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EFFECT OF METFORMIN AND ROSUVASTATIN ON ERYTHROCYTES OF PATIENTS WITH SICKLE CELL DISEASE: AN *IN-VITRO* STUDY

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Metformin, Statin, Sickle cell disease, Antioxidant, Deformability

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ABSTRACT: Objectives: Sickle cell disease (SCD) is a common hereditary blood disease with a progressive clinical course and multiple end organs damage. Due to its intercalated pathophysiology, several trials have been conducted targeting its pathophysiology in an attempt to modify the clinical course and minimize complications. Metformin and rosuvastatin are known to have pleiotropic effects such as antioxidant, anti-inflammatory and vasculoprotective properties making them a candidate for testing in SCD. **Aim:** To test possible effects of metformin and rosuvastatin on oxidative stress, erythrocytes deformability, %hemolysis in blood samples of patients with SCD. **Materials and Methods:** Blood samples were obtained from 36 SCD patients at stable conditions. The samples were incubated with metformin (1 mg/ml) or rosuvastatin (0.25 mg/ml). Oxidative stress was induced by incubating the samples with H_2O_2 for 30 min. Reduced glutathione (GSH), lipid peroxidation products (thiobarbituric acid reactive species TBARS), % hemolysis was determined by spectrophotometer and erythrocytes deformability by filtration method. **Result:** Treatment of blood with metformin or rosuvastatin resulted in a significant reduction in TBARS level (-32%, -37% for metformin and rosuvastatin respectively) with an increase in GSH level (+ 68%, + 75% respectively). Metformin and rosuvastatin reduced % hemolysis, P=0.01. Metformin treatment but not rosuvastatin significantly improved erythrocytes deformability, P=0.01. **Conclusion:** It is concluded that metformin and rosuvastatin, favorably changed TBARS and GSH levels in blood samples of SCD in which oxidative stress was induced by H_2O_2. These changes possibly resulted in reduced % hemolysis and improved erythrocytes deformability.

INTRODUCTION: Sickle cell disease (SCD) is a heterogeneous group of hereditary blood disorders that are characterized by the presence of at least one abnormal hemoglobin type (hemoglobin S) [1].

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It is a common blood disorder worldwide [1]. Although polymerization of HbS represents the initial step of the pathophysiology of the disease, SCD seems to have more complex pathophysiology with the emerging role of oxidative stress in the initiation and development of vascular complications of the disease [2].

Reactive oxygen species (ROS) result from different sources such as free heme that results from excessive hemolysis, a higher rate of HbS autoxidation and sickle erythrocytes that express a

high level of NADPH oxidase which is a major source of enzymatic generation of ROS [2, 3]. Free hemoglobin and ROS are potent scavengers of nitric oxide (NO) and can impair its bioavailability [2, 4]. This eventually can lead to a vicious cycle of coagulation and inflammation and finally vascular endothelial dysfunction with ultimate end-organ damage [4]. Therefore, targeting oxidative stress has been identified as one of the strategies of management of SCD [5]. In that context, SCD demonstrates remarkable similarities to changes observed in atherosclerosis and other chronic vascular diseases [6].

Metformin and statins, two distinct drugs that had been known to have multiple beneficial effects apart from their main pharmacological actions, like having antioxidant, anti-inflammatory and vasculoprotective properties [7, 8]. These effects can be extended to involve various aspects of SCD. In the last decade, several preclinical and clinical trials were conducted to explore the effects of these two drugs in patients with SCD [9, 10].

Recently, two *in-vitro* studies showed that metformin could induce fetal hemoglobin in erythroid cell culture [11, 12]. This effect can be beneficial in patients with SCD as it is well-known that the induction of fetal hemoglobin can decrease the rate of polymerization of HbS [5].

The aim of the study, therefore, was to test the possible effects of metformin and rosuvastatin on some parameters of oxidative stress, % hemolysis and erythrocytes deformability in blood samples obtained from patients with SCD.

