Evaluation of Antioxidant Status in Full-term Hyperbilirubinemic Neonates with Severe G6PD Deficiency in Najef: Iraq

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Abstract

Objective: The objectives of this study were an attempt to evaluate and to compare between some of the antioxidant and biochemical parameters investigated in severe G6PD-deficient neonates with hyperbilirubinemia with TSB≥ 15 mg/dl in Najef governorate : Iraq.

Methods: A total of 240 neonates who were admitted in Al-Zahra maternity and pediatrics hospitals in Najef governorate during 1st , Oct., 2007 and 12th, July, 2008 with age ranged between 1 – 28 days were screened for erythrocyte G6PD enzyme activity measurement to confirm the diagnosis of G6PD deficiency. Of these subjects, 61 (25.42%) neonates of them showed a normal enzyme activity levels ; whereas the remaining 179 (74.58%) neonates were found to have neonatal hyperbilirubinemia with TSB levels ≥ 15 mg/dl. Some of the antioxidant parameters have been measured in severe G6PD-deficient neonates with hyperbilirubinemia and compared with that found in control group.

Results: Among the hyperbilirubinemic neonates, only 21 (11.73%) neonates were diagnosed to have severe G6PD deficiency and its percentages of incidence identified was 11.73%. The results also indicated that there was a significant negative correlation ($r = -0.320$, $P < 0.05$) between the decreased G6PD activity levels and the elevated TSB concentrations in severe G6PD-deficient hyperbilirubinemic neonates with the TSB ≥ 15 mg/dl but not in control neonates. These data suggest that the G6PD-deficient neonates are at increased risk for hyperbilirubinemia even in the nursery free from agents that can potentially cause hemolysis to G6PD-deficient red cells. The mean ± SD of oxidative stress status parameters which include erythrocyte GSH, MDA, G-Red, G-Px and catalase were determined. There was a significant decrease in each of erythrocyte GSH, G-Red and catalase activity levels ($P<0.05$), whereas the lipid peroxidation end product MDA levels and G-Px activity levels were significantly increased in all hyperbilirubinemic neonates ($P < 0.05$) as compared with the control group. G6PD activity values identified were found to be positively correlated with each of GSH concentrations, G-Red and catalase activity levels in which their values were found to decreased in patient groups, while it was found to be negatively correlated with each of G-Px activity and MDA levels in which their values were elevated in severe G6PD-deficient neonates. These data indicates an increases in free radical generation and thus antioxidant defense mechanisms is impaired in peroxidation associated with a significant elevation in MDA levels in the erythrocytes of the hyperbilirubinemic neonates with severe G6PD deficiency than that found in the control group which demonstrate the presence of an increased oxidative stress due to reduction in NADPH which is generated in RBCs by HMP-shunt only.

Conclusion: The results of the present work indicate that severe deficiency of G6PD activity is associated with hyperbilirubinemia in full-term neonates and imbalance in oxidative stress parameters..

Keywords: hyperbilirubinemia, G6PD, glutathione, catalase , G-Px, G-Red , neonate.

Introduction

A homeostasis between rate of free radicals formation and the rate of their neutralization if not maintained, oxidative damage accumulates and is known as oxidative stress[1]. Neonatal jaundice is a normal physiological event that is being treated on a belief of pathology. Commonly neonatal jaundice occurs for two reasons. A) Infants have too many red blood cells. It is a natural process for the baby's body to break down these excess red blood cells, forming a large amount of bilirubin. It is this bilirubin causes the skin to take an yellowish color. B) A newborn's liver is immature and can not process bilirubin as quickly as the baby will be able to gets older. This slow processing of bilirubin has nothing to do with liver disease. It merely means that the neonates liver is not as fully developed as it will be, and thus, there is some delay in eliminating the bilirubin. Neonatal jaundice affects 60% of full-term infants and 80% of preterm infants in the first 3 days after birth [2]. Antioxidant activity in serum of term neonates is lower than that of adults and is still lower in preterm and low birth weight babies as compared to term babies[3]. Red blood cells are extremely susceptible to lipid peroxidation since they are rich in unsaturated membrane lipids, have rich supply of oxygen and transitional metal catalysts. Neonatal erythrocyte membrane is more susceptible to oxidative damage due to its predominant pro-oxidant potential[4]. The erythrocytes are particularly prone to the free radical damage since the membrane lipids are very rich in polyunsaturated fatty acids which play an essential role in generating free radicals. Free radicals, primarily the reactive oxygen species, superoxide and hydroxyl radicals which are highly reactive having an unpaired electron in an atomic or molecular orbit are generated under physiological conditions during aerobic metabolism. As free radicals are potentially toxic, they are usually inactivated or scavenged by antioxidants before they can inflict damage to lipids, proteins or nucleic acids. Alteration in the oxidant–antioxidant profile is known to occur in neonatal jaundice[5,6]. Moreover the body's defense mechanisms would play an important role in the form of antioxidants and try to minimize the damage, adapting itself to the above stressful situation. Antioxidants are compounds that dispose, scavenge, and suppress the formation of free radicals, or oppose their actions[1] and two main categories of antioxidants are those whose role is to prevent the generation of free radicals and those that intercept any free radicals that are generated[7].

