (a GPR30 antagonist). Conversely, in female mice, the impact of GPR30 activation varied based on ovarian status. In OVX females, GPR30 activation by G1 improved stroke outcomes with reduced infarct volume and neurological deficits via limiting apoptosis; these benefits were reversed by co-administration of G15.

However, in intact females, G1 had no significant effect on stroke outcomes, likely due to the high levels of circulating estrogen that may counteract the effects of GPR30 activation.

Several studies have shown that the severity of cerebrovascular disease is influenced by gender-specific differences in the production of ROS. For instance, female mice show significantly lower levels of hydrogen peroxide and superoxide production and exhibit weaker contractions in their cerebral arteries in response to angiotensin II stimulation than male mice (De Silva et al., 2009). This study showed that the differences in ROS production are partly linked to the activity of Nox2. In male mice, Nox2 deletion led to a significant reduction in superoxide production, whereas this genetic modification did not affect females, indicating a potential protective mechanism at play in females. Similar work done by Miller et al. (2007) revealed that Nox1 and Nox4 expressions in the basilar arteries of male SD rats were higher than that in intact female by 2.4- and 2.8-fold, respectively, which could be attributed to the reduced production of ROS in female rat arteries under 100 μmol/L NADPH stimulation. Also, male rats exhibited about 2-fold greater O_2^- production than females. Interestingly, OVX rats had 3 times higher O_2^- production than intact female. With the administration of 17β-estradiol, the superoxide level dropped and NADPH-induced relaxations were mitigated in OVX rats. These findings suggested that the lower NADPH-oxidase activity and reduced O_2^- production observed in female rats are closely linked to estrogen's influence on vascular function. Although this work did not directly compare OVX and male rats, the findings imply that the increased oxidative stress observed in OVX rats was comparable to males in terms of cerebrovascular risk factors, primarily due to the absence of estrogen.

1.4 The Role of Traditional Chinese Medicine in Treating Menopausal-Caused VaD

Traditional Chinese Medicine (TCM) is a holistic medical system with a history spanning over 5,000 years, rooted in the principles of balancing yin and yang and maintaining the free flow of vital energy (Qi) through meridians in the body (Matos et al., 2021). It employs practices such as acupuncture, herbal remedies, and mind-body exercises to restore harmony between physical, emotional, and environmental factors (Matos et al., 2021). TCM has gained in popularity for the management of AD in recent years because of its "multi-target, multi-system, multi-link, and multi-pathway capacity" (Chen et al., 2020). Little side effects associated with TCM also make it a popular medication for various diseases. According to the TCM theory, 'Qi" and "blood" are interdependent. "Qi" indicates a balanced immune and endocrine system, and it is responsible for moving and transforming blood in the body. If Qi is weak or blocked, it can lead to stagnation or deficiency of blood, causing various health problems (H. Q. Lin et al., 2017). Similarly, if the quality or quantity of blood is compromised, it can affect the flow and quality of Qi, leading to imbalances and health issues. Furthermore, kidney insufficiency and imbalance of kidney yin and yang are strongly related to menopausal symptoms (Scheid, 2007). Kidneys are responsible for a functional visceral system to maintain and regulate growth, maturation, and aging. While reduced kidney essence during aging resulted in the diminished vitality of yin-yang transformation, it is suggested to replenish kidney Qi to treat menopausal problems. Apart from that, these conditions also account for cerebrovascular diseases like VaD. (Bai & Zhang, 2021).

TCM offers a distinctive perspective on VaD, emphasizing the interconnectedness of the body's systems and the balance of vital energies. While modern medicine defines VaD as a neurocognitive dysfunction stemming from cerebrovascular disease, TCM interprets it as a disruption of overall bodily equilibrium (Bai & Zhang, 2021). The etiology of VaD in TCM is multifaceted, often attributed to kidney deficiency, liver depression, phlegm turbidity, blood deficiency, and blood stasis (Cao & Chen, 2018; Wang & LI, 2006; Zheng et al., 2014). Kidney deficiency is viewed as a fundamental factor since the kidneys store essence and produce marrow that nourishes the brain. Liver function is also critical, as it regulates emotions and ensures the smooth flow of Qi and blood essential for cognitive health. Phlegm turbidity refers to pathological fluid accumulation that obstructs energy and nutrient flow to the brain, while blood deficiency and stasis can impair nourishment to brain tissues. Consequently, TCM treatments aim to restore balance by nourishing the kidneys, strengthening the spleen and Qi, dissipating phlegm, promoting blood circulation, calming the liver, and nourishing the mind. This holistic approach is particularly relevant to cerebral small vessel disease (CSVD) which refers to conditions affecting the small blood vessels in the brain, for example, narrowing, thickening, or hardening of arterioles, venules, and capillaries (Gao et al., 2021). These conditions often lead to impaired blood flow and various neurological issues, including VaD. It is suggested that postmenopausal women are prone to these vessel lesions (Gao et al., 2021; Z. Wang et al., 2023). In TCM, cerebral small vessels are referred to as collaterals, which are crucial for Qi and blood circulation (Wu, 2009). To be specific, collaterals refer to the channels branching off from the main meridians for Qi and blood to circulate. Blockages in these collaterals can lead to various issues such as blood stasis and phlegm accumulation. TCM recognizes that issues

related to CSVD originate in the brain but are closely tied to the heart, kidneys, liver, and spleen (Hao et al., 2023). They also believed that proper functioning of these organs is essential for brain health.

For example, the Jiannao Yizhi Formula (JYF), which nourishes the mind and strengthens Qi, has been shown in controlled clinical studies to enhance cognitive performance in both Alzheimer's disease and VaD patients, with mechanistic studies indicating that JYF reduces neuronal apoptosis by modulating Bcl-2 and Bax protein levels (Bai & Zhang, 2021). Similarly, classic formulas such as Taohong Siwu decoction, which promotes blood circulation and removes blood stasis, have demonstrated neuroprotective effects in animal models of VaD by upregulating vascular endothelial growth factor (VEGF), enhancing angiogenesis, and increasing neuronal survival in the hippocampus (Tao et al., 2022)

TCM typically employs a multi-target approach in its treatment, using herbal formulas composed of various medicinal plants that act on multiple organs simultaneously. These formulas may target not only the brain's blood vessels but also the heart, liver, spleen, and kidneys, thus addressing the root causes of disease (Li et al., 2021). In contrast, there are no medications specifically approved by regulatory agencies such as the U.S. Food and Drug Administration (FDA) for the treatment of VaD and treatment in Western medicine for VaD only focuses on managing risk factors, especially controlling blood pressure, preventing stroke, and using medications such as antiplatelet agents, statins, and thrombolytics (Baskys & Cheng, 2012; Pan et al., 2022). Thus, the holistic and integrated

approach of TCM provides a comprehensive treatment strategy that considers both the brain and the interconnected organs, promoting overall wellness and preventing recurrence.

In TCM, both single herbs and complex formulations are being explored for their potential to alleviate memory issues, reduce anxiety, and provide neuroprotection. There is an increasing number of women who are seeking natural products or TCM to compensate for the loss of estrogen because of their phytoestrogen-rich compositions that mimic endogenous estrogen activity. Phytoestrogens are defined as "plant secondary metabolite that is structurally and functionally similar to mammalian estrogens, which have been shown to have various health benefits in humans." (Patra et al., 2023). TCM formulations are increasingly sought after for their potential to alleviate menopausal symptoms while minimizing risks associated with conventional HRT. Additionally, meta-analysis indicates that combining TCM with low-dose HRT could enhance efficacy while reducing hormonal side effects, underscoring its role as a synergistic or standalone alternative (Kou et al., 2016). The following section will introduce some of these herbal treatments and their emerging roles in addressing menopause-related cognitive decline.

1.4.1 Single Herbs for menopause-caused cognitive decline

Pueraria lobata

Pueraria lobata (kudza), which originated in Eastern Asia, is an ancient Chinese medicinal plant that could alleviate alcohol-induced liver poisoning and treat diabetes as mentioned in Bencao Gangmu (Compendium of Materia Medica). It is rich in various bioactive constituents, including isoflavones (e.g. daidzein, daidzin and puerarin). Previous research

has shown that puerarin extract from *Pueraria lobata* exhibits protection against oxidative stress apoptosis (Jiang et al., 2003; Xiong et al., 2006; H. Y. Zhang et al., 2011) as well as cognitive impairment (Wu et al., 2017; Zhang et al., 2015; Zhou et al., 2014). Li et al. (2010) found that intrahippocampally injected Aβ caused hippocampus neurons to undergo apoptosis and impairment in spatial memory, together with the activation of caspase 9. Yet, the Aβ (1-42)-induced cognitive impairment was lessened by puerarin administration, which also stopped the hippocampal apoptosis from increasing by activating Akt and phosphorylating BAD. Another *in vitro* experiment conducted by Li et al. (2017) explored the relationship between neuroprotection and estrogen receptors. Puerarin appeared to significantly suppress beta-amyloid-induced primary cortical neuron death and promote neurite growth through ERβ upregulation. The ER involvement was also further confirmed by ICI182,780 ER inhibition.

A considerable number of *in vivo* experiments have shown that puerarin is effective in attenuating cognitive deficits. It was found that puerarin alleviated bilateral common carotid arteries occlusion-induced VaD through TRPM2/NMDR and Bax/Bcl2 pathway, which was evidenced by the decreased ROS level, reversed neuron cells apoptosis, vasodilation, as well as the shortened escape latency in the Morris Water Maze test (Zhu et al., 2021). Furthermore, an animal study conducted by Anukulthanakorn et al. (2016) examined and compared the neurotherapeutic efficacy of puerarin and 17β-estradiol in OVX rats with early- and late-stage cognitive impairment. Results found that puerarin decreased amyloid precursor protein (APP) mRNA level and notably reduced ERβ mRNA

level in the OVX rats with early-stage cognitive impairment but not ERα mRNA level at either stage.

The evidence to support that puerarin could improve cognitive decline has been immensely researched in the current decades and various mechanisms are involved. The majority of the findings believed that puerarin can promote neuroprotection by reducing ROS levels, lowering the levels of Aβ deposition and preventing neuroinflammation.

Ligusticum chuanxiong

Ligusticum chuanxiong, commonly known as Chuanxiong or Sichuan Lovage, is a traditional Chinese medicinal herb deeply rooted in the history of TCM. Native to China, particularly in the Sichuan and Yunnan provinces, this plant is valued for its therapeutic properties concentrated in its roots and rhizomes. Throughout centuries, *Ligusticum chuanxiong* has been recognized for its role in promoting blood circulation and is often employed to invigorate the blood, addressing conditions associated with poor circulation (Ran et al., 2011). In TCM, this herb has historical significance in addressing cardiovascular health, including its application in stroke prevention due to its perceived ability to enhance blood flow and reduce clot formation (Chen et al., 2018). Its major chemical components include essential oil (EO), alkaloids, ligustilide, and ferulic acid (Liang et al., 2005). Among all the constituents, chuanxiongzine, which is also known as tetramethylpyrazine (TMP) has demonstrated antioxidant, anti-inflammation, anti-apoptotic and neuroprotective properties (J. G. Lin et al., 2022). A study evaluated the neuroprotection of TMP in permanent cerebral ischemic injured rats and demonstrated that

TMP (20 mg/kg) can indeed inhibit neutrophil activation through Nrf2/HO-1 pathway, Akt phosphorylation and ERK phosphorylation (Liang et al., 2005). The inhibition of neutrophils can prevent chronic inflammation in the brain and stop the progression of neurodegeneration. Additionally, TMP can slow down the inflammatory course of AD by significantly reducing the generation of NO, TNF-α, IL-1β, ROS, monocyte chemoattractant protein-1, and NF-κB activation in primary microglial cells when stimulated by Aβ25–35 and IFN-γ (Kim et al., 2014).

Although there is limited research available regarding the phytoestrogen content of *Ligusticum chuanxiong*, findings showed that the compound could demonstrate protective effect in menopause-mimicking models. A study investigated the effect of the ethanolic extract of *Ligusticum chuanxiong* on vascular protection in OVX rats revealed that it can protect vascular endothelium by upregulating eNOS expression and acetylcholine-induced vascular relaxation in the aorta (Li et al., 2013). The improvement in the cardiovascular system in OVX rats proposed that it could be a potential agent to treat menopausal-caused cognitive impairment, especially VaD where reduced blood flow is commonly found.

***Lycium barbarum* Linnaeus (Goji Berry)**

Lycium barbarum L. is brilliant orange-red, ellipsoid berries that are 1-2 cm long and found in China, Tibet, and other parts of Asia. It is commonly used as a health food in Asia family because of its well-known anti-aging and vision-improving properties (Amagase & Farnsworth, 2011). Besides, it is rich in a variety of bioactive compounds that contribute to its numerous health benefits. The fruit contains Lycium barbarum polysaccharides (LBP)

which make up 5-8% of the dried fruit and are considered key to its medicinal properties. LBP are complex glycopeptides that include monosaccharides such as xylose, glucose, and arabinose, as well as amino acids, and have been shown to possess a range of biological activities, including neuroprotection, anti-aging, immunomodulation, anti-fatigue, and anti-tumor effects (Gao et al., 2017; Jin et al., 2013; W. Ma et al., 2022; Y. Peng et al., 2022). Carotenoids, primarily zeaxanthin, give the fruit its reddish-orange color and play an essential role in protecting eye health, particularly in preventing macular degeneration. Other notable compounds include betaine, cerebrosides, beta-sitosterol, and various vitamins, along with minerals such as potassium, calcium, zinc, and magnesium (Amagase & Farnsworth, 2011). Additionally, phenolic compounds like quercetin and rutin contribute to its antioxidant properties.

A study investigated the effects of high dose LBP (200mg/kg) and low dose LBP (20 mg/kg) on anxiety in OVX Wistar albino rats (Soyturk et al., 2023). Results showed that rats had reduced anxiety in both high dose and low dose LBP group than OVX rats as demonstrated by the increased mobility in the open field test and elevated plus maze test. Furthermore, high dose LBP showed a significant effect on boosting antioxidant activity and promoting hippocampal health. Another study figured out that LBP exhibits anti-neuroinflammation through TLR4/NF-κB signalling pathway and decreases the release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (X. Zheng et al., 2021). The estrogen-like property of LBP was also evaluated. LBP increased estrogen level, maintained the structural integrity of the heart muscle, reduced ROS and enhanced Bcl2 expression in OVX rats, similar to estradiol valerate. These findings suggest that LBP could help

alleviate menopause-related cognitive and cardiovascular impairments by mimicking estrogen's effects, offering potential therapeutic benefits for managing menopause symptoms.

Ginkgo Biloba

Ginkgo biloba is a unique and ancient tree species that has captured attention for its historical significance and potential health benefits. The leaves of the ginkgo tree have been utilized in traditional medicine for centuries, and extracts from these leaves (EGb761) are now popularly consumed as a dietary supplement to treat age-related memory deficit problems. The predominant active constituents in ginkgo include flavanols (quercetin, kaempferol and luteolin, etc.), terpene trilactones and ginkgolides (Mohanta et al., 2014). In addition, *Ginkgo biloba* is renowned for its ability to improve blood flow, enhance antioxidant activity, and protect against ischemia/reperfusion injury (Tian et al., 2017).

There is mounting evidence that quercetin may be used therapeutically to prevent and treat a variety of illnesses, including cancer, neurological diseases, and cardiovascular disease (Bentz, 2017). It is reported that quercetin (20 μM) pretreatment on striatal astrocytes increased PON2 expression and exhibited antioxidant properties in the cells under H_2O_2 or 2,3-dimethoxy-1,4-naphthoquinoneoxidant challenge (Costa et al., 2013). The involvement of PON2 was further validated by using striatal astrocytes isolated from PON2 knockout mice, in which the cells did not show resistance to oxidants after quercetin pre-exposure. Furthermore, studies have indicated that estrogen can increase PON2 expression in astrocytes of female mice through activating ERα (Giordano et al., 2013). Together, it

can be postulated that quercetin's estrogen-like activity may partially mimic the neuroprotective effects of estrogen by modulating PON2 and offering a natural and potentially beneficial approach to counteract cognitive decline associated with menopause.

The neuroprotective effect and mechanism of action of EGb761 were also determined in OVX mice. Through the inhibition of caspase-3 cleavage and activation, downregulation of caspase-8 and TNF-α expression, and an increase in VEGF and endothelial NOS production, EGb 761 therapy suppressed the apoptotic cell death pathway in OVX mice with permanent ischemia (Tulsulkar et al., 2016). They also found it interesting that Egb 761 boosted the expression of androgen receptors in OVX mice but had no significance in regulating the HO-1/Wnt pathway. As androgen is present in women at a relatively low level compared to men, its importance in women might have been overlooked. Ahlbom et al. (1999) highlighted the ability of androgen in neuroprotection as it can exert protection to cerebellar granule neurons against oxidative stress induced by H_2O_2 or S-nitrosocysteine.

Table 1 Summary of the neuroprotective mechanisms and actions of single herbs for menopause-caused cognitive decline

Single herb	Main Bioactive Components	Key Pathways/Mechanisms	Neuroprotective Actions	References
Pueraria lobata	Isoflavones (puerarin, daidzein, daidzin)	- Akt/BAD phosphorylation - TRPM2/NMDAR - Bax/Bcl2 - Estrogen receptor β (ERβ) - GSK3β/Wnt/β-catenin	- Reduces Aβ-induced apoptosis - Lowers ROS - Prevents neuroinflammation - Promotes neurite growth - Inhibits tau hyperphosphorylation	(Anukulthanakorn et al., 2016; Jiang et al., 2003; Li et al., 2010; Li et al., 2017; Wu et al., 2017; Xiong et al., 2006; H. Y. Zhang et al., 2011; Zhang et al., 2015; Zhou et al., 2014; Zhu et al., 2021)
Ligusticum chuanxiong	Tetramethylpyrazine (TMP), ligustilide, ferulic acid, essential oils, alkaloids	- Nrf2/HO-1 - Akt/ERK phosphorylation - eNOS upregulation - NF-κB inhibition	- Antioxidant and anti-inflammatory - Reduces NO, TNF-α, IL-1β, ROS - Protects vascular endothelium - Improves blood flow	(Chen et al., 2018; Kim et al., 2014; Li et al., 2013; Liang et al., 2005; J. G. Lin et al., 2022; Ran et al., 2011)
Lycium barbarum	- Polysaccharides (LBP) - Carotenoids (zeaxanthin) - Betaine - Phenolics (quercetin, rutin)	- TLR4/NF-κB - Antioxidant pathways - Estrogen-mimetic effects	- Reduces pro-inflammatory cytokines - Boosts antioxidant activity - Increases estrogen, Bcl2 expression - Reduces anxiety, improves cognition	(Amagase & Farnsworth, 2011; Gao et al., 2017; Jin et al., 2013; W. Ma et al., 2022; Y. Peng et al., 2022)

Single herb	Main Bioactive Components	Key Pathways/Mechanisms	Neuroprotective Actions	References
Ginkgo biloba	- Flavanols (quercetin, kaempferol) - Terpene trilactones (ginkgolides, bilobalide)	- PON2 via ERα - Caspase-3/TNF-α inhibition - Androgen receptor upregulation - Increases CBF, inhibits inflammation	- Antioxidant, anti-apoptotic - Improves blood flow - Inhibits neuronal apoptosis - Modulates neurotransmitter and hormone pathways	(Ahlbom et al., 1999; Bentz, 2017; Costa et al., 2013; Giordano et al., 2013; Mohanta et al., 2014; Tian et al., 2017; Tulsulkar et al., 2016)

1.4.2 Herbal formula for menopause-caused cognitive decline

Xiao-Yao-San

Xiao-Yao-San is a traditional Chinese herbal formula that has been used for centuries in TCM. It consists of 8 herbs, including *Bupleuri radix* (Chaihu), *Angelicae radix* (Danggui), *Paeoniae radix alba* (Baishao), *Atractylodis rhizome macrocephalae* (Baizhu), *Poria cocos* (Fuling), *Zingiberis siccatum rhizoma* (Shengjiang), *Menthae haplocalycis* (Bohe), and *Glycyrrhizae radix* (Gancao). It is renowned for its ability to harmonize the body, ease emotional stress, and spleen gastric disease in China (Hao et al., 2019). Also, it is a popular herbal formula for relieving menopausal syndrome due to the rich content of phytoestrogen, for instance, ergosterol in *Poria cocus* and β-sitosterol in *Angelicae radix* (Liu et al., 2020). Besides, they evaluated the improvement of cognitive ability in OVX rats administrated with Xiao-Yao-San. It showed that the formula can moderately restore the estrogen level in the hippocampus and reduce neuronal damage as proved by the increased number of neurons and dendritic spine density through activation of the ER*α*-PI3K signalling pathway. Researchers have also determined the antioxidant effect of a single herb, *Bupleuri radix*, in SH-SY5Y cells (Seo et al., 2013). The treatment of the herb can help cells defend against

oxidative stress damage and promote cell proliferation. The investigation of Xiao-Yao-San on cognitive improvements is comparatively low, but these two findings proposed that Xiao-Yao-San is a candidate worthy of further investigation in the aspect of cognition.

Gengnianchun formula

Gengnianchun (GNC) is a formula developed to nourish the kidney and liver, and it has been used to treat menopausal symptoms (Gao et al., 2023; Y. Zhang et al., 2020). The GNC formula comprises 12 crude herbs: *Radix Rehmanniae, Rhizoma Coptidis, Radix Paeoniae Alba, Rhizoma Anemarrhenae, Cistanche Salsa, Radix Moridae Officinalls, Poria, Epimedium Brevicornums, Cortex Phellodendri Amurensis, Fructus Lycii, Semen Cuscutae, and Carapax et plastrum Testudinis*. In a study using OVX SD rats as a model for menopause, researchers investigated the effects of GNC, comparing them to those of estradiol (Y.-Q. Rao et al., 2015).

The study divided rats into four groups: Sham, OVX (saline-treated), OVX+GNC, and OVX+ estrogen. OVX was performed to induce menopause, and GNC (5 mg/g) and estradiol (0.4 mg/g) were administered daily for one month. The Morris water maze test was used to assess learning and memory capabilities. Besides, levels of neurotransmitters, cytokines, and leptin and endometrial thickness was also evaluated.

