The Toxicity Of Artesunate On Bone Developments: The Wistar Rat Animal Model Of Malaria Treatment

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Abstract

Artesunate is presently well acclaimed a potent anti-malaria, and hitherto, un-resisted by the malaria parasite plasmodium falciparum. Despite its beneficial effects, cases of toxicity had been reported in the off-springs of women that took the drug at pregnancy. Such effect is presently examined and observed on the fetal bones with the aim to find safe periods and dosages of administration of this promising anti-malaria drug.

INTRODUCTION

Falciparum malaria is a mass killer that went out of control. The drug treatments for this potentially lethal infection that have been most widely recommended and provided over the past 50 years (i.e., chloroquine and sulfadoxine–pyrimethamine) no longer work in most tropical countries. Resistance to these drugs emerged in Asia and South America and spread to Africa. Replacing the failing chloroquine and sulfadoxine–pyrimethamine with effective drugs necessitate the artesunate invention.

Pregnant women are a high-risk group in malaria attack, being more susceptible and likely to develop anemia, and if non-immune, are more likely to develop complications. Birth-weight is consistently reduced by malaria. Therefore, pregnant women desperately need effective and safe antimalarial treatments. The main concern surrounding the general deployment of the Artemisinin-based Combination Therapy (ACTs) is their safety in the first trimester of pregnancy. Work by Chinese scientists in rodents and rabbits conducted in the 1970s indicated that early pregnancy exposure could induce fetal resorption. Recent reproductive toxicity studies have confirmed that this is a class effect of the compounds and is seen in all experimental animal species studied. It results from a specific inhibition of fetal erythropoiesis. Fetal resorption would result in early pregnancy loss. Much more worrying is the potential to cause developmental abnormality. In rodents and rabbits at doses close to human therapeutic doses, artemisinins given in a critical period in the early stage of gestation, may also cause limb deformation. In primates, doses of 12–30 mg/kg daily given continuously between days 20 – 50 postcoitum which is equivalent to 20–56 days in human pregnancy caused fetal resorption and mild long bone shortening, but no abnormalities were seen.

In earlier studies, artemunate, an artemisinin antimalarial, had been reportedly embryolethal and teratogenic in rats, with the most sensitive days being 10 and 11 postcoitum. Pregnant rats were administered a single oral dose of 17 mg/kg artemunate on days 10-11 and conceptuses were evaluated through day 14. Among other defects observed, delay in limb and tail development occurred by day 13. While embryos were viable through day 13, about 77% of embryos had died by day 14, presumably due to hypoxia and/or cardiac abnormalities. The same adverse effects were seen in the brain and in a similar work to compare the developmental toxicity of structurally related artemisinins, dihydroartemisinin, artemether, and arteether to that of artesunate after oral administration to rats.

On the other hand, bones are rigid organs that form part of the endoskeleton of vertebrates. Bones function to move, support and protect the body; produce red and white blood cells and store minerals. The process of bone formation occurs in three stages, orchestrated by specialized bone cells that secrete and absorb materials as needed. First, a soft cartilage-based foundation is laid, upon which mature bone will solidify. Then, minerals containing calcium and phosphate are deposited throughout the foundation, creating a framework for the bone. Finally, this raw material is sculpted and hardened into bone. Errors in this process can
result in developmental defects and bone diseases\textsuperscript{10, 11}. The present work examines the embryotoxic effects of artesunate on the developing bones.

**MATERIALS AND METHODS**

**PROCUREMENT OF ANIMALS**

20 healthy adult Wistar rats comprising of 20 females and 5 males were obtained from the animal holdings of Faculty of Pharmacy, Ahmadu Bello University, Zaria. The females rats were confirmed virgin, and that none of the rats had been previously used for any experimental studies, from the attendants. The animals were brought into, and kept in the animal holdings of the Department of Human Anatomy, and allowed to adapt to the new environment and further breeding. They were fed on rat pellets (Sander Agro Feeds Ltd., Ibadan), with clean and pure drinking water provided liberally. The room was kept clean and well ventilated. 60 female and 15 male rats from the bred were used for the study. Daily assessment of the weights was done until the animals weighed between 200g and 250g; they were subsequently divided into 3 groups: Control - A; and Experimental - B and - C; with 20 rats in each group. The rats were caged in fours with one male, for mating. Confirmation of gestation was done according to the method of Adebisi\textsuperscript{12}.

**ADMINISTRATION OF DRUG**

One sachet of the drug artesunate (Guilin Pharmaceutical Works, China), which contains 12 tablets was purchased from Famex Pharmacy in Samaru, Zaria, Nigeria. One tablet, which contains 50mg of the active ingredient was dissolved in 50mls of distilled water. According to Eweka and Adjene\textsuperscript{9}, oral doses of 2mg/kg and 4mg/kg body weight were administered to pregnant rats in group B and C respectively from the 9th to the 11th day of gestation – being the active osteogenic period in this animal\textsuperscript{13}. The group A rats were used as the control which received 2.0ml/kg body weight of distilled water on the same days.

