In-Vitro Study on Effect of Henna on Normal and G6pd Deficient Blood Sample

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Abstract*—*Introduction: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme defect. G6PD deficient subjects have a diminished ability to withstand stress and therefore risk hemolysis. Most subjects with G6PD deficiency have no clinical symptoms until they are exposed to a hemolytic triggering agent. Henna application on human skin could trigger red blood cells destruction by inducing oxidative stress in G6PD deficient subjects as reported in case studies. This study aims to detect via in-vitro experiments, the effect of henna on red blood cells obtained from normal and G6PD deficient volunteer subjects.

Methods: Pure Henna powder from Sudan was fixed in plain test tubes. A 99.99% Methanol extraction of Henna was made to obtain the active ingredient "Lawson" and the mixture was left to stand overnight and then filtered. An 8ml, 6ml and 4ml filtrate was loaded in test tubes and allowed to dry. One ml of Heparinized blood from normal and G6PD deficient subject were applied on the dried tubes and incubated for 15 and 45 minutes in 37oC. The levels of Potassium; and Lactate Dehydrogenase enzyme; and the cytoskeleton of RBCs in the blood samples, were evaluated before and after henna exposure to detect the hemolytic process.

Results: The blood picture showed differences on RBC membrane between normal and G6PD deficient blood samples after exposure to Henna. The Potassium levels of the normal sample increased up to (14.3 mEq/l) at 45 min and in G6PD deficient sample it increased up to (15.7 mEq/l) at 15 min both with (8mg). LDH at 45 min in normal subject shows the highest reading (LDH= 24526 U\l) in Lowest concentration (4mg) at 45 minutes. While in G6PD deficient, shows the highest reading (LDH= 18755 U\l) in highest concentration (8mg) at 45 minutes.

Conclusion: The results of this in-vitro experiment re-iterates the findings of case reports, that Henna does influence the hemolytic process faster in G6PD deficient blood sample as compared to a normal subjects' blood sample.

***Keywords*—** Henna, Lawson, G6PD Deficiency, Hemolysis

I. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide. It is an X-linked, hereditary genetic defect due to mutations in the G6PD gene, which cause functional variants with many biochemical and clinical phenotypes. Ubiquitously expressed enzyme that has a role in all cells, and is particularly critical to the integrity and functioning of red blood cells (RBCs). G6PD deficiency selectively affects RBCs. RBCs are exquisitely susceptible to oxidative stress from exogenous oxidizing agents in the blood as well as the oxygen radicals continuously generated as haemoglobin cycles between its deoxygenated and oxygenated forms. When G6PD activity is deficient, they have a diminished ability to withstand stress and therefore risk of destruction (haemolysis). Fortunately, the large majority of G6PD deficient subjects have no clinical symptoms and the condition remains asymptomatic until they are exposed to a hemolytic triggering agent. For centuries, the most common known trigger of hemolysis has been fava beans. There are several types of compounds could trigger red blood cells hemolysis to G6PD Deficient.

Henna one of the compound that could trigger red blood cells destruction and induce oxidetive stress in G6PD deficient individuals. Henna which is a natural dye produced from the leaves of the henna plant, a small flowering shrub (scientific name: Lawsonia inermis). The henna plant grows all over the world in hot and dry areas, from North Africa across the Middle East and throughout Southern Asia. It has been used to create body art in cultures around the world for thousands of years. In some cultures, they use it as a cosmetic agent for dyeing hair, body, and nails, while others use it in Dermatological conditions as body or hair rash and fungal infection. When we see the prevalence of G6PD disease in the world the highest is among the people of African, Asian, or Mediterranean descent. Variants producing severe deficiency primarily occur in the Mediterranean population. In middle eastern region, also there is an excessive use of Henna in both sexes with different ages, in this case, the more incidence of Hemolytic crisis in G6PD deficient individuals in where henna be used.

By reviewing of some related studies we found there is an essential need to find the relationship between henna and G6PD deficient in-vitro. Henna which contain an active ingredient called (Lawsone), and it is a chemical composition (2-Hydroxy - 1,4-Naphthoquinone) each 10 g of Henna containing 1 gm of Lawsone (William H. Zinkham, etal, 1996). Which known to induce Oxidative Injury within Red Blood Cells. Several cases were reported with Hemolytic Crisis after topical application of Henna in UAE. Haemolytic crisis in glucose-6-phosphate dehydrogenase deficient individuals following topical application of henna occurred in children:

