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Green Coffee Extract Protects H9C2 Cardiomyocytes from Doxorubicin Induced Apoptosis

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ABSTRACT

Green Coffee Extract (GCE), an extract of *Coffea arabica* bean is a popular health supplement employed for anti-obesity and anti-diabetic effects. Here a hydroalcoholic extract of Green Coffee (GCE) was evaluated for its potential as a cardioprotective agent against Doxorubicin (Dox) induced cardiac insult in a H9C2 rat cardiomyocyte *in vitro* model system. The GCE was tested in an MTT viability assay using 1 μM Dox with and without GCE pretreatment at 50, 100 and 250 μg mL^{-1} concentrations. GCE was also tested for its free radical scavenging ability in a DPPH assay at 10 concentrations (500 μg mL^{-1} maximum concentration). To understand the mechanism of action of cardioprotection, mitochondrial membrane potential ($\Delta\psi m$) was compared between Dox treated cells with and without GCE pretreatment, using the JC-1 dye. Finally, the activation of caspase-3/7 was quantitated. Findings from the above experiments demonstrated that GCE rescued H9C2 cardiomyocytes from Dox induced loss of cell viability in a dose-dependent manner. While Dox treatment caused a clear decrease in the JC-1 ratio from 2 to 1.6 due to loss of $\Delta\psi m$, pre-treatment with GCE at 25, 50 and 100 μg mL^{-1} restored the JC-1 ratio to 1.6, 1.9 and 2.0, respectively. Dox treatment potently induced caspase 3/7 activity by 5 fold and pre-treatment with GCE at 100 and 500 μg mL^{-1} reduced this activation to 3.5 and 1.5 fold, respectively. This data clearly demonstrates that GCE is strongly cardioprotective against Dox induced cardiac insult and the mechanism of action is by blocking activation of intrinsic apoptotic pathway.

Key words: Green coffee extract, doxorubicin, MTT, JC-1, DPPH, caspase, H9C2, cardioprotection

INTRODUCTION

Coffee is the most popular beverage consumed all over the world. It is a complex mixture of about a thousand different components, which include many strong antioxidants (Higdon and Frei, 2006). It is the primary source of dietary antioxidants for the general population (Svilaas *et al*., 2004). Green coffee has been attributed with many therapeutic benefits. It has been shown to have anti-inflammatory effects (De Castro Moreira *et al*., 2013), hypotensive effects (Suzuki *et al*., 2002), anti-obesity effects and improvement of insulin resistance (Song *et al.,* 2014), neuroprotective effects (Jeong *et al*., 2013) among others.

The effect of coffee, either in its raw green form or in its roasted form has a controversial relationship with cardiovascular disease. An adverse effect between coffee drinking and

cardiovascular disease risk was found by Cornelis and El-Sohemy (2007). In contrast, Wu *et al*. (2009) in a meta-analysis of a number of cohort studies, found coffee drinking decreased the risk of cardiovascular disease. Chlorogenic acids are important components of coffee and are considered to be responsible for the beneficial effects of coffee, while caffeine is believed to mediate a number of harmful effects. Infact, a standardized green coffee extract with reduced caffeine and high chlorogenic acid content is being commercially sold under the name Svetol by Naturex, a French company as a health supplement for the prevention of obesity and metabolic syndrome.

Doxorubicin (Dox) is a potent chemotherapeutic compound, used extensively to treat a variety of cancers like lymphomas, breast cancers, ovarian cancers, etc. Cardiotoxicity is the most serious and often lethal side effect of this drug, thought to be mediated mainly by ROS generation (Vejpongsa and Yeh, 2014). The ROS affect mitochondria of the cells inducing apoptosis and cell death. Since cardiac cells depend heavily on mitochondria for their energy needs, Dox induced mitochondrial damage leads to cardiomyocyte cell death, leading to cardiac hypertrophy, ischemia and ultimately cardiac failure and death (Kalyanaraman *et al*., 2002). Dexrazoxane is the only FDA approved drug for countering Dox induced cardiotoxicity (Vejpongsa and Yeh, 2014). Yet, this drug's efficacy is not proven beyond doubt and it has been approved for certain types breast cancers only. There is a severe unmet medical need for cardioprotective agents that preserve cardiomyocyte cell health in the presence of Doxorubicin.

