In vitro hemolytic effect of sulfadoxine/pyrimethamine and artemether/ lumefantrine on malaria parasitized erythrocytes of female patients

Festus Chinyereugo Anaba, Blessing Osemengbe Ahiante and Dagogo John Pepple*
Department of Physiology, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria

Abstract: G6PD is an X-linked gene enzyme that protects erythrocytes from hemolysis when they are exposed to antimalarial drugs because of the effects of the free radicals generated by these drugs. We investigated the effects of Fansidar™ (Sulfatoxine/Pyrimethamine) and Coartem™ (Artemether/Lumefantrine) on the hemolysis of malaria parasitized female erythrocytes. Twelve (12) malarious patients attending the University of Benin Teaching Hospital, Benin City, Nigeria, were used in this study. Ten (10) apparently healthy female students from the Medical School, University of Benin, acted as control. Low, normal (the recommended adult dose) and high doses of Fansidar™ and Coartem™ were used to determine the percentage hemolysis by checking the absorbance of the various samples. Data was analyzed by the Student's t-test and ANOVA with $p<0.05$ indicating the level of significance. At low doses of Fansidar™ and Coartem™, no hemolysis occurred, while at normal doses, Fansidar™ showed no hemolysis but significant hemolysis ($p<0.05$) was observed in the Coartem™ group. At high doses, both Fansidar™ and Coartem™ caused significant ($p<0.05$) hemolysis. High doses of both drugs and normal dose of Coartem™ caused significant hemolysis. There was no hemolysis observed in the normal dose of Fansidar™ and low doses for both drugs, similar to the trend reported for male subjects.

Keywords: hemolysis, females, malarious, non-malarious, Fansidar, Coartem.

INTRODUCTION

When red blood cells are placed in hypotonic saline, water enters the cell down its osmotic gradient. Consequently, the cell becomes spherical and ultimately the red cell membrane gives way with liberation of hemoglobin into the surrounding fluid. This phenomenon is put into practical use in the red blood cell osmotic fragility test, which determines the release of hemoglobin from red cells in hypotonic sodium chloride (NaCl) solution (Kumar, 2002). The test is generally useful to ascertain the level of stability and functionality of erythrocyte membrane (Johanning and O'Dell, 1989).

Studies have shown that some anti-malarial agents such as Coartem™ and quinine cause increased hemolysis, while Fansidar™ and chloroquine sulphate cause reduced hemolysis in vitro (Chikezie *et al.,* 2009; Chikezie *et al.,* 2010).

The United States Army researchers identified glucose-6-phosphate dehydrogenase (G6PD) deficiency as the cause of hemolysis after administration of the related anti-malarial drug primaquine. However, deficiency of this enzyme is said to offer some protection against malaria (Beutler *et al.,* 2007).

Phenotypically, G6PD deficiency is most often seen in hemizygous males (Beutler *et al.,* 2007). Females are carriers of the G6PD gene and are therefore rarely deficient of this enzyme. In south-west Nigeria, the G6PD-deficiency prevalence is as high as 28.1% (Luzzatto, 2002).

Malaria is a disease caused by the infection of human erythrocytes with plasmodium falciparum by female anopheles mosquitoes (Snow *et al.,* 2005). The world Health Organisation reports that approximately 3 billion people in 109 countries, 45 of which are within the African region are affected by the disease (World Malaria Report, 2008). The emergence of multi drug resistant parasites has led to the recommendation of artemisinin based combination therapy now generally accepted as the best treatment for malaria (Nosten and White, 2007).

Previous studies have been done on malarious and non malarious male subjects who were not G6PD deficient (Chikezie *et al.,* 2009; Chikezie *et al.,* 2010). We therefore decided to investigate the effect of Fansidar™ and Coartem™ on hemolysis of malaria parasitized female erythrocytes to determine if there are any gender differences.

