https://doi.org/10.48047/AFJBS.6.13.2024.6456-6466

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

The influence of the severity of autoimmune hypothyroidism on insulin levels and other diabetes-related blood parameters in rats

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Volume 6, Issue 13, Aug 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

doi: 10.48047/AFJBS.6.13.2024.6456-6466

Abstract

It is supposed that there is a connection between autoimmune hypothyroidism (AIH) and diabetes mellitus (DM). The aim of this study was to identify the effect of AIH of varying severity on biochemical blood parameters associated with DM. The experiments were carried out on outbred white rats. AIH was induced by three injections of thyroglobulin (100μ/rat) and Freund's adjuvant. For the first injection complete adjuvant, for the second and third treatments, incomplete adjuvant was used on the sixth and seventh days after the first injection. Depending on the antibodies to thyroid peroxidase titer (anti-TPO) in the blood serum, rats were divided into three groups with mild, moderate and severe AIH. Results show the more level of some serum hormones, organic metabolites, enzymes and inorganic ions was changed as the disease severity increases. The serum thyroxine level was directly but insulin was inversely associated with the AIH severity. The more pronounced the AIH was, the more the biochemical blood parameters characteristic of both AIH and DM changed. So, parameters which change quite noticeably depending on AIH of varying severity, might also serve as metabolic blood markers for DM.

Keywords: autoimmune hypothyroidism, rats, thyroxine, insulin, serum biochemical parameters, diabetes mellitus.

Introduction

Autoimmune hypothyroidism (AIH) or hypothyroidism of Hashimoto is an organ-specific pathology that occurs as a result of the T lymphocytes attack on the own thyroid gland cells. Being the most common pathology of the thyroid gland, AIH leads to the destruction of the thyrocytes and the subsequent deprivation of the thyroxine and triiodothyronine hormones synthesis and secretion [18, 19]. Recently it was shown that autoimmunity is an important element in the relationship between AIH and type 1 diabetes mellitus (DM) [16, 27]. AIH has influence on glucose homeostasis which is associated with insulin resistance at DM [5, 19]. The results of examinations of patients with insulin-dependent DM for the presence of autoimmune thyroid disease showed that

the prevalence of Hashimoto's thyroiditis among them was 26.6%. 42.0% of them were euthyroid, and 58.0% had hypothyroidism [21]. A higher frequency of thyroid insufficiency is seen in patients with type 1 and type 2 DM compared to the general population [6]. It is shown that some common genetic factors, as well as many non-genetic issues, make a certain contribution to the pathogenesis of AIH and DM [9, 16]. Patients with hypothyroidism including AIH are usually prescribed L-thyroxine or synthetic levothyroxine daily. However, it has been shown that treatment with L-thyroxine or synthetic levothyroxine alone is not always desirable, and even in some cases is unacceptable [3]. In addition it has been shown that in patients with untreated primary hypothyroidism, levothyroxine replacement therapy did not improve insulin resistance [25]. These facts indicate the need for research aimed at a more in-depth biochemical analysis of the pathogenesis of AIH and associated changes in endocrine pancreas.

The effect of AIH of varying severity on the manifestation of diabetes mellitus has not been studied. We hypothesized that metabolic disorders in AIH of different severity may have specific effects on the occurrence or manifestation of DM.

The purpose of the study is to identify the effect of AIH of varying severity on biochemical blood parameters associated with DM.

1. Materials and methods Experimental animals

The experiments were carried out on the outbred white male rats weighing 200±20 g. The rats were housed under standard vivarium conditions with an unlimited access to water and standard food and natural light-dark regime. All animal procedures were performed according to the Guideline of the Ethical Committee on the use and care of laboratory animals at the Faculty of Biology of the National University of Uzbekistan.

Induction of AIH

Lyophilized bovine thyroglobulin (Merck KGaA, Darmstadt, Germany) was dissolved in distilled water at a concentration of 20 mg/ml. The emulsion consisting from thyroglobulin and complete Freund’s adjuvant (Pan-Eco, Russia) was prepared by mixing

2 volumes of thyroglobulin solution and 2 volume of adjuvant so that the final concentration of antigen was 10 mg/ml. Antigen-adjuvant mixture was injected subcutaneously into the left hind footpad (100 µg of thyroglobulin per rat). The volume of the preparation’s mixture administered to each rat was 0.1 ml. Second and the third injections was made on the sixth and seventh days after the first injection, respectively. In the repeated injections of the thyroglobulin and Freund's adjuvant mixture, instead of

a complete, an incomplete adjuvant (Pan-Eco, Russia) was applied. The control rats weresubcutaneously injected with an equal volume of 0.9% NaCl at the same time.

