Original article

Novel pharmaceutical rationale against human lymphatic filarial parasite: An oxidative premise

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Abstract

Objective: Mandate from WHO has boosted up anti-filarial drug research. Diethylcarbamazine citrate (DEC) was not known for any direct effect on filarial parasites. However, recent report proposed its direct apoptotic effect. Oxidative stress has been implicated in apoptotic impact. A study was designed to explore the possibility of oxidative rationale to be operative in the direct anti-filarial effect of DEC. **Methods**: Various doses of DEC and potent oxidant H_2O_2 alone were used *in vitro* to check for the effects on *B. malayi* microfilariae, followed by the use of DEC in combination with H_2O_2. Reversal of the oxidative impact of the drug was tested using the antioxidant, vitamin C and also lipid peroxidation levels in the post incubation culture supernatants were assayed. **Result**: As expected, DEC alone failed to record any anti-filarial effect. H_2O_2 alone also failed to show any significant effect until a very high dose was used. However, in combination significant anti-filarial effect was noticed, which allowed even 44% reduction in the dose of H_2O_2. Any significant lipid peroxidation was not found. Vitamin C demonstrated 30 % inhibitory effect. **Conclusion**: DEC and H_2O_2 combination being able to educe synergistic anti-filarial effect and inhibition of the same by vitamin C hinted towards covert oxidative component in the mechanism of DEC. Further implication of non-significant lipid peroxidation was addressed in the perspective of subtle oxidative nexus that seems to be operative in observed anti-filarial effect. Exploration of such rationale might lead to novel drug development.

Keywords: DEC; H_2O_2; Vitamin C; Oxidative stress; Anti-filarial drug

INTRODUCTION

Human lymphatic filariasis is recognized by the World health organization (WHO) as one of the six major infectious diseases. This disease is not life threatening but remains persistent research target because of limitations in mass drug administration with popular medicine, Diethylcarbamazine (DEC). Predictably WHO has emphasized for novel drug designing in this area [http://www.who.int/tdr/svc/research/drug-development-helminths-ntds]. DEC is quite prompt in clearance of microfilariae of *W. bancrofti* and *B. malayi* from the blood of human hosts. However there are reports of serious side effects[1] and more embarrassingly the mechanism through which it acts still remains unexplained. DEC failed to show any significant action on microfilariae in cell free system rather it is known to be active *in vivo*. The most accepted rationale of DEC involves the macrophage mediated response. Common motif of our body defense mechanism also utilizes recruitment of various immunocytes to induce oxidative burst on

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the invading microbes as inflammatory response. Hence it appears that DEC harnesses similar approach by promoting the innate response. Recently interesting reports suggested that DEC might incorporate apoptotic changes *in vitro* on filarial parasites[2,3]. Strikingly oxidative stress is an important triggering mechanism for apoptotic signal[4]. Thus, it seems possible that direct effect of DEC on the parasites might exploit certain oxidative modality. It can be envisaged that studying this aspect might yield a hypothetical pharmacological rationale involving targeted oxidative assault for novel drug designing. Hence, the present study was designed to explore the *in vitro* impact of DEC on filarial parasites and possibility of oxidative component, if any in such direct anti-filarial effect.

MATERIALS AND METHODS

Collection of *B. malayi* microfilariae

Microfilariae (mf) were obtained by the lavage of the peritoneal cavities of jirds with intraperitoneal filarial infection of 3 months or more duration. The mf were collected and washed with RPMI 1 640 medium and used for *in vitro* experiments[5]. The use of animals for the study was approved by Institute animal ethical committee which follows CPCSEA norms.

In vitro screening for anti-filarial activity of DEC/ H_2O_2

Approximately 100 mf were incubated in a total volume of 1 ml of RPMI 1640 culture medium (with various nutrients and antibiotic supplements; but without any added sera supplement) in sterile 24 well culture plates (Nunc, Denmark) as used in our lab earlier[6]. Previous study reported the use of DEC against microfilariae *in vitro* (up to a dose range of 50μg/mL)[2]. In this study we tried a wide dose range of Diethylcarbamazine citrate (DEC, Sigma) till the possible maximum effect was achieved. Hydrogen peroxide (H_2O_2) as potent oxidant was used in earlier study[7] with maximum dose of 50μM for microfilariae without any notable effect; hence we used wide dose range till significant effect was obtained. Microfilariae in RPMI medium alone was used as suitable control for the study. The plates were incubated at 37℃ and 5% CO_2 incubator for 4 hr as per previously standardized protocol. After 4 hr of exposure, the number of live and dead mf in each well were counted by microscopic examination (using Nikon Inverted microscope, Diaphot- TMD) and the loss of motility was calculated as number of immotile parasites out of the total parasites in percentage form. The entire procedure was repeated thrice for the reproducibility of the results.

