Abstract
Natural products with antioxidant capacity have recently caught the attention of researchers as well as members of the public. In this study, the possible protective effects of green tea and white pepper against oxidative damage induced by H$_2$O$_2$ were investigated on PC12 cells. Neither green tea nor white pepper was able to exert any protective effect. Indeed, green tea enhanced cytotoxicity, solely due to rapid generation of H$_2$O$_2$ in Dulbecco's Modified Eagles Medium. PC12 cells treated with green tea, H$_2$O$_2$ or their combinations had retarded cell proliferation, and showed morphological signs of apoptosis at higher H$_2$O$_2$ concentrations. Further analysis revealed that there was no evidence of DNA fragmentation or increased DNA base damage concurrent with cytotoxicity. These results suggest that confounding factors should be considered when drawing conclusions on the beneficial effects (or the lack of them) of natural products.

Introduction
In recent years, research had established a correlation between oxidative stress and numerous chronic human disorders such as cancers, arthritis and Alzheimer's disease, as well as normal processes such as aging (Weisburger, 2000). Oxidative stress usually results in the events of antioxidant depletion or excessive production of reactive oxygen species (ROS), during which the balance between ROS and antioxidant defence systems is shifted in favour of ROS (Gutteridge and Halliwell, 1994).

Hydrogen peroxide (H$_2$O$_2$) is often used as a potent inducer of oxidative damage in cell assays (Jang and Surh, 2001; Slamenová et al., 2002). It readily crosses biological membranes and penetrates into the nucleus to form highly reactive hydroxyl radical (•OH) by Fenton reaction in the presence of iron or copper ion (Halliwell and Gutteridge, 1999). •OH reacts rapidly with virtually all cellular components (e.g. DNA, lipids and proteins), producing functional and structural alteration to these biomolecules.

One way of preventing adverse effects of oxidative stress is to increase the level of antioxidant defences through dietary antioxidant supplements, such as the widely recognized vitamin C, vitamin E, and ß-carotene (Shikany et al., 2003) or antioxidant-rich natural products such as teas, red wine, soy product and cocoa (Weisburger, 2000). The potency of green tea (Camellia sinensis) stems from its polyphenolic constituents (Salah et al., 1995). White pepper (Piper nigrum) has a large array of constituent with major classes being alkaloids/amides, lignans, neolignans and terpenes (Parmar et al., 1997), any of which may give pepper antioxidant properties.

In this study, green tea and pepper were evaluated for their possible protective effects against oxidative damage and inhibition of cell proliferation induced by H$_2$O$_2$ in PC12 cells.
Materials and Methods

Reagents  Dulbecco’s Modified Eagles Medium (DMEM) containing bovine serum was from Gibco South America. Cat. No.: 10270-106. All reagents were purchased from Sigma Chemical Pte Ltd., Singapore unless otherwise stated.

Cell culture  PC12 cells (rat pheochromocytoma cell line) were cultured at 37°C and 5% CO\textsubscript{2} in DMEM culture medium supplemented with 10% horse serum, 5% foetal calf serum and 1 X Antibiotic Antimycotic colution. This cell line was maintained in a 75 cm\textsuperscript{3} tissue culture flask and was sub-cultured every 3-4 days at around 80% confluent.

Preparation of Stock Compounds  Stock green tea (OSK brand) was prepared at a concentration of 0.01 gram dried leaves per ml of hot (95°C) deionised water. The tea was left on a hot plate for 15 minutes to maintain the temperature. The white pepper (cultivated in Kuching, Malaysia) was ground into powdered form and its stock solution was prepared at 0.01 gram per ml of deionised water. It was then boiled on a hot plate for 5 minutes. Both compounds were prepared freshly and filtered with 0.2 µm filter before use.

Treatment and Assessment of Cell Viability  PC12 cells in DMEM were seeded into 96-well plates with density of 2×10\textsuperscript{4} cells/100µl/well. Following overnight incubation, the medium was replaced with DMEM mixed to give the desired concentration of green tea, pepper, H\textsubscript{2}O\textsubscript{2} or both. For experiments involving catalase, 10µl of catalase dissolved in PBS was added such that the final concentration was 1000 units/ml. After incubation for 24 hours, the medium was removed and the cells were rinsed with PBS and re-supplied with DMEM. The cells were incubated for another 24 hours, after which cell viability was determined by MTT reduction assay.

Hydrogen Peroxide Measurement  This was performed by the Ferrous ion oxidation-xylenol orange (FOX) method as described by Long et al. (2000).

Isolation of DNA and Analysis of Oxidative Base Damage by Gas-Chromatography-Mass-Spectroscopy (GC/MS)  DNA extraction was carried out as previously described by Spencer et al. (1995). Acid hydrolysis, derivatization and GC/MS analysis were essentially performed as described (Spencer et al., 2000).

Electrophoresis of DNA  Isolated DNA was suspended in TBE buffer (0.089 M Tris base, 0.089 M Boric acid, 0.002 M EDTA) and 1 µg DNA per lane was electrophoresed in 1% agarose gel for 2 h at 80 V. The gel was visualized with ethidium bromide.

