The antioxidant properties of a new concentrated fulvic acid product derived from a safe carbohydrate food source (CHD-FA^{new}) using cellular chemiluminescence and Trolox Equivalent Antioxidant Capacity (TEAC) assays.

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Introduction

Chronic excess of neutrophil derived reactive oxygen intermediates, such as hydrogen peroxide and superoxide radicals, have been implicated in the pathogenesis of various inflammatory conditions (Halliwell, 1994; Gafvert et al., 2000; Manzetti et al., 2001; Luokides et al., 2002). The cellular release of these reactive oxygen species can be measured by chemiluminescence *in vitro* using a sensitive chemiluminescence technique.

A new, concentrated fulvic acid product (CHD-FA^{new}) specially formulated for use in tablets or capsules has been formulated using the same carbohydrate derived fulvic acid product already tested for immune activity in this laboratory. In this study the effects of this concentrated fulvic acid on the lucigenin enhanced chemiluminescence of neutrophils either resting or stimulated by phorbol myristyl acetate (PMA) as well as on the oxygen scavenging properties thereof in a cell free system using the Trolox Equivalent Antioxidant Capacity (TEAC) assay were investigated.

Materials and methods

Measurement of the neutrophil generated reactive oxygen species

Neutrophils were isolated from heparinised venous blood obtained from normal healthy volunteers by density gradient centrifugation on Histopaque®-1077 (Sigma Diagnostics, St Louis, MO, U.S.A.) and suspended in phenol red free buffered Hanks balanced salt solution (HBSS) to a concentration of $1x10^7$cells/ml as described by Fernandes et al (2004).

Reactive oxidants generated by PMA-stimulated neutrophils were measured using lucigenin-enhanced chemiluminescence as previously described (Allen, 1986) with minor variations. A white 96 well luminescence plate was used for the assay. Briefly, neutrophils ($5x10^6$/ml), suspended in phenol red free HEPES buffered HBSS, were incubated at 4°C with lucigenin (0.5mg/ml) for 30min to load the cells with the luminescence enhancer. A homogenous aliquot of 40µl of this pre-loaded cell suspension was added to 120µl of HBSS in a white opaque 96 well luminescence plate. The plate was loaded into a LUMIstar OPTIMA chemiluminometer equipped with an automatic dispensing system and which was temperature controlled to 37˚C and incubated for 5 minutes. The plate was removed and 20µl of the relevant concentration of CHD-FA^{new} was added and the plate again incubated for a further 15 min at 37°C in the chemiluminometer.. The following final concentrations of CHD-FA^{new} were tested; 3, 6.3, 12.5, 25, 50 and 100µg/ml. Both negative and positive control wells had 20µl HBSS added instead of the fulvic acid product. All wells except the negative controls were then treated while shaking with 20µl of a freshly prepared solution of PMA (final concentration 25ng/ml). The negative controls were treated with 20µl HBSS. Reactive oxidant production was then monitored over a period of 800 sec as chemiluminescence in mV sec.

Measurement of the Trolox equivalent antioxidant capacity (TEAC)

Trolox is a synthetic water soluble vitamin E analogue which has been used as an antioxidant standard due to its strong antioxidant properties. The TEAC assay, commercialized by Randox Laboratories Ltd., is based on the decrease of the absorbance at 734 nm shown by the radical cation, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) but not the reduced parent compound. This reduction of the radical is due to antioxidants present in the test sample (Mariken et al, 2004). Briefly, 7 mmoles ABTS and 3 mmoles potassium persulfate ($K_2S_2O_8$) were dissolved in 500 ml water and allowed to stand in the dark at room temperature for 12–16 hrs to allow the highly coloured and stable ABTS radical cation ($ABTS^{\bullet+}$) to form. Required volumes of this stock solution was then diluted to give a final absorbance of 0.7 ± 0.02 A at 734 nm. The free radical scavenging properties of CHD-FA^{new} (at 3 µg/ml, 6.3µg/ml, 12.5µg/ml, 25µg/ml, 50µg/ml, and100µg/ml) was compared to a Trolox calibration curve generated with concentrations from 2 – 16 µM at a fixed time of 5 minutes. In this assay the total antioxidant capacity

represents the sum of the antioxidant capacity of all compounds capable of reducing the radical.

