Bioactive compounds in green tea leaves attenuate the injury of retinal ganglion RGC-5 cells induced by H$_2$O$_2$ and ultraviolet radiation

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Abstract: The Chinese commonly believe that tea helps maintain clear vision. This viewpoint has been recorded in Chinese medical books also. The key bioactive compounds in green tea leaves, (-)-epigallocatechin gallate (EGCG), L-theanine (theanine) and caffeine, were investigated for their abilities to attenuate the injury of retinal ganglion cells (RGC-5) induced by H$_2$O$_2$ and ultraviolet radiation. Theanine and caffeine promoted cell growth while concentrations of EGCG greater than 10µg/ml inhibited cell growth. The nine and caffeine both protected RGC-5 cells from injury as well as enhanced their recovery, while EGCG only protected the cells from injury and did not help them to recover. Tea is a unique drink, which is simultaneously enriched with EGCG, theanine and caffeine. The role of these compounds in optic nerve protection may partially explain why some tea drinkers feel enhanced vision.

Keywords: Tea leaves, bioactive compounds, retinal ganglion cells, protection, recovery.

INTRODUCTION

The tea leaves have many bioactive compounds in which the key components are (-)-epigallocatechin gallate (EGCG), L-theanine (theanine) and caffeine. EGCG, the most bioactive agent, has received extensive studies for its health protective effects (Yang et al., 2009; Boiling et al., 2008). EGCG could provide protective effect on the retina against transient ischemic injury by intraperitoneal or intra-ocular injection (Zhang et al., 2007). It could also attenuate retinal damage in vitro and in vivo (Chida et al., 1999; Zhang and Osborne, 2006). EGCG significantly reduced the apoptosis of retinal ganglion cell (RGC) induced by H$_2$O$_2$ (Zhang et al., 2007; Peng et al., 2010). In addition, EGCG could prevent the increase of caspase mRNA and protein levels in the retina from ischemic and oxidative damage in vitro (Zhang B et al., 2007; Zhang B et al., 2006). Reduction in the expression of neuronal nitric oxide synthase (nNOS) was another way to protect the RGCs by EGCG (Peng et al., 2008).

Theanine is a special amino acid with a content of 1-4% in dried tea leaves. It possesses an chemical structure analogous to glutamine (Gln) which is a key neurotransmitter in memory. The neuroprotective effects of theanine have been reported by research groups (Kakuda et al., 2000; Kakuda, 2011). Theanine intake may provide neuroprotective effects (Egashira IV et al., 2004; Cho et al., 2008, Di et al., 2010), and improve cognitive function (Takeda et al., 2011). A long-term theanine administration with a high concentration could suppress the progression of cognitive dysfunction (Kataoka et al., 2009). It has been reported that theanine impacts brain function (Haskell et al., 2008; Einother et al., 2010), and may help to slow the progression of Parkinson’s and Alzheimer’s diseases (Kim et al., 2009; Yamada et al., 2009). Caffeine also has neuroprotective properties. A decline in cerebral palsy and neurodevelopmental impairment was achieved by administration of caffeine to preterm infants (Schmidt et al., 2006; Schmidt et al., 2007; Davis et al., 2010; Rivkees and Wendler, 2011; Supcun et al., 2010).

The beneficial effects of caffeine on the brains have been observed in animal models of Parkinson’s and Alzheimer’s diseases (Kalda et al., 2006; Dall’Igna et al., 2007). Caffeine could improve arousal and alertness (Bryan, 2008; Jang et al., 2012). A synergistic effect of theanine and caffeine has been found to improve the ability of test subjects who carry out a task which was very challenging to visual spatial attention (Kelly et al., 2008). Caffeine could protect the neuronal cell injury induced by hyperoxia (Endesfelder et al., 2014), and the neuroprotective effects were mediated pro-inflammatory cytokines and histone deacetylase (Machado-Filho et al., 2014). The Chinese commonly believe that tea helps maintain clear vision. This viewpoint has been recorded in Chinese medical books over thousands of years including Chapu (Encyclopedia of Tea, 1530), Shennong Bencao (Classic Herbal Medicines, 220-280) and Bencao Tongyuan (Encyclopedia of Herbs, 1644-1911). Although there are reports showing that EGCG is beneficial to optic nerve health, few researchers have investigated the effects of theanine and caffeine on optic nerves.

In this study, we investigated the effects of EGCG, theanine and caffeine on the growth of RGC-5 (Retinal ganglion cells), and their protective effects on the RGC-5 injury induced by H$_2$O$_2$ and ultraviolet radiation, since RGC-5 cells are one of the three neuron types found in...
the retina and are a good model system for investigating retinal injury and recovery.

