

## Low-dose Insulin Treatment Ameliorate Glucose Metabolism in Type 1 Diabetic Rats

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### Abstract

**Study background:** Type 1 diabetes is an insulin-dependent diabetes mellitus (IDDM) because the body does not produce sufficient insulin for daily need. Persistent high glucose level in the blood circulation can stimulate the advanced glycation end products/receptor for AGEs (AGE/RAGE) pathway leading to diabetic complications. Currently, there is no preventive measure for IDMM with only treatment focusing on delaying the onset of diabetic complications. This study aimed to investigate how the different insulin regimens given affect the glycemic control and glucose metabolism in type 1 diabetes. The effects on the AGE/RAGE axis were determined as well.

**Methods:** Twenty-one male Sprague-Dawley rats were randomly assigned into three groups (Group A: streptozotocin-induced diabetic rats given high-dose insulin [5-7U per day], Group B: streptozotocin-induced diabetic rats given low-dose insulin [0-3U per day], Group C: non-diabetic control). Throughout the eight-week treatment, the body weight, systolic blood pressure and non-fasting blood glucose concentration were monitored. After eight weeks of treatment, fasting blood glucose concentration, glycosylated haemoglobin (HbA1c), nuclear factor kappa B (NF-κB), AGE level and RAGE expression were measured.

**Results:** High-dose insulin treatment impaired glucose metabolism. On the contrary, diabetic rats receiving low-dose insulin showed reduced glucose level and improved insulin sensitivity towards the end of experiment. HbA1c level was significantly lower in low-dose insulin treated rats while NF-κB level was significantly lower in high-dose insulin treated rats ( $p \leq 0.05$ ). There was no significant difference in AGE levels in all the groups ( $p > 0.05$ ) and only the heart tissues from the low-dose insulin treated group showed significant down regulation of the RAGE gene expression ( $p \leq 0.05$ ).

**Conclusion:** Low-dose insulin treatment was shown to be a better therapeutic intervention for type 1 diabetes.

**Keywords:** Glycated haemoglobin (HbA1c); Hyperinsulinemia; Nuclear factor kappa B (NF-κB); Glycemic control; Streptozotocin-induced diabetes

### Introduction

Diabetes mellitus is a global epidemic problem that causes public health crisis worldwide. In 2011, there were 366 million diabetic patients. This value is estimated to rise up to 522 million in 2030 [1]. In developed countries, this chronic disease is one of the leading causes of death and cardiovascular disease is the major cause of mortality in diabetic patients [2]. In Malaysia, the prevalence of diabetes among those 30 years old and above has increased from 8.3% to 14.9% to 20.8% in 1996, 2006 and 2011 respectively [3]. This is certainly an economic burden to the healthcare industries if the number continues to increase by leaps and bounds.

Diabetes mellitus is a group of metabolic diseases characterized by increased blood glucose levels (hyperglycemia). This abnormal elevation of blood glucose occurs due to defects in insulin secretion, insulin action or both [4]. There are different types of diabetes based on the aetiology and clinical stages. The four main categories identified up to date are type 1 diabetes, type 2 diabetes, gestational diabetes and other specific types [5]. Type 1 diabetes is also known as insulin-dependent diabetes mellitus (IDDM) because the body does not produce sufficient insulin for daily need and thus regular insulin injections are required [4]. Currently, there is no preventive measure for IDMM with only treatment focusing on delaying the onset of diabetic complications. Insulin therapy is a common treatment for IDMM. Although insulin dosage should be individualized, designing an ideal insulin regimen for reference are undoubtedly of utmost interest.