MATERIALS AND METHODS

Subjects

Twenty-two (22) subjects between the ages of 20 and 45 years were used for this study. They consisted of twelve (12) confirmed malarious female patients attending the

**Corresponding author:* e-mail: djpepple@gmail.com

Present address: Department of Basic Medical Sciences, The University of the West Indies, Mona Campus, Kingston 7, Jamaica.

University of Benin Teaching Hospital, Benin City, Edo State, Nigeria. Ten (10) apparently healthy female volunteers from the Medical School, University of Benin, acted as the control group.

Patients' identification/recruitment

The malarious patients were diagnosed by microscopic examination of their thick and thin blood smears with Giemsa stain for malaria parasites at the Haematology Department of the University of Benin Teaching Hospital, Benin City, Nigeria.

The control subjects had no malaria parasites in their thick and thin blood films.

Ethical approval/consent

Ethical approval was obtained from the Department of Community Health, University of Benin Teaching Hospital, Benin City. The informed consent of the patients and subjects were obtained before recruitment into the study. The study was conducted in accordance with the ethical principles of the Helsinki declaration.

Exclusion criteria

Individuals who were treated for malaria within a three week period before the study were excluded from the control group.

METHODS

Collection of blood samples

5 ml of venous blood was collected from the patient and control groups respectively and stored in K^+EDTA tubes to prevent clotting. They were used within three hours of sample collection.

Drug preparation

Two anti-malarial drugs namely Fansidar™ (Swiss Pharmaceutical Nigeria Ltd., Nigeria) and Coartem™ (Novartis Pharmaceutical Company, Beijing, China) were used in this study.

Normal adult doses of each drug were dissolved in 5 litres of physiological saline solution (PSS) (0.9% w/v). This translates to 315 mg/l of Fansidar™ and 140 mg/l of Coartem™ respectively. Stock solutions of 0.315% of Fansidar™ and 0.140% of Coartem™ (w/v) were prepared.

Three dilutions of the two anti-malarial drugs were prepared from the stock solutions as follows: For Fansidar™, twice the normal dose (Sulfatoxine 0.5mg/Pyrimethamine 0.025mg) was taken as the high dose; the normal being Sulfatoxine 0.3mg/Pyrimethamine 0.015mg and half of the normal dose (Sulfatoxine 0.125mg/Pyrimethamine 0.00625mg) taken as the low dose. Similarly for Coartem™, twice the normal dose (Artemether 0.12mg/Lumefantrine 0.02mg) was taken as the high dose; the normal dose being Artemether 0.09mg/ Lumefantrine 0.02 mg and half of the normal dose (Artemether 0.03mg/Lumefantrine 0.005mg) taken as the low dose.

In vitro hemolysis

The percentage hemolysis was calculated by a modification of the method used by Gupta and Saxena (1980). Briefly, 0.05 ml of each blood sample were put in six test tubes containing 5 ml of buffered physiological saline solution (PSS) in which the different doses, that is, low, normal or high of either Coartem™ or Fansidar™ was added. The solution was mixed carefully, without agitation, and left to stand for 30 minutes at room temperature, after which they were centrifuged by the use of low speed centrifuge (model; 80-20) at 1500 rpm for 5 minutes. The amount of hemoglobin released from the hemolysed erythrocytes was determined by checking the absorbance of the supernatant with a spectrophotometer (model T 70; PG Instrument Ltd, Japan) at a wavelength of 540nm. The absorbance of each sample in distilled water was used as the control with 100% with hemolysis. The percentage hemolysis was calculated from the formula:

$$\% \text{ Hemolysis} = \text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}} / \text{Abs}_{\text{control}} \times 100$$

Table 1: Percentage haemolysis (mean ± SEM) in malaria parasitized and non parasitized erythrocytes

