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Selective toxicity of dihydroartemisinin on human CD34+ erythroid cell differentiation

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ABSTRACT

Artemisinins are safely used in the combination therapy for uncomplicated malaria, but their employment during pregnancy is still controversial. In fact, animal studies reported that the active metabolite, dihydroartemisinin (DHA), causes embryonic erythrocytes depletion, when the treatment is performed during a critical period of time. The present study investigates the effect of DHA on human developmental erythropoiesis in order to characterize the target erythroid stage and to predict the window of susceptibility in human pregnancy. As a model for human developmental erythropoiesis, peripheral blood purified, CD34+ cells were committed towards erythrocytes and DHA (0.5 or 2 μM) was added to different erythroid stages during 14 days culture. Erythroid differentiation was investigated by cytofluorimetric analysis of Glycophorin A expression, by morphological analysis and erythroid globin gene expression analysis with real-time PCR. It was found that the effect of DHA was dependent on the maturation stage of erythroid cells. In fact when DHA was added to the pro- and basophilic erythroblasts caused a significant dose-dependent inhibition of cell proliferation and a significant delay of erythroid differentiation, as measured by morphological analysis, expression of Glycophorin A by immunofluorescence and of erythroid globin genes by real-time PCR. In contrast, the inhibition of stem cells and of early progenitors was transient and masked by the subsequent exponential cell growth. No effect was observed on mature erythroid stages. This is the first demonstration that DHA affects human erythropoiesis *in vitro*, in a dose- and time-dependent manner; the target population seems to be the pro-erythroblast and basophilic erythroblast stage, suggesting that DHA toxicity is limited to primitive human erythropoiesis. These findings outline the relevance of DHA dosage and timing to prevent embryotoxicity and support current WHO recommendations of avoiding malaria treatment with artemisinins during the first trimester of pregnancy.

1. Introduction

Malaria is a parasitic disease caused by protozoan parasites of the genus *Plasmodium* and it has a worldwide distribution with an incidence of more 500 million of new cases every year (WHO, 2008). During pregnancy, malaria causes serious clinical effects on the mother and the fetus. Women, particularly in their first pregnancy, are exposed to a very high risk of severe malaria complications such as maternal anemia, low birth weight of newborns and increased maternal and child mortality (WHO, 2006b). The World Health Organization (WHO) recommends Intermittent Preventive Treatment with Sulphadoxine–Pyrimethamine, but, since resistance is spreading, more information about the safety of new drugs that can be used for the cure of malaria during gestation is urgently needed (Menendez et al., 2007; Ward et al., 2007; WHO, 2006a).

Artemisinin is extracted from the leaves of Chinese *Artemisia annua* plants. It is a sesquiterpene lactone containing an endoperoxide bridge, which is considered essential for antimalarial activity. The artemisinin derivatives artemether, artesunate and dihydroartemisinin (DHA, both a drug on its own and a metabolite of artemisinin derivatives) are widely used in combination therapy (ACT) as first line treatment for uncomplicated malaria. The use of artemisinin derivatives is restricted during pregnancy to the second and third trimesters, when other chemotherapies fail (WHO, 2007, 2008). The basis for this recommendation is the experimental evidence that all current semi-synthetic artemisinin derivatives

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cause embryotoxicity during a time window in the first trimester of pregnancy: in mice on gestation days (GD) 9–11, in rats on GD 6–15, in rabbits on GD 7–18 and monkeys GD 26–36 (Chen et al., 1984; Clark et al., 2004, 2008a,b; Longo et al., 2006a,b, 2008; White et al., 2006; White and Clark, 2008). Various experiments concur to point to the primitive embryonic erythropoiesis (occurring in the yolk sack) as the primary target of the artemisinin drugs, with a marked reduction in the number of embryonic erythroblasts. These are class effects (at least for the available semi-synthetic derivatives) and not species-specific. However, the relevance of these findings to human pregnancy remains to be established and safety information about the potential toxicity for humans is urgently required to assess a safe use of artemisinins. Since for ethic reasons experiments cannot be conducted on pregnant women, *in vitro* assays and comparisons between animal and human organogenesis are the best ways to assess human toxicity.

The aim of the work was to investigate the effect of DHA on human erythropoiesis and to characterize the target erythroid stage in order to predict the window of susceptibility to DHA in human erythropoiesis. For this purpose, CD34+ stem cells, isolated from human peripheral blood are employed as standardized model to study the molecular mechanism controlling human developmental erythropoiesis, starting from progenitors towards committed erythrocytes (Fibach et al., 1989; Neildez-Nguyen et al., 2002; Timens and Kamps, 1997).

The effect of DHA was evaluated at different stages of human erythroid cell differentiation to determine the dose-related toxicity and the stage specificity.

2. Materials and methods

2.1. Drugs

Dihydroartemisinin (DHA), kindly provided by Prof. Haynes from Hong Kong University, was initially dissolved in ethyl alcohol (5 mg/mL stock solution) and diluted further with culture medium (ethyl alcohol less than 0.001% in the culture medium). DHA was freshly prepared for each experiment. The biological activity of DHA used in these experiments was constantly monitored against *P. falciparum* strains *in vitro*.