In this study, evidence is presented that a hydroalcoholic extract of Green Coffee (GCE) (*Coffea arabica*) prevents cardiomyocyte cell death induced by Dox treatment *in vitro*. Using H9C2 rat cardiac cell line as an *in vitro* model it is further elucidated that the mechanism of action by GCE is by blocking the intrinsic pathway of apoptosis induced by Dox. Results obtained here show that GCE prevents loss of mitochondrial membrane integrity induced by Dox, an early step in the apoptotic process. The GCE also inhibits Dox activation of the executioner caspases, caspase-3 and 7 preventing Dox induced apoptosis.

MATERIALS AND METHODS

Plant material: A hydroalcoholic Green Coffee Extract was kindly supplied by Sanat Products (Delhi, India). The procedure for preparation was as below:

- Green coffee beans from the plant *Coffea arabica* were milled and extracted for 24 h with 80:20 aqueous alcohol, concentrated to a thick liquid in vacuum, filtered. The filtrate was decaffeinated using chloroform for 2 h at room temperature. The resultant extract was spray dried and pounded to a fine powder. The extract was weighed out freshly for each experiment and dissolved in incomplete cell culture medium (DMEM) at the required concentration before cell treatment

Cell culture: H9C2 rat cardiomyocytes were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Steptomycin (all culture reagents from Invitrogen). The cells were sub-cultured every 3 days at a ratio of 1:4 and maintained at lower than 70% confluency in a cell culture incubator at 37°C and 5% CO_2.

Reagents: Doxorubicin, DMSO, DPPH, methanol were obtained from Sigma-Aldrich Chemicals. Carbonilcyanidem-cholorophenylhydrazone (CCCP) was obtained from Invitrogen.

Assessment of cell viability by MTT assay: Cell viability was determined by the MTT dye method. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium dye that is converted to purple formazan by living cells, which is quantitated as a measure of cell viability.

Procedure: H9C2 cells in log-phase were seeded at 10,000 cells per well in a 96 well cell culture plate and allowed to recover overnight in a cell culture incubator. The next day, GCE, solubilized in plain DMEM was added to the required concentration. After 1 h of pre-treatment with GCE, Dox was added to a final concentration of 1 μM (0.1% DMSO final concentration). Vehicle control cells received DMSO to 0.1% final concentration. The cells were incubated for 72 h. At the end of the incubation, media was removed and 100 μL of fresh medium containing 2 mg mL^{-1} of MTT dye was added and incubated for 4 h. The media was then removed after centrifugation for 2 min at 2000 rpm and the plates were allowed to air dry for 2-3 min. DMSO (100 μL) was added to the wells and the purple formazan was solubilized by shaking the plate for 5 min. The plate was read at 492 nm in a TECAN infinite M1000 pro plate reader. Cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

Assessment of DPPH radical scavenging activity: The free radical scavenging capacity of GCE was quantitated using 2,2-diphenyl-1-picrylhydrazyl (DPPH). This assay is commonly used to assess antioxidant potency.

Procedure: Ten different concentrations of GCE were prepared in methanol (500 μg mL^{-1} as highest concentration and 9 points 2 fold down). Then 180 μL of 0.39 mM freshly prepared DPPH solution in methanol was added to 20 μL of each sample and allowed to react in the dark at room temperature for 1 h. Absorbance of the resulting solution was measured at 517 nm. Methanol (180 μL) added to 20 μL of the lowest GCE concentration was used as blank, while 180 μL of 0.39 mM DPPH solution added to 20 μL of methanol served as a negative control. Percentage of DPPH scavenging activity was determined using the following equation:

$$\text{Antioxidant activity (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where:
A_s = Absorbance of sample (Trolox or GCE)
A_c = Absorbance of negative control

The antioxidant activity (%) was analyzed using GraphPad Prism software. Nonlinear regression analysis was performed using the Hill equation to obtain an IC_{50} value.

