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Hemoglobin HbA_1c and glucose blood levels in diabetic patients

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Abstract: Diabetes mellitus (DM) is defined as an absolute or relative lack of insulin, or a state of chronic hyperglycemia. Hemoglobin A_1c (HbA_1c) is a minor Hb form, produced *in vivo* by post-translational glycosylation. In the last 30 years, in biochemical laboratory practice, HbA_1c became a "gold standard" for clinical monitoring of DM. The aim of this study was to determine the glucose and HbA_1c levels in DM suffering patients at"Zavidovići" Health Center in different time periods, and estimate glucoregulation. The levels of HbA_1c and glucose were measured in 100 patientswith 3-month time period. The results were analyzed by appropriate statistical methods, to determine whether there are statistically significant differences between the two measurements. A spectrophotometric method was used to determine the level of HbA_1c, while glucose was determined using an enzymatic-colorimetric method on biochemical analyzer. It was found that in 61 of the total number of subjects, the levels of HbA_1c and glucose were significantly reduced ($p^{***}<0.001$) three months after the first measurement, which leads to the conclusion that their glucoregulation have improved. In the remaining 39 subjects the levels of HbA_1c and glucose were significantly increased ($p^{*}<0.05$) in the same time period, which leads to the conclusion that their glucoregulation worsened.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by elevated blood glucose levels (hyperglycemia) resulting from defects in insulins ecretion, insulin action or both (Njolstad *et al*., 2003; Janghorbani *et al*., 2007; Alimanovic-Halilovic *et al*., 2015).

The basic effect of insulin lack or insulin resistance on glucose metabolism is the worsened uptake and utilization of glucose by most cells of the body, except those of the brain (Guyton and Hall, 2006). As a result of this, blood glucose concentration increases, cell utilization of glucose falls increasingly and utilization of fats and proteins increases (Guyton and Hall, 2006, Ozougwu *et al*., 2013).

The chemical reaction of glucose with other compounds is termed glycation. If glucose reacts with hemoglobin, the resulting compound is named glycohemoglobin (Ibrahim *et al*., 2006; Selvin*et al*., 2010; Hinzmann *et al*., 2012). Hemoglobin A_1c (HbA_1c), which is irreversibly glycated on the *N*-terminal valine of the *β*-chain, is well known as the main diabetes marker protein used for clinically monitoring long-term glycemic control. (Rohlfing *et al*., 2002; Hinzmann *et al*., 2012; Alegre-Diaz *et al*., 2016).

The HbA_1c test is not recommended for diagnosis because there is not a standard assay for the HbA_1c and because many countries do not have ready access to the test (Montoya-Carralero *et al*., 2010; Mealey and Oates, 2006). Since red blood cells have an average lifespan of 3-4 months (80±10.9 days) in the blood circulation, $\%HbA_1c$ becomes a better indicator of patient glycemic control in that time frame (Saudek *et al*., 2006; Tanaka *et al*., 2007; Beltran Del Rio *et al*., 2016).

There is a linear relationship between the levels of $\%HbA_1c$ and the mean blood glucose concentrations (Hinzmann *et al*., 2012). Higher average blood glucose levels are reflected in higher HbA_1c values. The reference HbA_1c value is <6% (Janghorbani *et al*., 2007).

This study investigates blood levels of both,HbA_1c and glucose in all DM subjects, and in DM subjects divided in two main age groups (40-60, and 61-80 years), in two separate time periods: second measurement (M-II) was performed about 90 days after the first measurement (M-I). Our aim was to evaluate potential differences in glucoregulation in all DM subjects, and between the two age groups of diabetic patients. By comparing HbA_1c and glucose levels in M-II to the levels of these parameters in M-I, glucoregulation of these patients can be defined.

MATERIALS AND METHODS

Study population and design of experiment - Patients were males and females, aged 40-80 years at "Zavidovići" Health Center. A representative sample of this research was 100 subjects divided in two main age groups [40-60 years (Group 1, 34 patients) and 61-80 years (Group 2, 66 patients)]. The measurements were performed in two separate time periods: second measurement (M-II) was performed about 90 days after the first measurement (M-I). After obtaining the results, the main groups were divided into two subgroups (Group 1a and 1b, and Group 2a and 2b), according to the increase (a) or decrease (b) of HbA1c and glucose levels after M-II.

Sample collection and storage - From each patient, a few milliliters of venous blood samples were collected in a container with EDTA. HbA_1c and glucose were determined for each sample.

