IN VITRO-MODULATION OF HbS ERYTHROCYTE PARAMETERS BY PREDNISOLONE TESTING FOR Fe^{2+}/Fe^{3+} RATIO, HBS GELATION AND OSMOTIC FRAGILITY

ABSTRACT

Aim: It has been discovered that some drugs or medications taken for certain ailments are either prosickling or antisickling in nature. In this study, a corticosteroid by the name of prednisolone was investigated to determine its possible effects on human haemoglobin-S gelation, erythrocyte fragility and Fe^{2+} and Fe^{3+} concentrations.

Materials and method: Various concentrations of the drug (0.05, 0.1, 0.3, 0.5 and 1mg/ml) were used to determine the effects on human haemoglobin-s, gelation rate, erythrocyte fragility, Fe^{2+} & Fe^{3+} concentrations. Absorbance reading was taken at 540nm using a spectrophotometer.

Results: The results showed that Prednisolone increased haemoglobin S gelation at all concentrations ($p < 0.05$) when compared to the control. The Fe^{2+}/Fe^{3+} ratio showed a reduction in haemoglobin values at 0.3, 0.5 and 1.0mg/ml concentrations when compared to the control and a slight increase at 0.05 and 0.1mg/ml. For Erythrocyte Fragility, there was destabilization of red cell in all concentrations.

Conclusion: This study suggests that this drug could have some undesirable effects on sickle cell subjects.

KEY WORDS: Erythrocyte, Fragility, Gelation, Haemoglobin, Prednisolone, Sickle cell.

1. INTRODUCTION

Sickle-cell disease (SCD) is a group of genetically passed down blood disorders. The most common type is known as sickle-cell anaemia. It results in an abnormality in the oxygen-carrying protein haemoglobin found in red blood cells. This leads to a rigid, sickle-like shape under certain circumstances. Problems in sickle cell disease typically begin around 5 to 6 months of age. A number of health problems may develop, such as attacks of pain ("sickle-

cell crisis"), anaemia, bacterial infections, and stroke. Long term pain may develop as people get older. The average life expectancy in the developed world is 40 to 60 years [1, 2, 3].

Sickle-cell disease occurs when a person inherits two abnormal copies of the haemoglobin gene, one from each parent. Several subtypes exist, depending on the exact mutation in each haemoglobin gene. An attack can be set off by temperature changes, stress, dehydration, and high altitude. A person with a single abnormal copy does not usually have symptoms and is said to have sickle-cell trait. Such people are also referred to as carriers. Diagnosis is by a blood test and some countries test all babies at birth for the disease. Diagnosis is also possible during pregnancy. The care of people with sickle-cell disease may include blood transfusion. A small proportion of people can be cured by a transplant of bone marrow cells [4, 5].

As of 2013 about 3.2 million people have sickle-cell disease while an additional 43 million have sickle-cell trait. About 80% of sickle-cell disease cases are believed to occur in sub-Saharan Africa. It also occurs relatively frequently in parts of India, the Arabian peninsula, and among people of African origin living in other parts of the world. In 2013, it resulted in 176,000 deaths, up from 113,000 deaths in 1990. The condition was first described in the medical literature by the American physician James B. Herrick in 1910. In 1949 the genetic transmission was determined and in 1954 the protective effect against malaria of sickle-cell trait was described [6, 7]. In Nigeria about 10,000 sickle cell anemia are born yearly. The carrier frequency ranges between 10% and 40% across Africa [7, 8].

Sickle cell disease is also a single-gene aspect that results in sickle shaped red blood cells. Although the manifestations of sickle cell disease have been described, variations in the severity and a number of manifestation as well as interactions with other health events leads to significant gap in the understanding of the natural history of the disorder for as impairments to renal, cardiac and pulmonary organ function are known to occur in sickle cell

patients. However the descriptions of these outcomes is generally retrospective in nature and occurs when organ damage is severe [9, 10, 11].

In sickle hemoglobin (Hbs), a valine is substituted for glutamic acid on the surface of the Hbs molecule in the sixth codon of the betaglobin (HBBgluval). This change endows Haemaglobin s when deoxygenated with a new property, the capacity to polymerize conspires against an indispensable feature of the red cell [12, 13].

