



Studies on serum nitric oxide levels in subjects with high bilirubin value in comparison of normal healthy control subjects

Ramesh Kumar*, Ritika Jain

Department of Biochemistry, Bundelkhand University, Jhansi, Uttar Pradesh, (U.P.), India.

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Abstract: Bilirubin is excreted in bile and urine, and elevated levels may indicate certain diseases. Serum bilirubin are inversely related to risk of certain heart diseases. Since nitric oxide (NO) is produced by three types of Nitric Oxide Synthases (NOSs), rapid changes in stable oxidized metabolites (nitrite and nitrate) in the tissues and blood should be represented by the amount of stable forms in serum and may reflect changes in the body. Therefore, the aim of this study was to evaluate the correlation between nitric oxide (nitrite & nitrate) production and bilirubin levels in serum. The serum samples were collected from individuals with high levels of bilirubin and normal range controls. Nitrite was measured by a Griess reaction while nitrate was measured using the enzymatic one step assay with nitrate reductase. The total 36 samples (18 normal range (N) and 18 high bilirubin values (H)) were evaluated for the NO levels. The age group varies from 4-70 & 5-65 for normal & high levels of bilirubin, respectively. The levels of bilirubin in the normal range & high values varies from 0.81-0.98 (mean=0.88±0.01) & 1.12-20.18 (mean=5.96±2.07), respectively. When the nitrite (14.48±1.05µM versus 13.96±0.96µM, P>0.05) and nitrate (25.85±2.04µM versus 25.85±1.53µM, P>0.05) levels were compared between these groups no significant differences were observed. Results of this study reveal that there is no correlation between nitric oxide production and the serum bilirubin levels.

Key words: Bilirubin, Nitric Oxide, Nitrite, Nitrate

Introduction

Heme oxygenase (HO) is the primary enzyme responsible for heme catabolism and is found in several tissues with significant activity levels in the liver, spleen, and erythropoietic tissue (1). HO is the rate-limiting enzyme in the conversion of heme into carbon monoxide, iron, and biliverdin, which is immediately reduced to bilirubin by bilirubin reductase (2). The spleen plays a primary role in the degradation of hemoglobin and in bilirubin excretion. Bilirubin has long been regarded as a waste product, lacking any clear physiologic role. However, there is evidence suggesting that UCB is a potent antioxidant (3-6), and that mildly elevated serum UCB levels are associated with a better outcome in diseases involving oxidative stress (7). Because of the antioxidant properties of UCB, it is nowadays believed that physiologic jaundice of the neonate may have inherent benefits. Jaundice or hyperbilirubinaemia is the result of elevated serum levels of bilirubin. The production of bilirubin is regulated by the rate-limiting enzyme, heme oxygenase-1 (HO-1), which is induced rapidly by stimuli associated with oxidative stress and inflammation, such as hypoxia, cytokines and nitric oxide (NO) radicals (8-11). In vitro studies have shown that NO activates expression and activity of the isoform HO-1 (12). Several years ago, Stocker and co-workers demonstrated that bile pigments are potent scavengers of free radicals in vitro. Since then, it has been shown that bilirubin interacts with other biological processes, such as the ones involved in protein phosphorylation and nitric oxide (NO) signaling. The previous studies have shown that there is a link between bilirubin and nitric oxide.

Nitric oxide (NO) is heat-labile, unstable compound and is one of the few gaseous signaling molecules known (13). It is involved in many physiological and pathological processes within the body, both beneficial and detrimental (14,15). Appropriate levels of NO production are important in protecting organs from ischemic damage (16), whereas chronic expression of NO is associated with various malignancies and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (17,18). Genetic factors including endothelial nitric oxide synthase (eNOS) were implicated in pathogenesis of rheumatoid arthritis, and extra-articular manifestations of rheumatoid arthritis were significantly greater among the carriers (19).

Since NO is involved in various pathological states and is produced by three types of Nitric Oxide Synthases (NOSs), rapid changes in stable oxidized metabolites (nitrite and nitrate) in the tissues and blood should be represented by the amount of stable forms in serum and may reflect vascular activities and circulatory or inflammatory changes in the body (20). NO is produced in all tissues and organs by constitutive NOS (cNOS), which includes endothelial NOS (eNOS; isoform III) and neuronal NOS (nNOS; isoform I) and inducible NOS (iNOS; isoform II) (21). Therefore, pathophysiological changes such as atherosclerosis with coronary artery diseases (22,23), endothelial dysfunction (24), pro-inflammation and inflammation seen in various diseases (25-28) may be to some extent studied by measuring NO

*Corresponding Author:

Dr. Ramesh Kumar, Ph.D.