The findings revealed that ovariectomy impaired learning and memory, as indicated by an increased escape latency period and decreased time spent by rats across the platform in the Morris water maze test. However, treatment with GNC and estradiol significantly

improved learning and memory in OVX rats, decreasing escape latency period and increasing platform time. Furthermore, the trails in the OVX+GNC and OVX+E groups were concentrated near the platform, demonstrating improved spatial learning in comparison to the random trails observed in the OVX group. These results suggest that GNC has a similar effect on cognitive function as estradiol.

A significant decrease in serum estrogen concentration and endometrial thickness was found in the OVX model (Y.-Q. Rao et al., 2015). While estradiol treatment increased these parameters, GNC treatment did not significantly affect serum estrogen concentration or endometrial thickness (Y.-Q. Rao et al., 2015). This indicates that GNC may alleviate menopausal symptoms through phytoestrogen-mediated mechanisms that circumvent direct hormonal stimulation. Unlike synthetic estrogen, GNC is less potent and does not elevate systemic estrogen levels or induce endometrial hyperplasia, thereby mitigating risks associated with hormone-driven pathologies such as breast cancer and thromboembolism, suggesting it as a safer alternative to HRT for menopausal symptoms. In terms of neurotransmitters, OVX decreased the levels of norepinephrine (NE) and dopamine (DA) and increased the levels of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the hypothalamus. GNC and estradiol treatment significantly increased the levels of NE and DA and decreased the levels of 5-HT and 5-HIAA. GNC's ability to modulate neurotransmitter levels, specifically by increasing catecholaminergic neurotransmitters (NE and DA) and decreasing serotonergic neurotransmitters (5-HT and 5-HIAA), could be a mechanism by which it improves menopausal caused-cognitive symptoms. Furthermore, the study found that OVX

increased levels of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, while decreasing levels of anti-inflammatory cytokines, such as IL-2 and interferon-gamma (IFN-γ). Both GNC and estradiol treatment significantly decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines. This indicated that GNC, like estradiol, helps to restore the balance of the immune system that is disturbed by menopause, potentially preventing the development of chronic diseases.

Yin Huo Tang

Yin Huo Tang (YHT) is a TCM formula that has been utilized for centuries. This formula comprises five key herbs: *Rehmannia glutinosa, Morinda officinalis, Ophiopogon japonicus, Poria cocos, and Schisandra chinensi*s (Ye et al., 2022). YHT is particularly recognized for its therapeutic potential in addressing symptoms associated with menopause, including cognitive decline, which is often exacerbated by hormonal changes during this period. The formula has been clinically applied to treat conditions related to Yin deficiency syndrome, which aligns closely with the symptoms experienced during menopause, such as mood disturbances and cognitive impairments (Patel & W, 2022).

Recent studies have highlighted the efficacy of YHT in alleviating menopause-like symptoms in animal models, particularly in mice subjected to ovariectomy, a procedure that mimics the hormonal changes of menopause. The research demonstrated that YHT administration significantly improved behavioural outcomes, including reduced aggressive behaviours and enhanced exploratory activities, which are indicative of improved cognitive function (Ye et al., 2022). Furthermore, the formula was shown to positively influence

sleep patterns, a common issue faced by menopausal women, by decreasing the time taken to fall asleep and increasing the duration of uninterrupted sleep. These findings suggest that YHT not only addresses physical symptoms but also has a profound impact on psychological well-being, which is crucial for cognitive health.

The underlying mechanisms through which YHT exerts its effects have been explored using network pharmacology and molecular docking techniques. These analyses indicated that the estrogen signalling pathway plays a pivotal role in the protective effects of YHT against cognitive decline associated with menopause. Specifically, the formula was found to reverse the decrease in serum estradiol levels in menopausal mice, thereby potentially restoring some of the neuroprotective benefits typically conferred by estrogen. This restoration of estrogen levels is particularly significant, as estrogen is known to support cognitive function and neuronal health (Valera et al., 2015).

In addition to its hormonal effects, YHT has been associated with improvements in organ morphology and overall health. The formula was observed to mitigate the atrophy of the uterus and other reproductive organs, which is a common consequence of menopause. This morphological improvement was accompanied by a reduction in body weight gain, a frequent issue in postmenopausal women, suggesting that YHT may also help regulate metabolic changes associated with hormonal fluctuations. The combination of these physiological benefits underscores the multifaceted approach of YHT in addressing the complex symptoms of menopause. Moreover, the individual herbs within YHT contribute to its overall efficacy. For instance, *Rehmannia glutinosa* is known for its nourishing

properties, while *Poria cocos* has been recognized for its ability to promote tranquillity and reduce anxiety. The synergistic effects of these herbs enhance the formula's potential to improve cognitive function and emotional stability during menopause (Ye et al., 2022). Although the research on YHT's impact on cognitive improvements is still emerging, the existing evidence suggests that it is a promising candidate for further investigation in this area.

Table 2 Summary of the neuroprotective mechanisms and actions of herbal formulas for menopause-caused cognitive decline

Formula	Herbs involved	Key Pathways/Mechanisms	Neuroprotective Actions	References
Xiao-Yao-San	*Bupleuri radix*, *Angelicae radix*, *Paeoniae radix alba*, *Atractylodis rhizome macrocephalae*, *Poria cocos*, *Zingiberis siccatum rhizoma*, *Menthae haplocalycis*, and *Glycyrrhizae radix*	- ERα-PI3K signalling: Restores hippocampal estrogen levels and dendritic spine density - Antioxidant pathways: Bupleuri radix reduces oxidative stress in neurons	- Improves spatial memory - Reduces neuronal damage - Alleviates menopausal cognitive decline	(Hao et al., 2019; Liu et al., 2020; Seo et al., 2013)
Gengnianchun	*Radix Rehmanniae, Rhizoma Coptidis, Radix Paeoniae Alba, Rhizoma Anemarrhenae, Cistanche Salsa, Radix Moridae Officinalls, Poria, Epimedium Brevicornums, Cortex Phellodendri Amurensis, Fructus Lycii, Semen Cuscutae, and Carapax et plastrum Testudinis*	- Neurotransmitter modulation: Increases NE/DA and decreases 5-HT/5-HIAA1. - Cytokine balance: Reduces IL-1β/IL-6/TNF-α and increases IL-2/IFN-γ1.	-Enhances learning/memory in OVX rats - Mimics estradiol's cognitive benefits without estrogenic effects.	(Gao et al., 2023; Y.-Q. Rao et al., 2015; Y. Zhang et al., 2020)
Yin Huo Tang	*Rehmannia glutinosa, Morinda officinalis, Ophiopogon japonicus, Poria cocos, and Schisandra chinensis*	- Estrogen signalling: Reverses serum estradiol decline - Reduces uterine atrophy and metabolic dysregulation.	- Improves sleep - Reduces aggression - Supports neuroprotection via estrogen pathway restoration.	(Patel & W, 2022; Valera et al., 2015; Ye et al., 2022)

1.5 Danggui Buxue Tang

Danggui Buxue Tang (DBT) is an ancient Chinese herbal formula that has been used for centuries to promote blood circulation and nourish the “Qi”. The primary components of the mixture are Huangqi (*Astragali Radix,* AR) and Danggui (*Angelicae Sinensis Radix,* ASR), which together tonify the blood and increase the body's energy levels.

AR demonstrates significant potential for cardiovascular protection, particularly in myocardial ischemia-reperfusion injury and diabetic cardiomyopathy (Chen et al., 2010). Studies have shown that AR can alleviate myocardial ischemia-reperfusion injury. This is achieved through several mechanisms, including the regulation of inflammatory responses and the reduction of oxidative stress (Jiang et al., 2023). Specifically, AR has been found to decrease the levels of pro-inflammatory cytokines and improve heart function in animal models of myocardial ischemia-reperfusion injury (Chen et al., 2024). In diabetic cardiomyopathy, AR has shown promise in relieving symptoms. This is partly attributed to its ability to increase serum levels of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are crucial for vascular repair and angiogenesis (Du et al., 2023). In a clinical trial, diabetic foot patients treated with AR showed significantly higher serum VEGF and bFGF levels compared to a control group, highlighting its potential to improve vascular health. Furthermore, AR has been shown to reduce levels of matrix metalloproteinase-2 (MMP-2), which is involved in the degradation of the extracellular matrix and can hinder wound healing (Li et al., 2022).

The active components within AR, such as polysaccharides, saponins, and flavonoids, contribute to these cardioprotective effects through various mechanisms. For example, Astragalus polysaccharides have demonstrated the ability to upregulate VEGFA expression and inhibit caspase-3 activation, which together helps to prevent cardiac cell apoptosis. Additionally, these components exert anti-inflammatory, antioxidant, and immune-regulating effects, all of which are essential for maintaining cardiovascular health (Jiang et al., 2023).

The cognitive enhancement effect of astragaloside IV (AS-IV), a key bioactive compound of AR, has been demonstrated reported (Xue et al., 2019). It promotes hippocampal neurogenesis and reduces inflammatory markers associated with stroke recovery. In a study using photochemical ischemia-induced mice, AS-IV administration led to improved cognitive function, increased numbers of hippocampal neurons with normal morphology and increments in the length of apical dendrites and their spine density (Sun et al., 2020). Further, AS-IV treatment promoted hippocampal neurogenesis and neural stem cell proliferation (Chen et al., 2019). This suggests that AS-IV, and by extension, AR, has potential in neuroprotection. The mechanism behind this appears to be linked to the downregulation of interleukin-17 (IL-17) expression via the Wnt signalling pathway. Increased IL-17 expression is associated with poorer outcomes after stroke, and AS-IV can reduce the levels of IL-17, which is linked to improved neurogenesis. This highlights the diverse applications of *Astragali Radix* and its components in both cardiovascular and neurological conditions.

Another main component is isoflavonoids, including formononetin, ononin, and calycosin. Studies had found that these compounds possess anti-inflammatory and neuroprotective effects, which may help mitigate inflammation associated with menopause and vascular cognitive impairment (Al-Shami et al., 2023; Calis et al., 2020). Current studies have demonstrated its role in antioxidation. Research indicated that calycosin effectively reduces various markers of oxidative stress, including MDA, protein carbonyls, and ROS, which are typically overproduced during I/R injury and lead to oxidative damage in rat brain (Guo et al., 2012). Besides, AR was found to improve immune functions in OVX rats by increasing IL-2 and IL-8 level in serum and the effect is comparable to that of estrogen treatment group (Zheng et al., 2012). Interestingly, the immune function was boosted without impacting sex hormones levels, suggesting that a potential alternative therapy to address menopausal symptoms.

ASR has been utilized for centuries in TCM for its various therapeutic effects, particularly in women's health. ASR is renowned for its ability to nourish the blood and regulate the menstrual cycle, making it a popular choice for addressing issues related to menopause and other reproductive health concerns (Ling et al., 2012). The pharmacological efficacy of ASR can be attributed to its rich composition of bioactive compounds (Jin et al., 2016). Among these, ferulic acid is notable for its antioxidant properties, which help protect cells from oxidative damage and improve blood circulation. Ferulic acid also helps to improve blood circulation by promoting vasodilation, which enhances the flow of oxygen and nutrients to vital organs like the brain. This action not only supports general cardiovascular

health but also aids in enhancing cognitive function and reducing the risks associated with conditions such as stroke and dementia.

Another prominent compound in ASR is ligustilide, a member of the phthalide class, which exhibits significant anti-inflammatory and neuroprotective effects (Ma & Bai, 2012; D. Peng et al., 2022; Zhu et al., 2014). Ligustilide has been shown to reduce neuroinflammation and oxidative stress, thereby protecting neural tissues from damage and degeneration (Kuang et al., 2014). Furthermore, ligustilide enhances microcirculation, ensuring an adequate supply of blood, oxygen, and nutrients to brain cells, which is critical for maintaining cognitive performance (D. Peng et al., 2022). Ligustilide, Z-butylidenephthalide, and Tokinolide A, three phthalide compounds, have demonstrated significant neuroprotective effects against glutamate-induced injury in SH-SY5Y cells (Gong et al., 2023). At a concentration of 10 μM, these compounds effectively antagonize glutamate neurotoxicity, which is associated with the development of neurodegenerative diseases. These neuroprotective properties suggest its potential in mitigating the progression of neurodegenerative diseases, including Alzheimer's and Parkinson's disease. These neuroprotective effects suggest that ligustilide holds great potential for mitigating the progression of neurodegenerative diseases, where inflammation and oxidative stress are central to their pathophysiology.

1.5.1 Classic Ratio of DBT

Traditionally, the ratio of ASR to AR is 1:5. In this formulation, the larger proportion of AR ensures that the body’s Qi is adequately supported to enable it to effectively nourish

and circulate the blood tonified by ASR. Furthermore, a study comparing different ratio, from 1:1 to 1:10, has confirmed 1:5 as the optimal ratio in terms of osteoblast proliferation and differentiation, activation of estrogen promoter and peak chemical composition (Dong et al., 2006). Besides, the synergistic interaction between the two herbs markedly amplifies the medicinal benefits of DBT and surpasses the efficacy of either herb used alone in enhancing active compound dissolution, promoting angiogenesis, and alleviating endothelial dysfunction (P. L. Lin et al., 2022). Their study highlights that the classic 1:5 ratio optimizes the dissolution of key bioactive compounds, such as ferulic acid and calycosin-7-glucoside, which are released in higher concentrations in the combined decoction than in individual herb extracts. Furthermore, they suggested that 1:5 ratio as the classic formulation as it consistently demonstrated the greatest effectiveness across various tests, particularly in angiogenesis and endothelial function as shown in the vascular-injured zebrafish (P. L. Lin et al., 2022).

1.5.2 Neuroprotection, Cognitive Support, and Clinical Efficacy in Menopausal Health of DBT

DBT had demonstrated antidepressant-like effects and neuroprotection in diabetic rats suffering from depression as reported by W. K. Wang et al. (2021). DBT's therapeutic action is mediated through the activation of the CREB/BDNF/TrkB signalling pathway, which is involved in neuronal function and plasticity within the hippocampus. This work highlighted DBT's ability to improve the ultrastructure of hippocampal neurons, reversing damage such as membrane shrinkage and cell consolidation. Further investigation into the chemical components responsible for these beneficial effects led to the discovery that

ferulic acid, a key compound in DBT, was detected in substantial amounts in both the serum and hippocampus of treated rats. This finding suggests that ferulic acid may be one of the primary active ingredients contributing to neuroprotective properties of the decoction. Additional research has shown that DBT, at a concentration of 0.5 mg/mL, has the capacity to upregulate various neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) through MAPK/Erk signalling pathway in SH-SY5Y cells. The results interpreted DBT could maintain neural health and support cognitive functions. These results provide compelling evidence that DBT can maintain neural health and support cognitive functions by promoting the production of essential neurotrophic factors. Furthermore, DBT has also demonstrated significant antioxidant effects. Chiu et al. (2007) observed that DBT increased mitochondrial respiration in H9C2 cells subjected to oxidative stress. This finding implies that DBT may help protect cells against oxidative damage, which is implicated in various age-related diseases and cellular dysfunction (Chiu et al., 2007). The antioxidant properties of DBT could contribute to its overall health-promoting effects, potentially offering protection against conditions such as cardiovascular diseases, neurodegenerative disorders, and cancer.

Other research has shown that DBT and its chemical components possess a wide range of health benefits. One notable area of investigation is the effect of DBT on bone cell proliferation and differentiation. A study by Choi et al. (2011) revealed that DBT stimulated the proliferation and differentiation of MG-63 osteosarcoma cells in a dose-dependent manner. This effect was mediated through the activation of Erk-dependent and

ER-dependent pathways, suggesting a potential role for DBT in promoting bone health. Besides, the estrogenic properties of DBT have been further verified using transfected MCF-7 breast cancer cells. Qiu T Gao et al. (2007) found that DBT significantly induced estrogen-driven promoter activity, indicating its potential to mimic or modulate estrogen-like effects in the body. This estrogenic activity could have implications for various physiological processes, including bone metabolism, cardiovascular health, and menopausal symptoms. Additionally, the same study reported that DBT suppressed platelet aggregation, suggesting potential cardiovascular benefits.

DBT has emerged as a significant herbal supplement for alleviating menopausal symptoms. Three clinical trials with different designs and objectives were conducted by Wong et al. (2022) aimed at discovering the efficacy and safety of DBT in alleviating menopausal symptoms. The first trial was a single-center, randomized, double-blind, placebo-controlled study involving 103 symptomatic women over six months. This trial primarily aimed to assess the general efficacy of 3g DBT daily and their result revealed a significant reduction in mild hot flashes in the treatment group compared to the placebo group, although no significant difference was observed in severe hot flashes. The second trial was a multiple-dose escalation, where 60 women with menopausal symptoms were randomly assigned to receive low dose (1.5g), standard dose (3g) and double standard dose (6g) of DBT daily for 12 weeks. It was found that higher doses resulted in more substantial improvements in menopausal symptoms, with no reported adverse effect. After confirming the working dosage of DBT, third trial was carried out to observe the effect of DBT using menopause-specific measurement tool, Menopause-Specific Quality of Life, in 50 women

receiving high dose of DBT for 12 weeks. The results demonstrated significant improvements in the psychosocial, physical, and sexual domains, as well as the vasomotor domain. Besides, an inconsistent fluctuation of cytokine, specifically IL-6, IL-8, and TNF-α, was observed. Given the fact that these cytokines were linked to with estrogen activity, this finding may suggest that the beneficial effects of DBT on menopausal symptoms are not likely due to direct estrogenic activity, alleviating the concerns regarding potential adverse effects often linked to estrogen therapy.

Furthermore, anemia, menstrual irregularities, and postpartum weakness are some of the conditions that DBT could treat (Haines et al., 2008; Huang et al., 2016; Wong et al., 2022). The chemical compositions of DBT have been extensively studied by various researchers. According to C.-c. Ma et al. (2022), more than 30 active compounds are found in DBT, including wide range of flavones (formononetin, ononin, and calycosin), volatile oils, and polysaccharides. Some of these compounds have been shown to have anticancer, anti-aging, and beneficial effects on hematopoiesis and degenerative disease (Chaouhan et al., 2022; Kopustinskiene et al., 2020; Singh et al., 2024; Yan et al., 2009; Yang et al., 2009).

1.5.3 The Unique Simplicity and Therapeutic Potential of DBT

DBT is a relatively straightforward herbal decoction, consisting solely of two key ingredients. This simplicity distinguishes DBT from many other TCM formulas, which often include numerous herbs. This formulation emphasizes the synergistic effects of these two herbs, allowing for a targeted therapeutic approach without the complexity of multiple components. However, the simplicity of DBT does not compromise its therapeutic efficacy.

Instead, it highlights the potency of the 2 commonly used herbs working together. Also, the specific ratio used in DBT has been employed for centuries, and its effectiveness has been verified through modern research and practice. This formulation is particularly significant for menopausal women, as it addresses symptoms associated with hormonal fluctuations, such as hot flashes, mood swings, and cognitive decline. Besides, it does not only exhibit estrogenic properties that help alleviate menopausal symptoms but also demonstrate neuroprotective effects that are increasingly supported by various studies. Given its unique composition and the promising findings regarding its safety and neuroprotective capabilities, it is important to delve deeper into its mechanisms and potential applications in neuroprotection. Moreover, it is worthwhile to investigate the possibility of DBT as a viable natural alternative to HRT, especially considering the risks associated with long-term HRT use.

1.6 Hypothesis and Aim of the Study

In this study, we hypothesized that DBT could protect OGD/R injured cells by (1) promoting angiogenesis, (2) improving BBB integrity and (3) reducing oxidative stress via GPR30 and Nrf2/HO-1 signalling pathway. Also, it was hypothesized that DBT could protect against menopause-related cognitive decline. Therefore, this study was designed with the following specific aims: (1) to investigate the effects of DBT on cell proliferation and migration, tight junction molecules expression and oxidative stress level in OGD/R-insulted mouse brain endothelial (bEnd.3) cells and (2) to examine the effects of DBT on cognitive functions in ovariectomized SD rats.

Chronic hypoxia and ischemia, driven by transient disruptions in cerebral blood flow, are key contributors to VaD due to their role in progressive neuronal damage. To model these pathophysiological conditions, an oxygen-glucose deprivation/reoxygenation (OGD/R) *in vitro* system was employed. In this model, cells undergo initial deprivation of oxygen and glucose to simulate ischemic injury, followed by reintroduction to normoxic conditions to mimic reperfusion, a process critical for studying the cumulative effects of repeated hypoxic insults seen in VaD. By replicating the cycle of ischemia-reperfusion injury, this approach allows investigation of cellular responses such as oxidative stress, blood-brain barrier dysfunction, and neuronal apoptosis, thereby providing mechanistic insights into VaD progression and potential neuroprotective interventions. For the *in vivo* study, ovariectomized female SD rats were used. OVX is a surgery that remove the ovaries from female rats in order to mimic menopause, where a drop in estrogen levels is found. OVX rats experience symptoms similar to those seen in postmenopausal women, including altered mood, cognitive impairment, and increased susceptibility to neurodegenerative diseases. Previous research has shown that cognitive decline and related symptoms can begin to manifest as early as 8 weeks after OVX surgery (Qu et al., 2022; Tao et al., 2020). These effects include deficits in memory, learning, and spatial navigation, which are often used as key indicators of cognitive impairment in rodent models. In this study, OVX rats were allowed to undergo 8 weeks of hormone depletion following the surgery, after which they were evaluated for cognitive function by three behavioural tests.

By combining these *in vitro* and *in vivo* models, this study aimed to provide a comprehensive understanding of the potential neuroprotective effects of DBT. The OGD/R

model with bEnd.3 cells allowed for the investigation of BBB integrity and endothelial cell function under ischemic conditions, while the co-culture system with N2a cells provided insights into neurovascular interactions. The OVX rat model, along with a number of behavioural tests, allowed us to investigate the effects of DBT on cognitive performance in the context of estrogen deprivation. When combined, these methods provide a comprehensive analysis of DBT's potential as a treatment for cognitive decline linked to menopause.

2 Materials and Methods

2.1 Materials

High glucose DMEM (Cat#12800017), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Cat# M6494), PMSF Protease Inhibitor (Cat# 36978), Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) (Cat# 78440), ZO-1 Monoclonal Antibody (ZO1-1A12) (Cat# 33-9100), Claudin 5 Monoclonal Antibody (4C3C2) (Cat# 35-2500), GPR30 rabbit primary antibody (Cat# PA528647), Alexa Fluor® 594-conjugated goat anti-rabbit secondary antibody (Cat# PA528647), Goat anti-rabbit IgG (H+L) Secondary Antibody, HRP (Cat# 31460) were purchased from Invitrogen (Massachusetts, USA)

Fetal bovine serum (FBS) (Cat# A5256701), L-glutamine (200 mM) (Cat# A2916801), Penicillin-Streptomycin (Cat# 15140122), Glucose-free DMEM (Cat# 11966025) were purchased from Gibco. NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Cat# 78833), SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Cat# PI34577), Normal goat serum (10%) (Cat# 50062Z), Hoechst 33342, trihydrochloride trihydrate, 10 mg/mL (Cat# H3570) were purchased from Thermo Fisher Scientific (Massachusetts, USA).