	FL (% hemolysis)	FN (% hemolysis)	FH (% hemolysis)	CL (% hemolysis)	CN (% hemolysis)	CH (% hemolysis)
Parasitized erythrocytes (N=12)	-18.13±3.2 [a*]	-18.3±2.8 [a]	20.90±2.7[a]	-11.58±2.7[b]	13.72±3.7 [b*]	16.58±2.9 [b]
Non parasitized erythrocytes (N=10)	-16.50±3.2 [c]	-17.58±3.2 [c]	20.13±3.8[c]	-18.44±3.6[d**]	14.03±2.2 [d *]	17.13±4.2 [d]

FL = Fansidar™ Low, FN = Fansidar™, Normal, FH = Fansidar™ High, CL= Coartem™ Low, CN = Coartem™, Normal, CH = Coartem™ High, FH [a] vs FL [a] = p<0.05; FH [a] vs FN [a] = p<0.05, ANOVA, CL [b] vs CN [b] = p<0.05; CL [b] vs CH [b] = p<0.05, ANOVA, FH [c] vs FL [c] = p<0.05; FH [c] vs FN [c] = p<0.05, ANOVA, CL [d] vs CN [d] = p<0.05; CL [d] vs CH [d] = p<0.05, ANOVA, *FL vs CL = p<0.05, t-test; FN vs *CN = p<0.05, t-test (parasitized), FN vs *CN = p<0.05, t-test (non parasitized), **CN vs CN = p<0.05, t-test (non parasitized vs parasitized)

When the percentage hemolysis of the sample is less than that of the control, it connotes negative hemolysis.

STATISTICAL ANALYSIS

Data were analyzed by the student's t-test and ANOVA with SPSS version 17 package. The level of statistical significance was taken at $p<0.05$

RESULTS

Negative hemolysis occurred at low doses for Fansidar™ and Coartem™ in both malarious (fig. 1) and non malarious (fig. 2) subjects. However, the percentage of negative hemolysis was significantly ($p<0.05$) higher for Fansidar™ in the malarious group (fig. 1). At normal doses, Fansidar™ showed negative hemolysis while significant ($p<0.05$) hemolysis was observed in the

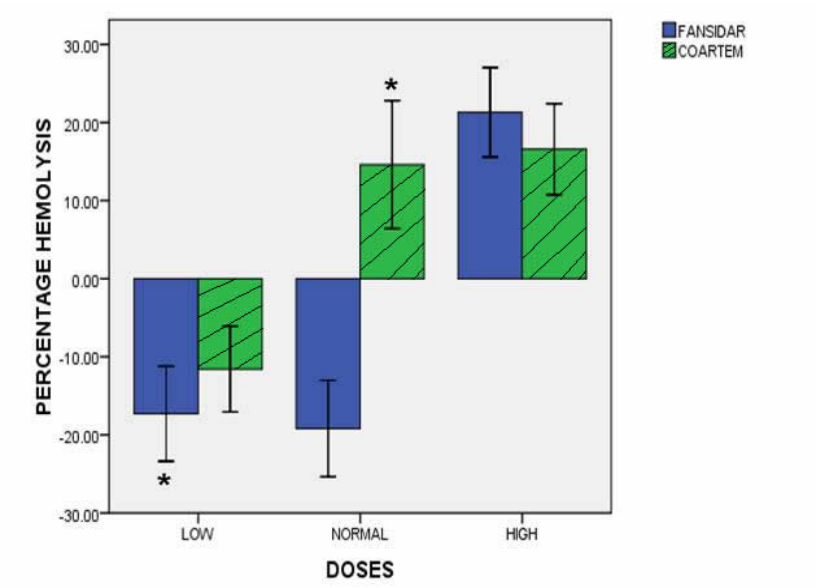

Fig. 1: Comparison of the percentage hemolysis of malaria parasitizied erythrocytes in the presence of low, normal and high doses of Fansidar™ and Coartem™
*Fansidar™ Low vs Coartem™ Low = $p<0.05$; t-test
Fansidar™ Normal vs *Coartem™ Normal = $p<0.05$; t-test