Advanced glycation end products (AGEs) are the stable products of glycation after the amino group of protein, lipid or deoxyribonucleic acid (DNA) is reduced by sugar. Hence, glucose is a main precursor for the formation of AGEs [6]. AGEs accumulation inside the body is thought to play a role in the pathophysiology of several diseases particularly those related to diabetes [7]. Besides modifying the intracellular proteins, AGEs can form specific interaction with certain receptors that eventually exert detrimental effect on the cellular system [8,9]. To date, there are several AGE receptors identified and among these, RAGE is the most extensively studied. AGE-RAGE interaction is the key event that brings about the deleterious effects in tissues. RAGE is a multi-ligand transmembrane receptor of the immunoglobulin superfamily [10]. Nuclear factor kappa B (NF-κB) is a transcription factor that binds onto the promoter region of RAGE to mediate inflammatory responses. Many vascular complications of diabetes have been implicated in the AGE-RAGE downstream signalling with the activation of NF-κB [11]. This study aimed to investigate how the

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different insulin regimens given affect the glycemic control and glucose metabolism in type 1 diabetes. Apart from that, it is crucial to know the effects on the AGE/RAGE axis as a primitive mean to ameliorate diabetic complications associated with type 1 diabetes.

## Materials and Methods

### Animal ethics and treatments

The procedure in using and handling animals in this research project had been approved by the Monash University School of Biomedical Sciences Animal Ethics Committee (AEC Approval Number: MARP/2013/082) in compliance to 2004 National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7<sup>th</sup> edition) along with Victorian legislations and Monash University policies, procedures and guidelines.

Twenty-one male Sprague-Dawley rats (*Rattus norvegicus*) with initial weights ranging from 180 g to 240 g were housed individually in plastic cage at room temperature of  $23 \pm 1^\circ\text{C}$  and subjected to 12:12 hours light:dark cycle. Paper was used as bedding and was changed regularly to provide a hygienic environment. Prior to treatments, the rats were acclimatized for one week with *ad libitum* feeding with standard rat chow (Gold Coin, Malaysia) and tap water. After that, the rats were randomly divided into three Groups, A, B and C with each group comprising of seven rats.

The rats from Groups A and B were given 55 mg/kg of streptozotocin (Sigma, USA) via intraperitoneal injection to induce diabetes. A 30 mg/mL streptozotocin stock was prepared by dissolving 30 mg of streptozotocin in 1 mL of 0.09M citrate buffer solution, pH 4.5 (Sigma, USA). Rats from Group C were non-diabetic control. The rats were left for 10 days without any treatment to stabilize their conditions. After 10 days, the rats from Group A were given high-dose insulin (total 5-7 U/day) (NovoMix<sup>®</sup> 30 Flex Pen, Denmark) with three times injection per day (at 0830, 1430, and 2130). On the other hand, rats from Group B were given low-dose insulin (total 0-3 U/day) with only one time injection per day (at 0830). No insulin treatment was given to the rats in Group C. All the rats were fed with normal diet consisting of standard rat chow and tap water. The duration of treatment was 8 weeks. Throughout the 8 weeks, the weight, systolic blood pressure and blood glucose concentrations were monitored.

### Measurement of blood pressure

The blood pressure of rats were measured once every week using the MRBP Mouse and Rat Tail Cuff Method Blood Pressure System (IITC Life Sciences, USA). The tail cuff with pulse transducer was applied onto the tail of the rat placed in a plastic restrainer. The systolic blood pressures were measured once the rat had calm down (e.g. no vigorous agitation).

### Sample collection

At the end of treatment, the rats were fasted for 12 hours prior to humane sacrifice. The rats were anaesthetized with a mixture of 75 mg/kg ketamine and 10 mg/kg xylazine via intraperitoneal injection. Whole blood, serum and red blood cell lysate were collected. The serum and red blood cell lysate were stored at  $-80^\circ\text{C}$  until required for analysis. Various target tissues were harvested which included the liver (L), kidney (K), heart (H), abdominal muscle (AM), subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). Extracted tissues were collected in individual microcentrifuge tubes and snap

frozen using liquid nitrogen. These tubes were then stored at  $-80^\circ\text{C}$  for further analysis.

### Blood and serum biochemical analyses

The non-fasting and fasting blood glucose of rats were determined using Accu-Chek Performa glucometer (Roche, Switzerland). The HbA1c levels of the rats were determined using Rat glycated haemoglobin A1c (GHbA1c) ELISA Kit (Cusabio<sup>®</sup>, China) while the serum NF- $\kappa$ B of rats was measured using Rat Nuclear Factor-kappa B (NF- $\kappa$ B) ELISA Kit (Cusabio<sup>®</sup>, China). The serum AGE level of each of the rat was determined using OxiSelect<sup>™</sup> Advanced Glycation End Product (AGE) ELISA Kit (Cell Biolabs, USA).

