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Original article

Serum levels of malondialdehyde and Bilirubin in neonates with different stages of hyperbilirubinaemia

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ABSTRACT

Objectives: Bilirubin, a haemoglobin metabolite acts as an efficient scavenger of reactive oxygen species. Increase of bilirubin production could be a response to an initial oxidative stress. Malondialdehyde (MDA) is a metabolite commonly used as indicator of free radical generation by lipid peroxidation. In this study relationship between the levels of serum bilirubin and malondialdehyde was investigated in neonates with different stages of hyperbilirubinaemia. **Materials and Methods:** The study included 110 neonates classified into four groups according the total bilirubin (TB) levels: G1 (n=31; TB < 5 mg/dl), G2 (n=31; TB > 5 and <10 mg/dl), G3 (*n*=31; TB > 10 and <15 mg/dl) and G4 (n=16; TB > 15 mg/dl). **Results:** The level of malondialdehyde was increased in stepwise manner in the hyperbilirubinaemia groups (G1=4.82 IU/l),(G2=9.21 IU/l), (G3=12.87IU/l), and (G4=17 IU/l).A significant positive correlation was observed between malondialdehyde and bilirubin concentrations in the studied groups G1, G2, and G3 (P<0.001). A significant negative correlation was found between malondialdehyde and haemoglobin (P<0.001) in the same groups. **Conclusions:** The increases of serum total bilirubin levels in the neonate could be a response to excessive free radical generation and lipid peroxide formation. That may indicate the possible protective effect of antioxidant administration to the mother during pregnancy on the neonate. Serum MDA levels in the neonate could be used as an indicator or predictorof the severity of neonatal jaundice.

KEYWORDS: Total bilirubin; malondialdehyde; hyperbilirubinaemia

INTRODUCTION

Free radicals and related metabolites have a great interest for the medicinal researchers [1]. They are generated in the body by different endogenous systems in response to a various physicochemical stimuli or pathophysiological condition. Therefore, many of the biological processes are combined with free radical reactions[2]. In all aerobic organisms, the oxygen molecules accept an electron resulting in the formation of high reactive oxygen species such as superoxide and hydroxyl radicals, which have unpaired electron in the atomic or molecular orbit of oxygen [3].

Generally, overproduction of free radicals is deleterious process which results in the formation of important mediators. These mediatorscan interact with many functional and structural molecules in the cell including lipids, proteins, and DNA forming highly mutagenic bases e.g. 8-oxo-guanine leading to the pathological changes, implicated in various human injury [4]. The defense mechanisms of the body are to minimize the cellular damage and overcome the above stressful conditions by the formation of antioxidants, which dispose, scavenge, and/ or suppress the formation of free radicals and thus oppose their actions [5].

There are two main categories of antioxidants including enzymes such as superoxide dismutase, glutathione peroxidase, and catalase or non-enzymes such as several antioxidant vitamins[6]. Numerous peroxidases can reduce lipid hydroperoxides and repair DNA lesions. In addition, the intracellular superoxide dismutases and catalase scavenge thereactive oxygen and nitrogen species and prevent their formation. In the same time, extra- and intracellular small molecules such as ascorbic acid (vitamin

C), glutathione (GSH), and tocopherols (primarily α-tocopherol; vitamin E) have important roles in signal transduction, apoptosis, gene expression, and gene regulation[7, 8]. Furthermore, a various of endogenous metabolites such as bilirubin, uric acid, and many others act as oxygen radical scavengers[4]. However, the knowledge of the basic mechanisms of oxidative/antioxidant balance has many gaps. The study of the optimal benefits from antioxidants for health promotion and disease prevention is needed to fill these gaps.

The disturbance in homeostasis between the rate of free radicals generation and the rate of their scavenging lead to the accumulation of oxidative damage; that is known as oxidative stress[9].Generally, this disturbance in the oxidant – antioxidant balance is known to occur in neonatal jaundice [1, 10].

