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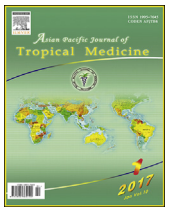

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The role of heme-oxygenase-1 in pathogenesis of cerebral malaria in the co-culture model of human brain microvascular endothelial cell and ITG *Plasmodium falciparum*-infected red blood cells

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ABSTRACT

Objective: To investigate the role of human host heme-oxygenase-1 (HO-1) in pathogenesis of cerebral malaria in the *in vitro* model.

Methods: The effect of human host HO-1 [human brain microvascular endothelial cell (HBMEC)] on hemoglobin degradation in the co-culture model of HBMEC and ITG *Plasmodium falciparum*-infected red cells (iRBC) through measurement of the enzymatic products iron and bilirubin.

Results: Following exposure to the HO-1 inducer CoPPIX at all concentrations, the HBMEC cells apoptosis occurred, which could be prominently observed at 15 μM of 3 h exposure. In contrast, there was no significant change in the morphology in the non-exposed iRBC at all concentrations and exposure time. This observation was in agreement with the levels of the enzymatic degradation products iron and bilirubin, of which the highest levels (106.03 and 1753.54% of baseline level, respectively) were observed at 15 μM *vs*. 20 μM at 3 h *vs*. 24 h exposure. For the effect of the HO-1 inhibitor ZnPPIX, HBMEC cell morphology was mostly unchanged, but significant inhibitory effect on cell apoptosis was seen at 10 μM for the exposure period of 3 h (37.17% of baseline level). The degree of the inhibitory effect as reflected by the level of iron produced was not clearly observed (highest effect at 10 μM and 3 h exposure).

Conclusions: Results provide at least in part, insight into the contribution of HO-1 on CM pathogenesis and need to be confirmed in animal model.

1. Introduction

Malaria remains one of the most important infectious diseases in the world, raiding developing countries in terms of morbidity and mortality [1]. Cerebral malaria (CM) is one of the most severe pathological complications of *Plasmodium falciparum* (*P. falciparum*) infection manifesting as coma that may lead to death [2]. However, mechanism of the pathogenesis of CM is not completely understood. Several factors associated with pathogenesis and severity of *P. falciparum* infections have been reported, but major factors involve the production of cytokines (IL-4 and IL-12) and tumor necrosis factor (TNF-α) [3,4]. The hypothetical role of heme-oxygenase-1 (HO-1) enzyme in pathogenesis of severe malaria has been proposed as one of the important factors that may be linked with susceptibility and severity of malaria infections [5]. HO-1 is the human enzyme involved in heme degradation process to release the nontoxic products iron, carbon monoxide, and biliverdin/bilirubin. This process therefore significantly influences iron supply that support the growth of *P. falciparum* [6]. It is hypothesized that polymorphisms in the promoter region of the *HMOX1* gene encoding HO-1 might confer protection against severe malaria [5,7–11]. Two single nucleotide

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polymorphisms (SNPs), *i.e.*,T(-1135A) and G(-1135)A, and a (GT)n repeat length polymorphism in the *HMOX1* promoter have been reported to link with susceptibility and severity of malaria disease. The prevalence of patients with short $(GT)_n$ alleles was significantly higher in patients with CM than those with uncomplicated malaria [8]. This suggests that short length $(GT)_n$ repeat in patients with CM possibly results in higher expression level and activity of HO-1 enzyme. Short $(GT)_n$ alleles in the promoter region may therefore represent a genetic risk factor for CM as it may directly enhance the transcription of HO-1 in malaria patients, and increase the products of heme degradation, *i.e.*, carbon monoxide, iron, and bilirubin in the brain [12]. Results from previous studies on the possible role of HO-1 and the risk of malaria infection and disease severity to CM are however, controversial. While the association between the short $(GT)_n$ repeat alleles and risk of severe malaria was reported in Gambia, Myanmar, and Angola[[5,7,8]], lack of such association was reported from Thailand [9] and Ghana [13]. The aim of the study was to investigate the role of human host HO-1 on CM pathogenesis in the *in vitro* model. Specifically, the effect of human host HO-1 (human brain microvascular endothelial cell: HBMEC) on hemoglobin degradation in the ITG *P. falciparum* ITG-infected red blood cells (iRBC) was investigated through the measurement of the enzymatic products iron and bilirubin.