HO-1 (E7U4W) Rabbit mAb (Cat# 82551), NRF2 (D1Z9C) XP ® Rabbit mAb (Cat# 12721), Lamin B1 (E6M5T) Rabbit mAb (Cat# 17416), GCLC (E2Y7D) Rabbit mAb

(Cat# 52183), Anti-mouse IgG, HRP-linked Antibody (Cat# 7076S) were purchased from Cell Signalling Technology (Massachusetts, USA).

RIPA lysis buffer (Cat#P0013B), Bovine serum albumin (BSA) (Cat# ST023), CD31 rabbit polyclonal antibody (Cat# AF6408), GFAP rat monoclonal antibody (Cat# AG259), DAB chromogen (Cat# P0203), Hematoxylin (Cat# C0107), EDTA buffer (Cat# P0085) were purchased in Beyotime (Shanghai, China).

Other materials include Dimethyl sulfoxide (DMSO) (Cat# D2650), Formalin Solution (Cat# HT501128-4L) from Sigma-Aldrich (Massachusetts, USA). Phosphate buffered saline (PBS Tablets) (Cat# T9181) from Takara Bio (San Jose, USA) and was prepared by dissolving the tablet in H_2O. β-actin rabbit mAb (Cat# AC026) from ABclonal. Anti-GCLM antibody [EPR6667] (Cat# ab126704) from Abcam (Cambridge, UK). G15 (Cat# HY-103449) from MedChemExpress. SlowFade™ Gold Antifade Mountant (Cat# S36936) from Life Technologies (California, USA). QuicKey Pro Rat Estradiol ELISA Kit (Cat# E-OSEL-R0001) from Elabscience (Texas, USA).

2.2 Cell work

2.2.1 Preparation of Danggui Buxue Tang Extract

AR and ASR were purchased from Wai Yuen Tong (Retail) Limited. To prepare the herbal extract, DBT (exact amounts of AR and ASR in a weight ratio of 5:1), 150 g of AR, and 30 g of ASR was weighed and immersed in a 20-fold amount of distilled water and boiled until half of the original amount was left. The extraction was repeated 3 times. The extract

was then filtered and lyophilized. The yield of the extract was approximately 26.69% (w/w). The freeze-dried DBT powder was stored at −20°C until use.

2.2.2 UPLC-Orbitrap-MS of DBT sample

2 mg of freeze-dried DBT sample was dissolved with 100 µL 70% methanol and sonicated for 30 minutes. The extract was then centrifuged for 15 minutes at 14,000 rpm at 4 °C. The supernatant was collected and re-dissolved in 95% methanol. The extract was filtered with a 0.22 µm PTFE syringe filter before analysis. Ultimate 3000 UHPLC System connected to Thermo Scientific Orbitrap IQ-X LC/MS (Thermo Fisher Scientific, Massachusetts, USA) was used for the orbitrap-MS analysis. The Waters ACQUITY UPLC HSS T3 column (2.1 mm x 100 mm, 1.8um) with HSS T3 pre-column (2.1 mm x 5 mm, 1.8 μm) was used for separation at 40 °C.

The mobile phase was a mixture of water (A) and acetonitrile (B), both containing 0.1% (V/V) formic acid, with a linear gradient elution as follows: initial 5% B, 0-1 min, 5% B; 1-1.3 min. 5 35% B; 1.5-3 min, 35-50% B; 3-6.5 min, 50-55% B; : 6.5-8.5 min, 55-95% B: 8.5 – 10.5 min, 95% B; 10.5 - 11 min, 95-3% B;11-14 min, 5% B. The injection volume was 3 μL. The flow rate was set at 0.30 mL/min. The sample chamber temperature was 4 °C. ESI-MS spectra were obtained in both positive and negative ion modes. The ESI parameters were as follows: Spray voltage, 3500V for ESI+ and 2300V for ESI-, sheath gas 35 arbitrary units nebulizer auxiliary gas 10 arbitrary units; Sweep gas 3 arbitrary units. General instrumental parameters were set as follows: ion transfer tube temperature. 300°C;

vaporizer temperature, 320°C. For the full scan MS, the muss range was set at 70-1200 m/z with 120,000 resolutions.

2.2.3 Cell culture and oxygen-glucose deprivation and reperfusion (OGD/R)

bEnd.3 cells (ATCC) and N2a cells (ATCC) were cultured with high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Cat#12800017) containing 10% heat-inactivated FBS (Gibco, Cat# A5256701), 1% L-glutamine (200 mM) (Gibco, Cat# A2916801) and 1% Penicillin-Streptomycin (10,000 U/mL) (Gibco, Cat# 15140122). For OGD/R, bEnd.3 cells were washed twice with phosphate-buffered saline (PBS) (Takara Bio, Cat# T9181) and cultured in glucose-free DMEM (Gibco, Cat# 11966025). The cells were then incubated in SCI-tive Dual Chamber Hypoxia Workstation (5% CO_2, 1% O_2, and 94% N_2 at 37°C) for 5 hours. Reperfusion was done by transferring to standard culturing condition and maintained in normoxic incubator for 19 hours. Cells cultured in normoxic conditions were considered as control.

2.2.4 MTT assay

A density of 1 x 10^4 cells/well of bEnd.3 were seeded into 96-well plate and incubated for 24 hours. Cells were treated with 0.01-100 mg/ml DBT for 24 hours in normoxic incubator. Then, the medium was discarded and 100 µl 1X MTT reagent (Invitrogen, Cat# M6494) was added to each well and the plates were incubated for 2 hours. The MTT reagent was aspirated and 100 µl DMSO (Sigma-Aldrich, Cat# D2650) was added to each well. Absorbance was measured at 590 nm by CLARIOstar® microplate reader of BMG LABTECH (Ortenberg, Germany). For assessing protection of DBT against OGD/R

condition, the steps for seeding, MTT addition and absorbance measurement were the same as described above except the cells were washed twice with 1 mL PBS and treated with 0.01-10 mg/ml DBT diluted in glucose-free medium (Gibco, Cat# 11966025). The cells were then incubated in SCI-tive Dual Chamber Hypoxia Workstation (5% CO_2, 1% O_2, and 94% N_2 at 37°C) for 5 hours. Reperfusion was done by transferring to standard culturing condition and maintained in normoxic incubator for 19 hours. Cells that incubated in normorxic incubator were used as control. Absorbance was measured at 590 nm by CLARIOstar® microplate reader of BMG LABTECH (Ortenberg, Germany).

2.2.5 Protein extraction

2.2.5.1 Total Protein extraction

Total protein will be extracted from the cells at the end of the experiment. RIPA lysis buffer (Beyotime, Cat#P0013B) containing 1% PMSF (Invitrogen, Cat#36978) and 1% haltprotease and phosphatase inhibitor cocktail (Invitrogen, Cat#78440) was prepared. The wells were washed with cold 1 mL PBS twice and 80 μL lysis buffer was added to each well for 30 minutes. The cells were scraped and collected in 1.5 mL Eppendorf. Then, it was centrifuged at 13,000 rpm for 15 minutes at 4°C. Supernatant was collected and placed on ice. Bradford assay was used to quantify the total protein concentration. Standard curve was constructed using BSA (Beyotime, Cat#ST023).

2.2.5.2 Nuclear protein extraction

NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Cat# 78833) was used to extract nuclear protein. Firstly, cells were detached by trypsin and collected into 1.5 mL tube. The cell pellet was suspended in CER I and vortexed vigorously for 15 seconds, followed by a 10-minute incubation on ice to allow complete lysis. Afterward, ice-cold CER II was added to the tube, which was then vortexed for 5 seconds and incubated on ice for an additional minute. The mixture was centrifuged at maximum speed (~16,000 × g) for 5 minutes, separating the cytoplasmic fraction from the intact nuclei. The supernatant, containing cytoplasmic proteins, was collected while the nuclear pellet was resuspended in NER and vortexed briefly. This mixture was incubated on ice for 30 minutes to facilitate the extraction of nuclear proteins.

2.2.6 Western Blot

The protein folding and protein-protein interactions was disrupted by the presence of SDS in the lysis buffer, leading to significant protein denaturation. To ensure complete protein denaturation, the collected lysate was immediately boiled at 95 °C for 5 minutes to simultaneously denature proteins and inactivate proteases. The amount of sample to be loaded onto polyacrylamide gel was calculated and 30 μg of total protein concentration was added onto polyacrylamide gel. Bio-rad Precision Plus Protein Dual Color Standards (Bio-rad, Cat# 1610374) was used as protein ladder. Gel was run at 50V for half an hour followed by 100V for one and a half hour. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immun-Blot® PVDF, Bio-Rad, USA, Cat# 1620177) using a wet tank transfer system. Before assembly, the PVDF membrane was activated by

soaking in 100% methanol for 1 minute. The transfer stack was carefully assembled to avoid air bubbles, starting with a pre-soaked fiber pad and filter paper placed beneath the gel. The activated PVDF membrane was then placed directly on top of the gel, followed by another layer of filter paper and a second fiber pad. The complete assembly was submerged in transfer buffer and electrophoresed at 100 V for 90 minutes at 4°C.

After protein transfer, the membranes were blocked by 5% BSA for one to three hours at room temperature. The membranes were then incubated with primary antibody against ZO-1 mouse mAb (Invitrogen, Cat# 33-9100, 1:1000), Claudin-5 mouse mAb (Invitrogen, Cat# 35-2500, 1:1000), HO-1 rabbit mAb (Cell Signaling Technology, Cat# 82551, 1:1000), Nrf2 rabbit mAb (Cell Signaling Technology, Cat# 12721, 1:500), Lamin B1 rabbit mAb (Cell Signaling Technology, Cat# 17416, 1:1000), GCLC rabbit mAb (Cell Signaling Technology, Cat# 52183, 1:1000) and GCLM rabbit mAb (Abcam, Cat# ab126704, 1:1000) overnight at 4 °C. β-actin rabbit mAb (ABclonal, Cat# AC026, 1:1000) was used as the loading control. The procedures were followed by the binding of goat anti-rabbit IgG (H+L) Secondary Antibody, HRP (Invitrogen, Cat# 31460) or anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology, Cat# 7076S). Thermo Scientific™ SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Cat# PI34577) was used for the detection of HRP. The signal was measured using ChemiDoc Imaging System (Bio-rad, USA).

2.2.7 Transendothelial electrical resistance (TEER) values

TEER values of mono- and co-culture 24 well systems were measured by epithelial-volt-ohm resistance meter according to manufacturer's instructions. Transwells with no cells seeded were considered as blank. For mono-culture system, 70,000 cells/well of bEnd.3 were seeded at the apical side of transwell. For co-culture systems, same density of bEnd.3 were seeded while 200,000 cells/well N2a were seeded in the basal side. The values were calculated by subtracting the blank resistance by total resistance in units of Ω, followed by multiplication of cell growing area of the transwell membrane. The reported values were in units of $\Omega.cm^2$ and it is calculated as: TEER= R_{tissue} (Ω) x Area of transwell (cm^2)

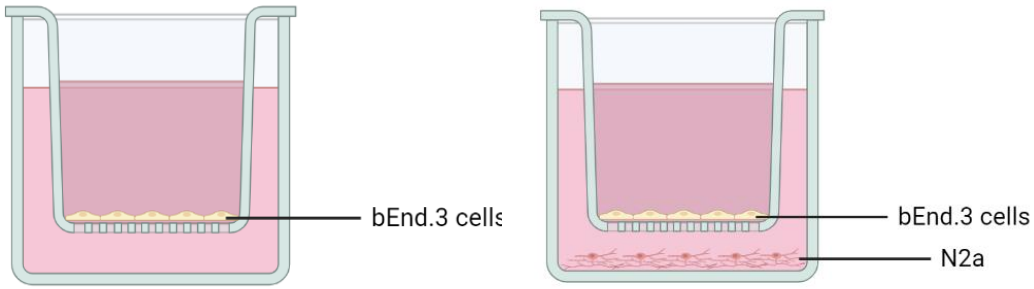


Figure 1 Illustration of *in vitro* mono- and co-culture transwell system

2.2.8 Wound healing assay

To evaluate the wound healing ability of DBT, 200,000 cells/well bEnd.3 cells were seeded on 12 well plate and incubated at 37 °C with 5% CO_2 overnight. Upon reaching confluence, a uniform wound was created by scratching the cell monolayer with a sterile 200 µL pipette tip. The pipette tip was held perpendicular to the bottom of the well, and a straight line was drawn across the center of the well with gentle and consistent pressure. The detached cells were washed away by 1 mL PBS twice. 30 ng/mL VEGF and different concentrations of

DBT was dissolved and diluted with serum-free DMEM. The cells were incubated for 24 hours at 37°C in a CO_2 incubator. At T0 and T24, images were taken by OLYMPUS inverted phase contrast microscope (model #CKX53) and analysed by ImageJ (NIH, USA). The percentage of wound healing was calculated to quantify the rate at which cells migrate to close the wound over time using the following formula:

% Wound Healing = (Initial Wound Area−Remaining Wound Area)/ Initial Wound Area ×100

2.2.9 Immunofluorescence staining preparation

5 x 10^5 cells/mL was seeded in coverslip in 6 well plate and incubated overnight. OGD/R was performed as mentioned in Part 1.3 while 5 μM G15 (MedChemExpress, Monmouth Junction, USA, Cat# HY-103449) was added to the cells 30 minutes before OGD/R. On the next day, cells were fixed with 1 mL 10% neutral buffered Formalin Solution (Sigma-Aldrich, Cat# HT501128-4L) for 10 minutes and washed three times with PBS. Afterwards, the cells were permeabilized with 0.1% Triton X-100 in PBS for 6 minutes and blocked by 1 mL of normal goat serum (10%) (Thermo Fisher Scientific, Cat# 50062Z) for 1 hour. Rabbit primary antibody against GPR30 (Invitrogen, Cat# PA528647, 1:500) was diluted in 1% BSA in PBS and 200 μL/well was added. The plate was then placed at 4°C overnight. After incubation, Alexa Fluor® 594-conjugaed goat anti-rabbit secondary antibody (Invitrogen, Cat# PA528647), was diluted by 1:1000 and was applied to the coverslip for an hour. The coverslip was washed twice with PBS to eliminate any residual secondary antibody. Counterstaining was performed by incubating the coverslip with 200 μL of Hoechst 33342 (0.2 μg/mL in PBS) (Thermo Fisher Scientific, Cat# H3570) for 7 minutes

at room temperature, ensuring protection from light. Following this, the coverslip was thoroughly washed with PBS and subsequently rinsed with ddH_2O to remove any non-nuclear staining and residual salts. 12 μL of SlowFade™ Gold Antifade Mountant (Life Technologies, Cat# S36936) was used to mount the coverslip on microscopic slide and sealed with clear nail polish.

2.3 Animal work

2.3.1 Animal experiment design

Adult female Sprague Dawley rats weighing 250–300 g, aged 6 months, were used in this study. All procedures were approved by the local legislation for ethics of experiments on animals (ASESC Case No.: 23-24/822-FSN-R-OTHERS). Rats were raised in individually ventilated cage with controlled temperature at 22-26°C and relative humidity of 30-90% and 12-hour light/dark cycle.

To investigate the effect of DBT on menopausal cognitive function *in vivo*, an OVX rat model was established. Rats were anaesthetized with 4% isoflurane and kept with 2% isoflurane by an anaesthetic machine, the anaesthetized rats were placed on a warm pad (30-35°C) to prevent hypothermia. The hair on the lower lumbar region was shaved with electric clippers. The skin was disinfected with 70% ethanol. A horizontal dorsolateral incision was made in the skin on the right side, and the musculature was separated with curved tip scissors. The ovarian fat was pulled out from the incision with a tweezer. The region below the ovary was tightly clamped by hemostatic forceps. Absorbable sutures were tied around the upper uterine horn to suppress the collateral blood supply to the ovary.

The ovary was removed by cutting above the ligated area with cauterizing scissors. The fat pad was relocated back into the peritoneal cavity, and the musculature was closed with absorbable sutures. The skin incision was then closed by 4-0 nylon sutures. The steps were repeated on the left-hand side. A standard sham ovariectomy model was prepared as described above regarding surgical site preparation, dissection of the lumbar region, exposure of the ovaries and ovarian fat, closing, and surgical site disinfection; but ovaries were not removed. The animal was placed on the warm pad (37°C) until recovery, and the condition of the animal was monitored throughout the experiment period. Animals with serious injury or signs of distress or pain were euthanized before the end of the study.

After surgery, meloxicam (0.5 mg/mL) and buprenorphine (0.06 mg/mL) were administered to all rats at 24 hours and 48 hours post-surgery. Meloxicam was administered again at 72 hours only if pain or discomfort was observed. The ovariectomized rat was housed individually for a week to prevent any contamination. Upon recovery for 2 weeks, the OVX-operated rats were randomly divided into 5 groups to receive the following interventions orally with reference to Zhou et al. (2018): Gp 1: Sham + Vehicle (0.9% saline); Gp 2: OVX + Vehicle (0.9% saline); Gp 3: OVX + 17β-estradiol (2 mg/kg/day); Gp 4: OVX + DBT low dose (1.5 g/kg/day); and Gp 5: OVX + DBT high dose (3 g/kg/day) for a consecutive 8 weeks. During the recovery and treatment period, the OVX rats were fed with phytoestrogen-free diets (AIN93-M) purchased from Research Diets, Inc. (New Brunswick, USA) to prevent the influences of phytoestrogen.

Table 3 Composition list of AIN93-M Diet

Class	Ingredient	Amount (g/kg diet)
Protein	Casein, Lactic, 30 Mesh	140.00
Protein	L-Cystine	1.80
Carbohydrate	Corn Starch	495.69
Carbohydrate	Lodex 10 (Dextrinized Corn Starch)	125.00
Carbohydrate	Sucrose, Fine Granulated	100.00
Fiber	Solka Floc, FCC200	50.00
Fat	Corn Oil	40.00
Mineral	Mineral Mix S10022M	35.00
Vitamin	Vitamin Mix V10037	10.00
Vitamin	Choline Bitartrate	2.50
Antioxidant	tert-Butylhydroquinone (tBHQ)	0.01
Dye	Red FD&C #40, Alum. Lake 35-42%	0.10

2.3.2 Blood perfusion and sample collection

To perform brain perfusion in SD rats, the animals were first anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), administered via intraperitoneal injection. Adequate anaesthesia was confirmed by checking the toe pinch response. Once anesthetized, the rats were positioned in a supine position on a dissection tray. Next, a midline incision was made in the abdominal cavity to expose the diaphragm and thoracic cavity. The ribs were then cut open to access the heart directly. Afterward, the diaphragm was cut to facilitate access to the chest and the heart. Before performing perfusion, blood was collected by cardiac puncture. The samples were allowed to clot for an hour before centrifugation for 15 mins

at 3000 rpm at 4 ℃. The supernatant was collected to 1.5 mL tube and stored at -80 ℃ until ELISA assay was carried out. The needle connecting the perfusion pump was carefully inserted into the left ventricle of the heart. Proper placement was ensured by checking for retrograde blood flow into the needle. The right auricle was cut to facilitate drainage. PBS perfusion was then initiated. When clear fluid emerged from the right auricle, blood had been adequately flushed from the system and the perfusion was stopped. To harvest the uterus, the abdominal cavity was cut opened and the intestines were moved cranially to locate the uterine horn. Once the horn was located, surrounding fat was removed and it was traced upwards to locate the ovaries. The uterus was carefully isolated by removing the fat or tissue attached to it. The harvested uterus was placed on a dish for photo taking and was transferred to 1.5 mL tube for weighing.

To extract the brain, a midline incision was made along the neck to expose the underlying tissues, followed by careful dissection to remove the skin and any muscle or fascia from the dorsal and posterior parts of the skull. The proximal end of the neck and vertebrae was cut by a straight scissor and the remaining muscles attached to the posterior and inferior parts of the skull was removed by forceps. The temporal and parietal bones were taken off carefully by inserting the tip of the scissors between the brain and the bones. Then, the cerebral cortex was exposed. The frontal bone was removed and the olfactory bulbs were exposed. The brain was extracted by a spatula.

2.3.3 Immunohistochemistry

The brain slides were baked in a drying oven at 65°C for 30 minutes. They were then passed through three xylene baths, each for 5 minutes, followed by three alcohol baths (100%, 95%, 80%) for 3 minutes each. The slides were rinsed under running water to remove the alcohol until they appeared clean and transparent. Antigen retrieval was conducted using a pressure cooker with 2000 mL of EDTA buffer (pH 9.0) (Beyotime, Cat#P0085). The slides were heated to boiling, pressurized for 2 minutes, then cooled under running water. Endogenous peroxidase activity was blocked by incubating the slides in 3% H_2O_2 for 10 minutes at room temperature, followed by three rinses with distilled water and PBS. Excess liquid was removed, and a hydrophobic barrier was drawn around the tissue. The primary antibody of CD31 rabbit polyclonal antibody (Beyotime, Cat# AF6408, 1:100 dilution) or GFAP (1:100, AG259, Beyotime) or GFAP rat monoclonal antibody (Beyotime, Cat# AG259, 1:100 dilution) was applied and incubated at 37°C for 60 minutes, followed by three PBS washes. The secondary antibody was then applied and incubated at 37°C for 30 minutes, again followed by three PBS washes. DAB chromogen (Beyotime, Cat#P0203) was applied, and the staining was monitored under a microscope. The reaction was stopped when positive staining was observed, and the slides were rinsed thoroughly with distilled water. Hematoxylin (Beyotime, Cat# C0107) counterstaining was performed for 2 minutes, followed by differentiation in 1% acid alcohol for a few seconds and bluing in lithium carbonate solution for 30 seconds. The slides were then dehydrated, cleared in xylene, and mounted. Finally, the results were observed under a microscope.

