Total bilirubin in athletes, determination of reference range

INTRODUCTION

The main cause of increased bilirubin in athletes is haemolysis and subsequent catabolism of haemoglobin [1]. The accelerated breakdown of red blood cells (RBC) is mainly caused by mechanical factors (marching haemolysis, damage caused by muscle work or RBC squeezing through capillaries) and the destructive effects of free radicals [2,3,4,5]. In recent years, there have been studies which indicate possible antioxidant function of bilirubin. It was also shown that its concentration correlates negatively with the risk of cardiovascular disease [6]. Other articles have described its anti-inflammatory properties, manifested in a negative correlation with the concentration of C-reactive protein (CRP) [7]. There were also several population studies indicating an inverse relationship between bilirubin levels and the incidence of metabolic syndrome [8,9]. It is therefore possible that the presence of elevated total bilirubin can be a kind of adaptation of training, and not just a transitional post-exercise effect of changes in body functions.

The main aim of this study was to determine a typical reference range for the population of athletes. An additional aim was to determine how frequently in athletes the total bilirubin concentration is outside the stated reference range for the general population.

In case of total bilirubin the clinical interest is only in high results, therefore, the work focused on determining the upper limit of reference range.

MATERIALS AND METHODS

We retrospectively analysed the results of blood tests in 339 athletes (82 women and 257 men, aged 18-37 years) performed in the Department of Biochemistry, Institute of Sport - National Research Institute (NRI) in Warsaw. The subjects were representatives of different sports disciplines. Venous blood was collected for testing in the morning on an empty stomach and transferred to the laboratory within 1 hour of collection. All assays were done on the same day. The programme of the study was approved by the Ethical Research Committee at the Institute of Sport - NRI in Warsaw. Haematological parameters were measured in whole blood collected into tubes containing K3EDTA.

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ABSTRACT: The purpose of this study was to determine a typical reference range for the population of athletes. Results of blood tests of 339 athletes (82 women and 257 men, aged 18-37 years) were retrospectively analysed. The subjects were representatives of different sports disciplines. The measurements of total bilirubin (BIT), iron (Fe), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) were made using a Pentra 400 biochemical analyser (Horiba, France). Red blood cell count (RBC), reticulocyte count and haemoglobin concentration measurements were made using an Advia 120 haematology analyser (Siemens, Germany). In groups of women and men the percentage of elevated results were similar at 18%. Most results of total bilirubin in both sexes were in the range 7-14 μmol·L−1 (49% of women and 42% of men). The highest results of elevated levels of BIT were in the range 21-28 μmol·L−1 (12% of women and 11% of men). There was a significant correlation between serum iron and BIT concentration in female and male athletes whose serum total bilirubin concentration does not exceed the upper limit of the reference range. Elevated concentrations of total bilirubin appear to be due to changes caused by regular exercise. The obtained upper limit of the reference range for total bilirubin concentration in the group of athletes is 29.0 μmol·L−1. It seems reasonable to use dedicated reference values for total bilirubin concentration in relation to the group of athletes.

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Blood samples for biochemical measurements were collected into tubes containing coagulation accelerator and serum separator. In order to obtain the serum for testing, blood samples were centrifuged at 2000g for 10 minutes.

The study was carried out in the laboratory of the Department of Biochemistry, Institute of Sport - NRI, having accreditation of the Polish Centre for Accreditation (no. AB 946).

The measurements of total bilirubin (BIT), iron (Fe), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) were made by spectrophotometric methods with a Pentra 400 Horiba biochemical analyser (France), using original manufacturer reagent kits. Red blood cell count (RBC), reticulocyte count and haemoglobin concentration measurements were made using an Advia 120 haematology analyser, Siemens (Germany). Reference value of total bilirubin for general population used in paper is regards to the manufacturer reagent kit instructions [10].

**Statistical analysis**

Values for total bilirubin, iron, alkaline phosphatase, alanine aminotransferase, gamma glutamyltransferase, red blood cells and reticulocyte count and haemoglobin concentration were log transformed. Correlations between total bilirubin and other parameters were conducted using Pearson’s r correlation coefficient. The level of p≤0.05 was considered significant. The upper reference range was set with the formula: uBIT = mBIT + 1.645xSD, where uBIT = total bilirubin upper reference range set, mBIT = mean total bilirubin concentration in analysed population of athletes, SD = standard deviation [11]. Because the clinical interest is only in high results one-sided reference intervals are defined that exclude only the 5% of the reference population [12].

