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Kenyan purple tea anthocyanins and coenzyme-Q_{10} ameliorate post treatment reactive encephalopathy associated with cerebral human African trypanosomiasis in murine model

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ABSTRACT

Human African trypanosomiasis (HAT) is a tropical disease caused by two subspecies of *Trypanosoma brucei*, the East African variant *T. b. rhodesiense* and the West African variant *T. b. gambiense*. Melarsoprol, an organic arsenical, is the only drug used to treat late stage *T. b. rhodesiense* infection. Unfortunately, this drug induces an extremely severe post treatment reactive encephalopathy (PTRE) in up to 10% of treated patients, half of whom die from this complication. A highly reproducible mouse model was adapted to assess the use of Kenyan purple tea anthocyanins and/or coenzyme-Q_{10} in blocking the occurrence of PTRE. Female Swiss white mice were inoculated intraperitoneally with approximately 10^4 trypanosome isolate *T. b. rhodesiense* KETRI 2537 and treated sub-curatively 21 days post infection with 5 mg/kg diminazene aceturate (DA) daily for 3 days to induce severe late CNS infection that closely mirrors PTRE in human subjects. Thereafter mice were monitored for relapse of parasitemia after which they were treated with melarsoprol at a dosage of 3.6 mg/kg body weight for 4 days and sacrificed 24 h post the last dosage to obtain brain samples. Brain sections from mice with PTRE that did not receive any antioxidant treatment showed a more marked presence of inflammatory cells, microglial activation and disruption of the brain parenchyma when compared to PTRE mice supplemented with either coenzyme-Q_{10}, purple tea anthocyanins or a combination of the two. The mice group that was treated with coenzyme-Q_{10} or purple tea anthocyanins had higher levels of GSH and aconitase-1 in the brain compared to untreated groups, implying a boost in brain antioxidant capacity. Overall, coenzyme-Q_{10} treatment produced more beneficial effects compared to anthocyanin treatment. These findings demonstrate that therapeutic intervention with coenzyme-Q_{10} and/or purple tea anthocyanins can be used in an experimental mouse model to ameliorate PTRE associated with cerebral HAT.

1. Introduction

Human African trypanosomiasis (HAT) or sleeping sickness is a tropical disease transmitted through the bites of infected tsetse flies of *Glossina* species and is a typical example of a neglected disease [1]. Treatment of HAT currently relies on a limited number of highly toxic drugs, but the disease is invariably fatal if untreated. Melarsoprol, a melaminophenyl based organic arsenical synthesized by complexing melarsen oxide with dimercaprol [2], remains the first line drug for treatment of late stage HAT caused by *Trypanosoma brucei rhodesiense* in spite of its extremely toxic side effects. Melarsoprol induces post treatment reactive encephalopathy (PTRE) in 10% of treated patients, resulting in death of up to 5% of all patients given the drug [3]. In modern medicine, this is quite unacceptable.

Murine models of experimental HAT have contributed immensely in understanding the neuropathogenesis of HAT and have also allowed studying the modulation of PTRE by different drug regimes [4]. Our study utilized mice infected with *T. b. rhodesiense* strain KETRI 2537 and treated sub-curatively with diminazene aceturate (berenil) to produce a marked exacerbation of the inflammatory response that closely mimics the fatal post treatment reaction seen in human patients [5,6]. Following the development of berenil-induced PTRE in an experimental mouse model, an intense inflammatory reaction occurs leading to the development of an acute meningoencephalitis [7]. The neuropathological features involved include infiltration of various cells in the brain including macrophages, lymphocytes, plasma cells and occasionally, morular cells. Perivascular cuffings and severe astrocyte and microglia

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activation are also common histopathological features associated with PTRE [8]. Moreover, a variety of inflammatory mediators are produced such as TNF-α, IL-1, IL-4, IL-6 and macrophage inflammatory protein (MIP)-1 [6].

There is strong evidence to suggest that the inflammatory response observed can be attributed to astrocytes which are brain cells with the capability of producing pro-inflammatory cytokines such as TNF-α and IL-1 when activated [6,9]. This phenomenon is validated by the fact that production of several cytokine transcripts within the CNS correlate with the onset of astrocyte activation [9,10]. However, other brain cells such as the microglia are also capable of producing inflammatory cytokines exacerbating the neuroinflammatory condition [11]. As a result, several anti-inflammatory agents have been evaluated with the aim of ameliorating the severity of PTRE complication. Hunter and colleagues [12] were able to establish that azathioprine, a potent anti-inflammatory agent and immunosuppressant significantly reduced the degree of CNS inflammation prior to the induction of PTRE but had no effect in an established PTRE. Kennedy and colleagues [13] also demonstrated that RP-67 580, an SP antagonist, significantly reduced the severity of an established meningoencephalitis as well as the degree of astrocyte activation in a PTRE mouse model. Steroidal anti-inflammatory drugs such as prednisolone have also been tested clinically and proved efficacious to some extent in ameliorating PTRE [14]. Furthermore, a recent study has been able to establish that inclusion complexes formed by complexing melarsoprol with anti-inflammatory oligosaccharide molecules, cyclodextrins can be used during late stage *Trypanosoma brucei brucei* infections to resolve CNS inflammation without any form of toxicity in female CD-1 mice [15].