They exist in both the aqueous and membrane compartment of cells and can be enzymes or non enzymes. The human body has a complex antioxidant defense system that includes the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (G-Px) and catalase (CAT). These block the initiation of free radical chain reactions[8].

The non enzymatic antioxidant components consists of molecules such as glutathione (GSH), vitamin E, ascorbic acid and beta-carotene that react with activated oxygen species and thereby prevent the propagation of free radical chain reactions.

In the present study, some of the oxidative stress parameters were assessed in the erythrocytes in hyperbilirubinemic neonates. Erythrocyte malondialdehyde (MDA) levels were measured as thiobarbituric acid reacting substances (TBARS) which serves as an index of extent of lipid peroxidation. Erythrocyte glutathione (GSH), serve as non-enzymatic antioxidant parameters. The antioxidant enzymes catalase, glutathione peroxidase (G-Px) and glutathione reductase (G-Red) in erythrocytes were estimated. This study is the first attempt to examine oxidative stress and the status of the protective antioxidants

under condition of stress due to the neonatal jaundice in Nejef governorate.

Materials and Methods

A total of 179 blood samples were collected from full-term deliveries male hyperbilirubinemic neonates and 61 control neonates with age ranged between 1-28 days which were admitted in Al-Zahra Maternity and Pediatrics Teaching Hospital / Najef during 1st , Oct., 2007 and 12th , July , 2008 and they received phototherapy when their TSB levels exceed 15 mg/dl. All the neonates were being breastfed and had no etiological factor for hyperbilirubinemia. Any G6PD-deficient neonates with other possible etiologies causing hyper-bilirubinemia, such as infants of diabetic mothers, polycythemia, perinatal infection, gastrointestinal obstruction, permaturity, ABO incompatibility, birth asphyxia, sepsis or those that had received intensive Phototherapy; those in which the TSB level rose by more than 5 mg/dl per day or was higher than 20 mg/dl within the first 24hours after birth; and those with signs and symptoms suggestive of serious illness were excluded.

Blood samples in a quantity of (2-3 ml) were taken from a peripheral vein in EDTA anticoagulant collecting tube (in 300 µL EDTA , 0.5 M) from both full-term male control and hyperbilirubinemic neonates which were centrifuged at 1500 rpm for 10 minutes within 20 minutes of collection. Serum samples were stored at – 20 °C and analyzed in duplicate for biochemical and oxidative stress parameters assays within two weeks.

Spectrophotometric G6PD activity levels was measured quantitatively in hemolysates by using Sigma kit (345-B) based on kinetic method recommended by WHO in 1967 and was modified Kornberg, and Horecker at 37 ^{o}C which involving the following reaction[9]:

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G6PD}} \text{6-PG} + \text{NADPH} + \text{H}^{+}$$

According to this method, the rate of the reduction of $NADP^{+}$ to NADPH when the sample was incubated with G6PD and the rate of formation of NADPH was monitored as an increase in absorbance at 340 nm which proportional to the G6PD activity. Production of a second molar equivalent of NADPH by erythrocyte 6-phospho-gluconolactonate dehydrogenase (6-PGD) was prevented by use of maleimide, a specified inhibitor of 6-PGD as shown in the following reaction:

$$\text{6-PG} + \text{NADP}^{+} \xrightarrow{\text{6PGD}} \text{Ribulose-5-Phosphate} + \text{NADPH} + \text{H}^{+} + \text{CO}_2$$

The activity was expressed in micromole of NADPH formed per minute per gram hemoglobin in hemolysates. Hemoglobin concentration was determined and the G6PD activity was expressed as international units per gram hemoglobin (U/g Hb) in erythrocyte hemolysate.

Total bilirubin in plasma of control and hyperbilirubinemic neonates was measured by adding caffeine reagent as accelerator followed by the addition of diazotized sulfanilic acid. During the incubation period, both conjugated and unconjugated bilirubin react with the diazo reagent to produce azobilirubin. After the addition of diazotized sulfanilic acid, solutions of ascorbic acid, alkaline tartrate, and dilute HCl to the reaction mixture, the absorbance of blue-green azobilirubin produced was measured at 600 nm[10].

To measure the conjugated bilirubin, the sample was acidified with dilute HCl and

then mixed with diazotized sulfanilic acid to produce azobilirubin. Only the conjugated form of bilirubin react with the diazo reagent in the absence of the accelerator caffeine-benzoate. The reaction was stopped by the addition of an ascorbic acid solution, then an alkaline tartrate solution was added to the reaction mixture to provide an alkaline pH and to produce the more intense azobilirubin blue color which followed by the addition of an aliquot of caffeine reagent..

