Association between glucose-lowering drugs and circulating insulin antibodies induced by insulin therapy in patients with type 2 diabetes

Running title: Glucose-lowering drugs and insulin antibodies
Abstract

Background: Blood glucose management is impacted by insulin antibodies (IAs) in patients on insulin therapy.

Aim: We looked into the relationship between various glucose-lowering medications and IAs in individuals with type 2 diabetes (T2DM).

Methods: In this retrospective, cross-sectional investigation, exogenous insulin therapy was being used by 1863 T2DM patients. IA levels were assessed using an iodine-125 array, and all patients had received stable antidiabetic medication within the previous three months.

Results: A total of 1863 patients were enrolled. There were 902 (48.4%) patients had positive IAs (IA level >5%), with mean IA level of 11.06 (10.39, 11.72)% IA levels were positively correlated with fasting blood glucose (OR=1.069, P<0.001). The proportion of positive IAs was lowest in patients using glargine only (31.9%) and highest in patients using human insulin only (70.3%), P<0.001. The IA levels in patients using sulfonylureas/glinides (8.3%), metformin (9.6%), and DPP-4 inhibitors (8.2%) were all lower than in patients without these drugs (P all <0.05).

Conclusions: IA levels should be measured in patients undergoing long-term insulin therapy. Insulin glargine and a combination of oral glucose-lowering drugs were correlated to lower IA levels.

Key words: insulin antibodies; insulin therapy; glucose-lowering drugs; glargine; type 2 diabetes
Core Tip:

In this study, we found that proportion of positive IAs was high in type 2 diabetes patients using exogenous insulin therapy, positive IAs was correlated with high fasting blood glucose, insulin glargine was associated with the lowest IA levels among insulin regimens, and the use of insulin secretagogues, metformin, and DPP-4 inhibitors was correlated with decreased IA levels.
INTRODUCTION

Many patients with type 2 diabetes mellitus (T2DM) eventually require insulin therapy once disease progression has overcome the effects of oral agents\(^1\). Insulin antibodies (IAs), which are seen in patients treated with exogenous insulin, may influence glycemic control due to their ability to bind and/or release insulin in an unpredictable manner\(^2-4\). Recently, Zhu et al. showed that a higher circulating IA titre was associated with increased glycemic variability and the risk of hypoglycemia\(^5\), which are considered potential risk factors for mortality\(^6\) and diabetes complication in patients with T2DM\(^7,8\). Therefore, endocrinologists should assess IA levels in patients receiving insulin therapy. Moreover, identifying the factors that influence IA levels may help us further understand blood glucose fluctuations and improve blood glucose control in patients with diabetes.

With the development of recombinant human insulin and insulin analogues, several studies have found that circulating IA levels are closely related to the mode of insulin injection and type of insulin\(^9-11\). IA levels usually increased during first 12 weeks after initiation of exogenous insulin treatment and then stabilized or gradually declined\(^10,12\). However, previous studies usually compared the IA levels between patients using two types of insulin. Previous studies have compared IA levels after the action of two similarly acting insulins, and comprehensive comparisons of different types of insulin are lacking, while the effect of the combination of oral hypoglycemic agents on IA after insulin administration has rarely been reported.

Therefore, we retrospectively analyzed the circulating IA levels in patients with T2DM using different insulin therapies and oral glucose-lowering drugs in this study.

MATERIALS AND METHODS

Study design

This retrospective cross-sectional study was approved by the Ethics
Committee of the First Hospital of Nanjing Medical University (KY20220124-01). The study was conducted in accordance with the guidelines of the Declaration of Helsinki. Informed consent was not required for this study because the risk to the subjects was minimal and there were no adverse effects on the rights and welfare of the subjects. All data were collected retrospectively from medical review databases with the consent of the relevant authorities and did not contain any identifiable private information.

Two researchers extracted data from consecutive medical records of patients hospitalized in Nanjing First Hospital. The data were analyzed from January 2017 to January 2019. The inclusion criteria were as follows: 1) patients diagnosed with T2DM; 2) patients treated with insulin who had not changed their glucose-lowering medication in the last 3 months prior to the end of the index date; and 3) patients who had tested IA levels. Patients were excluded if they had 1) infectious diseases which may promote antibody responses[33]; 2) admission with acute complications of diabetes, such as diabetic ketoacidosis and lactic acidosis, which affect serum IA concentrations through dehydration; 3) severe systemic disease (e.g., heart failure, myocardial infarction, acute pancreatitis, severe hepatic or renal dysfunction), or any other condition that may affect IA levels; 4) pregnancy; and 5) use of systemic glucagon in the past 3 months. Corticosteroids.