2. Material and methods

2.1. Study design

ITG *P. falciparum*-iRBC was co-cultured with HBMEC to investigate the role of human host HO-1 in the hemoglobin degradation process in iRBC. The model mimics at certain degree, the pathogenesis of CM. The co-culture experiment was divided to 6 groups (3 controls and 3 experimental groups) as follows: Group 1: HBMEC co-cultured with normal RBC (HBMEC/nRBC); Group 2: HBMEC co-cultured with normal RBC, with exposure to HO-1 inducer CoPPIX (HBMEC/nRBC + CoPPIX); Group 3: HBMEC co-cultured with normal RBC, with exposure to HO-1 inhibitor ZnPPIX (HBMEC/nRBC + ZnPPIX); Group 4: HBMEC co-cultured with iRBC (HBMEC/iRBC); Group 5: HBMEC co-culture with iRBC, with exposure to HO-1 inducer CoPPIX (HBMEC/iRBC + CoPPIX); Group 6: HBMEC co-cultured with iRBC, with exposure to HO-1 inhibitor ZnPPIX (HBMEC/iRBC + ZnPPIX).

2.2. Cultivation of ITG P. falciparum l iRBC

ITG strain *P. falciparum* used in the experiment was continuously maintained in culture at Liverpool School of Tropical Medicine, University of Liverpool, UK according to the method described by Trager and Jensen [14]. In brief, parasite was grown in group O^+ human RBCs in Malaria Complete Medium (MCM) consisting of RPMI 1640 fortified with 2 mmol/L glutamine (Thermo Fisher Scientific, Massachusetts, USA), glucose (10 mmol/L: Amresco, Ohio, USA), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (25 mmol/L: Research Organics, Ohio, USA), sodium bicarbonate (32 mmol/L: Research Organics, Ohio, USA), and albumax Ⅱ (0.5%, w/v: Life Technologies, California, USA). The parasite was cultured in a 25 cm^2 (T25) or 75 cm^2 (T75) flask (Corning, California, USA) with 5% hematocrit, and 5% O_2, 5% CO_2 and 90% N_2 (Core-gas, Michigan, USA). Three days before the experiment, the culture was synchronized with sorbitol. Schizonts and mature trophozoites were enriched via magnetic separation with an AutoMACSH (MiltenyiBiotec, BergischGladbach, Germany). The experiment was performed twice (triplicate each) using these two late stages of *P. falciparum* as they are the predominant forms that sequester in the brain microcirculation in CM.

2.3. Human brain microvascular endothelial cell culture

The line 5i (HBMEC) was cultured in a 25 cm^2 (T25) or 75 cm^2 (T75) flask (Corning, California, USA) pre-coated overnight with 1% gelatin (w/v in sterile PBS: Sigma-Aldrich, Missouri, USA) for 1 h in Dulbecco's Modified Eagle Media (Thermo Fisher Scientific, Massachusetts, USA). Nutrient Mixture F-12 (Thermo Fisher Scientific, Massachusetts, USA) was enriched with 10% (v/v) FBS and 30 μg/mL gentamycin (Life Technologies, California, USA) at 37 °C with 5% CO_2 in a humidified atmosphere.

2.4. Co-culture experiment

The nRBC and iRBC were co-cultured with HBMEC at the ratio of 100:2 cell density in a flat bottom microplate (Nunc, Roskilde, Denmark) under normoxic conditions. HBMEC was seeded at 2×10^4 cells per well into a 1% (w/v) gelatin (Sigma-Aldrish, Missouri, USA) pre-coated microplate and incubated at 37 °C for 1 h to achieve confluence. To investigate the time- and concentration-dependent inducing effect on HO-1, the co-cultures in group 2 (HBMEC/nRBC + CoPPIX) and 5 (HBMEC/iRBC + CoPPIX) were exposed to the HO-1 inducer Co-protoporphyrin Ⅸ (CoPPIX: Sigma-Aldrish, Missouri, USA) at the concentrations of 5, 10, 15 and 20 μM for 0, 3, 6, and 24 h. To investigate the time- and concentration-dependent inhibitory effect on HO-1, the co-cultures in group 3 (HBMEC/nRBC + ZnPPIX) and 6 (HBMEC/iRBC + ZnPPIX) were exposed to the HO-1 inhibitor Zn(Ⅱ) protoporphyrin Ⅸ (ZnPPIX: Sigma-Aldrish, Missouri, USA) at the concentrations of 5, 10, 15 and 20 μM for 0, 3, 6, and 24 h. In groups 1 (HBMEC/nRBC) and 4 (HBMEC/iRBC), the co-cultures were incubated for 0, 3, 6, and 24 h. At the end of each incubation period, the co-culture was centrifuged (25 °C, 300 ×*g* for 3 min) to separate cell supernatant. Morphology of the HBMEC in the cell sediment was examined under light microscope (×10). Concentrations of iron and bilirubin in cell supernatant were determined using QuantiChrom Iron Assay Kit (Bioassay Systems, California, USA) and QuantiChrom Bilirubin Assay Kit (Bioassay Systems, California, USA), respectively, according to the manufacturers' instructions. Concentrations of TNF-α and IL-10 in cell supernatant were determined using Human ELISA Kit (Pierce Biotechnology, California, USA) according to the manufacturer's instructions. The ratio of IL-10 and TNF-α levels was used as a criterion for severity of malaria pathogenesis. The ratio of greater than 1 is associated with non-severe malaria, while that of less than 1 is associated with severe malaria [1]. Data are present as mean and/or range (minimum–maximum) values where appropriate.

