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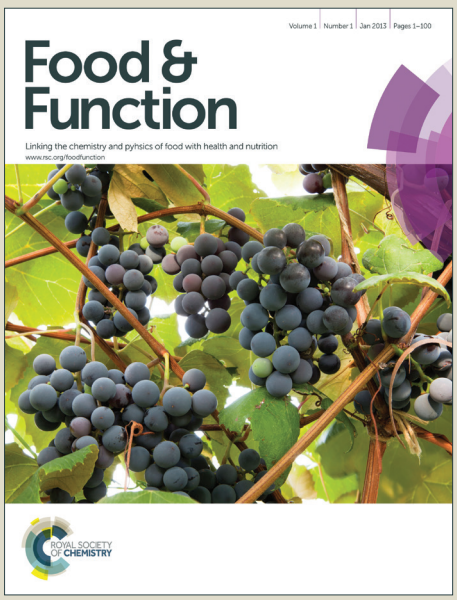

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Research Report

Effects of Edible Bird's Nest on hippocampal and cortical neurodegeneration in ovariectomized rats

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Abstract:

The aim of the present research was to investigate whether Edible Bird's Nest (EBN) attenuated cortical and hippocampal neurodegeneration in ovariectomized rats. Ovariectomized rats were randomly divided into seven experimental groups (n=6): ovariectomy (OVX) group had their ovaries surgically removed; sham group underwent surgical procedure similar to OVX group but ovaries were left intact; estrogen group had OVX and received estrogen therapy (0.2mg/kg/day); EBN treatment groups received 6%, 3%, and 1.5% EBN, respectively. Control group was not ovariectomized. After 12 weeks of intervention, biochemical assays, and markers of neurodegeneration and message ribonucleic acid (mRNA) levels of oxidative stress-related genes in the hippocampus and frontal cortex of the brain were analysed. Caspase 3 (cysteine-aspartic proteases 3) protein levels in the hippocampus and frontal cortex was also determined using western blotting. The results showed that EBNs significantly decreased estrogen deficiency-associated serum elevation of advanced glycation end-products (AGEs), and changed redox status as evidenced by oxidative damage (malondaldehyde content) and enzymatic antioxidant defense (superoxide dismutase and catalase levels) markers. Furthermore, genes associated with neurodegeneration and apoptosis were down regulated in the hippocampus and frontal cortex by EBN supplementation. Taken together, the results suggested that EBN had potential for neuroprotection against estrogen deficiency-associated senescence, at least in part, via modification of the redox system and attenuation of AGEs.

Key words: Edible Bird's Nest; Ovariectomy; Advanced glycation end-products; Oxidative stress; Neuroprotection

1. Introduction

The menopause is characterized by psychological and physical changes associated with termination of sex hormones secretion. The central nervous system (CNS) can be influenced by this loss of sex hormones via impaired neuronal plasticity [1,2] or mood and behavioral changes [3]. Additionally, the risk of neurodegenerative diseases is increased significantly post-menopause duet to loss of the sex hormones, and causes impairments in memory, cognition and quality of life [4]. The contribution of these sex hormones to these processes in the CNS is further underpinned by the increased susceptibility to dementia in young women who have received bilateral oophorectomy. Moreover, estrogen replacement is neuroprotective and delays the onset of neurodegenerative diseases like Alzheimer disease [5,6]. Recent studies have suggested the preventive effects of hormone replacement therapy (HRT) or phytoestrogen supplement therapy on oxidative stress-mediated neurodegenerative disorders [7]. However, it has been demonstrated that HRT in postmenopausal women can lead to the development of breast, cervix, and endometrial cancer [8]. Thus, alternatives to conventional HRT or phytoestrogens are direly needed.

Advanced glycation end-products (AGEs) formed by the non-enzymatic glycation of proteins, lipids, and nucleic acids, are involved in the development or worsening of many degenerative diseases [9,10]. Furthermore, depletion of cellular antioxidant mechanisms and the generation of free radicals by AGEs may play a major role in the pathogenesis of aging and aging related disease [11,12].

Edible bird's nest (EBN) is considered a precious food tonic by Chinese people ever since the Tang dynasty (618AD) [13], and has been referred to as "Caviar of the EAST" [14]. The usage of

EBN has principally been based on traditional hunch, and is thought to have anti-aging and immune-enhancing properties [15]. However, to date, there is a dearth of research and scientific evidence to substantiate the claims of health benefits associated with anti-aging despite EBN's long history of medicinal use.

In this study, we investigated whether EBN may attenuate AGEs, oxidative stress and improve neuro-dysfunction induced by ovariectomy. We also evaluated possible mechanistic basis for the neuroprotective effects of EBN.

Materials and methods

Materials

Rat estrogen and AGEs enzyme-linked immunosorbent assay (ELISA) kits were purchased from commercial companies (Adaltis, SRL, Milano, Italy, and Cloud-Clone Corp. Houston, USA, respectively) and insulin ELISA kit was from Millipore (Billerica, MA, USA). While, SOD and CAT ELISA kit was bought from Cell Biolabs (INC. USA). Glucometer strips were from Roche Diagnostics (Indianapolis, IN, USA). The GenomeLab™ GeXP Start Kit was from Beckman Coulter Inc. (Miami, FL, USA) and ribonucleic acid (RNA) extraction kit was from RBC Bioscience Corp. (Taipei, Taiwan). MgCl2 and deoxyribonucleic acid (DNA) Taq polymerase were purchased from Thermo Fisher Scientific (Pittsburgh, PA), while RCL2 Solution was purchased from Alphelys (Toulouse, France). Primary antibody and secondary antibody were from Abnova (Taipei, Taiwan). Rat chow was obtained from Specialty Feeds (Glen Forrest, WA, Australia). Ketamine/xylamine was from Sigma Chemical Co. (St. Louis, Missouri, USA), and other solvents of analytical grade were purchased from Merck (Darmstadt, Germany). Ready-to-

use EBN was supplied by Niah Bird's nest trading company (Sarawak, Malaysia), and was incorporated into standard rat chow for animal feeding

Animal treatment and operation procedure

Forty-two Sprague–Dawley rats (3-month old, female, 180-200g) were housed under controlled conditions (12h light/12h dark cycle, 20-22℃, 40-50% humidity and access to water and food ad libitum) two weeks prior to the experiments for acclimatization to the new environment. Use of animals was approved by the Animal Care and Use Committee (ACUC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (approval number: UPM/IACUC/AUP-R012/2014), and animals were handled as stipulated by the guidelines for the use of animals. All ovariectomy (OVX) procedures were performed as previously described [16] in our laboratory and were conducted under anesthesia after an injection of 10mg/60mg/kg xylazine/ketamine (i.p). The groupings in this study were as follows: Group 1 was normal control rats, while Groups 2 and 3 were OVX control and sham-operated control. Group 4 underwent OVX and received estrogen (0.2 mg/kg body weight/day). Groups 1-4 were given normal rat chow throughout the intervention period. Groups 5-7 underwent OVX and received semi-purified diets containing 6%, 3% and 1.5% EBN. Interventions lasted for 12 weeks, and food intake in each group was adjusted to the average intake according to the observation of OVX control group the day before. Weights were measured weekly, and the total amount of feed (gram) given was reviewed weekly based on the weekly weights of the rats. At the end of the experiment all animals were decapitated after anesthesia, and blood withdrawn. The hippocampus and frontal cortex were removed from the brain and quickly kept in RCL2 reagent

(Alphelys, Toulouse, France) for further analysis of molecular markers. The uterus and vagina were removed, weighed and the length measured.