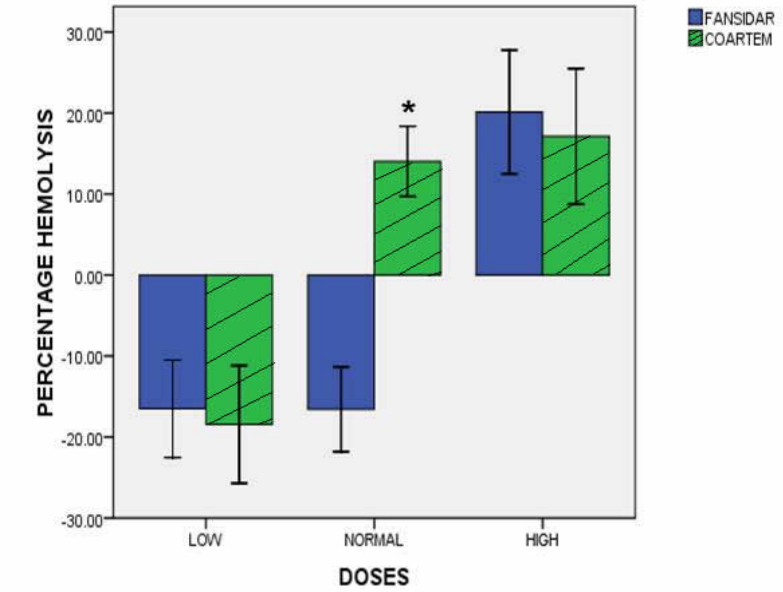

Fig. 2: Comparison of the percentage hemolysis of non parasitizied erythrocytes in the presence of low, normal and high doses of Fansidar™ and Coartem™
Fansidar™ Normal vs *Coartem™ Normal = $p<0.05$; t- test

Coartem™ group for the malarious (fig. 1) and non malarious (fig. 2) subjects. At high doses, both Fansidar™ and Coartem™ caused hemolysis, though they were not significantly different when both groups were compared.

There is no significant difference in the hemolysis pattern of the malarious and non-malarious groups for low, normal and high doses of Fansidar (table 1). Similarly, for the Coartem group the high and normal doses showed no statistically significant difference, though a significant difference ($p<0.05$) was observed in the low dose.

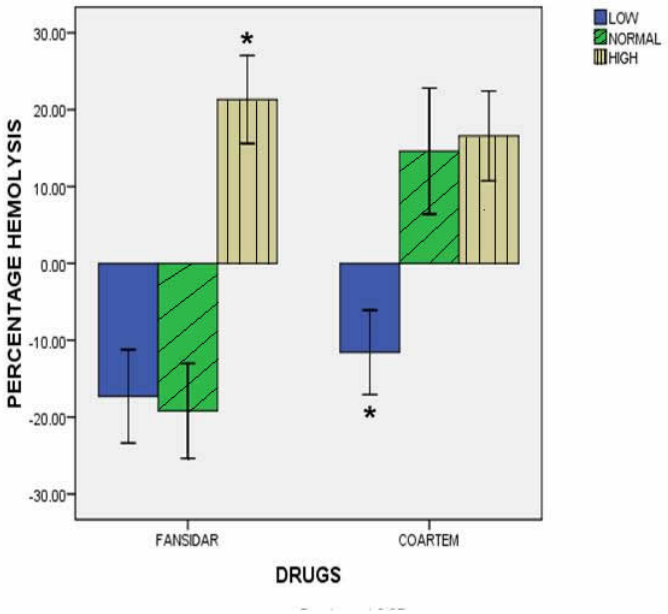

Fig. 3: Comparison of percentage hemolysis of malaria parasitizied erythrocytes in the presence of low, normal and high doses of Fansidar™ and Coartem™
Fansidar™ High vs Fansidar™ Low = $p<0.05$; ANOVA
Fansidar™ High vs Fansidar™ Normal = $p<0.05$; ANOVA
Coartem™ Low vs Coartem™ High = $p<0.05$; ANOVA
Coartem™ Low vs Coartem™ Normal = $p<0.05$; ANOVA