2.2. Cell culture

Peripheral blood from consenting healthy volunteers was collected into sterile heparinized tubes. Light-density mononuclear cells were obtained by centrifugation on a Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient and then enriched for CD34+ cells by positive selection using CD34+ microbeads (Miltenyi Biotech, Auburn, CA) according to the manufacturers' instructions. CD34+ cells were cultured at a density of 105 cells/mL in alpha-minimal essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 30% fetal bovine serum (FBS; GIBCO, Grand Island, NY), as previously described (Ronzoni et al., 2008). Briefly, to induce cell differentiation and erythroid maturation, cells were cultured with 20 ng/mL recombinant human (rH) stem cell factor (SCF, PeproTech, London, UK), 10 ng/mL rH interleukin-3 (IL-3, PeproTech, London, UK), 3 U/mL rH erythropoietin (rHuepo, Janssen-Cilag, Milan, Italy) and with 1 μg/mL Cyclosporin A (Sigma, St. Louis, MO) to inhibit lymphocyte growth. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 for 14 days; after 7 days of culture the medium was changed to ensure good cell feeding. DHA was added at days 0, 2, 4, 7 or 11 at 0.5 or 2 μM concentration, respectively. Due to the decay of the molecule, the treatment with DHA was repeated every 3 days up to day 14. Cell samples were collected on days 4, 7, 11 and 14. Cell number and viability were determined by Trypan Blue exclusion.

2.3. Morphological analysis

Cell morphology was analyzed on days 4, 7, 11 and 14 by light microscopy on cytocentrifuged (Shandon Astmoor, England) smears stained with May-Grunwald-Giemsa, by assessing and counting cells in 5 different fields of view, for a total of 500–600 cells. Hemoglobin-containing cells were identified by benzidine staining (Fibach, 1998).

2.4. Flow cytometry analysis

At specific times of erythroid differentiation (days 4, 7, 11 and 14), the expression of cell-surface antigens was analyzed by flow cytometry. Cells (10⁵) were first incubated in a phosphate-buffered saline (PBS, GIBCO, Grand Island, NY) solution with 0.1% sodium azide (NaN_3, Carlo Erba, Milano, Italy), 4% Newborn Calf Serum (Sigma–Aldrich, Milano, Italy) and 4% Mouse Serum (Sigma–Aldrich, Milano, Italy) to block Fc receptors and avoid non-specific antibody binding. Cells were then labeled with phycoerythrin-Cy7 (PE)-conjugated anti-CD34, APC-conjugate anti-CD45, fluorescein isothiocyanate (FITC)-conjugate anti-CD71, phycoerythrin (PE)-conjugated anti-Glycophorin A antibodies (BD, Becton Dickinson, San Jose, CA) at 4 °C in the dark. Appropriate isotypic controls for each fluorochrome were used to discriminate positive vs negative cells. Acquisition and analysis were performed on a FACSCan to flow cytometer using FACSDiva 5.0 software (BD).

2.5. Quantitative reverse-transcribed polymerase chain reaction (RT-PCR) of globin genes

Total RNA was purified from cell pellets collected on day 14 by the Chomczynski–Sacchi's method (Chomczynski and Sacchi, 1987). Reverse-transcription PCR from 1 μg total RNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a total final volume of 20 μL. The reaction mixtures for quantitative polymerase chain reaction (PCR) were prepared using Taqman PCR probes specific for the gene of interest: α-, β- and γ-globin (Applied Biosystems, Foster City, CA, USA) according to standard methods and processed using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Experiments were performed as triplicate and the data were normalized to GAPDH. The rate of single globin was expressed as percentage of the total globins.

2.6. Data analysis

Statistical comparisons were made using one-way ANOVA with Dunnet post test, two-way ANOVA with Bonferroni's post test analysis or Student's *t*-test, calculated with GraphPad Prism software.

3. Results

3.1. Experimental model

Liquid cultures of CD34+ human stem cells, isolated from peripheral blood were set up and committed towards erythrocytes using standardized conditions, previously described (Ronzoni et al., 2008). Based on the dosages employed in previous animal experiments, DHA at 0.5 or 2 μM was added at different steps of erythroid differentiation: on stem cells or early erythroid progenitors (day 0 or day 2 of culture), on erythroid precursors (pro-erythroblasts; day 4 or day 7 of culture), on mature erythroblasts (day 11 of culture). The treatment with DHA was repeated up to 14 days and the drug effect on the maturation process was followed, analyzing cell growth and the erythroid differentiation at several time points of the erythroid chain as shown in Fig. 1. For clarity, the results are subdivided into three sections describing the effects of DHA on: 1. stem cells and early erythroid progenitors (days 0–2); erythroid precursors (days 4–7); mature erythroblasts (day 11).