A term male neonate required phototherapy and was found

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to be G6PD deficient on screening. Although the parents had been instructed to avoid henna, the mother applied it to his palms and soles when he was 2 months old. Within 48 hours he passed red urine, became pale and jaundiced, and vomited; he was brought to the hospital after two further days. He was in shock: arterial pH was 6.64, PaCO2 12 mm Hg, Hb 28 g/l, hematocrit 8.9%, leucocytes 31 200/µl, platelets 657 000/µl, reticulocytes 18%, lactate dehydrogenase (LDH) 2322 U/l, SBR 231 µmol/l, conjugated 1 µmol/l; liver enzymes were unremarkable. Blood and urine cultures remained sterile, thin and thick blood film for malaria was negative. Despite transfusions, he remained anuric, developed notable uremia with disseminated intravascular coagulation and cerebral seizures, and died two days after admission (P Raupp, J et al 2001).

The aim of this study is to draw attention to henna and its effect on G6PD Deficiency individuals and to create an awareness among clinicians to give health education to affected family members.

Fig. 1: Henna Powder

II. Methodology

A. Tubes Preparation:

Pure Henna without any additives brought from Sudan was Fixed in a plain test tube. To prepare the mixture by adding 3000mg from pure Henna powder to 300ml of 99.99% Absolute Methanol and left overnight covered with aluminium foil and stored in the dark. After saturating the Methanol with henna, and mixed it with magnetic vortex for about a one hour the mixture then was completely filtered so no henna particles were present in the mixture. After filtration, the mixture poured into a clean sterile test tube and by labelling 2 racks and tubes with the sample ID No. and the concentration of poured mixture.

The first tube contained 8ml from the prepared solution of henna, the second and third were contain 6ml and 4ml respectively. As seen that 10 gm of henna contain 1 gm of Lawsone "active material" and in the experiment, we used 3 gm of henna so "contain 300mg of Lawsone" from this we load 8ml, 6ml and 4ml so 8mg, 6mg and 4mg of Lawsone respectively. Henna residual was fixed in the tube as a thick, heavy and sticky deposition, then the tubes stored in the dark.

Fig. 2: Fixed tube after methanol evaporation

B. Sample Collection:

After fixed tubes prepared, two blood samples were collected one for G6PD Normal and one G6PD Deficient in 2 ml Lavender coloured lid blood tube contains EDTA as an anticoagulant only to perform Complete Blood Count 'CBC' and make sure that the normal subject is healthy without any blood hemolytic abnormalities at the time of collection. The main blood samples were collected in Green coloured lid blood tube contains Lithium Heparin and was labelled with the time of collection and the state of the person if he/she G6PD deficient or Healthy. Four ml was drawn from a vein in Heparinized tube with a total of 12ml = 3 Heparinized tube for each subject. In Hematology lab at Qassim University the samples were evaluated for the initial Potassium, LDH levels and Cytoskeleton structure before exposing the blood to Henna.

C. Sample Testing:

After the initial measurement, 1ml was loaded in each prepared fixed tube. Then blood samples were incubated in a water-bath of 37oC and covered. One ml of the subject's blood sample was taken and incubated at the same condition except that ml it does not contain any henna and the purpose of this to make sure that changing in the parameters were due to Henna, not the temperature or delay since the Potassium very sensitive for any delay and used as control for this certain subject.

All the tubes were followed for 15 and 45 minutes by measuring LDH, Potassium, and Cytoskeleton. The Potassium and LDH were measured according to the Test Kit instruction given by the manufacturer and measure the absorbance and turbidity by Spectrophotometer and Colorimetric instruments provided by the college.

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Fig. 3: Blood Mixed with henna transferred to new test tubes for centrifugation and separate the plasma to be tested for Potassium and LDH

The final result for the cytoskeleton were detected with Wright's Stain by performing Peripheral Blood Smear (PBS). At first, the slides were fixed by immersing them in the methanol for about 2 mins then stained with double filtered Wright's stain for 3 mins and finally washed with Distilled water for about 7 mins. These steps were repeated in all remaining slides.

III. Results:

A. LDH and Potassium Readings for G6PD Deficient and G6PD Normal:

After Henna exposure the reading were estimated for 15 and 45 min.