MATERIALS AND METHODS:

Drugs, Chemicals and Instruments: Metformin HCl and Rosuvastatin calcium pure powder were donated by Pioneer company for pharmaceutical industries, Sulaymaniyah, Iraq, 2- thiobarbituric acid (Fluka AG, Switzerland), 5,5′-dithiobis(2-nitrobenzoic acid) (Himedia, India), Dimethyl sulfoxide DMSO (SDFCL, India), Hydrogen peroxide H_2O_2 50% (Scharlau, Spain), L- arginine pure powder (Nusci Institute and Corporation, USA), Phosphate buffered saline (Tissue pro technology, USA), Trichloroacetic acid (Thomas Baker chemicals, India), Tri-sodium citrate (Riedel-de Haen AG, Germany), Auto hematology blood analyzer (Mindray, Germany), Cyclopore polycarbonate membrane filter (GE Whatman, United Kingdom), UV- visible Spectrophotometer (Cecil Instruments, England).

Study Design: The study was conducted in the Department of Pharmacology at Basrah College of Medicine between January and May 2017. Patients with SCD were selected during their regular visit to Basrah center for hereditary blood diseases. The diagnosis of SCD was confirmed by Hb electrophoresis and Hb variant testing. Thirty-six patients with SCD were recruited for the study; their ages ranged between 5-15 years. The patients were symptoms free and stable. The study protocol was explained to the patients or parents with an emphasis that the outcome of the study conditions a direct benefit to the patients. All patients were willing to participate in the study; nevertheless, written consents were obtained from them or their parents.

The study protocol was approved by an Institutional Ethical Committee at Basrah College of Medicine (Approval no: 304/10/2016).

Exclusion Criteria Included: History of blood transfusion during the last three months, patients on hydroxyurea or other chronic medications apart from folic acid and zinc supplement, patients with G6PD deficiency and those in acute vaso-occlusive crisis.

Blood Sampling: Blood samples were collected from the patients by a qualified nurse during their visit to the center for a routine checkup. Blood was taken from antecubital veins, and an additional two milliliters of blood were collected for the present study. Aliquots of blood were placed in EDTA containing test tubes for complete blood count analysis using auto hematology blood analyzer at the hematological laboratory of the center. The remaining amount of the blood was placed in heparinized test tubes and stored at 4 ºC. After 2 h, deformability testing was performed and at 24 h, % hemolysis was determined, and at a maximum of 72 h, markers of oxidative stress were measured.

Preparation of Drugs: The final concentration used for the study was determined after a pilot study that involved the range of concentrations between 0.05-1 mg/ml for metformin and 0.02-0.25 mg/ml for rosuvastatin on GSH and TBARS levels

in blood samples. Based on these effects, a working concentration of 1 mg/ml for metformin and 0.25 mg/ml for rosuvastatin were found appropriate for *in-vitro* testing. Metformin was dissolved in 0.9% NaCl (normal saline) while rosuvastatin was dissolved in 2.5% dimethyl sulfoxide (DMSO). Normal saline was used as a control for metformin; DMSO was used as a control for rosuvastatin.

The stock solutions were kept at -20 ºC for one week.

Laboratory Measurements:

Estimation of Whole Blood GSH: The test was performed on 21 blood samples. A 0.2 ml of venous blood was incubated with an equal volume of metformin or rosuvastatin or their controls for 60 min at 37 ºC followed by 30 min incubation with 0.25% H_2O_2. GSH concentration was determined by using Ellman reagent as described by Layton and Roper, 2011 [13], through reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) by sulfhydryl compounds to form a yellow colored compound which was measured by a spectrophotometer at an absorbance of 412 nm. GSH concentration was expressed in µmol/L using a molar extinction coefficient ($13.6\times 10^3\ M^{-1}.cm^{-1}$).

