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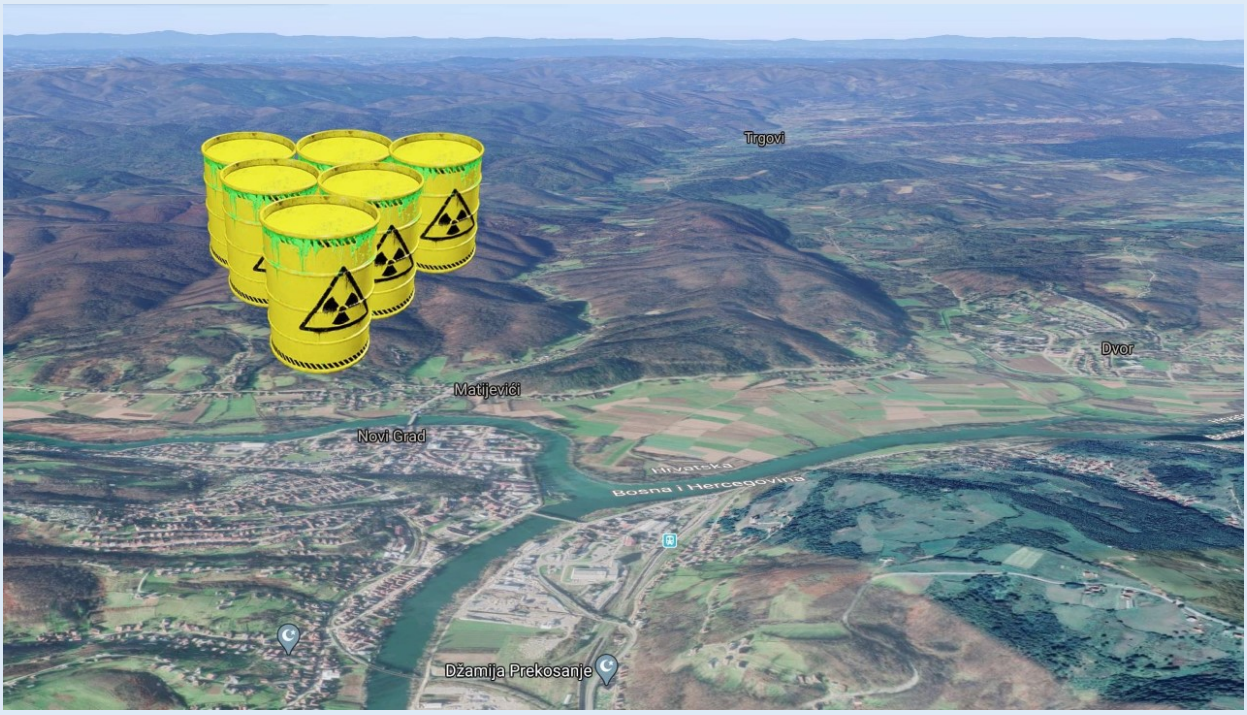

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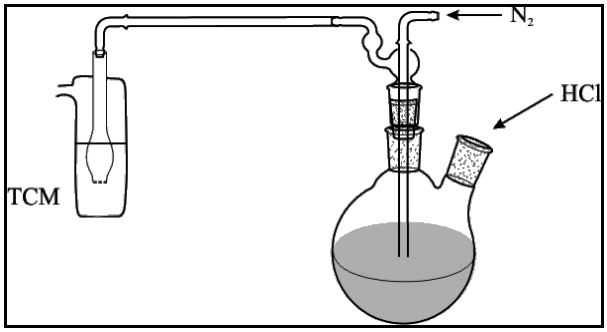

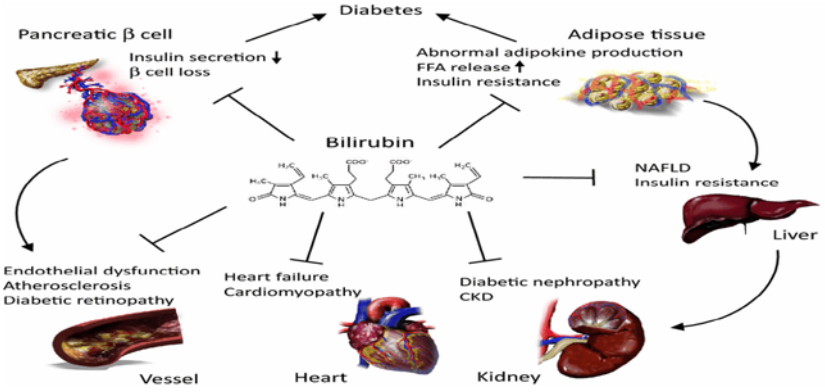

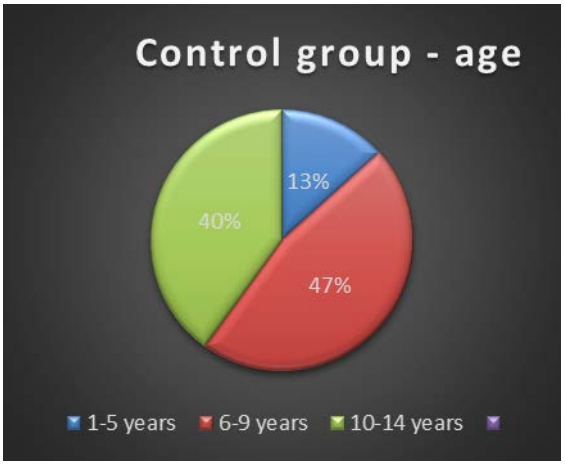

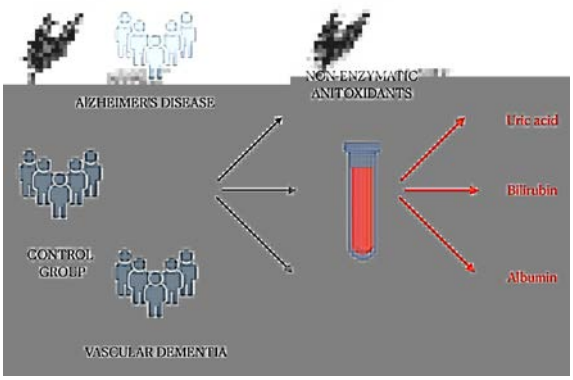

Editorial

The disposal of radioactive waste is the last stage in radioactive waste management and it is oftentimes considered as a permanent waste category. The main goals of the construction of radioactive waste repositories are the protection of people and environment from harmful radiological and non-radiological impact, as well as storing the waste in a way that leaves minimal responsibility for radioactive waste management to future generations.

In accordance with the recommendations of regulatory authorities in this field (IAEA, EU directives, Euratom directives,…), as a prerequisite for the construction of this type of repositories is the choice of location, or its characteristics (geological and geochemical structure, soil porosity, presence of surface water and groundwater – their flow and chemical composition, seismic characteristics of the location and environment, relevant climatic conditions, presence of populated areas, risk of fires,…). Also, in order for a repository to function properly, the radioactive waste classification is crucial, on the basis of which the criteria for the design and type of repository can be further defined.

Trgovska gora, ie. the Čerkezovac site, has been selected by the Republic of Croatia as a site for radioactive waste disposal (including the treatment, conditioning, manipulation, long-term storage and disposal of radioactive waste, in particular used radioactive sources and spent nuclear fuel). The site is located on the border with Bosnia and Herzegovina (Čerkezovac is located only 850 meters away from the Una National Park), which obliges the Republic of Croatia to adhere to the ESPOO Convention and the Aarhus Convention. According to the available data, the location of Trgovska gora is a terrain naturally unfavorable for radioactive waste disposal. The soil consists mainly of sedimentary rocks and a smaller share of metamorphic rocks, whose vertical development is insufficiently explored. There are surface water and groundwater, which in synergy with other factors (terrain slope, infiltration, vegetation, climatic conditions, floods, etc.) implies the direction of distribution and fast migration of contaminants to the protected area within the “Natura 2000” network, river Una, and the present settlements within the territory of Bosnia and Herzegovina. In addition, the seismic conditions of this location are unfavorable, and since the area is fich in forests, there is a possibility of fires of natural and anthropogenic origin. Also, the construction of radioactive waste landfills in the area of Trgovska gora will inevitably lead to an adverse impact on further prospects for the survival of the local population and development of the area, even in the best case scenario which assumes the impossibility of radionuclide leakage into the environment.

Editors

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1-6

Determination of Sulfites in Fruit Juices and Meals for Infants and Toddlers

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Abstract: Sulfites have a regular application in different technological procedures during food processing, storage and distribution. Yet their use in food industry can pose potential risk for human health since they can cause a various health effects including respiratory, dermatological and gastrointestinal signs and symptoms. Regulation regarding additive use in food in Bosnia and Herzegovina is in accordance with the EU Regulation 1333/2008. Since these bylaws do not allow a presence of sulfites in food products for children the aim of this work was to inspect some of commercially available foods intended for children (N=14) in order to determine content of sulfites and also to estimate possible exposure. Sulfites were determined by modified spectrophotometric method, upon releasing sulfur dioxide by acid addition and its capture in chloromercurate solution. Proposed analytical method had good linearity (R2= 0.999), LOD= 0.090 mg/L and LOQ= 0.271 mg/L, and recovery in range from 94.08% to 103.5 %. The content of SO2 was lower than LOQ in two samples (vegetable meal). In the rest of the samples, SO2 content was in range 1.82 to 23.6 mg/L. All calculated mean estimates of dietary exposure to sulfites were well below the ADI for both groups, infants and toddlers.

INTRODUCTION

Sulfites have been used as food preservatives for centuries, and since 1920 they had a regular application in different technological procedures during food processing, storage and distribution (Leclercq, Molinaro, Picinelli *et al.*, 2000). Sulfites or sulfiting agents represent a group of additives capable of releasing sulfur dioxide, as an active component under appropriate conditions. Sulfur dioxide has a great capacity of reacting with oxygen and so preventing oxidation along with its undesirable effect on the colour and flavour of different food products. It protects food not only against oxidation but also against a vast array of moulds, wild yeasts and bacteria, so it is most commonly used as an antimicrobial agent to control spoilage of food. Sulfites are able to inhibit the action of a wide variety of enzymes (including proteases, oxidases and peroxidases) thus preventing enzymatic browning during preparation, storage and distribution, and also can be used as bleaching agents in some types of food (Gould and Russell, 2003; Santos, Nunes, Saraiva, *et al*., 2012).

Sulfites and sulfiting agents are usually added in small amounts (ppm), and at these levels are generally harmless to the vast majority of consumers. Low levels of sulfur dioxide can also be produced naturally by yeast during fermentation, and can be present in various foods and beverages. Sulfites do not accumulate in the human body, because of the rapid biotransformation to sulfate metabolites, which are excreted via urine, thus chronic effects are not to be expected (Garcia-Fuentes, Wirtz, Vos, *et al*., 2015). The EFSA concluded that based on available results of chronic, carcinogenicity and reprotoxicity studies after oral exposure to sulfites, there are no reported effects, and there is no concern with

respect to genotoxicity. (EFSA, 2016) Adverse effects connected with the ingestion of sulfites or sulfiting agents occur mostly in individuals sensitive to sulfites. It is estimated that 3-10 % of all asthmatics are sensitive to sulfites (Timbo, Koehler, Wolyniak, *et al.*, 2004; EFSA, 2016), experiencing asthmatic reactions and bronchospasm after ingestion of food containing sulfites. It seems that the severity of adverse reactions varies, and those asthmatics on steroid therapy or with marked airway hyper responsiveness, and especially children with the diagnosis of chronic asthma, are at greater risk. (Vally, Misso, Madan, 2009) Other adverse reactions including anaphylactic reactions, dermatitis, urticaria, irritation of the respiratory and gastrointestinal tract, can be seen in non-sensitive individuals. The conclusion of the European food safety agency (EFSA) Panel on Food Additives and Nutrient Sources added to Food is that adverse reactions on sulfites as additives in food were not immune-mediated, and were mostly intolerance reactions (EFSA, 2016).

Sulfur dioxide–sulfites (E 220–228) are authorized in the EU according to Annex II to Regulation (EC) No 1333/2008 on food additives, with maximum permissible levels (MPLs) set on the amount of residual sulfur dioxide (ppm). Levels of sulfur dioxide above 10 ppm are subjected to mandatory labelling (EC, 2008; EC, 2011). Joint FAO/WHO Expert Committee on Food Additives (JECFA) on the safe use of food additives defined an acceptable daily intake (ADI) of sulfites (expressed as sulfur dioxide) as 0.7 mg/kg body weight (FAO/WHO, 2009). Regulation regarding additive use in food in Bosnia and Herzegovina (BiH) is in accordance with the aforementioned EU Directive (Rulebook, 2018). The use of additives, including sulfites is not allowed in foods for infants and toddlers. According to some previously conducted surveys on sulfite intake in different population groups, fruit juices are one of the most important sources of exposure to sulfites in children (FAO/WHO, 2009).
Thus, bearing in mind that children may be at higher risk due to their higher intake of food per kg body weight, in this work we have analyzed samples of commercially available ready to use fruit juices and meals for infants and toddlers in order to determine the content of sulfites in these products and to estimate possible exposure.

