EBN Lower Oxidative Stress: Lactoferrin and Ovotransferrin Contribute toward Antioxidative Effects of Edible Bird’s Nest Against Hydrogen Peroxide-Induced Oxidative Stress in Human SH-SY5Y Cells

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ABSTRACT

There are reports of improved redox outcomes due to consumption of Edible Bird’s nest (EBN). Many of the functional effects of EBN can be linked to its high amounts of antioxidants. Interestingly, dietary components with high antioxidants have shown promise in the prevention of aging and its related diseases like Alzheimer’s disease. In this study, the antioxidative potentials of EBN and its constituents, Lactoferrin (LF) and Ovotransferrin (OVF), were determined and protective effects against H2O2-induced toxicity on SH-SY5Y cells using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay and acridine orange and propidium iodide (AO/PI) staining with microscopy. Results showed that EBN and its constituent’s attenuated hydrogen peroxide (H2O2) induced cytotoxicity and decreased radical oxygen species (ROS) through increased scavenging activity. Furthermore, LF, OVF and EBN produced transcriptional changes in anti-oxidant related genes that tended towards neuroprotection as compared to H2O2 treated group. Overall, the results suggest that LF and OVF may produce synergistic or all-or-none anti-oxidative effects in EBN.

Abbreviations: AO/PI: Acridine Orange and Propidium Iodide; EBN: Edible Bird’s Nest; LF: Lactoferrin; ORAC: Oxygen Radical Absorbance Capacity; OVF: Ovotransferrin; ROS: Radical Oxygen Species; SOD: Superoxide Dismutase

INTRODUCTION

Aging is a slow and gradual biological process, associated with multiple physiological and pathological changes including altered redox status. The brain cells are normally sensitive to the effects of Reactive Oxygen Species (ROS) because they are a nidus for peroxidative molecules and because of their peculiar energetic demands [1]. In brain senescence, ROS starts to accumulate in neurons before clinically evident signs and symptoms of the disease can be detected [1]. When ROS accumulate, oxidative damage is normally prevented by induction of protective factors like antioxidants, which may not be effective if the insult is too overwhelming. In such cases, apoptotic mechanisms set in to remove neurons deemed irreparable [2]. Loss of neurons through these apoptotic deaths results in severe morphological and functional deficits, which manifest with progressive memory and cognitive decline.
Recently, due to concerns of side effects, researches have been focused on natural substances with neuro-protective potential that can scavenge free radicals and protect cells from oxidative damage, rather than synthetic chemicals [3]. Edible Bird’s Nest (EBN) is produced by swiftlets from their salivary glue, which is a cementing substance. Although EBN mainly contains carbohydrates, amino acids and mineral salts, its major ingredients are glycoproteins [5]. Due to its nutritious and medical properties, EBN has been deemed a precious food tonic in Chinese community ever since Tang (608-907AD) dynasties [6]. Despite the long history of using EBN for medicinal purposes, there has only been limited number of scientific reports on the health benefits of EBN. Recently, EBN has been found to potentiate mitogenic response of human peripheral blood monocytes that stimulate Deoxyribonucleic Acid (DNA) synthesis in 3T3 fibroblasts [7,8]. Furthermore, enzymatic hydrolysis can release active peptides, which have beneficial effects on a variety of biological systems including the cardiovascular, gastrointestinal, immune and nervous systems [9]. The ability of glycoprotein to interact with radical species or to inhibit oxidative reactions could lead to the development of novel food ingredients relevant in health promotion and disease prevention.