Results and Discussion

Analysis of H\textsubscript{2}O\textsubscript{2}-Mediated Oxidative Damage on PC12 cells by Viability Assay  There is a growing interest in the potential neuroprotective effects of dietary antioxidants, especially flavonoids (Youdim et al., 2002). Hence PC12 cell, which is often used as a neuronal model, was utilized in this study. H\textsubscript{2}O\textsubscript{2} was employed as the oxidative agent in this study due to its ease of conversion to the indiscriminately reactive hydroxyl radical (OH•) by interaction with transition metal ions in vivo (Halliwell and Gutteridge, 1999). The exposure of PC12 cells to H\textsubscript{2}O\textsubscript{2} (24 hours) was limited to the range within 50-200 µM for all the experiments performed. Metabolic status of the treated cells was determined using MTT reduction assay.
Effects of Green Tea on PC12 cells

Many in vitro and in vivo studies showed that green tea has strong antioxidant properties (Vinson and Dabbagh, 1998; Ho et al., 1992). These cytoprotective effects had been explained in relation to the polyphenolic compounds present. Other studies suggested these polyphenolic compounds can be oxidized and exhibited pro-oxidant effects in vitro under certain experimental conditions (Laughton et al., 1989; Yamanaka et al., 1997). Therefore the possible protection against oxidative damage by green tea was being studied on PC12 cells.

Preliminary experiments with different concentrations of green tea revealed that it was toxic to PC12 cells. The toxicity could be eliminated completely upon addition of catalase. Consistent with previously reported artefacts in cell culture (Long et al., 2000), this could be explained by the fact that green tea added to DMEM generated a substantial amount of \( \text{H}_2\text{O}_2 \) in a time dependent fashion over a 23h period with, probably due to oxidation of polyphenolic compounds. The correlation between concentration of green tea and the level of \( \text{H}_2\text{O}_2 \) formed in the medium was obvious. Interestingly, the study indicated that green tea was unable to protect PC12 cells against the toxicity of added \( \text{H}_2\text{O}_2 \) in contrast to the radical scavenging capability of its polyphenolic constituents (data not shown).

When assessed by phase contrast microscopy, PC12 cells treated with 4% green tea exhibited similar growth pattern as in those treated with 100 µM \( \text{H}_2\text{O}_2 \). There appeared to be an inhibition of growth in treated cells as fewer cells were observed with regard to untreated cells. Such growth inhibition may occur through \( \text{H}_2\text{O}_2 \) production in the medium as \( \text{H}_2\text{O}_2 \) can elicit DNA damage by •OH formation. As such, the cells are arrested by cell cycle checkpoint mechanisms while the repair process is taking place (Shiloh, 2001). Green tea treatment in the presence of catalase regained normal growth pattern to a similar extent as in control. Morphological alterations such as cell shrinkage and rounding up were apparent in cells exposed to 4% green tea along with 100 µM \( \text{H}_2\text{O}_2 \). However, no membrane blebbing or apoptotic bodies are observed after 24 h of treatment, which may suggest an early stage of apoptosis.

Interestingly, there is no DNA laddering effect that occurs concurrently with the cytotoxicity, even for PC12 cells treated with 4% green tea plus 100 µM \( \text{H}_2\text{O}_2 \) that shows morphological signs of apoptosis. Pertaining to this observation, Gardner et al. (1997) reported that low concentrations of \( \text{H}_2\text{O}_2 \) (at 0.1 and 0.5 mM) induced delayed cytotoxicity at 24 h in a pathway involving poly(ADP-ribose)polymerase without affecting DNA integrity. This suggests that the cytotoxicity measured with MTT could be a feature of inhibition of cell proliferation during which DNA strand breakage is repaired. It is also observed that green tea and \( \text{H}_2\text{O}_2 \) did not alter the level of base-damaged species present on the DNA molecule with the exception of two samples (100 µM \( \text{H}_2\text{O}_2 \) and 4% green tea plus 50 µM \( \text{H}_2\text{O}_2 \)) that appeared to have decreased xanthine level (data not shown). The seemingly lower level of xanthine could be an underestimation of the true extent of guanine deamination. The lack of DNA base modifications could be a consequence of elevated activity in DNA repair enzymes for cells exposed to \( \text{H}_2\text{O}_2 \) for long incubation times (Spencer et al., 1996). As discussed earlier, the level of \( \text{H}_2\text{O}_2 \) decreases in DMEM over the 24 h incubation period. Therefore the enhanced mechanisms of DNA repair soon after initial oxidative insult can probably cope well with reduced rate of base modifications.

White Pepper Does Not Prevent \( \text{H}_2\text{O}_2 \)-induced Loss of Viability of PC12

To ascertain possible cytotoxic effects of pepper (Piper nigrum), PC12 cells were treated with pepper. There was no significant difference in cell viability with incubation of cells in
DMEM containing 4% pepper (103.9 ± 4.5%) and 8% pepper (101.3 ± 2.5%) with reference to control. There was no evidence indicating that co-incubation of PC12 cells with pepper influenced H$_2$O$_2$-induced MTT reduction in cells as well. No enhancement of H$_2$O$_2$ was seen in DMEM containing pepper, which was a striking contrast to that containing green tea.

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