3.2. DHA added on stem cells and on early progenitors causes a transient inhibition of erythroid maturation

DHA at 0.5 or 2 μM, was added immediately after cell isolation and purification to investigate the effect on stem cells (day 0 of cell culture) or after 2 days of culture, to assess the drug effect on the early precursors (day 2 of culture shown in supplemental data). Cell growth of treated cells was expressed as percentage of control cells. In both the conditions, cell growth significantly decreased up to day 7 ($p < 0.05$; $p < 0.01$), but then, at days 11 and 14, cell proliferation resumed, reaching percentages higher than control values (Fig. 2A and Fig. 1A in supplemental data).

The differentiation over time of CD34+ cells, cultured with or without DHA, was documented by flow cytometry analysis. The expression of the transferrin receptor (CD71) and Glycophorin A (GpA) was followed as surface markers, respectively, of erythroid precursors and more mature erythroblasts (basophilic, polychromatic and orthochromatic erythroblasts). In normal conditions, CD34+ cells rapidly differentiate into CD71+ erythroid precursors. CD71 is constantly present from day 4 to day 7 of culture and then it

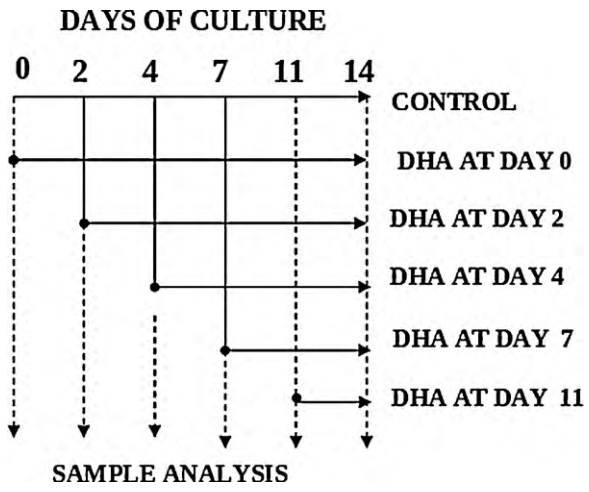

Fig. 1. Schedule of experiment. CD34+ cells from the peripheral blood of volunteers were immediately cultured (day 0) and committed towards erythroid progenitors (day 14). The bold line indicates the days of drug addition to cell culture. The treatment with DHA started at day 0 (on stem cells), at day 2 (on early erythroid precursors), at day 4 (in presence of early progenitors), at day 7 (on pro-erythroblasts) or at day 11 (on polychromatic erythroblasts). DHA was then added every 3 days, because of its short half life. Samples were collected and analyzed at several time points (dots), to follow the differentiation process.

decreases until day 14, when differentiation is almost complete. As cells become mature erythroblasts (basophilic, polychromatic and orthochromatic erythroblasts), CD71 is replaced by the GpA. The exposure of stem cells as well as of early progenitors to DHA did not change CD71 expression over time (Fig. 2 in supplemental data), whereas, the GpA expression slightly decreased at day 7, after the exposure to 2 μM DHA. No significant differences were observed at days 11 and 14 of culture (Fig. 2B and Fig. 1B in supplemental data). The morphological modifications of the cells exposed to DHA during the erythroid differentiation were analyzed using May-Grunwald-Giemsa staining. In normal conditions, without DHA, at day 7, 60% of cells in culture are pro-erythroblasts, becoming basophilic and polychromatic erythroblasts at day 11 and then orthochromatic erythroblasts at day 14. Stem cells showed an increased percentage of pro-erythroblasts at day 11 of culture, when treated with DHA 0.5 μM ($p < 0.01$) and to a more extent with DHA 2 μM ($p < 0.001$) (Fig. 2C). These effects disappeared at day 14, when drug treated cells and control had the same distribution of cell population, indicating that the percentage of polychromatic and orthochromatic erythroblasts was not altered by DHA treatment. Same results were observed when the early progenitors (day 2 of culture) were treated with DHA (see Fig. 1C in supplemental data). Globin analysis of control and DHA-treated cells was performed at day 14, which corresponds in this model, to the end of erythroid differentiation. In normal conditions, cells at the early stage of differentiation express γ-globin mRNA at high level; during maturation γ-globin mRNA declines and β-globin mRNA increases. Alpha-globin expression remains fairly constant. In DHA-treated cells, the percentages of β- and γ-globin mRNA were similar to controls, indicating that the cells were completely differentiated at day 14 of culture (Fig. 2D, and Fig. 1D in supplemental data).