EXPERIMENTAL

In this work, a total of 14 samples of ready-made foods for infants and toddlers were examined, 11 samples of fruit juices (6 samples of apple juice, 2 samples of orange juice, 2 samples of banana juice and one mixed red fruits juice) and three baby meals (two with mixed vegetables and one with rice and banana).
All the collected samples were ready to use juices and meals, and were purchased from the market in Sarajevo, Bosnia and Herzegovina.

Materials

All chemicals were of analytical grade. The nitrogen gas was over 98 % pure. The water was obtained from a Millipore Milli-Q water purification system. All glassware was cleaned and rinsed with double distilled water prior to use. The TCM (sodium tetrachloromercurate) solution was prepared by measuring 23.4 g of sodium chloride and 54.3 g of mercury (II) chloride in a volumetric flask of 2 L and dissolving with ultrapure water to mark. In order to construct calibration curve, standard stock solution of sulfites (equal to 193.6 mg/L SO_2) was diluted to get final standard concentrations of: 0 mg/L; 0.121 mg/L; 0.484 mg/L; 0.726 mg/L and 2.42 mg/L expressed as SO_2.

Method

In this study, we have used a conventional spectrophotometric method for the detection of sulfur dioxide in the atmosphere (Dasgupta, DeCesare, Ulrey, 1980), which was modified for the determination of sulfites in foods.
For that purpose, we have validated the modified method, and parameters such as linearity range, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) were analyzed and detected.

LOD and LOQ were calculated from the calibration curve using equations 1 and 2:

$$LOD = 3\ SD/S \quad (1)$$

$$LOQ = 10\ SD/S \quad (2)$$

Where SD is the standard deviation of the response based on the residual standard deviation of the regression line, and S is the slope of the calibration curve.

Precision (repeatability) was tested by consecutive measurement of 6 apple juice sample spiked with 0,500 mg/L SO_2 and expressed as the coefficient of variation (CV) calculated with formula in equation 3.

$$CV\ (\%) = SD \times 100/Mean \quad (3)$$

To determine the accuracy of the used method expressed as recovery factor (%) we have prepared 3 spiked samples (banana juice, mixed red fruits juice and orange juice) with a known amount (0.5 mL) of the standard solution of sulfites (equal to 193.6 mg/L SO_2).

Analysis of samples

In a glass apparatus shown in Figure 1, 100 mL of sample was measured, then 10 mL of hydrochloric acid was added and the apparatus was immediately closed. Nitrogen was aspirated through the sample for 20 minutes with a flow rate of 100 mL/minute. On the other end of the apparatus was an absorption bottle with 20 mL of TCM solution. An aliquot (5 mL) of this solution was transferred in a volumetric flask (50 mL) and treated with 5 mL of 0.01 % solution of pararosaniline and 10 mL of formaldehyde (0.015 % solution). Samples were

left on a dark place for 30 minutes, and then the absorbance on 550 nm was measured.
In order to prepare the calibration curve, five standard solution of sulfite-TCM were prepared and were treated on the same way as it was described for the samples. A calibration curve was prepared by plotting the absorbance values against sulfur dioxide concentration.

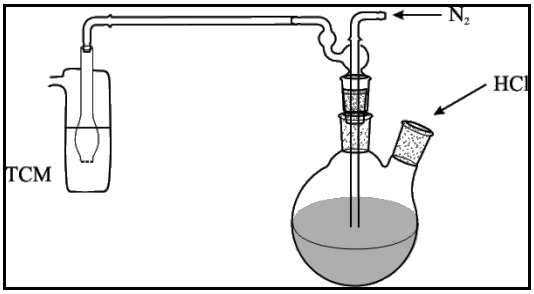

Figure 1. Glass apparatus used for determination of sulfites in samples

Bearing in mind that sulfites, when consumed, may trigger different types of allergic reactions, especially in children, consumption of samples analyzed in this study can pose a risk. Thus, we have calculated the risk as % of ADI, by comparing estimated daily exposure to sulfites in the population of infants and toddlers with respective ADI value. Estimated daily intake (mg/kg body weight/day) was calculated with the formula in equation 4.

$$EDI = \Sigma (Ci \times Fi) / b.w. \qquad (4)$$

Where EDI (mg/kg body weight/day) is the daily intake of the sulfites, Ci is the mean concentration of the sulfites in each food class (mg/L or mg/kg), Fi is the individual daily consumption of each food class (mL/day) and b.w. is the average body weight (kg).
For the calculation of EDI we have used the intake scenario with the average intake. Most of the recommendations for the daily intake of fruit juices in countries within EU vary from 25-30 mL to 150 mL. (Grammatikaki, Wollgast, Caldeira, 2019). Since, there is a lack of data regarding the dietary habits of the population in Bosnia and Herzegovina, especially in this population, for the Fi in calculations we have used the usual serving size of juices (100 mL) and meals (100 g). (EFSA, 2012). Two calculations were made for the group of infants (0-12 months), where the average body weight was 5 kg, and for the group of toddlers (1-3 years) with the average body weight of 12 kg, based on the recommendations of European Food Safety Authority (EFSA). (EFSA, 2012)

RESULTS

The linearity of the used method was tested with series of five standard solutions. The calibration curve is presented in Figure 2.

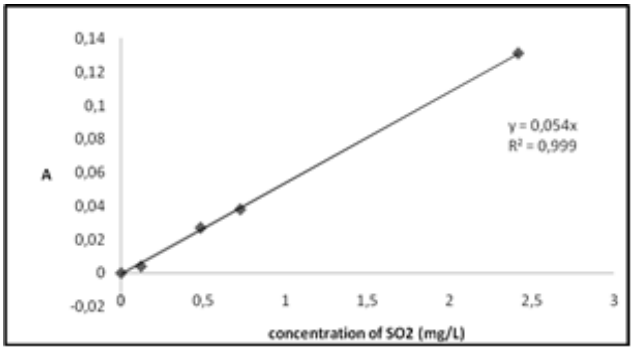

Figure 2. Calibration curve for determination of sulfites in samples

Details for analyzed method validation parameters are presented in Table 1.

Table 1. Method validation parameters

Validation parameter	Value
Linearity	R^2=0.999
Precision	CV (%) =3.89
Accuracy	Recovery (%)
spiked sample 1	95.96
spiked sample 2	94.08
spiked sample 3	103.5
LOD	0.090 mg/L
LOQ	0.271 mg/L

All the samples were analyzed in duplicate, and results are presented as the mean value.

The results of measurement are presented in Table 2.

Table 2. Content of sulfites (mg SO_2/L) in analyzed samples (mean± STD)

Sample	mg SO_2/L (mean±STD)	CV (%)[a]
Ready to drink fruit juice		
banana	14.8±0.09	0.61
banana	11.2±0.02	0.18
mixed red fruits	1.82±0.005	0.27
orange	6.77±0.01	0.15
orange	7.14±0.02	0.28
apple	9.46±0.03	0.32
apple	11.2±0.01	0.09
apple	9.15±0.02	0.22
apple	8.05±0.03	0.37
apple	10.3±0.02	0.19
apple	9.15±0.03	0.33
Ready to use meal		
mixed vegetable	0.20±0.00[b]	0.00
mixed vegetable	0.19±0.001[b]	0.53
rice with banana	23.6±1.22	5.17

[a]The coefficient of variation between analytical duplicates of the fruit juice samples
[b] bellow LOQ

For the calculation of the risk associated with consumption of analyzed samples of fruit juices and meals we compared estimated daily intake (g or mL/kg body weight/day) with the ADI for the sulfites in two groups of consumers, infants and toddlers. For the calculation mean concentration of sulfites in each group of samples was used. The results are expressed as % of ADI, and are presented in Table 3.

Table 3. Estimated daily intake (mg/kg body eight/day) of sulfite and % ADI

Sample	Infants (0-12 months)		Toddlers (1-3 years)	
	EDI mg/kg body weight/day	% ADI	EDI mg/kg body weight/day	% ADI
Ready to drink fruit juice				
banana	0.26	37.1	0.11	15.5
mixed red fruits	0.04	5.20	0.02	2.17
orange	0.14	19.9	0.06	8.27
apple	0.19	27.3	0.08	11.4
Ready to use meal				
mixed vegetable	0.00	0.54	0.00	0.22
rice with banana	0.47	67.5	0.20	28.1

DISCUSSION

The proposed modified method for the detection of sulfites in foods in this study showed a wide linear range (0.0-2.4 mg/L SO_2), with the linear regression coefficient of 0.999. The recoveries for all tested samples were acceptable (94.08-103.5) and in agreement with those reported by Li and Zhao (2006) for the modified p-rosaniline-formaldehyde method.
The precision of the method was good and below 5 %. The calculated LOD and LOQ (0.090 and 0.271 mg/L, respectively) of the tested method reflect good suitability for the determination of low levels of sulfites in different food samples.

The content of sulfites in analyzed samples, expressed as sulfur dioxide, ranged from 0.19 mg/L SO_2 in ready to use vegetable meal to 23.6 mg/L SO_2 in a meal with rice and banana (Table 2). The average content of sulfites in fruit juice samples was 9.0 mg/L SO_2, ranging from 1.82 mg/L in mixed red fruits juice to 14.8 mg/L SO_2 in the banana juice. In four out of 11 samples of fruit juices, the content of sulfites was above the regulation limit of 10 mg/L SO_2 (Table 2). This content was far below the content of sulfites found in similar samples (Machado, Toledo, Almeida, *et al.*, 2008). The content of sulfites in ready to use meals was above the regulation limit only in one sample (23.6 mg/L SO_2). In two samples of vegetable meals, the content of sulfites was below LOQ of the proposed method.

According to EU regulation on food additives content of not more than 10 mg/L is not considered to be present. When the content of sulfites exceeds this concentration, EU law requires food labels to indicate "contains sulfites" without specifying the amount (EC, 2008). None of the analyzed samples were declared to contain sulfites.
Although the content of sulfites in most of the analyzed samples was below the regulation limit, EU directive and also national legislation in BiH, banned the use of additives in food for infants and toddlers. This means that almost all analyzed samples, except two meals with the content below LOQ, do not comply with the regulation requirements (EC, 2011; Rulebook, 2018).
The overall exposure to sulfites did not exceed the ADI. All calculated mean estimates of dietary exposure to sulfites were well below the ADI for both groups, infants and toddlers (0.00-0.47 mg/kg body weight day^{-1} and 0.00-0.20 mg/kg body weight day^{-1}, respectively). This is similar to results reported in some previous studies (Mischek and Krapfenbauer-Cermak, 2012; Bemrah, Leblanc, Volatier, 2008; Soubra, Sarkis, Hilan, 2007; Leclercq, *et al*., 2000).
The highest risk was correlated to consumption of ready to use meal with rice and banana, where consumption of 100 g in infants would lead to 67.5 % of ADI and also banana fruit juice (37.1 % of ADI). The higher risk in group of infants is due to lower body weight.
Based on these results, calculated on an average intake, some high-end consumers can exceed ADI, especially if the content of sulfites is above MRL. However, the estimated intake levels of sulfites from fruit juices were low in both age groups and are within safe levels.

CONCLUSION

This study is to our knowledge, the first survey on levels of sulfites in foods for infants and young children in Bosnia and Herzegovina.
Despite the small sample size, results add to a base of knowledge in this field. It is obvious that there is a need to conduct further investigations on dietary intake and risk assessment of additives in foods, especially for the most sensitive populations groups.

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Summary/Sažetak

Sulfiti imaju značajnu primjenu u različitim tehnološkim procedurama tokom proizvodnje hrane, skladištenja i distribucije. Ipak njihova primjena u prehrambenoj industriji može predstavljati potencijalni rizik po zdravlje konzumenata, pošto mogu izazvati različite neželjene efekte na zdravlje, koji se mogu ispoljiti na nivou respiratornog trakta, kože ili gastrointestinalnog trakta. Zakonske odredbe koje se odnose na upotrebu aditiva u hrani u Bosni i Hercegovini usklađene su sa odredbama EU Direktive 1333/2008. Ove odredbe zabranjuju upotrebu sulfita kao aditiva u hrani za dojenčad i malu djecu, pa je cilj ovog rada bio ispitati prisustvo i sadržaj sulfita u komercijalnim proizvodima namijenjenim djeci (N=14), kao i procijeniti moguće izlaganje. Sulfiti u izabranim uzorcima određeni su modificiranom spektrofotometrijskom metodom, detekcijom oslobođenog sumpor dioksida. Predloženi analitički metod pokazao je dobru linearnost (R^2= 0,999), LOD= 0,090 mg/l, LOQ= 0,271 mg/l tačnost (*recovery)* u rasponu od 94,08% do 103,5 %. Sadržaj mjerenog SO_2 bio je niži od LOQ u dva uzorka (kašica sa povrćem). U ostalim uzorcima sadržaj SO_2 bio je u rasponu od 1,82 do 23,6 mg/l. Procijenjeni izračunati nivoi izlaganja sulfitima za obje dobne grupe, dojenčad i malu djecu bili su ispod prihvatljivog dnevnog unosa (ADI).