### Tissue total RNA extraction and cDNA synthesis

The total RNA in the liver (L), kidney (K), heart (H) and abdominal muscle (AM) were extracted using Qiagen RNeasy Mini Kit (Qiagen, USA) while the RNA of subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were isolated using Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, USA). The purity of the extracted RNA was assessed using a nanodrop program in the Infinite<sup>®</sup> 200 PRO (TECAN, Switzerland) by determining the ratio of absorbance at 260nm to 280nm. Agarose gel electrophoresis was carried out to assess the RNA integrity by examining the presence of 28S and 18S ribosomal bands. RNA extract was treated with RNase-free DNase using Fermentas DNase I, RNase-free (Fermentas, Canada) to remove contaminating DNA. The prepared RNA was used as a template for reverse transcriptase in cDNA synthesis. The process was carried out using the Qiagen Omniscript Reverse Transcription kit (Qiagen, USA).

### Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) of RAGE

The qRT-PCR was performed using Rotor-Gene Q (Qiagen, USA). The gene to be studied was RAGE while the endogenous control was  $\beta$ -actin gene (BAC). The products of qRT-PCR were confirmed by running agarose gel electrophoresis. The nucleotide sequences of primers and probes used are listed in Table 1.

### Statistical analysis

All data recorded were processed and analysed using Statistical Package for the Social Sciences (SPSS) version 22.0 with the exception of the relative expression of RAGE that was analysed using the Relative Expression Software Tool for Rotor-Gene 3000 and 6000 (REST-RG<sup>®</sup>) Version 3. Analysis of Variance (one way ANOVA) was used to analyse the data with parametric distribution. This was then followed by Post Hoc (Tukey) test when the results were significant. For non-parametric distribution data, Kruskal-Wallis test was used and when the results were significant, Mann Whitney U-test was carried out. Mixed Model ANOVA was used to analyse the weight, systolic blood pressure and non-fasting blood glucose. The relative expression of RAGE was evaluated based on the take-off points and PCR reaction efficiencies of the samples as analysed by the 'Comparative Quantitation' features of

Primer/Probe	Nucleotide sequence (5'-->3')
BAC forward primer	GTA TGG GTC AGA AGG ACT CC
BAC reverse primer	GTT CAA TGG GGT ACT TCA GG
BAC probe	[TET] CCT CTC TTG CTC TGG GC [BHQ1]
RAGE forward primer	CCT TGA CCT GTG CGA TCT CT
RAGE reverse primer	GGG TGT GCC ATC TTT TAT CCA
RAGE probe	[6FAM] CCC AGC CTC CCC CTC AAA TCC A [BHQ1]

Table 1: Nucleotide sequences of primers and probes for BAC and RAGE.

Rotor-Gene Q and interpreted by REST-RG® Version 3. This involved the calculation of the expression ratios of target to calibrator. In all the analyses, a p-value of  $\leq 0.05$  was considered statistically significant.

## Results

The initial weight ( $\pm$  SEM) of rats from Groups A, B and C were not significantly different until week 5 ( $p > 0.05$ ). Starting from week 6 onward, rats in Group B showed a significantly slower weight gain ( $p \leq 0.05$ ) compared to rats in Group A. The final body weight of rats from Group B was significantly lower than rats from group A ( $p \leq 0.05$ ). Rats in Group B also had the lowest weight gain. Rats in Groups A and C did not show any significant difference in body weight during the treatment period (Figure 1).

The initial mean systolic blood pressure ( $\pm$  SEM) of rats from Groups A, B and C were not significantly different until week 3 ( $p > 0.05$ ). Starting from week 4 to 6, rats in Group B and C started to show a difference in blood pressure. Rats in Groups A and B also showed significant difference in systolic blood pressure at week 5 (Figure 2). From the results, high-dose insulin treatment sustained the systolic blood pressure of the diabetic rats at a level similar to non-diabetic control rats.