Determination of AIH

The level of anti-TPO was determined 21 days after first immunization in the blood serum samples. For that after cutting gum with a sharp blade the releasing blood was collected into heparin-treated microtubes. Then the blood cells were sedimented in a centrifuge (DLAB D2012, China) at a speed of 3000 rpm for 10 minutes. The serum was carefully aspirated with an automatic pipette. Serum anti-TPO titer were determined using ELISA assay kits (Dublin, Ireland) for rat anti-TPO on the enzyme immunoassay analyzer RT-2100C Microplate Reader Rayto (China).

Experimental design

Rats were divided into 3 experimental and one control groups with 6 animals each. Only rats with the anti-TPO titer more than 0,5 were used in the experimental groups. Depending on the value of the anti-TPO titer, rats were divided into three equal experimental groups with mild (experiment 1), moderate (experiment 2) and severe (experiment 3) AIH. In the first, second and third experimental groups of rats, the serum anti-TPO titer was 0.59±0.01 IU/ml; 0.96±0.001 IU/ml and 1.31±0.09 IU/ml, respectively, versus 0.37±0.01 IU/ml in the control.

Blood samples

For the biochemical analyses, blood was collected in heparinized centrifuge tubes on the 30th day after the first immunization of rats during decapitation. Then the blood cells were sedimented in a DLAB D2012 centrifuge (China) at a speed of 3000 rpm for 10 minutes. The serum was carefully aspirated and used for biochemical analyses.

Biochemical analyses

The level of thyroxine and insulin in the serum was determined using a special reagent set ELISA Kits (Dublin, Ireland) on the immunoassay analyzer RT-2100C Microplate Reader Rayto (China). For analysis of some serum organic metabolites, enzyme activity and inorganic cations a biochemical analyzer RT 1904C (China) and a set of reagents Human Diagnostic firm (Germany) were applied.

Statistics

Calculation of the mean and standard error was carried out by pairwise comparison using Student's t-test. The arithmetic mean (M), the standard error of the mean (m), and the significant coefficient (P) were defined. If the P value was less than 0.05, the difference between the control and experimental rat groups was considered statistically significant.

2. Results

Thyroxine and insulin. As shown in **Figure 1** serum thyroxine levels in rats is decreased as the severity of AIH is increased. The level of thyroxine is decreased in rats with mild AIH by 1.5 times, with moderate hypothyroidism by 1.9 times, and with severe AIH by 6.0 times. But the level of insulin in the blood serum of rats, on the contrary, is increased by 1.2; 1.5 and 3.2 times in rats of the first, second and third experimental groups, respectively, compared to the control.

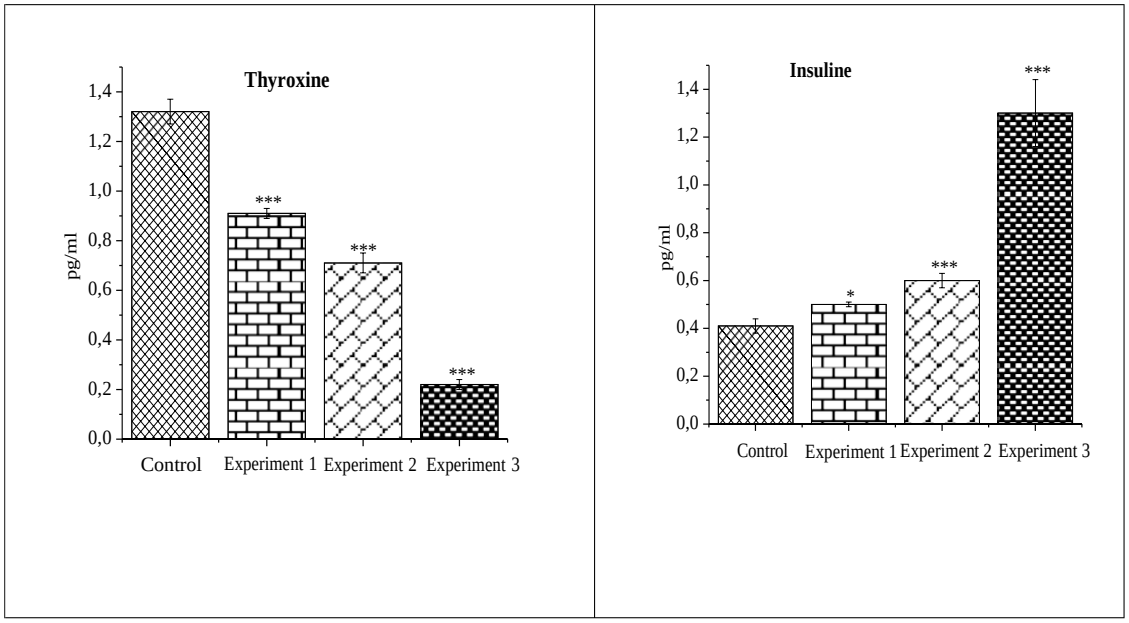

Figure 1 Level of thyroxine and insulin in the serum of rats with varying degrees of severity of AIH, (M±m; n=6); *<0.05, *** - <0.001 compared to control