In blood circulation only minimal molecules of bilirubin are free (free bilirubin) and have the pathophysiological properties, while the major fractions of bilirubin are bound to albumin (conjugated bilirubin) [11, 12]. At low concentrations and under physiological conditions, bilirubin plays a fundamental role as a natural antioxidant and scavenges oxygen free radical and its concentration is increasing in response to oxidative stress[8, 9][13]. The bilirubin at high plasma levels can passively diffuse through cell membrane and by targeting the central nervous system causes a serious of neurological disorders that are clinically known as bilirubin encephalopathy or kernicterus [14-16].

Many studies had observed that bilirubin is able to mediate the oxidative stress by its endogenous antioxidant activity whereas bilirubin prevents lipids from oxidation and cell death by the oxidant-specific fashion [19-21]. Furthermore, albumin bound bilirubin inhibits the oxidation of fatty acids and protects oxidizable substances from destruction in the intestinal tract [17, 18]. The interactions of bilirubin with free radicals or toxic oxygen products have been studied; unconjugated bilirubin efficiently scavenges singlet oxygen, reacts with peroxyl radicals and superoxide anions. Bilirubin also serves as a reducing substrate for peroxidases (in the presence of hydrogen peroxide or organic hydroperoxides) [19-22].

Malondialdehyde (MDA) is a highly reactive metabolite of lipid peroxidation induced by free radicals, and is commonly used as an index of lipid peroxidation[23]. Therefore, we studied serum malondialdehyde concentrations in newborn infants with or without hyperbilirubinaemia to determine lipid peroxide concentrations. On the other hand, red blood cells are particularlysusceptible to lipid peroxidation and free radical damage since they are rich in unsaturated membrane lipids; especially polyunsaturated fatty acids. These fatty acids play an important role in the generation of free radicals because they have rich supply of oxygen and transitional metal catalysts [24]. Therefore, we studied the correlation of serum MDA levels with RBCs count and with hemoglobin concentration in neonates with hyperbilirubinaemia.

This study hypothesized that an important factor in the state of hyperbilirubinemic neonates would be exacerbated oxidative stress, diminished antioxidants stores, or a combination of both. The aim of this study was to investigate the validity of this hypothesis by determining the level of bilirubin and MDA in neonates with different degree of hyperbilirubinemia. The relation between bilirubin and MDA as well as Hb and RBCs was investigated, in order to know if the release of bilirubin and MDA is dependable manner or not.

MATERIALS AND METHODS

This study was carried out in the period January 2011 to January 2013. The study included 110 neonates admitted to the nursing department, El-Nour Specialist Hospital, Makkah, Saudi Arabia. Infants with severe congenital malformation, sepsis, or birth asphyxia were excluded from this study. Neonates were divided into 4 groups according to the levels of total bilirubin (Table 1). The Ethics Committee of Umm Al-Qura University approved the study protocol. All mothers of neonates signed informed consent according to the declaration of Helsinki.

Table 1: Classification of the neonates according to the levels of total bilirubin

Groups	*N*	Male	Female	Criteria
G1	31	22	9	newborn with total bilirubin levels < 5 mg/dl
G2	31	19	12	newborn with total bilirubin levels >5 and < 10 mg/dl
G3	31	14	17	newborn with total bilirubin levels >10 and < 15 mg/dl
G4	17	9	8	newborn with total bilirubin levels > 15 mg/dl
	110	**64**	**46**	

n = number of neonates in each group

The description and classification of all subjects are summarized in Table 1. The neonates with total bilirubin levels < 5 mg/dl were considered as a control group (G1, *n*=31). The neonates with jaundice were 79 subjects (42 males and 37 females). They were classified into 3 groups according to the total bilirubin level, in group 2 (G2, *n*=31) total bilirubin (TB) levels was>5 and < 10 mg/dl. In the group 3 (G3, *n*=31), the TB levels was high (>10 and < 15 mg/dl). In the last group (G4, *n*=17), total bilirubin level (TB) was > 15 mg/dl.

Blood samples: Four ml blood was collected from neonate under complete aseptic conditions. It was divided into 2 parts; 2 ml were dispensed into a plain tube left to clot at 37°C for 15 min, centrifuged for5 min, then the serum was separated and stored at -80°C till measuring the MDA, direct and total bilirubin. The remaining 2 ml was collected on EDTA tubes for determination of complete blood count and hemoglobin.