Chemicals - Commercial enzyme assay kit (Glucose MR, Cat. No. 1129010), used as a reference method for glucose detection in real samples, was purchased from Cromatest (Barcelona, SPAIN). Another commercially available kit was used for the determination of HbA_1c (Glycated HbA_1c, Cat. No. 3155105; Barcelona, SPAIN).

Instrumental - HbA1c levels were determined using the ECOM-f6124 Eppendorf spectrophotometer, whereas glucose levels were determined using the Hitachi 902 Chemistry Analyzer.

Determination of HbA_1c

HbA_1c was extracted using a chromatographic ion-exchange method as described in the manual of the method for glycated HbA1c determination, supplied by Chromatest, LINEAR CHEMICALS S.L. A hemolyzed preparation of the whole blood was mixed continuously for 5 minutes with a weak binding cation-exchange resin (Hinzmann *et al*., 2012). During this time, HbA_0 binds to the resin. HbA_0 consists of all the other hemoglobins except A_1c which remains in the solution (Hinzmann *et al*., 2012). After the mixing period, a filter is used to separate the supernatant containing the A_1c from the resin (Hinzmann *et al*., 2012). The glycohemoglobin content (%) was determined by measuring the absorbance at 415 nm of the A_1c fraction and the total hemoglobin fraction, and using the formula:

$$\%HbA_1c = \frac{R_{(unknown)}}{R_{(standard)}} \cdot \ standard\ conc.$$

where

$$R_{(unknown)} = \frac{AbsofHbA_1c_{(unknown)}}{AbsofHbTot_{(unknown)}}$$

$$R_{(standard)} = \frac{AbsofHbA_1c_{(standard)}}{AbsofHbTot_{(standard)}}$$

Determination of glucose - The used model for quantitative determination of glucose in blood samples is based on the Trinder reaction (Lott and Turner, 1975). The glucose is oxidized to *D*-gluconate by the glucose oxidase (GOD) with the formation of hydrogen peroxide (H_2O_2). In the presence of peroxidase (POD), a mixture of phenol and 4-aminoantipyrine (4-AP) is oxidized by H_2O_2, to form a red quinone imine dye proportional to the concentration of glucose in the sample (Trinder, 1969; Raba and Mottola, 1995):

$$\beta - D - glucose + H_2O + O_2 \xrightarrow{GOD} D - gluconate + H_2O_2$$

$$4 - AP + phenol \xrightarrow{POD} quinoneimine + H_2O.$$

The used procedure is described in commercially available kit that was utilized for measurements of glucose levels in a tested subjects.

Absorbance of the samples and the standard was read at 500 nm against the blank.

Calculation of the glucose content was estimated using the following formula

$$c_{sample(\frac{mmol}{L})} = \frac{Abs_{sample}}{Abs_{standard}} \cdot c_{standard(\frac{mmol}{L})}$$

Statistical analysis - The one way ANOVA test was used to compare the differences in HbA_1c and glucose levels in two measurements (the second measurement was performed 90 days after the first measurement).

RESULTS AND DISCUSSION

Determination of HbA_1c –HbA_1c levels of the all subjects, and in both groups (Group 1 and Group 2) after the M-II were decreased, but without statistical significance ($p>0.05$, ANOVA test) as shown in Table 1.

Table 1. Average levels of HbA_1c (%) in the blood.

	$HbA_1c \pm SD$ (%) M-I	$HbA_1c \pm SD$ (%) M-II
All patients	8.49±1.56	8.24±1.32
Group 1 (34)	8.49±1.67	8.02±0.94
Group 2 (66)	8.45±1.50	8.34±1.47

In Group 1, 12 patients (subgroup 1a) showed an increase in HbA_1c levels after 90 days (M-II), while 22 patients (subgroup 1b) after the same time period showed lower levels of HbA_1c (Figure 1).

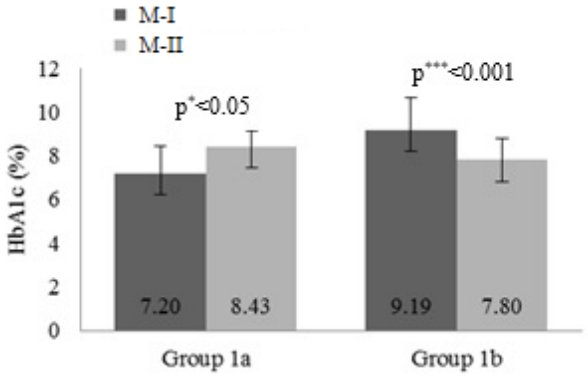

Figure 1. Changes of HbA_1c levels in the Group 1.
M-II: second measurement of HbA1c, about 90 days after the first measurement (M-I)

The ANOVA test showed that the levels of HbA_1c after the M-II were significantly higher in comparison to those in the M-I (subgroup 1a, $p^*<0.05$), indicating worsened glucoregulation. In patients with reduced HbA_1c levels after the M-II (subgroup 1b), ANOVA test confirmed that the levels of HbA_1c were significantly lower compared to the M-I ($p^{***}<0.001$), indicating improved glucoregulation. In Group 2, 27 patients (subgroup 2a) showed an increase in HbA_1c levels, while 39 patients (subgroup 2b) showed lower levels of HbA_1c (Figure 2).