In the early phase of the experiment (first five weeks), the rats in Group A (high-dose insulin treatment) achieved significantly lower mean blood glucose level as compared to Group B (low-dose insulin treatment) ( $p \leq 0.001$ ). Progressing towards the late phase of the experiment (week 6 to 8), the mean blood glucose level of rats in Group A started to increase. In contrast, the rats in Group B started to show a decrease in blood glucose level. At week 7, rats from both Groups A and B showed no significant difference in their mean blood glucose level ( $p > 0.05$ ). At week 8, the mean blood glucose of the rats in Group A ( $16.24 \pm 1.62$  mmol/L) was significantly higher than Group B ( $10.36 \pm 0.75$  mmol/L). The mean blood glucose of rats in Group C remained significantly lower than both Groups A and B throughout the experiment ( $p \leq 0.001$ ). The mean blood glucose was constant throughout the 8 weeks in Group C (Figure 3). For fasting blood glucose, the mean blood glucose concentration of the rats in Group B was significantly lower than Group A ( $p \leq 0.05$ ) at the end of the eight-week treatment. Concurrently, the mean fasting blood glucose concentration of rats in Group C was significantly lower than Group A as well ( $p \leq 0.05$ ) (Table 2).

At the end of the eight-week treatment, the mean HbA1c concentration of rats in Group B was significantly lower than Group C

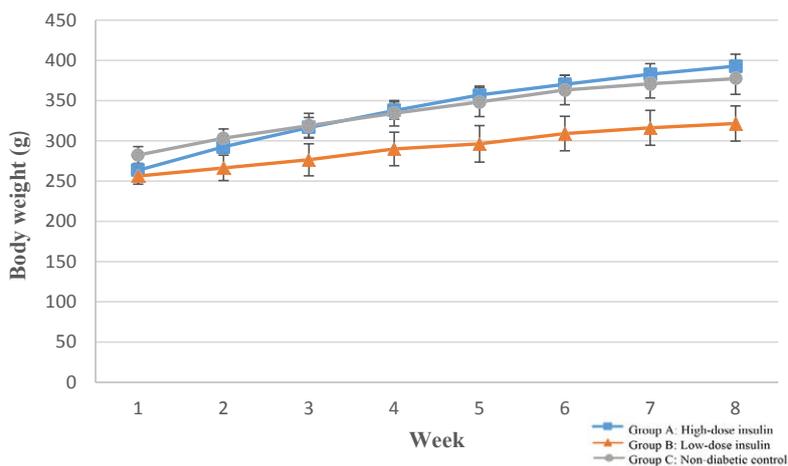


Figure 1: Mean body weight ( $\pm$  SEM) (g) of the rats in Groups A, B and C throughout the eight-week treatment.

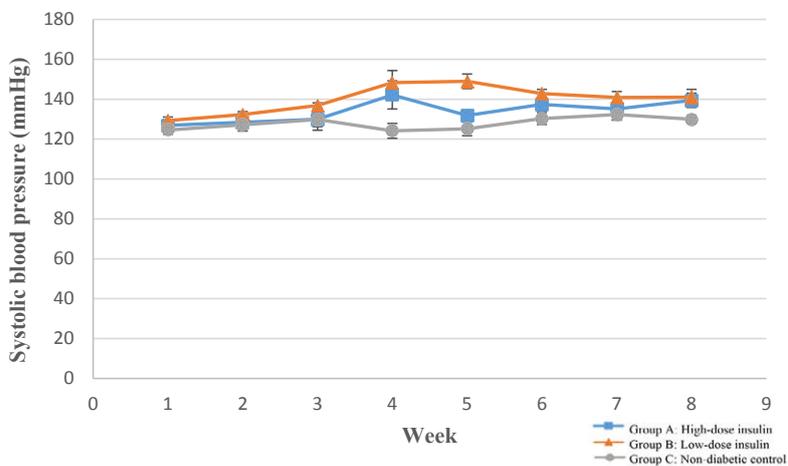
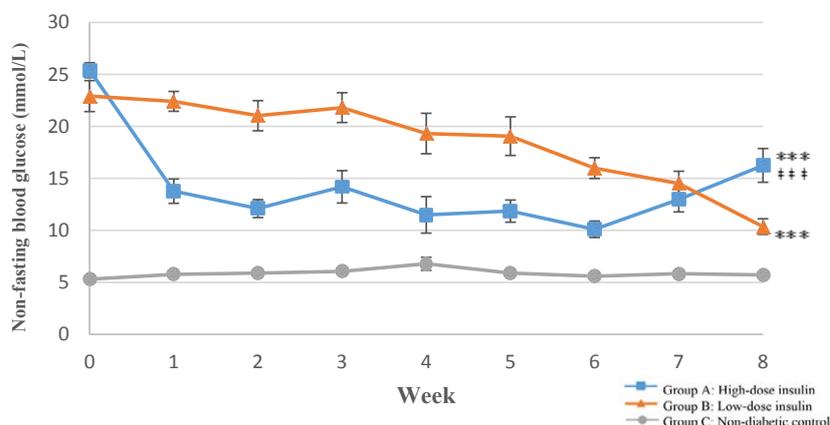