The polymerization of deoxy Hbs is the primary and indispensable event in the molecular pathogenesis of sickle cell disease. The polymer has the form of an elongated rope- like fibre which usually aligns with other fibres forming a fascile and distorting the red cell into the classic crescent or sickle shape, among other many abnormal shapes, and resulting in a marked degree in cell deformability [14].

Haemoglobin-S can polymerize when oxygenated and depolymerize when reoxygenated infinitely, however, the sickle erythrocyte membrane can withstand only a finite number of these cycles before it is irreversibly deformed and recognized via the many abnormal “sickle” shapes that circulate in patients [3].

Polymerization alone does not account for the physiology of sickle cell disease. Changes in red cell membrane structure and function, disordered red cell volume control, also contribute to the physiology of the disease. Furthermore, increased red cell adherence to vascular endothelium, misregulation of vaso-activity also contribute to sickle vaso-occulusion and haemolysis. In genetics, these are called pleiotropic effects, because they go beyond the immediate consequences of the abnormal gene [15].

It is known that most sickle cell patients usually experience severe painful crisis and over the years there has been the use of synthetic drugs to manage or alleviate this pain. This research work is concerned with the ultimate goal of determining the modulatory effects of the drug

commonly known as prednisolone and how it affects the human erythrocyte through its effect on Fe^{2+}/Fe^{3+} ratio, gelation rate and osmotic fragility in ranging concentrations

The objectives of this work are to determine the effect of predinosolone on human Hbs gelation rate, secondly to determine the effect of predinosolone on haemoglobin-S erythrocyte osmotic fragility and finally, to determine the effect of predinosolone on human Hbs erythrocyte haemoglobin (Fe^{2+}) and methaemoglobin (Fe^{3+}) ratios.

Prednisolone is a steroid under the class of corticosteroids, gluco corticoids and anti-inflammatory agent. The brand name is prelone. It prevents the release of substances in the body that causes inflammation. Prednisolone is used to treat many different conditions such as allergic disorders, skin conditions, ulcerative colitis, arthritis, lupus, psoriasis or breathing disorders. It is the active metabolite of the drugs prednisone and is used especially in patients with liver failure as these individuals are unable to metabolize prednisone into active prednisolone. Adverse effects are not generally seen with short term therapy, but weight gain, impaired immune response and disturbances in behaviour commonly occur with longer durations of treatment [16].

2. MATERIALS AND METHOD

The blood sample of 5ml was collected from adult male and female donors by vein puncture using a 5ml syringe and needle. The blood samples were confirmed as HbSS using standard haemoglobin electrophoresis. All experiments were carried out with fresh heparinized blood. Prednisolone tablets were bought from a Pharmacy in Port Harcourt Rivers State.

2.1. Preparation of five serial dilutions of prednisolone and tablet solution

Prednisolone of 50mg tablet was crushed using mortar and pestle then added to a 50ml of distilled water to form the solution. The solution was then stirred properly. After this, different concentrations of prednisolone were made using different reagent bottles.

Bottles	Conc/mg/ml	Vol. of Prednisolone (ml)	Volume of distilled water (ml)
1	0.05	1	19
2	0.10	2	18
3	0.30	6	14
4	0.50	10	10
5	1.00	20	0

The reagent bottles containing the solution were then stored in the refrigerator to preserve the freshness of the solution.

Control procedure for prednisolone:

Exactly 5ml of distilled water was put in a test tube using a micro pipette, 0.1 ml of blood sample was then added to the 5ml of water in the test tube using a micropipette. It was shaken and the mixture was transferred into a cuvette. The absorbance reading was taken at different wavelength of 540nm and 630nm using a spectrophotometer.

Test experiment for prednisolone:

Five test tubes were labeled at different concentrations in mg/ml and 5ml of distilled water was added to each of the test tubes.

Then 0.1 ml of the blood sample was added to the 5ml of water in the test tubes.

The test tubes were shaken by inversion until equal mixture was obtained. Exactly 0.1 ml of the test compound (prednisolone solution) was added into the 5ml of water and then 0.1 ml of blood was added into each of the test tubes with the different concentrations in (mg/ml).

The mixtures were transferred into a cuvette and then the absorbance reading were taken at 540nm using a spectrophotometer.