TABLE I: G6PD DEFICIENT READINGS:

Subject : G6PD Deficient	I	C 45 min	Conc. 8 mg		Conc. 6 mg		Conc. 4 mg	
			15	45	15	45	15	45
LDH	2004U/l	1122U/l	721	18755	4168	2084	160	5450
K^+	4.9mEq/l	5.43mEq/l	15.7	9.31	12	6.55	6.21	5.86
I= Initial, LDH= Lactate Dehydrogenase, K^+= Potassium, C= Control, Conc. = Concentration. **Reference Range for Potassium : 4.0-4.8 mEq/l / Reference Range for LDH : 225-450 U/l**								

TABLE II: G6PD NORMAL READINGS:

Subject : G6PD Normal	I	C 45 min	Conc. 8 mg		Conc. 6 mg		Conc. 4 mg	
			15	45	15	45	15	45
LDH	1603U/l	1443U/l	7454	3366	4248	1202	11221	24526
K^+	5.1mEq/l	5.43mEq/l	10.5	14.3	5.2	4.7	9.1	10.7
I= Initial, LDH= Lactate Dehydrogenase, K^+= Potassium, C= Control, Conc. = Concentration. **Reference Range for Potassium : 4.0-4.8 mEq/l / Reference Range for LDH : 225-450 U/l**								

B. Red Blood Cells Cytoskeleton Picture for G6PD Deficient and G6PD Normal :

The blood smear in panel (A) shows Reference PBS of G6PD deficient with bite cells "arrow"(Barbara J. Bain,2005), in panel (B) shows the Initial PBS of G6PD deficient before Henna exposure as intact membrane, central pallor and normal cell size, After Henna exposure (C) shows disturbed RBC membrane as irregular spikes "in arrow", in panel (D) shows hyperchromatic cells lost its pallor, anisocytosis and poikilocytosis, in panel (E) many intracellular granulation + Irregular Spikes, in panel (F) control "Without Henna after 45 mins incubation at 37oC normal intact membrane with central pallor and showing few Burr Cells "Fine Arrow".

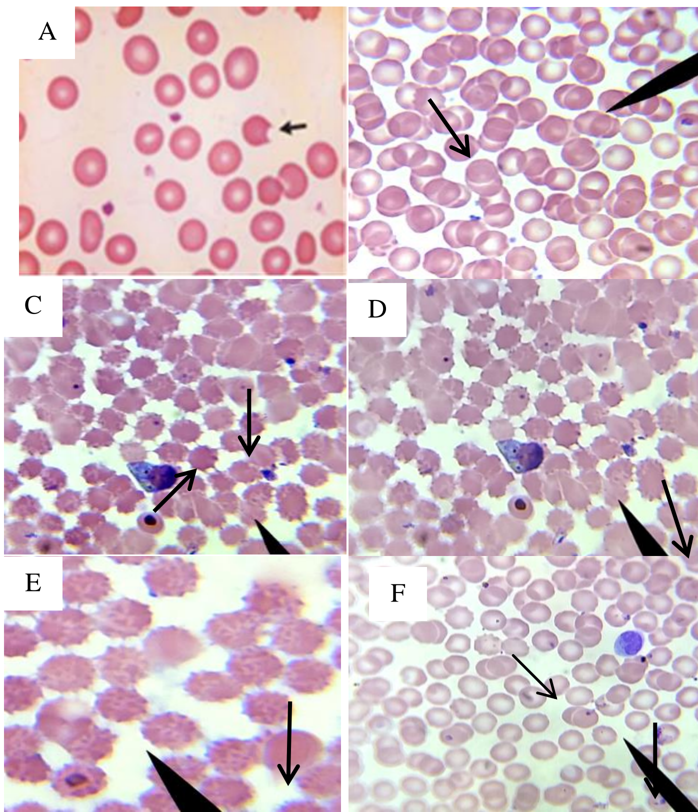

Fig. 4: Red Blood Cells changes in comparison to Reference blood picture of G6PD deficiency.