Lactoferrin (LF) and Ovotransferrin (OVF) are glycoproteins and family members of transferrin. Different studies have also shown that LF & OVF serve neuroprotective and antioxidant functions and thus showing good sources of functional food ingredients [10,11]. EBN is reported to have antioxidative properties and in view of the properties of LF and OVF. We hypothesize that, they may be contributing towards the overall antioxidative properties of EBN [12,13]. SY-SY5Y cell line can be differentiated by Retinoic Acid (RA) with similar characteristics to brain neurons. Thus, can be used to study neuronal cell activity invitro [3]. Hydrogen peroxide ($H_2O_2$) is a major reactive free radical that has been studied in a variety of neurodegenerative diseases that has been implicated as an important mediator of unbalanced redox reactions and apoptosis in various cells including neurons [4]. Consequently, this study was conducted to determine the concentration of LF and OVF in EBN, to evaluate their effectiveness against $H_2O_2$-induced oxidative stress on SH-SY5Y cells.

**MATERIALS AND METHOD**

**Materials**

SH-SY5Y human neuroblastoma cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Minimum essential Eagle’s medium, Ham’s nutrient mixture F-12(DMEM/F-12), fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, dimethyl sulfoxide (DMSO) and all other chemicals were purchased from Sigma (St. Louis, MO, USA). The GenomeLab™ GeXP Start Kit was from Beckman Coulter Inc. (Miami, FL, USA), and the Total ribonucleic acid (RNA) isolation kit was obtained from RBC Bioscience Corp. (Taipei, Taiwan). The OxiSelect™ Superoxide Dimutase Activity Assay (SOD) and OxiSelect™ Intracellular ROS Assay kit(ROS) were from Cell Biolabs, Inc.(USA). Lactoferrin (L4894) and ovotransferrin (C7786) were purchased from Sigma-Aldrich (St.Louis, Missouri United States)

**Preparation of EBN water-soluble protein**

House white Edible Bird’s Nest from Malaysia was dried in an oven at 50 °C for three days and finely ground with a food grinder (Waring Commercial, Torrington, CT, USA). As reported previously, the grounded EBN samples (1.0 g) were dissolved into 1000 mL of dd H$_2$O, followed by ultra-sonication (Power Sonic 505, Hwashin Technology Co. Seoul, Korea) in an ice bath (pulse on, 2 s, pulse off, 4 s, ampl, 80%, 30 min) and centrifugation (10000 rpm for 10 min), the supernatants were desalted and condensed in a dialysis bag with a 3500 cut off molecular weight, then the water-soluble protein stored at -20 °C until use [32].

**Lactoferrin and Ovotransferrin detection**

LF and OVF concentration in EBN water extract were detected using Chicken Lactoferrin Elisa kit and Chicken Ovotransferrin Elisa Kit, Biosource (San Diego, California, USA). All the protocols were based on manufacturer instructions.

**Cell culture**

The human neuroblastoma SH-SY5Y cells were grown in complete culture medium containing DMEM/F-12, 10% fetal bovine serum, 1% MEM (non-essential amino acids) and 50 µg/mL gentamicin. Cells were maintained at 37 °C with 5% CO$_2$ and 95% air.

**ABTS radical Cation scavenging assay**

ABTS radical cation was generated through oxidation of 7 mM ABTS with 2.45 mM potassium persulfate and incubated overnight in the dark at room temperature. ABTS radical cation solution was then diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm spectrophotometrically (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan). EBN, LF and OVF (50 µL each) were individually mixed with 950 µL of diluted ABTS solution and incubated for 10 min at room temperature. The absorbance was measured under 734 nm. All the determinations were carried out in triplicate and the readings were averaged. Trolox was used as standard and percentage of ABTS radical cation decolorization inhibition was calculated using the formula reported by Norsharina in 2012 [3]. ABTS radical cation scavenging activity of EBN, LF and OVF was expressed in mg Trolox equivalent per gram extracts as described before (mg TE/g extract).