Preparation of control sample for osmotic fragility:

Exactly 5ml of normal saline was measured into a test tube containing 0.05ml of blood this was thoroughly mixed by inverting the tube several times. The suspension was allowed to stand for 30minutes after which the content was centrifuged for 5 minutes at 1200rpm.

2.2. Osmotic fragility procedure

5ml of normal saline, was measured into 5 test tubes. Then 0.1ml of the test compound was also added into each of the test tubes. To each of the test tubes 0.05ml of blood sample was added and mixed thoroughly by inverting the tubes several times. The suspensions were allowed to stand at room temperature after which the content was centrifuged for 5 minutes at 1200rpm. The relative amount of haemoglobin released into the supernatant was determined using a Spectrophotometer at the maximum wave length of 540nm. The physiological saline solution and distilled water served as 100% lysis point and blank respectively [17, 18].

2.3. Haemoglobin-S gelation (polymerization) experiment.

Principle: Haemoglobin-S undergoes gelation when deprived of oxygen.

Sodium metabisulphite was used as a reductant. The haemoglobin polymerization (gelation) experiment was based on the method described by Noguchi and Schechter [19].

Control experiment: Sodium metabisulphite (4.4ml), 0.1ml of HbSS haemosylate and 0.1ml of normal saline were quickly mixed in a cuvette and the absorbance read at 540nm, and at 1 minute intervals for 5 minutes.

Test experiment: Sodium metabisulphite (4.4ml), 0.1ml of HbSS haemosylate and 0.1ml of test compound were quickly mixed in a cuvette and the absorbance read as was the control.

Preparation of control sample for Fe^{2+} and Fe^{3+} ratio:

Exactly 5ml of distilled water was measured in a test tube containing 0.1ml of blood. They were mixed and allowed to stand for 5 minutes. Fe^{2}+ and Fe^{3}+ concentration were measured using spectrophotometer at 540nm and 630nm respectively.

2.4. Procedure for Fe^{2+} and Fe^{3+} ratio of predinosolone

Five test tubes were labeled 0.05, 0.1, 0.3, 0.5, 1.0mg/ml respectively representing the different concentrations of the drug predinsolone. Then 5ml of distilled water was added into the five labeled test tubes.

Exactly 0.2 ml of normal saline was added into each of the test tubes containing the distilled water using a micropipette. Then 0.1ml of the erythrocyte haemosylate was added into each of the test tubes. Subsequently, 0.1ml of the test solution (the drug) was added into each of the test tubes with the respective concentration except the controls. The solution was properly mixed. The absorbance readings were taken respectively [17, 18, 19].

2.5. Statistical analysis

All data were subjected to statistical analysis. Values are reported as mean ± standard error of mean (SEM) while one way ANOVA was used to test for differences between treatment groups. The results were considered significant at p-values of less than 0.05, that is, at 95% confidence level ($p < 0.05$).

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3. RESULTS

The results of the *in vitro* modulation of some Hbs erythrocytes parameters by prednisolone are illustrated in the Tables 1 to 3.

Table 1: Effect of Predinosolone on Hbs gelation rate

Predinosolone	Erythrocyte fragility and Hb gelation (optical density) standard = 0.03. (Five minutes duration at one minute interval)

(mg/ml)	0	1	2	3	4	5
Control 0.00	0.00 ± 0.00	0.015 ± 0.00	0.015 ± 0.00	0.015 ± 0.00	0.015 ± 0.00	0.015 ± 0.00
0.05	0.00 ± 0.00	0.160 ± 0.00	0.161 ± 0.00	0.162 ± 0.00	0.163 ± 0.00	0.164 ± 0.01*
0.10	0.00 ± 0.00	0.180± 0.00	0.181 ± 0.00	0.182 ± 0.00	0.183 ± 0.00	0.184 ± 0.01*
0.30	0.00 ± 0.00	0.184 ± 0.00	0.185 ± 0.00	0.186 ± 0.00	0.187 ± 0.00	0.188 ± 0.00*
0.50	0.00 ± 0.00	0.186 ± 0.00	0.187 ± 0.00	0.188 ± 0.00	0.189 ± 0.00	0.190 ± 0.00*
1.00	0.00 ± 0.00	0.188 ± 0.00	0.189 ± 0.00	0.190 ± 0.00	0.191 ± 0.00	0.192 ± 0.00*

183 Results are means of three determinations ± standard deviation.

184 *Statistically significant at 95% confidence level, ($P < 0.05$).