Estimation of TBARS: This test was used for assessing lipid peroxidation of cell membranes. A 0.1 ml of venous blood was incubated with an equal volume of metformin or rosuvastatin or their controls for 120 min at 37 ºC, followed by 30 min incubation with 0.25% H_2O_2. The level of TBARS was measured according to a method described by Esterbauer and Cheeseman, 1990 [14], with the slight modification made on the reaction between TBARS and thiobarbituric acid TBA in acidic medium at 90ºC to produce purple color that can be estimated spectrophotometrically at 535 nm. TBARS was measured for 18 samples out of 21 and the results were expressed as nmol/ml using a molar extinction coefficient ($1.56 \times 10^5\ M^{-1}.cm^{-1}$).

Estimation of % Hemolysis: Percent hemolysis and erythrocytes deformability were estimated in blood samples obtained from 15 patients with SCD.

The principle of this test was when erythrocytes are hemolyzed, free hemoglobin released to the surrounding medium. Free hemoglobin can be assessed spectrophotometrically at 540 nm according to Repsold and colleagues, 2014 [15]. Fifty microliters of whole blood were incubated with two milliliters of normal saline containing either metformin or rosuvastatin or their controls at 37 ºC. As a baseline indicator of complete hemolysis, the same procedure was repeated using distilled water instead of normal saline. After 24 h of incubation, samples were centrifuged at 5000 rpm for five min at ambient temperature, free hemoglobin was assessed, and % hemolysis was expressed as:

$$\% \text{ hemolysis} = \frac{\text{Abs. of test or control}}{\text{Abs. in distilled water}} \times 100\%$$

Estimation of Deformability: The test was performed on 13 blood samples out of 15 (Two samples were lost during processing).

Two milliliters of blood was centrifuged at 3000 rpm for five min, the plasma and buffy coat were removed by aspiration. The packed RBC were washed three times in normal saline then re-suspended in normal saline containing 3 mmol of L- arginine at about 2% hematocrit. The final volume of two milliliters of erythrocytes suspension plus metformin or rosuvastatin or their corresponding controls were transferred to two-milliliter plastic tubes. The blood was deoxygenated by closing the tubes, incubated in water bath at 37 ºC for two h with gentle shaking and deformability was assessed by filtration. Filtration was made by placing two milliliters of erythrocytes suspension in an ordinary syringe fixed tightly on top of a small chamber fitted with a five microns membrane filter. A gauge monitored negative pressure of about 20 mmHg using a small vacuum pump was applied on the lower end of the chamber. The time required for filtration of one milliliter of blood suspension was taken as an index of deformability. This method was described by Reid *et al.,* 1976 [16].

Statistical Analysis: Statistical analysis was done using SPSS, version 20 (SPSS Inc., Chicago, USA, available at http:www.spss.com). Paired T-test was used for comparing dependent variables, P value < 0.05 is considered statistically significant.

RESULTS:

Patients Characteristic: Thirty-six patients were recruited for the study, with a mean age of 9.42 ± 3.06 year. They were anemic with mean

hemoglobin of 8.06 ± 1.26 g/dl. These data are presented in **Table 1**.

TABLE 1: PATIENTS CHARACTERISTIC

Parameter	Mean ± Std. Deviation
Age (year)	9.42 ± 3.06
Hemoglobin (g/dl)	8.06 ± 1.26
Hematocrit %	26.67 ± 3.52
MCV (fl)	79.49 ± 9.73
MCH (pg)	24.08 ± 4.04
MCHC (g/dl)	30.21 ± 2.57
Male/female	21/15
SS/SF	29/7

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; SS, homozygous for hemoglobin S; SF; heterozygous inheritance (sickle/βº thalassemia disease).

Effect of Treatment on H_2O_2- Induced Oxidative Stress:

Effect on GSH: In the control group, blood samples were treated with DMSO (2.5%) for rosuvastatin, normal saline for metformin. In these samples, oxidative stress is induced by incubating the blood in H_2O_2 (0.25%) for 30 min. The level of GSH in the control group was 89.4 ± 52 µmol/L which was significantly increased by rosuvastatin to 156 ± 68.8 µmol/L, P=0.001. Similarly, metformin treatment significantly increased the level of GSH from 91.6 ± 47 µmol/Lin the control group to153.6 ± 64.3 µmol/L in metformin-treated group, P=0.001 **Table 2**.