3.3. *DHA added on erythroid precursors causes cell growth inhibition and a delay in erythroid differentiation*

In normal condition, after 4 days, the cell culture consists of both early precursors and pro-erythroblasts, whereas after 7 days, the cells are predominantly pro-erythroblasts. The effects on cell growth and GpA expression obtained adding DHA at day 4 of culture were completely different from those observed on day 0 or

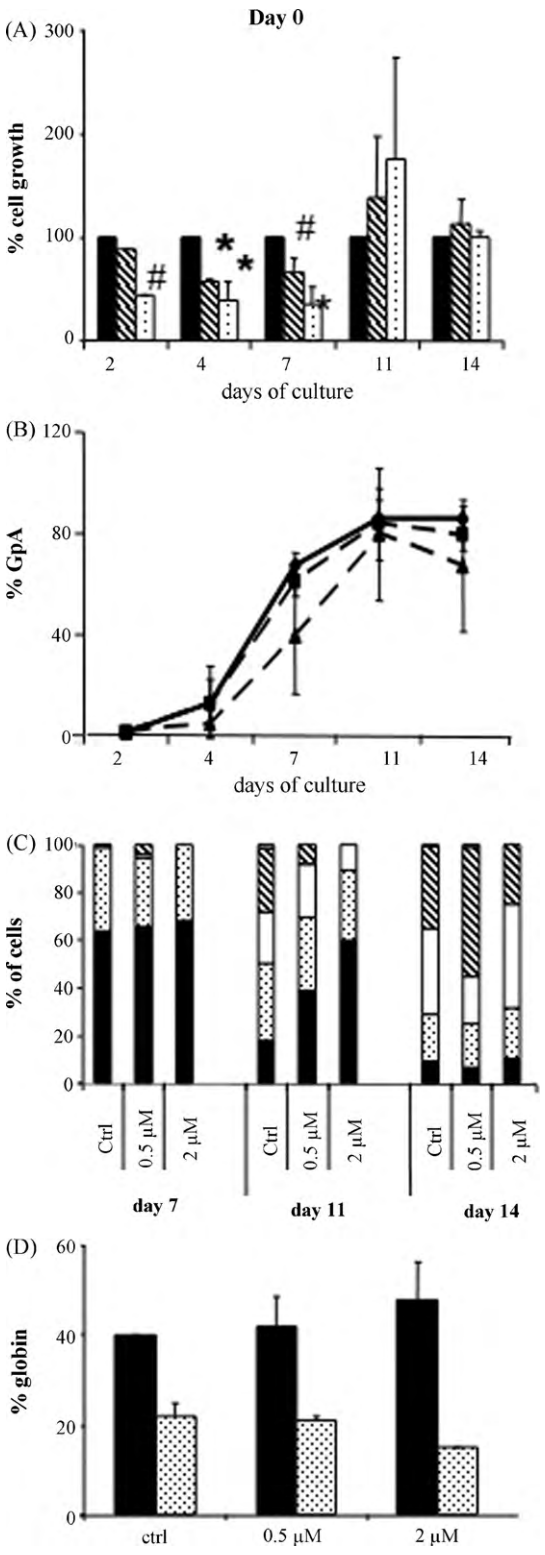

Fig. 2. Effects of DHA added on stem cells at day 0. Cells were counted by Trypan Blue exclusion from day 2 to day 14. (A) The proliferation of control (■), DHA 0.5 μM (▨) or DHA 2 μM (⊡) is expressed as percentage of control at each day. Error bars show the SD ($^{\#}p < 0.05$; $^{*}p < 0.01$ two-way ANOVA with Bonferroni post test). (B) The GpA analysis by flow cytometry showed control (♦), DHA 0.5 μM (■) or DHA 2 μM (▲). (C) Morphological analysis of samples with May-Grunwald-Giemsa staining of control and DHA-treated cells at days 7, 11 and 14. Pro-erythroblasts (■), basophilic erythroblasts (⊡), polychromatic erythroblasts (□), orthochromatic erythroblasts (▨) and erythrocytes (▩). (D) Globin gene expression by RT-PCR at day 14. Beta-globin (■) and γ-globin (⊡) mRNA are represented as percentages of total globin. Graph represents the mean of 3 independent experiments ± SD.

