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In vivo and in vitro investigations of the effects of the antimalarial drug dihydroartemisinin (DHA) on rat embryos

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Abstract

Artemisinin derivatives are clinically effective and safe antimalarials, but are not recommended during the first trimester of pregnancy because of the resorptions and abnormalities seen in animal reproduction studies. Understanding how, when and what toxicity occurs is crucial to any assessment of clinical relevance. Previously, DHA has been shown in the rat whole embryo culture (WEC) to primarily affect primitive red blood cells (RBCs) causing subsequent tissue damage and dysmorphogenesis. To verify the primary target of DHA in vivo and to detect consequences induced by early damage on embryo development, pregnant female rats were orally treated on gestation days (GD) 9.5 and 10.5 with 7.5 or 15 mg/kg/day DHA and caesarean sectioned on GD11.5, 12.5, 13.5, 15 and 20. A parallel in vitro WEC study evaluated the role of oxidative damage and examined blood islands and primitive RBCs.

In accordance with the WEC results, primitive RBCs from yolk sac hematopoiesis were the target of DHA in vivo. The resulting anemia led to cell damage, which depending on its degree, was either diffuse or focal. Embryonic response to acute anemia varied from complete recovery to malformation and death, depending on the extent of cell death. Malformations occurred only in litters with embryonic deaths. DHA induced low glutathione levels in RBCs, indicating that oxidative stress may be involved in artemisinin toxicity; effects were extremely rapid, with altered RBCs seen as early as GD10.

In establishing the relevance of these findings to humans, one should consider differences in the development of rodents and humans. While yolk sac hematopoiesis occurs similarly in the two species, early placentation and extent of exposure differ. In particular, early hematopoiesis takes only 7 days in rats (during which RBCs expand in a clonal fashion) compared with 6 weeks in humans; thus the susceptible period in relation to the duration of exposure to an artemisinin-based treatment may be substantially different.

Keywords: Dihydroartemisinin; Antimalarial drug; Embryolethality; Teratogenicity; Primitive red blood cells; Oxidative stress; Great vessels; Limb

1. Introduction

Options for prevention and treatment of malaria during pregnancy are limited. Artemisinin-containing combinations (ACTs) are not recommended for treatment of falciparum malaria during the first trimester of pregnancy because of embryolethality and dysmorphogenesis seen in early pregnancy in animals, and are only recommended for treatment of uncomplicated malaria during the second and third trimester of pregnancy in areas of multiple drug resistance [1–3]. To date embryotoxic effects have not been reported in humans [4–8].

The relevance of preclinical data for clinical treatment with artemisinin derivatives in pregnancy was considered in an Expert Panel convened in 2002 by WHO to review [3] all existing

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(largely unpublished) evidence on artesunate, arteether, dihydroartemisinin and artemether alone or in combination with other drugs during pregnancy. We recently reviewed [9] some of these studies carried out on rats, rabbits and mice, by different laboratories including those referred to Chen et al. [10], Li et al. [11] and Clark et al. [12]. Herein we highlight the relevant preclinical conclusions of these studies. A common class effect is the dose-related increase in post-implantation loss with total resorptions at higher dose levels [10–12]. Some studies show instances of morphological abnormalities at doses that cause fetal resorption, without maternal toxicity [11,12]. Clark et al. [13], using artemisinin, DHA, artemether or arteether demonstrated a steep dose–response curve and some have demonstrated marked sensitivity to the day of dosing between Days 10 and 14 of gestation in the rat. Gestation day (GD) 10 was identified as the most sensitive day for the induction of malformations and GD11 for the induction of embryolethality [13].

The experts convened by the WHO to review reproductive toxicology data judged that in animals "all the artemisinin analogues have been associated with embryo-lethality over a narrow dose range and that there are instances of effects on development of the cardiovascular system, the axial skeleton and the limbs", but that "these do not suggest the nature of the toxic action." They considered that "because the safety data are limited, artemisinin compounds should only be used in the second and third trimester when other treatments are considered unsuited" [3].

The review and its recommendations have obvious practical implications both for the use of artemisinin-containing treatments in pregnancy and for the development of new combination drugs based on these compounds. It is therefore crucial to understand the mechanism of the developmental toxicity observed in animal experiments, and to establish the relevance of the animal data to humans.