MATERIALS AND METHODS

Culture of RGC-5 cells

RGC-5 cells (Shanghai Xinyu Biology Ltd, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 25 mM glucose, and 100 U/ml streptomycin (Sigma-Aldrich) in a 95% humidified atmosphere air and 5% CO₂ at 37°C.

Assessment of the ability of bioactive compounds to enhance the growth of RGC-5 cells

RGC-5 cells (100 µl) were plated in 96-well plates at a density of 5 × 10³ cells/well. After 12 h growth, the cells were treated with tea bioactive compounds at a concentration of 10 µl for 24 h followed by CCK-8 reagent was added to assess cell viability. After 3 h, the absorbance was measured at 450 nm by a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). Cell viability was calculated by the following formula: Cell viability (%) = (ODtreatment - ODblank) / (ODcontrol - ODblank) × 100

Injury of RGC-5 cells induced by H₂O₂ or UV irradiation

The injury in the RGC-5 cells was achieved by treatment of different concentrations of H₂O₂ or different time period of exposure of UV irradiation. It could help to standardize the dose for subsequent experiments. In the case of H₂O₂, RGC-5 cells were treated with following concentrations such as 150, 200, 250, 300, 350, 400, 450 and 500 µM for 1 h. The UV irradiations were performed on a super-clean bench equipped with a 40 watt UV (254 nm) lamp tube (2.4 cm outer diameter & 1.2 m length). The culture dishes were placed directly under the lamp tube at a distance of 50 cm and irradiated for 5, 7, 9, 11, 13, 15, 17 and 19 min. The H₂O₂ concentration required in the culture medium which yielded 50% cell viability after 1 h of incubation was 350 µM and the length of UV irradiation which yielded 50% cell viability was 11 min. These conditions were selected for examining the ability of the tea bioactive compounds to protect and help RGC-5 cells recover from injury.

Assessment of the protective effects of bioactive compounds on RGC-5 cells injured by H₂O₂ or UV irradiation

To assess the protective efficacy of tea leaves bioactive compounds, RGC-5 cells were pre-treated with bioactive compounds than exposed to injury. Briefly, RGC-5 cells (5 × 10⁵ cell/ml, 100 µl) were plated and incubated with various concentrations of bioactive compounds for 24 h. Then the cells were exposed to 10 µl of H₂O₂ solution to a final concentration of 350 µM for 1 h (H₂O₂ treatment) or 11 min exposure of UV (UV treatment). After the injury induction period, the cells were continuously cultivated for another 24 h and then the medium was changed into fresh DMEM containing 1% FBS. Finally, 10 µl of CCK-8 reagent was added to assess cell viability.

Assessment of the ability of bioactive compounds to recover the RGC-5 cells from injury induced by UV irradiation

To study the recovery effect of bioactive compounds, RGC-5 cells were prior exposed to UV irradiation to induce injury. Briefly, the RGC-5 cells (5 × 10³, 100 µl) were plated in 96-well plates and exposed to UV irradiation for 11 min. Then the tea bioactive compounds were added to the culture medium and were continuously incubated for 24 h. After the incubation previous culture medium was replaced with new DMEM containing 1% FBS and 10 µl of CCK-8 reagent was added to test for cell viability.

STATISTICAL ANALYSIS

Significance analysis was performed by a one-way analysis of variance followed by a Tukey multiple-comparison test. A value of p < 0.05 was considered as significant.

RESULTS

Effects of tea bioactive compounds on RGC-5 cell growth

Both caffeine and theanine promoted the growth of RGC-5 cells while EGCG significantly inhibited the cell growth at concentrations more than 5 µg/ml (fig. 1). The growth of the cells was inhibited by 50% with the presence of 40 µg/ml EGCG. The enhancement effect of theanine and caffeine on the cell growth increased as their concentrations increased. The growth of the cells was increased by 45% and 36% after treatment with more than 40 µg/ml of theanine and caffeine, respectively. Growth of the cells continued to be enhanced up to the highest concentrations of theanine and caffeine tested (80 µg/ml and 160 µg/ml, respectively). At theanine concentrations less than 5 µg/ml and caffeine concentrations less than 10 µg/ml no significant enhancement in cell growth was observed.