3. Results

3.1. Effects of CoPPIX and ZnPPIX on HBMEC cell morphology in ITG P. falciparum-iRBC co-culture

In the co-culture of HBMEC with nRBC (group 1), and that with exposure to the HO-1 inducer CoPPIX (group 2), or the HO-1 inhibitor ZnPPIX (group 3), no significant change in the morphology of HBMEC cells was observed at all concentrations and exposure time. In the co-culture of HBMEC with iRBC (group 4), relatively low degree of HBMEC cell apoptosis (cell shrinkage with undefined cell boundary) was observed during the 3–24 h incubation period (Figure 1A). Following exposure to CoPPIX (group 5) at all concentrations on the other hand, a marked change in the morphology of HBMEC cells was observed at all concentrations during the 3–24 h incubation periods. Increase in cell apoptosis was clearly observed at 15 μM of CoPPIX at 3 h exposure (Figure 1B). Following exposure to ZnPPIX at all concentrations (group 6), no significant change in cell morphology was observed at all incubation periods. The decrease in cell apoptosis was more or less, best observed at 10 μM of ZnPPIX at 3 h exposure (Figure 1C).

3.2. Effects of CoPPIX and ZnPPIX on the production of iron and bilirubin by ITG P. falciparum-iRBC co-culture

We next investigated the time-dependent and concentration-dependent effects of CoPPIX and ZnPPIX on the activity of HO-1 in HBMEC cells co-cultured with iRBC (groups 4, 5, 6) compared with HBMEC cells co-cultured with nRBC (groups 1, 2, 3). The levels of the enzymatic products produced by iRBC and nRBC, *i.e.*, iron and bilirubin in cell supernatant were used as markers of HO-1 enzyme activity.

Baseline levels of iron (0.034 *vs.* 0.035 mM for nRBC *vs.* iRBC) and bilirubin (0.289 mM *vs.* 0.282 mM) produced by both nRBC and iRBC were comparable. For both the nRBC and iRBC co-cultured with HBMEC, the levels of iron produced were not significantly changed during the incubation period of 3–24 h in both the exposed and non-exposed cells to CoPPIX