Clinical and laboratory assessments

Patient data regarding height, weight, age, duration of diabetes, hypoglycemic treatment and use of other medications were collected at the time of hospital admission. Body mass index (BMI) was calculated as weight divided by the square of height (kg/m²). Blood samples were collected from all patients after fasting overnight (>10 h). HbA1c was measured using high-performance liquid chromatography (Bio-Rad Laboratories, Inc. CA, USA). C-peptide levels were measured using a chemiluminescent immunometric assay which employs the Modular Analytics E170 (Roche Diagnostics GmbH, Mannheim, Germany).
The IA titers were determined using the iodine-125 insulin antibody array kit (Beijing North Institute of Biological Technology, China) in accordance with the manufacturer's instructions, the procedure being similar to that in the previous studies[5, 14]. The method of IA measurement was routinely and clinically carried out in Nanjing Clinical Nuclear Medicine Center (ISO/IEC15189/17020) before Feb, 2019. The results were expressed in terms of bound radioactivity in the precipitate as a percentage of total counts (%B/T) in the assay. Blank values obtained by measurement of specific IA-zero serum were subtracted from sample values. IA ≤ 5% was identified as a negative result, and IA > 5% as positive[5]. The nonspecific binding rate should ≤ 15%, the binding rate with standard IA positive serum should > 25%, and the binding rate with standard negative serum should < 5%. The intra- and inter-assay coefficients of variation were ≤ 10% and ≤ 15%, respectively. In order to compensate for the lack of specificity and sensitivity of radioimmunoassay compared with chemiluminescence assay, we also analyzed the characteristics of patients with IA > 10% and IA < 1%.

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Statistical analysis

Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL, USA). All variables were tested for a normal distribution. Data are presented as mean (95% CI). Insulin resistance and β-cell function were evaluated using Homeostasis Model Assessment 2 (HOMA2-IR and HOMA2-β), which were calculated using fasting C-peptide and blood glucose by HOMA2 Calculator [15]. Differences between the positive and negative IA groups were examined using Student's unpaired t-test for parametric data or the Mann-Whitney u-test for non-parametric data, respectively. Logistic regression analysis was used to identify the factors which may affect IA levels. The differences in IA levels among the different blood glucose lowering drugs were analyzed using the analysis of covariance (ANCOVA) to adjust for influencing factors. Categorical data were examined using the Chi-square test.
All comparisons were 2-sided with a 5% significance level. A P value < 0.05 was considered to be statistically significant.

RESULTS

Study populations

Among 7426 patients with T2DM, a total of 2496 (33.61%) patients were using insulin therapy. Among these patients, two pregnant patients, two patients using systemic glucocorticoid, another 22 patients with changes in their glucose lowering drug treatment in the last 3 months, and 134 patients with infection were excluded. Moreover, a hundred patients with positive glutamate decarboxylase antibody (GAD-Ab) were excluded from the study. A total of 1863 patients completed the IA measurement and were finally enrolled in the analysis. There were 1015 (54.5%) males and the mean age was 63.7 (63.2, 64.3) years. The mean duration of diabetes was 13.0 (12.6, 13.3) years, and the mean HbA1c was 8.9 (8.8, 9.0)%. There were 902 (48.4%) patients with IA > 5% (Fig 1), and the mean IA level was 11.06 (10.39, 11.72)%.

Clinical characteristics of the patients with positive IAs

Compared with patients with negative IA (IA ≤ 5%), patients with positive IAs (IA > 5%) was associated with older people, diabetic duration, total cholesterol, low density lipoprotein-cholesterol (LDL-c), high density lipoprotein-cholesterol (HDL-c), fasting blood glucose (FBG), insulin levels, GAD-Ab levels, and the proportion of cancer. Alanine transferase (ALT), triglycerides and the proportion of smokers were lower in patients with IA > 5% than in the others (Table 1). To increase the specificity of IA, and reduce the false positives and false negatives, we also analyzed the characteristics of patients with IA > 10% or < 1%. The further analysis showed that the differences of age, ALT, total cholesterol, triglyceride, LDL-c, FBG, insulin levels, GAD-Ab, and the proportion of smoking and the complication of cancer between patients with IA > 10% and ≤ 1% were enlarged. Patients
having higher IA levels > 10% were having higher HOMA-IR as compared to those having IA < 1%. HbA1c seemed also higher (but not statistically significant) in patients with IA >= 10%. The prevalences of diabetic complications were all similar between the patients with positive and negative IA (Table 1).