Observation of estrous cycle

The phase of the estrous cycle was observed by vaginal swabs for 8 days prior to sacrifice to make sure the sham group's rats in estrus phase. The inspected stages were: diestrus stage, which present leukocytes, little nucleated cells, and mucus (around 2 days); proestrus stage, which only present nucleated cells (about 1 day); estrus stage, that only present cornified cells (1 day); and metaestrus stage, that observed leukocytes, some cornified cells and nucleated cells (1-2 days on average). The observed estrous cycle stages were consistent with reported [35].

Fasting blood glucose and serum insulin levels

At the time of sacrifice, fasting blood glucose was measured from tail blood of rats using glucometer (Roche Diagnostics, Indianapolis, IN, USA), while insulin was measured from serum of blood collected at sacrifice. Insulin ELISA kit, was used according to manufacturer's instruction, and absorbances were read on the Synergy H1 Hybrid Multi-Mode Microplate Reader ($y = 0.762x - 0.143$, $r^2=0.966$).

Serum estrogen and advanced glycation end-products (AGEs)

Serum estrogen and AGEs levels were determined by commercial ELISA kits (Adaltis, SRL, Milano, Italy, and Cloud-Clone Corp. Houston, USA, respectively) based on manufacturer instructions. The absorbances were read at 450nm immediately using the Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski VT, US), and results were calculated from

145 respective standard curves (AGEs, y = -0.6287x + 3.7395, r^2=0.9959; estrogen, y = 0.7355x +
146 0.017, r^2=0.9661)
147
148 **Superoxide dismutase (SOD) and catalase (CAT) activity assay**
149 SOD and CAT activities of hippocampal and frontal cortex lysates were performed by
150 commercial kit (Cell Biolabs, INC. U.S.) based on manufacturer instructions. Finally the
151 absorbances were read at 490nm on the Synergy H1 Hybrid Multi-Mode Microplate Reader
152 (BioTek, Winooski VT, U.S.), and results calculated from the standard curve (SOD, y = 0.143x -
153 0.017, r^2=1; CAT, y = 0.2787x - 0.3008. r^2=0.9953).
154
155 **Thiobarbituric acid reactive substances (TBARS) assay**
156 TBARS was determined according to the protocol reported by Chan et al. [17]. Briefly,
157 homogenized hippocampal and frontal cortical tissues (20mg/50µlPBS) were mixed with 0.25N
158 HCl, 15% 2,4,6-trichloroanisaole (TCA) and 0.375% 2,4,6-tribromoanisole (TBA), and then
159 incubated at 100°C for 10 min. The mixtures were centrifuged at 3000 rpm for 15 min. Finally,
160 the absorbance of supernatants were read at 540nm using the Synergy H1 Hybrid Multi-Mode
161 Microplate Reader (BioTek, Winooski VT, US). Tetramethoxypropane (TMP) was used as the
162 standard, y = 0.1982x - 0.1898 (r^2=0.9947)
163
164 **Ribonucleic acid (RNA) extraction, reverse transcription and multiplex polymerase chain**
165 **reaction (PCR) analyses**
166 RNA was extracted from rat hippocampus and frontal cortex using the Total RNA Isolation kit
167 (RBC Bioscience Corp., Taipei, Taiwan) according to the manufacturer's instructions. Primer

sequences (Table 2) were designed on the National Center for Biotechnology Information (NCBI) website, and supplied by Integrated DNA Technologies (Singapore), while the internal control (KanR) was supplied by Beckman Coulter (USA). Reverse transcription and PCR were performed according to the GenomeLab™ GeXP kit protocol (Beckman Coulter, Miami, FL, USA) in an XP Thermal Cycler (Bioer Technology, Germany). Furthermore, the PCR products were run on GeXP genetic analysis system (Beckman Coulter, Miami, FL, USA), and the results were analyzed by eXpress Profiler software based on the manufacturer's instructions.

Hippocampal and frontal cortical caspase 3 western blotting

Hippocampal and frontal cortical tissues were extracted by radio immune precipitation assay buffer (RIPA) with protease inhibitors, and protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Nacalai Tesque, INC. Kyoto, Japan). Then, 25mg protein was loaded per well on 10% resolving gel and 4% stacking gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with primary antibody caspase 3 (Abnova, Taipei, Taiwan) at 4℃ overnight. The horseradish peroxidase (HRP)-conjugated secondary antibody (Abnova, Taipel, Taiwan) was then added for 1 h at room temperature. The membrane was stripped once for 15–20 min with stripping buffer, and then re-probed with first/secondary antibody as described above for β-tubulin (Sigma, St. Louis, MO, USA) which was used as a loading control. Bands were visualized using 3, 3'-diaminobenzidine (DAB) kit (Nacalai Tesque, INC. Kyoto, Japan). The relative intensities of the immunoreactive bands were captured using a Molecular Imager, ChemiDoc XRS+System (Bio-Rad, Hercules, CA) and quantified with Quantity One Analysis Software, Version 4.6.4 (Bio-Rad, Hercules, CA).

191 **Statistical analysis**

192 Statistical analyes were calculated using one-way ANOVA with Tukey's HSD test, two-sided T
193 test, Pearson correlation and linear regression analysis using SPSS 20.0 (SPSS, Chicago). All
194 data are expressed as means ± S.E.M. $P \leq 0.05$ indicates statistical significance.

195

196 **Results**

197 **Food intake, body weight and biochemical level determination**

198 All rat groups had similar initial mean body weights ($F_{(6,35)}$=0.2756, P=0.9447; Table 3), and
199 mean food intake (Table 1) was similar throughout the intervention. At the end, the OVX group
200 showed significantly higher mean body weight in comparison with the sham group (t=5.432,
201 df=8, P=0.0006, Table 3). After 12 weeks of EBN supplementation, EBN groups showed
202 significantly decreased body weights in comparison with the OVX group ($F_{(3, 16)}$=10.9,
203 P=0.0004), with 6% EBN group showing the lowest weight gain (1.4±0.3g) among all groups,
204 which is significant lower than OVX group (t=12.28, df=8, P<0.0001). Fasting blood glucose
205 was similar among all groups ($F_{(6, 35)}$=2.042, P=0.0931), while the OVX group showed
206 significantly increased insulin level in comparison with the sham (t=4, df=8, P=0.0032) and
207 estrogen-treated (t=5.646, df=8, P=0.0005) groups. Furthermore, 6% EBN treatment group
208 lowered the insulin level compared with the OVX group (t=2.765, df=8, P=0.0245). Based on the
209 fasting glucose and insulin levels, OVX groups showed the highest tendency for insulin
210 resistance based on homeostatic model assessment of insulin resistance (HOMA-IR). As
211 expected, the mean uterine and vaginal length and weight of ovariectomized animals were
212 significantly lower than those of sham controls (length: t=11.25, df=8, P<0.0001; weight:
213 t=10.88, df=8, P<0.0001 Table 3). Estrogen treatment significantly increased the uterine and

vaginal length (t=5.770, df=8, P=0.0004) and weight (t=18.51, df=8, P<0.0001) of ovariectomized rats (p<0.05). Furthermore, treatment with EBN had modest stimulatory effects on uterine and vaginal length ($F_{(3, 16)}$=11.31, P=0.0003) and weight ($F_{(3,16)}$=61.31, P<0.0001) comparing with OVX group, in a dose-dependent manner.