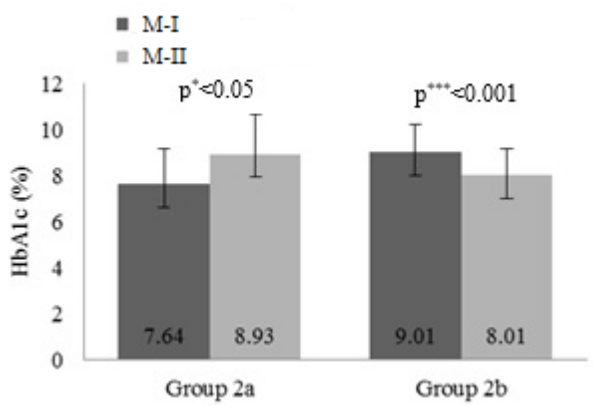

Figure 2. Changes of HbA_1c levels in the Group 2.
M-II: second measurement of HbA1c, about 90 days after the first measurement (M-I)

In the subgroup 2a, the ANOVA test confirmed significantly higher HbA1c levels in M-II compared to M-I (Figure 2, $p^*<0.05$). The statistical significantly increase of the HbA1c levels indicates worsened glucoregulation, whereas the statistical significantly decrease of the HbA1c levels (Figure 2, $p^{***}<0.001$), indicates improved glucoregulation.

Determination of glucose - glucose content in blood was analyzed for the same population. The average content of glucose for all patients and in both groups (Group 1 and Group 2) is presented in Table 2.

Table 2. Average levels of glucose (mmol) in the blood

	$c_{glucose} \pm SD$ (mmol/L) M-I	$c_{glucose} \pm SD$ (mmol/L) M-II
All patients	10.62 ± 3.78	9.76 ± 3.37
Group 1 (34)	11.36 ± 3.64	9.78 ± 2.30*
Group 2 (66)	10.41 ± 3.90	9.75 ± 3.82

* significantly decreased in comparison to M-I ($p<0.05$, one way ANOVA)
M-I: the first measurement of glucose
M-II: the second measurement of glucose
(in the same time as we done the second measurement of HbA1c)

The ANOVA test confirmed that the levels of glucose in Group 1 after 90 days (M-II) were significantly lower compared to the M-I ($p^*<0.05$), indicating improved glucoregulation.

Glucose levels in 12 patients of Group 1 (subgroup 1a) after the M-II were increased, while in 22 patients (subgroup 1b) glucose levels were decreased after the same time period (Figure 3).

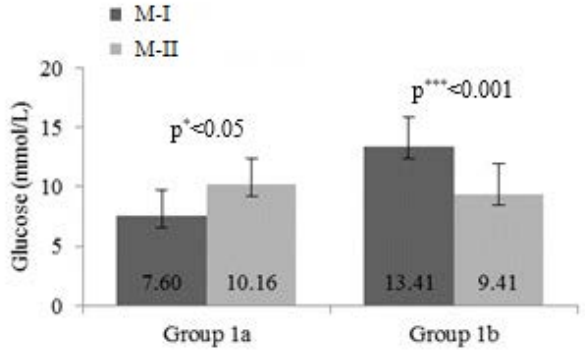

Figure 3. Changes of glucose levels in the Group 1
M-I: the first measurement of glucose
M-II: the second measurement of glucose
(in the same time as we done the second measurement of HbA1c)

It has been shown that both, the increase (worsening of glucoregulation) and the decrease (improvement of glucoregulation) of glucose levels were statistically significant ($p^{*}<0.05$ and $p^{***}<0.001$, respectively, ANOVA test, Figure 3) after the M-II compared to M-I.

Glucose levels in 27 patients of the total number of patients of the Group 2 (subgroup 2a), were increased, and in 39 patients (subgroup 2b) glucose levels were decreased (Figure 4).