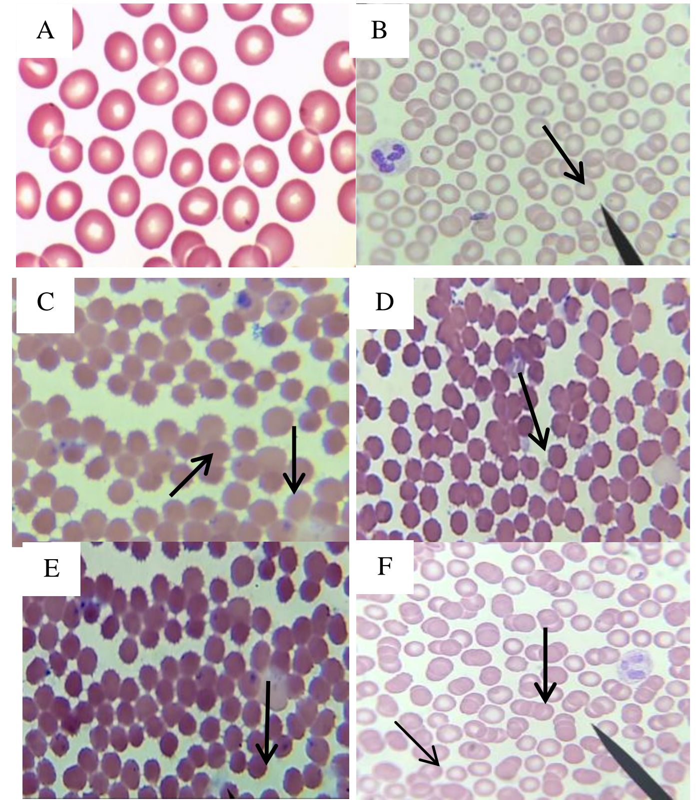

Fig. 5 :Red Blood Cells changes in comparison to Reference blood picture of Normal PBS

The blood smear in panel (A) shows Reference PBS of Normal blood picture (Barbara J. Bain,2005), in panel (B) shows the Initial PBS of Normal subject before Henna exposure as intact membrane, central pallor and normal cell size, After Henna exposure (C) shows disturbed RBC membrane as irregular spikes "in arrow", in panel (D) shows hyperchromatic cells lost its pallor, anisocytosis and poikilocytosis, in panel (E) Irregular Spikes, in panel (F) control "Without Henna after 45 mins incubation at 37oC normal intact membrane with central pallor and showing few Burr Cells "Fine Arrow".

TABLE III:
PBS FINDINGS IN BOTH G6PD DEFICIENT AND NORMAL SUBJECTS AFTER HENNA EXPOSURE IN OVERALL VIEW IN ALL THE DIFFERENT CONCENTRATIONS:

PBS	Anisocytosis	Poikilocytosis	Hyperchromatic	Spikes	Background	Size
G6PD Deficient	+	+	+	+/-	Dirty	↓
Normal	+	+	+	+/-	Dirty	↓

* +/- Depend on the Concentration.

IV. Discussion:

In our experiment, we depend on Potassium as hemolysis indicator due to its high sensitivity for any RBC membrane destruction and Blood picture give us the membrane integrity and valuable changes unlike for LDH which gave unreasonable readings. In G6PD deficient subject potassium gave the highest reading was 15.7 mEq/l at 15 min at the concentration of 8mg of lawsone then start to decrease and shows a normal graph of decreasing in proportional with the concentration decreasing. However, in the Normal subject, the highest reading was 14.3 mEq/l at 45 min this difference can be referred to the Anti-Oxidant absence in G6PD deficient made a faster action of Lawsone, unlike Normal subject who have normal Anti-Oxidant that protect the RBCs from the Oxidative stress. In the normal subject, the 6mg and 4mg show fluctuation but have the same pattern in both 15 and 45 min so for these concentration gave inconclusive result.

