Bacopa monniera extract plays a significant role in lipofuscin scavenging in cultured human umbilical endothelial cells

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Abstract
Introduction
We investigated the generation of lipofuscin granules in human umbilical vein endothelial cells that were exposed to hydrogen peroxide (H2O2, 100 µM) for 1 h. Lipofuscin granule scavenging was studied using the Bacopa monniera (Brahmi) extract (100 µg) for 2 h, 4 h and 6 h exposures. We used an endothelial cell culture model to investigate the antioxidant effects of the B. monniera extract on lipofuscin granules scavenging in cultured human umbilical vein endothelial cells.

Materials and methods
To understand the protective role of the B. monniera extract in post-oxidative stressed human umbilical vein endothelial cells, an experiment was set-up with the following exposure regimen: pre-B. monniera extract (100 µg) for 2 h plus post-H2O2 (100 µM) exposure for 1 h. To analyse whether pre-oxidative stressed human umbilical vein endothelial cells could be protected with exposure to post-B. monniera extract and whether the lipofuscin granules could be scavenged by post-B. monniera extract treatment, the following exposure regimen was provided: pre-H2O2 (100 µM) for 1 h plus post-B. monniera extract (100 µg) for 2 h.

Results
The presence of lipofuscin granule formation was confirmed by the Ziehl-Neelson's staining method and microscopic examination. Exposure to H2O2 (100 µM) showed the abundant generation and formation of lipofuscin granules. B. monniera extract exposures of 2 h, 4 h and 6 h (100 µg), showed the abundant generation and formation of lipofuscin granules. B. monniera extract exposures of 2 h, 4 h and 6 h (100 µg), showed a complete absence of lipofuscin granules. Pre-B. monniera extract (100 µg) exposure for 2 h plus post-H2O2 (100 µM) exposure for 1 h, resulted in extremely reduced lipofuscin granules. H2O2 exposure for 1 h and pre-H2O2 (100 µM) exposure for 1 h plus post-B. monniera extract (100 µg) exposure for 2 h, resulted in the moderate appearance of lipofuscin granules.

Conclusion
This study concluded that the B. monniera extract has potential antioxidant properties, which play a pivotal role in the analysis of lipofuscin granules scavenging in cultured human umbilical vein endothelial cells.

Introduction
Medicinal plants that have been useful in history are obvious choices as sources with significant pharmacological and biological activities1. One such medicinal plant is Bacopa monniera, commonly termed as Brahmi2. Studies in the past have focused on B. monniera’s cognitive enhancing effects that specifically include memory, learning and concentration. Post-mitotic cells accumulate a non-degradable, intralysosomal substance called lipofuscin which is formed due to the iron-catalysed oxidation/polymerisation of protein and lipid residues3. It is believed that these changes occur not only due to continuous oxidative stress (causing oxidation of mitochondrial constituents and autophagocytosed material) but also because of the inherent inability of cells to completely remove oxidatively damaged structures (biological garbage)4. Accumulation of lipofuscin granules is mostly observed in various neurological disorders5. There are no reported studies of B. monniera and its antioxidant activities, which play a significant role in lipofuscin scavenging in cultured human umbilical vein endothelial cells (HUVECs). Therefore, this study is the first to show that the B. monniera extract (BME) plays a pivotal role by strongly supporting the scavenging of lipofuscin granules in cultured HUVECs.

Material and methods
This work conforms to the values laid down in the Declaration of Helsinki (1964). The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed. All subjects gave full informed consent to participate in this study.

BME preparation
The dried herb was macerated in 95% methanol and the crude extract of B. monniera was prepared and filtered with Whatman no.1 filter paper6. The extract was stored at −30°C7.