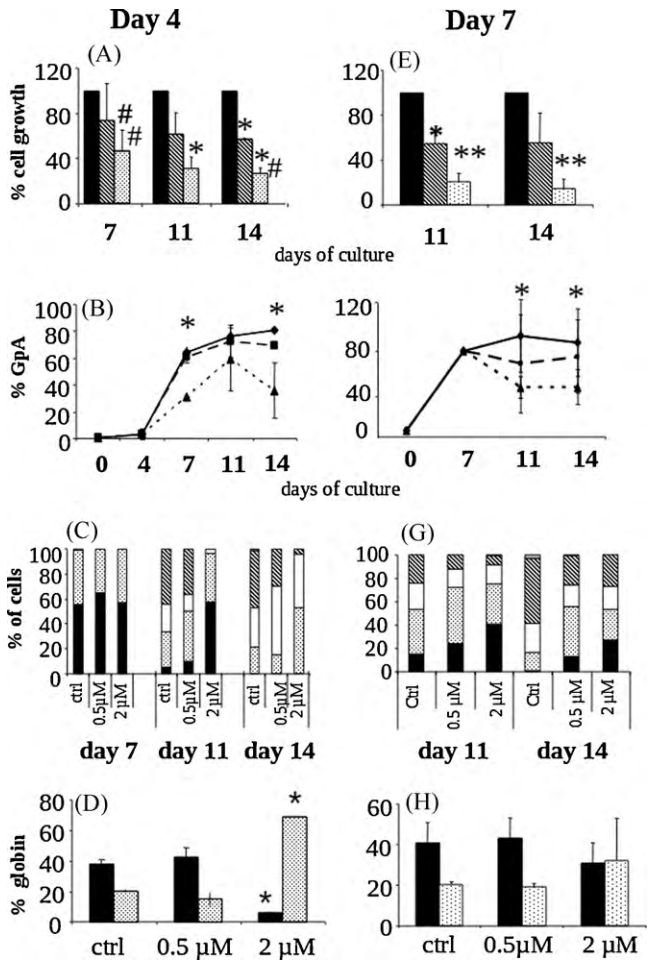

Fig. 3. Effects of DHA added on early precursors at day 4 (A–D) and on pro-erythroblasts at day 7 (E–H). (A/E) Cells were counted by Trypan Blue exclusion from day 2 to day 14. Proliferation of control (■), DHA 0.5 μM (▨) or DHA 2 μM (⊡) is expressed as percentage of control at each day (#$p < 0.05$; *$p < 0.01$ two-way ANOVA with Bonferroni post test). (B/F) The GpA expression by flow cytometry shows control (♦), DHA 0.5 μM (■) and DHA 2 μM (▲) (*$p < 0.05$ two-way ANOVA with Bonferroni post test). (C/G) Morphological analysis of samples with May-Grunwald-Giemsa staining of control and exposed cells at days 7, 11 and 14. Pro-erythroblasts (■), basophilic erythroblasts (⊡), polychromatic erythroblasts (□), orthochromatic erythroblasts (▨) and erythrocytes (▩). (D/H) Globin gene expression by RT-PCR at day 14. Beta-globin (■) and γ-globin (⊡) mRNA are represented as percentages of total globin. (*$p < 0.05$ one-way ANOVA with Dunnet post test). Data represent the mean of 3 independent experiments ± SD.

day 2 of culture. DHA, in fact, strongly reduced cell growth; more precisely: at day 7, cell growth was decreased to 74% or 47% by 0.5 or 2 μM DHA, respectively (compared to control 100%); at day 11, the reduction was to 62% or 31%; at day 14 to 57% and 27%, respectively ($p < 0.05$; $p < 0.01$) (Fig. 3A). GpA expression was also strongly reduced by DHA 2 μM not only at day 7 (from 64% to 31%, $p < 0.05$), but also at day 14 (from 81% to 36%, $p < 0.05$), as shown in Fig. 3B. Compared to day 0 cells, day 4 cells expressed higher percentages of CD71 and GpA (Table 1), suggesting that the erythroid maturation had started and that mature erythroid stages (different from those at day 0 or 2) were exposed to DHA. The morphological analysis also confirmed that the day 4 untreated culture consisted of 51% pro-erythroblasts, which became 43% of basophilic erythroblasts at day 7; mostly basophilic and mature polychromatic and orthochromatic erythroblasts were found at both days 11 and 14 (Fig. 3C). DHA-treated cells were similar to their controls at day 7. On the contrary, at day 11, the exposure to DHA 2 μM significantly increased the percentage of pro-erythroblasts ($p < 0.001$) and completely suppressed the polychromatic and orthochromatic

Table 1
Changes over time of erythroid cell-surface markers.[a].

Day	% Cell-surface antigens (mean ± SD)			
	CD34+	CD45+	CD71+	GpA+
0	50.3 ± 3.01*	71.6 ± 22.0*	9.50 ± 3.02**	0.40 ± 0.40**
4	22.5 ± 1.90*	11.4 ± 1.00*	78.6 ± 7.01**	16.3 ± 4.02**
7	18.4 ± 2.01	11.6 ± 1.50	87.6 ± 7.41	64.1 ± 4.40
11	18.7 ± 1.72	11.3 ± 1.49	81.8 ± 4.63	76.6 ± 3.82
14	12.4 ± 6.60	11.6 ± 1.15	66.5 ± 1.15	80.8 ± 1.13

[a] The expression of cell-surface antigens was analyzed by flow cytometry. The table reports the phenotype of untreated cells isolated and cultured up to day 14, to show the degree of differentiation over time. The mean and the SD were calculated from three independent experiments. Significant differences were observed between cell-surface marker expressions at days 0 and 4.
* $p < 0.05$ Student's *t*-test.
** $p < 0.001$ Student's *t*-test.

erythroblasts ($p < 0.05$). At day 14, basophilic erythroblasts were increased from 20% to 47% by DHA 2 μM as well as polychromatic erythroblasts ($p < 0.01$). At the same time, the number of orthochromatic erythroblasts was markedly reduced from 43% of control to 26% of DHA 0.5 μM ($p < 0.01$) or to 3% of DHA 2 μM ($p < 0.05$) (Fig. 3C). These results suggested that the differentiation of DHA exposed cells seemed to be at least one step delayed compared to control. The RT-PCR of globin genes indeed showed that DHA 2 μM exposed cells contained an increased percentage of γ-globin mRNA, confirming that at day 14, cells were significantly less differentiated than control ($p < 0.05$) as shown in Fig. 3D.