The erythrocyte lipid peroxidation end product malondialdehyde (MDA) level was determined by a method depends upon the reaction with thiobarbituric acid (TBA) at 90–100 °C [(11)].

The level of reduced glutathione (GSH) in erythrocytes was determined by Beutler method as a modification of the Ellman method[(12)]. Erythrocyte were deproteinated by addition of trichloroacetic acid (TCA). DTNB[5,5'-dithio-bis-(2-nitrobenzoic acid)] was added to supernatants cleared by centrifugation (10 min, 3000 rpm). The formation of 5-thio-2-nitrobenzoic acid, which is proportional to total glutathione concentration, was monitored at 412 nm at 25°C against reagent controls.

G-Red activity was measured by Randox G-Red assay kit" provides an indirect and highly reproducible method of quantifying the G-Red activity in hemolysates which is an important measure of the antioxidant status of the cell. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A_{340}), thus providing a spectrophotometric means of detection which is directly proportional to the G-Red activity in the sample[(13)].

G-Px activity was measured according to ZeptoMetrix diagnostic kit for G-Px activity measurements, USA, which is based on that of Paglia and Valentine in (1967), cumene hydroperoxide is used as the peroxide substrate (ROOH), and glutathione reductase (GSSG-R) and β-NADPH are included in the reaction mixture[(14)]. The formation of GSSG catalyzed by G-Px is coupled to the recycling of GSSG back to GSH using G-Red in which NADPH is oxidized to $NADP^+$. The rate of decrease in A_{340} due to NADPH oxidation is monitored spectrophotometrically and is directly proportional to the total activity of G-Px. Since all other reagents are provided in excess, the amount of G-Px in the test sample is the rate-limiting factor. Cumene hydroperoxide is suitable for the reaction because it has a low spontaneous reaction with GSH, low spontaneous hydrolysis and is not metabolized by Catalase, another universally present antioxidant enzymes. G-Px activity both in plasma and in RBC hemolysate can be determined with this kit which can also be adapted to G-Px activity determination in cells from culture and tissue homogenates[(15)].

Catalase activity in erythrocytes was assayed by a method described by Goth[(16)]. The rate of dismutation of H_2O_2 to water and molecular oxygen is proportional to the activity of catalase. Therefore, the sample containing catalase is incubated in the presence of a known concentration of H_2O_2. After incubation for exactly one minute, the reaction is stopped with ammonium molybdate. The amount of H_2O_2 remaining in the reaction is then determined by the oxidative coupling reaction between molybdate and H_2O_2.

All reagents used were of analytical reagent grade. DTNB and thiobarbituric acid were obtained from sigma chemicals, St. Louis, MO., USA. Statistical analysis between controls and hyperbilirubinemic neonates was performed by the student t – test using the stat-view package. The data were expressed as mean ± SD. $P < 0.05$ was considered as significant. Since our goals were to evaluate the differences of antioxidant status between hyper-bilirubinemic neonates and the healthy controls.

Results and Discussion

The number of neonates in healthy control group which are not jaundiced were 61 (25.42%) of the total neonates included (240 neonates) and their TSB levels were found to be 0.58 ± 0.22 mg/dl., whereas, the remaining neonates which include 179 (74.58%) of the total cases were associated with the appearance of severe neonatal hyperbilirubinemia and their TSB levels were significantly elevated ≥ 15 mg/dl as compared with the control group ($p < 0.05$) .

There were few studies on the incidence of severe G6PD deficiency and the status of oxidative stress parameters in neonatal jaundice in Iraq. A total of 240 neonates were screened for erythrocyte G6PD enzyme activity. These samples were randomly tested for G6PD deficiency to determine whether or not this deficiency could play an important role in the development of neonatal hyper-bilirubinemia. Of these subjects, 61 (25.42%) individuals showed a normal enzyme activity with normal TSB levels.

Among the hyperbilirubinemic neonates, only 21 of 179 (11.73%) cases with TSB ≥ 15 mg/dl was diagnosed and found to have severe G6PD deficiency which is the percentage of incidence in Najef governorate of Iraq. Their mean ± SD of G6PD activity levels were significantly decreased ($P<0.05$) to 0.46 ± 0.41 U/g Hb as compared with the control G6PD activity identified 10.91 ± 1.56 U/g Hb, see table 1. Serum conjugated bilirubin (SCB) was also determined as shown in the same table, and its mean ± SD values in severe hyperbilirubinemic neonates were significantly lower than that found in control ($P < 0.05$). SCB was undetectable in 9 of 21 of hyperbilirubinemic neonates (42.86%) with severe G6PD deficiency which imply a partial defect of bilirubin conjugation.