The ability of bioactive compounds from green tea to avoid injury of RGC-5 cells induced by H₂O₂

The viability of RGC-5 cells was 50% when they were incubated in the presence of 350 µM H₂O₂ in the culture medium. If the RGC-5 cells were first pre-cultured for 1 h with EGCG, theanine or caffeine, and then were exposed to 350 µM H₂O₂ in the culture medium, the cell viability was significantly improved (table 1). EGCG provided the best protection from H₂O₂ induced damage.
with 78.1% of RGC-5 cells remaining viable at 2.5µg/ml, which suggests that EGCG attenuates oxidative stress (H2O2) injury on RGC-5 in vitro. The protective effect of theanine on the RGC-5 cell viability enhanced with an increase in the theanine concentration. At the highest theanine concentration tested, 40µg/ml, the cell viability increased 36%, an increase of 72% relative to the untreated control group. The protective effect of caffeine on the cells was weaker than theanine although the cell viability also increased by 20% relative to the group without treatment.

![Fig. 1](image)

**Fig. 1**: The effects of green tea bioactive compounds on RGC-5 cell growth. *P<0.05, **P<0.01, compared to the untreated group.

**The ability of bioactive compounds from green tea to protect RGC-5 cells from injury induced by UV irradiation**

When the RGC-5 cells were pre-cultured for 1 h with EGCG, theanine and caffeine, and then injury was induced by exposure to 11 min of UV radiation, the cell viability showed a significant improvement (table 2) compared with the untreated control group. The results were very similar to the protective effects observed for cells injured by treatment with H2O2. As humans need to consume a large amount of oxygen, it is easy to generate reactive oxygen species in the retina such as superoxide anion free radical (O2-) and H2O2. In the presence of ultraviolet radiation, reactive oxygen species can immediately be converted into hydroxyl free radical (·OH), which causes serious damage to intracellular DNA and the cell membrane. Therefore, as H2O2 and UV treatment both induce damage in RGC-5 cells via reactive oxygen species, EGCG, theanine and caffeine are likely protecting RGC-5 cells from damage induced by similar mechanisms. Assessment of the ability of bioactive compounds to help RGC-5 cells recover from injury. To investigate the ability of the tea bioactive compounds to help RGC-5 cells recover from injury, we damaged the cells using an 11 min UV irradiation treatment resulting in a cell viability of 51% relative to the normal cultured group (CK). After UV irradiation, the cells were deformed, observed to clump together and overall, a decrease in the density of cells was observed, all which indicated lower RGC-5 cell viability (fig. 2). The UV treated RGC-5 cells were incubated with EGCG, theanine and caffeine. Both theanine and caffeine helped the injured cells to recover while EGCG did not. Incubation with theanine helped to increase RGC-5 cell viability better than caffeine (table 3). Cells incubated with theanine or caffeine after exposure to UV irradiation were observed to adopt a shape typical of untreated cells, and the cell density greatly increased (fig. 2). The ability of theanine and caffeine to help RGC-5 cells recovery from injury is likely related to their ability to enhance cell viability as shown in fig. 1.

![Fig. 2](image)

**Fig. 2**: The ability of green tea bioactive compounds to help RGC-5 cells recover from injury (a) induced by 11 min of UV irradiation followed by (b) incubation with theanine and (c) incubation with caffeine. (d) Normal cultured cells (CK), untreated and uninjured.

**DISCUSSION**

EGCG is a powerful antioxidant (Sutherland et al., 2006; Srividhya et al., 2008; Srividhya et al., 2004), and can influence the cell mechanisms, which relate to RGCs. For example, it decreases cytotoxicity induced by glutamate (Lee et al., 2004), blocks the NF-kB activation and prevent inducible NOS (nitric oxide synthase) reduction (Aktas et al., 2004; Xie et al., 2010; Xu et al., 2006), regulates cellular signaling pathways involved survival and differentiation of neuronal cells by mitogen-activated protein kinase (MAPKs) (Mandel et al., 2006). Therefore, a better understanding of the mechanism by
Bioactive compounds in green tea leaves attenuate the injury of retinal ganglion RGC-5 cells induced by H$_2$O$_2$

Table 1: Assessment of the ability of bioactive compounds from green tea to protect RGC-5 cells from injury induced by 350 µM H$_2$O$_2$.

<table>
<thead>
<tr>
<th></th>
<th>EGCG Con. (µg/ml)</th>
<th>Theanine Con. (µg/ml)</th>
<th>Caffeine Con. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell viability (%)</td>
<td>Cell viability (%)</td>
<td>Cell viability (%)</td>
</tr>
<tr>
<td>0</td>
<td>50.3±1.5</td>
<td>0</td>
<td>50.3±1.5</td>
</tr>
<tr>
<td>2.5</td>
<td>78.1±2.1**</td>
<td>10</td>
<td>64.6±1.8**</td>
</tr>
<tr>
<td>5</td>
<td>85.4±2.7**</td>
<td>20</td>
<td>77.2±2.5**</td>
</tr>
<tr>
<td>10</td>
<td>80.9±3.1**</td>
<td>40</td>
<td>86.3±3.5**</td>
</tr>
</tbody>
</table>

** indicate significant difference (P<0.01), compared to the untreated group.
a All values represent the mean ± SD (n=3)

Table 2: Assessment of the ability of bioactive compounds from green tea to protect RGC-5 cells from injury induced by UV radiation.