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7-12

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 off 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/Gróf. 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 affecteabout 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 immunosuppressive 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, Ganbardella, 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).

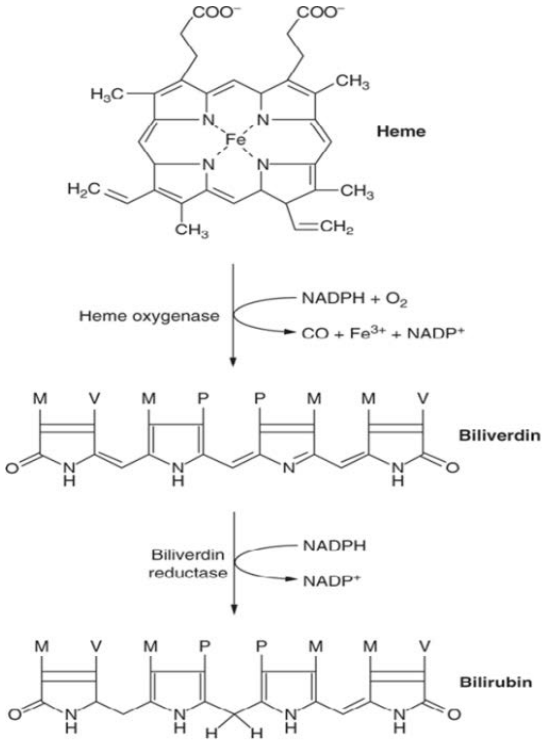

Figure 1: Conversion of heme to bilirubin is a two-step reaction catalyzed by heme oxygenase and biliverdin reductase. M, methyl; P, propionate; V, vinyl.

The aims of this work, was to determine serum total bilirubin concentration in Type 2 diabetes patients, and to investigatewhether 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 adisease with 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 andspectrophotometric 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 Gróf 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 Gróf) 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 darkin the refrigerator at low temperatures of 2-8^{0}C. The serum specimen for bilirubin determination should be used immediately or as possible on the same day. If is 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.

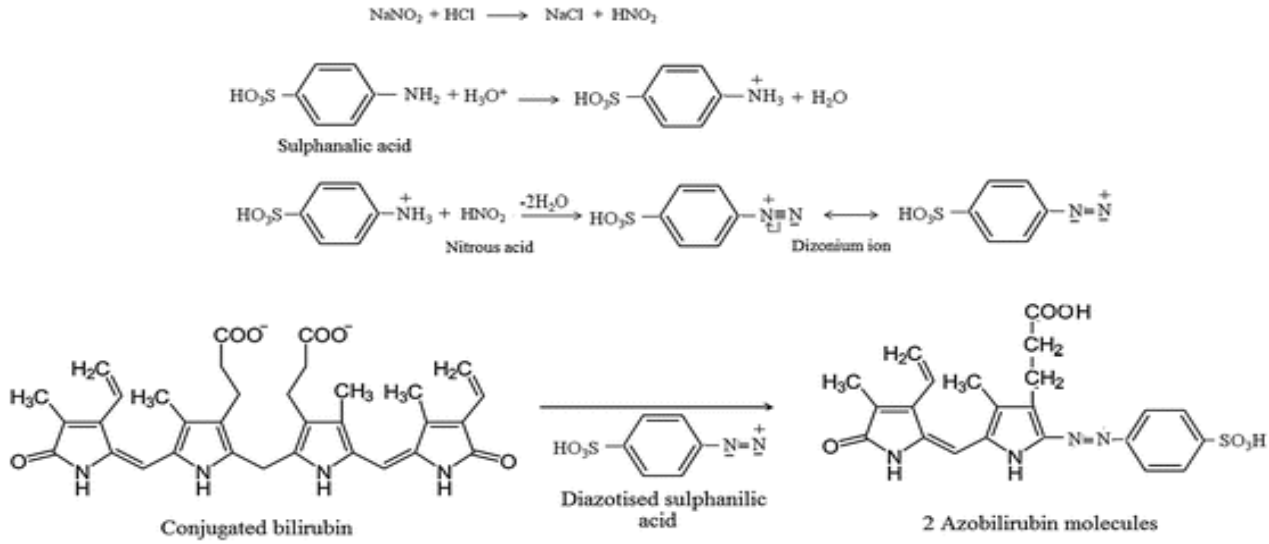

Figure 2: Reaction mechanism of measurement of bilirubin in serum sample.

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

.

Table 1: Age, gender, clinical and biochemical characteristics of Type 2 diabetic and control subjects in study population.

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).

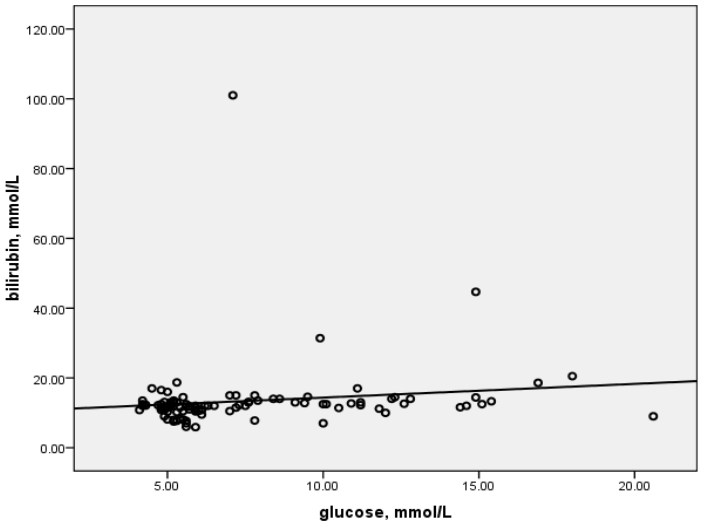

Figure 3: Correlation between concentration of bilirubin and glucose of the study population (rho = 0.235, $p< 0.05$).

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 intraperitonealy 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 in 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-Grof. Sve analize i mjerenja urađena su korištenjem hemijskog analizatora VITROS 350. Rezultati su pokazali značajnu razliku u koncentraciji glukoze, glikiranog hemoglobina (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 ispitjvanoj 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.

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The Content of Essential and Toxic Metals in the Hair of Children with Autistic Spectrum Disorders

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Abstract: The aim of this study was to assess the possible relationship between the content of essential and toxic metals in the hair samples with the etiology of autism spectrum disorder (ASD) in children from Bosnia and Herzegovina. Taking into account the age and gender of the child, in the study and control group, the samples were divided into three subgroups (1-5 years; 6-9 years; 10-14 years). Altered profiles of the values of the Cu, Fe, Mn, Zn, Cd, Co, Cr, Ni, Pb in the study group were observed in comparison with the control group children with typical neuromotor development. Higher values of toxic metal concentrations (Co, Ni, Cd, Pb) were found in boys, compared to the girls in the study group. The content of Pb in the study group was higher in all three ages compared to their controls, with the difference being especially pronounced in the age group 1-5 years (6.64 mg/kg; 1.89 mg/kg). A strong correlation between the content of Pb and Cd (0.93) was confirmed. Lower values of Cr concentration and higher of Ni, Cu and Fe were recorded in the study group. Statistically significant differences ($p<0.05$) were found in Zn concentrations (6-9 years; 10 -14 years) between the control and study groups. The findings help highlight the role of heavy metals as environmental factors in the etiology of ASD.

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous class of neurodevelopmental disorders characterized by deficits in social interaction, interests, and repetitive behaviors (Bishop, Havdahl, Huerta, *et al*., 2016). ASD is often associated with other mental and physical disorders such as: anxiety, ADHD (Attention Deficit Hyperactivity Disorder), intellectual disorders, epilepsy and motor coordination imbalance (Fiore, Barone, Copat, *et al*., 2020). There is currently no known cause for most ASD cases, and there are no psychological diagnostic tools or biomarkers for ASD to be reliably diagnosed.

Over 25% of people diagnosed with ASD have genetic disorders that are associated with the development of the nervous system (Wegiel, Kuchna, Nowicki, *et al*., 2010). In addition, mutations in genes closely related to metabolism, chromatin remodeling, mRNA regulation, protein synthesis, and synaptic function have been detected in individuals with ASD. Regardless of all the potentially detected factors associated with ASD, 80-90% of the entire autistic population remains without an identified cause (Kaushik and Zarbalis, 2016).

Many studies that follow the perinatal and neonatal periods list several risk factors that may be associated with ASD. The main risk factors are gestational diabetes in mothers, bleeding during pregnancy, intrauterine infections, gestational viral infections as well as the use of medications. Premature birth, trauma, low birth weight, neonatal anemia, incompatibility of ABO or Rh blood groups are also associated with an increased risk of autism. An increased risk of ASD in first-born children has also been reported (Modabbernia, Velthorst and Reichenberg, 2017).

Toxic metals are among the environmental factors associated with the development of ASD (Yassa, 2014; Mohamed, Zaky, El-Sayed, *et al*., 2015; Mostafa,

Bjørklund, Urbina, *et al*., 2016; Saghazadeh, Ahangari, Hendi, *et al*., 2017a). Toxic metals can cause epigenetic changes and thus play a role in neurodevelopmental disorders (Jakovcevski and Akbarian, 2012). Environmental pollutants act as neurotoxins of the central nervous system and adversely affect prenatal development. Essential minerals are crucial for the proper functioning of biological systems, thereforethe dysregulation of their content and the retention of toxic elements in the body can contribute to the genesis of ASD (Da Silva, Vellas, Elemans, *et al.*, 2014; Saghazadeh, Mahmoudi, Dehghani Ashkezari, *et al.*, 2017b).

Intensive global research in this field indicates that there are significant differences in the concentration of toxic and essential elements in children with autism spectrum disorders compared to the group of healthy children (Lakshmi Priya and Geetha, 2011; Li, Yang, Wang, *et al*., 2011; Blaurock-Busch, Amin, Dessoki, *et al*., 2012; De Palma, Catalani, Franco, *et al*., 2012; Adams, Audhya, McDonough-Means, *et al*., 2013; Tabatadze, Zhorzholiani, Kherkheulidze, *et al*., 2015; Fiore *et al*., 2020).

Biomonitoring, as a method that assesses the impact on living organisms of chemical elements present in the environment, is approved by mandatory legislation (The EU Water Framework Directive (2000/60/WE), US EPA-600 / 4-79-049. August 1979), (Mikulewicz, Chojnacka, Gedrangec, *et al*., 2013). In the case of human biomonitoring, non-invasive matrices (hair, urine, saliva) were approved. Interest in human hair as a clinical sample has recently increased due to the advantages of hair as a sample over blood or urine: sampling is relatively easy and non-invasive, the sample is stable, easy to store and transport in the laboratory, it is inert and chemically homogeneous (De Palma *et al*., 2012). Hair, as well as adipose tissue, is a place that can store substances from the body exposure, including heavy metals whose concentrations are up to 10 times higher than the concentrations determined in the blood or urine (Bader, Dietz, Ihrig, *et al*., 1999; Moreda-Piñeiro, Alonso-Rodríguez, López Mahía, *et al*., 2007). Hair grows about 10 mm per month, which allows long-term monitoring of past and recent exposure to metals, and an analysis of 2 cm of hair from the scalp gives a picture of the state of the organism in the last two months (Gil and Pla, 2001). Furthermore, there is not enough information to define heavy metal reference values, because the metal content in hair varies depending on age, gender, hair color, care, smoking habits, ethnic factors, geographical location of the population.

The analysis of metal content in hair has become an interesting diagnostic tool for biomonitoring exposure to toxic elements, in order to assess the health and nutritional status of the population of a particular geographical area. Research in this field is intensive and goes in the direction of highlighting the role of metals as environmental factors in the etiology of ASD. Given the number of factors that can affect the final results, the obtained values of metal content in one population may not correlate to that of another population.

Therefore, the aim of this study was to assess the possible relation between the content of essential and toxic metals with the etiology of ASD in children from Bosnia and Herzegovina.

MATERIALS AND METHODS

Materials

The study was conducted with the consent of the parents/guardians, whose children are the members of the "Support center for families of children/members with developmental difficulties - Colibri". Hair samples were collected during the year 2019. A total of 31 hair samples were analyzed from children in the age group of 1-14. From the total number of samples, 55% were taken from males and 45% from females. Hair samples were divided into two groups: a control group and a study group. The control group included children who, according to their parents, had a normal psychomotor development. The study group included children who, according to their parents, and according to their previous medical history, had elements or a disorder from the autism spectrum (ASD). All the hair samples were collected from children whose birthplace and place of residence were in the Sarajevo Canton. Table 1 provides an overview of samples based on their division into groups and gender.

Table 1: Review of the analyzed samples based on groups and gender

Total number of samples			
Gender	♀	♂	Total
Control group	9	6	15
Study group	5	11	16[1,2,3,4]
Total (N)	14	17	31

[1]Autism; [2]Pervasive Developmental Disorder With Elements Of Autism; [3]Disharmonious Development With Elements Of Autism; [4] Slowed Disharmonious Psychomotor Development

Out of the 15 samples in the control group, 60% were collected from females and 40% from males. In the examined group, there were more male samples 69%, while female hair samples accounted for 31% of the total number of examined samples. The percentage of samples in the study group is explained by the fact that gender is one of the risk factors when it comes to ASD (De Palma *et al*., 2012).