EBN lowered serum AGEs

AGEs were significantly increased in the OVX group in comparison with the sham group (Figure1; 182.24±62.45 pg/ml vs. 15.92± 4.51pg/ml; $F_{(6, 35)}$=32.24, P< 0.0001). Estrogen had a modest effect on AGEs (39.15± 23 ng/ml; $F_{(5, 24)}$=8.625, P=0.0001) while EBN reduced the levels much more significantly (6% EBN, 1.97 ± 0.17ng/ml; 3% EBN, 8.19 ± 4.42ng/ml; 1.5%EBN, 6.59±4.22ng/ml; $F_{(6, 35)}$=32.24, P< 0.0001).

Hippocampal and frontal cortical antioxidant enzyme activities

To evaluate the antioxidant effects of EBN treatment on OVX, hippocampal and frontal cortical SOD and CAT activities were measured. OVX significantly increased SOD activities in the hippocampus (t=7.373,df=8, P<0.0001; Figure 2A) and frontal cortex (t=5.971, P=0.0003; Figure 2A)of rats, and also increased CAT activities in hippocampus (t=6.506, df=8, P=0.0002; Figure 2B), and frontal cortex (t=8.550, df=8, P<0.0001; Figure 2B) comparing with sham group. However, comparing with OVX group, EBN treatment groups significantly ameliorated SOD activities in hippocampal ($F_{(3,16)}$=46.95, P<0.001; Figure 2A), and frontal cortical ($F_{(3,16)}$=13.28, P=0.0001; Figure 2A). For the CAT activities, there are no big differences between OVX group and 3% , 1.5% EBN groups in frontal cortex ($F_{(3,16)}$=3.871, P=0.0295; Figure 2B), whereas, in

hippocampus, only 6% and 3% EBN groups have significant differences ($F_{(3,16)}$=13.46, P=0.0001; Figure 2B). Interestingly, there are no significant differences between sham group and EBN treatment groups both in hippocampal and frontal cortical antioxidant activities, which consequently balanced SOD/CAT ratio ($F_{(3,16)}$=2.709, P=0.0798 in hippocampus; and $F_{(3,16)}$ =2.701, P=0.0804; Figure 2C). Furthermore, there are also no difference in EBN treatment groups comparing with OVX group either in hippocampus ($F_{(3,16)}$=3.329, P=0.0563) or in frontal cortex ($F_{(3,16)}$=1.539, P=0.2430). Still, comparing with estrogen group, EBN treatment groups have the same SOD/CAT ratio ($F_{(3,16)}$=1.508, P=0.2507) in frontal cortex.

Hippocampal and frontal cortical TBARs

The malondialdehyde (MDA) level in hippocampus and frontal cortex was measured because it is an indicator for oxidative damage. As depicted in Figure 3, OVX had higher levels of MDA in comparison with sham group, and estrogen group showed even higher levels, furthermore, EBN groups had lower MDA levels when compared with OVX and estrogen groups ($F_{(6,35)}$ =7.685, P<0.0001 in hippocampus; and $F_{(6,35)}$ =12.27, P<0.0001 in frontal cortex; Figure 2C).

mRNA levels of antioxidant and neurodegeneration-related genes

Figure 4 shows the effects of the interventions on the expression of SOD1, SOD2, SOD3 and CAT. In this study, OVX upregulated antioxidant genes, especially in the hippocampus (SOD1: $F_{(6, 28)}$=8.789, P<0.0001; SOD2: $F_{(6, 28)}$=15.86, P<0.0001; SOD3: $F_{(6, 28)}$=61.05, P<0.0001; CAT: $F_{(6, 28)}$=8.218, P=0.0006), while in the frontal cortex, OVX only significantly upregulated SOD3 ($F_{(6, 28)}$=12.07, P<0.0001). EBN decreased the expression of all the SOD1/SOD2/SOD3/CAT

genes in comparison with the OVX (SOD1: $F_{(3, 16)}$=40.01, P<0.0001 in hippocampus, and $F_{(3, 16)}$=5.943, P=0.0064 in cortex; SOD2: $F_{(3, 16)}$=37.38, P<0.0001 in hippocampus, and $F_{(3, 16)}$=6.835, P=0.0036 in cortex; SOD3: $F_{(3, 16)}$=129.4, P<0.0001 in hippocampus, and $F_{(3, 16)}$=23.23, P=0.0003 in cortex; CAT: $F_{(3, 16)}$=15.07, P=0.0012 in hippocampus, and $F_{(3, 16)}$=9.293, P=0.0055 in cortex;)and sham groups (SOD1: $F_{(3,16)}$=3.034, P=0.0597 in hippocampus, and $F_{(3, 16)}$=3.716, P=0.0336 in cortex; SOD2: $F_{(3, 16)}$=7.737, P=0.0020 in hippocampus, and $F_{(3, 16)}$=1.984, P=0.1571 in cortex; SOD3: $F_{(3, 16)}$=30.73, P<0.0001 in hippocampus, and $F_{(3, 16)}$=14.07, P=0.0015 in cortex; CAT: $F_{(3, 16)}$=8.474, P=0.0073 in hippocampus, and $F_{(3, 16)}$=2.347, P=0.1488 in cortex;). Furthermore, comparing with sham group, OVX upregulated the expression of presenilin (PSEN)1, PSEN2 and amyloid precursor protein (APP) genes in hippocampus (PSEN1: t=0.4015, df=8, P=0.0159; PSEN2: t=1.257, df=8, P=0.2771; APP: t=19.81, df=8, P<0.0001; Figure 5) and frontal cortex (PSEN1: t=1.094, df=8, P=0.3342; PSEN2: t=3.404, df=8, P=0.0272; APP: t=2.291, df=8, P=0.0512; Figure 5). While EBN groups had lower mRNA levels of these genes both in hippocampus (PSEN1: F(3,16)=1.228, P=0.3611; PSEN2: $F_{(3,16)}$=6.649, P=0.0145; APP: $F_{(3,16)}$=65.63, P<0.0001; Figure 5) and frontal cortex (PSEN1: $F_{(3,16)}$=2.181, P=0.1681; PSEN2: $F_{(3,16)}$=10.2, P=0.0042; APP: $F_{(3,16)}$=3.523, P=0.0393; Figure 5) comparing with sham group. The mRNA expression of insulin degrading enzyme (IDE) and Low density lipoprotein receptor-related protein (LRP)1 were significantly higher in OVX group both in hippocampus (IDE:$F_{(3,16)}$=46.87, P<0.0001; LRP1: $F_{(3,16)}$=59.77, P<0.0001; Figure 7) and frontal cortex (IDE:$F_{(3,16)}$=10.2, P<0.0001; LRP1: $F_{(3,16)}$=10.03, P<0.0001; Figure 7), while EBN groups suppressed the expression of the genes.