Assessment of mitochondrial membrane permeability by JC-1 dye method: Mitochondrial membrane permeability loss was assessed using JC-1 dye which allows comparative measurements of membrane potential that determines mitochondrial depolarization occurring in the early stages of apoptosis. The dye exists in two forms, aggregates and monomers, each possessing different excitation and emission maxima. In intact membranes, the dye accumulates and aggregates while

with loss of membrane integrity, it exists as monomers rather than as aggregates, resulting in a change in aggregate to monomer fluorescence ratio. Higher ratio signifies high membrane potential and integrity.

Procedure: H9C2 cells were seeded overnight at 10,000 cells per well in 96 well, black walled plates. The next day, cells were pretreated for 1 h with GCE (25, 50 and 100 μg mL^{-1}) followed by Doxorubicin addition to a final concentration of 1 μM for 16 h. Cells were stained with JC-1 dye (Invitrogen Cat # T3168) using the manufacturer's protocol. In brief, cells were incubated for 30 min at 37°C with JC-1 (5 μg mL^{-1} in DMEM) and then washed with PBS twice. Fluorescence was measured using TECAN Infinite M1000 Pro at excitation/emission 535/590 nm for the aggregate form and 485/530 nm for the monomer form. CCCP (500 μM) (an uncoupling agent that diminishes mitochondrial membrane potential) was used to confirm that the JC-1 response was sensitive to changes in membrane potential. The ratio between fluorescence at the two emission wavelengths was designated as the JC-1 ratio.

Evaluation of apoptosis using caspase-Glo 3/7 activity: The ability of GCE to prevent stimulation of the executioner caspase activation in Dox treated cells was determined using the Caspase-Glo 3/7 Assay kit (Promega G8090).

Procedure: H9C2 cells were seeded in white opaque 384-well plates at a density of 2500 cells per well and incubated overnight. The next day they were pre-treated with GCE at appropriate concentrations (100 and 500 μg mL^{-1}) for 1 h, followed by the addition of Dox to a final concentration of 1 μM. After incubation for 16 h, Caspase 3/7 activity was measured according to manufacturer's instructions using the CaspaseGlo 3/7 kit. Briefly, cells were subjected to 1 h of incubation with the caspase substrate at 22°C. Luminescence was quantified using TECAN Infinite M1000 Pro. Luminescence values were plotted and compared.

Study duration and year of study: This study was conducted in the period 2013-2014, for a duration of approximately 1 year.

RESULTS

Effect on cell viability (MTT cell viability assay): Doxorubicin (Dox) at 1 μM caused a drastic decrease in H9C2 cell viability by 64% (Fig. 1) compared to untreated controls. Pre-treatment with GCE for 1 h resulted in consistent protection against Dox-induced cytotoxicity. This protection was robust, dose-dependent and statistically significant. Dox reduced cell viability to 34% and pretreatment with 50, 100 and 250 μg mL^{-1} GCE restored viability to 47, 53 and 84%, respectively.

DPPH radical scavenging activity: DPPH is a strong free radical and GCE showed a strong potent free radical scavenging activity in a dose-dependent manner (Fig. 2). Ten concentrations of GCE were tested, starting from 500 μg mL^{-1} and 2 fold serial dilutions from thereon. At the top three concentrations of 500, 250 and 125 μg mL^{-1}, 100% activity was achieved. The IC_{50} of GCE was determined to be 39 μg mL^{-1}.

Effect on mitochondrial membrane potential (ψm): Loss of mitochondrial membrane potential (Δψm) is a key step in the initiation of apoptosis. Δψm was measured using JC-1 dye which

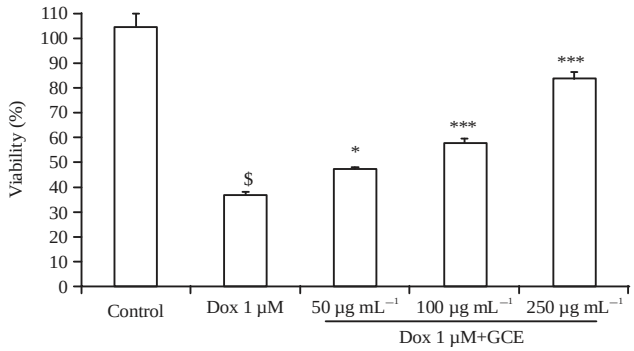

Fig. 1: GCE protects H9C2 cells from Dox-induced loss of cell viability in a dose dependent manner as measured by the MTT assay. Results analyzed by an Unpaired Student's t-test $ = $p<0.0001$, compared to vehicle control, ***$p<0.0001$ and *$p<0.05$, compared to Dox treatment at 1 µM