The staining was quantified using ImageJ (NIH, USA). The region of interest, hippocampal CA1) were captured. Digital images were subjected to color deconvolution to separate DAB staining from hematoxylin counterstain. Then, the DAB channel was thresholded to identify positive immunoreactivity. After that, the mean grey value of the image was measured. These values were then converted to optical density (OD) using the formula: OD = log (max intensity / mean intensity).

2.3.4 Estradiol ELISA

QuicKey Pro Rat Estradiol ELISA Kit (Elabscience, Cat# E-OSEL-R0001) was used in this assay. 50 µL of standard, blank and samples were added to each well in duplicate. Immediately after, 50 µL of HRP Conjugate working solution was added to each well, and the plate was covered with a sealer. The incubation was carried out for 60 minutes at 37°C. Following incubation, the solution was decanted from each well, and 350 µL of wash buffer was added to each well. The wells were soaked for 1 minute before the solution was aspirated, and they were patted dry against clean absorbent paper. Next, 90 µL of Substrate Reagent was added to each well, and the plate was covered and incubated for 15 minutes at 37°C. Finally, 50 µL of Stop Solution was added to each well in the same order as the substrate solution. Optical density was then determined using CLARIOstar® microplate reader of BMG LABTECH (Ortenberg, Germany) at 450 nm.

2.3.5 Behavioural tests

2.3.5.1 Rotarod test

Rats were placed on a rotating rod initially set at a speed of 4 revolutions per minute (rpm). Once the rats maintained balance on the rod, the rotation speed was gradually increased from 4 rpm to 40 rpm over a period of 300 seconds. The duration of time spent on the rod and the distance travelled were recorded.

2.3.5.2 Novel object recognition

During the familiarization phase, two identical objects were in the arena. Each rat was placed in the arena and allowed to explore freely. The process was recorded for 6 minutes. Following this phase, the rats were returned to their home cages for a retention interval of 1 hour. After this period, the test phase is commenced and one of the familiar objects was replaced by a novel object. Exploration was defined as either using one or both forepaws to touch the object or sniff it.

2.3.5.3 Morris water maze

Morris water maze test was lasted for 6 days consecutively. Tank with diameter of 160 cm and height of 40 cm was utilized. A circular rough surfaced platform with a diameter of 12 cm and height of 30 cm was put in the fourth quadrant. The water maze was placed in a temperature-controlled room maintained at approximately 24°C. The hidden platform experiment was designed to last for 5 days, with 4 training sessions each day. The rats were placed in one of the four quadrants against the wall of the tank and swam for a total of 60 seconds to search for the hidden platform. If the platform was not found within this time

frame, the rats were guided onto the platform and allowed to stand there for about 10 seconds. After completing the hidden platform experiment, the platform was removed and spatial probe test was conducted. The rats were gently placed into the water at the opposite quadrant, and their swimming paths were recorded for 60 seconds. The swimming trajectory, escape latency, distance of swimming, time spent at target quadrant and the number of crossing platform were recoded and analysed using ANY-maze (Stoelting Co., Wood Dale, USA)

3 Statistic

All obtained values are expressed as mean ± SEM. Experiments were performed in triplicate unless otherwise specified. Statistical analyses were conducted using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple group comparisons. Unpaired t test was performed to compare the difference between 2 groups.

4 Results

4.1 Effects of DBT on OGD/R-induced Injury in bEnd.3 Cells

4.1.1 Identification of main constituents in DBT

Constituents of DBT were separated and identified by UPLC-Orbitrap MS with simultaneous positive and negative ion mode acquisition in a single analytical run. Representative total ion chromatograms from both ionization modes were shown in Figure 2. A total of 11 flavones were identified, including wogonin 5-glucoside, glycitin, baicalin, ononin, cyrtopterin, protofarrerol, formononetin 7-O-glucoside-6"-O-malonate, 7-hydroxy-6-(3-methyl-2-buten-1-yl)-4-oxo-2-(2,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-5-yl hexopyranosiduronic acid, acacetin, glycitein, and 7-hydroxy-2'-methoxyisoflavone. The detected flavones were listed in Table 4 with its corresponding retention time (min) and formula.

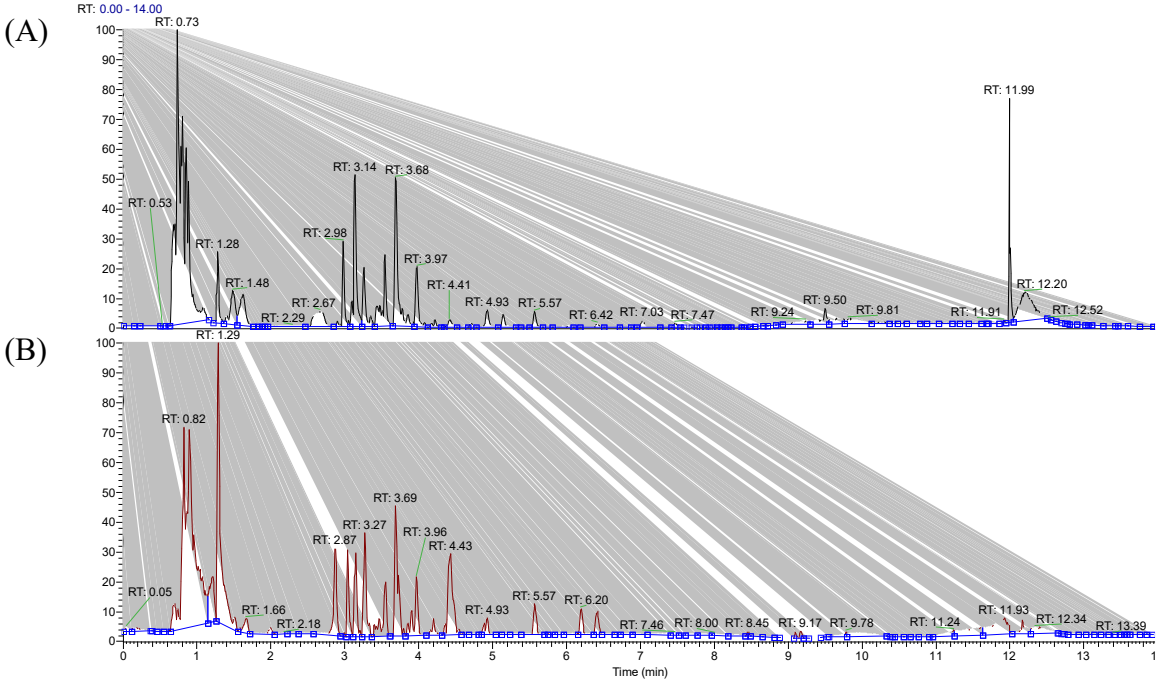


Figure 2 Total ion chromatograms of DBT water extract in positive and negative modes

Total ion chromatograms of (A) positive mode and (B) negative mode of DBT water extract.

Table 4 Flavone identified from DBT water extract by UPLC-Orbitrap-MS

	Retention time (min)	Compounds	Formula	Identification
1	3.14	Wogonin 5-glucoside	C22H22O10	Flavone
2	3.14	Glycitin	C22H22O10	Flavone
3	3.47	Baicalin	C21H18O11	Flavone
4	3.54	Ononin	C22H22O9	Flavone
5	3.69	Cyrtopterin	C23H26O10	Flavone
6	3.70	Protofarrerol	C17H18O	Flavone
7	3.74	Formononetin 7-O-glucoside-6"-O-malonate	C25H24O12	Flavone
8	3.86	7-Hydroxy-6-(3-methyl-2-buten-1-yl)-4-oxo-2-(2,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-5-yl hexopyranosiduronic acid	C26H28O13	Flavone
9	3.97	Acacetin	C16H12O5	Flavone
10	3.97	Glycitein	C16H12O5	Flavone
11	4.92	7-hybroxy-2'-methoxyisoflavone	C16H12O4	Flavone

4.1.2 Evaluation on cell proliferation effect and cytotoxicity of DBT under OGD/R

The effect of DBT on cell viability in normal and OGD/R condition was examined by MTT assay. Figure 3 showed that there was a gradual increase in cell viability from 0.01-10 mg/mL DBT. At 10 mg/mL, DBT markedly increased cell viability by 30%. The cell viability started to reduce at 30 mg/mL and only 10% of cell survived at 100 mg/mL. Therefore, maximum of 10 mg/mL DBT was used in all the subsequent experiments in this study. After examining the cell survival under normal condition, OGD/R was performed on bEnd.3 cells to imitate ischemia/reperfusion injury and 0.01-10 mg/mL DBT was used. Figure 4 showed that OGD/R induced a significant decrease (~50%) in cell viability compared to control group, whereas 1 mM NAC or 3 mg/mL DBT significantly increased cell survival. It showed that DBT could promote the proliferation of bEnd.3 and suppress OGD/R-induced cell death.

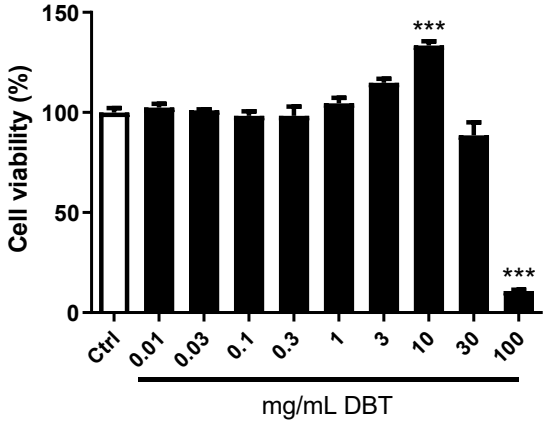


Figure 3 Examination of the cell proliferation of bEnd.3 cells after DBT treatment

Cells were treated with 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 mg/mL of DBT in serum-free HG DMEM for 24 hours, then analysed by MTT assay (n=3). One-way ANOVA followed

by Tukey's multiple comparison test was performed to compare the differences among group. ***$p < 0.001$ versus Ctrl.

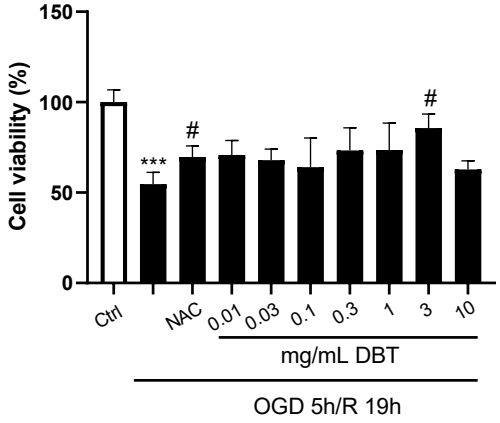


Figure 4 Examination of the cytotoxicity of DBT on bEnd.3 cells against OGD/R

Cells were treated with 1 mM N-acetyl cysteine and 0.01, 0.03, 0.1, 0.3, 1, 3, 10 mg/mL of DBT in HG DMEM before exposed to OGD for 5 hours, followed by 19-hour reoxygenation (n=3). It is then analysed by MTT assay. One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences among group. ***$p < 0.0001$ versus Ctrl and #$p < 0.01$ versus OGD/R model.

4.1.3 Effects of DBT on membrane integrity under OGD/R insult

The integrity of *in vitro* BBB model after OGD/R insult was assessed by western blotting of the expression of tight junctional proteins and TEER measurement. Western blot analysis showed that OGD/R challenge significantly reduced Claudin-5 expression by 50% in the bEnd.3 cells. DBT (0.1 – 3 mg/mL) restored the OGD/R downregulated Claudin-5 expression in a concentration dependent manner (

Figure 5A). Similarly, OGD/R challenge downregulated ZO-1 protein expression in the bEnd.3 cells by ~20% and it was significantly upregulated by DBT at 3 mg/mL (

Figure 5B). NAC (1mM) upregulated both Claudin-5 and ZO-1 expressions in bEnd.3 cells exposed to OGD/R. (

Figure 5).

TEER was used to assess the integrity and permeability of the mono- and co-culture BBB models. The co-culture system was established based on the tri-culture model for neuroinflammation described by Zheng et al. (2021), which incorporates microvascular endothelial cells (MVECs), N2a neuronal cells, and N11 microglia. The proposed model effectively recapitulates key features of neuroinflammation, including microglial activation, the release of proinflammatory mediators, disruption of the endothelial barrier, and neuronal injury. Besides, BBB is a highly interactive system composed of multiple cell types. Incorporating neuronal cells such as N2a enhances the model's ability to elucidate neurovascular interactions and neuronal responses to injury. OGD/R challenge caused significant reduction in BBB integrity as indicated by marked reduction of TEER values in both mono- (75%) and co-culture (50%) BBB models (Figure 6). DBT treatment (0.3-3

mg/mL) reversed these OGD/R-induced TEER reduction in concentration-dependent manner in both models (Figure 6). Similarly, positive control, NAC (1mM) also significantly increased the TEER values in both mono- and co-culture BBB models.

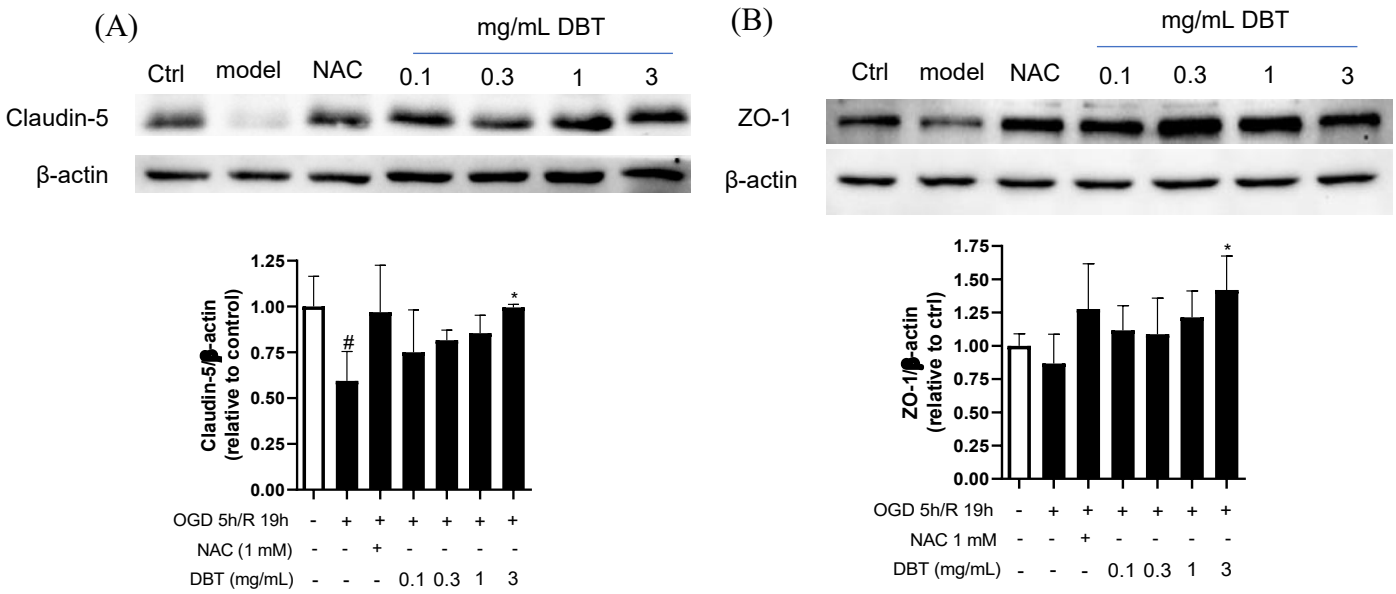


Figure 5 DBT showed restoration of (A) Claudin-5, (B) ZO-1 expression dose-dependently

Representative bands and densitometric quantification of Claudin-5 (n=3) and ZO-1 (n=3). Cells were treated with 1 mM N-acetyl cysteine and 0.1, 0.3, 1, 3 mg/mL of DBT in HG DMEM before exposed to OGD for 5 hours, followed by 19-hour reoxygenation. Unpaired t-test and One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences among group. #$p < 0.01$ versus Ctrl and *$p < 0.01$ versus OGD/R model.

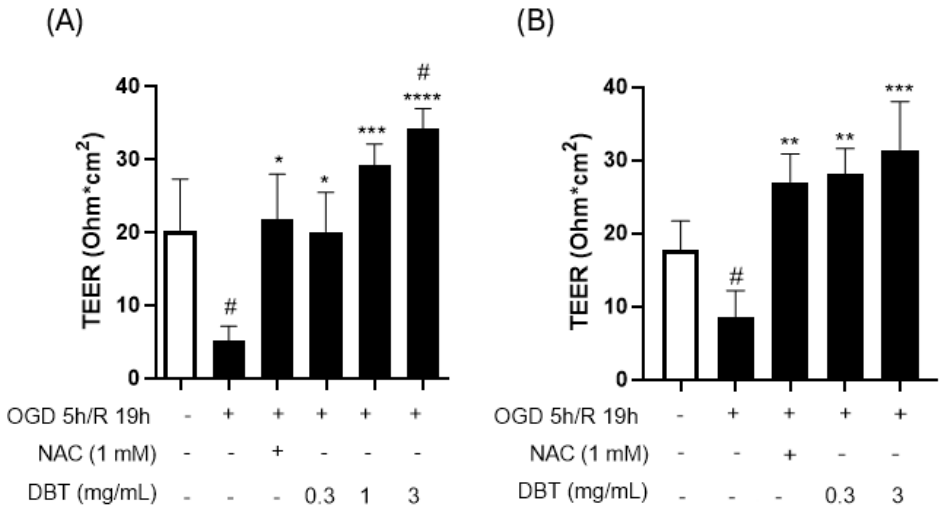


Figure 6 Increased TEER of (A) monoculture (bEnd.3) and (B) co-culture (bEnd.3+N2a) system treated with DBT dose-dependently.

(A) Only bEnd.3 cells were seeded on the apical side of the transwell (n=3). #$p < 0.05$ versus Ctrl. *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$ versus OGD/R model. (B) bEnd.3 cells were seeded on the apical side of the transwell while N2a cells were seeded on the basal part (n=3). #$p < 0.05$ versus Ctrl, **$p < 0.01$, ***$p < 0.001$ versus OGD/R model.

4.1.4 Effects of DBT on OGD-induced ROS release in bEnd.3 cells.

Intracellular ROS level was assessed using DCFDA staining (Figure 7). bEnd.3 cells were stained with DCFDA for 30 minutes followed by 5-hour OGD challenge with or without DBT treatment (0.3 mg/mL or 3 mg/mL). OGD significantly increased the intracellular ROS level in the bEnd.3 cells. Our positive control, NAC (1 mM) and DBT (0.3 mg/mL and 3 mg/mL) markedly suppressed the OGD-induced ROS generation in bEnd.3 cells in a concentration-dependent manner. The effect of DBT at 3mg/mL is comparable to the positive control, NAC (1 mM).

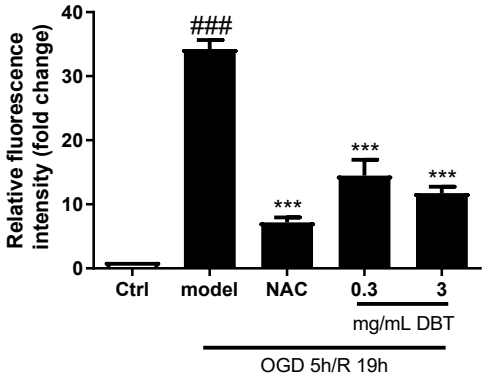


Figure 7 Decreased ROS level under OGD injury with administration of 1 mM NAC, 0.3 and 3 mg/mL DBT

DCFDA assay showed that 1 mM NAC and DBT remarkably reduced fluorescence intensity after OGD challenge, meaning that the ROS level is greatly suppressed (n=3). It might interpret that DBT has antioxidant effect. ###$p < 0.001$ versus Ctrl and ***$p <$ 0.001 versus OGD/R model.

4.1.5 Effects of DBT on bEnd.3 cell migration.

The migratory capacity of bEnd.3 cells was evaluated using a wound healing assay to assess the potential angiogenic effects of DBT. VEGF (30 ng/mL) was used as positive control, given its well-documented pro-angiogenic properties (Johnson & Wilgus, 2014). DBT was tested at 0.3 and 3 mg/mL to elucidate any dose-dependent effects on cellular migration (Figure 8). Digital images were captured at the start (T0) and 24 hours (T24) after treatment by inverted phase microscope. Quantitative analysis was performed by calculating the percentage of wound closure, utilizing the formula: [(Initial wound area - Wound area at time point) / Initial wound area × 100]. Our results showed that VEGF, at a concentration of 30 ng/mL, induced a statistically significant increase (40%) in wound closure compared to the untreated control. Notably, DBT exhibited a concentration-dependent effect on bEnd.3 cell migration. At both 0.3 mg/mL and 3 mg/mL concentrations, DBT promoted cellular migration into the wound area. The higher concentration of DBT (3 mg/mL) elicited a more pronounced effect (40%), suggesting a dose-responsive relationship between DBT concentration and migratory capacity.

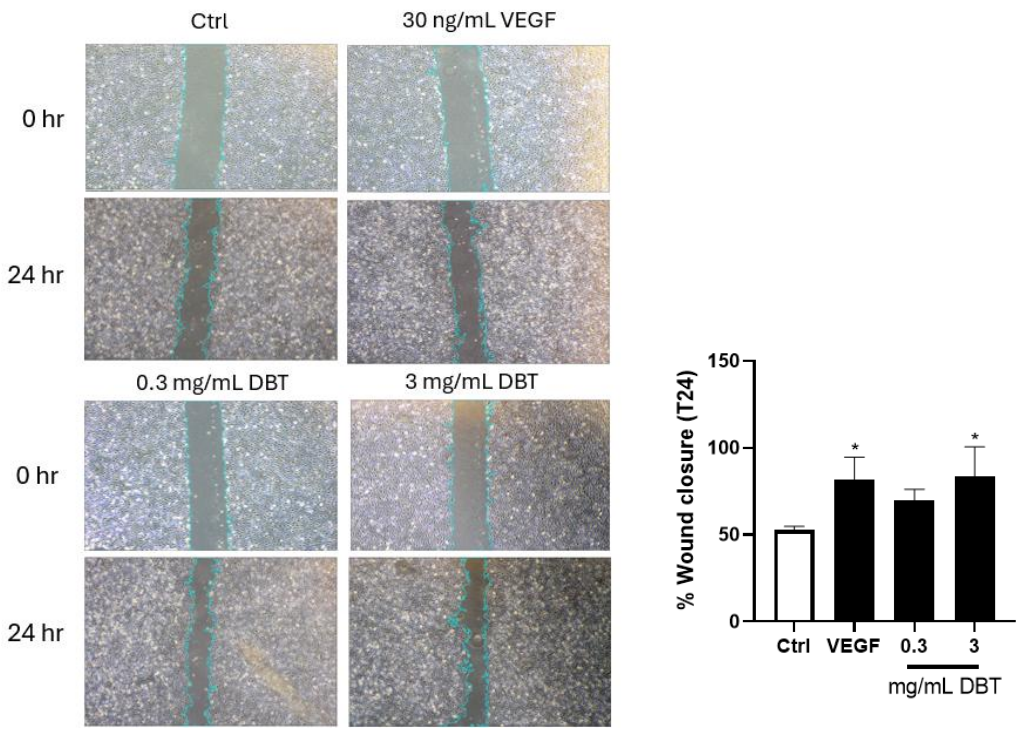


Figure 8 Effects of DBT on bEnd.3 cell migration

bEnd.3 cells were seeded in a 12-well plate and scratched with 200-ul tips. T hen, cells were treated with 30 ng/mL VEGF, 0.3 and 3 mg/mL DBT in serum-free medium (n=3). The images were taken immediately after the scratches and after 24h. (B) Percentage of wound closure of bEnd.3 cells after 24 h. *$p < 0.05$ versus Ctrl.