**Logistic regression analysis for risk factors of IA**

To identify the factors which were associated with IA levels, we performed a logistic regression analysis, including factors which were different between the IA-positive and IA-negative groups, such as age, diabetes duration, ALT, total cholesterol, triglyceride, LDL-c, FGB, GAD-Ab, HOMA2-IR, accompanied by cancer or smoking, and the types of oral hypoglycemic agents (OHAs) as independent variables. As a result, diabetes duration, FGB, and GAD-Ab were risk factors of positive IA (P all < 0.05, Table 2). ALT, triglyceride, types of OHAs, and history of cancer were protective factors of positive IA (P all < 0.05, Table 2).

**Distribution and IA levels of patients treated with different insulin regimens**

The proportions of different insulin therapies among the patients with T2DM using insulin therapy are shown in Fig 2A. Among the insulin therapy regimens with the population more than 1%, glargine with OHAs was the most used (28.8%), and the patients using biphasic insulin aspart 30/50 constituted the largest proportion of patients with positive IAs (13.7%). Patients using glimepiride alone had the lowest proportion of IAS positivity (31.9%) and those using human insulin alone had the highest proportion of IAS positivity (70.3%), P < 0.001 (Fig 2A). After adjusting for diabetes duration, ALT, triglyceride, FGB, types of OHAs, and cancer, which were risk factors in the logistics regression, the ANCOVA test showed that among the different insulin therapy regimens, patients on glargine therapy had lowest IA levels (6.7[5.8,7.5]), and patients receiving insulin aspart had highest IA levels.
3 (17.7[13.6,21.8]%), \( P < 0.001 \), Fig 2B.

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**Effects of OHAs on IA levels**

Among the OHAs, \( \alpha \)-glucosidase was the most used drug combined with insulin therapy (33.0%), and the proportion of positive IAs was highest in patients using thiazolidinediones (TZDs) (51.4%, \( P=0.121 \), Fig 3A). Patients using sulfonylureas/grignard, metformin, and dipeptidyl peptidase-4 (DPP-4) inhibitors all had significantly lower levels of IA than patients without these drugs (the controls were the patients using all the other OHAs and patients without any OHAs or glucagon-like peptide 1 receptor agonists (GLP-1RAs)) \((P \text{ all } < 0.01, \text{ Fig 3B})\), and patients using DPP-4 inhibitors had the lowest IA level (8.2[6.5,10.0]%, Fig 3B). After adjusting for diabetes duration, ALT, triglyceride, FGB, and cancer, ANCOVA test showed that the differences between patients taking or not taking these drugs remained significant (DPP-4 inhibitors, \( P = 0.026 \); metformin, \( P = 0.002 \); sulfonylureas/glinides, \( P < 0.001 \)). The proportion of patients treated with OHAs was 83.0% in patients taking insulin glargine, which was only 51.4% among patients using mixed insulin (biphasic aspart, biphasic lispro and biphasic human insulin) in the present study \((P < 0.001)\). On the other hand, the proportion of patients who were using glargine and deremir was similar in patients using different OHAs (42.0-55.1%) except sulfonylureas/glinides (75.0%) \((P < 0.001, \text{ Supplementary table 1})\).

**DISCUSSION**

The present study found that the positive IAs were significantly lower in patients using insulin glargine than in those using other insulin therapy regimens, while DPP-4 inhibitors, metformin, and insulin secretagogues may...
also have potential effects on reducing IA levels. To the best of our knowledge, there have been no previous reports of such findings. Although circulating IA rarely interferes with HbA1c levels, FBG levels and insulin resistance were higher in patients with positive IA than in the others in our study, and this result was in accordance with a previous study in patients with type 1 diabetes[16]. In the present study, increased HOMA2-IR in patients with positive IAs may have contributed to a higher FBG level rather than fasting C-peptide level. However, previous studies demonstrated that insulin resistance may result from increased IAs[17, 18]. The problem of high FBG and insulin resistance is more pronounced and needs to be addressed in patients with T2DM, which may lead to an increased insulin dose in these patients[17], and following increased financial cost of insulin and increased weight[19]. Moreover, nearly half of the patients using insulin had positive IAs, consistent with the results of previous studies[20, 21]. Therefore, attention should be paid to the detection of circulating IA level in patients with T2DM who are using insulin therapy, especially in elderly non-smokers with high LDL-c levels according to our study, and modification of therapeutic regimen in patients with positive IAs should be considered.