These results confirm with other studies performed in Isreal, Italy, and Taiwan which suggest that the G6PD–deficient neonates are at increased risk for hyperbilirubinemia. [17-19]. Therefore, data presented in this study may probably suggest that severe neonatal hyperbilirubinemia may continuously cause of a problem in this region of Iraq, which show that those neonates with severe G6PD-deficiency who developed higher maximal TSB values had significantly lower SCB fractions. Conversely, those with lower SCB values at the time of sampling were at higher risk for the subsequent development of hyperbilirubinemia. Serum bilirubin profile demonstrated in the subsequently hyper-bilirubinemic with severe G6PD-deficient neonates (high TSB, with low SCB) is a reminiscent of that seen in conditions of partial deficiency of the bilirubin conjugating enzyme UDP-glucuronosyl transferase1 A1 (UGT1A1), such as Gilbert's Syndrome[20].

Table 1. G6PD activity levels, TSB , SCB concentrations and oxidative stress profiles in full-term male normal and G6PD-deficient hyper-bilirubinemic neonates in Najef governorate : Iraq.

Measurements	Control n = 61	G6PD-deficient hyperbilirubinemic neonates n = 21	*P* value
G6PD , U/g Hb	10.91 ± 1.56	0.46 ± 0.41	< 0.05
TSB, mg/dl	0.58 ± 0.22	21.29 ± 5.43	< 0.05
SCB, mg/dl	0.18 ± 0.07	0.081 ± 0.051	< 0.05
GSH, µM/g Hb	5.55 ± 0.74	3.38 ± 2.38	< 0.05
MDA, nM/g Hb	36.53 ± 5.60	79.14 ± 14.08	< 0.05
G-Red, U/g Hb	10.56 ± 1.4	7.12 ± 1.22	< 0.05
G-Px, U/g Hb	39.74 ± 5.44	50.3 ± 14.15	< 0.05
Catalase, kU/g Hb	94.91 ± 6.91	69.91 ±19.27	< 0.05

These data support functionally the concept of the gene interaction demonstrated between G6PD deficiency and the variant promoter for the gene encoding the bilirubin conjugated enzyme UGT1A1 as suggested by[21,22] and then diminished bilirubin conjugation ability. Gene variants is reported to be in association with an increased risk for neonatal hyper-bilirubinemia include those of :

(1) The red blood cell enzyme (*G6PD*)[23] ;
(2) The hepatic bilirubin-conjugating enzyme UGT1A1[24] ;
(3) The hepatic organic anion transporter polypeptide1 B1 (*OATP1B1*) [25].

More recent findings suggested that gene polymorphisms of OATP1B1[26] a putative bilirubin transporter localized to the sinusoidal membrane of hepatocytes (i.e., the blood hepatocyte interface), may be a predispose to neonatal hyperbilirubinemia by possibly limiting hepatic bilirubin uptake. The primary site of the pathogenesis of the hyperbilirubinemia therefore appears to be localized to a deficiency in bilirubin conjugation. As a result, G6PD-deficient neonates who become hyperbilirubinemic have bilirubin conjugation ability which is even more inefficient than that of the physiological immaturity of conjugation normally found in neonates. Those with an excessively immature bilirubin eliminating capacity are more likely to develop hyperbilirubinemia than those with a more mature ability. This mechanism may exist to a certain extent in all neonates but may be exacerbated in the G6PD deficiency state because of increased hemolysis and the resultant additional bilirubin load[27]. Bhutani, et. al., in (1999) have demonstrate that measuring the TSB level and further testing (blood group, coombs and G6PD tests) at the time / or before infants are discharged from hospital is helpful in predicting which infants will experience severe hyperbilirubinemia and to evaluate the risk and to prevent it[28].

The results obtained also show that deficient bilirubin conjugation which was reflected by low SCB values measured, is a cardinal factor in the pathogenesis of G6PD deficiency associated with neonatal hyperbilirubinemia. In G6PD-deficient neonates who conjugate bilirubin less efficiently, hyperbilirubinemia is more likely to result.

It is unknown at present time whether the previous observations related to hemolysis and bilirubin production[27], or the deficient serum conjugated bilirubin fractions described above are unique to Sephardic Jews with G6PD Med or whether they have global implications for the hundreds of millions of people worldwide estimated to have G6PD deficiency[29]. Additional study of the pathophysiology of this process may lead to improved therapeutic or prophylactic interventions in the clinical management of G6PD deficiency associated neonatal hyperbilirubinemia**.**

The results obtained indicated that there is a significant negative correlation (r = -0.320 , $P < 0.05$) between the deceased in G6PD activity levels and TSB concentrations elevated in severe G6PD-deficient hyper-bilirubinemic neonates with the TSB $\geq$ 15 mg/dl but not in control individuals as shown in (Figure-1-).

