The Determination of Total Serum Bilirubin Concentration in Type 2 Diabetes patients

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Abstract: Bilirubin represent a natural end-product of heme metabolism and is used as as a marker in diagnosis of hepatobiliary diseases. Recent studies demonstrated that serum bilirubin levels are related to the risk of Type 2 diabetes mellitus (T2D) development and subsequent complications. The aim of this study was to analyzed serum total bilirubin concentrations and its relationship with biochemical and clinical characteristics in T2D patients. Total of 109 participants were included in this study, 54 controls and 55 diabetic patients, both gender, while ages ranged from 35 to 70 years. Biochemical parameters were analyzed by standard IFCC methods while serum total bilirubin concentrations was determined by the method of Jendrassik/Grőf. All analyses and measurements were provided by using the chemical analyzer VITROS 350. Results showed a significant difference in concentrations of glucose, glycated hemoglobin (HbA1c), lipid profile (total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol) and bilirubin between T2D patients and controls (p<0.05). Also, significant association was found between bilirubin and glucose concentrations in two investigated populations (p<0.05). It appears that elevated concentration of bilirubin and biochemical characteristics are associated with the progression development of Type 2 diabetes and its related vascular complications. Therefore, total serum bilirubin concentrations could be used as potential T2D biomarker and therefore, as new therapeutic target.

INTRODUCTION

Type 2 diabetes mellitus (T2D) is common type of diabetes mellitus, it is estimated that until 2035 will affect about 400 million people around the world (International Diabetes Federation, 2019). Underlying mechanisms involve insulin resistance (IR), oxidative stress, inflammation, dyslipidemia, and obesity but exact molecular mechanism is still not fully understood. Bilirubin belongs to the superfamily of tetra pyrrolic compounds which presents as a highly conserved groups of molecules. For many years, bilirubin has been considered to be waste product of heme catabolism in humans. Recent reported data pointed out that bilirubin has beneficial effects to various oxidative stress related diseases (Regino, Velasco, Sandova, 2009; Vitek, 2012; Inoguci, Sonoda, Maeda, 2016; Rani, Deep, Singh, et al., 2016). In addition, serum bilirubin is closely related to human health, although its proper mechanism remains largely unknown. It possesses anti-oxidative, anti-inflammatory and immuno-suppressive properties, and acts as a central molecule in the pathogenesis of many diseases. Previous studies showed important role of bilirubin as risk factor in development and incidence of T2D and related vascular complications (Vona, Ganhardell, Cittadini, et al., 2019; Yang, Ni, Chang, et al., 2019). Bilirubin represents a natural end-product of heme metabolism and is used in clinical practice as a marker for hepatobiliary diseases and its related disturbances. In the body, bilirubin formation is based on breakdown reaction of heme present in hemoglobin, myoglobin, cytochromes, catalase, peroxidase, and tryptophan pyrrolase. This reaction is catalyzing by the ubiquitously expressed heme oxidase-1 (HO-1) which
participates in heme breakdown to generate biliverdin, free ferrous ion and carbon monoxide. Then, biliverdin is rapidly converted to bilirubin by biliverdin reductase, and further processed in the liver by uridine diphosphate-glucuronosyltransferase1A1 (UGT1A1) to a water-soluble form of bilirubin for elimination from the body (Figure 1) (Vitek, 2012; Peng, Goyal, Xu, 2017). Bilirubin is nonpolar molecule thus insoluble in plasma and circulates in a body by binding to plasma albumin. This form is called unconjugated or indirect bilirubin or free bilirubin. Conjugated bilirubin or direct reacting bilirubin represent form of bilirubin bonded to glucuronic acid. Total bilirubin is the sum of conjugated (direct) and unconjugated (indirect) bilirubin and generally ranges from 3 to 20 μmol/L in healthy individuals both, men and women (Fevery, 2008).

The aims of this work, was to determine serum total bilirubin concentration in Type 2 diabetes patients, and to investigate whether elevated total bilirubin is associated with clinical and biochemical characteristics of Type 2 diabetics.