The metal content in hair varies depending on a number of factors, primarily the age structure of the observed population (Chojnacka *et al*., 2006; Mikulewicz *et al*., 2013). In order to achieve consistency in the interpretation of the results in the study and control groups, the samples were divided into three subgroups, taking into account the age (1-5 years; 6-9 years; 10-14 years). The percentual representation of the samples in the control and study groups by age is presented in Figures 1 and 2.

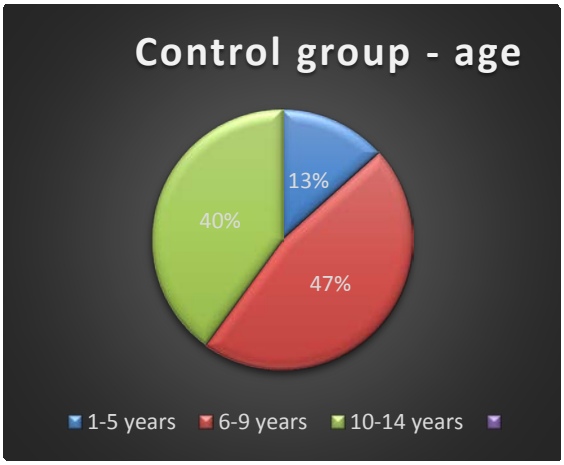

Figure 1: Representation of samples by age in the control group

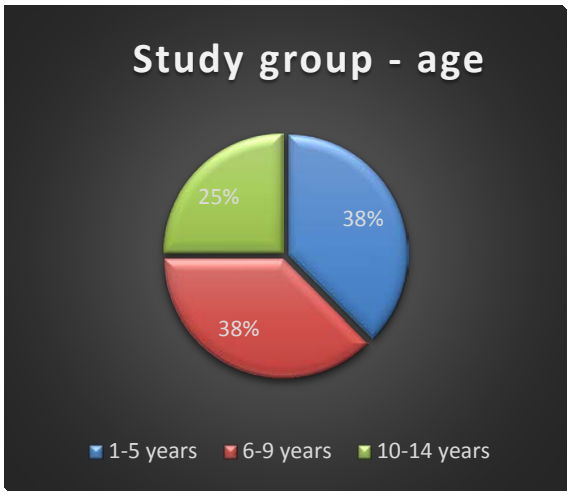

Figure 2: Representation of samples by age in the study group

Methods

Sample preparation and analysis

Determination of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn content in the hair samples was performed using atomic absorption spectrometry (AAS), flame technique (Varian AA240FS) in the Laboratory for Analytical Chemistry, Department of Chemistry, Faculty of Natural Science, University of Sarajevo. Before dissolution the hair samples were cut into 0.5 cm long hair pieces. Hair washing, in order to remove grease and surface impurities, was carried out according to the IAEA (International Atomic Energy Agency) procedure with ethanol and distilled water, upon which the hair samples were dried in an oven at 80°C for 2 hours. and dried sections of hair were subjected to wet digestion. The volume of 10 mL of concentrated nitric acid was added to the defined weight – of approximately 0.5 g with the precision of 0.1 mg – of previously prepared samples and gradually heated until brown nitrogen oxide vapors separated. Heating continued further with the addition of 1-2 mL of 30% hydrogen peroxide. After cooling, the samples were filtered through Whatman No.42 filter paper into 25 mL flasks and diluted with distilled water. In the obtained filtrates, the metal levels were determined using the method of calibration curve, constructed by measuring the absorbance value for a series of standard solutions (1000 mg/L, Merck) of the tested metals. A comparative analysis by groups and subgroups between the monitored categories was used for the interpretation of the results.

Analytical quality control

All used reagents had the analytical grade of purity (Merck, Germany). Precision of the method was checked by performing two or three indenpendent determination (depending on the available sample mass) for each sample and calculating the value of repeatability standard deviation. The traceability of the measurement results was established using standard metal solutions traceable to NIST (National Institute of Standards and Technology, USA). Method blanks are prepared and analyzed along with the samples in order to correct results for systematic errors.

Statistical data analysis

All statistical analyses were performed using Statistics 12 software (Copyright © StatSoft, Inc. 1984-2014). Descriptive statistics of the content of essential and toxic metals in hair included mean, standard deviation (SD), minimum (MIN) and maximum values (MAX). One-way ANOVA was usedfor comparative analysis by groups, followed by the Post-hoc LSD test and the Newman-Keuls test. The Pearson product-moment correlation coefficient was used to examine the correlations between groups and variables. A statistical significance level of $p<0.05$ was applied to all analyses. ORIGIN version 8.1 software (originLab Corporation, USA) was used to graphically display the results.

RESULTS AND DISCUSSION

The content of essential and toxic metals in the control and study groups by gender

Table 2 shows the values of concentrations of essential and toxic metals in the control and study groups by gender. Preforming a comparative analysis of the obtained results by gender, we can observe that the mean values of metal concentrations in the control group are quite equal, except for the content of Cu, Co and Zn, whose values are slightly higher in females, while Cr is higher in males. In the examined group, a higher value of the content of the essential metal Cu was observed in females compared to its values in males. With regard to the values of toxic metals between the genders in the examined group, higher values of Cr were found in females, compared to males. If we compare the values of Cr in the control and study groups in females, we can conclude that they do not differ significantly. Higher values of concentrations of toxic metals Co, Ni, Cd, Pb were found in males, compared to the same values in females in the study group. Observing the results by gender, we notice that the values of Cd in the study group males were statistically significantly higher than in the control group males. In the control and study groups, a statistically significant correlation was found between Pb and Cd contents (0.90; 0.89). On the other hand, a negative correlation in the control group males was found between Fe and Zn (-0.85). This result was recorded in a meta-analysis by De Palma *et al*. (2012).

Table 2: Elements content (mg/kg)±standard deviation in hair samples of males and females in the control and study groups

Gender	Control group				Study group			
Group	Male		Female		Male		Female	
Data	Mean±SD	(MIN, MAX)	Mean±SD	(MIN, MAX)	Mean±SD	(MIN, MAX)	Mean±SD	(MIN, MAX)
Cu (mg/kg)	9.89±2.15	7.5, 12.32	11.65±6.14	7.08, 27.05	11.63±1.76	8.13, 14.29	14.07±3.23	10.26, 18.91
Fe (mg/kg)	52.52±18.42*	37.7, 80.31	41.69±7.37	33.69, 55.04	57.48±16.99	35.28, 79.38	57.76±5.04	52.50, 64.39
Mn (mg/kg)	2.04±0.44	1.35, 2.52	1.71±0.71	0.43, 2.57	1.90±0.78	0.22, 2.92	1.69±0.67	1.28, 2.86
Zn (mg/kg)	110.24±34.36*	53.94, 156.33	139.67±82.64	33.94, 279.10	123.64±43.90	49.36, 198.81	110.28±34.71	67.75, 144.28
Cd (mg/kg)	0.15±0.05^{a*}	0.09, 0.19	0.26±0.24	0.08, 0.74	0.48±0.32^{a*}	0.15, 1.34	0.33±0.07	0.26, 0.42
Co (mg/kg)	0.26±0.18	0.13, 0.39	1.74±1.97	0.34, 3.13	1.28±1.41	0.22, 3.81	0.62±0.49	0.27, 0.96
Cr (mg/kg)	3.56±0.87	2.64, 5.11	2.50±1.70	0.13, 5.82	1.88±1.04	0.30, 4.30	2.89±2.27	0.96, 6.14
Ni (mg/kg)	2.32±0.67	0.98, 2.75	2.50±1.01	1.03, 4.83	3.15±1.14	1.98, 5.89	2.34±0.77	1.75, 3.67
Pb (mg/kg)	2.59±1.84*	0.33, 5.51	2.96±3.21	0.68, 11.30	4.68±4.82*	2.21, 19.05	4.43±1.73	2.98, 7.31

Values sharing the same letter differ statistically significantly within one parameter at a significance level of $p<0.05$ after Post-hoc analysis of variance by the LSD test and the Newman-Keuls test

*correlations are statistically significant at the level of $p<0.05$

Content of essential and toxic metals in the control and study groups by age

The metal content in hair varies according to age (Chojnacka *et al*., 2006; Mikulewicz et *al*., 2013). Table 3 shows the values of essential metals in the samples of the control and study groups by age (1-5 years; 6-9 years; 10-14 years).

Table 3: Content of essential metals in the control and study group presented by age of the sample

Age	Group	Data	Cu (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Zn (mg/kg)
1-5 years	Control group	Mean±SD	9.70±3.71	54.68±22.92	1.93±0.10	123.0±27.44
		(MIN, MAX)	7.08,12.32	38.47, 70.89	1.86, 2.00	103.63, 142.44
	Study group	Mean±SD	12.46±1.79	62.40±14.96	1.92±1.02	122.4±55.08
		(MIN, MAX)	10.26, 14.92	37.44, 77.95	0.22, 2.87	49.36, 198.81
6-9 years	Control group	Mean±SD	9.90±1.41	48.39±15.62	1.87±0.59	76.29±34.49a
		(MIN, MAX)	7.68, 12.11	33.69, 80.31	0.9, 2.52	33.94, 123.69
	Study group	Mean±SD	11.51±8.13	51.35±15.74	1.90±0.64	124.5±25.79a
		(MIN, MAX)	8.13, 14.29	35.28, 79.38	1.20, 2.92	77.85, 154.26
10-14 years	Control group	Mean±SD	12.57±7.54	40.37±6.06	1.78±0.81	189.7±53.17b
		(MIN, MAX)	7.5, 27.05	34.22, 48.51	0.43, 2.57	125.68, 279.10
	Study group	Mean±SD	13.62±3.86	59.66±71.50	1.60±0.43	107.6±41.56b
		(MIN, MAX)	10.09, 18.91	52.50, 71.50	1.28, 2.22	67.8, 155.36

Values that share the same letter differ statistically significantly with in one parameter at a significance level of $p<0.05$ after Post-hoc analysis of variance by the Newman-Keuls test.

* correlations are statistically significant at the level of $p <0.05$

Analyzing the results of Cu, Fe, Zn and Mn levels in children's hair, it can be concluded that the levels of essential metals in the control and study groups, for all three age groups, follows the pattern: Zn> Fe> Cu> Mn (Table 3). The highest Zn level was found in the control group of the age 10-14 in the amount of 189.7 mg/kg. In the same age study group, the Zn level was 107.6 mg/kg, which also represents the lowest Zn concentration recorded in the work. In the age group of 6-9, the study group had a higher Zn level (124.5 mg/kg), compared to the control group (76.29 mg/kg). Statistically significant differences ($p<0.05$) were found in the Zn content between the control and study groups in the age group of 6-9 as well as in the group of 10-14. Lower concentrations of trace elements such as zinc are significantly associated with autism spectrum disorders (De Palma *et al*., 2012; Tabatadze *et al*., 2015). However, there are results in which zinc levels in hair were inversely related to age, and a negative significant association was found between zinc levels in hair and the severity of autistic symptoms (De Palma *et al*., 1012; Fiore *et al*., 2020). However, the mean values of Zn

levels for the age of 1-5, in the control and study groups, were almost identical: 123.0 mg/kg and 122.4 mg/kg.

The Cu content in the study group, for all three age groups, was higher compared to the same age groups of the control group, which is in line with research conducted by Lakshmi Priya and Geeth (2011). In literature, this trend of higher Cu levels in the hair of children with ASD is associated with increased oxidative stress and increased "cytokine" levels (Bjørklund, 2013). Since Cu and Zn are metabolic antagonists, there are many results in this field confirming that the Cu/Zn ratio in the blood is increased in children from the autism spectrum. Higher values of Cu concentration and less Zn in the blood were recorded by Saghazadeh *et al*. (2017). By analyzing the obtained results in our study, it can be seen that the Cu/Zn ratio is higher in the study group aged 1-5 as well as the group 10-14 compared to the control groups of the same age.

The Fe content in the control group decreases with the age of the sample, while in the study group the highest Fe content was recorded in the youngest children (age group 1-5). A comparative analysis of the Fe content by age structure of samples in the study groups found a higher concentration compared to the control groups. These results are in line with other studies that report a higher Fe content in children with autism spectrum disorders (Yasuda,Yonashiro, Yoshida,*et al*., 2005).

The smallest differences between the control and study groups were confirmed for the Mn levels. In the control group, there was a decreasing trend of the Mn concentration with age (from 1.93 to 1.78 mg/kg). The same trend was observed in the study group with a tendency to increase with age (from 1.92 to 1.60 mg/kg). This trend is in line with the results of a 2012 study that found a deficit and negative correlation of manganese with age (De Palma *et al*., 2012).

The content of toxic metals in the control group aged 1-5 follows the pattern: Cr> Ni> Pb> Cd, while at the age of 6-9 and 10-14 this pattern is different: Pb ~ Cr ~ Ni >> Cd. For all three age groups of the study group the content of toxic metals follows the pattern: Pb> Ni> Cr> Cd (Table 4).