So, experimental AIH leads to a decrease in the level of thyroxine and, on the contrary, to an increase in the level of insulin in the blood serum of rats. This tendency intensifies as the severity of the AIH increases.

Organic metabolites. The level of organic metabolites in the rat blood serum with AIH of varying severity changed ambiguously, as shown in Table 1.

In rats with AIH, the serum level of glucose was kept at control level in rats with mild AIH, but it was increased in animals with moderate and severe disease by 1,3 and 1,5 times respectively. The increase in total protein content in the three experimental groups compared to the control was also depended on the severity of AIH. The level of total protein in rats with both a mild and moderate AIH was 1.2 times higher, and in rats with a severe form of the disease it was 1.3 times higher compared to the level in the control group. The level of the serum albumin was also increased with rising severity of AIH. The albumin level in the rat blood serum with mild, moderate and severe AIH was 1.2; 1.4 and 1.4 times more, respectively, compared to the control group parametrs. The level of serum uric acid in rats with mild AIH was 2.7 times more, with moderate AIH was 3.8 times higher, and with severe AIH was 4.5 times higher than in the control group rats.

Table 1: Level of serum metabolites in rats with varying severity of AIH (M±m, n=6)

Serum Parameters	Animal groups			
	Control	Experiment 1	Experiment	Experiment
Glucose (mmol/l)	4.5±0.2 -	4.7±0.2 >0.5	6.0±0.1 <0.001	6.9±0.2 <0.001
Total protein (g/l) P	101.4±2.8 -	118.1±2.6 <0.001	124.1±3.2 <0.001	123.9±3.8 <0.001
Albumin (g/l) P	40.2±0.3 -	49.6±0.4 <0.001	55.1±0.8 <0.001	57.6±0.6 <0.001

Cholesterol (mmol/l) P	2.0±0.3 -	2.3±0.1 >0.2	2.8±0.2 <0.01	3.7±0.3 <0.001
Uric acid (µmol/l P	151.1±23. -	415.1± <0.001	577.2±51.4 <0.001	680.1±87.5 <0.001

P - statistically significant difference between control and experimental groups.

Enzyme activity. As **Table 2** shows the activity of α-amylase, alanine aminotransferase and aspartate aminotransferase in the rat blood serum with AIH statistically significantly increased compared to healthy rats. The activity of α-amylase in rats with mild, moderate and severe AIH compared with control values was increased by 3,9; 17,5 and 18,0 times respectively. The activity of alanine aminotransferase was also 2.8 times higher in rats with mild AIH; 4.7 times more in animals with moderate pathology and 4.1 times higher in rats with severe autoimmune thyroid gland disease compared with the enzyme activity in rats that were injected with saline. Aspartate aminotransferase activity was increased by 1.5; 1.7 and 2.0 times in animals with mild, moderate and severe AIH respectively compared with control values. The activity of blood creatine phosphokinase did not increase, but, on the contrary, decreased. The decrease in creatine phosphokinase activity in rats with mild, moderate and severe AIH was by 5.9; 8.3 and 10.4 times lower, respectively, compared with the parametrs in the control group rats. The activity of alkaline phosphatase was also decreased by 4.2; 8.0 and 10.3 times, respectively, in animals with mild, moderate and severe AIH respectively compared with control values.

Table 2: Enzyme's activity in the serum of rats with varying severity of AIH (M±m; n=6)

Enzymes	Animal groups			
	Control	Experiment 1	Experiment 2	Experiment 3
α- Amylase P	321.7±22.1 -	1244.4±98.2 <0.001	5623.1±324.1 <0.001	5789.2±222.3 <0.001
ALT P	61.2±4.1 -	172.4±4.9 <0.001	222.0±14.4 <0.001	285.4±22.6 <0.001
ACT P	50.4±4.2 -	101.4±3.4 <0.001	84.7±3.1 <0.001	76.6±6.7 <0.001
CPK P	430.3±68.6 -	103.3±3.6 <0.001	54.1±4.4 <0.001	41.6±2.3 <0.001
ALP P	49.5±3.5 -	37.5±2.1 <0.001	34.0±1.2 <0.001	30.2±1.4 <0.001

Note: P - statistically significant difference between control and experimental groups; ALT - alanine aminotransferase; AST – aspartate aminotransferase; CPK – creatine phosphokinase; ALP – alkaline phosphatase.