Anthocyanins are a diverse group of naturally occurring polyphenol compounds conferring hues of blue, purple or deep red to several plants especially in leaves, flowers and fruits [16]. Human consumption of anthocyanins is increasing because of their potential health benefits including being powerful antioxidants [17] and having antimicrobial, anti-carcinogenic and anti-inflammatory properties [18,19]. On the other hand, coenzyme-Q_{10} (Co-Q_{10}), or ubiquinone, is an endogenously synthesized lipid and an essential component of the electron transport chain where it shuttles electrons from complexes I and II to complex III (ubiquinol cytochrome c oxidase) [20]. Co-Q_{10} has widely been implicated as having potent anti-inflammatory properties by inhibiting the expression of IL-6, TNF-α, and NF-κB [21]. In addition, Co-Q_{10} exerts its anti-inflammatory effects by gene expression modification reducing the activity of inflammatory markers [22]. This has seen the supplementation of clinical populations with Co-Q_{10} to modulate inflammatory conditions such as cancer and diabetes. These observations raised the possibility that oral administration of tea ACNs and/or Co-Q_{10}, compounds whose protective effects have widely been ascribed to their anti-inflammatory properties, may have positive effects in preventing and/or reducing the severity of the meningoencephalitis in PTRE complication.

Consistent with our earlier hypothesis, we report that Kenyan purple tea ACNs and/or Co-Q_{10} can be used to assuage CNS inflammation resulting in a significant reduction of clinical features associated with *T. b. rhodesiense* infection and PTRE complication in an experimental mouse model.

2. Materials and methods

2.1. Tea samples

Purple tea used to extract anthocyanins was obtained from the Tea Research Foundation of Kenya, Timbilil Estate in Kericho (latitude 0°22′S, longitude 35°21′E, altitude 2180 m a.m.s.l). Anthocyanins were extracted from the purple tea variety TRFK 306. Young tender shoots comprising two leaves plus a bud were harvested, dried using a microwave and pulverized with a grinder into fine powder.

2.1.1. Extraction and purification of anthocyanins

Extraction and purification of tea anthocyanins were carried out as described by Kerio and others [23]. Five grams of powdered leaves of purple *Camellia sinensis* were weighed into 250 mL conical flasks covered with foil to prevent photo degradation and mixed with 50 mL methanol/formic acid at a ratio of 99:1 volume/volume (v/v). The sample was magnetically stirred for 4 h at room temperature at a speed of 900 rpm. The resultant solution was filtered and methanol and formic acid were removed using a rotary evaporator (Buchi Rotavapour R-300, Switzerland) at 35 °C under vacuum, and the residue was reconstituted to 10 mL with distilled water. The extract was then passed through a membrane filter 0.45 μM and kept at 4 °C for analysis.

The tea extracts were passed through reverse phase (RP) C-18 solid phase extraction (SUPELCO, SPE) cartridges (Sigma-Aldrich, USA) previously activated with acidified methanol (10% HCl/methanol v/v). Anthocyanins were adsorbed into the column while sugars, acids and other water soluble compounds were washed out using 0.01% HCl in distilled water. The anthocyanins were recovered using acidified methanol (10% formic acid/methanol v/v). The cartridges were washed with ethyl acetate (Fischer Scientific) to remove phenolic compounds other than anthocyanins. The purified extracts were then stored at −10 °C until further analysis.

2.1.2. Lyophilization of anthocyanin extract

Prior to the lyophilization process, methanol and formic acid were removed using a rotary evaporator at 35 °C under vacuum and the residue was reconstituted with distilled water. Pre-freezing of the extract was done before it was placed on the drying accessory. A volume of 200 mL of the anthocyanin sample were placed in dehydration flasks and rapidly frozen by spinning the round bottom flasks in a dry ice–acetone bath. Temperature and pressure of the lyophilizer were allowed to reach appropriate levels of −40 °C and 100×10^{-3} M Bar, respectively before the freeze drying process was initiated. Lyophilization was done using a Modulyo freeze dryer (Edwards, England) producing a free flowing powder that was weighed and stored in airtight containers at room temperature until use.

2.1.3. HPLC analysis of anthocyanins

Qualitative and quantitative analyses of the tea extract and anthocyanin profiles of purple tea variety TRFK/306 were carried out in triplicate by high performance liquid chromatography (HPLC) as described by Guisti and Wrolstad [24] with modifications [23]. Briefly, 1 mL of the anthocyanin sample was pipetted into separate tubes and diluted to 2 mL with mobile phase A solution (87:3:10 water/acetonitrile/formic acid v/v/v) filtered and loaded into 2 mL vials. A Shimadzu LC 20 AT HPLC fitted with a SIL 20A autosample and a SPD-20 UV–visible detector with a class LC10 chromatography workstation with UV detection at 520 nm was used for analysis of the prepared samples. A Luna TM 5 μM, C18, 25 cm × 4.6 mm internal diameter (Phenomenex, Torrance, CA, USA) column fitted with a Rheodyne precolumn filter of 7335 model was used. Mobile phase solutions were filtered through a 0.45 μm nitrocellulose filter on a membrane filter disk and degassed before injection into the HPLC system.

Gradient elution was employed for analysis using the following solvent: The eluents were mobile phase A (water/acetonitrile/formic acid at a ratio of 87/3/10 v/v/v) and mobile phase B (100% HPLC grade acetonitrile). The flow rate of the mobile phase was set at 1 mL/min, column temperature at 35 ± 0.5 °C and injection volume at 20 μL. Chromatographic conditions were set as follows: 3% B in A at the time of injection, at 45 min 25% B in A, at 46 min 30% B in A and at 47 min 3% B in A. The conditions were set at 3% B for 10 min before the next injection to allow for equilibration.

Identification of individual anthocyanidins was carried out by comparing the retention times from sample chromatographs and absorbances of unknown peaks with the peaks obtained from the individual and mixed standards under similar conditions. The standards used