Harvest and expansion of HUVECs
The isolation protocol for HUVECs was adapted from Baudin et al. 20077. The umbilical cord was collected immediately and the cord was rinsed

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and washed with sterile phosphate buffered saline (PBS). One end of the umbilical vein was clamped using artery forceps and an enzyme cocktail of 0.15% collagenase type IV (Sigma Aldrich, St. Louis, Missouri, USA) and dispase II (Roche, Nutley, New Jersey, USA) was filled into the lumen cavity of the vein for dislodging the endothelial cells (ECs). The ECs were incubated for 20 min at 37°C. After completion of incubation, the vein was flushed with M199 medium (Invitrogen, Carlsbad, California, USA). The cells in suspension were centrifuged at 1500 rpm for 10 min. Cell pellets were refreshed twice by centrifugation and the final pellet was again resuspended in an endothelial cell growth medium (ECGM) (PromoCell GmbH, Germany) containing 20% foetal bovine serum (FBS) (Invitrogen, Carlsbad, California, USA); isolated HUVECs were plated in tissue culture flasks. After 12 h of incubation, the HUVECs were fed with complete ECGM containing 20% FBS, 2 mM L-glutamine, 10 µg/mL heparin and penicillin 5 unit/mL and incubated at 37°C with 5% CO₂.

The ECs were incubated for 20 min (100 µM) for 1 h (100 µM) and were fixed with 4% paraformaldehyde and refreshed with PBS 1 × (5 min × 3 washes). The HUVECs were washed with 70% isopropanol (10 min × 2 washes) and stained with carbolfuscin for 30 min at 60°C. The HUVECs were further rinsed in acid-alcohol (1% HCl in 70% ethanol) until they turned light pink. They were washed in distilled water for 5 min, counter stained (0.5% toluidene blue) for 10 min and rinsed again with distilled water for 5 min. Isopropyl alcohol (70%) was used to dehydrate the cells. The cover slips were mounted with dibutyl phthalate xylene (DPX). Stained HUVECs were observed under a bright field microscope for lipofuscin granules.

Exposure to BME for 2 h, 4 h and 6 h, pre-BME exposure for 2 h plus post-H₂O₂ for 1 h and pre-H₂O₂ exposure for 1 h plus post-BME for 2 h to cultured HUVECs to study the effect of BME on lipofuscin granules

HUVECs were cultured on cover slips until they reached a cobble stone morphology. HUVECs were further processed as follows: BME exposure for 2 h, 4 h and 6 h, pre-BME exposure for 2 h plus post-H₂O₂ for 1 h and pre-H₂O₂ exposure for 1 h plus post-BME for 2 h.

Results

B. monniera crude extract

A 12% yield of sticky, crude extract was obtained from B. monniera and further used for experimental work.

Isolation and culture of HUVECs

HUVECs were isolated in clusters of 30. HUVECs were attached to the plate surface immediately after seeding for 2 h. HUVECs started expanding in clusters and reached confluence on the 10th day (Figure 1). On 70% confluence, the HUVECs resembled the perfect cobblestone morphology, and the cell density was 3 × 10⁵ cells/cm².

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Lipofuscin granules in the control cells and in cells exposed to oxidative stress (H₂O₂ for 1 h)
The current investigation reveals the appearance and occurrence of lipofuscin granules in cultured HUVECs. HUVECs without any exposure served as controls (normal) and showed a much lower presence of lipofuscin granules (Figure 2).

Oxidant exposures (H₂O₂ for 1 h) to cultured HUVECs, led to lipofuscin granule generation. Exposure of H₂O₂ for 1 h at a concentration of 100 µM resulted in maximum damage to the cultured HUVECs; the cells showed distorted cell and nuclear membranes with copious formations of lipofuscin granules in the cytosol region (Figure 3).

Exposure to BME for 2 h, 4 h and 6 h, pre-BME exposure for 2 h plus H₂O₂ for 1 h and pre-H₂O₂ exposure for 1 h plus post-BME for 2 h to cultured HUVECs to study the effect of BME on lipofuscin granules
Exposure to BME (100 µg) treatment for 2 h (Figure 4), 4 h (Figure 5) and 6 h (Figure 6), showed the absence of or the lack of lipofuscin granule formation and an intact cell and nuclear membrane. Pre-BME (100 µg) exposure for 2 h plus post-H₂O₂ (100 µM) for 1 h, showed the presence of very reduced amounts of lipofuscin granule formation with intact nuclear membranes and very little injury to the cell membranes (Figure 7). Pre-H₂O₂ (100 µM) exposure for 1 h plus post-BME (100 µg) for 2 h, resulted in the moderate appearance of lipofuscin granule formation with little nuclear and cell membrane damage (Figure 8).