In vitro screening for anti-filarial activity of combination with DEC and H_2O_2

As per the results obtained in the screening with DEC alone, the maximal effective dose of DEC (300 μg/mL) was used to combine with various concentrations of H_2O_2 over the dose range used in this study to evaluate their combined effect. The plates were incubated at 37℃ in 5% CO_2 incubator for 4 hr. The observations were made as described previously.

In vitro anti oxidant effect of Vitamin C against combination of DEC and H_2O_2

Variable concentrations of vitamin C as anti-oxidant were used to evaluate the regression effect on the maximal effective dose of DEC and H_2O_2 combination on the microfilariae. After obtaining the maximum reversal effect, dose of vitamin C was optimized at 20 μg/mL. Further experimentation was carried out by pre-incubating the microfilariae (for 30 minutes) with this concentration of vitamin C against DEC and H_2O_2 combination along with suitable controls (microfilariae in RPMI medium with and without vitamin C).

Estimation of Malondialdehyde (MDA) by TCA- TBA method

Culture supernatants of the DEC and H_2O_2 combination treated microfilariae were collected by centrifuging at 2 500 rpm for 10 minutes to estimate malondialdehyde level as a marker for lipid peroxidation. To each ml of culture supernatants and also the MDA standards, 2.5 ml of 20 % TCA and 1 mL of TBA were added and mixed thoroughly by using vortex. Then the mixture was boiled in hot water bath for 30

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min. After cooling in cold water bath, the resultant chromogen was extracted with 4 mL of n- butyl alcohol and separation of organic phase was done by centrifugation at 3 000 rpm for 10 min.

Absorbance of the butyl alcohol extracts of standards and samples were measured at 530 nm against n-butyl alcohol as blank. The standard curve was plotted and concentration of total MDA in samples were calculated and expressed as nM/mL malondialdehyde from the standard graph[8].

Statistical analysis:

Mean and Standard error of mean were calculated from the values of triplicate observations. Student's *t*-test was used for the statistical comparison of means of the sample values against respective controls.

RESULTS

Effect of DEC on motility of mf *in vitro*

Microfilariae of *B. malayi* incubated with different concentrations of DEC were examined microscopically after 4 hours and the mean percentage of loss of motility and standard error of mean was calculated from three observations. After studying over the wide dose range (5-500μg/mL) we found that maximum loss of motility was found to be 13 % at a concentration of 300μg/mL. Further increase in the concentration had no significant additional effect. For lower concentrations, the loss of motility was negligible.

Effect of H_2O_2 on motility of mf *in vitro*

Different concentrations of H_2O_2 starting from 50 μM were used to evaluate the effect of H_2O_2 against *B. malayi* mf *in vitro*. Even up to a very high dose of 1 mM loss of motility of parasites was negligible. At a further higher dose of 1.25 mM sudden 100% loss of motility of all parasites was recorded.

Effect of DEC and H_2O_2 combination on motility of mf *in vitro*

The evidences presented in Table 1 shows linear increase in activity of this combination as a function of H_2O_2 concentration starting from 0.4 mM below which the response was very meager. The doses of H_2O_2 and DEC in combination, required to obtain complete loss of motility of all parasites was found to be 0.7 mM H_2O_2 (compared to 1.25 mM with H_2O_2 alone) with the fixed dose of 300 μg/mL of DEC. Thus an overall 44% decrease in the requirement of this potent oxidant to achieve comparable complete loss of motility, emphasizes on the synergistic effect of DEC and H_2O_2 on mf motility *in vitro*.

Table 1 Anti-filarial effect (% Loss of motility) of either DEC/H_2O_2 alone or in combination and also DEC and H_2O_2 combination with vitamin C along with suitable control (RPMI).