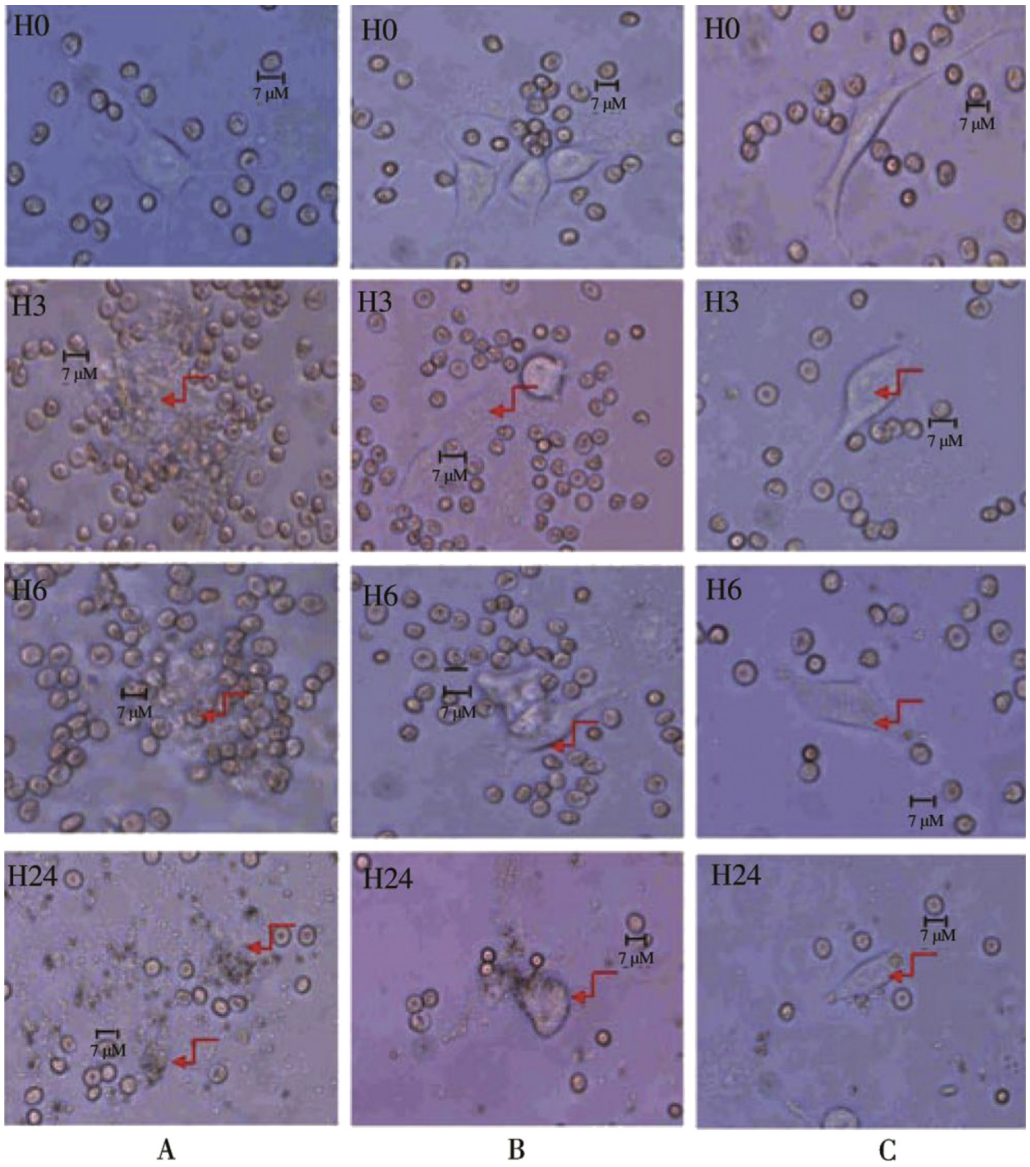

Figure 1. Effects of CoPPIX and ZnPPIX on HBMEC cell morphology in ITG *P. falciparum*-iRBC co-culture during 24 h observation period.
(A) non-exposure; (B) exposure to 15 μM CoPPIX; and (C) exposure to 10 μM ZnPPIX. Red arrows indicate the change in morphology of HBMEC cells.

and ZnPPIX at almost all concentrations (94.4%–106.67% of baseline). For the iRBC co-cultured with HBMEC (group 5), the highest production of iron (106.3%) was observed with 15 μM CoPPIX for an exposure period of 3 h (Figure 2A). The lowest production of iron (88.97%) was observed with 10 μM ZnPPIX for an exposure period of 3 h (Figure 3A). At 20 μM ZnPPIX however, the level was markedly increased at 6 h (93.18% of baseline) and 24 (98.02% of baseline).

The production of bilirubin in both the exposed nRBC and iRBC on the other hand, markedly fluctuated at various concentrations of CoPPIX and ZnPPIX during the incubation periods of 3–24 h. Bilirubin levels in both the nRBC and iRBC in the non-exposed and CoPPIX exposed cells varied from 22.06% to 187.67% of baseline during the incubation periods of 3–24 h. For the nRBC (group 1), the increase in bilirubin level was found at 3 h exposure to 5 μM (118.18%) and 10 μM (187.67%) CoPPIX. Decrease in the level (22.03%–27.56%) was observed following exposure to CoPPIX at higher concentrations (15 and 20 μM) during 3–24 h incubation. The production of bilirubin following exposure of iRBC to CoPPIX at all concentrations (group 5) however, markedly fluctuated between 127.23% and 1753.45% of the baseline levels (Figure 2B). The highest level (1753.45%) was observed with 20 μM CoPPIX for an exposure period of 24 h. The production of bilirubin in the non-exposed iRBC (group 4) varied from 106.59% to 124.53% of the baseline level (Figure 2B).