Figure 2: Mean systolic blood pressure ( $\pm$  SEM) (mmHg) of the rats in Groups A, B and C throughout the eight-week treatment.



**Figure 3:** Mean non-fasting blood glucose concentration ( $\pm$  SEM) (mmol/L) of the rats in Groups A, B and C throughout the eight-week treatment. † † † significant compared to Group B ( $p \leq 0.001$ ); † † † † significant compared to Group C ( $p \leq 0.001$ ).

Biochemical analyses	Group A Diabetic rats given high dose insulin (5-7U per day) n= 7	Group B Diabetic rats given low dose insulin (0-3U per day) n= 7	Group C Non-diabetic control n= 7
	Fasting blood glucose (mmol/L)	14.16 $\pm$ 2.11**	6.6 $\pm$ 1.05
HbA1c ( $\mu$ g/mL)	4.07 $\pm$ 0.35	3.18 $\pm$ 0.19*	5.07 $\pm$ 0.55
NF- $\kappa$ B (pg/mL)	13.89 $\pm$ 4.63**	40.55 $\pm$ 1.50	41.27 $\pm$ 3.14
AGE ( $\mu$ g/mL)	282.74 $\pm$ 28.46	260.53 $\pm$ 24.52	275.46 $\pm$ 43.69

Data are means  $\pm$  SEM. \*significant compared to C ( $p \leq 0.05$ ); † significant compared to B ( $p \leq 0.05$ ). HbA1c: glycated haemoglobin; NF- $\kappa$ B: nuclear factor kappa B; AGE: advanced glycation end product.

**Table 2:** Blood and serum biochemical parameters for rats in Groups A, B and C measured at the end of eight-week treatment.

( $p \leq 0.05$ ) whereas the mean NF- $\kappa$ B concentration of the rats in Group A was significantly lower than both Groups B and C ( $p \leq 0.05$ ) (Table 2).

There was no significant difference in the mean serum AGE concentration between all the groups despite the differences in blood glucose level ( $p > 0.05$ ) (Table 2). In a short term period, insulin treatments given to the diabetic rats had countered the expected accumulation of AGE. Using Group A as the calibrator in the experiment, the RAGE expression ( $\pm$  SEM) of Group B was up-regulated in the abdominal muscle (AM), subcutaneous (SAT) and visceral adipose tissue (VAT) by 1.10  $\pm$  1.20 fold, 2.70  $\pm$  3.78 fold and 1.02  $\pm$  1.29 fold respectively, but was down-regulated in the liver (L), and kidney (K) by 2.13  $\pm$  2.79 fold and 1.45  $\pm$  0.74 fold respectively. Nevertheless, the changes in RAGE expression in all these tissues was not statistically significant ( $p > 0.05$ ). Only in the heart (H) tissue, the RAGE expression significantly down-regulated by 2.04  $\pm$  1.17 fold ( $p \leq 0.05$ ) (Figure 4A). On the other hand, the relative expression of RAGE for Group B (target) to Group C (calibrator) as well as Group A (target) to Group C (calibrator) showed no significant up-regulation and down-regulation ( $p > 0.05$ ) (Figure 4B and 4C).