We previously reported [9] the effects of dihydroartemisinin (DHA) in rat whole embryo cultures (WEC) exposed in vitro on GD9.5–11.5 to DHA at 0.01–2 μg/mL for the entire 48 h culture or 1.5 h at the beginning or at the end of the culture. DHA was studied as the common bioactive metabolite of all artemisinin derivatives in use, and because of its greater intrinsic antimalarial activity [14]. DHA primarily affected red blood cells (RBCs) during yolk sac hematopoiesis with higher concentrations and longer exposure inhibiting angiogenesis. Tissue damage (cell death) and effects on embryo morphology (neural tube, branchial arches, somites and caudal region defects) were attributed to these events.

The previous study identified the early target of DHA in the embryo but did not draw conclusions on the consequence of the embryonic RBCs damage on embryofetal viability and morphology. Although the degree of defective hematopoiesis and angiogenesis seen in embryos exposed to the high doses of DHA were expected to lead to eventual embryofetal death, all embryos survived exposure to DHA at 48 h. Therefore, any additional effects on morphology prior to death could not be confirmed using the WEC model alone. To verify these in vitro findings and to assess the consequences of RBC damage on lethality and dysmorphogenesis throughout gestation, we conducted an in vivo study. The conditions of the WEC experiment were reproduced by exposing pregnant rats to DHA on GD9.5 and 10.5 and then assessing the embryos on different days during gestation.

Doses of 15 and 7.5 mg/kg/day were selected as likely to be embryolethal (expected to induce close to 100% resorption) and 50% embryolethal (to allow for exposure-related morphological changes), respectively. Dose selection was based on published and unpublished data made available to WHO/TDR: Clark et al. [13] showed a steep dose-response with artesunate between 10 (15% resorptions) and 15–17 mg/kg (100% resorptions) when administered on GD10; 15 mg/kg artesunate (38 μmol/kg) produced similar results to 11.1 mg/kg DHA (38 μmol/kg). Studies on DHA given to pregnant rats from GD6 to 16 showed a similar dose-response between doses $\leq$10 and $\geq$20 mg/kg/day [3].

Blood samples for measuring drug exposure were taken 30 min post-dosing at the anticipated time of maximum concentration (T_{max}) in rats [12,15]. Absorption and elimination are very rapid; $t_{1/2}$ for DHA is 50–80 min for artesunate/DHA in malaria patients after administration of either artesunate or DHA [16–18].

The in vivo study was complemented with a parallel study using the WEC model to assess the possible role of reactive oxygen species in DHA-induced developmental toxicity by measuring glutathione (GSH) in visceral yolk sac, embryo proper and embryonic RBCs. Embryos and embryonic blood were also collected at different time-points during the 48 h culture to observe possible DHA effects on Wolffian blood islands and embryonic RBCs by electron microscopy. The use of the in vitro model avoided interference or contamination of embryonic RBCs by maternal blood cells.

2. Materials and methods

2.1. Test article

In the in vivo study DHA (artenimol, Holleykin, Guanzhou, China) was administered at 7.5 and 15 mg/kg/day suspended in 0.5% Methocel + 1% Tween 80. Concentrations of suspensions were 0.75 and 1.5 mg/mL, respectively. The volume of administration was 10 mL/kg.

In the in vitro study DHA was dissolved in DMSO (Sigma) at the concentrations of 0.05 and 0.1 mg/mL immediately before use, and then diluted 1:1000 in the culture medium (added to the culture medium in a volume of 5 μL DHA solution in 5 mL culture medium) to reach the final concentrations of 0.05 and 0.1 μg/mL.

2.2. Animals

Crl:CD(SD)BR virgin male and female rats were supplied by Charles River, Calco, Lecco (Italy), 11–12 weeks old on arrival and maintained under standard conditions (room temperature 21.5 ± 1.5 °C; humidity 55 ± 5% and artificial light 6.00 a.m. to 6.00 p.m.) with food (4RF25 GLP pelletted rat feed supplied by Mucedola, Settimo Milanese, Milano) and tap water ad libitum. Female rats were mated overnight with male breeder rats. The paired animals were left in cohabitation from 4.00 p.m. to 9.00 a.m. the next morning. Copulation was ascertained by vaginal smear. The day on which spermatozoa were found in the vaginal smear was considered as Day 0 of pregnancy.

2.3. In vivo study

DHA was administered orally once daily to pregnant rats on Days 9.5 and 10.5 of pregnancy (i.e., in the afternoon of Days 9 and 10). Control animals received the vehicle alone. There were 14, 16 and 12 pregnant females in the