**Oxygen radical absorbance capacity assay**

The abilities of extracts to scavenge peroxyl radical capacity were determined by Oxygen Radical Absorbance Capacity
(ORAC) according to the method described by Huang et al. [30]. Briefly, 150 µL fluorescein and 25 µL sample (1000 µg/ml EBN, 5 µg/ml LF and 10 µg/ml OVF) or standard (Trolox) were added into each well and incubated 37 °C for 10-15 min. After incubation, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every one min to determine the background signal. After 3 cycles, 25 µL of freshly prepared 2, 2’-Azobis (2-amidinopropane) dihydrochloride (AAPH) at final concentration 81.6 nM was added manually with a multi-channel-pipette. Flourescence readings for each cycle were saved in Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski VT, US), as they would all be used to calculate areas under the curves and trolox equivalents.

**MTT assay**

SH-SY5Y cells were seeded into 96-well culture plates at density of 2 × 10^5 cells/mL and were allowed to attach. After 2 days, cells were differentiated with retinoic acid (10 µM) for 7 days prior to treatment. To examine the possible toxic effects, the cells were treated with EBN (10-100000 µg/ml), LF (0.05-500 µg/ml) and OVF (0.1-1000 µg/ml) individually for 24 h. To determine the neuroprotective ability, cells were pretreated with 1000 µg/mL EBN and the equivalence 5 µg/mL LF and 10 µg/mL OVF diluted in serum-free medium for 24 h and then incubated with H_2O_2 for another 2 h. Then, MTT was added to all the wells and standardised in incubator for 4 h. The amount of MTT formazan product was dissolved by DMSO and measured absorbance at 540 nm using a Micro plate reader (All the MTT assays were performed in triplicate).

**Acridine orange and propidium iodide (AO/PI) staining**

SH-SY5Y cells (2 × 10^5) were seeded into 6-well culture plates, treated with 1000 µg/mL EBN and the equivalence 5 µg/mL LF and 10 µg/mL OVF, then followed by 250 µM H_2O_2 for 2 h. Protocols for the ELISA tests were based on manufacturer instructions. For SOD assay, absorbance of samples were finally read at 490 nm on the Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski VT, US), while fluorescence for ROS assay were read at 480/530 nm by Synergy H1 Hybrid Multi-Mode Micro plate Reader (BioTek, Winooski VT, US).

**SOD and ROS ELISA Assays**

SH-SY5Y cells (2 × 10^5) were seeded into 12 well culture plates, treated with 1000 µg/mL EBN and the equivalence 5 µg/mL LF and 10 µg/mL OVF, then followed by 250 µM H_2O_2 for 2 h. Protocols for the ELISA tests were based on manufacturer instructions. For SOD assay, absorbance of samples were finally read at 490 nm on the Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski VT, US), while fluorescence for ROS assay were read at 480/530 nm by Synergy H1 Hybrid Multi-Mode Micro plate Reader (BioTek, Winooski VT, US).

**RNA extraction, reverse transcription and Multiplex PCR analysis**

SH-SY5Y treated cells were extracted by the Total RNA Isolation kit (RBC Bioscience Corp., Taiwan) according to the manufacturer’s instructions. Primer sequences were designed using GenomeLab eXpress Profiler software based on Homo sapiens sequence from the NCBI website (Table 1). The primers were supplied by Biosune (Shanghai, China), while the internal control (KanR) was supplied by Beckman Coulter. Reverse transcription and PCR were performed according to the GenomeLab™ GeXP kit instruction (Beckman Coulter) in an XP Thermal Cycler (Bioer Technology, Germany). The PCR products were finally analyzed by the GeXP genetic analysis system and the results were normalized using GeXpress Profiler software based on the manufacturer’s instructions.