The logarithmic mean values and reference values were back-transformed. All calculations were done with Statistica 10 software (StatSoft, Inc., Tulsa, OK, USA).

**RESULTS**

Table 1 shows the results of determinations of total bilirubin. In the general group of athletes 18.9% of total bilirubin results exceeded the upper limit of the reference values adopted by the laboratory (21 μmol·L⁻¹). In individual groups of women and men the percentage of elevated results were similar at 18%. Most results of total bilirubin in both sexes were in the range 7-14 μmol·L⁻¹ (49% of women and 42% of men). The highest results of elevated levels of BIT were in the range 21-28 μmol·L⁻¹ (12% of women and 11% of men).

**TABLE 1.** Average total bilirubin concentration in subgroups of participants with a normal or elevated level of bit, by gender

|         | Women   | Men     |
|---------|---------|---------|
| Normal BIT level (≤ 21.0μmol/L) | 12.2 ± 4.1 | 12.7 ± 4.3 |
| Elevated BIT level (> 21.0μmol/L) | 27.6 ± 4.8 | 29.3 ± 8.1 |

**TABLE 2.** Average value of selected blood parameter results in specific subgroups of participants with a normal or elevated level of bit, by gender.

| Parameter | Reference range for general population | Normal BIT level (≤ 21.0μmol/L) | Elevated BIT level (> 21.0μmol/L) |
|-----------|----------------------------------------|----------------------------------|-----------------------------------|
| RBC       | M: 4.7-6.1 W: 4.2-5.4                  | 4.81 ± 0.30 M: 12.7 ± 4.3        | 5.33 ± 0.33 M: 29.3 ± 8.1         |
| [x10⁹· uL⁻¹] |                                       |                                  |                                   |
| Haemoglobin| M: 140-180 W: 120-160                 | 139.8 ± 7.2 M: 154.2 ± 8.8      | 139.1 ± 5.2 M: 158.2 ± 6.3       |
| [g/L]     |                                       |                                  |                                   |
| Iron      | M: 40-155 W: 37-165                   | 77.1 ± 36.1 M: 84.9 ± 36.8      | 103.6 ± 32.1 M: 122.9 ± 48.9     |
| [μg/dl]   |                                       |                                  |                                   |
| Reticulocyte | M: 22-139 W: 22-139                | 63.8 ± 12.8 M: 67.8 ± 18.2      | 63.6 ± 15.0 M: 73.0 ± 20.1       |
| [x10⁶· L⁻¹] |                                      |                                  |                                   |
| ALP       | M: 52-171 W: 47-119                  | 87.1 ± 27.4 M: 114.3 ± 36.2     | 77.9 ± 23.3 M: 109.6 ± 24.5      |
| [U· L⁻¹]  |                                       |                                  |                                   |
| ALT       | M: ≤ 45 W: ≤ 34                      | 19.6 ± 7.3 M: 26.7 ± 10.2       | 17.8 ± 5.7 M: 29.2 ± 12.2        |
| [U· L⁻¹]  |                                       |                                  |                                   |
| GGT       | M: ≤ 55 W: ≤ 38                      | 16.9 ± 4.8 M: 22.9 ± 7.6        | 17.6 ± 4.3 M: 25.0 ± 9.1         |
| [U· L⁻¹]  |                                       |                                  |                                   |

**Note:** the values represent mean ± SD; BIT = total bilirubin.
TABLE 3. Values of the correlation coefficient of selected blood parameters in specific subgroups of participants with a normal or elevated level of total bilirubin, by gender.

|                  | Sex | RBC    | Haemoglobin | Reticulocyte | Iron   | ALP    | ALT    | GGT    |
|------------------|-----|--------|-------------|--------------|--------|--------|--------|--------|
| Normal BIT level (≤ 21.0μmol·L⁻¹) | W   | 0.13   | 0.20        | 0.01         | 0.60*  | -0.02  | 0.08   | 0.18   |
| Elevated BIT level (> 21.0μmol·L⁻¹) | W   | 0.48   | -0.1        | 0.26         | 0.11   | 0.03   | 0.34   | -0.36  |
| Normal BIT level (≤ 21.0μmol·L⁻¹) | M   | 0.07   | 0.04        | -0.01        | 0.51#  | -0.01  | 0.01   | 0.02   |
| Elevated BIT level (> 21.0μmol·L⁻¹) | M   | 0.24   | 0.08        | 0.003        | 0.004  | -0.12  | -0.04  | -0.11  |

Note: BIT = total bilirubin; w = women, m = men; significant correlation: * p=0.0034, # p=0.0057.