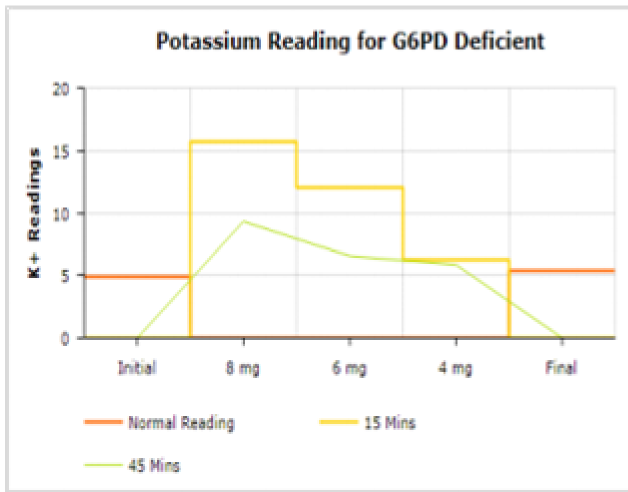

Fig. 6: K+ Readings for G6PD Deficient

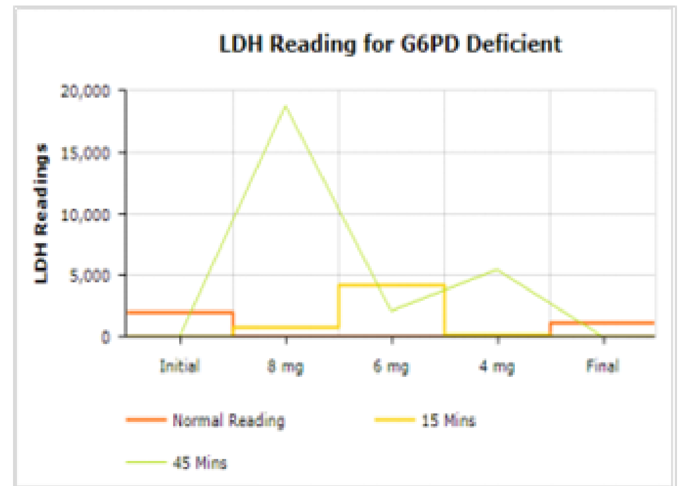

Fig. 8 :LDH Readings for G6PD Deficient

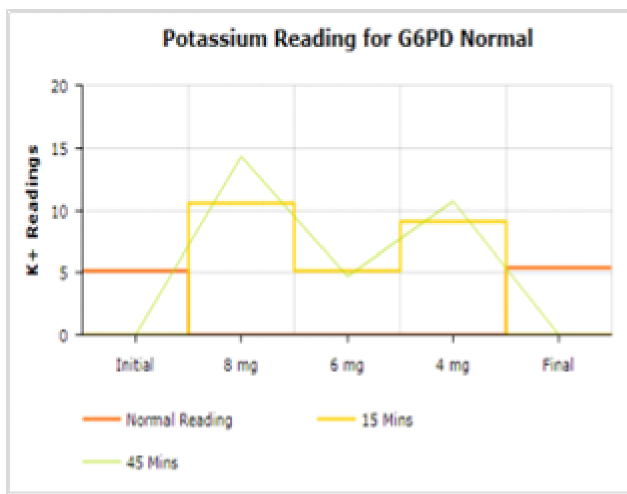

Fig. 7: K+ Readings for G6PD Normal

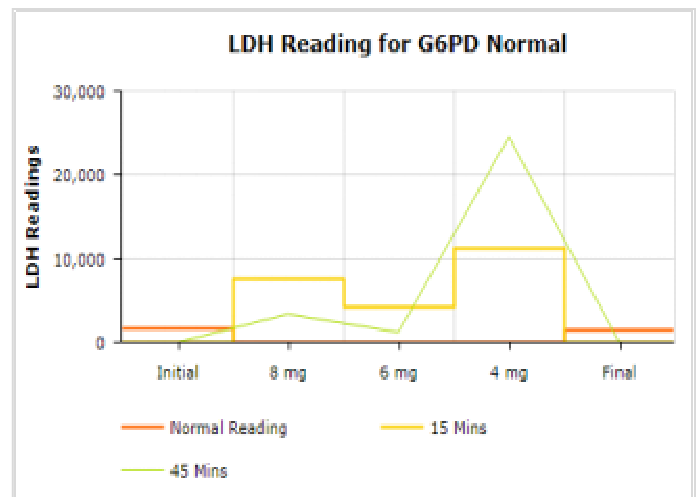

Fig. 9: LDH Readings for G6PD Normal

Lactate Dehydrogenase readings in both G6PD deficient and Normal subjects gave so many unreasonable fluctuations seen from the beginning of the experiment it was double checked result to rule out the errors and it gave the same fluctuation, normally they used to use plain test tube to take the serum rather than plasma and in the LDH test kit protocol it mentioned that the plasma from Heparinized tube also acceptable, since from the initial readings the results did not make sense this is the only current explanation for it. For high-value readings, it shows there is something going on with henna but still the result inconclusive until repeating the experiment with using a serum instead of plasma but in this case following the fact of red blood cells rupture will be so weak due to the absence of anticoagulant so blood will be clotted.

Peripheral Blood smears show a very clear reasonable findings that similar microscopic to case reports such as Anisocytosis and poikilocytosis. In our findings, hyperchromatic, spikes with irregular length and shape, dirty background, reduce in RBCs size, clumps together and the most important is the intracellular granules that likely suggest is due to the Oxidative Stress. In Macroscopic findings, the PBS with henna shows hard spreading in PBS preparation, many holes and impurities suggest also hemolytic samples unlike the initial and the control for 15, 45 mins and final which gave very smooth, easy and clean spreading.

V. Conclusion:

The results of this in-vitro experiment re-iterates the findings of case reports. Henna does some changes in the red blood cells and shows that the hemolytic process faster in the G6PD deficient blood sample as compared to a normal subjects' blood sample with direct exposure to Henna. Also, show that the red blood cells destruction could occur even in adults red blood cells and not only related to children the main difference here is the penetration of the Henna to the circulation by the skin whether the skin is soft and thin as seen

in babies and the opposite in adult. Also, show the hemolysis could occur even in G6PD Normal but weaker and longer in time than in G6PD Deficient as direct Exposure.

For Biochemicals Testing further studies have to be done with greater blood samples and more biochemical parameters.

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