Effect on Lipid Peroxidation: The level of TBARS in the control group in which oxidative stress was induced by H_2O_2 (0.25%) for 30 min, was 13.4 ± 5.6 nmol/ ml which was significantly decreased to 8.4 ± 4.6 nmol/ ml in the blood samples treated with rosuvastatin, P=0.001. The similar reduction in TBARS level was observed with metformin **Table 2**.

TABLE 2: THE EFFECT OF ROSUVASTATIN, METFORMIN ON BLOOD GSH AND TBARS LEVELS

Parameter	Rosuvastatin			Metformin			P value
	Control	Treatment	% change	Control	Treatment	% change	
GSH (µmol/L) (n= 21)	89.4±52	156±68.8*	+74.5%	91.6±47	153.6±64.3*	+67.7%	0.001
TBARS (nmol/ml) (n= 18)	13.4±5.6	8.4±4.6*	-37.3%	12.6±4.2	8.6±3.5*	-31.7%	0.001

GSH: reduced glutathione; TBARS, thiobarbituric acid reactive species
Control group for rosuvastatin (2.5% DMSO); control group for metformin (normal saline)
* Significantly different from the corresponding control

Effect on % Hemolysis: Incubation of blood from patients with SCD with rosuvastatin at a concentration of 0.25 mg/ ml for 24 h showed a significant reduction in % hemolysis from 39.7% ± 23.5 in the control group to 27.5% ± 20.5 (-30.7%) in the rosuvastatin-treated group, P=0.002. In the same setting of the experiment, metformin at a dose of 1 mg/ml significantly reduced % hemolysis from 36.6% ± 19.7 in control to 20.2% ± 14.2 in the group treated with metformin (-44.8 %), P=0.001. Metformin produced the significantly higher reduction in % hemolysis compared to rosuvastatin, P=0.026. These data are presented in **Fig. 1**.

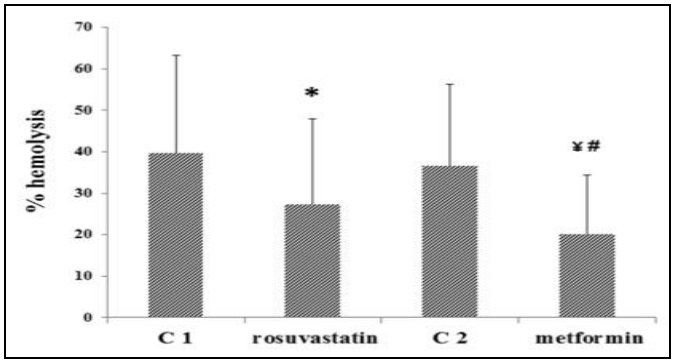

FIG. 1: THE EFFECT OF ROSUVASTATIN, METFORMIN ON % HEMOLYSIS (n = 15). C1, control group of rosuvastatin (2.5% DMSO); C2, control group of metformin (normal saline). *significantly lower than the corresponding control (P=0.002); ¥ significantly lower than the corresponding control (P=0.001); # significantly different from rosuvastatin (P=0.026). Data are presented as mean ± SD

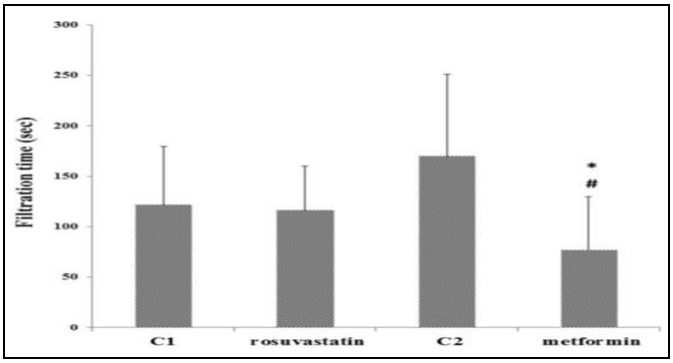

FIG. 2: THE EFFECT OF ROSUVASTATIN, METFORMIN ON FILTRATION TIME (DEFORMABILITY) (n = 13). C1, control group of rosuvastatin (2.5% DMSO); C2, control group of metformin (normal saline). *significantly different from the corresponding control, P=0.001; # significantly different from rosuvastatin – treated group, P=0.028. Data are presented as mean ± SD

