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A prospective strategy to restore the tissue damage in malaria infection: Approach with chitosan-trypolyphosphate conjugated nanochloroquine in Swiss mice

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ABSTRACT

Accumulating evidence indicates that wide range of polymer based nanoconjugated drug have the ability to overcome the microbial infection. The present study was to evaluate the effects of nanoconjugated chloroquine (Nch) against *Plasmodium berghei* NK65 (*P. berghei*) infection on selective makers of oxidative damage, antioxidant status, pro-inflammatory and anti-inflammatory cytokines in liver and spleen. *P. berghei* infected Swiss mice were treated with Nch (250 mg/kg bw for 15 days) compared with chloroquine. The stress markers, pro-inflammatory cytokines were increased significantly ($P<0.05$) and the anti-oxidant enzymes level, redox ratio (GSH/GSSG), anti-inflammatory markers were decreased significantly ($P<0.05$) in liver and spleen of infected mice compared with uninfected mice. Chloroquine and Nch effectively decreased the stress markers, pro-inflammatory cytokines, as well as, increased antioxidants level in liver and spleen of the infected mice. Moreover, the favorable effect Nch is better than the chloroquine defending the tissue damage during malarial infection. These findings suggested that the potential use and prospective role of Nch than only chloroquine against *P. berghei* induced pathology as well as oxidative damage in liver and spleen.

1. Introduction

Malaria is the most devastating infectious parasitic disease, still imposes a heavy burden inflicting both death and economic losses on at least half the world's population. Numerous attempts have been made to control the disease by using vector control measures and/or chemoprophylaxis, but they have had limited success (Trigg and Kondrachine, 1998; Martins et al., 2013). Parasite virulence factors associated with severe malaria are multiplication capacity, red cell preference, and ability to induce cytokines, adhesivity, antigenicity and anti-malarial drug resistance (Sherman, 1999). After a short period of development and multiplication, these parasites leave the liver and invade erythrocytes where they multiply by schizogony, undergoing development through ring, trophozoite and schizont stages that are responsible for malaria pathogenesis (Srivastava et al., 1991).

The multiplication of the parasite in the blood causes respiratory distress anemia (Martins et al., 2013) and damages of essential organs of host. The spleen is believed to participate in both the clearing of parasites from the circulation as well as providing a strong hematopoietic response during acute infection. The rapid and characteristic enlargement of the spleen has been postulated to be associated with an increased capacity to remove parasitized erythrocytes from circulation (Sherman, 1999; Shah et al., 2003). Like other microbes, *Plasmodium berghei* (*P. berghei*) is also a causative agent to change the normal condition of host by utilizing or protecting defense factors. It is generally accepted that ROS, nitric oxide (NO) and peroxynitrite kill intra-erythrocytic malarial parasites (Brunet, 2001; Stevenson and Riley, 2004) but parasite protect itself by own antioxidant enzyme system.

Moreover, treatment of malaria is becoming difficult due to emergence of resistance against the commonly used drug like

Abbreviations: BW, body weight; CAT, catalase; DTNB, 5′, 5′-dithio (bis)-2-nitrobenzoic acid; EDTA, ethylene diamine tetra acetate; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-tranferase; IF, infection; MDA, malondialdehyde; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; Nch, nanochloroquine; NO, nitric oxide; OPD, *O*-diamisidine; FSC, forward scatter; PBS, phosphate buffer saline; SOD, super oxide dismutase; TBA, thiobutiric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloro acetic acid

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chloroquine, pyremethamine, artesunate etc. Now, World Health Organization has recommended artemisinin for uncomplicated malaria treatment in combination with other anti-malarials as artemisinin combination therapies (ACTs). If resistance emerges against ACTs, then result will be catastrophic (Bhattacharyya et al., 2014). Furthermore, artemisinins are known for their very short half-life (Lindegardh et al., 2008; Thanh et al., 2009). Considering the findings above, there is an urgent urge to develop efficacious formulations able for malaria treatment and also to limit the progression of drug resistance.

Nanomedicine has potential to restore the use of old and toxic drugs by modifying its bio-distribution, improve bioavailability and reducing toxicity (Santos-Magalhães and Mosqueira, 2010). Chitosan is deacetylated form of chitin, an aminopolysaccharide found in exoskeletons and fungal cell wall of various arthropods including insects, crabs and shrimp (Omara et al., 2012). This natural linear biopolyaminosaccharide was used as a nanodrug carrier for development a potent anti-malarial compound with conjugating the different age-old drug such as pyrimethamine (Ibezim et al., 2011) and chloroquine (Tripathy et al., 2012b). Chitosan, recognized as biocompatible, have no toxic effect on liver, the most detoxifying organ. It is also reported that chitosan nanoparticles inflicted extensive damage to the cell morphology (Kean and Thanou, 2010).