4.2 Role of GPR30 in DBT-Mediated Protection: G15 Antagonist Studies

4.2.1 Effects of G15 on GPR30, ZO-1, and Claudin-5 in bEnd.3 Cells

The expression of GPR30 and the effects of OGD/R on its expression in bEnd.3 cells was examined using confocal microscopy (Figure 9). The role of GPR30 was examined with G15 (5 μM), an antagonist of GPR30. Our results showed that GPR30 is expressed in the bEnd.3 cells. OGD/R alone only caused a modest increase of GPR30 expression in the bEnd.3 cells. Interestingly, G15 (5 μM) reduced the GPR30 expression by approximately 50% in bEnd.3 cells subjected to OGD/R, indicating that GPR30 expression under OGD/R condition is sensitive to G15.

After confirming the presence of GPR30 in the bEnd.3 cells, Western blot analysis was employed to assess the effects of DBT on expression of GPR30 in OGD/R-insulted bEnd.3 cells. Similarly to the confocal results above, G15 markedly reduced GPR30 expression in the OGD/R-insulted bEnd.3 cells. Interestingly, this G15-mediated GPR30 downregulation was reserved by DBT (3 mg/mL) in the OGD/R-insulted bEnd.3 cells (Figure 10A&B). We then accessed the role of GPR30 in DBT-mediated junctional proteins (ZO-1 and Claudin-5) expression in OGD/R-insulted bEnd.3 cells. Our results showed that OGD/R markedly reduced (more than 50%) the expression of both ZO-1 and Claudin-5 in the bEnd.3 cells (Figure 10A&B). These reductions were exacerbated in the present of G15, underscoring the potential role of GPR30 in maintaining junctional protein expression during OGD/R. DBT (3 mg/mL) restored the ZO-1 and Claudin-5 downregulation caused by both OGD/R in the bEnd.3 cells. Moreover, these effects were not affected by the

presence of G15, suggesting that the protective effects of DBT may involve pathways independent from GRP30 signalling pathway.

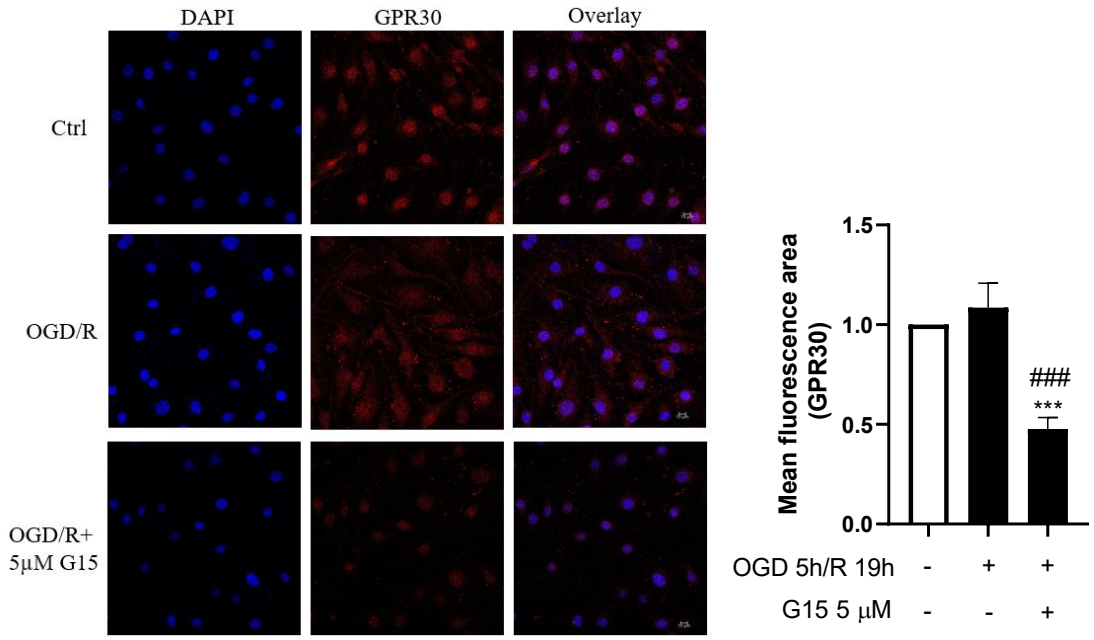


Figure 9 Immunofluorescence staining of GPR30 expression in bEnd.3 under OGD/R condition

Cells were treated with 5μM G15 for 30 minutes before exposing to OGD 5h/R 19h. Immunofluorescence staining signal was visualized by laser confocal microscopy at 40X (n=3). The nuclei are stained with Hoechst33342 (blue) and GPR30 was detected by Alexa Fluor® 594 conjugated secondary antibody (red). GPR30 slightly increased under OGD/R condition and G15 suppressed its expression by nearly half. Scale bar = 10 μm. ***$p <$ 0.001 versus Ctrl and ###$p < 0.001$ versus model.

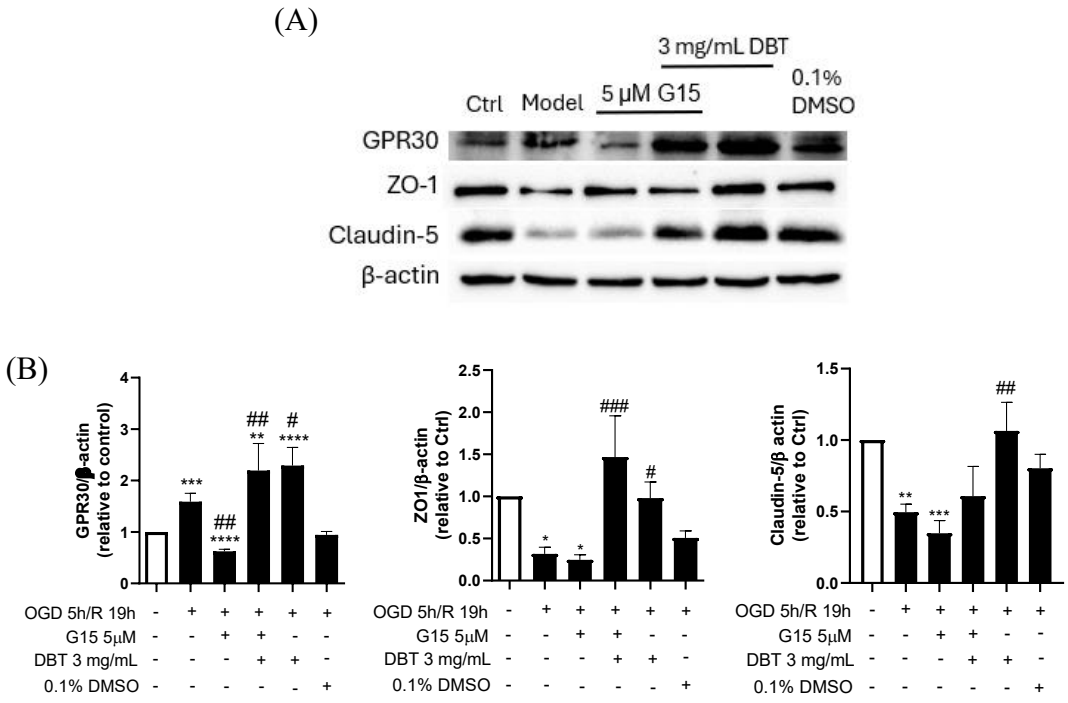


Figure 10 Expression of GPR30, ZO-1 and Claudin-5 in OGD/R G15-treated cells

(A) Representative bands and densitometric quantification of GPR30 (n=3), ZO-1 (n=3) and Claudin-5 (n=3).

(B) Cells were treated with 5 μM G15 for 30 minutes. Subsequently, 3 mg/mL of DBT in HG DMEM was added to the G15+DBT and DBT alone wells before exposure to OGD conditions. Unpaired t-test and One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences among group. *$p < 0.01$, ** $p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ versus Ctrl and #$p < 0.01$, ## $p < 0.01$, ###$p < 0.001$ versus OGD/R model.

4.2.2 Impact of G15 on Nrf2/HO-1 Signalling Pathway in bEnd.3 Cells

Figure 11B&C demonstrate that OGD/R-induced stress markedly reduced both nuclear and cytoplasmic Nrf2 expression, indicating a compromised antioxidant defence mechanism. This reduction was further exacerbated in the presence of G15, suggesting a potential role of GPR30 in maintaining Nrf2-mediated cellular protection against oxidative stress. Conversely, the reduced total and nuclear Nrf2 protein levels were restored by the treatment of DBT (3 mg/mL) in the bEnd.3 cells. We then investigated the effects of DBT on the downstream effectors of the Nrf2 signalling cascade in OGD/R-insulted bEnd.3 cells. Specifically, the expression levels of HO-1, GCLM, and GCLC were assessed. Similar to Nrf2, our results showed significant reduction of HO-1 (Figure 11D), GCLM (Figure 11E), and GCLC (Figure 11F) expressions in OGD/R and G15 challenged bEnd.3 cells, corroborating the observed decrease in Nrf2 activation. More importantly, DBT (3 mg/mL) reversed these OGD/R-mediated effects with marked promotion of these antioxidant proteins. Furthermore, the upregulation of these antioxidant proteins were not sensitive to GRP30 inhibition by G15.

Given the well-established role of the Nrf2/HO-1 pathway in modulating oxidative stress, we evaluated the functional consequences of these protein-level changes by assessing intracellular ROS levels using the DCFDA assay. Our results revealed a dramatic increase in ROS levels following OGD/R insult and G15 treatment, with both conditions elevating ROS by approximately 20-fold compared to the control group. The treatment with 3

mg/mL DBT demonstrated remarkable antioxidant efficacy, significantly suppressed the OGD/R- and G15-increased intracellular ROS level in bEnd.3 cells (Figure 12).

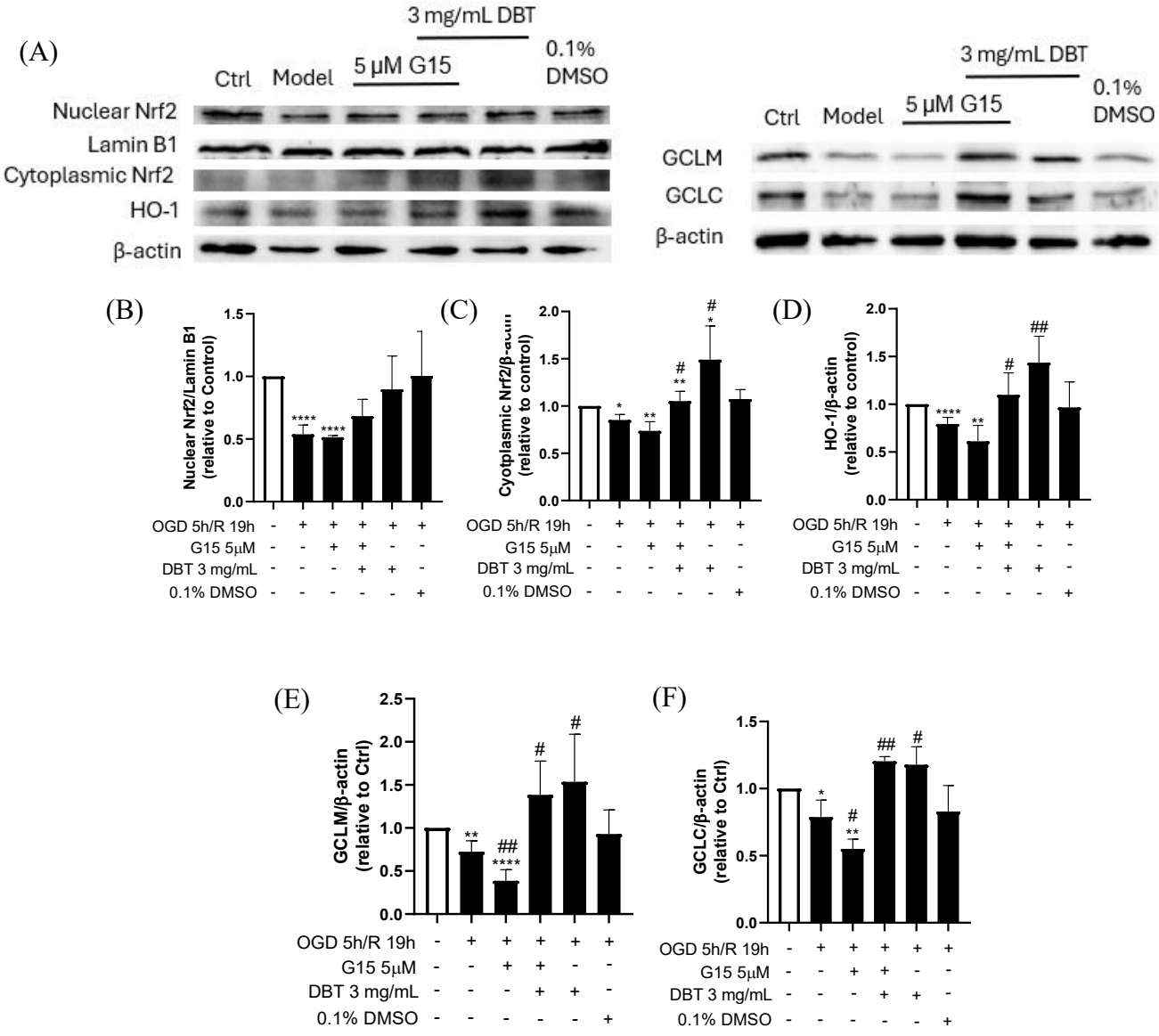


Figure 11 Expression of nuclear Nrf2, total Nrf2, HO-1, GCLC and GCLM in OGD/R G15-treated cells

(A) Representative bands and densitometric quantification of (B) nuclear Nrf2 (n=3), (C) cytoplasmic Nrf2 (n=3), (D) HO-1 (n=4) and (E) GCLM (n=3) and (F) GCLC (n=4).

(B) Cells were treated with 5 μM G15 for 30 minutes. Subsequently, 3 mg/mL of DBT in HG DMEM was added to the G15+DBT and DBT alone wells before exposure to OGD conditions. Unpaired t-test and One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences among group. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ versus Ctrl. #$p < 0.01$ and ## $p < 0.01$ versus OGD/R model.

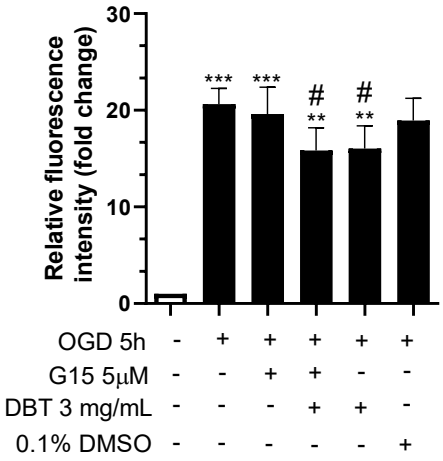


Figure 12 ROS level of OGD/R bEnd.3 cells in OGD/R G15-treated cells

DCFDA assay was performed and fluorescence intensity was measured after OGD/R (n=3). OGD conditions resulted in the highest levels of ROS fluorescence intensity, which were comparable to those observed with G15 treatment. Notably, the addition of DBT led to a significant reduction in ROS fluorescence intensity, suggesting a potential protective effect against oxidative stress following DBT administration. **$p < 0.01$ and ***$p < 0.001$ versus Ctrl. #$p < 0.01$ versus OGD model.

4.3 Neuroprotective Properties of DBT in OVX-induced Cognitive and Motor Impaired Rat Model

4.3.1 Effects of OVX and DBT treatment on uterus and body weight

We established a rat model to assess the effects of DBT on menopause-associated cognitive decline by OVX. To assess the effectiveness of OVX, the uterus was harvested and weighed, while serum estradiol levels were measured using ELISA. Additionally, body weight changes were recorded. In the sham group, the ovaries were intact, and the uterine horns appeared thick and healthy. In contrast, significant shrinkage of the uterine horns was observed in the ovaries isolated from the OVX and treatment groups (Figure 13A). The weight of the uterus showed a marked reduction in both the OVX and treatment groups (Figure 13B). Furthermore, estradiol levels in both the model and treatment groups were significantly lower than the sham group (Figure 13C). These alterations in uterine morphology and the reduction in hormone levels confirmed that the OVX surgery successfully mimicked menopause in the rats. Conversely, body weight in the OVX group was mildly higher than in the sham group, while the treatment group exhibited a significant increase in body weight (Figure 13D).

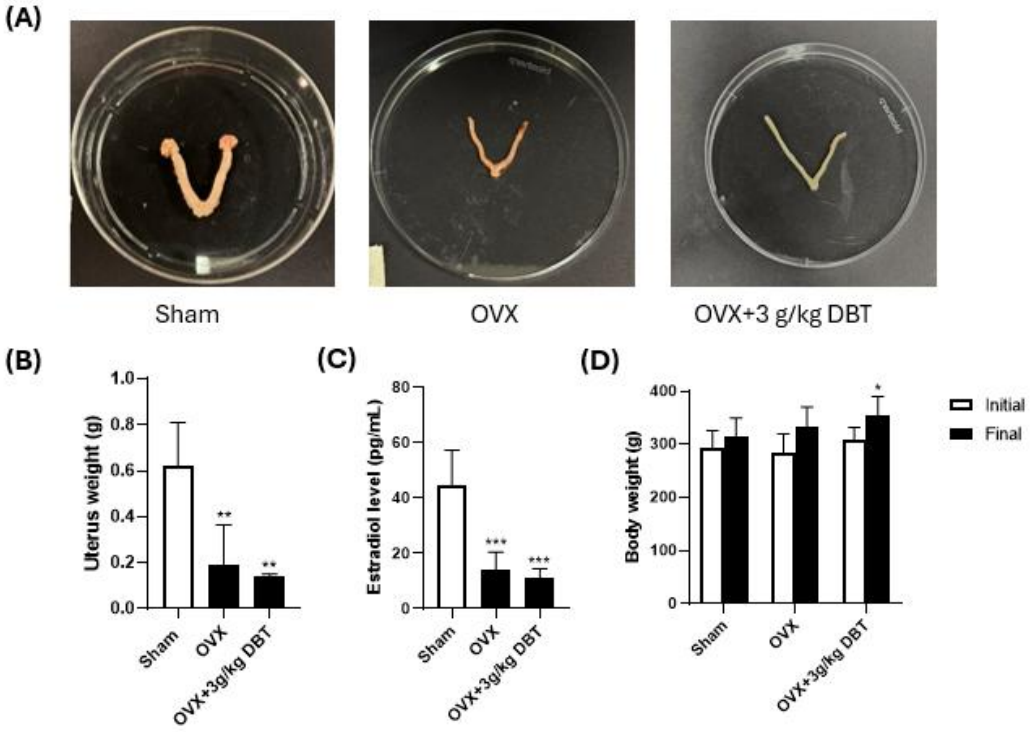


Figure 13 Impact of DBT Treatment on uterine, body weight and estradiol levels in OVX Rats

The OVX rats were treated with 3 g/kg/day DBT for 8 weeks. (A) Representative uterine phenotype photos (B) uterus tissue weight, (C) Serum estradiol level and (D) Change of body weight between the first day and final day of treatment of Sham, OVX and OVX+ 3 g/kg DBT. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus Sham.

4.3.2 Immunohistochemical Analysis of GFAP and CD31 in OVX Rat Brain following DBT Treatment

The effects of OVX and DBT treatment on astrocyte activity and vascular integrity were examined by immunohistochemical analysis of GFAP and CD31in the hippocampus of the OVX rat, respectively. These markers provide crucial insights into neuroinflammation and blood-brain barrier function in the OVX animal.

Firstly, GFAP expression in the CA1 region revealed distinct morphological differences across experimental groups (Figure 14A&B). In sham-operated controls, GFAP-positive astrocytes displayed characteristic morphology with slender, well-defined processes extending from small cell bodies, indicating a normal, non-reactive state. In contrast, OVX rats exhibited marked changes in astrocyte morphology, characterized by cellular hypertrophy with thickened processes, suggesting enhanced astrocyte reactivity in response to estrogen deficiency. Estrogen administration showed the most pronounced effect, with astrocytes returning to a morphology similar to that observed in sham controls. The effects of DBT treatment were dose dependent. Low-dose DBT (1.5 g/kg) partially attenuated the OVX-induced astrocyte reactivity, with cells showing slightly hypertrophic features. High-dose DBT (3 g/kg) demonstrated more pronounced effects, with astrocytes returning to a slender, non-reactive morphology comparable to sham controls.