Table 4: Values of toxic metal content in the control and study group presented by the age of the sample

Age	Group	Data	Cd (mg/kg)	Co (mg/kg)	Cr (mg/kg)	Ni (mg/kg)	Pb (mg/kg)
1-5 years	Control group	Mean±SD	0.16±0.02[a]	0.13±0 (<LOD)	3.47±0.42	2.69±0.08	1.90±1.40
		(MIN, MAX)	0.14, 0.17	0.13, 0.13	3.17, 3.77	2.63, 2.75	0.91, 2.88
	Study group	Mean±SD	0.59±0.38[a*]	1.81±1.81	2.80±2.11	2.81±1.53	6.64±6.28*
		(MIN, MAX)	0.27, 1.34	0.27, 3.81	0.3, 6.14	1.85, 5.89	3.05, 19.05
6-9 years	Control group	Mean±SD	0.18±0.06	0.37±0.04	2.72±1.52	2.35±0.25	2.81±1.39
		(MIN, MAX)	0.10, 0.27	0.34, 0.39	0.13, 5.11	1.95, 2.67	1.70, 5.51
	Study group	Mean±SD	0.34±0.16	0.76±0.91	1.73±0.61	3.31±0.76	3.51±1.07
		(MIN, MAX)	0.22, 0.65	0.22, 1.81	1.09, 2.77	2.14, 4.14	2.21, 4.73
10-14 years	Control group	Mean±SD	0.27±0.32	3.13±0 (<LOD)	2.98±1.79	2.43±1.41	3.13±4.10
		(MIN, MAX)	0.08, 0.74	3.13, 3.13	0.91, 5.82	0.98, 4.83	0.33, 11.30
	Study group	Mean±SD	0.34±0.50	0.59±0.52	2.01±1.54	2.41±0.58	3.19±0.65
		(MIN, MAX)	0.15, 0.50	0.22, 0.96	0.96, 4.30	1.75, 3.15	2.37, 3.76

Values that share the same letter differ statistically significantly within one parameter at a significance level of $p<0.05$ after Post-hoc analysis of LSD variance and the Newman-Keuls test.

*correlation is significant at $p<0.05$

There is an increase in the level of Ni compared to the content of Cr, whose level is declining in the study samples, especially in older children (6-9 years). The results of similar studies indicate that the content of Ni in the hair of children aged 3-9 with ASD was higher compared to the control group (Blaurock-Busch *et al*., 2012; Fiore *et al*., 2020). The level of Pb in the control group increases with age, while the case is opposite in the study group. The level of Pb in the study group is higher in all three age groups compared to the levels in the control groups, with this difference being especially highlighted in the age group 1-5 (study group - 6.64 mg/kg; control - 1.89 mg/kg). The increased lead concentration and its positive association with ASD have been confirmed in other studies (Lakshmi Priya and Geetha, 2011; De Palma *et al*., 2012; Fiore *et al*., 2020). Significantly increased lead levels in children with autism spectrum disorders compared to healthy children (78% and 16%) were observed at the age of 4 to 5 in a study conducted by Tabatadze et al. (2015) which corresponds with our results. The negative correlation of the Pb content with the age in the hair of children with ASD suggests that younger children are more susceptible to toxic metal retention compared to older children, as confirmed by the results of similar studies (Ballesteros, Serrano and Álvarez, 2017; Skalny, Skalnaya, Grabeklis

et al., 2018; Fiore *et al*., 2020). The Cd content in the control group increased from 0.15 mg/kg (1-5 years) to 0.27 mg/kg (10-14 years). The highest Cd content (0.59 mg/kg) was recorded in the study group aged 1-5. The content of Cd in the study group aged 1-5 was statistically significantly higher ($p<0.05$) compared to the control group of the same age according to LSD and Newman-Keuls test. Significant correlations in this study group were found between Pb and Cd (0.926936), which is in agreement with the results of research conducted by De Palma et al. (2012). In the study group of 6-9 and 10-14 years of age, Cd has the same mean value (0.34 mg/kg), which is higher compared to the same age groups of the control group. The association and significant differences in cadmium values in children with ASD compared to healthy ones have previously been observed (De Palma *et al*., 2012; Tabatadze *et al.* 2015; Fiore *et al*., 2020). The Cr content in the study group for all three age groups was lower compared to the controls. The lowest level of Cr in the control group is 2.71 mg/kg for the age 6-9. In the same age of the study group the Cr level was 1.70 mg/kg. The highest Cr content in the control group was 3.47 mg/kg for the age of 1-5, while in the same age of the study group it was 2.80 mg/kg. Lower Cr concentrations in the hair of children with ASD compared to the control group were noted by Saghazadeh *et al.* (2017). Chromium is a component of the "glucose-tolerance" factor and playsa role in cellular sensitivity to insulin, so its deficiency may indicate a disorder of glucose metabolism, leading to disruption of cell activity (Yasuda *et al*., 2005). The Co content in the control groups was below the detection limit for 90% of the samples. Therefore, the obtained results were not interpreted. The literature states that the Co content is lower in ASD compared to the control group of children Saghazadeh *et al*. (2017).

Comparative analysis of the content of essential and toxic metals in the control and study groups based on gender and age

Analyzing the values of essential and toxic metals and taking into account the gender and age structures, altered profiles of the values of metals were observed between the study group and the control group of children (Figure 3). The content of Co, Cd and Pb in the hair of children aged 1-5 in the control group is lower than the values determined in the study group. The largest difference is recorded in the content of Pb, where the control group has values of 0.91 mg/kg in females and 2.88 in males, while in the study group these values are 5.37 mg/kg and 7.28 mg/kg, respectively. The values for Cr levels in the control group aged 1-5 are similar in both genders (mean value 3.47 mg/kg), but in the study group the Cr content in females is statistically significantly higher than in males. A strong positive correlation (0.99) was observed in the levels of Cd and Pb in the study group in males aged 1-5. It is also characteristic that the Fe level in the hair of the control group females increases with age, while in the study group it decreases. The Cr level in the hair of males aged 6-9 was statistically significantly different between the control and study groups. In male hair, the Pb levels in the control and study groups were similar, but in females of the same age it was about 2 times higher in the study group compared to the control group. The Zn levels in the hair of females of this age differed statistically significantly between the control and study groups.

In the hair of children aged 10-14, the Zn level was lower in the study group compared to the control group. In females of this age, the Zn levels differed statistically significantly in the control and study groups. The Pb level in the hair of the study group males was 3.3 times higher than that in the control group.

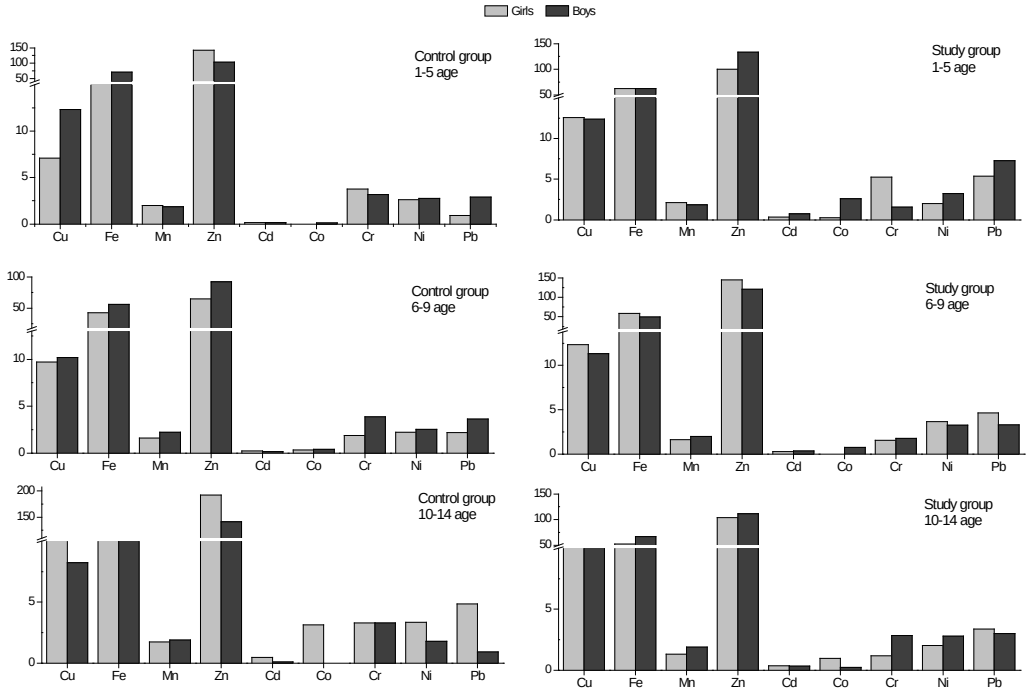

Figure 3: Comparative analysis of metal content by groups in relation to gender and age

CONCLUSION

Our research has shown different concentrations of essential and toxic metals in hair samples of children with autism spectrum disorders compared to healthy children. There is a higher content of toxic metals in the hair of children with ASD, primarily of Pb and Cd, but also a higher content of essential metals Cu and Fe, and a deficit of Zn compared to the control group of children. Strong correlations between Pb and Cd were observed in a large number of monitored groups.

Several factors may play a role in explaining the differences in the content of toxic and essential metals in children's hair. In our study, it was expected that children grew up in the same environment, i.e. that they had similar ways of exposure to pollutants, so that the observed differences are not only due to different exposure to toxic metals, but also due to a difference in retention or greater absorption. In addition, poor eating habits are often recorded in children with ASD, so that some of the results can be explained by inadequate intake of certain nutrients as well as their excess if children who have consumed some of the supplements.

Further research in this field is extremely important, primarily for establishing the reference values for cross-linked metals in a given population, possible positive correlations between elevated levels of heavy metals in hair and autism spectrum disorders, as well as biomonitoring, i.e. assessment of the effects on human health of chemical elements present in the environment. Such research helps to highlight the role of such elements as environmental factors in the etiology of ASD. The results of this research represent a significant scientific contribution given that this is the first research in Bosnia and Herzegovina.

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Summary/Sažetak

Cilj ovog istraživanja bio je procijeniti moguću povezanost sadržaja esencijalnih i toksičnih metala u uzorcima kose s etiologijom poremećaja autističnog spektra (ASD) kod djece iz Bosne i Hercegovine. Uzimajući u obzir spolnu i dobnu strukturu, u ispitivanoj i kontrolnoj skupini uzorci su podijeljeni u tri podskupine (1-5 godina; 6-9 godina; 10-14 godina). Uočeni su izmjenjeni profili vrijednosti Cu, Fe, Mn, Zn, Cd, Co, Cr, Ni, Pb u ispitivanoj skupini u poređenju sa djecom kontrolne skupine tipičnog neuromotorog razvoja. Kod dječaka utvrđene su veće vrijednosti koncentracija toksičnih metala (Co, Ni, Cd, Pb) u poređenju s djevojčicama u ispitivanoj skupini. Sadržaj Pb u ispitivanoj skupini bio je veći u sva tri uzrasta u poređenju s njihovom kontrolom, s tim da je razlika posebno izražena u dobnoj skupini 1-5 godina (6,64 mg/kg; 1,89 mg/kg). Potvrđena je snažna korelacija između sadržaja Pb i Cd (0,93). Niže vrijednosti koncentracije Cr i veće Ni, Cu i Fe zabilježene su u ispitivanoj skupini. Utvrđene su statistički značajne razlike ($p < 0,05$) u koncentraciji Zn (6-9 godina; 10 -14 godina) između kontrolne i ispitivane skupine. Rezultatiovog istraživanja ukazuju na ulogu teških metala kao faktora okoliša u etiologiji ASD-a.

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21-28

Serum levels of non-enzymatic antioxidants in female dementia patients with respect to the degree of cognitive impairment

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Abstract: The aim of this study was to investigate the correlation between the severity of cognitive impairment in Alzheimer's disease (AD) and vascular dementia (VD) and the serum antioxidant status of uric acid (UA), albumin (ALB) and bilirubin (BIL) in female patients. The cross-sectional study included 90 subjects, aged ≥65, divided into three groups: 30 patients with AD, 30 patients with VD and 30 control subjects. For cognitive assessment, all participants underwent the Montreal Cognitive Assessment (MoCA). Serum concentrations of ALB, UA and BIL were determined spectrophotometrically. The AD patients had a significant decrease of UA and increase of serum BIL. Upon stratification according to the degree of cognitive impairment, lower UA concentrations were found in patients with severe cognitive impairment, whereas increased BIL was found in patients with moderate cognitive impairment. Patients with VD were characterized by hypoalbuminemia and upon stratification this finding was evident among patients with severe cognitive impairment. The MoCA score correlated positively with BIL in AD patients. The obtained data supports the protective role of serum antioxidants in the pathogenesis of dementia. Further on, we suggest further longitudinal research to confirm the combined use of these parameters as potential biomarkers in AD and VD.