The mechanism of the relationship between G6PD activity and neonatal hyperbilirubinemia is not clear. The presence of another genetic factors has been postulated in the pathogenesis of neonatal hyperbilirubinemia in G6PD deficiency. Kaplan, et. al. in (1997) reported that UGT1A1 gene mutation, diminishing the activity of the conjugated enzyme UGT1A1 , was associated with neonatal hyperbilirubinemia in G6PD deficiency[21]. Weng, et. al., in (2002) reported that the expression of heme oxygenase-1 , a rate-limiting enzyme in the production of bilirubin and inducible under the exposure to oxidative stress, was increased in G6PD

deficiency[19]. Recent studies suggest that bilirubin was a strong endogenous antioxidant. Therefore, it is reasonable to suggest that the neonatal hyperbilirubinemia caused by increased heme oxygenase-1 in G6PD deficiency is the consequence of genetic interaction to compensate the decreased antioxidant activity. Therefore, the low levels of G6PD activity in male infants may play a role in the interaction of different genes, such as UGT1A1 and heme oxygenase-1 , and subsequently aggregative the high TSB levels. Phototherapy has been documented as an effective treatment to reduce neonatal hyperbilirubinemia in G6PD deficient neonate[30]. Therefore, the difference in the incidence of hyperbilirubinemia between G6PD deficient and G6PD normal neonates may be masked by early phototherapy. It seems that, in severe hyperbilirubinemia (TSB ≥ 15 mg/dl) the prevalence of G6PD deficiency is more than moderate hyperbilirubinemia (TSB < 15 mg/dl) that may be a risk factor for some complication and kernicterus.

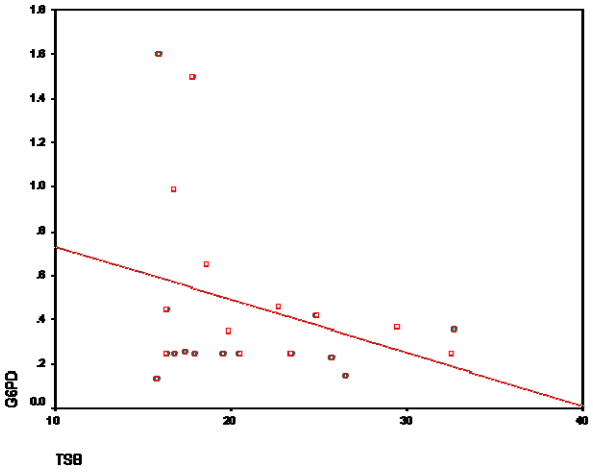

Fig 1.The correlation between G6PD activity levels and TSB conc. in severe G6PD-deficient male hyperbilirubinemic neonates in Najef Governorate ($P < 0.05$; r = - 0.320)

Erythrocytes are the first react to increased activity of free radical oxidation and to exhaust their compensatory potential. Previous studies on erythrocyte antioxidant capacity and human disease relation showed that some changes in activities of the antioxidant enzymes in the cell may occur[31].

In this study, the mean ± SD of erythrocyte GSH, MDA concentrations and G-Red, G-Px and Catalase activity levels were determined in Iraqi hyperbilirubinemic neonates with severe G6PD deficiency and compared with the control group , and the results of this study to our knowledge is being reported for the first time in this literature.

There was a significant decrease in the erythrocyte GSH levels in neonatal hyperbilirubinemia with severe G6PD deficiency (P<0.05) in Najef region governorate as compared with the control group (Tables-1-). Whereas the erythrocyte lipid peroxidation product MDA levels was significantly increased (P<0.05) and reached to 79.14 ± 14.08 nM/g Hb as compared with control values 36.53 ± 5.60 nM/g Hb .

The activities of erythrocyte antioxidant enzymes G-Red, and catalase were significantly decreased in neonatal hyperbilirubinemia with severe G6PD deficiency (P<0.05) , whereas the activity of the other antioxidant enzyme G-Px is

significantly increased ($P<0.05$) as compared with control group (Table 1).

The data obtained from this study indicate that there is increases in free radical generation and the antioxidant defense is impaired in peroxidation which is in agreement with other report that concerned with jaundice neonates[32] ; and other studies published in Italy[33,34], while it is in disagreement with others seen in Kurdish Jews, China and Saudi Arabia[35-37]. The lipid peroxidation product i.e. malondialdehyde (MDA) levels have been increased significantly in erythrocytes of the hyperbilirubinemic neonates with severe G6PD deficiency than that found in control group. This may indicate the presence of increased oxidative stress. Rise in MDA could be due to increased generation of reactive oxygen species (ROS) due to the excessive oxidative damage generated in these patients. These oxygen species in turn can oxidize many other important biomolecules including membrane lipids. The raised MDA level in severe G6PD-deficient neonates reflects the oxidative injury due to neonatal hyperbilirubinemia, which is attributed to free radical formation that abstracts of hydrogen atoms from lipoproteins causing lipid peroxidation, of which MDA is the main product (Halliwell, 1994). The membrane phospholipids, specifically polyunsaturated fatty acids are converted to MDA, which can be analyzed by reactivity to thiobarbituric acid[32].