The results of other measured biochemical and haematological parameters were within accepted ranges of reference values for general population, as regards to the manufacturer reagent kit instructions, both in women and men (Table 2). There was a significant correlation between serum iron and BIT concentration in female and male athletes whose serum total bilirubin concentration does not exceed the upper limit of the reference range (r=0.60 and 0.51 respectively) (Table 3). There was no significant correlation in relation to other parameters in groups of men and women, regardless of the division into groups with higher or normal concentration of BIT.

The calculated upper limit of the reference concentration of total bilirubin for the group of athletes showed no significant difference between women and men (respectively 27 μmol·L⁻¹ and 30 μmol·L⁻¹).

**DISCUSSION**

Total bilirubin largely depends on the degree of haemolysis, whose intensity increases with increasing intensity of physical efforts. Fallon et al. [13], studying 100 athletes from 11 sports, found that an elevated level of BIT was the second parameter by number which gave results outside the reference range. On the other hand, in a study of 37 marathon runners, after the competition elevated levels of BIT occurred in 19 persons, which the authors explained by the severe haemolysis [14]. Swift et al. [15], examining the effect of different volumes of exercise on the level of bilirubin, found that only a large physical training load increases its concentration. Hammoud et al. [16] observed an increase in total bilirubin after a maximal exercise of short duration (30-second Wingate test).

Most of the studies refer to the phenomenon of haemolysis caused by a specific exercise. A few however cover observation of a longer period of time. Banfi et al. [17] observed 24 rugby players throughout the league season. They observed a significant increase in total bilirubin concentration at the end of the league season with a simultaneous significant decrease of haptoglobin concentration. These authors noted a steady increase in the severity of haemolysis throughout the observation period.

In the present study the examined haematological parameters did not indicate the occurrence of increased haemolysis. There was no significant relationship between the total bilirubin concentration and the number of red blood cells, haemoglobin or iron levels in the blood.

The concentration of total bilirubin is one of the parameters used in the evaluation of liver function, and an increased value may suggest disturbances in the functioning or hepatic injury [18]. Additional parameters used for the evaluation of liver function include alkaline phosphatase (ALP), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) [19]. In the present study the activity of liver enzymes was within the accepted limits of the reference range for general population, which may indicate the proper functioning of the liver in the examined athletes. The above-cited Pettersson et al. [18] stated in the conclusions of their paper that the causes of the asymptomatic increase in the value of such parameters as total bilirubin are largely unknown, and the impact of exercise on its level requires further study.

In addition to haemolysis and liver function disorders, other mechanisms seem to significantly affect the concentration of bilirubin in the body. It is believed that bilirubin is a potential anti-inflammatory and antioxidant agent [6]. It is also an inhibitor of oxidative changes in LDL and other lipids, takes part in neutralizing free radicals and prevents oxidative stress [20]. There are authors who state that bilirubin more effectively inhibits oxidation of LDL than α-tocopherol and has a greater protective effect on monocytes than vitamins C and E [21]. In addition, bilirubin concentration is in inverse proportion to the risk of metabolic syndrome and insulin resistance [22]. In the present study, parameters relating to antioxidant defence were not evaluated. However, physical activity increasing daily intake of oxygen causes enhanced production of reactive oxygen species (ROS) [23]. Also, increasing metabolism due to muscle activity results in increased ROS production [24,25].

At the end it should be noted that the reference ranges depends not only on the population, but also on other factors, such as methods and test procedures used in laboratory. Therefore, according to
Clinical and Laboratory Standards Institute (CLSI) guidelines, each laboratory should establish its own reference ranges.

CONCLUSIONS

In summary, these elevated concentrations of total bilirubin appear to be due to changes caused by regular exercise. The obtained upper limit of the reference range for total bilirubin concentration in the group of athletes is 29.0 μmol·L⁻¹. Further research is required to clarify whether these changes can be considered as training adaptation. However, it seems reasonable to use a dedicated reference range for total bilirubin concentration in relation to the group of athletes.

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