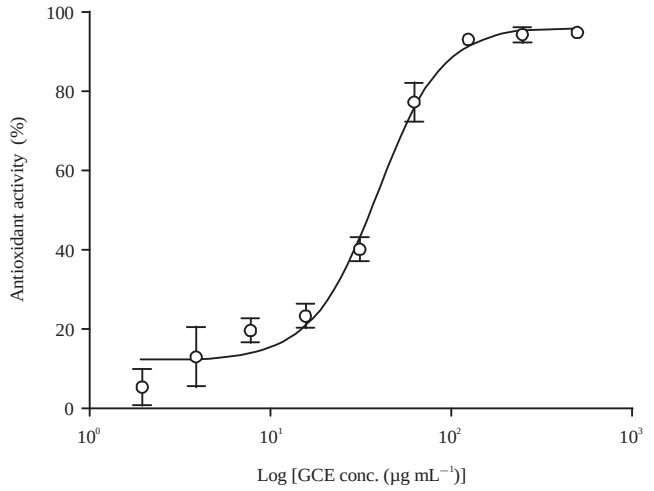

Fig. 2: GCE acts as a potent antioxidant as measured by the DPPH assay. All the 10 concentrations tested showed potent activity, with an IC_{50} value of 39 µg mL^{-1}

exists in an aggregated form (fluorescing red) in intact mitochondria or in a monomer form (fluorescing green) in cells with reduced mitochondrial transmembrane potential. A higher ratio between aggregate to monomer fluorescence (JC-1 ratio) indicates high Δψm. Dox at 1 µM caused a clear and statistically significant decrease in JC-1 ratio indicating loss of Δψm (Fig. 3). Pre-treatment with GCE restored Δψm and JC-1 ratio in a dose-dependent manner, clearly indicating the protective activity of GCE against Dox induced mitochondrial toxicity. As a positive control, CCCP was used. This compound dissipates the proton gradient across the mitochondrial membrane and causes a drastic loss of Δψm. JC-1 ratio in untreated control cells was 2.03, which decreased to 1.64 upon Dox treatment. Pre-treatment with GCE at 25, 50 and 100 µg mL^{-1} restored the JC-1 ratio to 1.62, 1.93 and 2.0, respectively. This increase was statistically significant compared to Dox treated cells at 50 and 100 µg mL^{-1} of GCE. CCCP decreased the JC-1 ratio to 0.6, confirming that the system was responsive to changes in mitochondrial membrane potential.