Results

Measurement of the neutrophil generated reactive oxygen species

CHD-FA^{new} , at all the concentrations tested, significantly decreased the lucigenin enhanced chemiluminescence response of neutrophils stimulated using PMA to that of the negative controls. The results obtained with the lowest concentration tested, i.e. 3µg/ml, is illustrated in Figure 1.

No significant luminescence was seen in the neutrophil response of CHD-FA^{new} treated but not PMA stimulated cells.

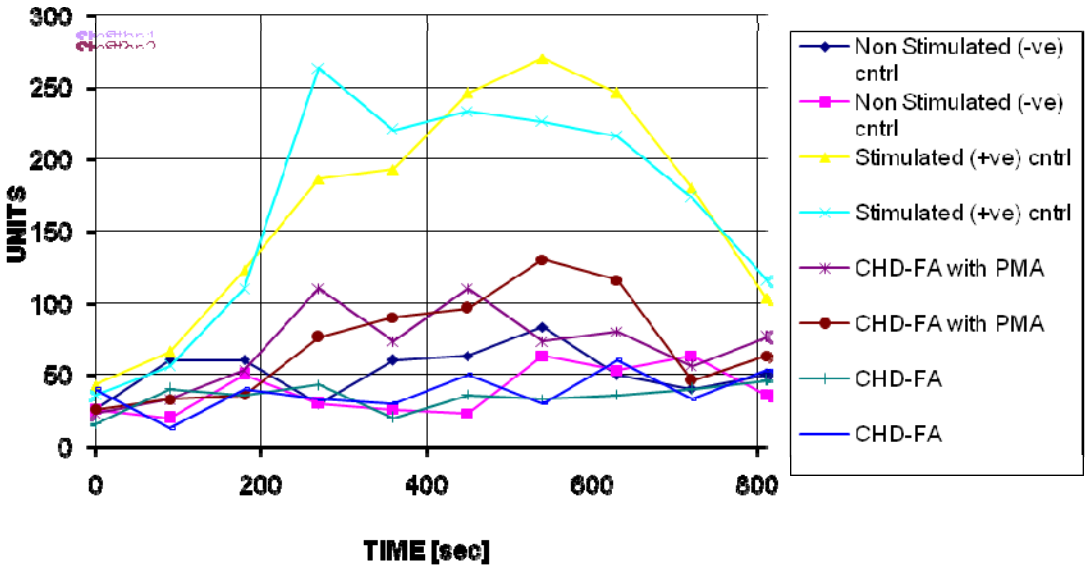

Figure 1. Inhibitory effects of CHD-FA^{new} , at 3 µg/ml, on lucigenin enhanced chemiluminescence response mediated either by resting or PMA-stimulated human neutrophils. Results are expressed as Units (mV). Results are for the average of duplicate wells of each treatment.

Measurement of the Trolox equivalent antioxidant capacity (TEAC)

According to the results obtained with the TEAC assay it was found that the highest concentration of CHD-FA^{new} used in this study which was 500 µg/ml, resulted in antioxidant activity which was comparable to Trolox at 1mg/ml. At the lowest concentrations (125 µg/ml and below) there was a possibility of radical generation instead of elimination as CHD-FA^{new} yielded an absorbance value higher than the starting solution. This implies that fulvic acid could be pro-oxidative for neutrophils at very low concentrations but becomes anti-oxidative at higher concentrations.

Discussion

According to the results obtained it was found that CHD-FA^{new} had strong antioxidant activity which was detectable at concentrations as of 500mg/ml, when using lucigenin enhanced chemiluminescence of stimulated neutrophils. Scavenging of the, 2,2-azinobis(3-ethylbenzothiazoline 6-sulfonate) radical cation ($ABTS^{\bullet+}$) using the TEAC assay confirmed that the antioxidant activity was due to chemical activity and not to the inhibition of the membrane associated NADPH oxidase system of neutrophils.

The results obtained with the TEAC assay indicate that the inhibition observed in the chemiluminescence assay by the product is possibly due to the scavenging of free radicals rather than a direct effect on the neutrophil NADPH system.

In conclusion, it has been shown that CHD-FA^{new} possesses some antioxidant properties *in vitro*. This property suggests that this product may be an effective immunomodulator for the treatment of diseases associated with an overproduction of reactive oxidants by human phagocytes. This however needs to be confirmed in *in vivo* systems.

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