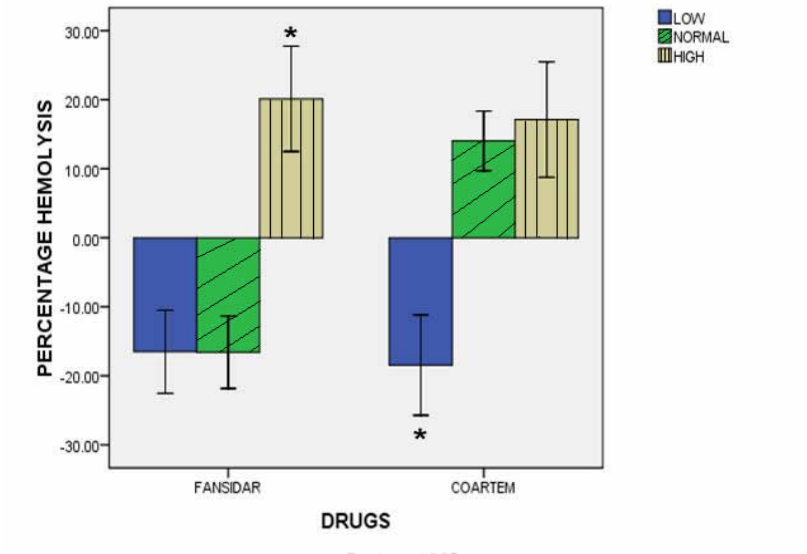

Fig. 4: Comparison of the percentage hemolysis of non parasitizied erythrocytes in the presence of low, normal and high doses of Fansidar™ and Coartem™
Fansidar™ High vs Fansidar™ Low = $p<0.05$; ANOVA
Fansidar™ High vs Fansidar™ Normal = $p<0.05$; ANOVA
Coartem™ Low vs Coartem™ High = $p<0.05$; ANOVA
Coartem™ Low vs Coartem™ Normal = $p<0.05$; ANOVA

Comparing the three drug doses, that is, high, normal, and low for Fansidar™, significant differences ($p<0.05$, ANOVA) were observed between the high versus normal and high versus low doses in both malarious (fig. 3) and non malarious (fig. 4) groups. For Coartem™, significant differences ($p<0.05$, ANOVA) were observed between the low dose versus normal and low dose versus high dose in both malarious and non malarious subjects (fig. 3 and 4).

DISCUSSION

The result from this study showed that Fansidar™ caused negative hemolysis in the low doses of both malarious and non-malarious erythrocytes. This means that the hemolysis observed was less than the one hundred percent observed with distilled water. However, a significant increase in hemolysis was observed at the high dose, suggesting that the hemolytic effect of aqueous solution of Fansidar™ increased with increasing concentration of the drug in the malarious and non-malarious erythrocytes. This observation agrees with the report of Ali and Kadaru (2005) that blood samples incubated in normal dose of Fansidar™ for 48 hours did not show appreciable hemolysis. Hemolysis was observed only in the samples incubated with higher doses of the drug.

Coartem™ caused negative hemolysis in the low doses for both malarious and non malarious subjects. A significant increase in hemolysis was observed at the normal and high doses, also suggesting that the hemolytic effect of Coartem™ increased with increasing concentration of the drug. Meshnick *et al* (1991) had reported that Coartem™ release toxic reactive oxygen species which may hemolyse the erythrocyte membrane.

The foregoing observations from the result of this study could therefore be interpreted to mean that at the normal recommended doses, Fansidar™ had less hemolytic effect compared with Coartem™ .

CONCLUSION

The trend in the pattern of hemolysis observed in the present study with female subjects is similar to previous studies done with male subjects who had been screened for G6PD deficiency (Chikezie *et al.*, 2009; Chikezie *et al.*, 2010), suggesting that there is no gender difference in the hemolytic effect of the anti-malarial drugs Fansidar™ and Coartem™.

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