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Transdermal delivery of oleanolic acid attenuates pro-inflammatory cytokine release and ameliorates anaemia in *P. berghei* malaria

Happiness Sibiya*, Cephas T. Musabayane[1], Musa V. Mabandla

School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa

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ABSTRACT

Malaria remains a major health problem in many tropical areas. Severe malaria infection is associated with secondary complications including anaemia leading to a need for the search of affordable antimalarial agents that can clear the parasitaemia and ameliorate anaemia during infection. The current study investigated the effects of transdermally delivered OA on malaria parasites, HCT and selected plasma cytokine concentrations in *P. berghei*-infected male Sprague-Dawley rats. The study was carried out over a period of 21 days, divided into pre-treatment (day 0–7), treatment (day 8–12) and post-treatment (day 13–21) periods. Parasitaemia, HCT, RBC count, Hgb, plasma TNF-α, IL-6 and IL-10 concentrations were monitored in non-infected and infected rats following a once-off application of an OA-pectin patch (34 mg/kg). Animals treated with drug-free pectin and CHQ (30 mg/kg, p.o) twice daily for 5 consecutive days acted as negative and positive controls respectively. Infected control animals exhibited increased percentage parasitaemia, TNF-α, IL-6, IL-10 and a reduction in HCT. Interestingly, OA-pectin patch application cleared the malaria parasites and increased HCT values back to normalcy. Furthermore, TNF-α, IL-6 and IL-10 were reduced by day 12 of the study. These findings suggest that the OA-pectin patch delivers therapeutic doses of OA which are able to attenuate cytokine release and ameliorate anaemia during malaria infection. Therefore, transdermally delivered OA may be a potent therapeutic agent for malaria and amelioration of anaemia.

1. Introduction

Severe malarial anaemia (SMA) is a major cause of mortality in children and pregnant women, living in malarial endemic areas (Ekvall, 2003). The pathophysiology of is complex and multifactorial. The increased destruction of RBCs by the *plasmodium* parasites is thought to be one of the factors that are involved in severe malaria anaemia (Price et al., 2001). During acute malaria infection, there is increased proliferation and hyperactivity of macrophages in the reticuloendothelial system (RES) (Davis et al., 1990; Phillips and Pasvol, 1992). The deposition of *P. falciparum* ring surface protein 2 (RSP-2), a protein which mediates adhesion of parasitized RBCs (pRBCs) to endothelial cells, and the opsonisation of these RSP-2-bearing unparasitized RBCs plays a major role in the removal of unparasitized RBCs (Layez et al., 2005). The phagocytosis of non-infected RBCs is believed to be a major cause of persistent anaemia during infection (Totino et al., 2010).

Increased inflammation and unregulated production of inflammatory cytokines have been implicated in SMA (Clark and Chaudhri, 1988; Kurtzhals et al., 1998). Studies have reported increased plasma tumour necrosis factor alpha (TNF-α), interleukin 10 (IL-10) and interferon gamma (IFN-γ) in malaria patients (Kurtzhals et al., 1998; McGuire et al., 1999; Othoro et al., 1999). TNF-α has been implicated in multiple biological actions during malaria infection including the production of other pro-inflammatory cytokines such as IL-6 (Kern et al., 1989). The production of IL-6 is reported to induce the expression of hepcidin, an iron-regulating hormone synthesised by the liver (Park et al., 2001). Hepcidin subsequently disrupts iron metabolism, resulting in hypoferremia, which ultimately contributes to anaemia (Nemeth et al., 2004a). Chloroquine (CHQ) has been used as first-line treatment for malaria in many tropical areas including Sub-Saharan Africa (Foster, 1995). However, a decrease in efficacy of this antimalarial drug has been reported (Ekvall et al., 1998). Furthermore, CHQ has been reported to interfere with iron metabolism which may subsequently aggravate anaemia (el Hassan et al., 1997).

Abbreviations: NIC, non-infected control; IC, infected control; O CHQ, orally administered chloroquine; TD OA, transdermally delivered oleanolic acid.

* Corresponding author at: Discipline of Human Physiology, Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.
E-mail address: 208512882@stu.ukzn.ac.za (H. Sibiya).
[1] Deceased.

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Therefore, there is a need to search and develop novel antimalarial agents which will attenuate anaemia. Natural triterpenoids, betulinic acid, ursolic acid and oleanolic acid (OA), are found in many medicinal plants and possess multiple pharmacological activities (Singh et al., 1992; Wolska et al., 2010). These triterpenes have also been reported to possess antiplasmodial activities against *P. falciparum* (Steele et al., 1999; Innocente et al., 2012; Simelane et al., 2013). Current literature evidence only demonstrates the *in vitro* antiplasmodial activity of OA. The current study investigated for the first whether the antimalarial effects of OA are able to ameliorate anaemia *in vivo*. Oral administration of OA is associated with poor solubility which results in low drug bioavailability (Jäger et al., 2007; Jeong et al., 2007). Hence, in the current study, OA was administered through the transdermal route using an OA-pectin matrix patch. Accordingly, the effects of transdermally administered OA on parasitaemia, hepcidin, the expression of cytokines (TNF-α, IL-6 and IL-10) and haematological parameters (RBC, HCT, Hgb) was evaluated in *P. berghei*-infected rats.