Concentration of agents	% Loss of motility (Mean ± SEM)
DEC (300 μg/mL)	13.00 ± 0.47 *
H_2O_2 (1.25 mM)	99.50 ± 0.57 *
DEC (300 μg/mL) + H_2O_2 (0.4mM)	13.50 ± 0.33 *
DEC (300 μg/mL) + H_2O_2 (0.5mM)	16.00 ± 0.16 *
DEC (300 μg/mL) + H_2O_2 (0.6mM)	33.00 ± 0.33 *
DEC (300 μg/mL) + H_2O_2 (0.7mM)	99.00 ± 0.67 *
DEC (300 μg/mL) + H_2O_2 (0.7mM) + vitamin C (20 μg/mL)	70.00 ± 0.63 *
RPMI (Control)	3.00 ± 0.23

* $P < 0.05$; significant as compared to control

Effect of vitamin C on the *in vitro* response to DEC and /or H_2O_2

The optimum dose of vitamin C in this experimental setup was found to be 20 μg/mL. Further increase in the concentration was not able to reverse the effect any more. Vitamin C (20 μg/mL) showed 30% reduction in the highest level of loss of motility caused by DEC + H_2O_2 combination (Table 1). At this dose however, no toxicity was found to be induced upon the microfilariae due to vitamin C alone.

Estimation of Lipid peroxidation in the culture supernatants

When checked for lipid peroxidation in the parasite culture supernatants treated with DEC + H_2O_2 combination (the maximum effective dose), it was found that there was no significant change in the MDA levels between the test and control groups (mean ± SEM for DEC alone: 13.93 ± 2.786 nM/mL, H_2O_2: 10.30 ± 1.545 nM/mL, DEC + H_2O_2: 16.44 ± 2.794 nM/mL, Control (RPMI): 10.00 ± 1.734 nM/mL respectively).

DISCUSSION

This study was designed to detect the presumptive role of oxidative stress associated with DEC activity. In accordance with the earlier reports, DEC alone failed to record any anti-filarial effect *in vitro*. However, when it was complemented with H_2O_2 it was found to achieve complete loss of motility. The potent oxidant H_2O_2 when used alone was strikingly devoid of any activity and finally at an exceptionally high level showed complete loss of motility of the parasites. This unusually high dose requirement to obtain loss of motility of parasites in the *in vitro* cell free setup with this potent oxidant reflected relative resistance of the filarial parasites towards oxidative attack and supported the earlier work which validated such special attribute of filarial parasites[9]. Interestingly, the combination of H_2O_2 with otherwise unproductive DEC not only achieved complete loss of motility but was also found to bring definitive reduction (44%) in the dose of H_2O_2 required against the high dose required to achieve comparable effect when used alone. This experimental evidence does not in itself warrant any claim to use such combination for therapy. However, the observed synergism of DEC with a potent oxidant is definitely a proof of principle that hinted towards involvement of probable oxidative means behind the observed anti-filarial activity.

Thus, to validate this oxidative hypothesis further, vitamin C was used as an anti oxidant in the same experimental setup after optimizing for the concentration to study the regression of the anti-filarial effect reproducibly. It was found to bring forth a maximum reduction of 30 % from the observed anti-filarial effect of DEC and H_2O_2 combination, supporting such oxidative rationale.

In search of the direct mechanistic effect of oxidative impact, the culture supernatants obtained from the filarial parasites incubated with this combination were tested for the oxidative damage in terms of lipid peroxidation. The result was not found to be significantly different with these agents against suitable control. However, this was in concurrence with the previous findings which suggest that the cuticular membrane lipids of *Brugia malayi* lack susceptibility to oxidative stress generated by the oxidant attack *in vitro*[9]. This resistance is mainly attributed to less number of unsaturated fatty acyl residues and the presence of lipid soluble anti oxidants in the neutral lipid fraction of the parasites[7, 10].

In this context it may be relevant to consider that oxidative stress apart from overt macromolecular damage may trigger apoptotic response more subtly[11]. H_2O_2 is well known to induce intracellular response leading to programmed death[12]. Moreover, as already mentioned recent evidence showed that DEC may exert damage to the sheath of filarial parasite *in vitro* and even may induce apoptotic changes[2, 3]. However these studies deployed either electron transmission microscopy or LM-PCR and TUNEL method respectively for detection of apoptosis but relatively less sophisticated method like DNA ladder experiment failed to record any such evidence. These works were able to demonstrate the impact of the drug only by means of superior technical modality; in our experimental condition we were unable to detect any substantial effect of DEC alone

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through direct microscopy. However, when augmented with the potent agent H_2O_2 in combination, the marked synergistic effect was demonstrated revealing the tale-tell evidence.