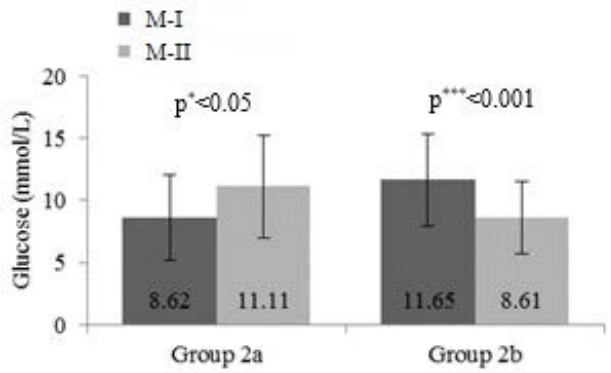

Figure 4. Changes of glucose levels in the Group 2
M-I: the first measurement of glucose
M-II: the second measurement of glucose
(in the same time as we done the second measurement of HbA1c).

The ANOVA test confirmed that the levels of glucose in subgroup 2a after the M-II were significantly higher than in the M-I (Figure 4, $p^{*}<0.05$). According to the HbA1c levels for the same subgroups, statistical significantly increase of glucose levels indicates worsened glucoregulation, whereas statistical significantly decrease of glucose levels in subgroups 2b (in according to the HbA1c levels for the same subgroups) after the M-II (Figure 4, $p^{***}<0.001$), leads to the conclusion that the glucoregulation process has improved.

Our results showed that younger patients (Group 1) can have a better glucoregulation than the older (Group 2), because their glucose levels after 90 days were statistically lower ($p^{*}<0.05$), and also the HbA1c decrease after 90 days was higher than in older patients (0.47% and 0.14%, respectively), but without statistical significance.

Rohlfing *et al*. (2002), established the linear relationship between mean plasma glucose and HbA1c (the study was performed on 1439 patients with type 1 diabetes). In our study HbA1c also correlates with glucose: in both subgroups of patients where levels of glucose were increased (1a) or decreased (1b), HbA1c levels were also increased or decreased.

In the study by Larsen *et al*. (1990) it was shown that regular monitoring of measured levels of HbA_1c accompanied by appropriate diet and living habits leads to a fall in the value of the same.

CONCLUSIONS

Based on collected results and the statistical analysis, it was concluded that, after 90 days, in 61patients (22 in the Group 1, and 39 in the Group 2) out of the total number of patients, lower HbA_1c and glucose levels were recorded, while in 39 patients (12in the Group 1, and 27 in the Group 2) an increase in HbA1c and glucose levels was registered. Given that, a significant percentage of patients (61%) manifested improved glucoregulation.

Also, the differences in all subgroups with increase or decrease of HbA1c and glucose levels after 90 days, were statistically significant.

Possible causes of unfavorable results in a smaller, but significant percentage of subjects (39%), in which an increase in HbA_1c and glucose levels was found, should be sought in the unadjusted therapies, irregular controls, social status or insufficient education of patients (Snorgaard, *et al*., 2017).

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Summary/Sažetak

Diabetes mellitus (DM) se definira kao potpuni ili relativni nedostatak inzulina, ili stanje hronične hiperglikemije. Hemoglobin A_1c (HbA_1c) je manje zastupljeni, oblik Hb koji nastaje *in vivo* posttranslacijskom modifikacijom sa glukozom. U biohemijskoj laboratorijskoj praksi u prošlih 30 godina HbA_1c je postao "zlatni standard" za kliničko praćenje DM. Cilj ovog izučavanja bio je odreditinivoe glukoze i HbA_1c kod 100 bolesnika sa DM u Domu zdravlja „Zavidovići" u različitom vremenskom intervalu, te na osnovu rezultata procijeniti glukoregulaciju. Nivoi HbA_1c i glukoze mjereni su u 3-mjesečnim vremenskim periodima. Dobiveni rezultati su analizirani odgovarajućom statističkom metodom, da se utvrdi da li postoje statistički značajne razlike između nivoa glukoze, odnosno HbA_1c, mjerenih u krvnoj plazmi i punoj krvi u dva različita vremenska perioda. Za određivanje HbA_1c korištena je spektrofotometrijska metoda, a glukoza je određivana enzimatsko-kolorimetrijskom metodom na biohemijskom analizatoru. Od ukupnog broja bolesnika, kod njih 61 je nađeno da su nivoi HbA_1c i glukoze bili značajno sniženi ($p^{***}<0.001$) tri mjeseca nakon prvog mjerenja, što navodi na zaključak o poboljšanju glukoregulacije. U preostalih 39 bolesnika nivoi HbA_1c i glukoze bili su značajno povišeni nakon istog vremenskog perioda ($p^{*}<0.05$, ANOVA), pri čemu se može zaključiti da je njihovaglukoregulacija pogoršana.