2. Materials and methods

2.1. Drug and chemicals

Amidated low-methoxyl pectin was kindly donated by Dr Hans-Ulrich Endress of Herbstreith and Fox KG, Neuenburg, Germany. Drugs were sourced from standard pharmaceutical suppliers. All other chemicals which were of analytical grade quality were purchased from standard commercial suppliers.

2.2. Patch preparation

Amidated pectin hydrogel OA matrix patches were prepared using a well-established protocol by Musabayane et al. (2003) with slight modifications. Briefly, amidated low methoxyl pectin was dissolved in deionized water (4.4 g/110 mL) followed by adding OA (1.44 g dissolved in DMSO, 3 mL) (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA), followed by agitation for 30 min. Subsequently, antioxidants, vitamin E (1.65 mL, Pharmacare Ltd, Johannesburg, South Africa) and eucalyptus oil (1.65 mL, Barrs Pharmaceutical Industries cc, Cape Town, South Africa) were added to the mixture and spun for 1 h 30 min to allow for proper mixing. Vitamin E and eucalyptus oil protect the rats from skin irritation when the patch is applied. Following this, an aliquot (11 mL) was transferred to a petri dish of known diameter and frozen at −4 °C for 18 h. After freezing, 1.5 mL of a 2% $CaCl_2$ solution was added to allow for cross-linking and formation of the matrix patch. The patches were then stored in a refrigerator at 4 °C until use.

2.3. Animals

Male Sprague-Dawley rats (90–120 g body weight) bred and maintained at the Biomedical Research Unit, University of KwaZulu-Natal were used in this study. The animals had free access to standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) as well as water and were kept under a 12 h light/12 h dark cycle. Procedures involving animal care and handling were conducted in conformity with institutional guidelines of the University of KwaZulu-Natal. All animal experimentation was reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal, Ethics number: **095/14/Animal**.

2.4. Induction of malaria

Malaria was induced in male Sprague-Dawley rats with a single intraperitoneal injection of *P. berghei* parasitized RBCs (10^5) (Gumede et al., 2003). Control animals were injected with phosphate buffered saline. Successful malaria induction was confirmed by microscopic examination of Giemsa-stained thin smears of rat tail blood. Percentage parasitaemia greater than or equal to 20% was considered a stable malaria state before commencing any experiments involving infection.

2.5. Application of the hydrogel matrix patch

Rats were shaved on the dorsal region of the neck using the Oster Golden A5 heavy duty single-speed animal clipper (Oster Professional products, McMinnville, Tennessee, United States) 24 h prior to the application of hydrogel matrix patches. The dermal patch was secured in place with adhesive hydrofilm (Hartman-Congo Inc, Rock Hill, South Carolina, USA) and a rat jacket (Braintree, Scientific, Inc, Braintree, Massachusetts, USA) which was adjusted according to the size of the animal. The patch was applied for a period of 12 h.

2.6. Short-term studies

The short-term effects of transdermal application of OA pectin patch (34 mg/kg) were evaluated in *P. berghei*-infected rats. The dose used in the current study was selected based on preliminary studies, where 3 doses (9, 17, 34 mg/kg) of transdermally administered OA were evaluated for antiplasmodial activities *in vivo* (Supplementary file 1). The OA patch containing 34 mg/kg and showed the most potent antimalarial activity and was used for further investigations. OA pectin patches (34 mg/kg) were applied once at 9h00 on day 7 of the study. The patch was applied for 12 h. Oral CHQ (30 mg/kg) was administered twice daily at 9h00 on day 7 for 5 consecutive days. Animals treated with drug-free pectin containing vitamin E, eucalyptus oil and DMSO acted as negative controls while CHQ treated animals acted as positive controls.

2.7. Parasitaemia monitoring

Malaria parasite density was monitored daily by the counting of Giemsa-stained thin tail-blood smears of *P. berghei*-infected animals throughout the 21-day experimental period using a microscope (Olympus cooperation, Tokyo, Japan) with an x50–x100 oil immersion objective.

2.8. Experimental design

On selected days (days 0, 7, 9, 12 and 21), separate groups of animals were sacrificed by exposure to halothane (100 mg/kg) for 3 min via a gas anaesthetic chamber. Blood was collected by cardiac puncture into individual pre-cooled heparinized tubes for measurement of haematological parameters, plasma and inflammatory cytokines. Due to ethical considerations, untreated infected control animals were sacrificed on day 12 of the study.

2.9. Haematological measurements

On days 0, 7, 9, 12 and 21 blood was collected from control and treated animals by cardiac puncture into pre- cooled heparinized tube. The blood samples were then analysed immediately for RBCs, HCT and Hgb concentrations using an automated cell counter (Coulter Electronics, Luton, Bedfordshire, UK) with standard calibration according to the manufacturer's instruction (1979) (supplementary file 2).

2.10. Hepcidin concentration measurements

Plasma hepcidin concentrations were measured in plasma samples collected from separate groups of controls and animals treated