Determination of bilirubin: The direct and total bilirubin was analyzed according to Jendrassik and Grof (diazo) method on Dimension® RxL-Max® Integrated Chemistry System (Dade Behring, USA) [25].The serum bilirubin reacts with diazotized sulphanilic acid to form an azo compound, the color of which is measured at 546 nm and is proportional to the concentration of bilirubin. The bilirubin concentrations were expressed in mg/dl.

Quantitation of malondialdehyde (MDA):

The concentration of MDA as indication of lipid peroxides in serum was measured using the Quanti-Chrome TBARS Assay Kit (DTBA-100, BioAssay Systems-USA). The reaction of thiobarbituric acid reactive substances (TBARS) with thiobarbituric acid (TBA) forms a pink colored complex. The concentration of TBARS in the sample is proportional to the change in absorbance at 535 nm [26].

Complete blood picture: Complete blood picture was performed to evaluate RBCs and Hb concentration by sysmex XT 2000iblood counter (Sysmex Corporation of America, Long Grove, Illinois, USA).

Statistical analysis: The Statistical analysis of this study was done using SPSS program version 21(IBM, USA). Quantitative data were described in the form of mean ± SE, median and range. The Comparison between groups was performed by using Kruskall-Wallis test. In addition, Spearman correlation was also used. A $P>0.05$ was considered statistically not significant, while a $P<0.05$ and <0.001 were considered significant and highly significant, respectively.

RESULTS

Concentrations of bilirubin, MDA, Hb and RBCs

Total bilirubin, indirect bilirubin, MDA, hemoglobin concentrations, and RBCs were evaluated for each group. The results are summarized in Table 2.The level of MDA was gradually increasing in the hyperbilirubinaemia groups from 9.21 IU/l (G2) to 17 IU/l (G4) in comparing to the control group 4.82 IU/l(G1). Comparisons between the different groups were performed using the Kruskall-Wallis test. Highly significant differences between the groups in age, total bilirubin levels ($P<0.001$) were observed. The levels of indirect bilirubin and MDA were significantly increased in G3 and G4 groups compared to control group ($P<0.001$). Hemoglobin concentration was significantly decreased in G4 compared to the control ($P<0.05$). No significant differences in RBC counts were found ($P>0.05$).

Table: 2 The serum levels of bilirubin (total, direct and indirect), malondialdehyde, hemoglobin, and RBC count in the all studied groups

		G1	G2	G3	G4
Age (days)	Mean± SE Median(range)	19.8 ± 1.7 23 (0-30)	7.9 ± 1.6‡ 4 (0-30)	5.94 ± 0.62‡ 5 (2-14)	6.2 ± 1.8‡ 4 (1-30)
Total bilirubin (mg/dl)	Mean± SE Median(range)	1.8 ± 0.2 1 (0.4-5.0)	7.6 ± 0.3‡ 7 (6-10)	13.10 ± 4.25‡ 13 (11-15)	17.7 ± 0.4‡ 18 (16-20)
Direct bilirubin (mg/dl)	Mean± SE Median(range)	0.6 ± 0.1 0.1 (0.0-3.7)	0.8 ± 0.3 0.2 (0.1-5.5)	0.52 ± 0.08 0.4 (0.1-1.9)	9.6 ± 1.3† 12 (0.3-15)
Indirect bilirubin (mg/dl)	Mean± SE Median(range)	1.2 ± 0.2 0.8 (0.3-4.5)	6.8 ± 0.4 6.8 (0.5-9.9)	12.57 ± 0.21‡ 12.6 (10.5-14.7)	8.5 ± 1.1‡ 8 (4-15.7)
MDA (IU/l)	Mean± SE Median(range)	4.8 ± 0.3 4.1 (2.8-8.0)	9.2 ± 0.4 9.1(4.5-13)	12.87 ± 0.58‡ 13(8-18)	17.0 ± 0.7‡ 17 (12-21)
RBC ($\times 10^{12}$/l)	Mean± SE Median(range)	4.4 ± 0.12 4.3 (2.8-6.2)	4.6 ± 0.2 4.6 (3.5-6.2)	4.7 ± 0.1 4.7 (3.3-6.2)	4.1 ± 0.5 4.2 (2.5-6.1)
Hb (g/dl)	Mean± SE Median(range)	13.1 ± 0.4 13 (8-20)	13.4 ± 0.5 14 (7-19)	15.0 ± 0.4 15 (11-20)	9.8 ± 0.3* 10 (8-12)