Associate Professor,
Department of Biochemistry,
Bundelkhand University,
Jhansi (U.P.), India.

metabolites in the peripheral blood (29-32). The aim of this pilot study was to compare the serum levels of bilirubin and NO as well as to estimate whether NO serum levels differ between healthy controls and the subjects/individuals with increased serum bilirubin levels.

Materials and Methods

Study group: Serum samples from eighteen subjects (6 males and 12 females) having high Bilirubin values and 18 individuals (10 males and 8 females) who have normal Bilirubin values were collected and stored at -80°C .

Nitrite determination: Nitrite was measured by using a Griess reaction which is described elsewhere (33). The results were given as μM .

Nitrate determination: Nitrate was measured using the enzymatic one-step assay with nitrate reductase (34). This method is based on the reduction of nitrate to nitrite by nitrate reductase in the presence of $\beta\text{-NADPH}$. Tubes containing $250\ \mu\text{l}$ of $100\ \text{mmol/l}$ potassium phosphate buffer ($\text{pH}\ 7.5$), $50\ \mu\text{l}$ of $12\ \text{mmol/l}$ $\beta\text{-NADPH}$, and $100\ \mu\text{l}$ sample were equilibrated at 25°C . To start the enzymatic reaction, $40\ \mu\text{l}$ of $500\ \text{U/l}$ nitrate reductase was added. The samples were incubated for 45 min in the dark. The oxidation of $\beta\text{-NADPH}$ was monitored in terms of the decrease in absorbency at $340\ \text{nm}$. The method of standard addition was used to minimize the effect of interfering substances from the serum. The results are given as μM . Samples with internal standard, and serum and reagent blanks were also analyzed.

Statistical analysis

Null Hypothesis: There is no correlation between nitric oxide levels and serum bilirubin conc. To compare differences in nitrite-nitrate levels in different groups (normal bilirubin vs high bilirubin values), all values were expressed as mean \pm standard of means (SEM) unless stated otherwise. Statistical significance level was set to 0.05 for all calculations. Nitrite and nitrate have no correlation at 5% level of significance.

Results

The total 36 samples (18 normal range (N) and 18 high bilirubin values (H)) were evaluated for the NO levels. The age group varies from 4-70 & 5-65 for normal & high levels of bilirubin, respectively. The levels of bilirubin in the normal range & high values varies from $0.81\text{-}0.98$ (mean= 0.88 ± 0.01) & $1.12\text{-}20.18$ (mean= 5.96 ± 2.07), respectively. When the nitrite ($14.48\pm 1.05\ \mu\text{M}$ versus $13.96\pm 0.96\ \mu\text{M}$, $P>0.05$) and nitrate ($25.85\pm 2.04\ \mu\text{M}$ versus $25.85\pm 1.53\ \mu\text{M}$, $P>0.05$) levels were compared between these groups no significant differences were observed (Fig. 1 & 2).

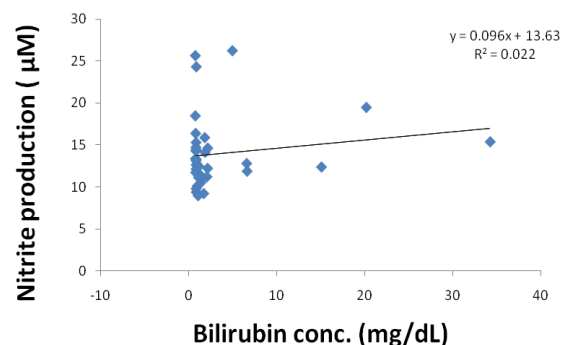


Figure 1: Serum levels of Nitrite in subjects with normal and high values of Bilirubin

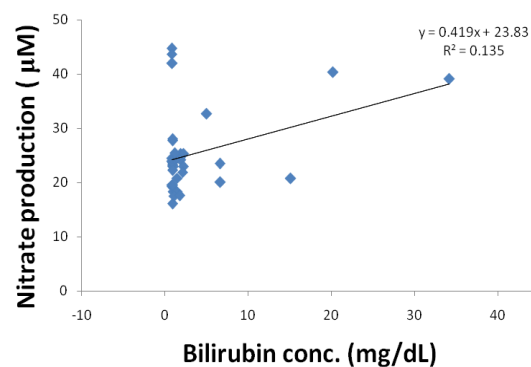


Figure 2: Serum levels of Nitrate in subjects with normal and high values of Bilirubin