INTRODUCTION

The causes of dementia are still incompletely understood, but it is certain that the accumulation of reactive oxygen species results in the damage of brain cellular structures. Low content of antioxidants, the abundance of polyunsaturated fatty acids, and high oxygen consumption are the main predispositions of brain tissue vulnerability towards oxidative damage (Wang and Michaelis, 2010). Alzheimer's disease (AD)

and vascular dementia (VD) are the two most common types of dementia that affect physiological, cognitive, and behavioral functions in the elderly population. The burden these diseases place on patients and society urge for the identification of risk factors and preventive strategies.Plasma systemic antioxidants, including homocysteine, UA, albumin ALB and BIL, are strong free radicals scavengers and hence described as non-enzymatic laboratory parameters associated with oxidative stress (Wayner, Burton, Ingold et al., 1987; Cascalheira, João, Pinhanços *et al.*, 2009). The antioxidant functions of serum albumin are related to its chemical structure that conveys ligand-binding and free radical-trapping properties (Halliwell and Gutteridge, 2015). The hydrophilic UA is known for suppressing $O_2^{\cdot-}$ and $^1O_2$ and protecting ascorbic acid from oxidation through metal chelation (Waugh, 2008). On the other hand, bilirubin's lipophilic nature makes it effective against some lipophilic reactive oxygen species (Kim and Park, 2102). As a cytotoxic waste product and the end product of heme metabolism, bilirubin is, therefore, an important antioxidant, which has anti-inflammatory and immunosuppressive properties, too (Jangi, Otterbein and Robson, 2013). While the antioxidant activities of UA, ALB, and BIL are universally acknowledged, recent studies have produced equivocal or conflicting results regarding their association with cognitive impairment. It is partially due to the concentrations achieved in biological fluids and the fact that different biological activities may depend on the chemical microenvironment. In addition, it appears that a single serum antioxidant may exhibit different roles, depending on the type of dementia. The aim of the study was to investigate serum levels of non-enzymatic antioxidants in female patients with AD and VD. To the best of our knowledge, this is the first study of this kind in our country. In clinical practice, serum levels of ALB, BIL and UA are routinely measured, however our aim was to determine whether there was an association between their serum levels and cognitive impairment in these patients.

EXPERIMENTAL

Patients and study design

This controlled, cross-sectional study included 90 female subjects, aged ≥65, divided into three groups: 30 patients with AD, 30 patients with VD and 30 control subjects. The study was designed to include female patients only to exclude the influence of gender on the study results but also due to the low prevalence of male patients**.** Patients were recruited from a specialized unit at the Health-Care Hospice for persons with disabilities in Sarajevo, B&H, and their baseline characteristics are presented in Table 1. Subjects diagnosed with AD met the NINCDS-ADRDA criteria (McKhann, Drachman, Folstein *et al.*, 1984) and subjects diagnosed with VD met the NINDS-AIREN criteria (Román, Tatemichi, Erkinjuntti *et al.*, 1993). For the differentiation between AD and VD patients, the Hachinski ischemic score (HIS) was used (Lončarević, Mehmedika-Suljić, Alajbegović *et al.*, 2005). For cognitive assessment, all participants underwent the Montreal Cognitive Assessment (MoCA). All subjects in the AD and VD groups had a score ≤20, while the control group (CG) subjects had a score between 27 and 30. The patients with AD and VD were further classified as those with severe cognitive impairment (MoCA score: <10) and moderate cognitive impairment (MoCA score: 10-17) (Claveau, Presse, Kergoat *et al.*, 2018).

The exclusion criteria were chronic inflammatory diseases (asthma and rheumatoid arthritis), hepatic or renal insufficiency and cancer.

The study was approved by the local research Ethics Committee (protocol number 0305-28838) and conducted according to the Helsinki Declaration of 2013. Informed consent was obtained from caregivers for the dementia patients upon careful explanation of the study procedure. The control subjects were also explained the study procedure and they all signed the inform consent.

METHODS

After overnight fasting, venous blood samples were drawn from the median cubital vein, allowed to coagulate and centrifuged (5 min, 2000 g). The serum samples were stored and frozen at -80°C until analysis.

Serum concentrations of ALB (reference range 35-50 g/L), UA (reference range 155-428 μmol/L) and BIL (reference range 1.7-20.5 μmol/L) were determined on automated apparatus (Cobas 600 Roche Hitachi) at the Clinical Centre of the University of Sarajevo, Laboratory for clinical chemistry and biochemistry, using standard spectrophotometric methods.

Statistical Analysis

Statistical analysis was performed using the SPSS 16.0 software. The distribution of variables was tested by the Shapiro-Wilk test. Values with normal distribution were expressed as mean ± standard deviation, while those without normal distribution were shown as median and interquartile range. Depending on the distribution of variables, a comparison between the groups was performed by the ANOVA test Bonferroni post hoc test and Kruskal–Wallis test followed by Mann-Whitney U-test. Additionally, since variables were not normally distributed, correlations were assessed by Spearman's test. Multiple linear regression was used to assess antioxidants as predictors of the MoCA score in all patients.

To determine optimal cutoff values of potential biomarkers for differentiation between AD patients and CG, as well as for differentiation of patients with VD and CG, receiver operating characteristic (ROC) curves and their corresponding areas under the curve (AUC) were used. The accuracy rate for ROC curves was calculated with a 95% confidence interval (95% CI). Statistical significance was set at $p < 0.05$.

RESULTS

The baseline characteristics of the three study groups are shown in Table 1. There were no differences in age, systolic and diastolic blood pressures, WHR and BMI between the groups (Table 1).

Table 1. Baseline characteristics of patients with AD or VD and the control group.

Variables	CG (n=30)	AD group (n=30)	VD group (n=30)
Age (years)	80.5 (77.75-83.0)	82.5 (79.75-87.0)	79.0 (76.75-87.0)
BMI (kg/m^2)	26.18 ± 4.25	24.79 ± 3.69	24.76 ± 5.92
WHR	0,89 (0,87-0.91)	0,88 (0,83-0,92)	0,90 (0,86-0,93)
SBD (mmHg)	132.5 (125.0-152.5)	135.0 (115.0-150.0)	130.0 (120.0-140.0)
DBP (mmHg)	85.0 (70.0-90.0)	80.0 (70.0-90.0)	82.5 (75.0-90.0)

Data as mean ± SD and as median and interquartile range. AD: Alzheimer's disease; VD: Vascular dementia; BMI: Body mass index. WHR: Waist-Hip Ratio. SBP: systolic blood pressure; DBP: diastolic blood pressure

Patients with AD had significantly lower UA and significantly higher BIL concentrations compared to controls. Such differences were not evident among patients with VD. The only statistically significant difference regarding ALB concentrations were its lower values in VD patients compared with controls. Uric acid and BIL concentrations were also significantly different between the two groups of dementia patients (Table 2).

Table 2. Serum concentrations of non-enzymatic antioxidants in patients with dementia and the control group

Parameter	CG (n=30)	AD group (n=30)	VD group (n=30)
Uric acid (μmol/L)	353.50 (265.00-535.00)	253.00 * (193.75-362.00)	324.50 $^{\#}$(236.25-503.50)
Albumin (g/L)	37.13±0.84	35.23±0.71	34.33±0.98 *
Bilirubin (μmol/L)	5.65 (4.67-6.70)	6.65 *(5.25-8.82)	5.30 $^{\#}$(4.25-7.37)

CG, control group; AD group, patients with Alzheimer's disease; VD group, patients with vascular dementia; *p<0.05, in comparison to the CG; $^{\#}$p<0.05, in comparison to the AD group

Optimal cutoff values, area under the curve (AUC), sensitivity, specificity, positive and negative predictive value of serum antioxidants in for differentiation purposes are presented in Table 3. Results regarding the correlation between serum non-enzymatic antioxidants and MoCA in patients with dementia and the CG showed a statistically significant positive correlation in the case of BIL in patients with AD (Rho=0.375; p<0.05). The multiple linear regression model that included all non-enzymatic parameters and confusing variables, did not reveal any significant predictors of the cognitive MoCA score in AD and VD patients.

The differences in non-enzymatic antioxidants were further evaluated upon stratifying the dementia diseases into degrees of moderate and severe cognitive impairment (Table 4). The serum UA concentrations were significantly lower in the AD group with severe cognitive impairment compared to the AD group with moderate cognitive impairment and compared to the CG, but no significant difference in UA levels was found between AD patients with moderate cognitive impairment and CG. There were no differences in ALB concentrations between the groups. The serum BIL concentrations were significantly higher in the AD group with moderate cognitive impairment compared to the AD group with severe cognitive impairment and compared to the CG, but no significant difference in BIL levels was found between AD patients with severe cognitive impairment and CG.

The only statistically significant difference in serum ALB concentrations was found between patients with severe cognitive impairment in comparison to the CG (Table 5.)

Table 3. Optimal Cut-off, Area under the curve (AUC), sensitivity, specificity, positive and negative predictive value of serum non-enzymatic antioxidants in differentiating between AD patients and control subjects, as well as in differentiating between AD and VD patients.

Parameter	Optimal Cut off (μmol/L)	AUC (95% CI)	SEN	SPE	PPV	NPV	p
Uric acid AD *vs.* CG	≥ 310.50	0.723 (0.594-0.851)	66.7	73.3	71.4	68.8	0.003
Bilirubin AD *vs.* CG	≥ 7.05	0.665 (0.528-0.802)	46.7	83.3	73.7	60.9	0.028
Bilirubin AD *vs.* VD	≥ 6.25	0.671 (0.534-0.808)	60.0	66.6	64.2	62.5	0.023

AD, patients with Alzheimer's disease; VD, patients with vascular dementia; CG, control group; AUC, area under curve; CI, Confidence interval; SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value

Table 4. Serum non-enzymatic antioxidant concentrations in patients with AD stratified according to the degree of cognitive impairment and in control subjects

Parameter	CG (n=30)	AD group with severe cognitive impairment (n=19)	AD group with moderate cognitive impairment (n=11)
Uric acid (μmol/L)	353.50 (265.00-535.00)	215 *(151-305)	300 $^{\#}$(259-408)
Albumin (g/L)	37.13±0.84	34.84±0.90	35.90±1.19
Bilirubin (μmol/L)	5.65 (4.67-6.70)	6.1 (4.8-7.1)	7.6 * $^{\#}$(7.2-12.5)

CG, control group; VD group, vascular dementia, *p <0.05 - in comparison to the CG; $^{\#}$p<0.05 in comparison to the AD group with severe cognitive impairment

Table 5. Serum non-enzymatic antioxidant concentrations in patients with VD stratified according to the degree of cognitive impairment and in control subjects

Parameter	CG (n=30)	VD group with severe cognitive impairment (n=11)	VD group with moderate cognitive impairment (n=19)
Uric acid (μmol/L)	353.50 (265.00-535.00)	314 (231-387)	390 (275-509)
Albumin (g/L)	37.13±0.84	32.81±1.89 *	35.21±1.09
Bilirubin (μmol/L)	5.65 (4.67-6.70)	5.7 (4.1-8.5)	4.9 (4.3-7.3)

CG, control group; VD group, vascular dementia; *p<0.05 - in comparison to the CG

DISCUSSION

The obtained results showed a significant decrease in UA and a significant increase of BIL in AD patients compared to controls. The same trend was observed upon stratification of cognitive impairment, whereby UA was decreased in severe cognitive impairment, and increased levels of BIL were noted in moderate cognitive impairment. In all cases, the UA and BIL levels significantly differed between the two degrees of cognitive impairment. The clinical significance of serum UA is still debated. Many studies have reported decreased UA concentrations in AD patients (Cankurtaran, Yesil, Kuyumcu *et al.*, 2013; Euser, Hofman, Westendorp *et al.*, 2009). Particularly comprehensive was a large cohort study by Euser *et al.* (2009), who concluded that elevated UA reduced dementia risk, independent of the cardiovascular risk. The study of Hong, Lan, Tang *et al.* (2015) included 28760 gouty patients with both non-vascular and VD. After age, gender, and comorbidities adjustment, subjects with hyperuricemia were found to be at lower risk of non-vascular dementia, including AD (Tana, Ticinesi, Prati *et al.*, 2018). Our results are in line with these studies; however, there were also a few reports which imply a significant association between UA and white matter atrophy (Verhaaren, Vernooij, Dehghan *et al.*, 2013) or even no significant difference of UA values between AD patients and controls (Chen, Guo, Huang *et al.*, 2014). The biosynthetic pathways are the reason why UA is considered both as potentially neuroprotective and as potentially damaging. Depending on the concentration in the cerebrospinal fluid and the chemical micro-environment, UA may have detrimental effects (Desideri, Gentile, Antonosante *et al.*, 2017).

Unlike AD, the correlation between UA and VD is more precise. By accelerating vascular diseases, UA could contribute to cognitive decline (Vannorsdall, Jinnah, Gordon *et al.*, 2008). One study showed that elevated serum UA could lead to inflammation and oxidative stress processes, resulting in severe atherosclerosis, cerebral ischemia, and hypoxia, thereby increasing the risk for cognitive impairment in female patients (Perna, Mons, Schöttker *et al.*, 2016). Collectively, recent

research data suggest that serum UA may have different physio-pathological roles and clinical utility, depending on the type of dementia.