EBN attenuated caspase 3 protein level

Neuronal loss was tested via caspase 3 activity as a marker of apoptosis-induced neurodegeneration of hippocampal and frontal cortical cells. Cleaved caspase3 level was increased in OVX group compared with sham group both in hippocampus ($F_{(6,28)}$ =68.56, P<0.0001; Figure 7B) and frontal cortex ($F_{(6,28)}$ =20.62, P<0.0001; Figure 7B), while EBN groups exhibited lower caspase3 protein comparing to OVX group ($F_{(3,16)}$ =70.55, P<0.0001 in hippocampus; and $F_{(3,16)}$ =17.04, P<0.0001 in frontal cortex, Figure 7B). At 6% EBN, cleaved caspase 3 level was lower than estrogen group (t=8.519, df=8, P<0.0001 in hippocampus; t=4.193, df=8, P=0.0030 in frontal cortex) and similar to that of the sham group (t=0.5002, df=8, P=0.6304 in hippocampus; t=0.3502, df=8, P=0.7352 in frontal cortex).

Discussion

Although the exact pathogenesis of neurodegenerative diseases is mostly still not clear, estrogen deficiency has long been associated with the pathological development in menopause women, such as Alzheimer, Parkinson and stroke [18]. Moreover, it seems likely that memory and cognitive function in hippocampus were subject to fluctuation of estrogen level because of the effect of estrogen on synaptic density between hippocampal neurons [36]. The best rodent model that induce experimental menopause is the bilateral surgical OVX. With the deficit of endogenous estrogen, the OVX rat model represents the best characterized clinical hallmarks of postmenopausal induced nervous system aging in the menopause women [19]. In the present study, the rats in sham group addressed in estrus stage which expressed highest estrogen lever among estrous cycle, and the OVX showed an increase weight gain and higher risk of insulin resistance, which are consistent with estrogen deficiency-associated menopause changes. Furthermore, the used dose of estrogen was based on our earlier work [16], and in agreement with previous studies showing

304 that estrogen feeding rat present anti-oxidant ability [35]. In addition, other changes of worsening
305 lipid profile and atrophy of the uterus and vagina as documented in the present study have been
306 reported for OVX [20, 21,22]. These changes in OVX group, therefore, confirmed the induction of
307 estrogen deficiency and showed increased risk of accelerated aging process. Based on the present
308 data, elevated estrogen levels and improvements in other indices may have been due to presence
309 of estrogen-like compounds in EBN.

310 AGEs play pathogenetic roles in neurodegenerative disease, including Alzheimer, Parkinson, and
311 dementia. Their accumulation in tissues may contribute to increased oxidative stress and
312 impairment of organ function [23], and a positive feedback mechanism for production of more
313 AGEs by free radicals has been described [24]. They have also been reported to be higher in the
314 elderly, even with lower dietary intake of AGEs [25]. Based on the present results, therefore, we
315 proposed that the elevated AGEs in the ovariectomized rats indicates their natural history of
316 increased AGEs in estrogen deficiency [26], which EBN supplementation is able to reduce.
317 Moreover, AGEs are higher in insulin resistant conditions [27], in keeping with the higher risk of
318 insulin resistance in the OVX group in this study. Furthermore, the reduced AGEs in EBN-
319 treated groups may have been contributed by antioxidant effects of EBN and its improved insulin
320 sensitivity status.

321 To our knowledge, this is the first time to investigate the function of EBN supplementation on
322 redox profile central in nervous system via OVX and sham operation rat. Oxidative damage on
323 neurons is often initially followed by increased antioxidant enzymes and their activity, and may
324 even be followed thereafter by apoptotic cell death if the stimulus overwhelms the cellular
325 machinery for repair [28]. In this study, increased hippocampal and frontal cortical MDA contents
326 in OVX rats suggested increased oxidative stress damage [29]. The higher levels of MDA in the

estrogen group indicates that it may promote oxidative stress in the brain, and may in fact underlie some of the side effects associated with estrogen therapy in menopausal women. Antioxidants like SOD and CAT are involved in clearance of free radicals responsible for oxidative damage [30], and as can be recalled, their levels are elevated during neuronal oxidative stress as seen in the present study, but consistent with the oxidative damage level. Pearson correlation and linear regression analysis indicated the MDA levels did not correlated with SOD/CAT ratio both in hippocampus (r=-0.6978, P=0.0813) and frontal cortex (r=0.5114, P=0.2407). This may indicate the brain protection of EBN mediated by directly scavenging toxic radicals, and it is compatible with previous reported anti oxidant [34]. The normalization of their levels by EBN coupled with the lower MDA levels suggested that EBN lowered oxidative damage on the brain, which may have contributed to the lower AGEs observed earlier. Hence, these results indicate that the effect of EBN against oxidative stress may be mediated by modification of the redox system via AGEs in ovariectomy rats.

The transcriptional changes in hippocampus and frontal cortex in the OVX group in this study were consistent with increased risk of neurodegenerative diseases. Similarly, EBN induced changes that tended towards neuroprotection, although the activities detected did not all together reflect the transcriptional activity observed. It is likely that the overall effects of EBN on these processes including post-transcriptional modifications will only produce an all-or-none-effect, which is further supported by the lack of significant differences in MDA levels and that of antioxidant enzyme activities between the EBN groups despite some differences in antioxidant gene expression. Furthermore, APP, PSEN1 and PSEN2 genes have been reported to play a direct role in Alzheimer's disease pathogenesis [31]. Increased cytoplasmic PSEN promotes APP expression, and higher APP levels would increase mitochondrial PSEN expression, which leads

to mitochondrial dysfunction. Meanwhile, high amounts of APP may upregulate LRP1. These and other reported changes for APP, PSEN1, PSEN2 and LRP1 that promote neurodegeneration[32] are consistent with what we observed for OVX group, while EBN supplementation produced changes that tended towards neuroprotection. Additionally, neurodegenerative diseases often result when oxidative damage induces apoptosis of neurons because the endogenous defenses are unable to counter the stimuli. Caspase 3 is an effector caspase that signals apoptosis, and has been reported to trigger early synaptic dysfunction in rodent Alzheimer model once it is activated [33]. Interestingly, OVX in this study promoted activation of caspase 3, while EBN attenuated this activation in a dose dependent manner. Although this is not in keeping with other effects of EBN observed in this study, this effect on caspase attenuation may have been due to differential effects of EBN constituents on different pathways, with a cumulatively better effect on neuroprotection with increasing concentrations of EBN (Figure 8).