It was also observed that there is a significant decrease in the levels of erythrocyte reduced glutathione (GSH), in hyperbilirubinemic neonates with severe G6PD deficiency when compared to controls (Table1). GSH is important in chain breaking antioxidants responsible for scavenging the free radicals and suppression of peroxidation in aqueous and lipid region of the cell[38]. The decrease in the levels of GSH observed may be due to the increased turnover, for preventing oxidative damage in these neonates suggesting an increased defense against oxidant damage in hyperbilirubinemic neonates. Similar reports that were associated with a decreased levels of GSH concentrations in hyper-bilirubinemic neonates were reported by various studies[6].

In this study, the erythrocyte antioxidant enzyme glutathione peroxidase was slightly elevated in severe G6PD-deficient hyper-bilirubinemic neonates as compared with that found in control group (Table1). G-Px is an oxidative stress inducible enzyme that plays a significant role in the peroxyl scavenging mechanism and in maintaining functional integration of cell membranes[3]. The rise in the activity levels of G-Px could be due to its induction to counter the effect of increased oxidative stress. G-Px provides an effective protective mechanism against cytosolic injury because it eliminates H_2O_2 and lipid peroxide products by reduction reactions utilizing GSH. Decrease in the activities of antioxidant enzyme status was reported in various studies[39,40].

In the present study, it was also observed a significant decrease in the activity levels of Catalase and G-Red in hyper-bilirubinemic neonates with G6PD deficiency as compared to controls (Table -1-). Catalase is the enzyme which protects the cells from the accumulation of H_2O_2 by dismutating it to form water and oxygen or by using it as an anti-oxidant in which it works as a peroxidase. Reports in the literature have shown that the decreased activity of G-Red is the result of changes in normal activity of G6PD whose deficiency may limit NADPH synthesis[41].

The relationship between G6PD deficiency, oxidant damage and mechanical impairment is quite expected and well known one. In RBC, like in most other cells, the only source of NADPH is HMP-shunt [29]. Glucose-6-phosphate (G6P) is converted into 6-phospho-gluconolactone, catalyzed by G6PD, and accompanied by a reduction of

$NADP^+$ into NADPH. A sufficient amount of NADPH is essential for the integrity of RBC, because it reduces glutathione, which plays important roles in the anti-oxidant defense mechanisms of RBC[42]. NADPH is the coenzyme of G-Red enzyme which regenerate GSH , which in turn takes part in the conversion of H_2O_2 [29]. Therefore, the deficiency of G6PD leads to increased oxidant damage manifested by increased methemoglobin percentage, lipid peroxidation, cross linking between membrane skeletal proteins, hemoglobin attachment to the membrane skeleton and altered membrane protein structure and function[43]. Reactions of bilirubin involving free radicals or toxic oxygen reduction products have been well documented: unconjugated bilirubin scavenges singlet oxygen with high efficiency, reacts with superoxide anions and peroxyl radicals, and serves as a reducing substrate for peroxidases in the presence of H_2O_2 or organic hydroperoxides [44].

The results showed that positively significant correlation was found between G6PD activity levels with GSH concentrations ($r = + 0.442$, $p < 0.05$),(Table-2-) and presented in (Fig. -2-), whereas a negative significant correlations was found between G6PD activity levels and MDA concentrations ($r = - 0.448$, $p < 0.05$) (Table-2-) and presented in (Figs. -3-). The results of each G6PD and G-Red activity levels identified in hyperbilirubinemic neonates with severe G6PD deficiency indicated that the decreased levels of G6PD activity was significantly positively correlated with the decreased levels of G-Red activity ($r = + 0.447$, $p < 0.05$) as shown in (Fig-4-), and the results obtained from G6PD and G-Px activity determination indicate that G6PD activity levels were non significant negatively correlated with the elevated levels of G-Px activity ($r = - 0.310$, $p > 0.05$) as indicated in (Table-2-) and presented in (Figs. -5-). The data obtained from this study also indicated that decreased G6PD activity levels was significantly positively correlated with the reduced catalase activity levels ($r = + 0.441$, $p < 0.05$) as indicated in the same table and presented in (Fig.-6-)

Table 2. The correlation between G6PD activity levels and TSB with different oxidative stress parameters in 21 severe G6PD-deficient hyperbilirubinemic neonates in Najef Governorates .

