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Effects Of Different Insulin Injection Types on IgE, IgG, and Histopathology In Injection Site Of Mice

Ali Saleh Ali Alzidani[1], Edi Widjajanto[2], Hidayat Sujuti[3]

[1]Department of Immunology science and Technology, faculty of Biomedical technology, Brawijaya University, Malang 65145, East Java, Indonesia
[2]Laboratory of biomedical, Department of Immunology science and Technology, faculty of Biomedical technology, Brawijaya University, Malang 65145, East Java, Indonesia
[3]Laboratory of biomedical, Department of Biochemistry, faculty of Biomedical technology, Brawijaya University, Malang 65145, East Java, Indonesia

Abstract:. An insulin allergy is a hypersensitive reaction to insulin. It usually reacts to its protein components. The aim of this study was to analyze the effect of two insulin types for 1 week in blood stream as shown by IgE, IgG and histopathology on skin in injection site of mice. Eighteen wistar male mice were randomly divided into three groups, namely Group A (injected by X insulin), group B (injected by Y insulin) and group C (control) which was injected by irrigation water. The doses for 3 groups were given for 1 week depending on the body weight of each mouse. Blood samples and histopathology skin site of injection were taken on the last day for analysis. The levels of IgE, IgG were statically analyzed by One Way Anova test and followed by observation of histopathology site under microscope. After 1 week of treatment, the mean of IgE, IgG levels between groups were not significantly different. In contrast, there was a significant difference in histopathology site of injection between two insulin-treated groups compared with control group
Keywords: Types of insulin, IgE and IgG antibodies, Histopathology of skin injection, mice.

Introduction

Diabetes mellitus (DM) is a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyper glycaemia with disturbances of carbohydrate, fat and protein metabolism. It is the most common endocrine disorder and by the year 2010, it is estimated that more than 200 million people worldwide will have DM and 300 million will subsequently have the disease by 2025. As the disease progresses, tissue or vascular damage ensues and leads to severe diabetic complications such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration. Thus, diabetes covers a wide range of heterogeneous diseases. Diabetes mellitus may be categorized into several types but the two major types are type 1 and type 2. Drugs are used primarily to save life and alleviate symptoms[1].

Type 1 diabetes is a chronic inflammatory disease caused by a selective destruction of insulin producing-cells in the islets of Langerhans. The incidence of type 1 diabetes has consistently increased worldwide during the last decades, especially in children and developed countries[2]. People diagnosed with type 1 diabetes usually start with two injections of insulin per day of two different insulin types and generally progress to three or four injections per day of different insulin types. The types of insulin depend on their blood glucose levels. Studies have shown that three or four injections of insulin a day give the best blood glucose control and can prevent or delay the eye, kidney, and nerve damage caused by diabetes[3].

Adverse reactions to insulin have significantly decreased since the introduction of human insulin preparations. However, cases with insulin allergy continue to present in the clinic. Symptoms range from local injection site reactions to severe generalized anaphylactic reactions. Allergic reactions to insulin include immediate type of IgE-mediated reactions, type 3 immune complex (Arthus reaction-localized or serum sickness-generalized type) or delayed type of hypersensitive reactions. Furthermore, reactions with a delayed onset, i.e. 6 h after injection of insulin may develop. These delayed reactions include durations in the injection site with histological signs of leukocytoclastic vasculitis. Finally, some reactions are even more delayed with onset after 8–24 h and may be on account of delayed hypersensitivity[4].

Experimental

This study applied two types of insulin injection. The animals (n=18) were grouped as A and B (experimental), and C (control), The experimental animals in group A were administered with 0.3 -1 U/Kg body weight of mice by first insulin for 7 days, group B was administered with 0.3 -1 U/kg body weight of mice by second insulin for 7 days, and group C was controlled with sterile water irrigation. This study used two types of insulin as research material by means of 18 male mice whose IgG and IgE test applied ELISA and Histopathology site of injection was observed by microscope.

ELISA Method

IgE and IgG serum were measured by using ELISA method. Then, all reagents were at room temperature before use. 50 ml of 10x concentrated wash solution was diluted in 450 ml deionized water to reach 500 ml of the diluted wash solution which was stable for at least 3 months in bayonet room temperature. Thus, storing unopened reagents at 2-8 C would be stable until expiration date and reagents were not allowed to be in use beyond this date. Next, opened reagents must be stored at 2-8 C. Micro titer wells had to be stored at 2-8 C and the foil bag was sealed tightly.