In summary, the present study demonstrates that EBN is neuroprotective against estrogen deficiency-induced damage. This is evidenced by decreased serum AGEs, and reduced hippocampal and frontal cortical caspase3 protein and MDA levels, and balanced activities of anti-oxidant enzymes in the hippocampus and frontal cortex of ovariectomized female rats. The data suggests that EBN may serve as an attractive candidate and novel strategy for clinical treatment of neurodegenerative diseases in menopause.

Conflict of interest statement

The authors declare that they have no competing interests.

372

373 ***Author contributions***

374 Study design: HZ, MI.

375 Supervision of the study: MI, AI and RM

376 Primer design for gene expression study: NI and MUI

377 Conduct of experimental parts: HZ, ZY and NS

378 Data analyses and preparation of manuscript: AI, RM, MUI and HZ

379 Review of manuscript and final approval for submission: MI and MUI

380

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384 Biomedicine for their help during the study.

385

386 **List of Abbreviations:** OVX, Ovariectomy; CASP3, Caspase3; PSEN1, Presenilin-1; PSEN2,

387 Presenilin-2; APP, Amyloid Precursor Protein; IDE, Insulin-Degrading Enzyme; LRP1, Low

388 Density Lipoprotein Receptor-Related Protein1; SOD, Superoxide Dismutase; CAT, Catalase.

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Figure caption

Figure 1. Serum advanced glycation end-products (AGEs) in ovariectomized rats after 12 weeks of intervention with edible birds' nest (EBN) or estrogen. Ovariectomy group (OVX) had their ovaries surgically removed while sham control group had the same surgical procedure as ovariectomized rats but ovaries were left intact, and EBN high, EBN middle and EBN low received 6, 3 and 1.5% EBN in semi-purified diet, respectively. [a]P<0.05 *VS* OVX group; [b]P<0.05 *VS* estrogen group.

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Figure 4. mRNA levels of superoxide dismutase (SOD) 1, SOD 2, SOD 3 and catalase (CAT) in hippocampal and frontal cortical tissue of ovariectomized rats after 12 weeks of intervention with edible birds' nest (EBN) or estrogen. Groupings are the same as Figure 1. [a]P<0.05 *VS* OVX group; [b]P<0.05 *VS* sham group; [c]P<0.05 *VS* 6%EBN treatment group; [d]P<0.05 *VS* estrogen group.

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571 Figure 8. Proposed schematic showing how edible birds' nest (EBN) may prevent estrogen
572 deficiency-associated neurodegeneration. Estrogen is known to modulate various metabolic
573 processes including glucose homeostatis through maintaining insulin sensitivity. Loss of
574 estrogen will promote insulin resistance which drives up the production of advanced glycation
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580 as indicated on the schema. SOD: superoxide dismutase; CAT: catalase.
581

Table 1. Food composition and animal groups

Food composition (total 1000g) / Animal group	Normal pellet	Estrogen	Starch	EBN
Normal control	1000g			
Sham	950g		50g	
OVX	950g		50g	
OVX+estrogen	945g	5g	50g	
OVX+6% EBN	890g		50g	60g
OVX+3%EBN	920g		50g	30g
OVX+1.5%EBN	935g		50g	15g

OVX: ovariectomy; EBN: edible birds nest. All rat groups were ovariectomized except the control group, and all groups received standard rat chow for 12 weeks thereafter. In addition, the estrogen treated group received 0.2mg/kg/day, while EBN groups received 6, 3 or 1.5% EBN in their rat chow, respectively. EBN: edible bird's nest; OVX: ovariectomy.

Table 2. Names, accession number and primer sequences used in the study

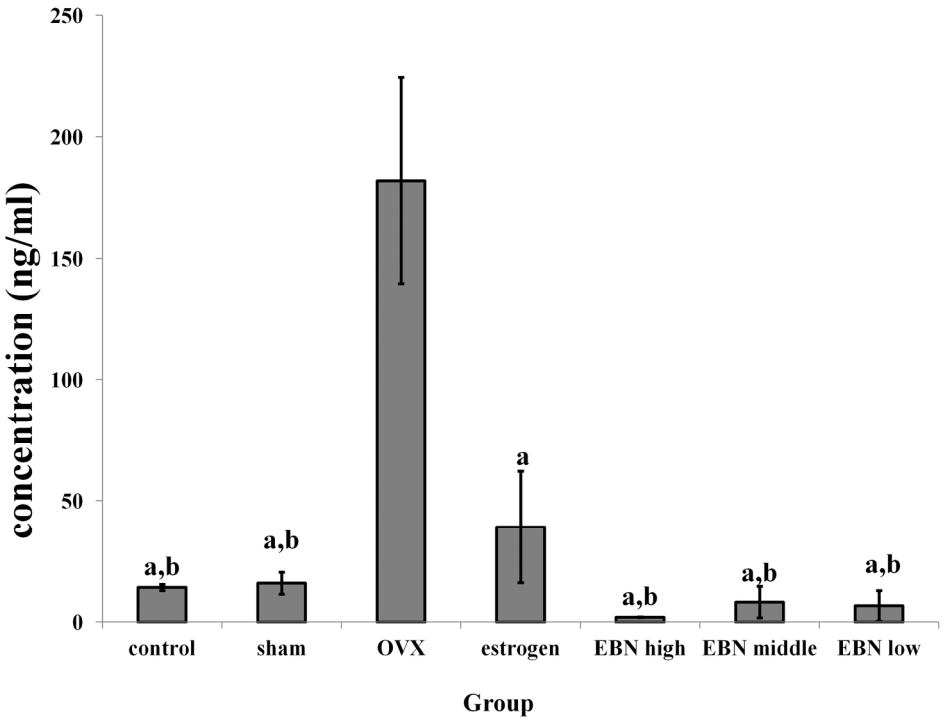
Gene Name	Accession number	Left sequence	Right sequence
IDE	NM_013159	AGGTGACACTATAGAATATGGCAACATAACAAAGCAGG	GTACGACTCACTATAGGGAGTTCCTCCGCTGGTAAACAA
LRP1	NM_001130490	AGGTGACACTATAGAATAGGCATCTCAGTAGACTATCA	GTACGACTCACTATAGGGATCACTCCAGTAGATGAAATC
PSEN1	NM_019163	AGGTGACACTATAGAATATATACCCCATTCACAGAAGA	GTACGACTCACTATAGGGATTCCCCTAAGTAAATGAATG
PSEN2	NM_031087	AGGTGACACTATAGAATACCATCTCTGTGTACGATCTC	GTACGACTCACTATAGGGAAAACTGTCATAGGAGTCTTCTT
Gapdh*#	NM_017008	AGGTGACACTATAGAATACTGAGGACCAGGTTGTCTCC	GTACGACTCACTATAGGGAGAGGGCCTCTCTCTTGCTCT
APP	NM_019288	AGGTGACACTATAGAATAATGCTTGCAGAGTTAAACA	GTACGACTCACTATAGGGATGCATAAAATATTTAAGGTAAGA
SOD1	NM_017050	AGGTGACACTATAGAATATCAATATGGGGACAATACAC	GTACGACTCACTATAGGGATACTTTCTTCATTTCCACCTT
SOD2	NM_017051	AGGTGACACTATAGAATATGTATGAAAGTGCTCAAGAT	GTACGACTCACTATAGGGAGCCCTCTTGTGAGTATAAGT
SOD3	NM_012880	AGGTGACACTATAGAATATCGAACTACTTTATGCCC	GTACGACTCACTATAGGGAGAAGACAAACGAGGTCTCTA
Kan(r)**			
CAT	NM_012520	AGGTGACACTATAGAATAACTGCAAGTTCCATTACAAG	GTACGACTCACTATAGGGAGTTCAACTTCAGCAAAATAAT
Beta-actin*	NM_031144	AGGTGACACTATAGAATAGGCATCCTGACCCTGAAGTA	GTACGACTCACTATAGGGAAGACGCAGGATGGCATGAG