TSB and oxidative stress parameters	Full-term Neonates with Severe Hyperbilirubinemia TSB ≥ 15 mg/dl			
	Mean ± SD	G6PD activity Mean ± SD U/g Hb	*P* Value	r Value
TSB conc., mg/dl	21.29 ± 5.43	0.46 ± 0.41	< 0.05	-0.320
GSH conc. , µM/g Hb	3.38 ± 2.38	0.46 ± 0.41	< 0.05	+0.442
MDA conc., nM/g Hb	79.14 ± 14.08	0.46 ± 0.41	< 0.05	- 0.448
G-Red activity , U/g Hb	6.98 ± 2.63	0.46 ± 0.41	< 0.05	+0.447
G-Px activity, U/g Hb	50.3 ±14.15	0.46 ± 0.41	> 0.05	- 0.310
Catalase activity , kU/g Hb	69.91 ±19.27	0.46 ± 0.41	< 0.05	+0.441

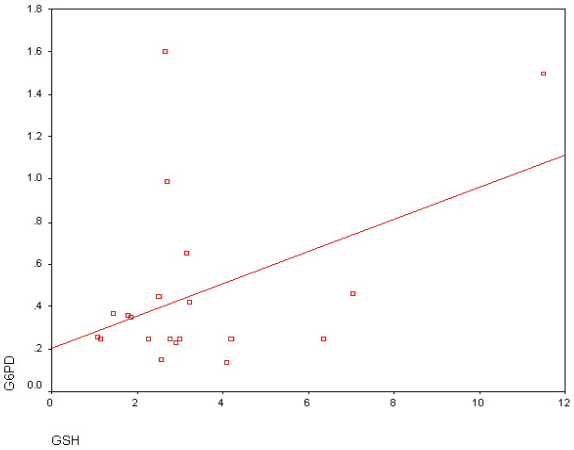

Fig 2. The correlation between G6PD activity levels and GSH conc. in severe G6PD-deficient male hyperbilirubinemic neonates in Najef Governorate ($P < 0.05$; r = + 0.442)

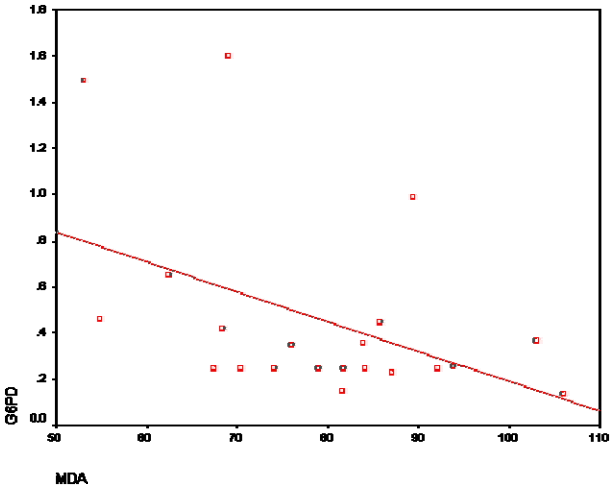

Fig 3. The correlation between G6PD activity levels and MDA conc. in severe G6PD-deficient male hyperbilirubinemic neonates in Najef Governorate (P < 0.05 ; r = - 0.448)

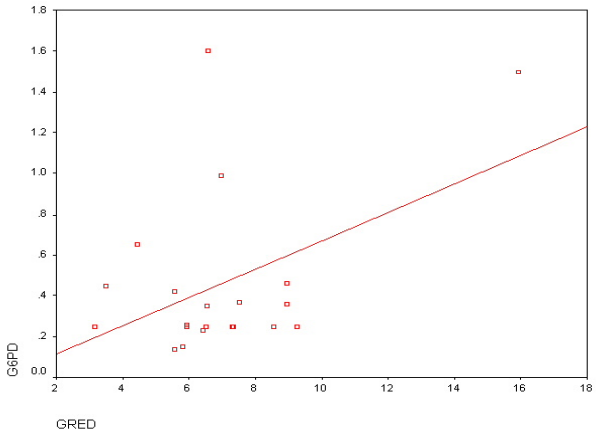

Fig 4. The correlation between G6PD activity levels and G-Red activity in severe G6PD-deficient male hyperbilirubinemic neonates in Najef Governorate ($P < 0.05$; r = + 0.447).

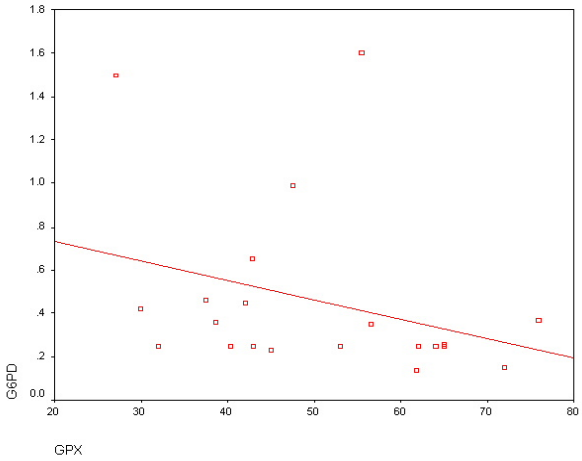

Fig 5. The correlation between G6PD activity levels and G-Px activity in severe G6PD-deficient male hyperbilirubinemic neonates in Najef Governorate ($P > 0.05$; r = - 0.310).