Discussion

Bilirubin, a major product of heme catabolism, belongs to compounds with pleiotropic biologic effects. Although for decades it was considered as a metabolite dangerous for human health, recent data indicate that bilirubin exhibits potent antioxidant properties with substantial positive clinical consequences. Vitek *et al.*, (2002) reported that total serum antioxidant capacity was found to correlate with serum bilirubin levels in adult Gilbert syndrome (GS) patients, and this relation was confirmed in an *in vitro* study (35). It has been demonstrated a close correlation between these markers (called biopyrrins) and many other pathologic conditions including ischemic heart disease (36,37) congestive heart failure (38), atopic dermatitis (39), and surgical (40,41) or even psychological stress (42). The same relationship was also found for asbestos-induced oxidative stress (43). Close associations between biopyrrin levels in cerebrospinal fluid and childhood meningitis (39) and Alzheimer's disease (44) were also demonstrated, suggesting that bilirubin consumption during oxidative stress may belong to the major pathways, preventing deleterious effects of oxidative stress. It is interesting to note that low urinary biopyrrin excretion in patients with GS was described recently (45) which is consistent with low oxidative stress in patients with benign hyperbilirubinemia (35).

The anti-proliferative effects of bilirubin were proved in an *in vitro* study by Zucker *et al.*, (46) who showed that proliferation of a breast cancer cell line is inhibited in a dose-dependent manner by unconjugated bilirubin at physiologic concentrations through the induction of apolipoprotein D. Zucker and co-workers, who demonstrated in *in vitro* (47) and *in vivo* epidemiologic

(48) studies protective effects of bilirubin against colorectal cancer. On the other hand, the lower expression alleles of *UGT1A1*, the bilirubin conjugation gene in the liver tissue, were found to be associated with increased risk of breast cancer in premenopausal black (49) and Chinese (50) women. However, these results were not confirmed in a subsequent study on white women, suggesting that other factors might be responsible (51).

Cerne *et al.*, (52) showed that decreased serum bilirubin levels were associated with peripheral atherosclerosis only in smokers. Low serum bilirubin in patients with peripheral vascular disease (PVD) was detected also in a small study by Kangas *et al.*, (53) It is known that patients with impaired glucose tolerance, diabetes (54), or arterial hypertension (55), risk factors known to be closely associated with atherosclerosis, have lower serum bilirubin levels. Furthermore, low serum bilirubin levels were found to be associated with lupus nephritis (56), indicating that serum bilirubin may contribute to protection against oxidative stress-mediated diseases, presumably in a general manner. Moreover, low serum bilirubin also may be associated with mental illnesses, as suggested by studies by Oren *et al.*, (57), who reported that patients with winter depression exhibited lower nocturnal bilirubin levels compared with controls and suggested that bilirubin may serve as an important chronobiological photoreceptor.

In individuals with normal hepatic function, elevated plasma bilirubin levels have been correlated with decreased risk for a number of health disorders, including coronary heart disease (58-65) This protection is particularly notable in subjects with Gilbert syndrome, who for genetic reasons maintain plasma bilirubin above 20 μ M. It is now known that, in submicromolar concentrations, bilirubin can inhibit certain isoforms of NADPH oxidase (66-70) – a key source of pathogenic oxidative stress in many disorders (62). Moreover, there is other recent evidence that bilirubin may exert anti-inflammatory immunomodulatory effects that likely are independent of its antioxidant activity (71-73). These findings cast a fresh light on the situation, and render more plausible the notion that the plasma bilirubin pool could be providing antioxidant protection to the body's cells, or working in other ways to dampen inflammation.

The elevated plasma bilirubin may correlate with improved health outcomes. For example, obesity tends to associate with decreased plasma bilirubin, whereas bilirubin tends to rise after weight loss, for unknown reasons (74). Hence, plasma bilirubin may serve as a marker for obesity.

It may be postulated that serum bilirubin is a major clinically relevant cytoprotectant, contributing substantially to protection against oxidative stress. However, additional studies are necessary to uncover all the pathophysiologic associations and mechanisms involved. Abd El-Azem M Ali *et al.*, (75) evaluated the role of NO and endothelial function in patient's portal hypertension patients with compensated decompensated liver cirrhosis. They observed portal hypertension was associated with significant increase in NO and significant decrease in endothelin-1 levels. Further, there were no significant correlation between serum albumin, bilirubin, ALT and prothrombin time with NO

production.

There is not much study has been explored to see the association/relation of serum nitric oxide and bilirubin levels. Keeping in view of the vast role of bilirubin in various pathological conditions we performed the experiments to know the association between bilirubin and nitric oxide. Our findings are further supported by the previous report by Abd El-Azem M Ali *et al.*, (75). Results of this study reveal that there is no correlation between nitric oxide production and the serum bilirubin levels. However, those results are preliminary and have to be confirmed in sample of larger size.

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