Samples were allowed to clot for 30 minutes before centrifugation for 15 minute at 1000x g. Then, serum and assay were immediately removed or aliquot and samples were stored at -20 C to avoid repeated freezing-thawing cycles. To collect plasma, the citrate, EDTA, or heparin were used as an anticoagulant, centrifuged for 15 minutes at 1000x g within 30 minutes, then assayed immediately oe aliquot and samples were stored at -20 C. The use of plasma as specimen could result in a diminished precision of this assay.

To accommodate calibrators and samples in duplicates, a sufficient number of micro plate wells were prepared. Next, 10 ul of each calibrator and sample with new disposable tips into appropriate wells were dispensed. 100 ul of incubation Buffer was dispensed into each well, 50 ul Enzyme was conjugated into each well and incubated for 60 minutes at room temperature on a micro plate mixer. The optimal reaction in this assay markedly depended on the shaking of micro plate on absorbent paper. Then, the content of well was discarded and the wells were rinsed 4 times with diluted wash solution (300 ul per well). Wash solution was removed by beating the micro plate on absorbent paper.

After that, 200 ul of substrate solution was added to each well and incubated without shaking for 30 minutes in the dark. The reaction was stopped by adding 50 ul of solution to each well. The absorbance of each well was determined at 450 nm and read the wells within 15 minutes to calculate the average absorbance values for each set of calibrators, controls and patient sample. Semi logarithmic graph paper was used to construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (y) axis and concentration on the horizontal (x) axis. By using the mean absorbance value for each sample, the corresponding concentration from the calibration curve was determined.

Histological Preparation for differential counts of cell Observation

The tissue cutaneous sections were fixed in 10 % formalin, embedded in paraffin and 5 um sections were cut and stained with hematoxylin and eosin stain. The tissue sections were observed under a research light microscope for qualitative change in different count of cell. The animals of group A, B and C were executed on day 7, then the cutaneous section were removed and examined for histological change of differential count of cell under microscope.

Animals preparation

18 mice (animal experiment) were divided into 3 randomized groups and placed in different cages. Before grouping, the mice were in the lab for adaptation in 7 days and given a normal diet plan. Group For a week, a control group was injected by PBS saline, group B was injected by X Insulin dosage from (0.3 to 1) U/kg, and group C was injected by Y Insulin dosage from (0.3 to 1) U/kg. animals of group A and B were

given their dosage daily for the same period as the control group. After 7 days, the serum blood ware taken from mice to see the concentration of IgE and IgG by Elisa method and see the histological change of differential count of cell by microscope.

Data Analysis

Data analysis of IgE and IgG in blood stream was conducted by one way Anova test statistic and that of histopathological was done by description. Data analysis was considered done with statistical product when the service solution program for windows version 12.0 was at the trusted rate 95 % ($\alpha = 0.05$) and probability rate of ($p=0.05$).

Results

Preliminary data of six insulin injection types in histopathology site

This study conducted 6 types of insulin as the preliminary data and the data on histopathology of injection site were observed to understand which 2 types of insulin could make more allergy in the injection site.

Histopathology of six types of insulin injection

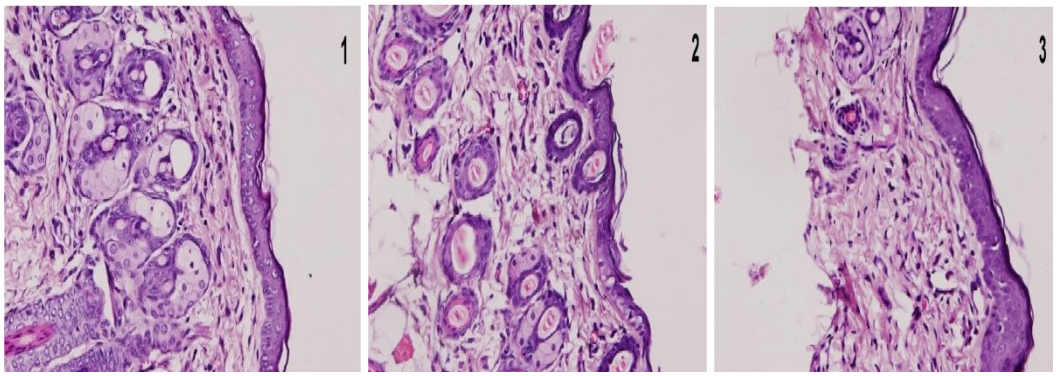

Figure 1. Subepidermis layer of first insulin injection (1), subepidermis layer of second insulin injection (2), subepidermis layer of third insulin injection (3) 400x magnification each