<table>
<thead>
<tr>
<th></th>
<th>EGCG Con. (µg/ml)</th>
<th>Theanine Con. (µg/ml)</th>
<th>Caffeine Con. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell viability (%)</td>
<td>Cell viability (%)</td>
<td>Cell viability (%)</td>
</tr>
<tr>
<td>0</td>
<td>50.8±1.2</td>
<td>0</td>
<td>50.8±1.2</td>
</tr>
<tr>
<td>2.5</td>
<td>76.1±2.2**</td>
<td>10</td>
<td>73.3±1.8**</td>
</tr>
<tr>
<td>5</td>
<td>87.0±3.6**</td>
<td>20</td>
<td>81.7±2.5**</td>
</tr>
<tr>
<td>10</td>
<td>83.4±2.9**</td>
<td>40</td>
<td>92.0±3.6**</td>
</tr>
</tbody>
</table>

** indicate significant difference (P<0.01), compared to the untreated group.
a All values represent the mean ± SD (n=5)

Table 3: The ability of bioactive compounds from green tea to help RGC-5 cells recover from injury induced by H$_2$O$_2$

<table>
<thead>
<tr>
<th>Cells</th>
<th>EGCG Con. (µg/ml)</th>
<th>Theanine Con. (µg/ml)</th>
<th>Caffeine Con. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Injury</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>0</td>
<td>50.6±0.9</td>
<td>0</td>
<td>50.6±0.9</td>
</tr>
<tr>
<td>2.5</td>
<td>56.3±1.3</td>
<td>10</td>
<td>62±1.6**</td>
</tr>
<tr>
<td>5</td>
<td>57.7±1.9</td>
<td>20</td>
<td>74±2.0**</td>
</tr>
<tr>
<td>10</td>
<td>55.8±1.7</td>
<td>40</td>
<td>87±2.6**</td>
</tr>
</tbody>
</table>

** indicate significant difference (P<0.01), compared to the control group.
a All values represent the mean ± SD (n=3)

which EGCG attenuates oxidative stress (H$_2$O$_2$) injury on RGC-5 cells is expected to advance research relevant to optic injury. The cerebro-neuro-protective effects of theanine were reported by several groups (Kakuda, 2011; Egashira et al., 2004; Cho et al., 2008). Their results showed the neuroprotective effects are partly contributed by its antagonistic action on kainate and glutamate receptors. Theanine is also known to strongly interact with the glutamine transporter and inhibit neurons to take in glutamine, which suppresses transforming glutamine into glutamate by glutaminase (Egashira et al., 2004). However, no reports exist on its involvement with the optic nerve. The present study shows that theanine promotes the growth of RGC-5 cells, and can protect and help the cells recover after injury induced by H$_2$O$_2$ and UV irradiation, indicating that treatment with theanine is beneficial to the optic nerve. Caffeine possesses neuroprotective effect since it is a non-selective inhibitor of adenosine receptors. Caffeine has anti-pain, anti-inflammatory and neuroprotective pharmacological properties as observed in several in vivo and in vitro models of Parkinson’s disease (Kalda et al., 2006; Dal'lIgna et al., 2007). Also caffeine can improve arousal and alertness (Bryan, 2008). Therefore, a combination of theanine and caffeine simultaneously improved speed and accuracy when checked in a task of attention-switching and reduced the sensibility of distracting information in a memory (Jang et al., 2012; Kelly et al., 2008). Our investigation indicated that caffeine promoted the growth of RGC-5 cells, and protected and helped the cells to recover from injury induced by H$_2$O$_2$ and UV irradiation.

CONCLUSION

Theanine and caffeine can promote RGC-5 cell growth and protect them from injury induced by H$_2$O$_2$ and ultraviolet radiation as well as recover from the injury once it is induced. EGCG also protected the cells from H$_2$O$_2$ and ultraviolet radiation induced injury. Green tea is
naturally enriched with EGCG, theanine and caffeine, all of which may play a role in optic nerve protection which may explain why some tea drinkers observe that their eyesight is enhanced.

ACKNOWLEDGEMENTS

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