**Statistical Analysis**

All the data (n=3) was conducted by one-way ANOVA, Tukey’s multiple comparisons and Student’s t-test using Statistical Package for Social Science (SPSS) version 20 (SPSS Inc., Chicago, IL). p<0.05 was considered as statistically significant difference.

| Table 1: Gene name, accession number, reverse and forward primer sequences used in GeXP multiplex gene expression analyses |
|---|---|---|---|---|---|
| Gene Name | Accession number | Prime sequences with universal tag | For | Rev | Primer sequence with universal tag |
| KanR | | AGGTGACACTATAGAATAATCATCAGTCTTCTTGG | GTCAGCTCTACATAGGGAATTC-CGACTGTCCACATC |
| 18sRNA | M10098 | AGGTGACACTATAGAATAATGGGAGCCCTT-GGGCTTAA | GTACGACTCATATAGGATAGCAGCATGC |
| NF-kB2 | NM_001077493 | AGGTGACACTATAGAATAGGAGTGGGCTTCAAAATCTTG | GTACGACTCATATAGGATAGCAGCATGC-CACGGTACC |
| P53 | NM_001126117 | AGGTGACACTTAGAATAGGAGGAGGAGGCACTCAAAAATCTTG | GTACGACTCATATAGGATAGCAGCATGC-CACGGTACC |
| P38MAPK | NM_001315 | AGGTGACACTATAGAATAGGAGGAGGAGGCACTCAAAAATCTTG | GTACGACTCATATAGGATAGCAGCATGC-CACGGTACC |
| Akt | NM_001014431 | AGGTGACACTATAGAATAGGAGGAGGAGGCACTCAAAAATCTTG | GTACGACTCATATAGGATAGCAGCATGC-CACGGTACC |
EBN, LF and OVF attenuate $H_2O_2$ Induced Cytotoxicity on SH-SY5Y Cells

The abilities of EBN, LF and OVF to protect SH-SY5Y cells from $H_2O_2$ were determined by MTT assay, an indicator of cell viability. EBN (10-100000 µg/mL) and equivalent concentrations of LF and OVF in EBN (0.05-500 µg/mL and 0.1-1000 µg/mL, respectively) displayed above 80% viability on SH-SY5Y cells and showed no significant differences when compared with the control group (P>0.05) (Figure 1).

Hydrogen Peroxide ($H_2O_2$), is an oxidant that can induce intracellular defence mechanisms to attenuate $H_2O_2$-induced oxidative damage. As reported in our previous paper, SH-SY5Y cells exposure to 250 µM $H_2O_2$ for 2 h resulted in approximately

Figure 1: Toxic and neuroprotective effects of EBN, LF and OVF on SH-SY5Y cells determined by MTT assay. (A) Toxic effect of EBN on SH-SY5Y cells. Human SH-SY5Y neuroblastoma cells were incubated with EBN (0.001--100mg/ml) for 24h; (B) Neuroprotective effect of EBN on $H_2O_2$-induced SH-SY5Y cells. Series of EBN (0.001--100mg/ml) pretreated SH-SY5Y for 24h followed by $H_2O_2$ (250 µM) for an additional 2 h. (C) Toxic and neuroprotective effects of 1mg/ml EBN equivant LF(5µg/ml) and OVF(10µg/ml). LF and OVF pretreated SH-SY5Y cells for 24h, with or without followed by 2h $H_2O_2$ incubation. Results are presented as the mean ± SD in triplicates. *p<0.01 vs $H_2O_2$, #p<0.01 vs control. EBN, Edible Bird’s Nest; LF, lactoferrin; OVF, ovotransferrin.
50% cell death compared with untreated control cells (p<0.01) [3]. As shown on Figure 1B, EBN produced a hormetic effect on the viability of the cells in the presence of H$_2$O$_2$, with 1000 µg/ml EBN producing the best result. Although antioxidants are known to relieve oxidative stress and improve cell survival, it is also suggested that higher concentrations of antioxidants may in fact disrupt the natural mitohormetic capacity of cells resulting in more damage [19]. It is also likely that at higher concentrations, the oxidants simply became provident [22,23]. Therefore, for our subsequent experiments, 1000 µg/ml EBN and equivalent concentrations of LF and OVF (5 µg/ml and 10 µg/ml, respectively) were used.