The values are expressed as the mean ± SE, median, rangeof the neonate number of each group. All comparisons were done with control subjects. Significant differences are indicated as * for $P<0.05$, † for $P<0.01$, and ‡ for $P<0.001$.

The correlations between MDA and investigated parameters

Pearson R-value was used as indication of the degree of correlations between MDA and the investigated parameters. There is significant positive correlation between malondialdehyde and each of total bilirubin ($r=0.91$, $P<0.001$), indirect bilirubin ($r=0.70$, $P<0.001$), and direct bilirubin ($r=0.50$, $P<0.001$) in all neonates as shown in (Fig. 1). Non-significant negative correlation was observed between MDA and haemoglobin in all neonates. Significant positive correlation was observed between MDA and the total bilirubin in G1 (r=0.61, $P<0.001$), G2 (r=0.61, $P<0.001$), and G3 (r=0.80, $P<0.001$) Fig. 2 (A, B, and C). While a non-significant correlation in G4 (Fig. 2D)was observed.

Fig. 1: Significant correlations between malondialdehyde and total bilirubin (A), indirect bilirubin (B), direct bilirubin (C), and haemoglobin (D) in all neonates. Pearson *R*-value indicate the degree of correlation, *p*-value < 0.05 was considered as a significant difference, and *n* =the number of samples.