* Housekeeping genes. # Normalization gene. Underlined sequences are left and right universal left and right sequences (tags). ** internal control supplied by Beckman Coulter Inc (Miami, FL, USA) as part of the GeXP kit. RT conditions were: 48 °C for 1 min; 37 °C for 5 min; 42 °C for 60 min; 95 °C for 5 min, then hold at 4 °C. PCR conditions were initial denaturation at 95 °C for 10 min, followed by two-step cycles of 94 °C for 30 sec and 55 °C for 30 sec, ending in a single extension cycle of 68 °C for 1 min. IDE: insulin degrading enzyme; LRP: low density lipoprotein receptor-related protein 1; SOD: superoxide dismutase; CAT, catalase; PSEN: presenilin; APP: amyloid precursor protein. Gapdh: glyceraldehyde-3-phosphate dehydrogenase; Kan(r): kanamycin resistance.

Table 3. Body weight, tissue weight and length, and serum biochemical parameters in ovariectomized rats

	Control	Sham	OVX	Estrogen	6% EBN	3% EBN	1.5% EBN
Body weight(g) before treatment	221.5±33.5	217.8±44.0	230±29.7	236±33.3	213.1±41.8	228.1±53.9	216±31.5
Body weight(g) end point	227.5±6.0[a]	224.6±6.8[a]	283.1±23.1	239±3.0[a]	214.5±1.4[a]	242.8±14.6[a]	242.6±26.7[a,b]
Body weight (g) incensement	6.0±0.6[a]	6.8±0.9[a]	43.1±7.6	3.0±1.0[a]	1.4±0.3[a]	14.6±2.0[a,b,c,d]	26.7±3.7 [a,b,c,d]
uterus+vagina (mm) length	54.27±3.50[a]	59.07±1.78[a]	39.81±3.39	58.18±6.26[a]	57.41±4.76[a]	54.21±5.45[a]	52.74±6.48[a]
uterus+vagina (g) weight	1.02±0.12[a]	1.16±0.17[a,c]	0.31±0.04	0.84±0.05[a]	0.84±0.10[a,b]	0.72±0.04[a,b]	0.63±0.06[a,b,c,d]
Serum estrogen (pg/ml)	151.1±8[a]	156.7±13[a]	35.6±0.9	169.8±11.4[a]	150.4±7.4[a]	147.8±8.7[a,c]	143.3±13.4[a,c]
Serum fasting glucose (mmol/L)	5.13±0.36	5.37±0.21	5.3±0.14	4.94±0.57	5.1±0.36	5.0±0.32	5.5±0.23
Serum fasting insulin (ng/ml)	1.08±0.21	0.99±0.16[a]	1.35±0.11	0.92±0.13[a]	1.12±0.15	1.2±0.11	1.22±0.19
HOMA-IR	5.12±1.0	4.90±0.79[a]	6.74±0.55	4.01±0.57[a]	5.17±0.69	5.54±0.51[c]	6.4±0.99[c]

Values are Mean ± SD, n=6 or 5. All rat groups were ovariectomized except the control group, and all groups received standard rat chow for 12 weeks thereafter. In addition, the estrogen treated group received 0.2mg/kg/day, while EBN groups received 6, 3 or 1.5% EBN in their rat chow, respectively. EBN: edible bird's nest; OVX: ovariectomy. [a]P<0.05 *VS* OVX group; [b]P<0.05 *VS* sham group; [c]P<0.05 *VS* estrogen group; [d]P<0.05 *VS* 6% EBN treatment group.



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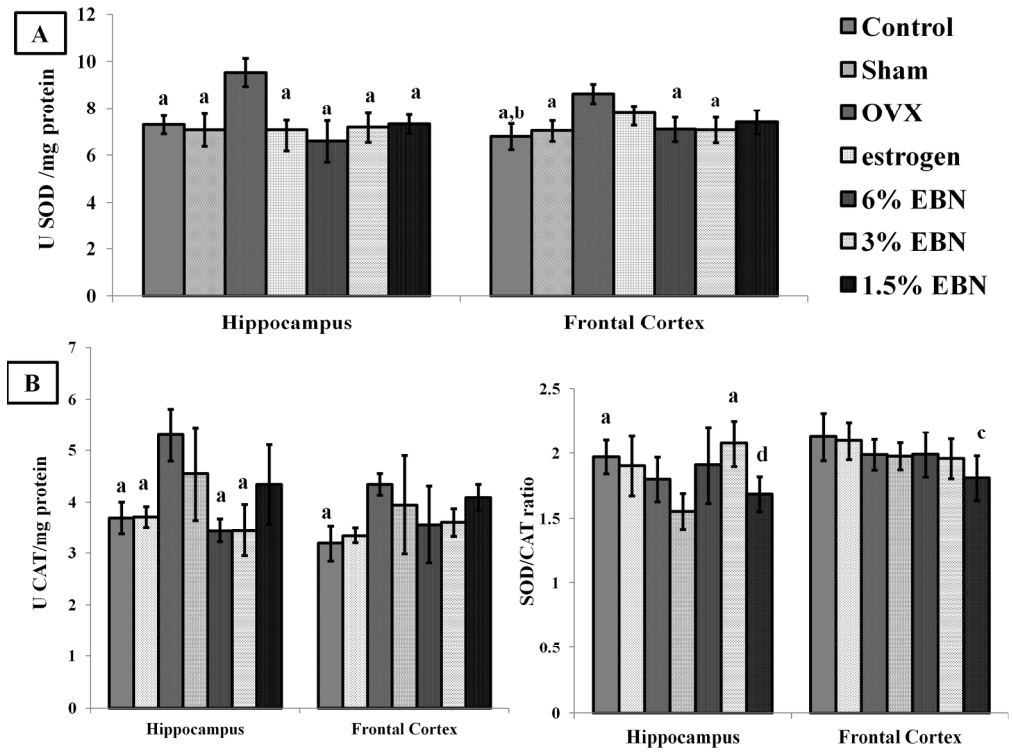