Discussion
Cells are constantly subjected to free-radical attack. Free radicals are scavenged in the cells by the antioxidant system present in the cell. The unscavenged free radicals exert their cytotoxic action on cell membrane lipids. These peroxidised membranes are engulfed by lysosomes which may bring about damage to the lysosomal enzymes. The residual body of lysosomes that are unable to digest cell membranes turns into lipofuscin granules. In advanced aging, the rate of accumulation of lipofuscin granules increases in tissues like the heart and brain².

_B. monniera_ is a herb, and a variety of studies have been carried out to study its antioxidative properties. Past research investigating BME exposure in neurons, established the lipofuscin scavenging property of _B. monniera_.² We tried to investigate the role of BME in lipofuscin scavenging in cultured HUVECs. HUVECs without any exposure served as the controls and showed very little presence of lipofuscin granules. Exposure to 1 h of oxidative stress (H₂O₂) at a concentration of 100 µM resulted in...
maximum damage to the cultured HUVECs, such as distorted cell and nuclear membranes and abundant formation of lipofuscin granules in the cytosol region. This firmly explains that dose-dependant H$_2$O$_2$ exposure for 1 h brings about cellular damage and is involved in the formation of lipofuscin granules in cultured HUVECs.

Exposure to BME (100 µg) for 2 h, 4 h and 6 h, showed the absence of lipofuscin granule formation and intact cell and nuclear membranes. Pre-BME (100 µg) exposure for 2 h plus post-H$_2$O$_2$ (6.25 µM to 100 µM) for 1 h, showed negligible/reduced amount of lipofuscin granule formation with intact nuclear membrane and very little injury to the cell membrane. This illustrates that pre-BME exposure for 2 h provides the necessary anti-oxidative protection to cultured HUVECs while the results from the post-H$_2$O$_2$ exposure for 1 h illustrate that there was a lack of or minimal lipofuscin granule formation. Therefore, it is deduced that pre-BME exposure has a protective role against lipofuscin granule formation even after post-H$_2$O$_2$ exposures. Pre-H$_2$O$_2$ (100 µM) exposure for 1 h plus post-BME (100 µg) exposure for 2 h, resulted in the moderate appearance of lipofuscin granules. This investigation shows that BME is an antioxidant which plays a pivotal role in lipofuscin scavenging in stressed and normal HUVECs. H$_2$O$_2$ (100 µM) treatment is responsible for oxidative impair and brings about the extreme generation or formation of lipofuscin granules. BME exposures for 2 h, 4 h and 6 h (100 µg), bring about scavenging of lipofuscin granules at different time intervals. Pre-BME (100 µg) exposure for 2 h brings about reduced lipofuscin formation. After post-H$_2$O$_2$ (100 µM) exposure for 1 h, there is no further rise in lipofuscin. Pre-H$_2$O$_2$ (100 µM) exposure for 1 h plus post-BME (100 µg) exposure for 2 h, resulted in the moderate appearance of lipofuscin granules.

**Abbreviations list**

BME, *Bacopa monniera* extract; ECs, endothelial cells; ECGM, endothelial cell growth medium; FBS, foetal bovine serum; PBS, phosphate buffered saline.

**Conclusion**

This antioxidant plays a pivotal role in lipofuscin scavenging in stressed and normal HUVECs. H$_2$O$_2$ (100 µM) treatment is responsible for oxidative impair and brings about the extreme generation or formation of lipofuscin granules. BME exposures for 2 h, 4 h and 6 h (100 µg), bring about scavenging of lipofuscin granules at different time intervals. Pre-BME (100 µg) exposure for 2 h brings about reduced lipofuscin formation. After post-H$_2$O$_2$ (100 µM) exposure for 1 h, there is no further rise in lipofuscin. Pre-H$_2$O$_2$ (100 µM) exposure for 1 h plus post-BME (100 µg) exposure for 2 h, resulted in the moderate appearance of lipofuscin granules.

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**References**