Bilirubin, the second parameter of this study that showed a significant difference between AD patients and controls, has been reported to have more potent antioxidant activity than α-tocopherol, superoxide dismutase and catalase (Stocker, Yamamoto, McDonagh *et al.*, 1987). Impaired liver functions are common in older adults and AD patients. These alterations, including changes in BIL metabolism, have been widely observed; however, the exact association with AD pathogenesis is insufficiently elucidated (Grimm, Zimmer, Lehmann *et al.*, 2013). Still, several authors (Cankurtaran *et al.*, 2013; Kim, Pae, Yoon *et al.*, 2006) observed lower BIL levels in AD patients. On the other hand, using an animal model, Chen, Liang, Xu *et al.* (2019) provided evidence that strengthens BIL as an endogenous pathogenic factor of AD. This is in agreement with our results that have shown increased levels of BIL in female patients with AD. The same authors postulated that neuronal BIL may be increased by low serum ALB concentration, hyperbilirubinemia and abnormally increased permeability of the blood brain barrier. In the present study, the MoCA score was correlated with all three oxidative stress parameters, but a positive correlation was established for BIL only. Vasantharekha, Priyanka, Swarnalingam *et al.* (2017) also found a positive correlation between cognitive function and BIL. However, the same study reported a decline in BIL concentrations in AD patients. Similarly, de Leeuw, van der Flier, Tijms *et al.* (2020) conducted a study where low bilirubin and zinc levels were associated with cognitive decline.

According to the obtained results, serum ALB concentrations were significantly decreased in VD patients, but not in AD patients. Upon stratifying the degree of cognitive impairment, a significant reduction in ALB content was evident only among patients with severe cognitive impairment. The ALB concentrations did not differ significantly between the groups of VD patients with moderate and severe cognitive impairment. Lower ALB concentrations imply impaired blood supply to the central nervous system and imbalance in the redox system - predispositions for cognitive impairment (Mao, 2013). Hypoalbuminemia was reported to be independently associated with poor cognitive performance and dementia in the elderly by several authors (Duarte, Duarte, Pelichek *et al.*, 2017; Murayama, Shinkai, Nishi *et al.*, 2017). We classify our finding of decreased ALB concentrations in VD patients as expected considering the ALB roles mentioned above and studies which reported an association between hypoalbuminemia and increased risk of coronary heart disease and venous thromboembolism (Vázquez-Oliva G, Zamora A, Ramos *et al.*, 2018; Kunutsor, Seidu, Katechia *et al.*, 2018). A highly important role of ALB is the ability to inhibit the formation of amyloid beta-peptide fibrils (Milojevic, Esposito, Das *et al.*, 2007). That is the reason why low levels of ALB in the brain and cerebrospinal fluid may lead to increased Alzheimer's type pathology (Galeazzi, Galeazzi, Valli *et al.*, 2002). Our results also showed decreased serum ALB concentrations in AD patients compared to controls; however, the difference was of no statistical significance. Albumin could not be considered as a specific biomarker of cognitive decline since minor to severe hypoalbuminemia is observed in common geriatric diseases. As a single parameter, it does not provide substantial assistance in the differential diagnosis, but rather perhaps, in conjunction with other antioxidant parameters.

The main limitation of this study was the cross-sectional design with a rather small sample size that did not assess the nutritional and educational status of the female subjects. While the exact mechanistic role of various antioxidants and their interplay in the pathogenesis of dementia diseases is still obscure, our results indicate that antioxidant deficiencies pose a risk for cognitive decline. The novelty of this study is reflected in the comparison of several non-enzymatic antioxidants in female patients with AD and VD and their evaluation according to the level of cognitive impairment. These parameters can be used as predictive tools in the progression of dementia diseases. Prospective studies with a larger cohort would be required to verify the suggested utility.

CONCLUSION

Patients with VD have significantly lower ALB concentrations. Upon stratification, according to the degree of cognitive impairment, a decline in concentrations of ALB and UA was found in patients with dementia with severe cognitive impairment. Such a finding can be attributed to their consumption in defense against the damage caused by free radicals reactions. On the other hand, BIL was increased in patients with AD, and its concentration was positively correlated to the MoCA score. In summary, the obtained data support the hypothesis that there is an association between natural oxidative and antioxidant markers and the type of dementia.

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Summary/Sažetak

Cilj ovog rada je bilo istražiti korelaciju između stepena kognitivnog oštećenja kod pacijenata ženskog spola oboljelih od Alzheimerove bolesti (AD) i vaskularne demencije (VD), te serumske koncentracije mokraćne kiseline (UA), albumina (ALB) i bilirubina (BIL). Presječna studija je obuhvatala 90 ispitanika, starosne dobi ≥65 godina podijeljenih u tri grupe: 30 pacijenata sa AD, 30 pacijenata sa VD i 30 kontrolnih ispitanika. Za procjenu kognitivnih sposobnosti svi ispitanici su podvrgnuti Montreal Cognitive Assessment testu (MoCA). Serumske koncntracije ALB, UA I BIL su određene spektrofotometrijskim metodama. Pacijenti sa AD su imali značajno niže serumske koncentracije UA, te povišene koncentracije BIL. Nakon stratifikacije prema stepenu kognitivnog oštećenja, niže UA koncentracije su zabilježene kod pacijenata sa teškim kognitivnim oštećenjem, dok je povišen BIL zabilježen kod pacijenata sa umjerenim kognitivnim oštećenjem. Za pacijente sa VD je bio karakteristična hipoalbuminemija, a nakon stratifikacija taj nalaz je bio evidentan kod pacijenata sa teškim kognitivnim oštećenjem. Bodovi MoCA testa su pozitivno korelirali sa BIL kod AD pacijenata. Dobiveni rezultati podržavaju zaštitnu ulogu serumskih antioksidanasa u patogenezi demencije. Nadalje, predlažu se dodatne longitudinalne studije da bi se potvrdila kombinirana upotreba ovih parametara kao potencijalnih biomarkera AD i VD.

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29-34

Relationship between platelet indices and lipid status in chronic hookah consumption

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Abstract: Hookah smoking is a growing trend, both in the world and in Bosnia and Herzegovina. The aim of this study was to determine the value of platelet indices in hookah smokers and find out associations with lipid profile. Cross-sectional study included 60 students (30 chronic hookah smokers and 30 non-smokers). The complete blood count (erythrocytes, leukocytes, platelets, hemoglobin, hematocrit, erythrocyte and platelet indices), lipid parameters (total cholesterol, triglycerides, LDL-C, HDL-C) were determined. The platelet count, mean platelet volume and MPV/Platelets ratio were statistically significantly higher in chronic hookah consumers in the student population than in the control group ($p < 0.001$). In addition, platelet count was in significant positive correlation with values of total cholesterol, LDL-C and negative correlation with value of HDL-C, while there was a significant negative correlation between mean platelets volume, and MPV/Platelets ratio with HDL cholesterol levels in chronic hookah smokers ($p < 0.05$). These findings suggest that chronic hookah consumption could be associated with the development of atherosclerotic changes in blood vessels, which could lead to the development of long-term consequences on the cardiovascular system's function.

INTRODUCTION

The hookah, also known as the shisha, nargile, is a tobacco, long, flexible pipe that draws smoke through the water contained in the vessel from which it comes out (Bou Fakhreddine et al.,2014). It is estimated that around 1.1 billion people use hookah, which is the most popular smoking product, along with cigarettes (Badran et al., 2020). The prevalence of hookah smoking among students is extremely high in the Eastern Mediterranean countries (> 30%), followed by over 20% in the Czech Republic, Estonia, Latvia, and Jordan, over 10% in Hungary, Poland, Slovakia, and Ukraine (Jawad et al., 2015). Hookah users are exposed to many of the same toxic compounds as cigarette users, but at dramatically higher levels, leading to more serious negative health effects. Data from previous studies have shown that hookah smoke contains 7 carcinogens, 39 central nervous system depressants, and 31 respiratory irritants. (Elsayed et al., 2016). Hookah smoking leads to

significant acute disorders o of the cardiovascular and respiratory systems, which are characterized by an increase in heart rate by 6-13 beats, systolic pressure by 3-16 mmHg, diastolic pressure 2-14 mmHg, and the number inspiration of 2 per minute (Haddad et al., 2016). There is a lack of research in regards to the connection between hookash smoking and emerging chronic effects on the cardiovascular system.

In that sense, an increasing number of researchers analyze the potential proatherogenic effects of chronic hookah consumption in order to prevent the development of other diseases of the cardiovascular system, such as acute coronary syndrome.

Atherosclerosis is a multifactorial disease of medium and large arteries, in which there is a focal accumulation of deposits composed of lipids, carbohydrates, blood products, fibrous tissue, and calcium on the inner wall of the arteries. Due to the accumulation of deposits, the wall hardens and loses elasticity. (Rafieian-Kopaei et al., 2014). In the development of initial atherosclerotic changes, the most important event is changes in lipid fractions concentrations with proatherogenic lipids' dominance. Dyslipidemia is a disorder of fat metabolism, which results in a disorder of the concentration of certain lipids (hyperlipidemia, hypercholesterolemia, hyperlipoproteinemia) and is considered the main cause of atherosclerosis and associated diseases such as : cardiovascular disease, ischemic cerebrovascular disease, and peripheral vascular disease. The most important risk factors for atherosclerosis are elevated LDL, decreased HDL. (Nelson, 2013). Platelets, oval or round cells formed by the fragmentation of megakaryocytes in the bone marrow, liver, spleen, and lungs, from where they are released into the bloodstream, play a significant role in the development of atherosclerotic plaque. The most important physiological function of platelets is active participation in all phases of hemostasis, both by physical and chemical processes and by the release and activity of special platelet factors. Also, they have a role in the processes of maintaining endothelial integrity, phagocytosis, detoxification of the organism, and transport of substances (Periayah et al., 2017). In conditions of endothelial dysfunction in atherosclerosis, e.g., plaque rupture, there is a reduced synthesis of platelet aggregation inhibitors, which with endothelial damage caused by mechanical, chemical, immune mechanisms lead to platelet interaction with endothelium and subendothelial structures and consequent activation, adherence and platelet aggregation (Schäfer and Bauersachs 2008).

The aim of this work is to assess the hazardous effect of chronic smoking tobacco by hookah on lipid profile and platelet indices, as the main factors in the development of atherosclerotic disorders.

EXPERIMENTAL

Subjects

The cross-sectional study included 60 consecutive participants from September 2019 to May 2020 at the Medical Faculty of the University of Sarajevo. The study included 60 students of the University of Sarajevo divided into two groups: 30 chronic hookah consumers of both sexes, average age 24 years, and 30 healthy participants, average age 25 years, who never consumed hookah. Criteria for inclusion in the study were: respondents who consumed a hookah for at least a year at least 2 times a week, respondents who voluntarily agreed to participate in the study, a properly completed questionnaire, respondents aged 18-30, students of the University of Sarajevo. Exclusion criteria were: participants who did not complete the questionnaire correctly, participants older than 30 years, participants who consumed hookahs for less than a year, participants who smoked cigarettes, participants who had an acute or chronic illness or used therapy or supplements, which may affect the values of the examined parameters. The study was carried out in accordance to Declaration of Helsinki, as revised in 2000. Written informed consent for inclusion in the study was obtained from all participants.

Sample analysis

After taking the medical interview and physical examination, the subjects had their blood taken for laboratory tests by puncturing the cubital vein. Serum was extracted from blood samples after coagulation and centrifugation for 10 minutes at 4000 rpm and stored until the required results were obtained.

Blood samples were taken from all participants by cubital vein puncture for the following laboratory analyzes: complete blood count (erythrocytes, leukocytes, platelets, differential blood count, erythrocyte indices) and lipid profile (total cholesterol, triglycerides, LDL-C, HDL -C).

Platelet count, mean platelet volume (MPV), platelet distribution width (PDW), and plateletcrit (PCT) were determined using an automatic Beckman Coulter STKS Hematology Analyzer. Based on the obtained values, the MPV /platelets ratio (MPV/Plt) is calculated.

Lipid profile

Total cholesterol, HDL-cholesterol (high-density lipoprotein cholesterol), and serum triglycerides were determined by standard enzyme methods on an Olympus 2700 analyzer (Beckman Coulter, USA). The results were read automatically on the instrument.

Principle of estimation total cholesterol.

The recommended method for determining total cholesterol is the photometric method after hydrolysis and cholesterol extraction. Enzymatic determination of cholesterol concentration is specific and sensitive. The reaction principle is as follows: cholesterol esterase catalyzes the hydrolysis of cholesterol esters to free cholesterol and free fatty acid. In the presence of cholesterol oxidase, cholesterol is oxidized to cholest-4-en-3-one to form hydrogen peroxide. Phenol and 4-aminoantipyrine with hydrogen peroxide in the presence of peroxidase give the red colored product quinonymine. The intensity of staining is directly proportional to the concentration of total cholesterol in the sample.