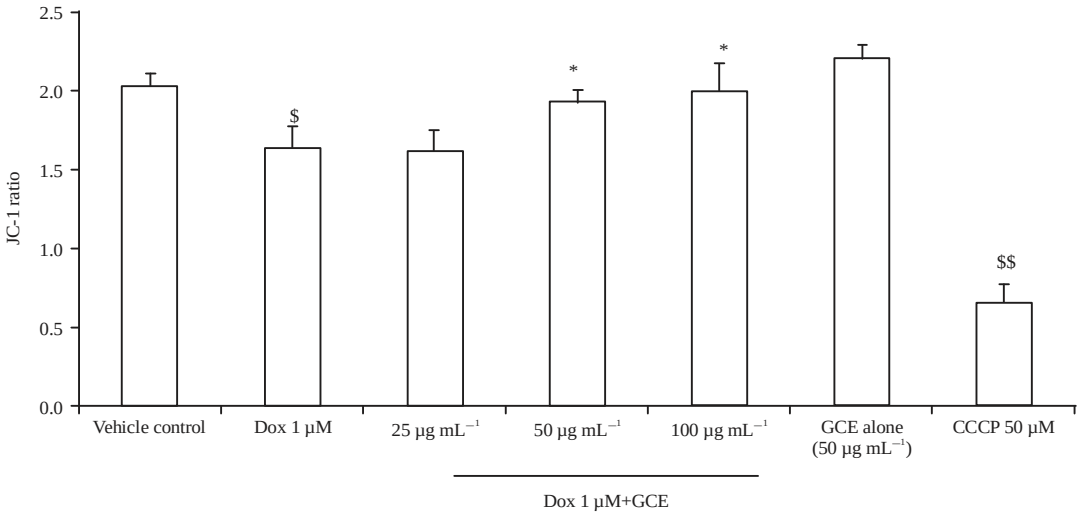

Fig. 3: GCE prevents loss of mitochondrial permeability induced by Doxorubicin in H9C2 cardiomyocytes. JC-1 dye was employed to measure mitochondrial membrane potential. CCCP was used as the reference control. Results analyzed by an Unpaired Student's t-test $ = $p<0.05$ and $$ = $p<0.01$, compared to vehicle control, *$p<0.05$, compared to Dox treatment at 1 M

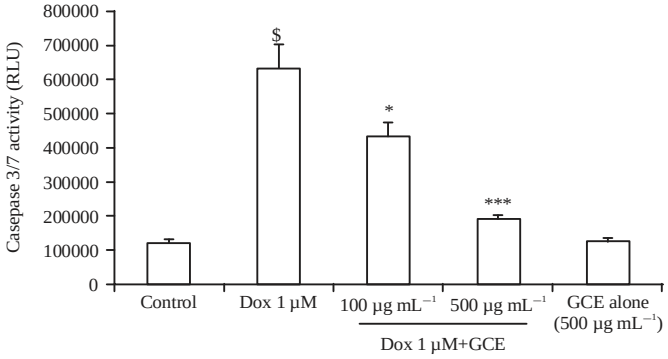

Fig. 4: Effect of GCE on Dox-induced caspase 3/7 activation in H9C2 cardiomyocytes. Results analyzed by an Unpaired Student's t-test $ = $p<0.0002$, compared to vehicle control, *$p<0.05$ and ***$p<0.0001$ compared to Dox treatment at 1 M

Effect on activity of executional caspases 3/7: Caspase-3 and 7 belong to the caspase family of proteases that play key roles in the apoptotic process. These enzymes are known as executioner caspases and are essentially the effector proteins for cellular apoptosis. Caspase-Glo 3/7 kit provides the DEVD peptide substrate for detection of enzyme activity with luminescence readout. Dox treatment for 18 h induced a high level of caspase 3/7 activity (Fig. 4). GCE potently prevented caspase 3/7 activation in a dose-dependent manner. Caspase 3/7 activity was induced 5 fold upon Dox treatment and this induction was attenuated in cells pre-treated with 100 and 500 µg mL^{-1} to 3.5 and 1.5 fold, respectively. The effect was dose-dependent and statistically significant, indicative of potent protective effects by GCE. GCE by itself did not change caspase activity as compared to untreated controls.

DISCUSSION

Drug hunters have always been in search of better and safer medicines and have often turned to natural plant products for answers. Coffee is one of the most commonly consumed beverages worldwide and the extract of the green coffee bean is sold as a popular health supplement. Doxorubicin, a potent chemotherapeutic drug is strongly cardiotoxic and cardiomyocyte death in patients treated with Dox leads to a variety of serious and often lethal heart conditions. Dexrazoxane, the only FDA approved drug to fight this serious side effect is nevertheless not proven to be completely effective (Vejpongsa and Yeh, 2014). There is a strong unmet medical need for safe drugs that can effectively combat cardiac insult by Dox. In the present study, it is demonstrated conclusively that a hydroalcoholic extract of green coffee bean confers protection of cardiomyocytes against Dox-induced apoptosis. H9C2 cells are widely used as a cardiomyocyte *in vitro* cell model. In this system, Dox potently caused cell death in as measured by MTT dye assay and GCE pretreatment was able to dose-dependently prevent this death. While toxicity induced by Dox involves multiple factors, the primary mechanismof cardiomyocyte cell death is widely believed to be due to Dox-induced generation of ROS (reactive oxygen species) and concomitant destructive effects of these free radicals (Singal *et al*., 2000). Dox undergoes redox cycling by dehydrogenases like the NADH dehydrogenase enzyme, which is part of the mitochondrial complex I. Since cardiomyocytes have an abundance of mitochondria, they are especially susceptible to Dox induced ROS generation (Govender *et al*., 2014). Several groups have demonstrated that treatment of cardiomyocytes with antioxidant compounds prevent ROS formation and toxicity induced by Dox (Chularojmontri *et al*., 2005; Lou *et al*., 2015; Quiles *et al*., 2002; Spallarossa *et al*., 2004). To understand if GCE is capable of behaving as an antioxidant to combat Dox induced ROS, its anti-oxidant capacity was quantified in the DPPH radical scavenging assay. DPPH is a strong free radical and GCE was able to show potent anti-oxidant activity with an IC_{50} value of 39 μg mL^{-1}, proving that GCE is capable of scavenging free radicals that might be generated by Dox.