**EXPERIMENTAL**

**Subjects**

The study sample consisted of 54 healthy subjects and 55 patients diagnosed with Type 2 diabetes mellitus, with age span of 35 to 65 years. All human subjects involved in this study were patients of General Hospital in Tešanj, BH. Protocols of research involving human subjects and material derived from human subjects in this study were done in accordance with the ethical recommendations and practices of the Tešanj General Hospital and complied with ethical principles outlined in World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects (initiated in June 1964, last amendment in October 2000). Patients were selected for the study on the basis of presence of history of diabetes for more than five years and were receiving standard antidiabetic drug therapy of 250 mg Metformin. Initial diagnosis of T2D was established by a specialist of internal medicine who used American Diabetes Association (ADA, 2016) criteria for diagnosis of the disease. Controls as healthy subjects were of approximately same age (35-65 years old), with normal glucose tolerance (fasting plasma glucose less than 6.2 mmol/l and two-hour postprandial glycaemia less than 7.8 mmol/l). They also had no abdominal obesity as clinical criteria for insulin resistance and adipose tissue inflammatory component. Participants excluded from study where the ones with clinically confirmed hepatitis B or C viral infection or active liver and kidney damage. All subjects involved in this study gave their written informed consent for participation.

**Sample Analysis**

The subjects gave venous blood samples between 8 and 10 in the morning after overnight fasting. All samples, after collection in sterile tubes, were centrifuged at 3000 rpm for 10 minutes and serum was stored at 4°C until analysis. Fasting blood glucose concentration was measured by an enzymatic glucose hexokinase method while ion-exchange high performance liquid chromatography was used for measurement of hemoglobin A1c (HbA1c). Lipid profile (total cholesterol, triglycerides (TGs), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C)) were determined by standard clinical laboratory protocols. Total bilirubin (TBIL) concentration was measured by the Jendrassik and Gróf method and all measurement were performed using chemical autoanalyzer VITROS 350, (Rome, Italy). Standard IFCC (International Federation for Clinical Chemistry) protocols and recommended methods were used for all analyses and measurements.

**Serum total bilirubin method**

The common methods for the analysis of total bilirubin in serum are enzymatic and spectrophotometric methods based on a coupling reaction with different diazo dyes in the presence of an acceleratory agent (Rand, di Pasqua, 1962; Cherian, Soldin, Hill, 1981; Garber, 1981; Westwood, 1991; Choosongsang, Bodhikul, Musigavon, et al., 2010). Nowadays, a specific assay for the measurements of bilirubin levels in serum uses HPLC method dry-slide technique (Ngashangva, Bachu, Goswani, 2019). In this work, the Jendrassik and Gröf method (1938) was used for determination of serum total bilirubin concentration. Although a number of modifications have been made, the Doumas, et al. (1973) method (with minor alterations of the original protocol given by Jendrassik and Gröf) is currently being applied in clinical trials.
The main advantages of Jendrassik and Gróf method are:

a) not affected by pH changes of type of specimen (plasma, serum),
b) maintains optical sensitivity,
c) insensitive to high protein concentration.

It is very important that the sample is not hemolyzed and lipemic otherwise the measurement is burdened with serious errors. Also, bilirubin is photo-oxidized when exposed to artificial light or sunlight, and serum should be protected from direct light and stored in the dark in the refrigerator at low temperatures of 2-8°C. The serum specimen for bilirubin determination should be used immediately or as possible on the same day. If not tested immediately samples should be stored in dark colored bottle at 2-8°C for not more than 24 hours. Gross contamination at any stage makes the specimen unsuitable for bilirubin determination. The samples should be brought to room temperature before use. No prior patient preparation is required.

Principle of method: The total bilirubin in serum or plasma is determined with the using the automated method of Jendrassik and Gróf by coupling with diazotized sulfanilic acid after the addition of caffeine, sodium benzoate and sodium acetate. A blue azobilirubin is formed in alkaline Fehling’s solution II and is measured at 578 nm (Figure 2) using the Chemical autoanalyzer VITROS 350. Reference interval of serum of total bilirubin for adults and infants >1 month is 3.4-17 μmol/L.

![Figure 2: Reaction mechanism of measurement of bilirubin in serum sample.](image)

Statistical analysis

All statistical analyses were done by SPSS (version 17.0 for Windows, SPSS Inc; Chicago, IL, USA). P values smaller than 0.05 were accepted as significant. Data are expressed as mean ± SEM. The significance of differences among groups was analyzed statistically by Student’s t test, followed by Spearman’s coefficient correlation and nonparametric Mann-Whitney U-test was used in order to estimate differences in glucose, hemoglobin A1c, lipid profile, and bilirubin concentration between groups.