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186 This result shows that Prednisolone increased Hbs- Gelation in a concentration and time
187 dependent manner.

188 **Table 2: Effect of Predinosolone on Hbs Fe^{2+}/Fe^{3+}**

Prednisolone mg/ml	Fe^{2+} /OD 540 nm	Fe^{3+} /OD 630 nm

Control	0.142 ± 0.00	0.142 ± 0.00
0.05	0.171 ± 0.00	1.184 ± 0.00
0.10	0.174 ± 0.01*	0.187 ± 0.00
0.30	0.178 ± 0.00	1.194 ± 0.01*
0.50	0.179 ± 0.01*	1.200 ± 0.01*
1.00	0.183 ± 0.01*	1.215 ± 0.00

189 Results are means of three determinations ± standard deviation.

190 *Statistiscally significant at 95% confidence level, ($P < 0.05$).

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192 **Table 3: Effect of predinosolone on Hbs osmotic fragility**

Predinosolone mg/ml	OD	% Hemolysis
Control	0.400	0
0.01	0.352+ ± 0.02	4.60 ± 0.02*
0.05	0.462 ± 0.02	6.00 ± 0.02*
0.3	0.581 ± 0.02	18.00 ± 0.02*
0.5	0.623 ± 0.02	22.00 ± 0.02*
0.1	0.672 ± 0.03	27.00 ± 0.03*

193 Results are means of three determinations ± standard deviation.

194 *Statistically significant at 95% confidence level, ($P < 0.05$).

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4. DISCUSSION

Sickle cell disease is genetic disorder which has over the years proved difficult to manage which results from the abnormal haemoglobin S.

Sickle cell diseases has caused lots of problem to human life, that is why efforts are constantly being made to find a permanent cure to the disease.

For Fe^{2+} and Fe^{3+} the result indicates a progressive effect in the values obtained with increasing concentration of the drug from 0.01mg/ml to 0.1 mg/ml. The results also show that Prednisolone inhibited Hbs- Gelation at increasing concentration and so a reduction in the polymerization rate.

In Erythrocyte fragility, at 1mg/ml concentration, Prednisolone stabilized the red cell, but destabilized the cells at lower concentrations which include 0.5mg/ml, 0.3 mg/ml, 0.05mg/ml and 0.1mg/ml. this indicates that Prednisolone inhibits Erythrocyte fragility in Haemoglobin-S patient but can only be administered at 1mg/ml concentration if so desired.

Several works have been carried out in the past to determine the effect of various drugs taken for other ailments on sickle cell haemoglobin gelation [20, 21, 22, 23, 24, 25]. The biochemistry of haemoglobin S and the hypothesized pathophysiologic mechanisms of complications provide principles for treatment of complications in sickle cell syndromes. These principles must be applied in preventing and treating almost every complication observed during the clinical course of patients. The pathophysiology of pain and principles of pain management are shown to provide a rational basis for the use of analgesics to treat pain associated with complications [26, 27].

Also good medical as well as nutritional management has helped immensely in reducing the incidence of death among sicklers. Most of these treatments are aimed at alleviating one or

more of the complications that accompanies crisis [28, 29]. At present time, the important aspect of treatment is supportive care with fluids and analgesics and the judicious use of transfusion. Most currently some chemicals have been found to effective in preventing or managing sickle cell disease in various way. These includes nutritional supplement "sicklevit" an antisickling formula have been formulated. It exerts its effects in reducing the frequency of crisis occurring and act as a maintenance formula [7, 27, 30].

5. CONCLUSION

In conclusion, the present study has shown that Prednisolone has a sickling effect through its inhibition of Haemoglobin-S Gelation rate. Furthermore, the drug had negative effect on Hbs erythrocyte fragility where there was a gross destabilization of the erythrocyte at varying concentrations except at 1mg/ml and Fe^{2+} and Fe^{3+} concentration which was also seen to decrease when compared to the control. Therefore Prednisolone shouldn't be administered to HBS patients.

Competing Interests

Authors have declared that no competing interests exist.

6. ETHICAL APPROVAL:

This research work was carried out with the approval of the University of Port Harcourt research ethics committee.

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