2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation scavenging activities and Oxygen Radical Absorbance Capacity (ORAC) Assay

ORAC assay is a simple, sensitive, and reliable method to quantitate the radical absorbing capacity of antioxidants in serum or other biological fluids, especially natural products [24]. Additionally, ABTS is also widely used for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidants in many fields [25]. To determine antioxidant potentials of EBN, LF and OVF, ABTS and ORAC were performed on the samples (Figure 2). EBN (1000 µg/mL) exhibited the best scavenging effect on ABTS (5.23 ± 0.18 mg Trolox equivalent/g extract), followed by LF (5 µg/mL) and OVF (10 µg/mL) at concentrations similar to what is contained in 1000 µg/mL of EBN (4.67 ± 0.40 and 2.79 ± 0.18 mg Trolox equivalent/g extract, respectively).

Results of the ORAC assay also showed that EBN had the highest antioxidant capacity (3.04 ± 0.23 mg Trolox equivalent/g extract) when compared with LF and OVF (2.14 ± 0.10 and 1.22 ± 0.08 mg Trolox equivalent/g extract, respectively). The results also indicated significant differences (p<0.05) between LF and OVF and also when each is compared with EBN.

Results of EBN, LF and OVF have demonstrated consistent data in scavenging activities by ORAC and ABTS radical scavenging assays. EBN demonstrated the highest antioxidant activity followed by LF and OVF via ORAC and ABTS radical cation scavenging methods (Figure 2). The results, so far suggest that LF and OVF possess antioxidant capacities and even though the capacities of any one of the compounds may not explain the antioxidant capacity of EBN, it is likely that they contribute towards the overall capacity in EBN. Additionally, the overall antioxidant capacity of EBN may be

![Figure 2](image-url)

**Figure 2**: SH-SY5Y cells were captured under inverted light microscope, 20 X magnification; (A) Untreated control group; (B) Treatment with 250µM H$_2$O$_2$ for 2 h; (C) Cells pretreated with 1mg/ml Edible Bird’s Nest (EBN) and followed with 250µM H$_2$O$_2$ 2 h. (D) Cells pretreated with 5µg/ml lactoferrin (LF) and with 250µM H$_2$O$_2$ 2 h; (E) Cells pretreated with 10µg/ml ovotransferrin (OVF) and with 250µM H$_2$O$_2$ 2 h.
contributed by other compounds in addition to LF and OVF, since LF and OVF do not entirely reflect the same activity as EBN. Different studies have already shown that due to synergistic effects of compounds in crude extracts, crude plant extracts usually exhibit superior health oriented properties than purified compounds [26]. It is also likely that synergy between the different bioactive compounds in EBN may potentiate their activity when they are bound together in a single matrix [27].

**Acridine Orange (AO)/Propidium Iodide (PI) staining**

AO and PI are among the most used stains for analyses of cell apoptosis. Using a combination of these stains, cells appear green to indicate they are apoptosis, while intact cells appear orange/red. As shown in Figure 3, control cells appeared green, suggesting normal structure and viability, while H2O2 treated cells appeared orange, representing early apoptosis. As for the EBN-treated cells, majority of the cells were stained green, showing normal appearance of intact cells. Similarly, LF and OVF groups showed mostly normal cells, with little signs of apoptosis.

**ROS and Superoxide Dismutase (SOD) assay**

SOD belongs to the endogenous antioxidant system that can scavenge ROS. The disruption in the balance between ROS and the endogenous antioxidant systems contributes to oxidative damage of cellular macromolecules ultimately leading to cell death in [28]. In this study, the relative activity of SOD in the SH-SY5Y cells exposed to 250 μM H2O2 for 2 h decreased to 69.6% compared with the control group. Pre-treatment with EBN, LF and OVF for 24 h recovered the SOD activity to 90.7%, 87.8% and 80.9%, respectively (Figure 4A).

To investigate whether EBN, LF and OVF have the effect to scavenge intracellular ROS generation induced by H2O2, intracellular ROS kit was used to examine intracellular H2O2/hydroxyl radical. DCFH-DA (2',7'-dichlorofluorescin diacetate) fluorescence staining indicated that the value of H2O2-treated cells was increased about 2-fold compared with untreated control cells. However, the increase in intracellular H2O2/hydroxyl radical was reduced by EBN, LF and OVF treatment (Figure 4B).