Previously we reported that successive *P. berghei* infection develops in experimental Swiss mice after ten days (Tripathy et al., 2012a) and Nch treatment by 250 mg/kg-bw for 15 days eliminates the parasite and restore the cellular functions of lymphocytes (Tripathy et al., 2012b). In this study we evaluate the protective efficacy of Nch comparing the only CQ against oxidative damage and antioxidant defense system during parasite infection in the liver and spleen.

2. Material and methods

2.1. Chemicals and reagents

Tris buffer, Sodium chloride (NaCl), Triton-X 100, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), ethylene diamine tetra acetate (EDTA), sodium hydroxide (NaOH), chloroform, sodium acetate, ammonium acetate, potassium hydroxide (KOH), methanol, Giemsa, Tris–HCl, formaldehyde, alcohol, diphenylamine (DPA) were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. 5′,5′-dithio (bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), glutathione reductase (GR), NADPH, Na_4, NADPH, oxidized glutathione (GSSG) were procured from Sigma (St. Louis, MO, USA). Commercially available Histopaque 1077 was purchased from Sigma Chemical Co., USA. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest purity grade available.

2.2. Animals, inoculums preparation and parasite density determination

Swiss male mice (6–8 weeks old, weight 20–25 g) were used to full fill the experiments. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Vidyasagar University. The animals were fed standard pellet diet with vitamins, antibiotic and water was given ad libitum and housed in polypropylene cage (Tarson) in the departmental animal house with 12 h light and dark cycle under standard temperature (25 ± 2 °C). The animals used in this study did not show any sign of malignancy or other pathological processes.

P. berghei NK65 was used in the study and collected from Department of Biotechnology, Delhi University, South campus, Delhi, India and maintained into sex and age-matched wild type mice by weekly passage and blood stage parasites were stored at −80 °C. Parasite infection was developed in Swiss male mice by intraperitoneal (i.p.) injection of 200 μl of infected blood containing 1×10^5 parasites according to our previous laboratory report (Tripathy et al., 2012a).

2.3. Preparation and characterization of nanochloroquine

Nanochloroquine was prepared by ionotropic gelation and characterized by FTIR, DLS and zeta potential according to our previous laboratory report (Tripathy et al., 2012b).

2.4. Separation of red blood cell (RBC) and isolation of parasitic DNA

The infected blood samples were collected in a vacutainer (BD falcon) coated with an anticoagulant (EDTA) and washed in folate and *p*-amino benzoic acid-free RPMI 1640 medium for several times; followed by centrifugation at $2000 \times g$ for 10 min at 4 °C, an aliquot of 1.5–2 ml of the red blood cell pellet was obtained (Basco and Ringwald, 2000).

Erythrocytes were suspended in 15 ml of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and lysed with 0.015% Saponin (Sigma). The lysate was centrifuged at $2000 \times g$ for 10 min at 4 °C and the pellet was transferred to a 1.5-ml micro centrifuge tube and suspended in 500 μl of NET buffer. The mixture was treated with 1% *N*-lauroylsarcosine (Sigma) and RNAse A (100 μg/ml) at 37 °C for 1 h and proteinase K (200 μg/ml) at 50 °C for 1 h. DNA was extracted three times in equilibrated phenol (pH 8), phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1) and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at −20 °C until use (Basco and Ringwald, 2000).

2.5. Polymerase chain reaction of Pbcrt gene

The regions of the *Pbcrt* gene were amplified by the polymerase chain reaction using the Eppendorf thermal cycler under the following conditions: approximately 200 ng of genomic DNA, 15 pmol of primers (*Pbcrt*-F: 5′CGAAGTGACGAGCAACAAAA3′ and *Pbcrt*-R: 5′ATCGAGTCTGCATTGGTCAG3′), reaction buffer (10 mM Tris, 50 mM KCl, pH 8.3), 2.5 mM $MgCl_2$, 250 μM dNTP, and 1 unit of Taq DNA polymerase in a 25 μl reaction mixture at 95 °C for 5 min for the first cycle and 30 s in subsequent cycles, 50 °C for 30 s in all cycles, and 72 °C for 1 min in all cycles, for a total of 40 cycles. 5 μl of the amplification product then mixed with 1 μl of bromophenol blue and loaded on a 2% agarose gel; subjected to electrophoresis and stained with ethidium bromide, and visualized under ultraviolet transillumination to confirm the presence of the particular amplicon according to base pair size (Basco and Ringwald, 2000; Ghobakhloo et al., 2008).

2.6. Experimental design

After infection development the work design was done in different groups. Group ‘A’: control, Group ‘B’: Infected control group; Group ‘C’: infection + CQ; Group ‘D’: infection + Nch. Here, CQ was charged intra-peritoneal by the actual drug content, 68.4 mg/kg bw (27.36%) in the effective dose of nanochloroquine (250 mg/kg bw) for 15 days (Tripathy et al., 2012b). After the