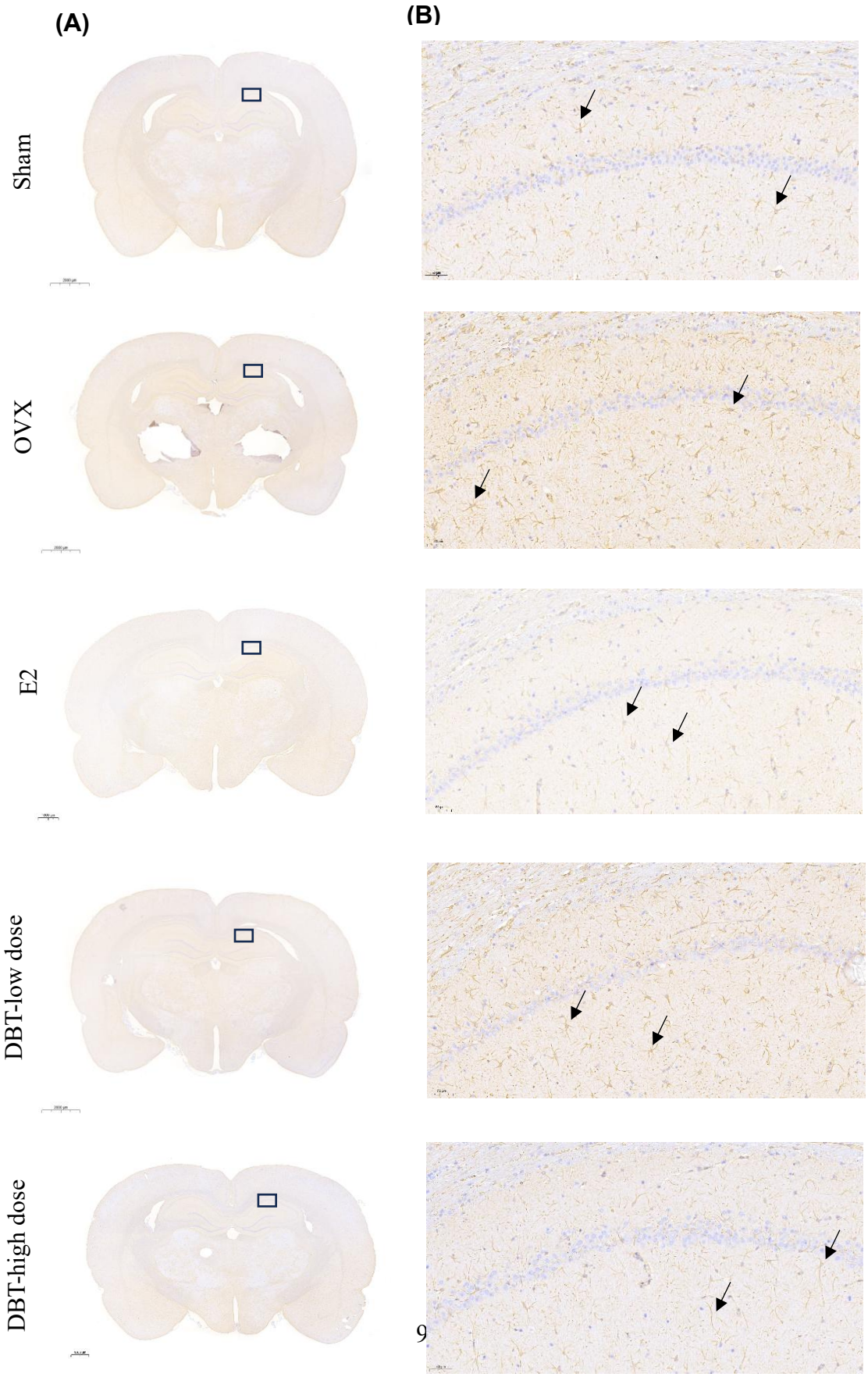
Quantitative analysis through optical density measurements confirmed these morphological observations (Figure 14C). OVX rats exhibited the highest optical density

values, confirming increased GFAP expression and astrocyte reactivity. While E2 treatment resulted in the most substantial reduction. High-dose DBT demonstrated marked efficacy in reducing GFAP expression, with values approaching those observed in estrogen-treated animals, while low-dose DBT yielded values comparable to sham controls.

On the other hand, the assessment of vascular integrity through CD31 immunostaining revealed complementary findings (Figure 15A&B). In the CA1 region, sham-operated rats exhibited evenly distributed microvessels with clear CD31-positive endothelial cell linings. This normal vascular pattern was disrupted in OVX rats, which showed reduced CD31 expression, indicating compromised vascular integrity. While E2 treatment effectively restored the vascular network to patterns similar to sham controls, both doses of DBT showed only partial preservation of vascular structure, with few delineated endothelial cell linings visible.

Optical density measurements of CD31 immunoreactivity provided quantitative support for these observations (Figure 15C). OVX rats showed significantly decreased values (approximately 0.05) compared to sham controls (0.08). E2 treatment normalised this reduction to sham-comparable levels. However, neither low-dose nor high-dose DBT treatment significantly increased CD31 optical density, suggesting limited effectiveness in restoring vascular density despite some preservation of vessel morphology.

GFAP



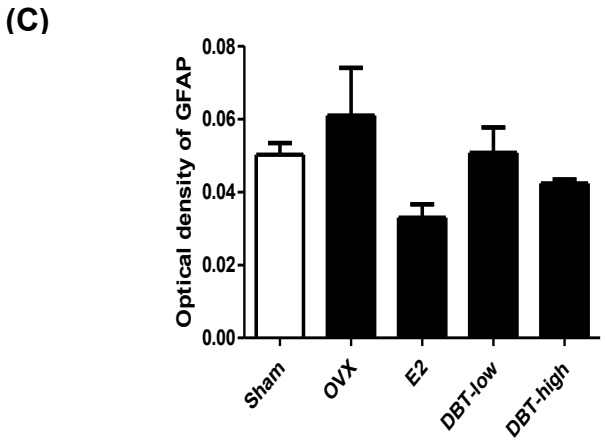
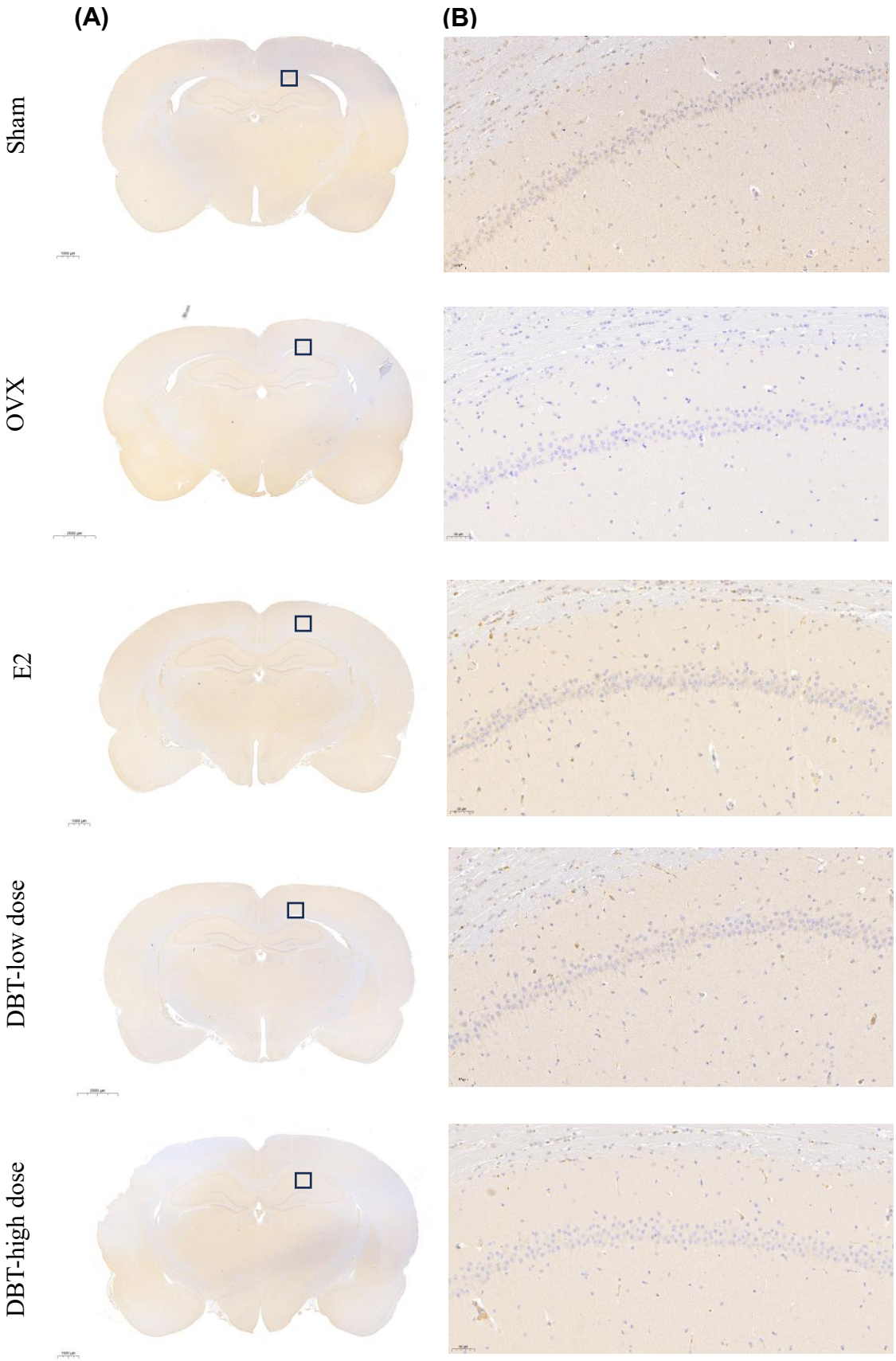


Figure 14 Immunohistochemical staining and optical density (OD) analysis of GFAP expression in the hippocampal CA1 region (n=3)

(A) Representative brain sections of GFAP immunostaining visualized with DAB and counterstained with hematoxylin in Sham, OVX, E2, OVX+ low dose DBT (1.5 g/kg), and OVX+ high dose DBT (3 g/kg) groups (Scale bar= 2000μm). GFAP immunostaining was performed using a rat monoclonal anti-GFAP antibody (Cat# AG259, Beyotime; 1:500). (B) The boxed areas in the full-size brain images are magnified (20X) to show detailed astrocyte morphology in the CA1 region. Representative astrocytes are marked with arrows. (Scale bar= 50μm). GFAP-positive astrocytes showed morphological differences across groups, with hypertrophic features prominent in OVX rats and normalized morphology in treatment groups. (C) Quantification of GFAP immunoreactivity by optical density measurements (mean gray value) obtained through colour deconvolution analysis.

CD31



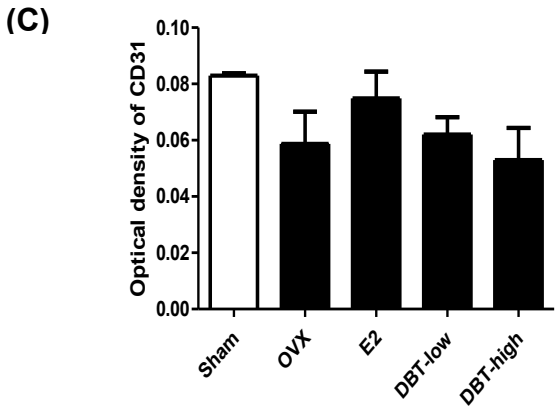


Figure 15 Immunohistochemical staining and optical density (OD) analysis of CD31 expression in the hippocampal CA1 region (n=3)

(A) Representative brain sections of CD31 immunostaining visualized with DAB and counterstained with hematoxylin in Sham, OVX, E2, OVX+ low dose DBT (1.5 g/kg), and OVX+ high dose DBT (3 g/kg) groups (Scale bar= 2000μm). CD31 immunostaining was performed using a rabbit polyclonal anti-CD31 antibody (Cat# AF6408, Beyotime; 1:200). (B) The boxed areas in the full-size brain images are magnified (20X) to show detailed vascular structures in the CA1 region. (Scale bar= 50μm). CD31-positive endothelial cells were evenly distributed in Sham, reduced expression in OVX, restored vascular networks in E2-treated groups, and partially preserved vessel structure in DBT-treated groups. (C) Quantification of CD31 immunoreactivity by optical density measurements (mean grey value) obtained through colour deconvolution analysis.

4.3.3 Effects of DBT on Cognitive and Motor Performance on OVX rats

Firstly, the spatial learning and memory in rats of different treatment groups were investigated using the Morris Water Maze. The results demonstrated that the OVX rats had slightly fewer platform crossings and significantly shorter distances travelled in the target quadrant compared to the sham group, indicating a tendency of impaired spatial memory (Figure 16B& C). Conversely, treatment with E2 and both low and high doses of DBT reversed these deficits, leading to improved performance in these measures (Figure 16B&C). Furthermore, latency to enter the target quadrant was modestly longer in the OVX group, while E2 and both DBT treatment groups exhibited significantly reduced latencies (Figure 16D). These findings highlight the effectiveness of E2 and DBT in enhancing spatial learning and memory in OVX rats.

Secondly, novel object recognition (NOR) test was performed. NOR test revealed that OVX significantly reduced the discrimination ratios when compared to the sham group, indicating impaired recognition memory (Figure 17A). E2 and low-dose DBT resulted in a modest increase in the discrimination ratio, while high-dose DBT significantly improved the discrimination ratio in the OVX rats. Additionally, the time spent exploring the novel object was shortest in the OVX group, with increased exploration times observed in both the E2 and DBT-low groups, and a significant increase in the DBT-high group (Figure 17B).

Finally, rotarod test was conducted to assess the effects of DBT on the motor function in OVX rat. The duration of time spent on the rod and the distance travelled were recorded.

Our results showed that OVX caused significant motor deficit in rat as indicated by marked reduction in the duration and travel distance (Figure 17C&D). Like the NOR test, DBT markedly restored the OVX-induced motor impairment in rats. E2 treatment also caused a small improvement to the motor functions in the OVX-rat (Figure 17C&D). Overall, these results highlight the potential of DBT in improving memory and motor coordination in models of cognitive impairment.

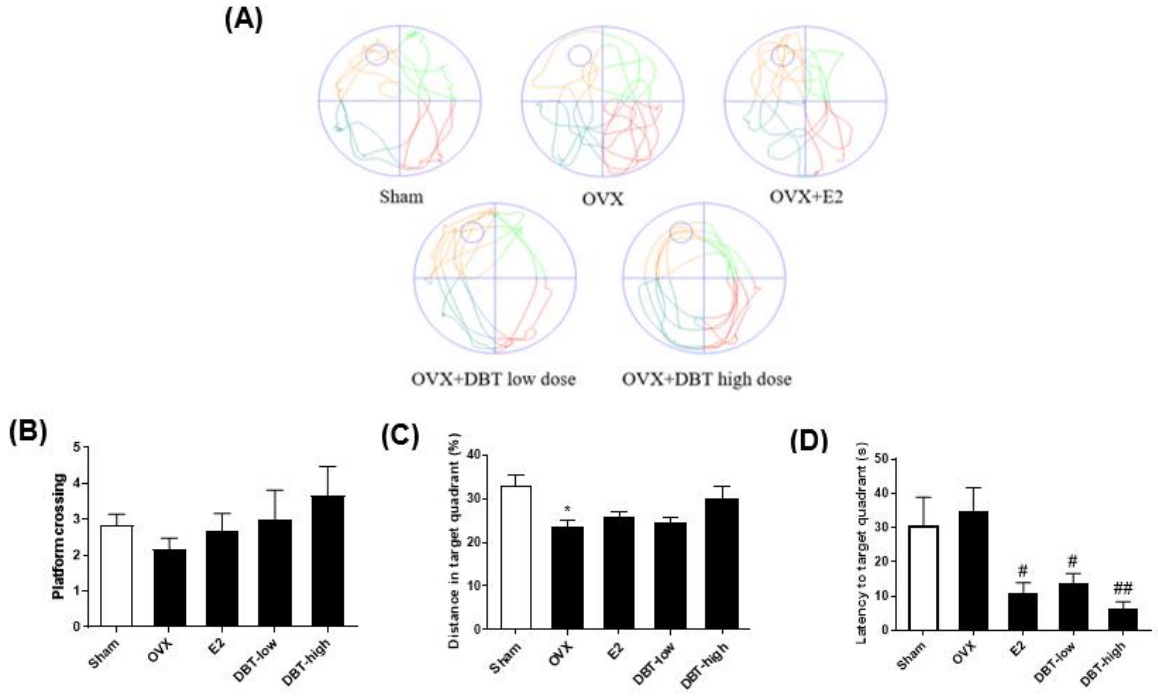


Figure 16 Effect of DBT on spatial learning and memory in OVX SD rats assessed by the Morris water maze test

Spatial learning and memory were tested in MWM (n=6) (A) The swimming trajectory of SD rats (B) Number of crossing the platform (C) Distance travelled in target quadrant during probe test (D) The latency to cross platform location during probe test. *$p < 0.05$ versus Sham. #$p < 0.05$, ##$p < 0.01$ versus OVX.

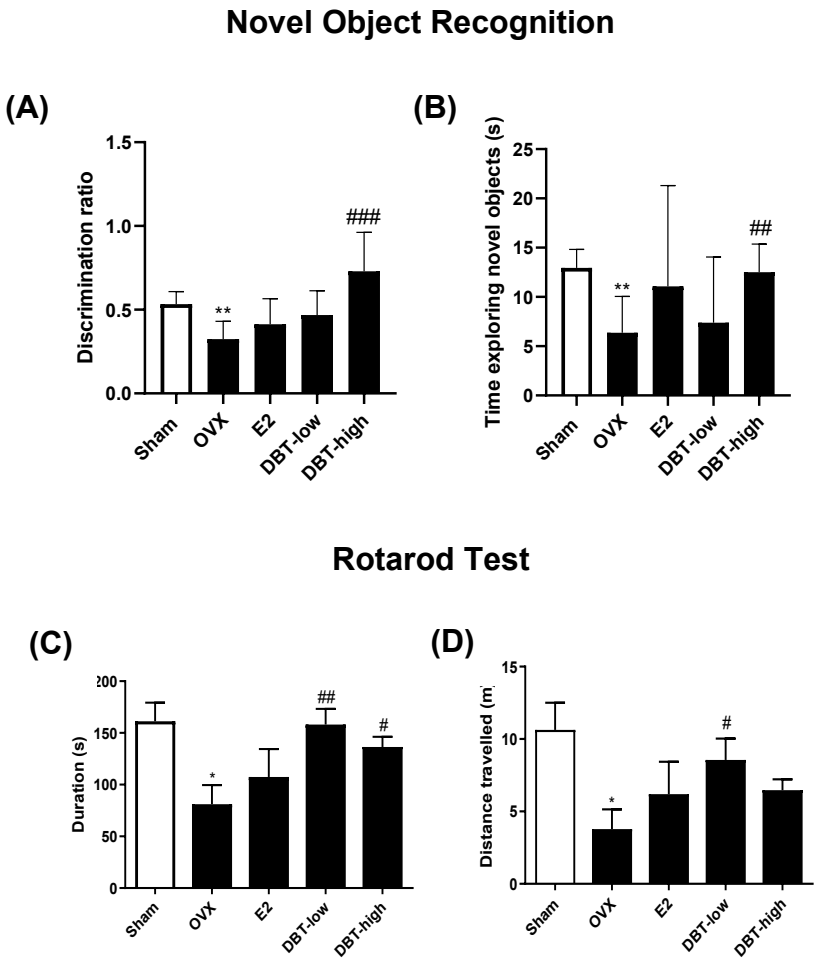


Figure 17 Effect of DBT on recognition memory and motor coordination in OVX SD rats assessed by the novel object recognition test and rotarod test (n=6)

(A) Discrimination ratio was calculated by [time exploring novel object/ total time exploring both objects]. Values above 0.5 indicate a preference for the novel object, while values below 0.5 indicate a preference for the familiar object. (B) Time exploring novel object. (C) Duration and (D) Distance travelled on the rod. *$p < 0.05$, **$p < 0.01$ versus Sham. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus OVX.

5 Discussion

Our findings indicate that DBT improved tight junctional protein expression, reduced oxidative stress and promoted angiogenesis in OGD/R bEnd.3 cells. In addition, DBT treatment alleviated the injury by increasing ZO-1 and Claudin-5, as well as the suppression of oxidative stress through Nrf2/HO-1 activation. Our *in vivo* study using OVX SD rats showed that DBT improved spatial learning and memory and enhanced recognition memory. Also, high-dose DBT reduced astrocyte reactivity with effects comparable to estrogen treatment.

Chemical analysis of DBT water extract

Previous reports have shown that AR is rich in flavonoids like formononetin, ononin and glucoside and therefore these active ingredients could also be found in DBT (H. Q. Lin et al., 2017). Our UPLC-Orbitrap-MS results verified that our DBT extract consists of the chemical markers of AR and identified 11 flavones (Table 1.). Flavone, which is a subgroup of flavonoids, is abundantly found in DBT, to be specific, isolated from AR. It is classified as phytoestrogen due to its high structural similarity with E2, which allows it to interact with E2 receptors (Kiyama, 2023).

Large bodies have evidence have shown that phytoestrogen has multi-target action on various aspects of neuroprotection, including modulating oxidative stress, regulating inflammatory responses, enhancing synaptic plasticity, and inhibiting neuronal apoptosis with few side effects (Al-Shami et al., 2023; Lal & Sutradhar, 2024). It is suggested that flavonoids could cross the highly selective semi-permeable BBB and promote

neuroprotective effect by various actions, for example, increasing synaptic plasticity, reducing neuroinflammation and inhibiting autophagy of neuronal cells (Vauzour et al., 2008). Several protein kinases and lipid kinase signalling pathways have been proven to be modulated by flavones. Yan et al. (2022) demonstrated that the administration of ononin remarkably promoted SH-SY5Y cell survival and release of anti-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Furthermore, their work also showed that ononin limited infarct size and brain edema in I/R rats.

The synergistic effects of the various flavonoids found in DBT should not be overlooked. The combination of multiple compounds may produce amplified therapeutic outcomes than individual flavonoids acting alone. This combination may strengthen the protective effects on the BBB and cognitive function, underscoring the significance of using whole extracts in therapeutic applications as opposed to separating their constituent parts. A study explored the synergistic effects of 2 active flavones, baicalein from *Scutellaria baicalensis* and daidzein from soybean extract on both estrogenic and neuroprotective activities (Ji et al., 2020). It was found that each compound stimulated estrogen-responsive element (ERE) transcriptional activity in MCF-7 cells dose-dependently, with daidzein displaying greater potency than baicalein. Notably, cotreatment with both flavonoids resulted in significantly greater activation of ERE and phosphorylation of ERα at serine 118 than either compound alone, as confirmed by combination index analysis (CI = 0.04587) indicating strong synergism. Also, their combination at lower doses produced a markedly enhanced neuroprotective effect against Aβ toxicity in PC12 neuronal cells. These findings suggest that the combined use of baicalein and daidzein may offer a promising approach for

developing supplements or therapeutics targeting estrogenic pathways and neurodegeneration (Choi et al., 2013).