To analyze the effect of DHA on pro-erythroblasts, cells were cultured for 1 week and DHA (0.5 and 2 μM) was added for the first time at day 7. As described in Fig. 3E, DHA dose-dependently inhibited cell growth at both days 11 and 14 ($p < 0.05$; $p < 0.001$, respectively). The flow cytometric analysis showed that the GpA expression was reduced from 85% to 60% by DHA 0.5 μM and to 39% by 2 μM at day 11 and from 79% to 66% and to 39%, respectively, at day 14 as shown in Fig. 3F ($p < 0.05$). The delay in cell differentiation was confirmed by the morphological analysis, which showed an increased percentage of pro-erythroblasts by DHA 2 μM ($p < 0.01$) with the consequent reduction of orthochromatic erythroblasts at days 11 and 14 ($p < 0.05$) (Fig. 3G). On the contrary, control cells differentiated almost completely, since, at day 14, 53% of cell population consisted of orthochromatic erythroblasts, together with few erythrocytes. Also in this case, the RT-PCR of globin genes showed that DHA 2 μM increased γ-globin mRNA whereas decreased β-globin, confirming that these cells were less differentiated than control (Fig. 3H).

3.4. DHA added on mature erythroblasts did not affect erythroid differentiation

Cells were cultured for 11 days and committed towards mature erythroblasts; then DHA was added to investigate the effect on the mature erythroblasts (polychromatic and orthochromatic erythroblasts). The cell growth and differentiation were analyzed at day 14. The cell proliferation was reduced to 45% by DHA 2 μM only (compared to control), probably due to its antiproliferative effect (Fig. 4A). In contrast, no effects were detected on either CD71 and GpA expression by DHA treatment (Fig. 4B). Moreover, no significant morphological differences were observed in the DHA exposed cells compared to control, suggesting that the erythroid differentiation was completed and DHA has no effect on mature erythroblasts (Fig. 4C). At day 11, in fact, both DHA-treated and control cells were mostly polychromatic erythroblasts, which become orthochromatic erythroblasts and erythrocytes at day 14. The globin mRNA analysis also confirmed that cells exposed to DHA were all differentiated with percentages of γ-globin and β-