**SACRIFICE OF ANIMALS**

Two rats were sacrificed each day from day 12 to day 21 by chloroform inhalation and the foetuses retrieved through maternal abdominal incision. The abdominal walls were opened and the full extent of both uterine horns exposed promptly. Before opening either horn, however, a careful count was made of both the foetal swellings and of the metrial glands. Metrial glands are highly vascularised, yellowish nodules, which are found along the meso-metrial margins of the uterine horns where they mark any original implantation site, whether the embryo or the foetus associated with that site survive or not. Records of litter size, number resorbed, dead, living or malformed foetuses were taken. The retrieved foetuses were subsequently weighed and fixed in 10% formol saline. Under stereoscopic microscope, detailed morphological examinations were done to observe any distortion of a gross nature. The foetuses were processed in alizarin red S stains according to the methods of Dix\textsuperscript{14} with sodium alizarin sulphonate, (BDH chemicals Ltd., Poole, England), to monitor and score the daily sequence and extent of calcification in the bones. Photographs of the specimens were taken on Nikkon-M-35 597767 camera.

**PROCEDURES FOR BONE MINERAL ANALYSIS**

The total skeletal calcium and phosphorus contents were determined by the following wet digestion methods in the Soil Science Department, Ahmadu Bello University, Zaria, following these procedures:

The dried bone was ground in to powder, and weighed out 0.5 to 1.0g of the ground sample in to the digestion tube into which was added 25ml of the HNO\textsubscript{3} and mix the contents. Place the tube into the digestion block inside a fume hood and set the temperature control of the digestor to 150 ° C. Digest for 1 ½ hours. Increase the temperature to 230 ° C and digest for another 30 minutes. Reduce the digestor temperature back to 150 ° C. Add 1ml of the HCl to the tube and heat the content at 150 ° C for about 30 minutes. Switch off the digestor. Remove the tubes and add about 30 ml of distilled water to the tube within a few minutes. Add more water to the tube and mix to make up to mark on the tube and mix the content, filter content if necessary or make up to 100ml volumetric flask. Transfer 10ml aliquot from digest into a 50ml volumetric flask. Add 10ml of ammonium molybdate – ammoniumvandase reagent. After 30 minutes read intensity of colour at 470ml wavelength on spectrophotometer to determine calcium level in the solution; and also the phosphorus level, colorimetrically using atomic absorption spectrophotometer SP 192 (PYE UNICAM).

**RESULTS**

**ASSESSMENT OF BONE CALCIFICATION**

Following alizarin red S staining, various skeletal dysgenesis were seen as indicated by impairment of, and retarded calcification, severest in the group C rats. These exhibited non-calcified palate, vertebrae, spine and arches; skull, sternabrae and ribs. Group B foetuses exhibited few cases of
missing ribs, vertebrae, phalanges, non-ossified tarsals and metacarpals (Figs. 1 and 2).

Daily monitor of the sequence of calcification shows that there was delay of about 36 to 48 hours or more in the group B rat bones, and delay of about 12 to 24 hours in the group C foetal bones, as compared with the control rat timing; the longest delay being in the skull and limb bones (Tables 1.1 - 1.3).

ASSESSMENT OF FOETAL RAT MORPHOLOGY

Structural defects observed in the ethanol rat foetuses include microcephally, adactyly, stunted of growth and stature and wrinkled skin. Death in the extremely malformed foetuses in the experimental rats occurred (Table 2).

ASSESSMENT OF BONE MINERAL VALUES

Calcium and Phosphorus are expressed as milligram per gram dry bone weights. In the group C, the calcium content was low throughout the foetal age. Likewise the phosphorus, the levels apparently plateaued through the gestational days and were significantly lower than the control and group B (Table 3). Calcium level was relatively higher in the control and group B foetuses than in the group C throughout the gestational days. Phosphorus contents were higher in the control than group B rats. In the group C, the mineral levels remained relatively lowest throughout the foetal days.

Figure 1

Table 1.1 The earliest days of calcification (Ö) in the skull bone of Wistar rats’ foetuses monitored from day 12 to day 21 of gestation (foetuses, n = 6/group/day)
Figure 2
Table 1.2 The earliest days of calcification (Ö) in the axial bone of Wistar rats’ foetuses monitored from day 12 to day 21 of gestation (foetuses, n = 6/group/day)

Figure 4
Table 1.3 The earliest days of calcification (Ö) in the appendicular bones of Wistar rat foetuses monitored from day 12 to day 21 of gestation (foetuses, n = 6/group/day)
Figure 6
Table 2. Percentages of Surviving foetuses of Wistar rats with malformations

<table>
<thead>
<tr>
<th>TYPES OF DEFORMITIES</th>
<th>GROUP A CONTROL (N=24)</th>
<th>GROUP B (N=20)</th>
<th>GROUP C (N=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maxillo-mandibular dysplasia</td>
<td>0%</td>
<td>6%</td>
<td>10%</td>
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<tr>
<td>Syndactyly</td>
<td>0%</td>
<td>4%</td>
<td>8%</td>
</tr>
<tr>
<td>Missing ribs, sternum and vertebrae</td>
<td>0%</td>
<td>3%</td>
<td>7%</td>
</tr>
</tbody>
</table>