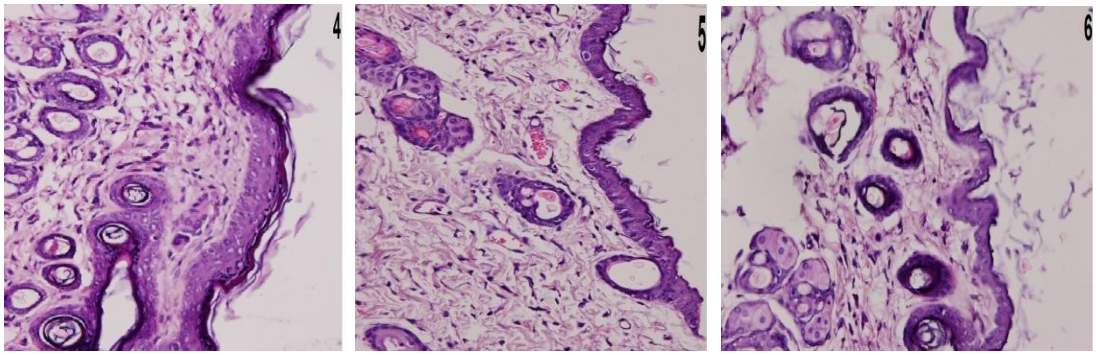

Figure 2. Subepidermis layer of fourth insulin injection (4), subepidermis layer of fifth insulin injection (5), subepidermis layer of sixth insulin injection (6) 400x magnification each.

After we have done the preliminary data of 6 insulin types and observed the histopathology site of injection, we continued the experiment of insulin I and II to compare which one had more ability to cause inflammation reaction and compared with control group.

The result of IgG levels after treatment with two insulin types compared with the control group was not significantly different. The results can be seen from Table and Figure showing that the average of each treatment was not too much different. The lowest average in treatment B was 11.768±6.2158, and the highest average in treatment C was at 18.522±2.0395. Overall average of obtained IgG was 15.485±5.0667.

Table 1. Serum IgG concentration of mice treated with different types of insulin injections.

Treatment	Average	SD
A	16.166	4.0354
B	11.768	6.2158
C	18.522	2.0395
Average	15.485	5.0667

A: Type I insulin injection
B: Type II insulin injection
C: Control group

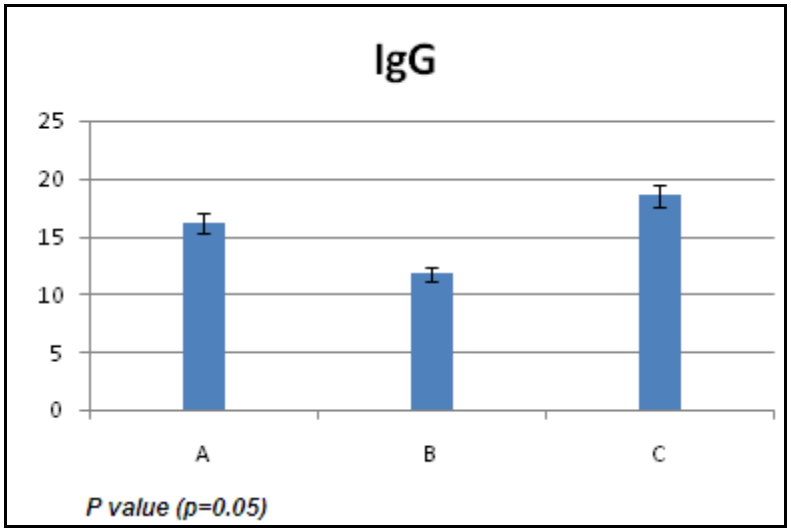

Figure 3. Serum IgG concentration of mice treated with different types of insulin injections

The result of IgE levels after treatment with two types of insulin compared with control group was not significantly different. The results can be seen from Table 2 and Figure 4 showing that the average of each treatment was not too much different. The lowest average in treatment A was 6.430±0.9986, and the highest average in treatment C was at 8.202±1.8674. The overall average of obtained IgE was 7.168±1.9074.

Table 2. Serum IgE concentration of mice treated with different types of insulin injections.