For the effect of ZnPPIX, bilirubin levels in both the nRBC and iRBC in the non-exposed (77.31%–101.99% *vs*. 100%–124.53% for nRBC and iRBC, respectively) and ZnPPIX exposed cells (20–56–89.74% *vs*. 37.17% *vs*. 547.46% for nRBC and iRBC, respectively) markedly varied during 3–24 h incubation. For the exposed nRBC, the level was decreased from baseline at all concentrations and exposure time (20.56%–89.74%), with lowest level at 6 h (20.56%). For the exposed iRBC, the level was on the other hand, increased from baseline at almost all concentrations and exposure periods (123.4%–547.46%), except at 15 μM of 3 h exposure period (37.17%). The highest level was observed at 20 μM ZnPPIX with 6 h exposure period (547.46%) (Figure 3B).

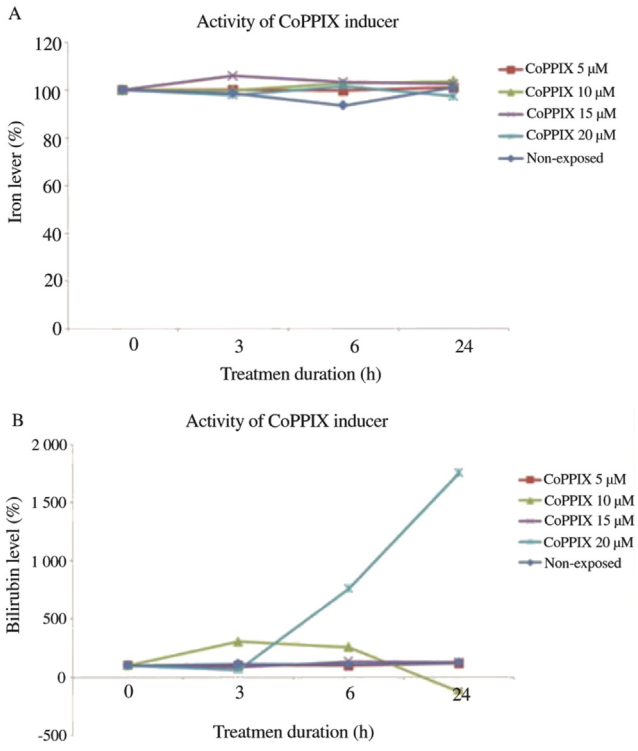

Figure 2. Mean value of iron (A) and bilirubin (B) levels measured in cell supernatant following exposure of HBMEC-iRBC co-culture with the HO-1 inducer CoPPIX at 5, 10, 15, and 20 μM compared with the non-exposed cells.

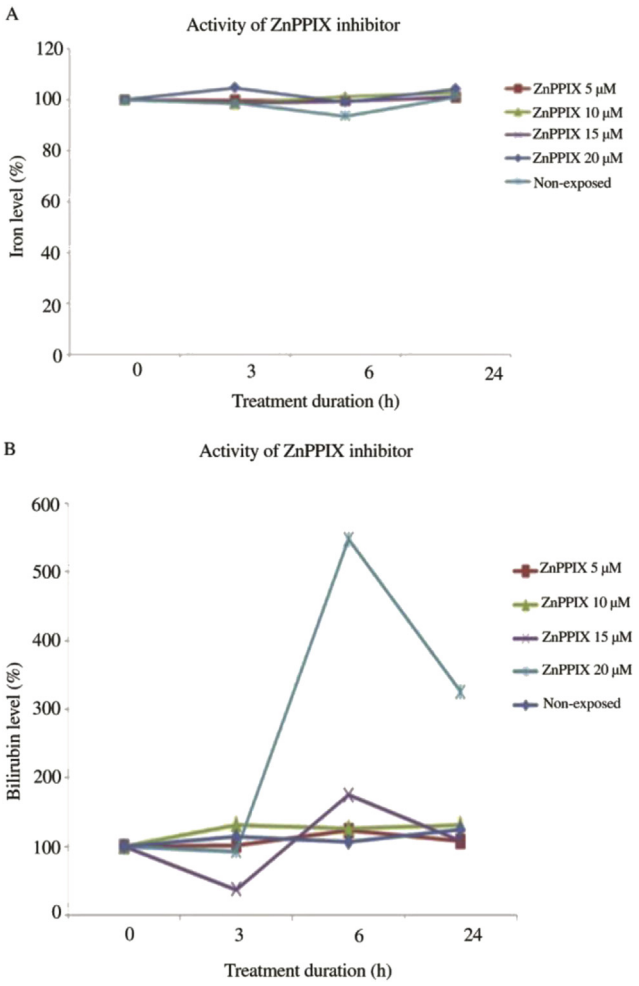

Figure 3. Mean values of iron (A) and bilirubin (B) levels measured in cell supernatant following exposure of HBMEC-iRBC co-culture with the HO-1 inhibitor ZnPPIX at 5, 10, 15, 20 μM compared with the non-exposed cells.