Effect on Erythrocyte Deformability: The time required for filtration of one milliliter of erythrocytes suspension through a five-micron filter in the presence a negative pressure is taken as an index of deformability of erythrocytes. The time required for transit of erythrocyte in the control group of metformin was 170 ± 81.4 sec, which was significantly decreased to 76.8 ± 53.4 sec. in blood samples incubated with metformin, P=0.001. Rosuvastatin resulted in a small and insignificant effect on the time of filtration compared to its control (121.5 ± 58.93 sec. *vs*. 116.3 ± 44.17 sec.). These results are presented in **Fig. 2**.

DISCUSSION: SCD is a common hereditary blood disorder worldwide. It represents a major health problem that requires specific care and attention in addition to its impact and burden on patients and health services. As a genetic disease, there is no specific cure. Up till now, two medications were approved by FDA for management of SCD; hydroxyurea was the first and quite recently glutamine, a second medication was also approved for the management of the disease for its antioxidant properties [5]. Still, there are continuous trials to use agents that can modify the clinical course of the disease or halt the progression of complications.

These trials are focusing upon using agents with various pharmacological effects like those with anti-sickling and antioxidant properties like niprisan, those with anti-inflammatory properties like regadenoson and others with NO restoring ability as arginine [5]. Thus, the use of agents that combine multiple beneficial effects in addition to their antioxidant effects like metformin and statins may represent new research lines in the management of SCD.

The results of the present study revealed that metformin and rosuvastatin decreased the levels of TBARS with an increase in the levels of GSH and thus demonstrated a significant *in-vitro* antioxidant effect with a possible beneficial effect in SCD. The antioxidant effects of metformin and rosuvastatin could be attributed to direct free radical scavenging activities, particularly hydroxyl radicals [17, 18], or to binding ability with metals like iron and copper [19, 20]. H_2O_2 in the presence of metal ions can be broken down rapidly leading to the generation of hydroxyl radical which may in turn initiate membrane lipid peroxidation [2].

Metformin and rosuvastatin possibly chelate free iron released from erythrocytes hemolysis under oxidative conditions so decreasing its availability to generate ROS and membrane damage [2, 19, 20]. In an *in-vitro* study, Bai *et al*., 2013 [21], found that metformin can inhibit intracellular ROS generation through inhibiting NADPH oxidase enzyme which is present in high levels in sickle erythrocytes [3].

Impaired erythrocytes deformability is one of the initiating factors of the vascular events of SCD. Upon exposure to a hypoxic condition, HbS undergoes polymerization leading to alteration in the shape of erythrocytes and subsequent impairment of deformability [2, 4]. Nondeformable erythrocytes cause microvascular occlusion with inadequate tissue perfusion and organ infarction.

The present study showed that incubation of erythrocytes suspension with metformin in L-arginine containing medium under brief hypoxic condition was associated with improvement in erythrocytes deformability. Filtration time which is considered as an index of deformability of erythrocytes is decreased by 54.8%. The reason for adding L-arginine to the medium is to facilitate uptake of metformin by erythrocytes thus augmenting its intracellular effect [22]. The observed improvement of erythrocytes deformability by metformin could be attributed to its antioxidant properties which ultimately minimize HbS polymerization. Free heme within sickled erythrocytes plays an important role in adversely affecting hemoglobin polymerization [23]. As an antioxidant, metformin might decrease the rate of Hb oxidation and subsequent free heme formation and hemoglobin polymerization. It has been found that erythrocytes NO have a regulatory effect on erythrocytes deformability [24]. In the presence of L-arginine, a substrate of nitric oxide synthase (NOS), metformin might enhance the production of NO and improving deformability of erythrocytes [7].