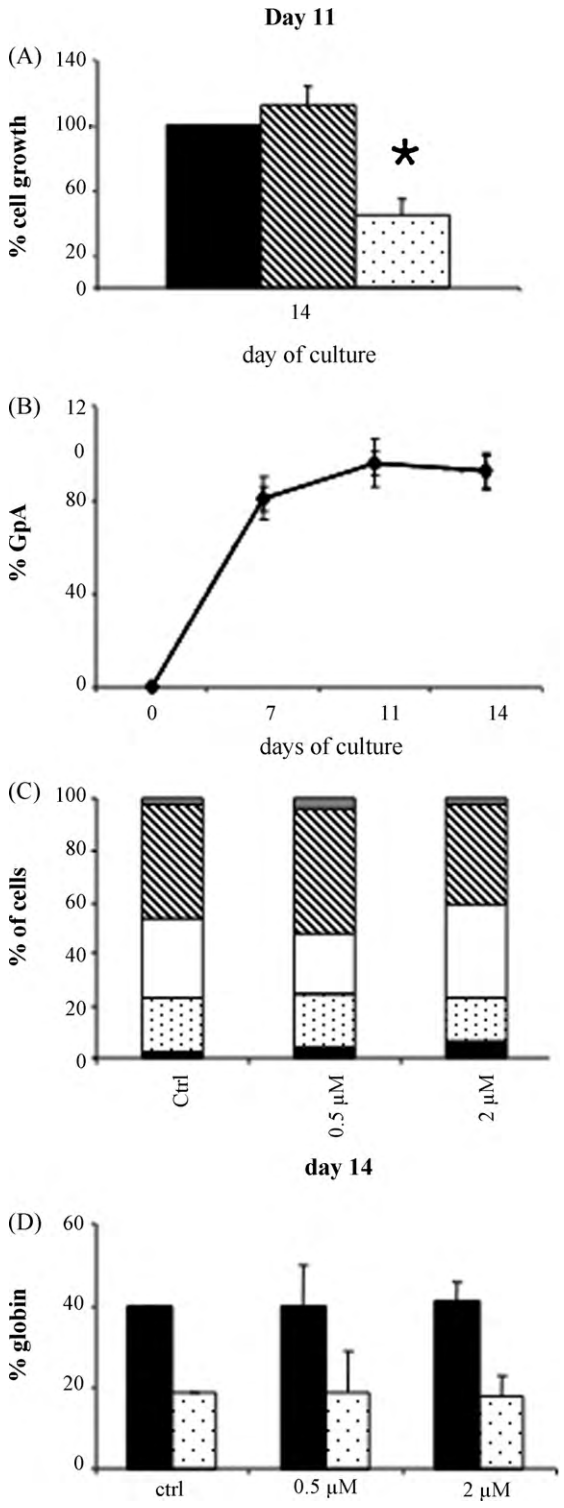

Fig. 4. Effects of DHA added on polychromatic erythroblasts at day 11. (A) Cell proliferation of control (■), DHA 0.5 μM (▨) or DHA 2 μM (⊡) is expressed as percentage of control at each day (*$p < 0.05$; **$p < 0.001$ two-way ANOVA with Bonferroni post test). Proliferation was analyzed by Trypan Blue exclusion and the normalized data represent the average of 3 experiments ± SD. (B) The GpA analysis by flow cytometry shows control (♦), DHA 0.5 μM (■) or DHA 2 μM (▲). Data represent the mean of 3 independent experiments ± SD (*$p < 0.05$ two-way ANOVA with Bonferroni post test). (C) Morphological analysis using May-Grunwald-Giemsa staining at days 11 and 14. Pro-erythroblasts (■), basophilic erythroblasts (⊡), polychromatic erythroblasts (□), orthochromatic erythroblasts (▨) and erythrocytes (▩). Data represent the mean of 3 independent experiments. (D) Globin gene expression by RT-PCR at day 14. Beta-globin (■) and γ-globin (⊡) mRNA are represented as percentages of total globin. Data represent the mean of 3 independent experiments ± SD.

globin mRNA similar to the control. Therefore, DHA did not affect the erythroid differentiation when added to mature stage cells (Fig. 4D).

4. Discussion

CD34+ cells are a suitable model for studying the mechanisms regulating human erythropoiesis *in vitro* (Fibach, 1998). They differentiate from early erythroblasts (pro-erythroblasts and basophilic) towards mature erythroblasts (polychromatic and orthochromatic) over 14 days in response to erythropoietin stimulus (Fibach et al., 1989; Timens and Kamps, 1997).

In the present study, we investigated the effect of DHA, which is the active metabolite of many artemisinins, on human erythroid cell differentiation, to help inform decision as to the use of artemisinin-type compounds to treat malaria during the first trimester of human pregnancy. The DHA concentrations (0.5 and 2 μM) were selected to be in the range of plasma concentration (Cmax) detected in humans after oral administration of DHA or artesunate (McGready et al., 2006a,b; Ward et al., 2007). The same doses were also used in previous animal reproductive studies (Clark et al., 2008a,b; Longo et al., 2006a,b, 2008). Our results indicated that DHA inhibited human erythroid cell differentiation in a dose- and erythroid cell stage-dependent manner. When early erythroid progenitors dominated in the culture, (i.e. DHA added at days 0 and 2), DHA strongly inhibited cell growth and delayed cell differentiation, but only in a transient manner. The effect, in fact, disappeared around day 7, when control and DHA exposed cells started growing and switching to mature erythroblasts. From this time on, DHA-treated cells seemed to become less sensitive to the drug and continued to grow exponentially. Even in the presence of freshly added drug, the differentiation was completed at day 14.

A completely different scenario occurred when DHA was added to pro- and basophilic erythroblasts (i.e. DHA added at days 4 and 7). Specific toxic effects were evident at days 11 and 14: cell proliferation decreased, the erythroid cell differentiation was delayed, GpA expression reduced and the expression of γ-globin increased compared to the controls.

In contrast, no effect on the erythroid cell maturation, but only a reduction of cell growth, were observed when DHA 2 μM was added to mature erythroblasts (day 11).

The first conclusion is that a window of susceptibility to DHA exists also in human developmental erythropoiesis, as described in animal models. The dose is crucial, since most of the toxicity was observed at 2 μM DHA and much less at 0.5 μM. The exact timing for human toxicity cannot be defined *in vitro*, but the target population seems now identified as the pro-erythroblasts and the basophilic erythroblasts. The reason why these erythroblasts are particularly susceptible to DHA compared to other erythroid stages is not clear, yet. We tend to exclude any non-specific interference of the drug on freshly cultured CD34+ cells for several reasons. First, different classes of drugs (like HDAC inhibitors or immunomodulatory compounds) selected to induce and/or increase the synthesis of hemoglobin in CD34+ cultures were previously investigated, and no growth inhibition, neither a delay of the cell differentiation were observed treating freshly isolated stem cells (Aerbajinai et al., 2007; Moutouh-de Parseval et al., 2008). Moreover, DHA resulted inhibitory when the treatment was performed on days 0 and 2, but then cell growth and differentiation resumed normally, whereas a significant and long lasting delay in differentiation was observed when the treatment was done on days 4–7 when the cultures are already stabilized. No effects were seen on later stages. Therefore, the effects seem to be specific for DHA and restricted to a particular differentiation step of the erythroid lineage. Experiments are in progress to define the molecular target for DHA. Preliminary data

indicate that the treatment with DHA interferes with the transcription factors GATA-1/GATA-2 that regulate erythropoiesis (Finaurini S. personal communication).