3.3. Effects of CoPPIX and ZnPPIX on the production of IL-10 and TNF-α by ITG P. falciparum-iRBC co-culture

The IL-10/TNF-α ratios in the non-exposed nRBC and iRBC were less than 1, but the ratio was relatively lower in the iRBC (3.42–32.05 and 1.74–5.81 for nRBC and iRBC, respectively). Dramatic decrease in the ratio was found in the iRBC (0.023–0.375), but significantly observed at 15 μM of 3 h exposure to CoPPIX (0.111). In the presence of ZnPPIX, the ratio was relatively higher (0.478–0.974) during 3–24 h exposure. The ratios in the nRBC either in the presence or absence of CoPPIX or ZnPPIX were not significantly changed (2.23–11.44).

4. Discussion

In this preliminary study, the role of human host HO-1 and malaria disease severity was investigated using the *in vitro* co-culture model of HBMEC and ITG *P. falciparum*-iRBC that mimics CM pathogenesis in humans. The severity of malaria

stage (CM) was confirmed by the ratio of IL-10 and TNF-α which was relatively lower in HBMEC/iRBC (1.74–5.81) compared with HBMEC/nRBC (3.42–32.05). The levels of the degradation products iron and bilirubin were used as markers of HO-1 enzyme activity.

Following the exposure of the HBMEC/iRBC to the HO-1 inducer CoPPIX at all concentrations (group 5), the HBMEC cells apoptosis occurred which could be prominently observed at the concentration 15 μM of 3 h exposure. In contrast, there was no significant change in the morphology in the non-exposed cells (group 4) at all concentrations and exposure time. The observed apoptotic cells (HBMEC/iRBC) following exposure the HO-1 inducer could be a consequence of a defense mechanism when the cells were destroyed by noxious stimuli [1]. This was generally correlated with the levels of the enzymatic degradation products iron and bilirubin, of which the highest level of iron was observed at 15 μM of 3 h exposure (106.03%) and the highest level of bilirubin was observed at 20 μM of 24 h exposure (1753.54%).

For the effect of the HO-1 inhibitor ZnPPIX, HBMEC cell morphology was mostly unchanged, but significant inhibitory effect on cell apoptosis was seen at 10 μM for the exposure period of 3 h. The degree of the inhibitory effect as reflected by the levels of iron produced was not clearly observed (highest effect at 10 μM and 3 h exposure: 98.47%). The inhibitory effect on bilirubin production on the other hand, was clearly observed only at 15 μM and 3 h exposure (37.17% of baseline); at other concentrations however, increased in bilirubin levels was found.

Although the results from the present study could not provide definite conclusion on the concentration- and time-dependent inducing effect of CoPPIX as well as the inhibitory effect of ZnPPIX on the production of iron and bilirubin, it provides at least in part, insight into the contribution of HO-1 on CM pathogenesis. The modulation of enzyme activity by CoPPIX and ZnPPIX was specific to the induction or inhibition of HO-1 expression in the HBMEC cells and in turn, modulation of the levels of heme degradation to release the non-toxic products (iron, carbon monoxide, and biliverdin/bilirubin) by both the iRBC and nRBC. This process therefore significantly influences iron supply that support the growth of *P. falciparum* and thus contributing to disease pathogenesis and severity [6]. This was indicated by the observed HBMEC cell death when co-cultured with iRBC, particularly in the presence of the HO-1 inducer CoPPIX. These effects did not occur in HBMEC co-cultured with nRBC. The limitation of the current study is that, measurement of the direct change of HO-1 was not performed due to technical problem related to the analysis of the expression of HO-1. Nevertheless, results from a previous study demonstrated significant changes in the expression of HO-1 at transcriptional and translational levels by HO-1 inducers and inhibitors [15]. Further study in animal model in HO-1 knock-out mice should provide direct evidence to confirm the possible role of HO-1 in malaria pathogenesis and severity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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