Metformin was found to enhance membrane fluidity in a suspension of erythrocytes obtained from healthy and diabetic volunteers which is attributed to the direct effect of metformin on membrane lipids [25]. It is not yet known if

metformin can increase the fluidity of membranes of sickle erythrocytes because of the rigid nature of these cells.

On the contrary to metformin, rosuvastatin in the present study lacks activity on erythrocytes deformability. This is probably due to the use of rosuvastatin calcium salt and at a very high concentration. High calcium level may accumulate inside erythrocytes or in extracellular compartment leading to erythrocytes rigidity [26]. Another explanation for the lack of effect of rosuvastatin could be attributed to the use of DMSO as an organic solvent for dissolving rosuvastatin which is poorly soluble in water [19]. DMSO at a concentration ranging from 2-40% can increase intracellular calcium levels by increasing membrane permeability to calcium and may also increase the release of calcium from intracellular pools [27, 28]. These mechanisms may ultimately change the shape of erythrocytes leading to cell rigidity **.**

To the best of our knowledge, there is only one *in-vitro* study that evaluated the effect of rosuvastatin on deformability in erythrocytes obtained from healthy volunteers in the absence of oxidative stress [29]. The concentration of rosuvastatin used in that study was near its therapeutic plasma concentration and found to improve deformability of erythrocytes, possibly through upregulation of erythrocytes NOS.

The results of the present study showed that % hemolysis in metformin or rosuvastatin treated samples was significantly lower than their corresponding controls. This by itself is a favorable result and, may additionally indicate that both drugs do not possess direct hemolytic activity *in-vitro*. Metformin appeared slightly better than rosuvastatin in reducing % hemolysis (44.8% reduction with metformin *vs.* 30.7% with rosuvastatin.

Although the present study did not show an association between hemolysis and deformability, it can be speculated that a drug which reduces hemolysis and release of free hemoglobin and arginase from hemolyzed erythrocytes, can improve NO bioavailability and deformability. Free hemoglobin and arginase can decrease NO bioavailability through the generation of ROS and depletion of its precursor [4]. An important observation recognized in the present study is that the antioxidant properties may occur at a very high concentration compared to therapeutic plasma concentrations of metformin and rosuvastatin. In several previous studies, the pleiotropic effects of rosuvastatin, particularly the antioxidant effects, were achieved at concentrations near to that used in the present study [18, 30]. Most of the studies that used low concentrations, utilized specific cell cultures for the long duration rather than acute short term treatment**.**

In general, for metformin to exert intracellular effects, it needs to cross the erythrocytes cell membrane and becomes available at high concentrations inside the cells. Metformin is a highly hydrophilic compound, and its entry to the erythrocytes in vitro is considerably slow and time-dependent where less than 1% of the extracellular drug can cross the membranes over several hours [31]. This situation may not be applied in vivo due to the presence of specific active transporter systems as organic cation transporters that might not be well functioning *in-vitro* [32]. The same thing may be applied for rosuvastatin as it is also a hydrophilic compound and requires for crossing cell membrane, active transporters such as anion organic transporters [33].

The effects of metformin were achieved at a concentration lower than that required for rosuvastatin when corrected for their plasma concentration *in-vivo*. Plasma concentration of metformin does not exceed 5 µg/ml; however, it can reach to 12 µg/ ml in portal circulation [32], while the concentration of rosuvastatin ranged between 20-40 ng/ml after a single oral dose [34].

Despite high concentrations, metformin and rosuvastatin did not appear to have a pro-oxidant or hemolytic effect and exhibit relative safety to be started at small doses in patients with SCD especially those with a high risk of vascular complications like ischemic stroke or pulmonary hypertension**.**

Metformin and rosuvastatin belong to two unrelated classes of drugs with a unique ability to decrease cardiovascular morbidity and mortality.

CONCLUSION: Metformin and rosuvastatin have beneficial effects on SCD. Part or most of these protective effects are attributed to their pleiotropic effects like antioxidants, anti-inflammatory, antithrombotic and vasculoprotective effects.

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