RESULTS AND DISCUSSION

Clinical and biochemical characteristics of the investigated population are shown in Table I. All of the measured metabolic parameters were significantly different between the diabetic patients and controls.

| Parameters                     | Type 2 diabetics | Controls | p-value* |
|--------------------------------|------------------|----------|----------|
| Number                         | 55               | 54       |          |
| Age (years)                    | 49               | 51       | p < 0.001|
| Gender (M/F)                   | 27/28            | 20/37    |          |
| Glucose, mmol/L                | 9.98±3.54        | 5.25±0.58| p < 0.001|
| HbA1c, %                       | 6.85±1.35        | 4.79±0.67| p < 0.001|
| Total cholesterol, mmol/L      | 5.23±1.14        | 5.65±1.05| p = 0.05 |
| HDL-C, mmol/L                  | 1.20±0.58        | 1.63±0.36| p < 0.001|
| LDL-C, mmol/L                  | 2.93±1.03        | 3.10±1.06| p < 0.05 |
| TGs, mmol/L                    | 3.00±2.06        | 2.16±1.15| p < 0.05 |
| Bilirubin, mmol/L              | 15.87±13.67      | 11.11±2.24| p < 0.05 |

Data are presented as mean±SEM. HbA1c-glycated hemoglobin; HDL-C-high density lipoprotein cholesterol; LDL-C-low density lipoprotein cholesterol; TGs-triglycerides. * All differences were tested using Mann-Whitney U test.
Total bilirubin concentrations are significantly higher in Type 2 diabetics when compared with healthy subjects (p<0.05). In this study we discovered a positive significant association between total bilirubin and glucose levels in a study population (Figure 3).

![Figure 3: Correlation between concentration of bilirubin and glucose of the study population (rho = 0.235, p< 0.05).](image)

These results are in line with reported data of previous studies in T2D patients.

In the body, serum total bilirubin levels are influenced by many factors as gender, age, smoking status, alcohol use, consumption of drugs or plant products, race, ethnicity, and liver disorders, which could be the reason for inconsistency in the results of some of the provided studies. Recent investigations have indicated that bilirubin at moderate or slightly elevated levels acts as a cytoprotectant molecule in diabetes subjects with vascular complications (Zhu, Wu, Bi, et al., 2017; Takei, Inoue, Sonoda, et al., 2019). In this study, significant increased levels of bilirubin were showed in T2 diabetic patients. Previous studies demonstrated that higher concentrations of bilirubin lead to decrease oxidative stress and improve insulin sensitivity and insulin synthesis in diabetes (Jaynathi, Srinivasan, Maran, 2019). Also, lower bilirubin concentrations are associated with development of T2D and vascular complications; role was dependent on ethnicity and age of study population and positively associated with the risk of incidence of diabetes. Low bilirubin concentrations in T2D patients indicates poor regulation of disease and glycemia. According to HbA1c measurement, elevation of serum bilirubin may be useful in managing disease and diabetic control in T2D patients (Zhu, Wu, Bi, et al., 2017; Erkus, Aksas, Kocak, et al., 2018).

Increased bilirubin and glucose levels as well as a positive correlation between bilirubin and glucose shown that patients included in this study have a good regulation of diabetes (HbA1c less than 7.0% which cut off for good control glucose and managing of disease), and therefore, good glycemic control (Farasat, Sharif, Manzoor, et al., 2017). Di Nicolantonio, et al. (2018) have found that higher bilirubin concentrations or injections of bilirubin or biliverdin could prevent deterioration of glucose tolerance. Chronic hyperglycemia leads to the generation of the highly dangerous reactive oxygen species (ROSs) that have been implicated in vascular dysregulation (this is a major underlying feature in diabetic complications). Previous studies have been demonstrated that hyperglycemia could act as predisposing factor to increased LDL glycation and associated with higher levels of other lipids (free fatty acids, FFAs; TGs, total cholesterol) and pointed out that good lipid control means and better glycemic control (Xu, Lee, Baek, et al., 2014). Serum bilirubin as endogenously produced natural antioxidant can scavenge ROSs and reactive nitrogen species (RNSs) produced by elevated glucose in body and therefore, inhibit of the oxidation of LDL (Cherryath, Gonepatia, Petersh, et al., 2010; Nano, Muka, Cepeda, et al., 2016). Results of present work for lipid profile i.e. concentrations of total cholesterol, TGs, HDL cholesterol, LDL cholesterol (Table 1), showed significant difference between cases and controls. Concentrations of total cholesterol, TGs and LDL were slightly lower in diabetics compared with healthy subjects while HDL level was significant higher in controls than T2D patients. One of the explanations for these results is protective role of elevated total bilirubin concentrations in serum of diabetic populations. Provided investigations have reported that a relationship between elevated serum bilirubin and decreasing lipid profile concentrations although significant, also depends on the experimental conditions of the measurement.
In the study Peng, et al. (2017) have found that patients with higher bilirubin have reduced total cholesterol, TGs and LDL levels in serum. Also, animal model confirms this finding showing that bilirubin treatment with 60 mg/kg, 3 times per week injected intraperitoneally decreased levels of total cholesterol, TGs and FFAs in the liver tissues.