In the previous studies, the proportions of positive IAs were 27.6-29% in patients with glargine only[22, 23], and 24.5-49.2% in patients using glargine plus aspart or lispro[10], which was lower than in patients with biphasic insulin aspart (68%), biphasic human insulin (66%) [14], and insulin detemir plus aspart (40-50%) [24]. Yki-Järvinen et al. found that antibodies against insulin glargine and human insulin were both significantly lower in patients treated with insulin glargine than in those treated with neutral protamine hagedorn (NPH)[25], while the difference in IA levels between these two types of insulin was not statistically significant in patients with type 1 diabetes[26]. Moreover, Hattori et al. found that glargine and aspart induced insulin antibodies more frequently than the others based on a small sample size of 381 patients with T2DM, and only 17 patients used glargine in their study[15].
We confirmed that patients on insulin glargine had less IA levels than other insulin regimens for the first time in such a large population. The decreased IA levels in patients using insulin glargine may contribute to its pharmacokinetic and pharmacodynamic characteristics. Following injection, insulin glargine forms a depot in the subcutaneous tissue, from which it is slowly absorbed. Thus, a relatively uniform, peak-free concentration could be obtained within about 24 hours after administration\(^\text{[27]}\). Insulin glargine treatment results in a lower daily basal insulin dose and fewer injection site reactions than those with insulin detemir\(^\text{[28]}\) and neutral protamine hagedorn. \(^\text{[29]}\). Insulin degludec is another long-acting (>42 h) basal insulin that elicits less immunogenic responses than insulin glargine\(^\text{[23]}\). However, there were only 7 patients (<1% of the population) using insulin degludec in the present study, and we did not include them in the statistical analysis of IA levels. On the other hand, the proportion of patients treated with OHAs was much higher among those taking insulin glargine than among those treated with mixed insulin in the present study. We demonstrated that the use of OHAs was a protective factor against IAs, and may also reduce IA levels in patients using glargine.

We unexpectedly found that IA levels in patients using α-glucosidase and TZDs were similar with the patients who were not using these drugs, although they had similar effects on reduction of insulin resistance and insulin dose compared with metformin, DPP-4 inhibitors, and SGLT2 inhibitors\([30,32]\). Therefore, the association between OHAs and IA levels may not be attributed to their effects on insulin dose reduction only. Elevation of endogenous insulin may play an important role in the prevention of IA positivity. Because our study showed that a combination of insulin secretion promoters was associated with decreased IA levels in patients with T2DM. Moreover, Bae et al. found that DPP-4 inhibitor users secreted more insulin than TZD users\([33]\). Their findings partially explained the decrease in IA levels in patients using insulin combined with DPP-4 inhibitors but not with TZDs.
in our study. Francesco et al. suggested that metformin could inhibit B cell activation and antibody production in autoimmune diseases by increasing the activation of adenosine monophosphate activated protein kinase, with a subsequent decrease in the phosphorylation of mTOR\textsuperscript{[34]}. In the present study, low IA levels in patients using sulfonylureas/glinides may be correlated with the high proportion of long-acting insulin analogues in these patients. A case report showed that Liraglutide might also have effect on decreasing IA levels in patient with T2DM\textsuperscript{[35]}. However, we did not observe IA reduction in patients treated with GLP-1RA because of the small number of patients taking a combination of GLP-1RA and insulin. Moreover, the number of patients using SGLT2 inhibitors was small, and the relationship between SGLT2 inhibitors and IA levels requires further confirmation in a large sample size. Therefore, the mechanisms underlying the effects of different OHA\textsuperscript{s} on IA levels are complex and require further investigation.

In the present study, old age was another relevant factor for IA levels, with the exception of OHA\textsuperscript{s} types. The incidence and prevalence of many common autoimmune diseases are increasing in the elderly, although autoimmune diabetes is more common in young people and children. Donna et al. suggested that aging is associated with chronic inflammation and increased likelihood of developing autoimmune diseases\textsuperscript{[36]}. Aging can have a negative effect on the immune system, known as immune aging, which increases the susceptibility of older people to infections, autoimmune diseases and cancer\textsuperscript{[37]}. One limitation of our study was that the cross-sectional design could not explain the causality between the use of blood glucose lowering drugs and changes in IA levels. Other factors, such as the duration of insulin use, age, and blood lipid levels, may simultaneously affect drug prescription and IA levels. Moreover, we observed hospitalised patients with type 2 diabetes in the present study, most of whom had poor glycemic control. The association
between IA levels and glucose-lowering drugs in insulin-treated patients with good glycemic control requires further investigation.

CONCLUSIONS

In conclusion, IA levels should be tested in patients with T2DM receiving insulin therapy, especially in elderly non-smokers with lipid metabolic disorders. The use of insulin glargine, DPP-4 inhibitors, metformin, insulin secretagogues, and SGLT2 inhibitors was associated with lower IA levels. The causal relationships between the changes in IA levels and these drugs requires further investigation.
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