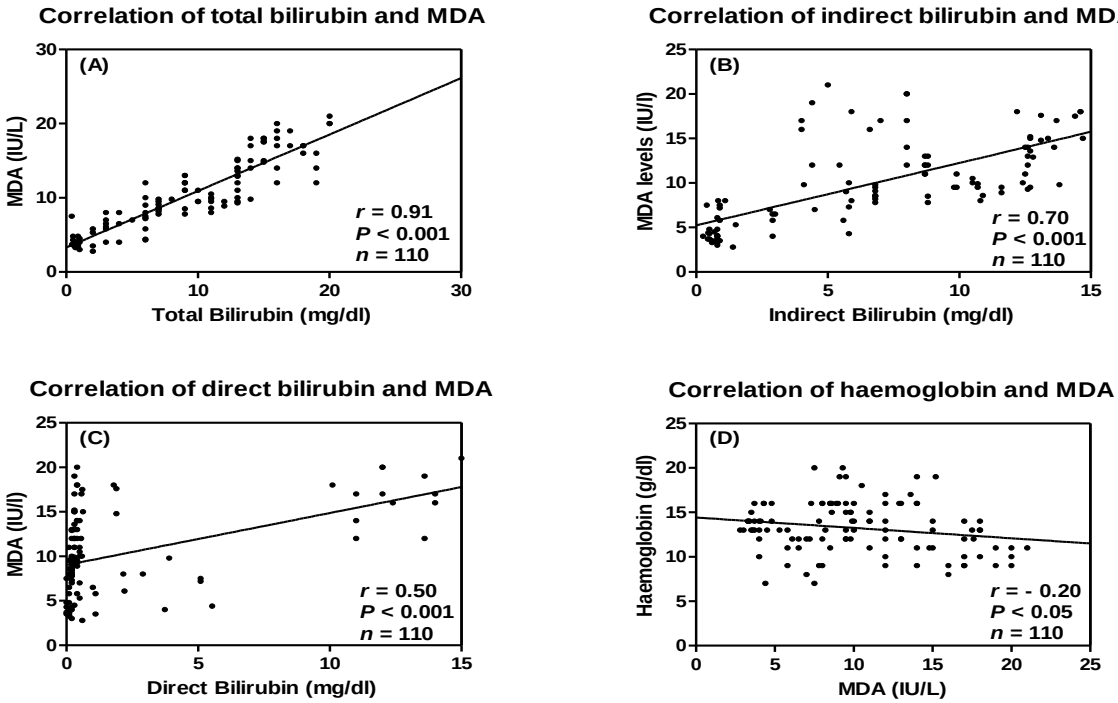
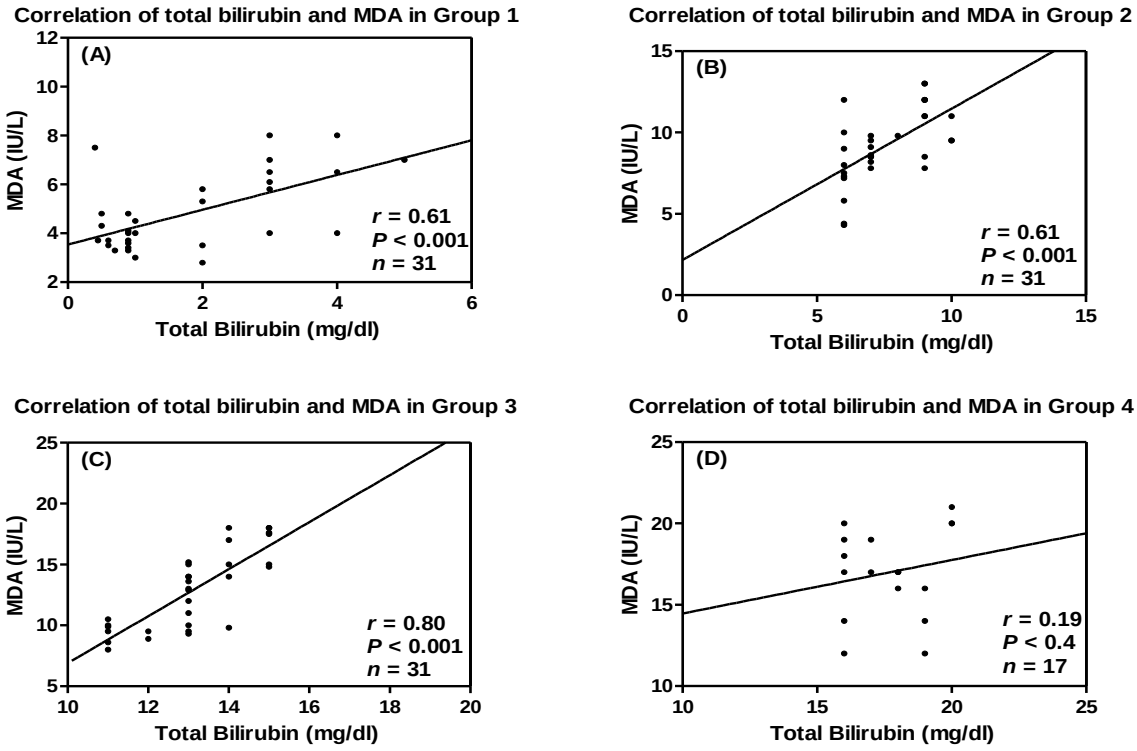


Fig 2: Significant correlations between the levels of malondialdehyde and total bilirubin in-group 1 (A), group 2 (B), group 3 (C), and group 4 (D).Pearson *R*-value indicate the degree of correlation, *p*-value < 0.05 was considered as a significant difference, and *n* =the number of samples.



DISCUSSION

The oxidative stress and lipid peroxidation play important causative role in the development of many serious neurological diseases of the newborn and they are considered as hallmarks of bilirubin-induced neurotoxicity [27]. MDA is frequently used as a biomarker of oxidative stress because it is the end-product of the radical-initiated oxidative decomposition of polyunsaturated fatty acids or lipid peroxidation [28]. MDA was found to be increased in neonatal diseases associated with oxidative damage [29, 30]. Bilirubin has both cytoprotective and cytotoxic properties [31].The exact molecular mechanism and the selective toxicity of bilirubin on certain neuron cells remains not fully understood [32-34].