In vitro study of DBT on OGD/R-injured bEnd.3 cells

DBT has been shown to promote cell proliferation. Q. T. Gao et al. (2007) showed that DBT promoted T-lymphocytes with a submaximal effect at 0.1 mg/mL. Similar to this study, our current data showed that cell proliferation was promoted from 1 mg/mL and reached the maximum effect at 10 mg/mL. Furthermore, DBT induced cell death from 30 mg/mL to 100 mg/mL in the bEnd.3 cells under normoxic condition. Interestingly, OGD/R injury reduced cell viability by ~50% which was suppressed by 3 mg/mL DBT. Overall, our study showed that DBT could promote proliferation in brain endothelial cells and improve viability against OGD/R injury.

BBB is a specialized semi-permeable structure that is responsible for the molecules or pathogens exchange between the blood and the brain. It is formed by endothelial cells from the capillary wall, surrounded by the end feet of astrocytes and pericytes on the basal lamina, as well as neurons. The endothelial is tightly connected by various tight junction proteins, like, ZO-1 and claudins. Maintaining the integrity of endothelial cells is essential for controlling the passage of hazardous chemicals from penetrating the CNS. The breakdown of BBB is often considered as one of the causes of VaD. To mitigate the environment of BBB *in vitro*, a mono- and co-culture transwell system is designed to measure TEER with reference to Y. F. Zheng et al. (2021). TEER is the measurement of electrical resistance across endothelial monolayer and the determination of the integrity.

The cells were treated with different concentrations of DBT before undergoing OGD 5h/ R 19h. In both mono- and co-culture systems, the result showed that OGD/R injury significantly reduced TEER. With the administration of 1 mM NAC and 0.3-3 mg/mL DBT, TEER was restored in a dose-dependent manner. TEER is often associated with endothelial tight junction proteins, which are found to be disrupted and decreased during OGD/R challenge. Indeed, our western blot results showed that ZO-1 and Claudin-5 expression were reduced in bEnd.3 cells subjected to OGD/R and more importantly, these reductions were restored by DBT.

Our experimental findings demonstrate that DBT significantly suppressed oxidative stress in bEnd.3 under OGD/R injury. The OGD/R challenge induced a marked increase in intracellular ROS levels, consistent with the oxidative burst characteristic of ischemic injury. However, treatment with DBT at 0.3 and 3 mg/mL reduced ROS levels in a concentration-dependent manner, with the higher dose achieving efficacy comparable to the antioxidant NAC (1 mM). These results align with the established role of oxidative stress in exacerbating endothelial dysfunction and BBB disruption, where excess ROS damages endothelial cells and tight junction proteins (e.g., Claudin-5, occludin), increasing BBB permeability (Schreibelt et al., 2007). ROS could lead to the alteration of tight junction proteins, followed by increased endothelial monolayer permeability and thus, BBB disruption. By mitigating ROS, DBT preserved tight junction integrity as presented in Western blot results, thereby maintaining BBB function and limiting neurotoxic infiltration, which is a critical mechanism in VaD pathology. Furthermore, ROS overproduction reduces nitric oxide bioavailability, impairing vasodilation and cerebral

blood flow regulation (Bennett et al., 2009). DBT's antioxidant activity could restore NO signalling, improving endothelial function and perfusion, which is essential for mitigating hypoperfusion-driven neurodegeneration in VaD.

Although our study did not directly measure matrix metalloproteinase (MMP) activity, existing literature highlights ROS as a key activator of MMP-2 and MMP-9, enzymes that degrade extracellular matrix components and tight junctions, exacerbating BBB breakdown (Pun et al., 2009). By reducing ROS, DBT may indirectly suppress MMP activation, thereby attenuating BBB disruption. This hypothesis warrants further investigation to confirm DBT's dual role in antioxidant and MMP modulation. The antioxidative efficacy of DBT parallels its documented benefits in myocardial ischemia-reperfusion injury, where oxidative stress is a primary driver of tissue damage (Mak et al., 2006). In the context of VaD, DBT's ability to mitigate ROS could protect cerebral microvasculature, slow disease progression, and preserve cognitive function.

Menopausal cognitive impairment is a significant concern for many women, as it is often associated with disrupted vascular tone, which can compromise cerebral blood flow (CBF) and contribute to the development of VaD. During menopause, fluctuations in E2 levels disrupt the balance of vascular homeostasis, impairing endothelial function and vascular tone regulation. E2 is known to play a protective role in the vasculature by reducing inflammation, enhancing nitric oxide production, and promoting endothelial cell migration and angiogenesis. However, as E2 levels decline during menopause, these protective mechanisms are weakened, leading to endothelial dysfunction, reduced vascular reactivity,

and increased risk of ischemia, an insufficient supply of oxygen and nutrients to tissues. Ischemia in the brain, resulting from impaired vascular function, can trigger a cascade of pathological events that damage neurons and the vasculature. This damage contributes to cognitive decline, as neurons are deprived of oxygen and nutrients necessary for normal function. In this context, the impaired blood flow caused by disrupted vascular tone during menopause may initiate or accelerate the development of VaD, a condition characterized by cognitive dysfunction due to compromised cerebral circulation. The brain's ability to maintain adequate blood supply is critical for supporting neuronal health, neuroplasticity, and cognitive function, all of which are jeopardized in the presence of ischemic injury.

Recent evidence suggested that promoting endothelial repair and angiogenesis could be a promising therapeutic strategy for preventing or alleviating menopausal-related cognitive decline. In this study, we examined the angiogenic property of DBT using wound healing assay. The wound healing assay is a widely used method for evaluating the migratory capacity of cells, particularly endothelial cells, and their role in angiogenesis. By mimicking tissue injury, this assay offers valuable insights into the mechanisms of endothelial repair, including cell migration and proliferation, which are the processes essential for restoring vascular integrity. Endothelial cell migration toward the site of injury is a critical component of angiogenesis, facilitating the reestablishment of blood flow and the delivery of oxygen and nutrients, which are vital for tissue recovery following ischemic events. In our study, 0.3 and 3 mg/mL DBT was examined, and VEGF was used as a positive control. It was revealed that 3 mg/mL DBT markedly promoted the closure of wound, with effects comparable to those induced by VEGF, a well-established growth

factor known to promote angiogenesis and endothelial cell migration. This result demonstrated that the comparable efficacy of DBT and VEGF in enhancing cell migration highlights the potential of DBT as a therapeutic agent for promoting endothelial repair and angiogenesis, Besides, MTT assay results revealed that DBT significantly enhanced bEnd.3 cell proliferation, indicating its ability to not only promote migration but also amplify the endothelial cell population necessary for vascular repair. This dual action highlights DBT's potential as a therapeutic agent for endothelial repair and CBF improvement. Enhanced CBF is essential for delivering oxygen and nutrients to brain tissues, supporting cognitive functions, and potentially slowing the progression of dementia. This finding is particularly noteworthy in the context of stroke and VaD, where endothelial dysfunction and inadequate blood flow to the brain contribute significantly to disease progression.

The active compounds in DBT, such as calycosin, have also been shown to have pro-angiogenic effects, including the induction of endothelial cell proliferation and the enhancement of angiogenesis in other study It was demonstrated that calycosin promotes blood vessel formation in zebrafish, where it increases the diameter of blood vessels and supports vascular development. (Tang et al., 2010). Besides, baicalin, which was detected in UPLC-MS of DBT, has been verified its pro-angiogenic property in chick embryo chorioallantoic membrane model and wound healing assay using human umbilical vascular endothelial cells (K. Zhang et al., 2011; Zhu et al., 2016). These findings are consistent with the observed effects of DBT on endothelial cell migration in the current study, providing further evidence that DBT may act as a potent inducer of angiogenesis. Additionally, Lin et al. (2022) evaluated the effects of DBT and its individual components

on vascular repair, in a model of vascular injury and showed that DBT contributed to the restoration of vascular integrity, particularly by alleviating the effects of vascular repair inhibitors, thereby enhancing the ability of blood vessels to heal and regenerate. Taken together, these studies provide a strong foundation for understanding the potential of DBT as a multi-faceted therapeutic agent that may not only promote endothelial migration and angiogenesis but also support overall vascular health in the brain, offering a promising approach for treating VaD and related cerebrovascular disorders.

The wound healing assay results, combined with previous studies, suggest that DBT may offer a multi-faceted approach to addressing VaD. By promoting endothelial cell migration and proliferation, DBT could improve CBF through enhanced angiogenesis and vascular network restoration. Moreover, the comparable effects of DBT to VEGF highlight its potential as a novel treatment strategy to address the vascular causes of cognitive impairment, offering a complementary or alternative approach to conventional therapies for menopausal VaD.

Role of GPR30 and Nrf2/HO-1 Pathway in OGD/R bEnd.3 cells

The neuroprotective role of GPR30, a novel G protein-coupled estrogen receptor involved in mediating rapid, non-genomic estrogen signalling, has emerged as a significant area of research in understanding and potentially treating ischemic brain injuries. Recent studies have extensively investigated and verified the neuroprotective effects of GPR30 using both agonists (G1) and antagonists (G15) in various *in vitro* and *in vivo* models. A particularly noteworthy study utilizing primary astrocytes demonstrated that G1 prevented neuronal

damage by retaining autophagy levels and decreasing the release of inflammatory cytokines under glutamate-induced excitotoxic injury (Wang et al., 2020). Meanwhile, these protective effects were reversed by pharmacological inhibition of GPR30 using G15 treatment, highlighting the specificity of GPR30's involvement. The study further solidified these findings through *in vivo* experiments with GPR30 knockout models, where the neuroprotective effect of G1 was completely blocked, underscoring the critical role of GPR30 in neuroprotection. Furthermore, the potential of GPR30 in addressing menopause-associated cognitive decline has also gained significant attention recently. Activation of GPR30 has been shown to alleviate memory impairments in OVX mice. This activation enhances synaptic plasticity and supports cognitive function, indicating that GPR30 could be a potential therapeutic target for addressing cognitive decline in peri- and postmenopausal women (Luo et al., 2023).

In this study, we demonstrated the presence of GPR30 in bEnd.3 cells and the changes of its expression following OGD/R and G15 treatment using confocal microscopy. G15 (5 μM) treatment on bEnd.3 cells prior to undergoing OGD/R significantly reduced the expression of GPR30 as indicated by both immunofluorescence staining and Western blot analysis (Figure 9 & Figure 10). This reduction in GPR30 expression was accompanied by an exacerbation of tight junction protein loss (Figure 10) and increased ROS production (Figure 12), highlighting the protective role of GPR30 in maintaining BBB integrity and reducing oxidative stress. Notably, the addition of DBT reversed these detrimental effects while simultaneously elevating GPR30 levels. These results align with previous studies that reported a slight elevation of GPR30 in neuronal ischemic models and demonstrated

that GPR30 blockade by G15 intensified neuronal impairment (Shen et al., 2021; Zhang et al., 2018). The ability of DBT to overcome G15-induced GPR30 inhibition and elicit protective effects on BBB integrity is particularly noteworthy. While we did not directly compare DBT with the known GPR30 agonist G1 in this study, it is important to point out that our current results clearly demonstrated that DBT produced significant protective effects on BBB integrity even in the presence of G15. This finding suggests that DBT's antioxidant actions are not dependent on GPR30-mediated signalling and may involve other, as yet unidentified, mechanisms.

Our investigation further delved into the mechanisms underlying GPR30's neuroprotective effects, focusing on the Nrf2/HO-1 signalling pathway. This pathway plays a vital role in combating oxidative stress-related detrimental effects in vascular diseases (Zhang et al., 2021). Activation of this pathway has shown promising results in various models of oxidative stress-related disorders, including chronic inflammation diseases, cardiovascular disease and neurodegenerative conditions (Liu et al., 2023; Luo et al., 2018; Y. M. Wang et al., 2021; Zhang et al., 2021). When activated, Nrf2 translocated to the nucleus to upregulate the expression of antioxidant and detoxification genes, including HO-1, GCLC and GCLM. Several studies have shown that the induction of HO-1 can provide protection to neuronal cells against oxidative stress in Alzheimer's disease or Parkinson disease (Zhan et al., 2013). HO-1 is an enzyme that catalyzes the breakdown of heme into biliverdin, carbon monoxide (CO), and iron. Biliverdin is then converted into bilirubin, which is an antioxidant that helps to scavenge ROS and protect cells from oxidative damage (Chiang et al., 2021). The elevation of HO-1 could have increased the breakdown of heme into

biliverdin, which would result in an increase in the levels of bilirubin, reducing the ROS caused during OGD/R injury. Besides, GCLC and GCLM is involved in the synthesis of glutathione, a key antioxidant in the body (R. Wang et al., 2023). These proteins form glutamate-cysteine ligase, which catalyse the first and rate-limiting step in glutathione production. Increased glutathione synthesis enhances the cell's capacity to neutralize ROS and protect against oxidative damage. By promoting the expression of HO-1, GCLC, and GCLM, DBT appears to maintain cellular redox balance and safeguard neuronal health, particularly under OGD/R conditions. Several studies have demonstrated the neuroprotective effects of Nrf2/HO-1 pathway activation. For instance, Xu et al. (2019) found that activation of the Nrf2/HO-1 pathway by melatonin protected against traumatic brain injury in mice by reducing oxidative stress and neuroinflammation. Similarly, Zhang et al. (2017) reported that curcumin-activated Nrf2 signalling provided neuroprotection in a rat model of Parkinson's disease by upregulating antioxidant enzymes and reducing dopaminergic neuron loss. the neuroprotective effects of GPR30 activation, mediated through the Nrf2/HO-1 pathway, offer a promising avenue for the development of new therapies for ischemic brain injuries and neurodegenerative diseases. The ability of DBT to activate this pathway and protect against oxidative stress and BBB disruption in bEnd.3 cells under OGD/R conditions further supports its potential as a therapeutic agent.

Although DBT treatment was associated with increased GPR30 expression, our findings indicated that its protective effects were not dependent on GPR30 signalling. This conclusion was supported by the observation that DBT continues to restore barrier function and reduce oxidative stress even in the presence of the GPR30 antagonist G15, suggesting

that DBT's beneficial actions occur through alternative mechanisms rather than direct GPR30 activation. The upregulation of GPR30 observed after DBT treatment should not be interpreted as a direct signal for Nrf2/HO-1 pathway activation. Instead, this increase in GPR30 expression may reflect an indirect or compensatory cellular response to injury or stress. It is plausible that other signalling pathways, such as the PI3K/Akt, are involved in mediating the effects of DBT. GPR30 can activate downstream pathways including PI3K/Akt, which are known to regulate gene expression and cellular stress responses. Activation of the PI3K/Akt pathway may, in turn, influence the activity of transcription factors such as Nrf2, leading to upregulation of antioxidant genes like HO-1 (Peng et al., 2024). This multi-component, multi-target mechanism likely allows DBT to sustain robust antioxidant defences and maintain BBB integrity even when GPR30 signalling is compromised. Collectively, these findings underscore the therapeutic potential of DBT under ischemic injury, where effective treatment relies on the interplay and reinforcement of multiple protective mechanisms.

In vivo study of DBT on cognitive performance in OVX rats

The vascular changes associated with menopause, including endothelial dysfunction, arterial stiffening, and reduced cerebrovascular integrity, represent a critical pathway to increased risk of VaD (Moreau & Hildreth, 2014; Moreau et al., 2012) . During menopause, the decline in E2 levels leads to the loss of its vasoprotective effects, resulting in heightened oxidative stress and dysregulation of BBB function. These changes collectively contribute to chronic cerebral hypoperfusion and microvascular damage, which are key drivers of VaD pathogenesis. Several studies have demonstrated alterations in GFAP and CD31

expression in VaD models (Denver et al., 2019; Edgerton-Fulton & Ergul, 2022; Jia et al., 2020; Sierra et al., 2007). The mechanisms underlying these changes are further elucidated by using OVX rat models in this study, which mimic the estrogen-deficient state of postmenopausal women. The menopausal vascular alterations as well as neuroinflammation was evidenced by the elevated GFAP and reduced CD31 in hippocampal tissue.

GFAP (glial fibrillary acidic protein) is a key intermediate filament protein primarily expressed in astrocytes, serving as a specific marker for astrocyte reactivity and neuroinflammation (Hol & Pekny, 2015). Increased GFAP expression typically occurs in response to various forms of brain injury and it reflects an enhanced activation of astrocytes (Hol & Pekny, 2015). Under normal conditions, GFAP expression in astrocytes is relatively low, but in response to various forms of stress or injury, such as ischemia, neuroinflammation, or neurodegenerative diseases, GFAP expression increases significantly, reflecting the activation of astrocytes in the affected area. This increase in GFAP expression is often associated with the phenomenon of astrogliosis, a reactive process in which astrocytes undergo structural and functional changes in response to damage. In conditions such as VaD, which is a form of cognitive decline linked to impaired blood flow to the brain, GFAP expression is upregulated as part of the brain's attempt to repair and compensate for the disrupted vasculature. Astrocyte activation and proliferation are particularly significant because of the direct impact they have on the BBB (Abbott et al., 2006). When brain regions experience ischemia or reduced blood flow, as is common in VaD, astrocytes become activated and increase GFAP expression in response to the

neuronal damage. On the other hand, CD31 is a marker for endothelial cells that plays a crucial role in maintaining vascular and BBB integrity. Its expression patterns provide valuable insights into vascular structure and function, with changes in CD31 levels potentially indicating alterations in BBB integrity and vascular health.

The interplay between GFAP and CD31 underscores the neurovascular coupling critical to brain health and its disruption in VaD (Price et al., 2018). The elevated GFAP expression is part of a compensatory response to neuronal injury and oxidative stress. Reactive astrocytes release pro-inflammatory cytokines (e.g., TNF-α, IL-6) and matrix metalloproteinases (MMPs), which exacerbate endothelial dysfunction by degrading tight junction proteins (e.g., Claudin-5, ZO-1) and reducing CD31 expression, which is a glycoprotein essential for endothelial cell adhesion, angiogenesis, and BBB stability. Concurrently, diminished CD31 levels impair vascular repair mechanisms, further compromising cerebral blood flow and perpetuating a cycle of neuroinflammation and vascular decay.

In this study, our immunohistochemical analysis of GFAP and CD31 in the hippocampus of OVX rats provides compelling evidence for the successful establishment of a model that mimics key aspects of VaD pathology associated with menopause. Our results revealed an increase in GFAP expression in OVX rats, characterized by cellular hypertrophy and thickened astrocytic processes, demonstrates enhanced astrocyte reactivity in response to estrogen deficiency. This heightened neuroinflammatory state is consistent with the increased risk of cognitive decline and VaD associated with estrogen loss in

postmenopausal women. Concurrently, the reduced CD31 expression in OVX rats, evidenced by disrupted vascular patterns and decreased optical density measurements, indicates compromised vascular integrity and BBB dysfunction. These changes collectively support the validity of the OVX model in replicating key pathological features of VaD.

The results for DBT treatment are particularly intriguing. High-dose DBT (3 g/kg) demonstrated marked efficacy in reducing GFAP expression, suggesting its potential to attenuate astrocyte reactivity by suppressing pro-inflammatory pathways such as NF-κB and TNF-α signalling (Li et al., 2020). This effect was comparable to E2 treatment, indicating that DBT might mimic estrogen's neuroprotective properties, possibly through its phytoestrogen content (e.g., ligustilide from ASR), which binds to estrogen receptors to modulate neuroinflammatory responses (Zhang et al., 2019). However, the effects of DBT on vascular integrity, as measured by CD31 expression, were less pronounced. While DBT showed partial preservation of vascular structure, it did not significantly increase CD31 expression, a critical marker of endothelial repair and angiogenesis (Wang et al., 2021). These findings suggest that DBT exerts differential effects on astrocyte reactivity and vascular integrity. Its ability to modulate GFAP expression highlights its neuroprotective potential, likely through estrogen receptor-mediated suppression of astrogliosis and oxidative stress (Chen et al., 2022). However, its limited effect on CD31 expression implies that DBT may not fully replicate estrogen's vascular protective effects, which include upregulating VEGF and promoting endothelial cell survival (Bake & Sohrabji, 2004). It is important to note that the small sample size (n=3) in this experiment is a significant

limitation. Future studies with larger sample sizes are necessary to confirm these findings and establish the reproducibility of the observed effects on vascular density.

E2 plays a critical role in modulating synaptic plasticity and memory processes in the hippocampus (Lu et al., 2019). In light of this, there is a growing interest in exploring alternative therapeutic approaches that can mimic the neuroprotective effects of E2. Recent studies have highlighted the protective properties of estrogenic plants in neuroprotection, particularly concerning cognitive decline and neurodegenerative conditions (Echeverria et al., 2021). These plant-derived compounds may offer a natural means of supporting cognitive health by mimicking the neuroprotective actions of E2, potentially providing therapeutic benefits for those experiencing cognitive deficits related to hormonal changes. Research has shown that phytoestrogens can positively influence cognitive function and provide neuroprotective benefits. For instance, soy extract (0.2 mg/kg) prevented kainic acid-induced neuronal loss in OVX Wistar albino rats. Furthermore, it has been shown that the herbal compounds in AR exhibited therapeutic effects in myocardial and cerebral ischemia and atherosclerosis (Li et al., 2022) while ASR promoted cognitive recovery by promoting synaptic plasticity and neurogenesis (Deng et al., 2015; Xin et al., 2013). The neuroprotective effect of DBT has also been shown *in vitro* and *in vivo*. The decoction can mitigate spatial memory deficits and synaptic plasticity impairment in Alzheimer disease mice model through the upregulation of synaptic proteins and reduction in Aβ deposition (Chai et al., 2023). Also, it decreases β-amyloid-induced apoptosis dose-dependently by regulating the expression of Bcl2 and Bax proteins and influencing caspase activity in cultured cortical neurons (Gong et al., 2019).

The loss of E2 not only affects synaptic plasticity and memory processes but also diminishes the brain's ability to cope with oxidative stress and inflammation, both of which are exacerbated in VaD. Consequently, the cognitive deficits observed in OVX rats serve as a model for understanding similar impairments in postmenopausal women, who experience a natural decline in E2 levels. In this study, Morris water maze, novel object recognition and rotarod test were performed to assess cognitive and motor functions in OVX rats. The Morris water maze is a recognised method for evaluating spatial learning and memory by measuring the time taken for rodents to locate a hidden platform in the pool. Besides, novel object recognition focuses on recognition memory by examining the tendency of exploring novel objects. Last, rotarod test measures the ability of rats to remain on a rotating rod, which is related to the motor coordination. Together, these tests provide comprehensive understanding of the cognitive and motor impairments resulting from OVX and DBT treatment.