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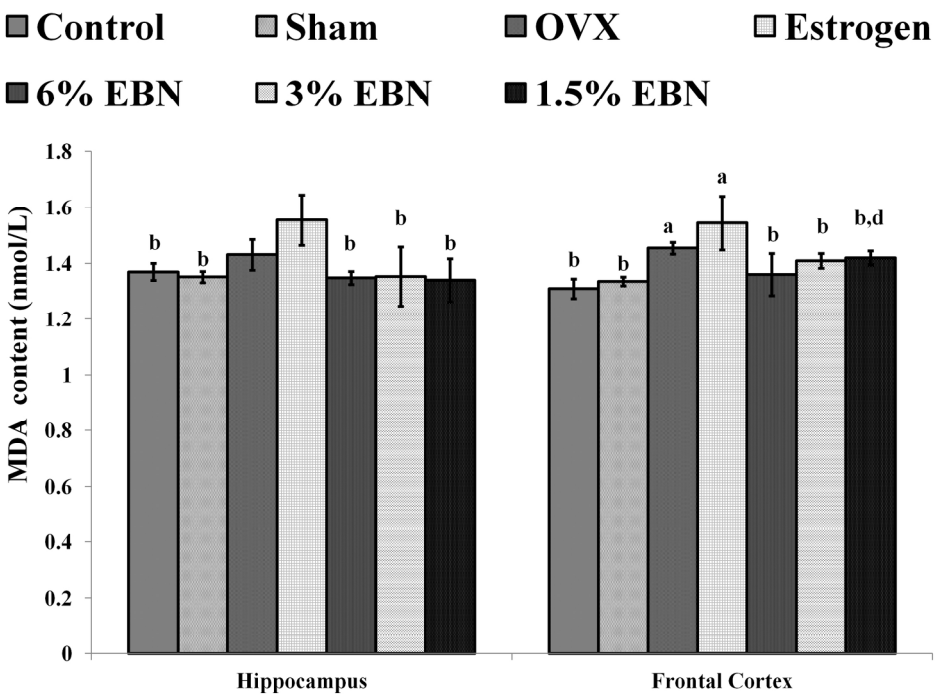

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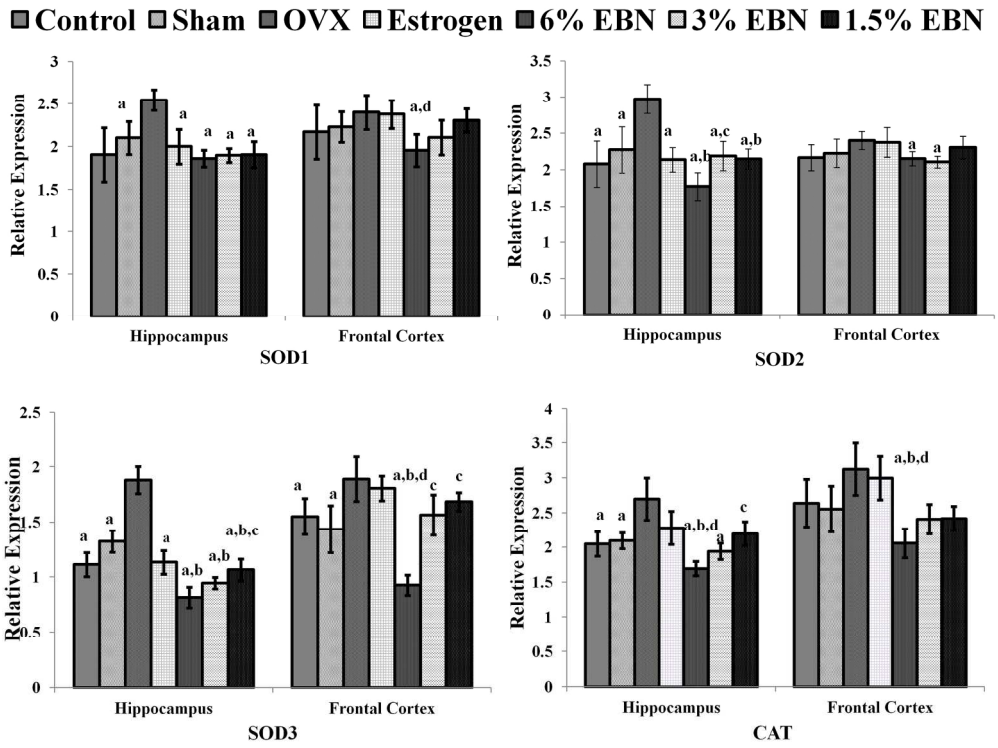

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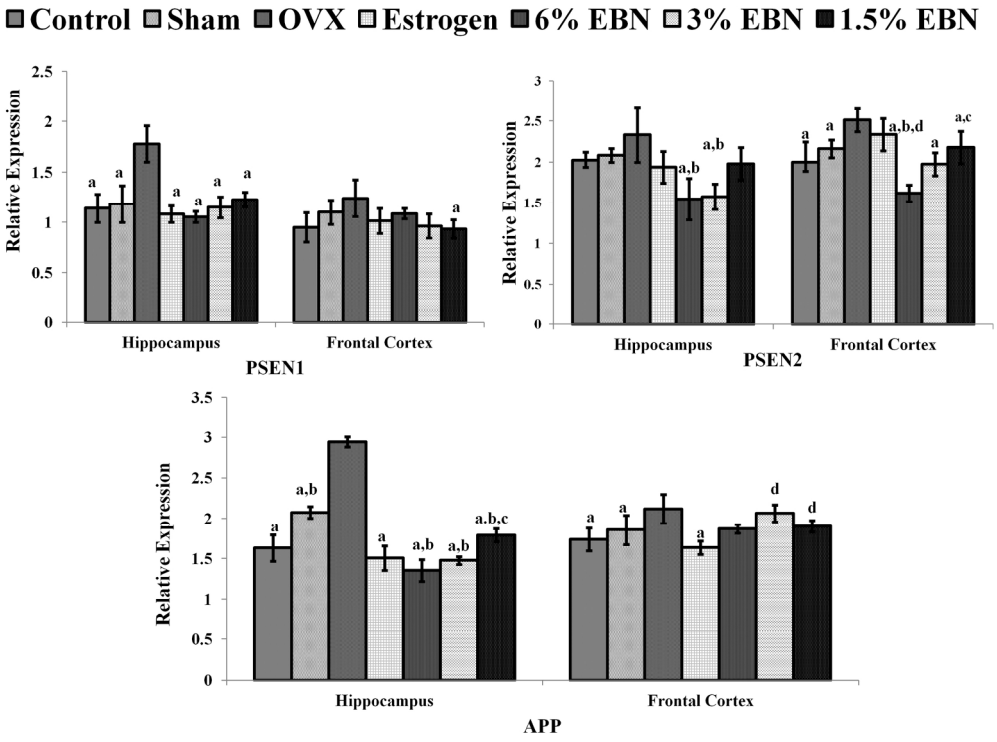