Principle of estimation HDL. The recommended method for determining HDL-cholesterol is

ultracentrifugation and precipitation with heparin and $MnCl_2$ and the determination of cholesterol in the supernatant. The determination of HDL-cholesterol is based on the separation of HDL from lipoproteins containing apolipoprotein B (LDL and VLDL) by ultracentrifuge, electrophoresis, or specific precipitation with polyanions and divalent cations. The concentration of HDL-cholesterol is determined by one of the methods for determining total cholesterol. LDL - (low-density lipoproteins) and VLDL - cholesterol (very low-density lipoproteins) are precipitated by the addition of dextran sulfate solution and serum magnesium chloride. Negatively charged groups on the polyanion, which react with positively charged groups of lipoprotein molecules, are probably significant for the reaction. The present divalent cations accelerate the formation of insoluble LDL- and VLDL-cholesterol complexes. Insoluble complexes sediment by centrifugation due to higher density and HDL-cholesterol remains in the supernatant, determined by one of the methods for determining total cholesterol.

Principle of estimation triglycerides.

The recommended method for determining triglycerides is the photometric method after extraction, saponification, and glycerol oxidation. Methods for determining triglyceride concentration are based on the measurement of released glycerol. Reaction principle: the resulting glycerol-1-phosphate is oxidized to dihydroxyacetone phosphate in the presence of glycerol-1-phosphate dehydrogenase and NAD. The resulting reduced coenzyme, NADH, reduces the color 2- (p-iodophenyl) -3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) to a red colored formazan whose concentration is measured photometrically.

The LDL-C concentration was determined by the Friedwald formula, [LDL] = [K] – [HDL] – ([TG] / 5) in which the LDL-C concentration was calculated from the concentration of total cholesterol, HDL-C, and triglycerides.

Statistical analysis

Statistical data processing was done using the computer program Excel (Microsoft Office Excel 2010) and SPSS computer program for statistical analysis (SPSS-Statistical Package for Social Sciences) version 13.0. Data were processed by standard statistical methods and presented in the form of tables and graphs. Shapiro-Wilk test was used to assess the normality of continuous variables' distribution.

The mean value (X) and standard deviation (SD) for continuous independent variables that followed the normal distribution were determined, ie the median and interquartile range for independent continuous variables that did not follow the normal distribution. The Student t-test tested the significance of the difference for the continuous independent variables that followed the normal distribution. In contrast, the Mann - Whitney test tested the significance of the difference for the independent continuous variables that did not follow the normal distribution for independent samples. The correlation coefficient (rho) was determined by the Spearman method. Values of $p < 0.05$ are considered statistically significant.

RESULTS

The characteristics of hookah smokers and control subjects are presented in Table 1. The values of hemoglobin, hematocrit, platelets, MCV, MCH, MCHC, PDW, MPV, PCT, and MPV/Platelets ratio were statistically significantly higher in chronic hookah smokers compared to the control group of subjects ($p<0.001$).

Table 1: Laboratory values of blood count parameters in chronic hookah smokers in relation to the control group

Variables	Hookah smokers (n=30)	Control group (n=30)	p
Leukocytes ($x10^9$)	7.01 ± 1.54	7.27 ± 1.92	0.894
Erythrocytes ($x10^{12}$)	5.1(5.02 – 5.36)	5.25(4.47 - 5.74)	0.099
Hemoglobin (g/L)	164.97(160.25 – 176)	154(133 - 173)	<0.001
Hematocrit (%)	46.82(45.52 – 49.6)	45.46(38.3 - 50.3)	0.021
MCV (fL)	92.09(90.75 – 94.22)	85.98(65.3 - 97.2)	<0.001
MCH (pg)	32.76(32.07 – 33.9)	29.23(26.9 - 30.8)	<0.001
MCHC (g/L)	353.03(345 – 360.25)	337.63(313 - 360)	<0.001
Platelets ($x10^9$)	242.50(197.5 – 288.25)	192.6(173.25 – 211.25)	<0.001
RDW-CV (%)	12.31(11.7 – 12.32)	12.4(11.4 - 13.8)	0.097
PDW	13.8(11.7 - 14.9)	13.64(11.7 - 16.9)	0.233
MPV (fL)	9.45(8.5 - 10.2)	9.078(8.0 - 10.2)	<0.001
PCT (%)	0.160(0.124 - 0.244)	0.157(0.118 - 0.246)	0.324
MPV/Plt	0.038 ± 0.003	0.047 ± 0.002	<0.001

*data presented as mean ± standard deviation (X ± SD) and as median with 25-75 percentile interquartile range;
Mean Corpuscular Volume, MCV; Mean Corpuscular Hemoglobin, MCH; Mean Corpuscular Hemoglobin Concentration, MCHC; Red blood cell Distribution Width, RDW-CV; Platelet Distribution Width, PDW; Mean Platelet Volume, MPV; Plateletcrit, PCT; MPV/Plt, MPV/Platelet ratio; probability, p

Relationship between basal values of lipid status in chronic hookah smokers and control group are presented in Table 2. Chronic hookah smokers had statistically significantly lower HDL-cholesterol values than the control group (p <0.001), while other parameters did not show statistical significance.

Table 2: Lipid status in chronic hookah smokers and control group

Variables	Hookah smokers (n=30)	Control group (n=30)	p
Cholesterol (mmol/L)	3.9 (3.58 - 4.62)	4.2 (3.9 - 4.3)	0.307
Triglycerides (mmol/L)	1.14 (0.78 - 1.43)	0.95 (0.76 - 1.15)	0.211
LDL (mmol/L)	2.38 (2.02 - 2.68)	2.31 (2.05 - 2.65)	0.941
HDL (mmol/L)	1.09 (0.9 - 1.25)	1.28 (1.15 - 1.5)	0.001

*data presented as mean ± standard deviation (X ± SD) and as median with 25-75 percentile interquartile range;
Low-density lipoproteins, LDL; High-density lipoproteins, HDL

The correlation between platelet index values and plasma lipid fractions is shown in Table 3. Platelet counts were positively correlated with total cholesterol (p <0.05),, LDL (p <0.05), and were negatively correlated with HDL values. MPV and MPV /platelet values were significantly negatively correlated with HDL values (p <0.001).

Table 3: Correlation of platelet indices and lipid status in chronic hookah smokers

Variables		Plateletes	PDW (%)	MPV(fL)	PCT(%)	MPV / Plt
Cholesterol (mmol/L)	Rho	0.396*	0,089	0.332	0.126	0.145
Triglycerides (mmol/L)	Rho	0.062	0.075	0.031	0.061	0.083
LDL (mmol/L)	Rho	0.433*	0.122	0.397	0.101	0.146
HDL (mmol/L)	Rho	-0.718**	-0.002	-0.668*	-0.092	-0,553*

Platelet Distribution Width, PDW; Mean Platelet Volume, MPV; Plateletcrit, PCT; Low-density lipoproteins, LDL;
High-density lipoproteins, HDL; Rho – Spearman correlation coefficient ; *p<0.05. **p<0.001

DISCUSSION

The growing trend of hookah smoking has slowly and imperceptibly begun to have its effect on the overall health of people who enjoy it. Smoking with the use of water pipes causes a number of adverse effects on the smallest structures in the human body, such as blood cells, to the multiple destructions of organs and organ systems. Data from numerous studies have shown that smoking classic cigarettes has a significant influence on the promotion of the proatherogenic effect in the body. In case if hookah smoking, data, due to the limited number of studies, are still not significant to explain all the mechanisms involved in the pathogenesis and promotion of atherosclerotic changes.

The pathophysiological mechanism by which the products released during hookah consumption induce atherosclerosis development and the subsequent consequences has not yet been sufficiently elucidated. The main candidate is considered to be the activation of oxidative stress reactions, with consequent endothelial dysfunction. Previous research has shown that the possibility of vasodilation of blood vessels is significantly reduced in hookah smokers compared to cigarette smokers and non-smokers, which shows a direct impact of hookah toxins on the development of endothelial dysfunction. Hookah smokers, active or passive, are exposed to higher doses of toxic particles, such as polycyclic aromatic hydrocarbons and benzene aldehydes, which are important triggers for oxidative stress reactions (Al-Amri et al. 2020).

Increased synthesis of reactive oxygen species, through the process of lipid peroxidation, is associated with changes in the concentration of individual lipid fractions, which can be expected in hookah smokers. In addition to the influence of reactive oxygen species, a potential cause of dyslipidemia is nicotine, one of the components in the taste hookah, which leads to the activation of catecholamines, with consequent lipolysis of adipose tissue triglycerides (Andersson K., Arner P., 2001).

Our results showed that HDL-C values were statistically significantly lower in chronic hookah consumers, while other lipid status parameters did not differ significantly. The present study findings are similar to the finding of

Trupti and co-works who found that mean HDL-C was significantly lower among smokers than control. In contrast to our study, other studies have shown an increase in hookah smokers' proatherogenic lipid fractions (Sami Alkubaisy et al., 2020; Shafique et al., 2012), that suggests that the research results are not consistent. The reason can be sought in the length of hookah consumption, which implies the need for further research that would elucidate all the pathophysiological mechanisms in developing lipid disorders in hookah use.

By analyzing platelet parameters, our results showed that the number of platelets, MPV, MPV / Plt ratio were statistically significantly higher in chronic hookah consumers compared to the control group, which may indicate increased thrombotic activity in proatherogenic processes. Previous studies have shown that the metabolites of nicotine (cotinine), acrolein, and aldehyde, from hookah smoke, affect platelet activation and lead to increased prothrombotic expression, consumption, and producing new platelets, which explains the increase in their number in chronic hookah consumers. (Alarabi et al., 2020). Activated platelets exert their function on the endothelium, where they modulate the inflammatory response and participate in the formation of atherosclerotic changes and later thrombotic complications (Badimón et al., 2009).

Platelet size, shown as MPV, reflects platelet activity and appears to be a useful predictive and prognostic biomarker of cardiovascular events. Increased MPV values correlate with diseases based on the atherosclerotic process, such as acute coronary syndrome and cardiovascular disease. Larger platelets are known to be metabolically, enzymatically, and functionally more active and produce more thromboxane A2, leading to potentially increased thrombogenic activity and promotion of atherosclerosis (Khode et al., 2012).

The MPV / Plt ratio is easily measured biomarker, suitable for routine use to demonstrate increased platelet activity in atherosclerosis. Increased MPV/Plt ratio, in contrast to platelet count and MPV alone, is a better predictor of long-term mortality in many diseases, including ischemic cardiovascular diseases and nonalcoholic fatty liver disease (Slavka et al., 2011).

We found that MPV and MPV / Plt ratio values were significantly negatively correlated with HDL values by analyzing the relationship between platelet parameters and lipid status. According to data from the literature, HDL inhibits several procoagulation and prothrombotic processes. Low HDL-cholesterol levels are an independent predictor of acute thrombus formation (van der Stoep et al., 2014). The inhibitory effect of HDL particles on platelet activity depends on apo E's presence in these lipoproteins, which stimulates the synthesis of nitric oxide (NO) in platelets. Recent studies have established the presence of LRP8 receptors on the platelet surface. It is a receptor for apo E protein, The binding of HDL particles via apo E to this receptor stimulates the production of nitric oxide synthase(iNOS). HDL stimulates endothelial production of nitric oxide and prostacyclin, potent inhibitors of platelet activation (Holtzman et al., 2012), which leads us to conclude that subjects from our study, where HDL is statistically significantly lower in chronic hookah consumers, have a higher chance of thrombotic clot formation and higher cardiovascular risk.

CONCLUSION

Recently, hookah consumption has been identified as an important factor in the progression and outcome of several important disorders. In addition to numerous systemic effects, hookah consumption products lead to the initiation of changes in the wall of blood vessels, which, combined with the disturbance of lipid concentration, promotes the accelerated development of atherosclerotic damage. A better understanding of the mechanisms involved in these disorders will provide additional opportunities to prevent the development of long-term effects on other organ systems. The results of the current study indicate the potential involvement of hookah on platelet function and lipid metabolism. To validate our findings, further major prospective population-based studies are still required.

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Summary/Sažetak

Pušenje nargile je rastući trend, kako u svijetu, tako i u Bosni i Hercegovini. Cilj ovog istraživanja bio je utvrditi vrijednost tromobcitnih indeksa kod pušača nargile i utvrditi povezanost sa lipidnim profilom. U presječnu studiju je uključeno 60 studenata (30 hroničnih pušača nargile i 30 nepušača). Određena je kompletna krvna slika (eritrociti, leukociti, trombociti, hemoglobin, hematokrit, indeksi eritrocita i trombocita), lipidni parametri (ukupni holesterol, trigliceridi, LDL-C, HDL-C). Broj trombocita, srednji volumen trombocita i omjer MPV /trombociti bili su statistički značajno veći kod hroničnih potrošača nargile u odnosu na kontrolnu grupu ($p < 0,001$). Pored toga, broj trombocita bio je u značajnoj pozitivnoj korelaciji sa koncentracijama ukupnog holesterola, LDL-C i negativnoj korelaciji sa koncentracijom HDL-C, dok je postojala značajna negativna korelacija između srednjeg volumena trombocita i odnosa MPV / trombocita sa nivoom HDL holesterola kod hroničnih pušača nargile ($p < 0,05$). Ovi rezultati sugeriraju da bi hronična konzumacija nargile mogla biti povezana s razvojem aterosklerotskih promjena u krvnim žilama, što bi moglo dovesti do razvoja dugoročnih posljedica na funkciju kardiovaskularnog sistema.

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