Obviously, these data indicate that LF and OVF have a strong tendency to scavenge ROS and may in fact be contributing to the ROS scavenging ability of EBN. These results corroborate those of the antioxidant capacities of LF and OVF, which indicated that they had high antioxidant capacities that may be contributing to that of EBN.

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**Figure 3:** Expression levels of ORAC in SH-SY5Y cells, following treatment with 1mg/ml EBN water extract, 5µg/mL LF, 10µg/mL OVF and subsequent treatment with 250µM H2O2, in comparison to H2O2 group. A: kinetic data; B, trolox equivalence. Results are presented as the mean ± SD in triplicates. ap< 0.01 vs H2O2, bp< 0.01 vs EBN treatment group. AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride; ORAC, oxygen radical absorbance capacity; EBN, Edible Bird’s Nest, LF, lactoferrin; OVF, ovotransferrin.
Effects of EBN, LF and OVF on mRNA levels of antioxidant and apoptosis genes

The mRNA levels of three antioxidant and apoptosis genes (PARP1, SOD1 and SOD2) were studied using Multiplex GeXP genetic analysis system, with KanR as the internal control. The target genes and housekeeping gene are shown on Table 1.

As shown in Figure 5, treatment with 250 μM H₂O₂ downregulated the mRNA levels of SOD1, SOD2 and PARP1 genes. Treatments with 1000 μg/ml EBN water extract, 5 μg/mL LF or 10μg/mL OVF unregulated the expression of the three genes even in the presence of 250 μM H₂O₂. In the case of SOD1 and SOD2, the EBN, LF and OVF treated-cells unregulated the gene significantly higher than H₂O₂-treated controls (P<0.01), although the expression levels were lower than in non-treated control cells (P<0.01). The mRNA level of PARP1 in EBN treated cells was higher than H₂O₂ treated and normal untreated cells (P<0.05), but those of LF and OVF treated cells were not different from H₂O₂-treated cells, and were lower than in untreated controls (P>0.05).

Unregulation of antioxidant genes (SOD) is an endogenous mechanism for boosting antioxidant defences in the presence of oxidative insults and could be the basis for the increased SOD activity observed in this study when SH-SY5Y cell
were treated with EBN, LF or OVF (Figure 4). Moreover, the mRNA levels for the EBN, LF and OVF groups are reflective of the SOD activities observed in the respective groups, as no significant differences in SOD mRNA levels or activities were observed between the groups. The results, therefore, suggest that EBN boosts SOD levels and its activity through increased transcriptional regulation of the gene and that LF and OVF in EBN contribute to this effect. However, combination of these compounds and others in EBN may not have produced additive effects since there are no differences between EBN, LF and OVF treatments. Furthermore, activation of PARP1 is reported to improve cell survival after oxidative damage from H$_2$O$_2$ [29]. Its increased expression by EBN but not LF or OVF suggest that other constituents in EBN may be contributing to this effect other than LF or OVF, or that their synergistic effect with or without other compounds may be contributing to expression level seen in EBN-treated cells. The overall data from this study indicates that EBN constituents interact in different ways to produce its activity. The presence of multiple compounds may produce additive or synergistic effects as suggested by the antioxidant capacity tests and expression of PARP1, while in some cases it may produce an all-or-none effect as seen with the activity and expression of SOD.

**CONCLUSION**

In summary, the present study demonstrated that EBN protects SH-SY5Y cells against H$_2$O$_2$-induced cytotoxicity and cell oxidative stress. LF and OVF contributed to the antioxidative effects of EBN in different ways. They were also able to inhibit early apoptosis in response to H$_2$O$_2$ treatment. The findings indicate that EBN constituents contribute towards producing synergistic antioxidative effects of EBN.

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