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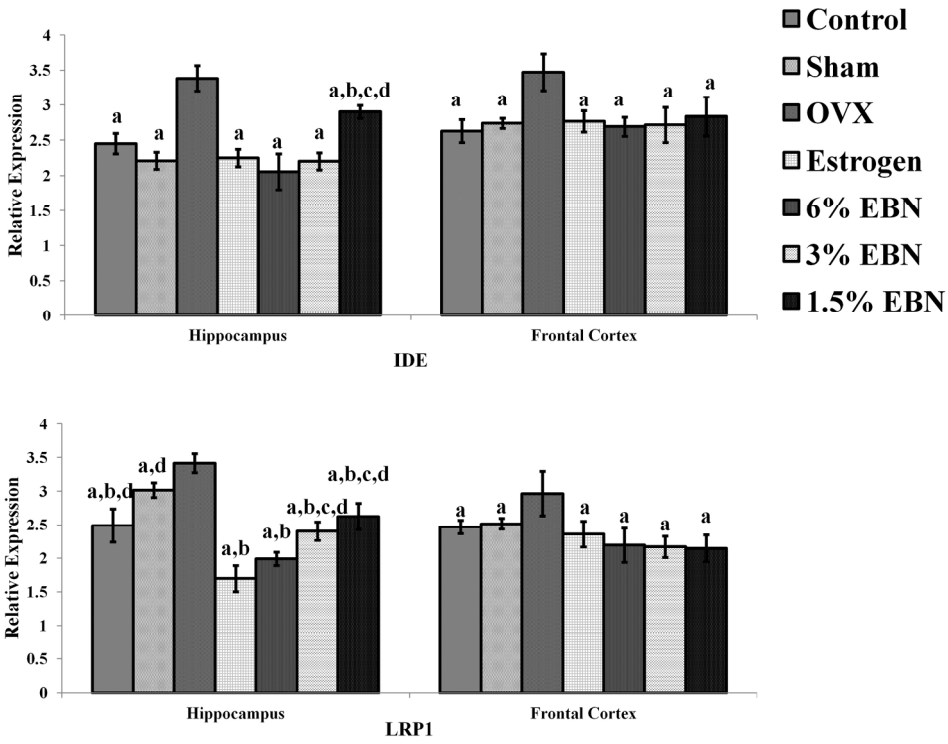

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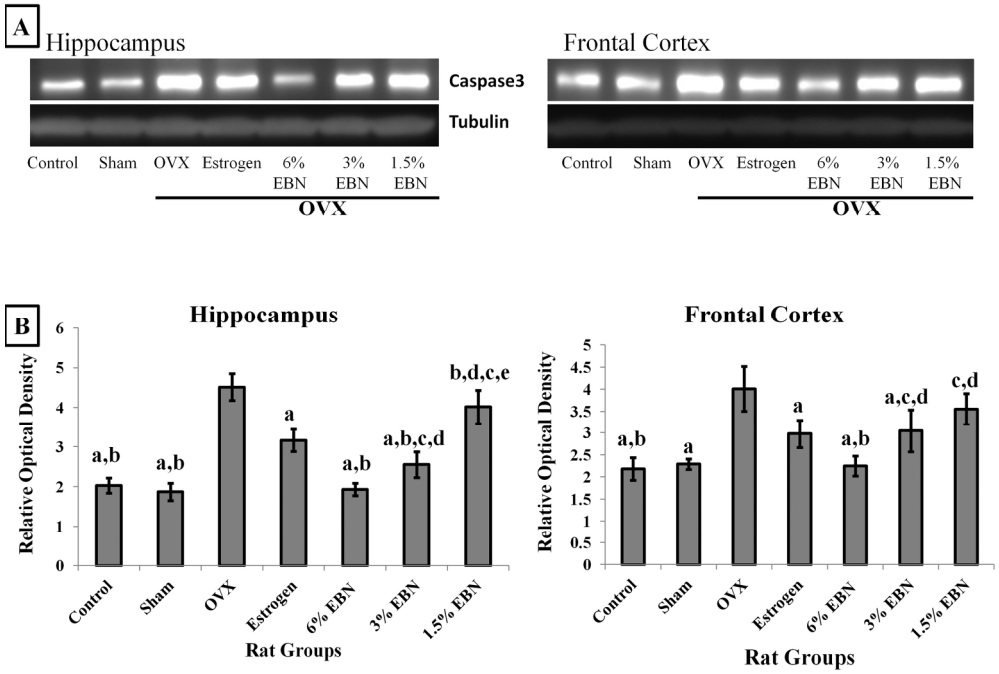

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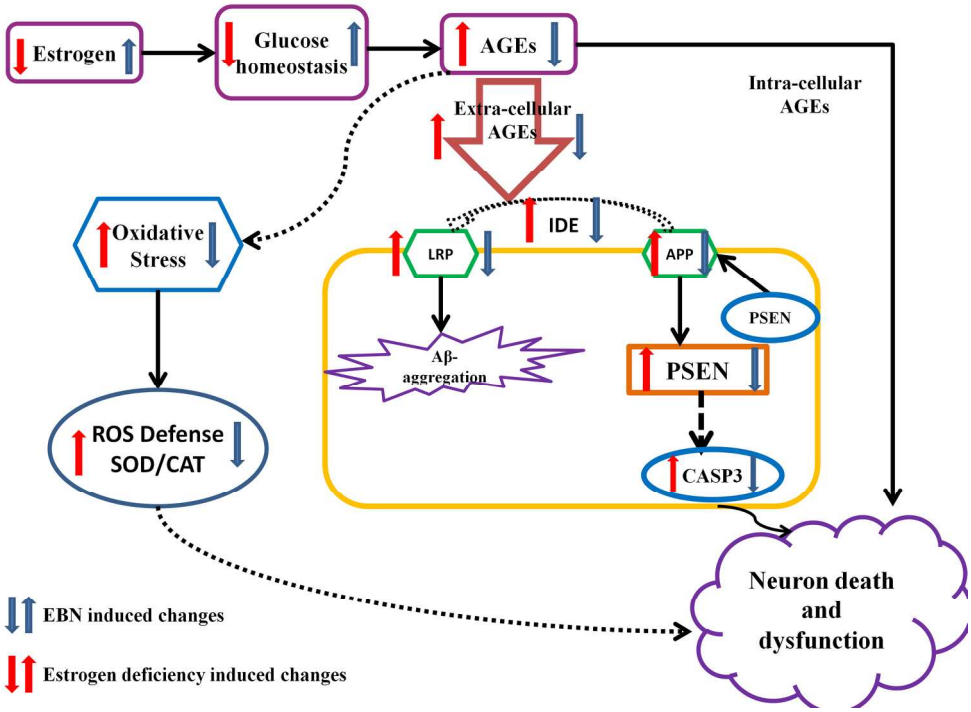

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