In this study, the OVX model was successfully established as shown by the significant shrinkage of uterine horn, sharp reduction in E2 level and increase in body weight. The behavioural tests conducted revealed that OVX rats exhibited cognitive deficits. This was demonstrated through reduced distance travelled in the target quadrant during the MWM test, indicating impaired spatial memory. Additionally, OVX rats spent less time exploring novel objects and showed a lower discrimination ratio in the NOR test, further supporting the presence of recognition memory deficits. Moreover, the diminished duration and distance travelled in the rotarod test highlighted impaired motor function among OVX rats. Yet, the group treated with estradiol did not show better performance compared to the

vehicle-treated OVX rats and DBT administration only had slight improvement on OVX rat memory.

The absence of E2, which typically controls the activity of hormones including ghrelin, leptin, and insulin, is thought to be the cause of weight increase in OVX samples. E2 has been shown to suppress the appetite-stimulating effects of ghrelin, a hormone produced primarily in the stomach that promotes food intake. In OVX rats, the lack of E2 leads to increased ghrelin signalling, potentially contributing to hyperphagia and subsequent weight gain (Clegg et al., 2007). Furthermore, E2 enhances the circulation and signalling of leptin, an adipocyte-derived hormone that promotes satiety and increases energy expenditure (Burch et al., 2022). Insulin, another key metabolic hormone, is also influenced by E2. E2 has been shown to enhance insulin sensitivity in various tissues, including the brain. In OVX rats, the absence of E2 can lead to reduced insulin sensitivity, potentially contributing to altered glucose metabolism and increased adiposity. A study by Riant et al. (2009) found that OVX mice exhibited impaired glucose tolerance and insulin resistance, which were reversed by E2 treatment. The disruption of these regulatory processes in OVX rats typically results in a significant increase in food intake and body weight gain. For instance, a study by Witte et al. (2010) reported that OVX rats exhibited a 20-30% increase in body weight compared to sham-operated controls within 4-6 weeks post-surgery. This weight gain is often accompanied by an increase in adipose tissue mass, particularly in the abdominal region.

Concurrently with weight gain, OVX rats experience a marked reduction in uterine weight due to the absence of E2's trophic effects on uterine tissue. E2 plays a crucial role in maintaining uterine growth, vascularization, and function (Storment et al., 2000). Without E2, uterine tissue undergoes atrophy, resulting in decreased size and weight of the uterus (Graves et al., 2011). A study by Westerlind et al. (1998) reported a 75-80% reduction in uterine weight in OVX rats compared to intact controls within two weeks of ovariectomy. This uterine atrophy is a reliable indicator of successful ovariectomy and is often used to confirm the effectiveness of the OVX model. The OVX model's validity was further supported by changes in circulating hormone levels. Typically, OVX rats exhibit significantly reduced serum E2 levels. In addition, the pronounced reduction in uterine weight not only confirmed the loss of endogenous estrogen but also highlighted the sensitivity of uterine tissue to hormonal status. The treatment of high dose DBT in OVX rats failed to restore uterine suggested a lack of potent estrogenic stimulation in the uterus. This is particularly relevant for evaluating the safety profile of potential therapies for postmenopausal women. The absence of uterotrophic effects with DBT administration in OVX rats implied that, although DBT may confer benefits such as reduced neuroinflammation and improved memory in our study, it does not mimic estrogen's action in the uterus. This tissue-selective action is advantageous, as excessive uterine stimulation by estrogen from HRT is associated with increased risks of endometrial hyperplasia and cancer (Feeley & Wells, 2001). Therefore, therapies like DBT that do not promote uterine growth may offer a safer alternative for long-term management of postmenopausal symptoms, especially for women at risk of hormone-sensitive cancers.

Our results of behavioural tests are aligned with previous research indicating that E2 deprivation following ovariectomy can lead to cognitive impairments (Y. Q. Rao et al., 2015; F. G. Wang et al., 2023; W. Y. Zhang et al., 2020). It also indicated that DBT led to a moderate improvement in cognitive function among OVX SD rats. This finding aligns with the hypothesis that herbal treatments can provide neuroprotective benefits, particularly in models of cognitive decline associated with E2 deprivation. Although the mechanisms underlying the DBT-induced cognitive improvements in our *in vivo* study have not been fully elucidated, data from our *in vitro* study and Li et al. (2023) suggested that is most likely mediated via the antioxidative effects through activation of the Nrf2/HO-1 pathway. Li et al. (2023) also found that DBT markedly alleviated cardiovascular dysfunction and reduced markers of vascular senescence (p16, p21, p53 and SIRT1) in mice aortic endothelial cells. These results suggest that DBT's beneficial effects extend beyond direct neuroprotection to include vascular health, which is critical for maintaining cognitive function. Vascular senescence is associated with endothelial dysfunction, inflammation, and reduced blood flow, all of which can contribute to cognitive decline. In addition, another study incubated rat aortic rings with Lysoph-Osphatidyl choline to induce endothelial dysfunction (P. L. Lin et al., 2017). DBT treatment showed dose-dependent improvement in the relaxant function in the damaged aortic rings, with the classic ratio showing the most pronounced effect. This improvement in vascular function could translate to better cerebral blood flow, which is essential for cognitive health. The findings regarding vascular senescence and endothelial dysfunction are particularly relevant as they could be a critical factor in the pathogenesis of VaD. The ability of DBT to alleviate these conditions may be fundamental to its neuroprotective effects. Specifically, vascular

senescence leads to endothelial cell dysfunction, which can compromise the integrity of the BBB and reduce cerebral blood flow (Jia et al., 2019). Together, these insights underscore the importance of targeting both neuroprotective and vascular pathways when developing therapeutic strategies for managing cognitive impairments associated with menopause, especially VaD. The multi-faceted effects of DBT observed in these studies highlight its potential as a comprehensive treatment for menopause-associated cognitive decline. By targeting both neuronal and vascular pathways, DBT may offer advantages over treatments that focus solely on neuronal protection. The antioxidant effects mediated through the Nrf2/HO-1 pathway could protect neurons from oxidative damage, while the vascular protective effects could ensure adequate blood flow and nutrient delivery to the brain.

It is interesting to point out that our current result showed that 17β-estradiol treatment did not have notable improvement in cognitive function while there are studies demonstrating that E2 may ameliorate memory deficit in OVX rodents. The discrepancies of the effect of E2 could attribute to the timing of hormone administration. Gresack and Frick (2006) suggested that intermittent E2 regimen, in which E2 was administrated every 4 days, appeared to improve memory in OVX mice. This is probably because this regimen closely mimics the natural fluctuation of E2 levels during the estrous cycle.

In the *in vitro* experiments conducted on bEnd.3 cells exposed to OGD/R, DBT demonstrated several key protective effects that are critical for maintaining brain health. The treatment significantly increased cell viability and proliferation, suggesting that DBT

may help mitigate cellular damage caused by hypoxia or ischemic conditions. Additionally, DBT was shown to restore the integrity of the BBB by upregulating tight junction proteins, which is crucial for protecting the brain from harmful substances and maintaining neurological function. The compound also exhibited potent antioxidant properties, reducing oxidative stress, which plays a key role in the progression of VaD. Furthermore, DBT promoted cell migration, an effect that could potentially support tissue repair and regeneration in neurodegenerative conditions. Our results also showed that DBT produced its beneficial effects at least partly via the GPR30 pathway and the activation of the Nrf2/HO-1 signalling pathway, highlighting its estrogenic and antioxidant actions.

The OVX rat model successfully recapitulated key features of menopausal VaD, including neuroinflammation, endothelial dysfunction, and cognitive deficits, validating its utility for studying estrogen-deficient states. DBT treatment could reduce astrocyte reactivity and partially preserving vascular density. Besides, the decoction resulted in significant improvements in cognitive function without impacting uterine weight or morphology. It suggests that DBT may provide neuroprotective benefits typically associated with estrogen therapy, but without the adverse effects on reproductive tissues that are often seen with long-term estrogen exposure. Specifically, DBT enhanced spatial learning and memory, recognition memory, and motor function in OVX rats, as evidenced by various behavioral tests. These improvements in cognitive and motor coordination indicate that DBT could address the cognitive decline and motor dysfunction that often accompany menopause, making it a potentially safer and equally effective alternative to HRT in women at risk for menopause-related cognitive impairments.

The multi-target approach of DBT—targeting antioxidant pathways, BBB integrity, and potential estrogenic-like activity—aligns with the holistic treatment philosophy of TCM, which emphasizes treating the whole body rather than focusing solely on isolated symptoms. This comprehensive approach may offer more well-rounded neuroprotection, particularly for conditions like menopause-associated VaD. Moreover, the lack of significant effects on uterine tissue further suggests that DBT may have a more favourable safety profile than traditional HRT, reducing the risk of side effects such as endometrial hyperplasia or breast cancer, which are associated with prolonged estrogen use.

Despite these promising findings, several considerations must be addressed before DBT can be widely adopted as an alternative to HRT. Further research is necessary to optimize the dosage of DBT and to assess its long-term effects. Additionally, the precise mechanisms by which DBT exerts its effects, including its interaction with estrogen receptors and other signalling pathways, remain to be fully elucidated. A deeper understanding of these mechanisms is essential to confirm the potential of DBT as a targeted therapy for menopause-associated cognitive decline.

Overall our current results demonstrating that DBT emerges as a promising alternative therapy to HRT for the management of menopause-associated VaD. This study provides compelling evidence of DBT's neuroprotective and cognitive-enhancing effects across both *in vitro* and *in vivo* models, indicating that DBT may offer a viable treatment option for cognitive decline linked to menopause without the associated risks of traditional HRT. Its

neuroprotective properties, combined with a potentially favourable safety profile, position it as an attractive candidate for further clinical investigation. However, more comprehensive studies, particularly in human populations, are needed to validate these findings and determine whether DBT can be recommended as a widely used alternative to traditional HRT in the treatment of menopause-related cognitive decline.

6 Future potential studies

6.1 Cell work

In this study, while DBT was shown to activate GPR30, a notable limitation is the absence of a positive control to validate these findings. Incorporating GPR30 agonist G1 would allow us to compare and enhance the reliability of the results as well as confirm the specificity of DBT's effects on GPR30 activation. This addition would allow for direct comparison between DBT's effects and those of a known GPR30 activator, thereby enhancing the reliability and interpretability of the results. Such a comparison would also help to confirm the specificity of DBT's effects on GPR30 activation. Additionally, future work could benefit from employing siRNA techniques to selectively knock down GPR30 expression instead of using G15 that may have off-target effects. This approach would allow for a more precise evaluation of GPR30's role in mediating the effects of DBT.

Another important aspect for future cell work is the exploration of non-genomic signalling pathways mediated by GPR30. While the current study focused primarily on genomic effects, GPR30 is known to mediate rapid cellular responses through various kinase cascades. Future research should aim to investigate these non-genomic pathways, examining aspects such as calcium mobilization, cAMP production, and the activation of protein kinases like ERK and Akt. In addition to GPR30, it is essential to consider the role of classical estrogen receptors (ERα and ERβ), which are also capable of mediating both genomic and non-genomic estrogen signalling. Comparing and contrasting the functions of GPR30 and classical estrogen receptors, as well as exploring potential crosstalk or synergistic effects between them, will provide a more comprehensive understanding of

their roles in cellular responses, particularly in the context of DBT. By systematically studying both genomic and non-genomic pathways and integrating the involvement of both GPR30 and classical estrogen receptors, future research can offer a more complete picture of estrogen signalling and its multifaceted relationship with DBT.

Additionally, future cell studies could benefit from exploring the effects of DBT on different cell types within the neurovascular unit, such as astrocytes and pericytes, in addition to endothelial cells. This broader approach would provide a more comprehensive understanding of DBT's effects on the blood-brain barrier and overall brain health.

6.2 Animal work

In this study, OVX SD rats were utilized; however, some findings like cognitive deficits did not reach statistical significance. To enhance the validity and sensitivity of our model, the implementation of an ameroid constrictor is proposed to induce a more precise and consistent reduction in cerebral blood flow. The ameroid constrictor induces a gradual occlusion of the common carotid artery, leading to chronic cerebral hypoperfusion, which is a key factor in the development of VaD. This approach may better replicate the vascular contributions to cognitive decline commonly observed in postmenopausal conditions. Furthermore, increasing the sample size is recommended to improve the reliability of the results and strengthen the conclusions drawn regarding cognitive deficits in OVX SD rats.

Additionally, understanding how DBT enhances neurogenesis and promotes synaptic health will be crucial for elucidating its potential as a therapeutic agent. The

neuroprotective effects of DBT in our study warrant further exploration into its specific mechanisms of action. the inclusion of both agonists and antagonists of GPR30 or knockout rats in subsequent experiments is proposed to elucidate the receptor's involvement in cognitive function. By examining the effects of GPR30 modulation on cognitive performance, we can gain valuable insights into its role in neuroprotection and memory processes, potentially informing novel therapeutic strategies aimed at addressing cognitive deficits associated with menopause.

7 Conclusion

TCM has played a vital role in health protection and disease control in China and other Asian countries for thousands of years. In recent years, TCM has gained increasing attention from both the public and scientific communities as a promising approach for managing various conditions, including ischemia, menopausal symptoms, and stroke (Borud & White, 2010; Gong & Sucher, 1999). The growing interest in TCM stems from its multi-target effects, generally low toxicity, and high efficacy profiles.

The classic decoction, DBT, has long been a research target for its potential in treating osteoporosis with well-documented antioxidant properties. Our study expands on this body of knowledge by demonstrating the neuroprotective effects of DBT in both *in vitro* and *in vivo* models of VaD associated with estrogen deficiency. Specifically, we investigated DBT's effects on OGD/R injured bEnd.3 cells and OVX rats, elucidating a possible mechanism of action through the GPR30-mediated Nrf2/HO-1 pathway.

Our comprehensive investigation identified four main targets of DBT action in bEnd.3 cells. Firstly, DBT demonstrated a remarkable ability to promote cell proliferation, suppress OGD/R-induced cell death and promote wound healing, suggesting its potential to enhance cellular resilience under ischemic conditions and facilitate vascular restoration. Secondly, DBT effectively restored tight junction proteins between endothelial cells, indicating its capacity to maintain and potentially repair BBB integrity. Thirdly, the DBT water extract exhibited potent antioxidant properties, significantly depleting ROS levels. The observed neuroprotective effects of DBT may be attributed to this antioxidant effect, as oxidative

stress is a key driver of cellular damage in ischemic conditions and neurodegenerative diseases. This study also revealed that DBT could activate the Nrf2/HO-1 pathway and upregulate GPR30 expression. However, while DBT increased GPR30 expression, the use of the GPR30 antagonist G15 did not appear to diminish DBT's neuroprotective effects. This suggests that DBT is unlikely to act directly through the GPR30 receptor, and that other, as yet unidentified, mechanisms may contribute to its observed benefits. These findings highlight the complex and potentially multi-faceted mechanisms of DBT, reinforcing the importance of further research to elucidate its precise molecular targets.

Our *in vivo* study using OVX rats further supported DBT's potential as a therapeutic agent for VaD. DBT treatment resulted in moderate improvements in cognitive function across various behavioural tests, including the Morris water maze, novel object recognition, and rotarod tests. These improvements were accompanied by the decrease in astrocyte activation, as demonstrated by reduced GFAP expression, alongside with the partial maintenance of vascular integrity, evaluated through CD31 levels in the hippocampus. Importantly, DBT did not induce uterine growth in OVX rats, indicating a lack of estrogen-like effects on uterine tissue. This suggested that DBT may offer cognitive and neuroprotective benefits without the risks associated with estrogenic stimulation of the uterus, supporting its potential as a safer option for postmenopausal women.

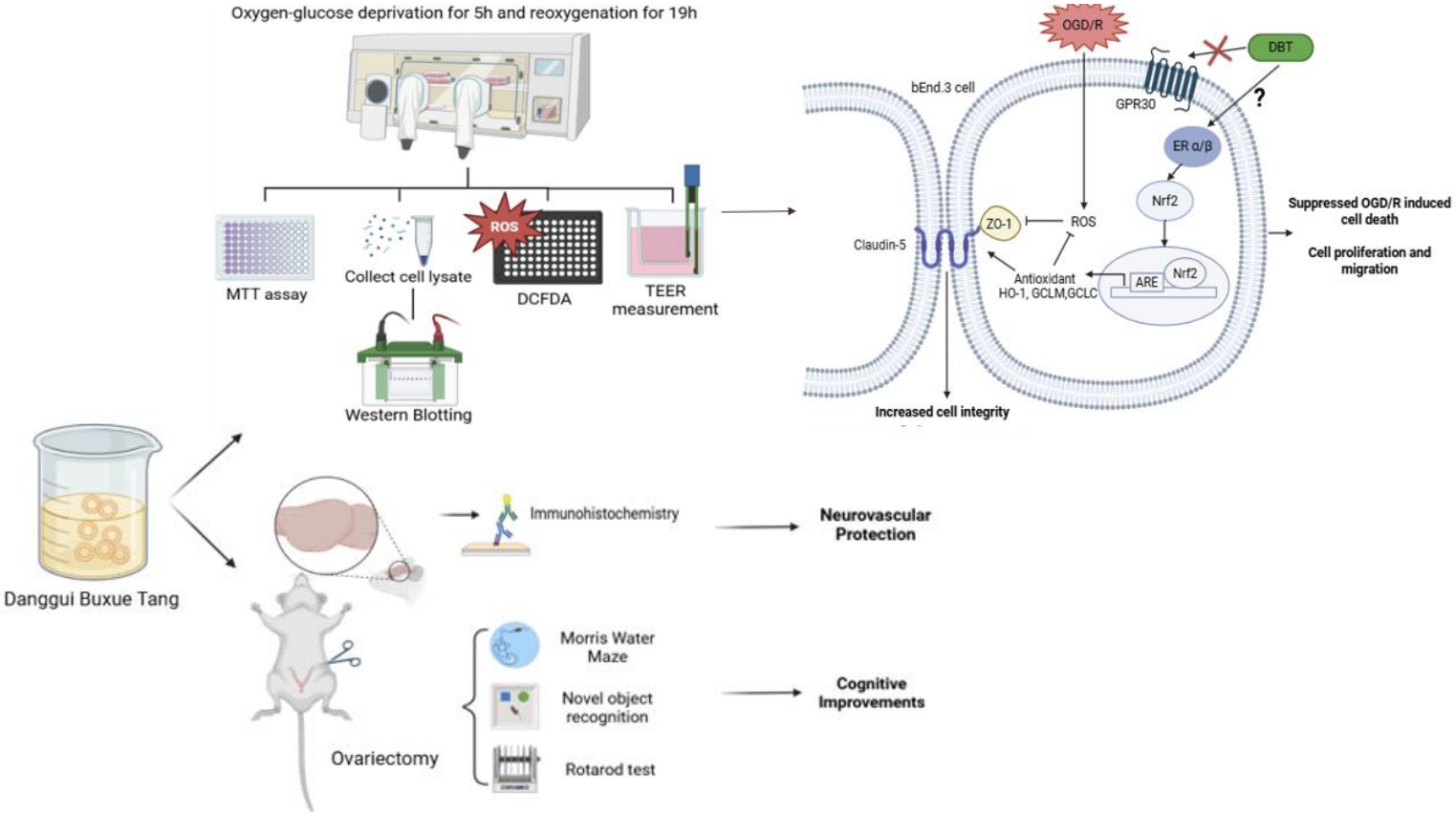
While our study provides compelling evidence for DBT's neuroprotective effects, it also highlights the need for further investigation. Future research directions include further

investigating the molecular mechanisms of DBT and exploring its effects on different cell types within the neurovascular unit.

In conclusion, our findings contribute significantly to the growing body of evidence supporting the use of TCM, specifically DBT, in managing complex health conditions such as VaD. By demonstrating DBT's multi-target effects on cellular health, antioxidant status, and cognitive function, this study paves the way for innovative approaches to neuroprotection and cognitive enhancement in the context of estrogen deficiency. Importantly, our research suggests that DBT could serve as a promising alternative to HRT for postmenopausal women at risk of VaD. While HRT has been traditionally used to alleviate menopausal symptoms and protect against cognitive decline, it is associated with potential risks such as increased chances of breast cancer and cardiovascular events. DBT, on the other hand, demonstrated neuroprotective and cognitive-enhancing effects in our study without significantly impacting uterine weight or morphology, suggesting a potentially more favourable safety profile. This makes DBT an attractive option for women seeking to manage menopause-related cognitive decline without the risks associated with long-term estrogen exposure.

Graphical Abstract

Overview of the effects of DBT on OGD/R bEnd.3 cells and menopause-associated cognitive decline and neurovascular health in OVX rats.



Abbreviations

5-HIAA	5- hydroxytryptamine
5-HT	5- hydroxyindoleacetic acid
AR	*Astragali Radix*
ASR	*Angelicae Sinensis Radix*
BSA	Bovine serum albumin
CBF	Cerebral blood flow
CD31	Platelet endothelial cell adhesion molecule 1
CO	Carbon monoxide
CSVD	Cerebral small vessel disease
DA	Dopamine
DBT	Danggui Buxue Tang
E2	Estrogen
EO	Essential oil
FSH	Follicle-Stimulating Hormone
GFAP	Glial Fibrillary Acidic Protein
HO-1	Heme Oxygenase-1
HRT	Hormone replacement therapy

IFN	Interferon
IL	Interleukin
LBP	*Lycium barbarum* polysaccharides
LH	Luteinizing Hormone
MAPK	Mitogen-Activated Protein Kinase
MCAO	Middle Cerebral Artery Occlusion
MMP	Matrix metalloproteinase
MWM	Morris Water Maze
NAC	N-acetyl cysteine
NE	Norepinephrine
NO	Nitric oxide
NOR	Novel Object Recognition
OVX	Oxygen glucose deprivation and reperfusion
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation Assay
ROS	Reactive Oxygen Species
TCM	Traditional Chinese Medcine

TMP	Tetramethylpyrazine
TEER	Transendothelial electrical resistance
TNF-α	Tumor Necrosis Factor-alpha
VaD	Vascular dementia
VEGF	Vascular endothelial growth factor

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