Inorganic ions

As shown in **Figure 2** the content of calcium ions was increased progressively as the severity of AIH was raised. Thus, in rats with mild AIH, the level of calcium ions was increased by 1.3 times; in animals with moderate AIH it was raised by 1.5 times, and in animals with severe AIH calcium level was increased by 1.6 times compared to animals injected with saline.

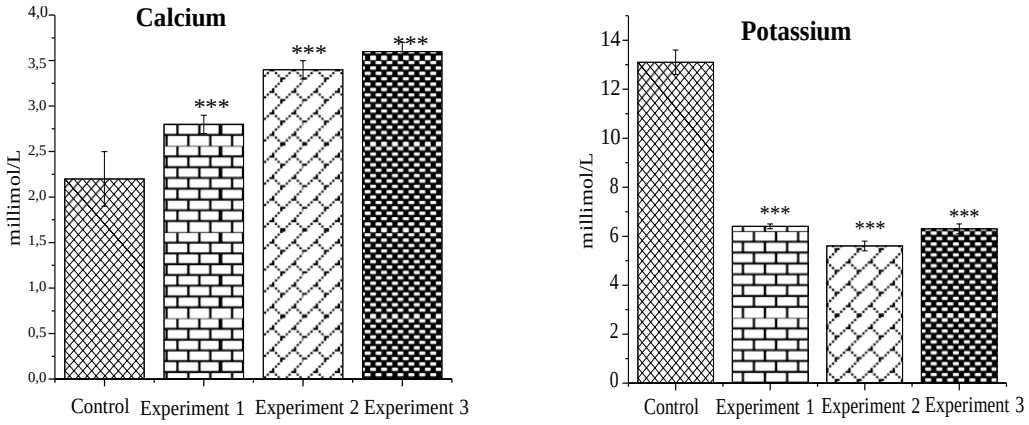

Figure 2 Calcium and potassium ions level in the serum of rats with varying degrees of severity of AIH; *** - <0.001 compared to control

The concentration of potassium ions was in contrary decreased in all three groups of rats with AIH. In rats with mild, moderate and severe AIH, the concentration of potassium in the serum blood was 2.0, 2.3 and 2.1 times lower, respectively, compared to the control.

3. Discussion

The main goal of this study was to identify diabetes-associated signs in rats with AIH of varying severity. Obtained data show that when rats are immunized with a mixture of thyroglobulin and Freund's adjuvant the susceptibility of rats to AIH is not the same, which is manifested in different levels of anti-TPO titer in the animal blood serum. Depending on the level of anti-TPO titer, i.e. severity of AIH, the content of hormones (thyroxine, insulin), organic metabolites (glucose, total protein, albumin, cholesterol, uric acid), enzyme activity (α-amylase, alanine aminotransferase, aspartate aminotransferase, creatine phosphokinase and alkaline phosphatase) and the concentration of inorganic cations (calcium and potassium) change ambiguously. The more level of anti-TPO titer increases the more level of insulin, glucose, total protein, albumin, cholesterol, uric acid, calcium ions, as well as the activity of α-amylase and alanine aminotransferase increases. In rats with AIH the level of thyroxine, activity of aspartate aminotransferase, creatine phosphokinase, and alkaline phosphatase, as well as the content of potassium ions in the blood serum, on the contrary, decrease compared to the control.

Thyroid insufficiency often manifests itself in the form of an autoimmune lesion of the thyroid gland [10]. The association of AIH with a wide range of organ-specific and non-organ-specific autoimmune diseases has been proven. The list of these diseases includes pernicious anemia, celiac disease, Addison's disease, dermatitis herpetiformis, multiple sclerosis, rheumatoid arthritis, type 1 DM and others [11]. The current experiment findings also confirm that AIH causes a wide range of metabolic changes associated with type 2 DM.

Thus, the results show that in rats with different severity of AIH, the insulin level changes in inverse proportion to the thyroxine level, what is manifests itself in a parallel increase in hypothyroidism and hyperinsulinemia. It should be noted that the glucose content increased in groups with moderate and severe AIH compare with control. An increase in

hyperinsulenemia in parallel with an increase in glucose levels in the blood serum of rats indicates the development of type 2 DM in rats with AIH. Some researchers suggest that insulin resistance triggers thyroid antibody production and Hashimoto disease development and if so, that it might even lead to progression to hypothyroidism [2]. In our observations, progression of insulin resistance may also cause increased hypothyroidism. This assumption is consistent with the opinion of authors who believe that AIH contributes to the development of insulin resistance and related disorders [23, 27].