Figure 7
Table 3. Analysis of bone mineral values (mg/g, from day 12 – 21 of gestation) in the Wistar rats’ foetuses

<table>
<thead>
<tr>
<th>FOETAL AGE (Days)</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (GROUP A CONTROL)</td>
<td>3.40</td>
<td>4.50</td>
<td>4.42</td>
<td>4.30</td>
<td>5.67</td>
<td>4.82</td>
<td>4.92</td>
<td>4.86</td>
<td>5.00</td>
<td>5.30</td>
</tr>
<tr>
<td>Calcium GROUP B</td>
<td>3.00</td>
<td>3.20</td>
<td>3.30</td>
<td>3.40</td>
<td>3.64</td>
<td>3.40</td>
<td>3.30</td>
<td>4.10</td>
<td>4.40</td>
<td>4.40</td>
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<tr>
<td>Phosphorus</td>
<td>8.25</td>
<td>8.87</td>
<td>7.85</td>
<td>8.87</td>
<td>8.25</td>
<td>8.22</td>
<td>8.25</td>
<td>6.42</td>
<td>8.86</td>
<td>8.94</td>
</tr>
<tr>
<td>Calcium GROUP C</td>
<td>2.10</td>
<td>2.30</td>
<td>2.50</td>
<td>2.20</td>
<td>2.00</td>
<td>3.00</td>
<td>2.70</td>
<td>2.80</td>
<td>2.50</td>
<td>3.00</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>4.60</td>
<td>4.46</td>
<td>4.65</td>
<td>4.42</td>
<td>4.46</td>
<td>4.23</td>
<td>3.45</td>
<td>4.86</td>
<td>4.58</td>
<td>4.59</td>
</tr>
</tbody>
</table>

Figure 8
Fig. 1: Skeletal defects in the group B rat indicating delay ossification in the caudal bone-(DC); tarsal bone-(DT) and occipital bones-(DO). Alizarin red S stains (x4)
DISCUSSION

Artesunate is a potent blood schizonticide agent for Plasmodium falciparum. It is effective against Plasmodium falciparum resistant to all other antimalarial drugs. It does not have hypnozoiticidal activity. Artesunate has been reported to clear fever in patients with severe falciparum malaria 16 - 25 hours after parenteral administration. Artemisinin and its derivatives inhibit erythropoiesis in the early foetus, this could possibly explain the paleness, wrinkle skin observed and fetal resorption seen in the experimental animals. In its malarial combat quest, artesunate binds tightly to parasitized erythrocyte membranes. The functional group responsible for antimalarial activity of artesunate is endoperoxide bond. Release of an active oxygen species from this bond kills the malaria parasite if accumulated in the erythrocytic cells. Artesunate has demonstrated the fastest clearance of all anti-malarials currently used and acts primarily on the trophozoite phase, thus preventing progression of the malaria disease. It is converted to active metabolite dihydroartemesinin that then inhibits the sarcoplasmic/endoplasmic reticulum Calcium ATPase encoded by P. falciparum. The mechanisms of its osteo-toxicity could not yet be ascertain presently; but it is possible that, like ethanol, artesunate could facilitate the action of the calcification inhibitors, that is, a family of inorganic pyrophosphatases, phosphonates and diphosphonates; these act normally to prevent calcium deposits from forming on soft tissues. Although artesunate produced lesser assaults compared to other drugs, this work is indicative and confirms the adverse effect profiles of the artemisinin-based treatments, its toxicity cum teratogenicity, hence, a caution in its use particularly in early pregnancy. Such fears of its toxicity and a possible emergence of resistance of the malaria parasite to artesunate had of recent posed a threat to the use of this much preferred drug. Hence, Artemisinin-based combination treatments (ACTs) are now generally accepted as the best treatments for uncomplicated falciparum malaria. The combination – depending on the location and prevalence of malaria parasites could be: artesunate–mefloquine, artemether–lumefantrine, and dihydroartemisinin–piperaquine. They are rapidly and reliably effective. Efficacy is determined by the drug partnering the artemisinin derivative. Artesunate–sulfadoxine–pyrimethamine and artesunate–amodiaquine are effective in some areas, but in other areas, resistance to the partner precludes their use. There is still uncertainty over the safety of artemisinin derivatives in the first trimester of pregnancy when they should not be used, unless there are no effective alternatives. Most malaria endemic countries have now adopted artemisinin-based combination treatments as first-line treatment of falciparum malaria, but in most of these, only a minority of the patients that need artemisinin-based combination treatments actually receive them. Because these drugs have not been evaluated extensively in early pregnancy in humans, they should be avoided in patients in the first trimester of pregnancy with uncomplicated malaria until more information is available. There is no evidence for...
adverse effects on the foetus exposed in the second and third trimesters, when these drugs are recommended depending on the safety profile of the partnering drug.

References
12. Adebusi, S. S. Effects of prenatal ingestion of alcohol and folic acid supplementation on foetal osteo-morphology: The Wistar rat model
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