Artemisinins compounds are sesquiterpene lactones characterized by the presence of a trioxane pharmacophore crucial for the antimalarial activity (Meshnick et al., 1996) and for the antitumor and the antiangiogenic effects on mammalian cells (Efferth, 2007). The endoperoxide bridge is considered responsible for the toxic side effects, neurotoxicity and embryotoxicity (Mercer, 2009). The prevailing hypothesis is that, in order for the molecule to act as an antimalarial, it must be "activated" through the cleavage of the peroxide bridge by an electron transfer (Robert et al., 2002; Stocks et al., 2007). The final target has yet to be identified. Iron (II) either free or within the heme molecules is considered the most likely source of electrons. Like the malaria parasite, erythrocytes contain high amounts of iron and free heme to synthesize hemoglobin and this may activate the peroxide bridge of artemisinin (WHO, 2006a). However, our results seem to support the theory that heme iron does not play a major role in the biological activity of DHA (Longo et al., 2008; Parapini et al., 2004). While cells at all stages of the erythroid maturation contain and use iron (as demonstrated by the expression of the transferrin receptor CD71 both in control and DHA-treated cultures in supplemental data), the toxicity of DHA is limited to the pro- and basophilic erythroblasts. We cannot exclude however that, compared to other stages, the high level of hemoglobin synthesis and iron content of these erythroblasts, might increases their sensitivity to DHA (Gunsilius et al., 2001). Alternatively, since artemisinins seems to target directly the mitochondria (Wang et al., 2010) and erythroblasts are critically dependent on mitochondrial function for high level heme biosynthesis, as well as elevated ATP production for globin gene transcription and translation, this could be the critical point worth to investigate more deeply.

The cell growth rate of all the erythroid stages is impaired by DHA and this is consistent with the observation that artemisinins primarily damage actively dividing cells, such as tumor cells or stimulated endothelial cells (Anfosso et al., 2006; D'Alessandro et al., 2007; Efferth, 2005, 2007). In conclusion, in human erythroid cells and in several animal models, the effects of DHA exposure seems to be limited to early, but not to mature erythroid cells. Our results are consistent with those recently observed in animal model (Clark et al., 2008a), in which artesunate was demonstrate to cause embryo death only during the window of time corresponding to the primitive erythropoiesis occurring in the animal yolk sac. Outside of this period of time and with lower doses, no effects were observed. Our finding together with the extrapolation from animal data would indicate a sensitive period in human beings on weeks 2–6 of pregnancy. In fact, DHA could affect human primitive erythropoiesis occurring during the yolk sac erythropoiesis (weeks 4–6 of gestation), when the fetal blood mainly consisted of pro- and basophilic erythroblasts, but not later, when the liver erythropoiesis predominates. This means that, if treatment with DHA or artemisinin derivatives is performed during the first trimester of human pregnancy, toxic effects on embryo could occur. The loss of erythroblasts, which cannot be replaced, would generate a strong anemia at the yolk sac level leading to the death of embryo (Palis, 2008; Palis and Yoder, 2001). However, at this stage in the absence of clinical data, this extrapolation to human pregnancy remains highly speculative. In conclusion, in human erythroid cells and in several animal models, the effects of DHA exposure seems to be limited to early erythroid cells. Therefore our data support current WHO recommendations for use in pregnancy and therefore, no risk occurs in the second and the third trimester of gestation. Nevertheless, further investigations on DHA toxicity and molecular target are needed. Artemisinin treatment is well tolerated by adult patients and at present, there is no clinical evidence of artemisinin-induced embryotoxicity in human pregnancy. This is reassuring, but definitive data are still lacking (Li and Weina, 2010; Menendez et al., 2007; Ribeiro and Olliaro, 1998; Ward et al., 2007; WHO, 2006a). Moreover, two clinical observations support that DHA toxicity is unlikely to occur in human gestation: the dose and the duration of the treatment. In fact, the DHA plasma concentration is reduced by the increased clearance occurring during pregnancy (McGready et al., 2006b). In addition, differently from experimental conditions both *in vivo* and *in vitro*, exposure to DHA in humans is short compared to the duration of the sensitive window. Treatment is given once daily for 3 days, and the half-lives of these products are ~1–2 h (Nosten et al., 2006). Therefore, both DHA reduced plasma concentration and the short time of exposure may limit the toxic effects. Similar conclusions were reported in a recent review on the embryotoxicity of artemisinin derivatives (Li and Weina, 2010). There is general consensus that, in order to prevent toxicity to pregnant women, further investigations on toxicity and pharmacokinetics as well as an accurate pharmacovigilance are needed to better define the risk-benefit of artemisinin therapy during pregnancy. At present, our data support current WHO recommendations of avoiding the usage of DHA in the first trimester of pregnancy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2010.07.016.

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