However, it is known that functional deficiency of the thyroid gland leads to hypoglycemia [5]. Increasing of glucose level in rats with AIH assumes the development of non-insulin-dependent DM could be a reason of glucose rising. The opposing effects of hypothyroidism and diabetes on glucose concentration possibly cause relative slight increase of glucose levels in rats with AIH.

Almost all changes in blood parameters that were recorded in rats with experimental AIH were noted by other researchers in DM. Disorders of protein metabolism in our studies were manifested in increased concentrations of protein, albumin and uric acid, associated with the severity of AIH, were observed in other researchers both at AIH and DM [14]. showed a near linear, positive and independent association tendency between serum albumin and type 2 DM and in patients with type 2 DM [14]. Total serum protein was increased in diabetic patients with and without complications but these parameters remained within normal limits in non-diabetic patients with retinopathy [7]. It was discovered, that increased serum albumin level was also associated with both insulin resistance [1] and Hashimoto's hypothyroidism [32, 15]. found out that AIH was associated with elevated serum uric acid [15], and discovered association between albuminuria and thyroid antibodies in newly diagnosed type 2 DM patients with Hashimoto's hypothyreodism and euthyroidism [34, 15].

Lipid metabolism disorders in our studies manifested themselves in increased cholesterol concentrations associated with the severity of AIH in experimental rats. The same tendency expressed in elevated serum cholesterol level in Hashimoto's hypothyroidism has noted in clinical observations [15]. The serum cholesterol and triglycerides levels were also increased in experimental type 2 DM [29]. Critically high cholesterol levels in patient who was diagnosed with type 2 DM and primary hypothyroidism was noted by [26]. The relationship between thyroid autoimmunity and dyslipidemia with elevated cholesterol levels was demonstrate even in the euthyroid phase [4,8]. The serum cholesterol and triglycerides level was also increased in experimental type 2 DM [29].

Changes in enzyme activity also suggest that AIH may be a cause or prerequisite for DM. In our experiments, an increase in the level of α-amylase activity in experimental AIH was noted as the severity of the disease increased. In large study of Steinberg et al. (2014) of type 2 diabetic patients it was also found that nearly 25% had elevated lipase or amylase levels without symptoms of acute pancreatitis [30]. Increase of amylase activity was also observed in experimental alloxan-indused and / or streptozotocin-induced diabetic in rats [13, 28]. The results showed that an increase in alanine aminotransferase activity was observed on the base of decrease of aspartate aminotransferase activity as the severity of the disease increases in a rats with AIH. Consequently, the ratio of alanine aminotransferase activity to aspartate aminotransferase activity increases as the severity of the disease increases. Clinical studies conducted in Japan showed that the ratio of alanine aminotransferase to aspartate aminotransferase was associated with insulin resistance and β-cell function even in non-obese women, suggesting a pathophysiologic

basis in its prediction of diabetic risk [22]. Thus, recently published studies reported that the activity of alanine aminotransferase in patients with type 2 DM is increased [17]. The activity of creatine phosphokinase in rats with AIH in our observation was decreased as well as it was decreased in rats with streptozotocin diabetes [33]. These findings in enzyme activities also suggest a cause-and-effect relationship between AIH and diabetes mellitus.

The association between AIH and diabetes manifests itself in the increase of calcium ions level in the rat serum blood with thyroid autoimmune pathology. In fact, it was shown that diabetes can lead to hyperfunction of the parathyroid glands and appropriate hypercalcemia [31]. Cases of severe hypercalcemia, as well as cases of severe hypokalemia, have been reported in patients with diabetic ketoacidosis [20; 24].‘

Conclusion

Consequently, data on analysis of serum parameters in AIH indirectly indicate that the metabolic pathways of AIH and DM is intersected. As AIH progresses, changes in blood biochemical parameters intensify. We hypothesized that changes in blood biochemical parameters in AIH are responsible for many of the damages associated with diabetes. Changes associated with diabetes, in turn, affect the progression of AIH. Shifts depending on AIH of varying severity, may also serve as metabolic markers for severity of type 2 DM. Understanding the mechanism of this interplay will be crucial for designing new, mechanism-based therapies for AIHD and DM.

Acknowledgements

The authors are grateful to the Ministry of Higher Education, Science and Innovation of the Republic of Uzbekistan for financial support of project number IL-4821091572 within the framework of which the work was performed.

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