Evidence indicates that bilirubin is closely related to lipid metabolism. Lin, et al. (2016) found that bilirubin increases insulin sensitivity and glucose tolerance by regulating cholesterol metabolism in mice as consequences of role of HO-1 (heme oxygenase-1) in adipose tissue through different molecular mechanisms. The one of these processes is neutralization of free radicals of oxygen and nitrogen formed under hyperglycemia and prevention of oxidation of intracellular lipids. Also, some authors demonstrated that middle-aged and elderly individuals have increased concentrations of total bilirubin in serum compared to healthy people (Boland, Doug, Bettencourt, et al., 2014). Age difference between cases and controls were also demonstrated in the present study (Table 1).

Experimental and human studies have shown that elevated bilirubin levels are associated with decreased risk of T2D and diabetes complications. Abbasi, et al. (2015) observed that increased levels of bilirubin for about 25% are associated with lower risk of T2D.

Metabolomics research together with epidemiologic and genetic analysis highlight importance of bilirubin in pathogenesis of Type 2 diabetes. Obtained data suggests that bilirubin may be important biomarker for the development of diabetes and its complications as well as a newly potential therapeutic target (Abbasi, 2015; Peng, et al., 2019; Vona, Ganbardella, Cittadini, et al., 2019; Mandal, 2020). In support of this, bilirubin measurement is inexpensive, performed routinely, and accessible to most medical laboratories.

This study has some limitations. First, number of participants was small and further analysis should be done on a larger number of participants. Second, in this study, total bilirubin was measured while provided studies and examinations demonstrated that direct bilirubin had clinical significance. Third, analysis of total bilirubin in serum was determined on a subject of Caucasian descents although it is well known that bilirubin is affected by ethnicity and race of study population.

CONCLUSION

In summary, results show that elevated bilirubin concentrations were negatively associated with fasting blood glucose and glycated hemoglobin, while positive association between bilirubin with TG and HDL levels was detected. These findings may play an important role in explaining glycemic control and intracellular lipids metabolism.

The results of this preliminary study indicate a beneficial effect of the total bilirubin on the progression and management of Type 2 diabetes and its related vascular complications.

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Summary/Sažetak

Bilirubin predstavlja prirodni krajnji produkt metabolizma hema i njegova klinička primjena je kao markera u dijagnosticiranju hepatobilijarnih bolesti. Nedavna ispitivanja pokazala su da su koncentracije bilirubina u serumu povezane s rizikom od *diabetes mellitusa* Tipa 2 (T2D) i pratećim komplikacijama. Cilj ovog istraživanja bio je analizirati koncentraciju ukupnog bilirubina u serumu i njegova povezanost s biohemijskim i kliničkim karakteristikama u T2D bolesnika. Ukupno je 109 učesnika bilo uključeno u ovu studiju, od čega su bile 54 kontrole i 55 bolesnika s dijabetesom, oba spola i starosne dobi od 35 do 70 godina. Biohemijski parametri analizirani su standardnim IFCC metodama, dok je koncentracija ukupnog bilirubina u serumu određena metodom Jendrassik-Gróf. Sve analize i mjerenja urađena su korištenjem hemijskog analizatora VITROS 350. Rezultati su pokazali značajnu razliku u koncentraciji glukoze, glikiranog hemoglobin (HbA1c), lipidnog profila (ukupnog holesterola, triglicerida, HDL-holesterola, LDL-holesterola) i bilirubina između T2D bolesnika i kontrola (p <0.05). Također, nađena je značajna povezanost između koncentracije bilirubina i glukoze u ispitivanoj populaciji (p <0.05). Čini se da su povišena koncentracija bilirubina i biohemijske karakteristike povezane s rizikom od razvoja dijabetesa Tipa 2, a ukupne koncentracije bilirubina u serumu mogu se upotrijebiti kao potencijalni T2D biomarker te prema tome i kao novi terapeutski cilj.