Treatment	Average	SD
A	6.430	0.9986
B	6.873	2.5835
C	8.202	1.8674
Average	7.168	1.9074

A: Type I insulin injection
B: Type II insulin injection
C: Control group

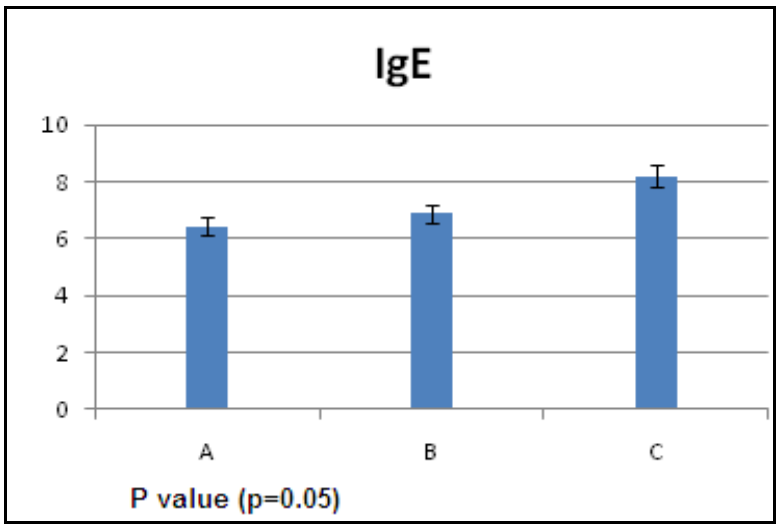

Figure 4. Serum IgE concentration of mice treated with different types of insulin injections

The histopathological examination of group C (control group) mice showed a normal structure of two layers (sub epidermis, Dermis) and lower rate of neutrophil (+) in (subcutaneous) skin under the microscope and there were no any other types of inflammatory cells such as lymphocyte and plasma cell.

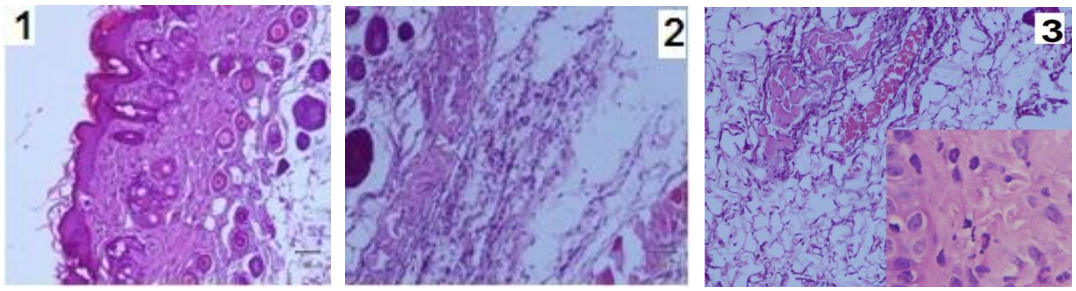

Figure 5. subepidermis layer of control group (C1), Dermis layer of control group (C2), subcutaneous layer of control group (C3) (by microscope 400x)

The histopathological examination of group A (First insulin group) mice showed a normal structure of sub epidermis layer and higher rate of mixed inflammatory cells such as neutrophil, lymphocyte and plasma cell in Dermis layer and subcutaneous layer (++) which was observed under the microscope.

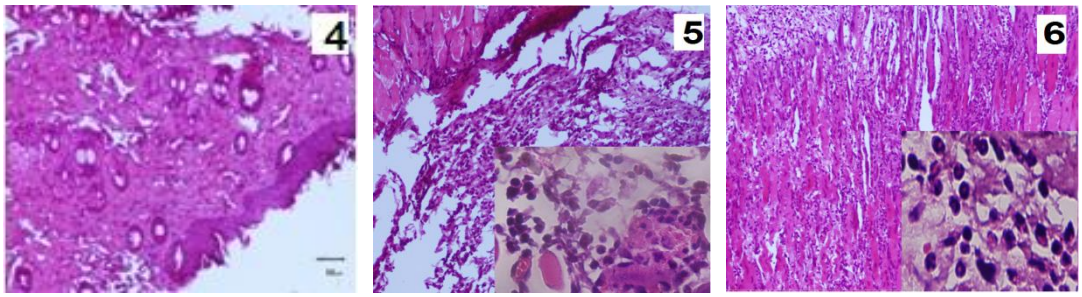

Figure 6. Sub epidermis layer of first insulin group (A4), Dermis layer of first insulin group (A5), Subcutaneous layer of first insulin group (A6) (by microscope 400x)

The histopathological examination of group B (second insulin group) mice showed a normal structure of sub epidermis layer and higher rate of mixed inflammatory cells such as neutrophil, lymphocyte and plasma cell in Dermis layer (++) and highest rate in subcutaneous layer (+++) of skin which was observed by the microscope.