It has been demonstrated that mitochondria are the gateways to cellular apoptosis. One of the key events in induction of apoptosis is the loss of mitochondrial membrane integrity. The intrinsic apoptotic pathway, stimulated by ROS causes a loss of mitochondrial membrane potential, leading to the opening of pores in the membrane and the leakage of cytochrome c into the cytoplasm. This starts a cascade leading to activation of executioner caspases like Caspase-3 and ultimately cellular apoptosis (Kalyanaraman *et al*., 2002). The loss of mitochondrial membrane potential gradient is recognized as the point of no return in the apoptotic pathway (Zamzami *et al*., 1995), underlining the importance of this event. One of the ways Doxorubicin is thought to mediate cardiac cell apoptosis is through the intrinsic pathway, initiated in the mitochondria. Hsu *et al*. (2014) have demonstrated that Dox causes a loss of $\Delta\psi m$ in H9C2 cardiomyocytes and restoration of $\Delta\psi m$ by treatment with polyunsaturated fatty acids prevents cell death induced by Dox. Treatment with Dox caused loss of $\Delta\psi m$ in the present study. JC-1 ratio was reduced by Dox, which is a measure of how much dye leaks out of the mitochondria due to pores in the membrane. The GCE treatment was able to attenuate this loss of mitochondrial membrane potential, demonstrating that GCE was working by inhibiting a very early step in the apoptotic cascade.

In addition to restoring cell viability and protecting mitochondrial integrity, GCE also potently inhibited Caspase-3 activation in H9C2 cardiomyocytes. The activation of this enzyme, downstream of loss of membrane potential is a required step in the apoptotic cascade mediated by Dox (Kalyanaraman *et al*., 2002). Preventing Caspase 3/7 activation is a strong indicator of

anti-apoptotic capacity. Silva *et al*. (2010), among many others have demonstrated that prevention of Caspase-3 activation protects H9C2 cells from apoptosis. GCE prevented Dox from activating Caspase-3/7 robustly and in a dose-dependent manner.

GCE prevented H9C2 cell loss of viability, loss of mitochondrial membrane potential and blocked caspase-3 and 7 activation by Dox. Taken together, these results point to strong cardioprotective capabilities of GCE. Recently, Mandziuk *et al*. (2015) have shown that purified chlorogenic acid protects H9C2 cells from morphological changes in response to Doxorubicin. They also demonstrated that ROS generated in H9C2 cells due to Dox treatment were reduced due to treatment with purified Chlorogenic acid. Chlorogenic acids form a large component of GCE (Svilaas *et al*., 2004) and it is a strong possibility that this component is responsible for some or all of the cardioprotective and anti-apoptotic effects seen here.

Green coffee has been demonstrated to have anti-inflammatory effects (De Castro Moreira *et al*., 2013), hypotensive effects (Suzuki *et al*., 2002), anti-obesity effects (Song *et al*., 2014) and neuroprotective effects (Jeong *et al*., 2013). As a result, it has been explored for therapeutic activity in a variety of disease models like diabetes, obesity, hypertension, etc., but the present study is the first to determine that this plant product is strongly cardioprotective against Dox induced insult. The results obtained here demonstrate that GCE protects cardiomyocytes from cell death and that the mechanism is by inhibition of the intrinsic pathway of apoptosis. Future studies involving identification of the main component of this extract that is responsible for the protective effects would be highly beneficial.

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