Since, there is a close association between oxidative stress and apoptosis[13], it might be surmised that an oxidative nexus is probably underlying direct apoptotic effect of DEC observed earlier[2]. As a corollary to that it is quite tempting to contemplate further for the possibility of similar oxidative stress associated apoptotic motif to be operative in the observed synergism between DEC and H_2O_2 in the present study. However, it is subjected to be proven by direct evidence of apoptosis. Work on this aspect is already in progress in our laboratory.

Importance of apoptosis in host parasite relationship is not itself a unique concept and was also highlighted for exploiting the same in future drug designing research[14]. However, it is yet to be explored in anti-filarial therapeutics. The present study underscores that in depth work towards novel anti-filarial drug development involving potential apoptotic impact associated with targeted oxidative stress may be rewarding.

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REFERENCES

1 **Ghosh M**, Sinha babu SP, Sukul NC. Anti-filarial effect of two triterpenoid saponins islolated from *Acacia auriculiformis*. *Indian J Exp Biol*. 1993; 31: 604-606.

2 **Florencio MS**, Peixoto C. A. The effects of diethylcarbamazine on the ultrastructure of microfilariae of *Wuchereria bancrofti*. *Parasitol*. 2003; 26: 551-554.

3 **Peixoto CA**, Santos ACO, Ayres CFJ. Molecular evidence for apoptosis in microfilariae of *Wuchereria bancrofti* induced by diethylcarbamazine. *Parasitol Res*. 2008; 103: 717- 721.

4 **Buttke TM**, Sandstrom PA. Oxidative stress as a mediator of apoptosis. *Immunol Today* . 1994; 1 :7-10.

5 **Bhandary YP**, Krithika KN, Kulkarni S, Reddy MVR, Harinath BC. Detection of enzyme dehydrogenases and proteases in *Brugia malayi filarial* parasites. *Indian J Parasitic Diseases*. 2006; 21: 1-7.

6 **Sahare KN**, Anandhraman V, Meshram VG, Meshram SU, Reddy MVR, Tumane PM, et al. *Anti-microfilarial* activity of methanolic extract of *Vitex negundo* and *Aegle marmelos* and their phytochemical analysis. *Indian J Exp Biol*. 2008; 46 :128-131.

7 **Ou X**, Thomas GR, Chacone MR, Tang L, Selkirk ME. *Brugia malayi*: differential susceptibility to and metabolism of hydrogen peroxide in adults and microfilariae. *Exp Parasitol*. 1995; 80: 530-540.

8 **Satoh K**. Serum lipid peroxide in cerebrovascular disorders determined by a new calorimetric method. *Clin Chem Acta*. 1978; 90: 37-43.

9 **Selkirk ME**, Smith VP, Thomas GR, Gounaris K. Resistance of filarial nematode parasites to oxidative stress. *Int J Parasitol*. 1998; 28: 1315-1332.

10 **Smith VP**, Selkirk ME, Gounaris K. *Brugia malayi*: Resistance of cuticular lipids to oxidant induced damage and detection of α-Tocopherol in the neutral Lipid Fraction. *Exp Parasitol*. 1998; 88: 103- 110.

11 **Clutton S**. The importance of oxidative stress in apoptosis. *Brit Med Bull*. 1997; 53: 662-668.

12 **Masanuri K**, Yoshihisa I. Oxidant induced apoptosis of glomular cells: intracellular signaling and its intervention by bioflavonoids. *Kidney Int*. 1999; 56: 1223-1229.

13 **Haddad JJ**. Redox and oxidant-mediated regulation of apoptosis signaling pathways: immuno-pharmaco-redox conception of oxidative siege versus cell death commitment. *Int Immunopharmacol*. 2004; 4 :475-93.

14 **Barkinski MA**, Dosreis G. Apoptosis in parasites and parasite induced apoptosis in the host immune system: a new approach to parasitic diseases. *Braz J Med Biol Res*. 1999; 32: 395-401.