In this study the level of MDA was increased in stepwise manner in the hyperbilirubinaemia groups(G1=4.82 IU/l),(G2=9.21 IU/l),(G3=12.87IU/l), and (G4=17 IU/l) (Table 2). The plasma MDA concentration was significantly increased in all groups of neonates with hyperbilirubinaemia(P<0.001) comparing to group 1 (Fig. 3), indicating that the increase of bilirubin may be a response to oxidative stress in these neonates. This data indicates that there are direct relationships between bilirubin levels and MDA concentrations in oxidative stress conditions such as neonatal jaundice.

These results are in agreement with the previous studies that determined malondialdehyde concentrations in neonates with or without hyperbilirubinaemia during the first 10 days of life. Previously, it has been reported that serum malondialdehyde concentrations were higher in infants with hyperbilirubinaemia than in controls[35, 36]. Furthermore, it has been reported that the plasma MDA concentrations in neonates with nonhemolytic jaundice were significantly higher than those in healthy infants and MDA level was reduced after phototherapy[37, 38]. Recently, it has reported that the levels of MDA were significantly high in newborn with neonatal jaundice in comparison with its levels in healthy neonates.

The study focused on the detection of the frequency of the different types of neonatal jaundice but did not evaluate the correlation of MDA and bilirubin levels[39]. The results of current study confirmed these findings, but it clearly establishes a positive correlation between serum MDA and serum bilirubin concentrations in all neonates with hyperbilirubinaemia (Fig.1). Our study results are in agreement with previous studies that reported an increase in serum MDA in infants with hyperbilirubinaemia with positive correlations between MDA and bilirubin concentrations in their study group [35, 38, 40].

The authors in previous study have been classified the studied neonates into 2 groups: neonates with haemolysis and without haemolysis. They reported that there was clear significant positive correlation between MDA and bilirubin levels in the group with haemolysis while the situation of neonates without haemolysis is less obvious [36]. They suggested that the positive correlation found between malondialdehyde and bilirubin concentrations in neonates with haemolysis may be due to the effect of haemolysis itself that leads to increase in oxidative stress and so increase the bilirubin concentration. Additionally, bilirubin; a product of the haemolytic process protects against the potential harmful effects of haemolysis process. This may explain why haem is excreted as bilirubin rather than as biliverdin[36]. In the non-haemolytic neonates, the absence of apparent correlation between the high concentrations of bilirubin and malondialdehyde may be indicative of a more complex interaction between them. Such an interaction could explain why bilirubin toxicity is rare in full term babies without haemolysis[36].

In the present study, neonates with hyperbilirubinaemia with low haemoglobin and low RBCs as in group 4 (G4)had a non-significant positive correlation between MDA and bilirubin concentrations (P<0.4) (Fig. 2). The increase in bilirubin levels may be explained by the effect of oxidative stress in these groups. However, in group 4 the correlation between the high concentrations of bilirubin and MDA may be explained by the defense mechanisms of hyperbilirubinaemia that is often seen in neonates when antioxidant mechanisms are not yet fully developed.

Plasma MDA levels of newborn infants is indicative of oxidative stress during the perinatal period as reported in previous study [41]. They observed that the MDA levels were significantly higher in infants who were born after spontaneous vaginal delivery, compared to those born by cesarean section[41].

CONCLUSION

The present study shows that hyperbilirubinaemia in neonates is associated with increased generation of ROS reflected in the increased level of serum MDA. The serum MDA levels could be used as indicator for predicting the severity of neonatal jaundice. Future research studies including measurement of parameters of oxidative stress and inflammatory markers should be carried out to investigate the possible causative factor for hyperbilirubinaemia in neonates. The possible benefits of antioxidants in hyperbilirubinemia should be considered in further study.

The results of this study highly recommend starting of clinical trials and studies dealing with the administration of antioxidants vitamins such as vitamin A and E to the pregnant mothers, especially in the last trimester, as the development of antioxidant stores in the liver of the neonate may decrease the possibility of developing neonatal jaundice or at least decrease the severity of hyperbilirubinaemia.

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