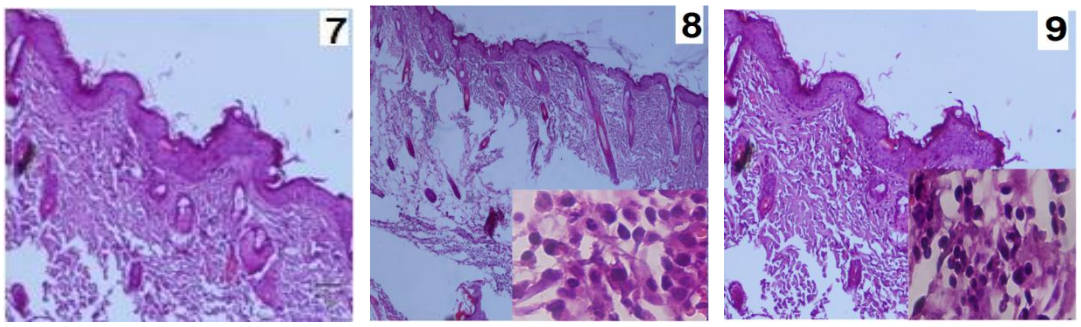

Figure 7. Sub epidermis layer of second insulin group (B7), Dermis layer of second insulin group (B8), Subcutaneous layer of second insulin group (B9) (by microscope 400x)

Discussion

The study required 7 days to accomplish the treatment on 18 male mice to find the effect of different types of insulin, compare with control group of serum by ELISA method, and observe any signs of allergy which might be caused by insulin. The effect of insulin was noticed in the site of injection by histopathology slide under microscope. There were two types of insulin injection for treating the animals (n=18) which were grouped as A and B (experimental), while C was the control group,. The experimental animals in group A were administered with (0.3 -1) U/Kg body weight of mice by first insulin for 7 days and group B was administered with (0.3 -1) U/kg body weight of mice by second insulin for 7 days, while group C was controlled with sterile irrigation water,This study used two types of insulin as research material by means of 18 male mice in which the IgG and IgE test used ELISA and Histopathology site of injection was observed via the microscope.We used two types of insulin appllicable for type 1 or type 2 diabetes mellitus patients. However, in this study we used normal mice to observe the sign of allergy in blood stream and site of injection to control the blood glucose level.

During this study we divided our experiments in two stages. The first stage was ELISA method which measured IgG and IgE in blood serum concentration, the second one was histopathology in skin injection site to observe any presence of inflammatory cells.In the first stage we measured IgG and IgE in two insulin groups and compared them with the control group. We noticed no significant difference between three groups, and the average of each treatment was not too much different. We compared that the lowest average in treatment A was 6.430±0.9986, and the highest average in treatment C was at 8.202±1.8674. The overall average of IgE obtained by 7.168±1.9074 and the lowest average in treatment B of 11.768±6.2158, and the highest average in treatment C at 18.522±2.0395. Overall average of IgG obtained by 15.485±5.0667.

After the first stage was done, there was no reaction of allergy in blood serum concentration analysed by ELISA method. The two groups of insulin were in the normal range with control group. It meant two types of insulin in this study did not cause allergy to mice in blood stream. It is only a small number of generalised reactions to insulin, localised reactions to insulin still occurs in 5% of patients receiving insulin, despite it is now being available in a highly purified state and having the same molecular structure as human insulin[5]. More recently, allergies to the insulin components protamine, metacresol and phenol have been reported in a series of five patients[6].

In the second stage we compared the site of injection in three groups by preparing the histopathology slides and observed it under microscope. The purpose of this stage was to determine the presence of inflammatory cell in the site of injection. We also noticed any change in the layers of skin after injecting it by different types of insulin.

During the examination of skin layers in control group (C), the sub epidermis layer and dermis layer were normal and no presence of inflammatory cells shown, but in the subcutaneous layer we observed a few amount of neutrophil(+). In the first group of insulin (A) during the examination of skin layers, the sub epidermis layer was natural. Dermis and subcutaneous layers showed an increasing amount of inflammatory cells (++) such as neutrophil, lymphocyte and plasma cell. In the second group of insulin (B) during the examination of skin layers, the sub epidermis layer was natural. Dermis and subcutaneous layers showed higher amount of inflammatory cells (+++) such as neutrophil, lymphocyte and plasma cell. In most of previous studies, they mentioned about the side effect of insulin injections to diabetic patient such as hypoglycemia or hyperglycemia and another side effects to some organs including heart, kidney, eye, and nerve damage.

However, during our experiment we noticed inflammatory reaction in injection site after one week of injection by two types of insulin available in market.

As a conclusion, the study proves that different types of insulin injected in the same site for long time cause inflammatory reaction which may lead to cause insulin allergy after a period of time.

Acknowledgment

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