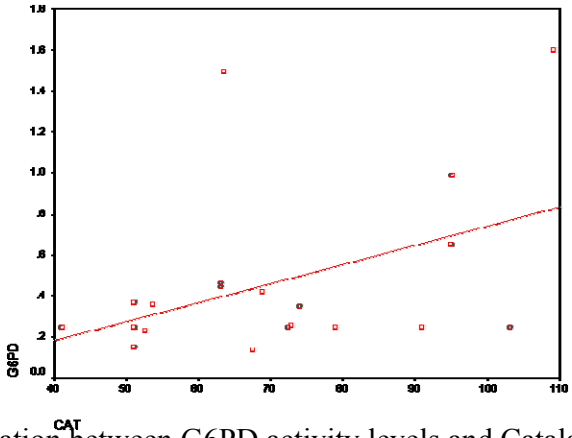

Fig 6. The correlation between G6PD activity levels and Catalase activity in severe G6PD-deficient male hyperbilirubinemic neonates in Najef Governorate ($P < 0.05$; r = + 0.441)

The antioxidants are classified into : primary, secondary and tertiary defense. The primary antioxidants work by preventing the formation of new free radical species. These include SOD, G-Px and metal-binding proteins (e.g. ferritin or ceruloplasmin). Secondary antioxidants trap radicals thereby preventing chain reactions. These include vitamin E, vitamin C, beta-carotene, uric acid, bilirubin and albumin. Tertiary antioxidant repair biomolecules damaged by free radicals. These include DNA repair enzymes[45].

In the present study, various enzymatic and non-enzymatic antioxidant defense system have been determined in severe G6PD-deficient hyperbilirubinemic neonates and compared with that identified in control full-term neonates.

G-Red, and catalase activity levels , which are well known antioxidants enzymes were significantly lower in neonatal hyper-bilirubinemia with severe G6PD deficiency as compared with that found in control group, whereas GSH concentration levels was also decreased. Interestingly, the other activity levels of antioxidant enzyme G-Px

and the oxidant marker lipid peroxidation end product MDA, were increased in neonates with severe G6PD-deficient hyperbilirubinemic neonates. There was also a significant positive correlation between MDA and TSB in severe G6PD-deficient neonates.

This study, revealed the presence of an association between serum oxidant / antioxidant parameters in full-term hyper-bilirubinemic neonates with severe G6PD in Middle Euphrates Province of Iraq. In a healthy human being, the formation and inactivation of reactive oxygen species are balanced at a level at which the compounds can play their physiological role without any toxic effects. This balance can be unstable in the neonatal period following rapid changes in tissue oxygen concentration, immature antioxidant mechanism and considerable neonatal developmental changes in antioxidants. This deterioration is especially evident in the presence of oxidative stress such as phototherapy.

Neonatal hyperbilirubinemia affects 60% of full term infants and 80% of preterm infants in the first 3 days after birth . Although transient, the condition accounts for upto 75% of hospital re-admissions in the first week after birth[46]. Antioxidant activity in the serum of term neonates is lower than that of adults and is still lower in preterm and low birth weight babies as compared to term babies[3]. Red blood cells are extremely susceptible to lipid peroxidation since they are rich in unsaturated membrane lipids, have rich supply of oxygen and transitional metal catalysts. Neonatal erythrocyte membrane is more susceptible to oxidative damage due to its predominant pro-oxidant potential[4].

The erythrocytes are particularly prone to the free radical damage since the membrane lipids are very rich in polyunsaturated fatty acids which play an essential role in generating free radicals. Free radicals, primarily the reactive oxygen species, superoxide and hydroxyl radicals which are highly reactive having an unpaired electron in an atomic or molecular orbit are generated under physiological conditions during aerobic metabolism. As free radicals are potentially toxic, they are usually inactivated or scavenged by antioxidants before they can inflict damage to lipids, proteins or nucleic acids. Alteration in the oxidant – antioxidant profile is known to occur in neonatal jaundice[39]. Moreover the body's defense mechanisms would play an important role in the form of antioxidants and try to minimize the damage, adapting itself to the above stressful situation.

Conclusions

Glucose-6-phosphate dehydrogenase deficiency is a major public health problem. Geographically, it is heterogenous among Iraqi population.

High percentage of incidence of severe G6PD deficiency with TSB $\geq$ 15 mg/dl was observed Najef region of Iraq (11.73%). The results obtained concluded that severe neonatal hyperbilirubinemia continues to be a problem in Najef..

The data obtained indicate that severe G6PD deficiency play an important role as a common etiologic factor in neonatal hyperbilirubinemia in this region of Iraq.

Decreased levels of GSH , Catalase and G-Red were observed in severe G6PD-deficient neonatal hyper-bilirubinemia. Increased levels of G-Px and lipid peroxidation product MDA were observed in severe G6PD-deficient hyperbilirubinemic neonates which indicate an increase in ROS formation due to different causes. Negative correlations were observed between G6PD activity and each of MDA and G-Px in severe G6PD-deficient hyperbilirubinemic neonates.

Positive correlations were observed between G6PD activity and each of GSH, G-Red, and catalase .

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