## Discussion

### Chronic hyperglycemia hinders normal growth by reducing weight gain

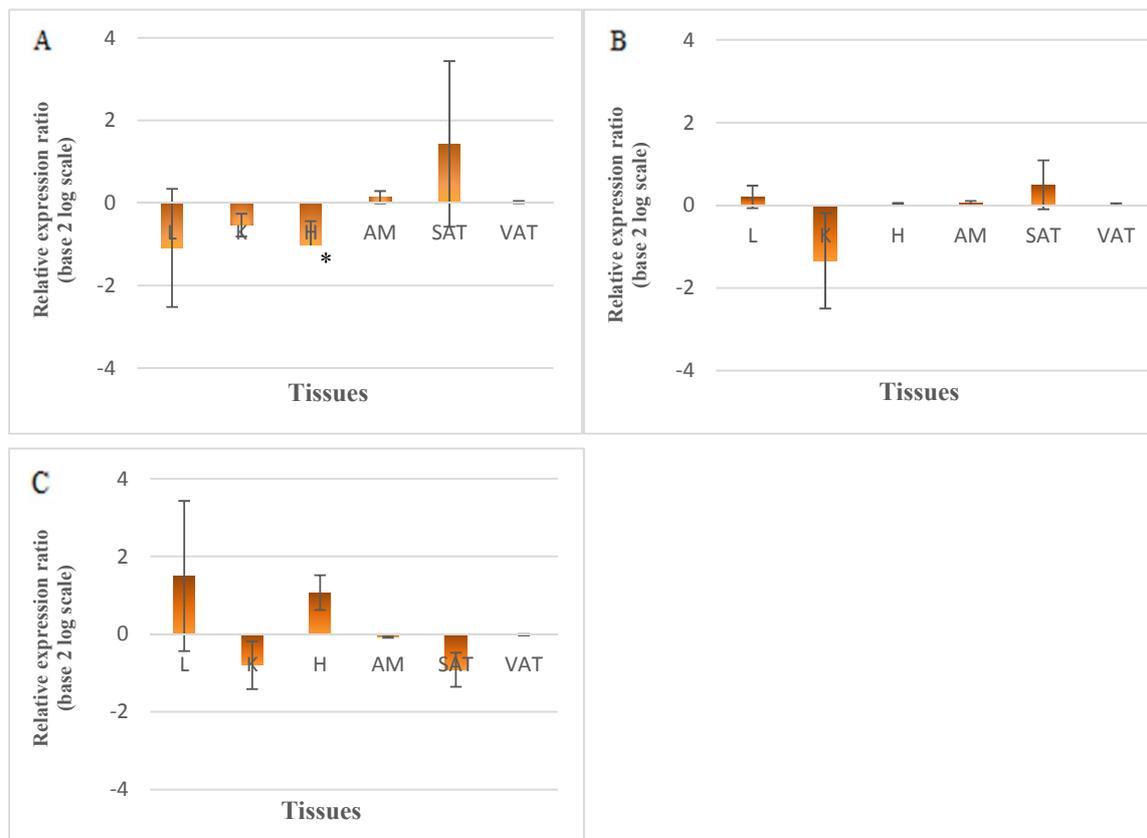
Body weight is one of the parameters to be considered in the development and growth of the laboratory rat model [12]. During the first five weeks of treatment, rats in Group B had high blood glucose concentrations but were given only low-dose insulin. The insulin dosage given was not sufficient to reduce the blood glucose to

physiological relevant level. This condition of chronic hyperglycemia had significantly reduced the total weight gain of the rats. Insulin plays an important role in anabolic processes and the loss of its effects is the main cause for weight reduction observed in diabetic patients [13,14]. The liver, muscle and adipose tissues are insulin responsive tissues. They respond differently to insulin action. Firstly, insulin stimulates glucose uptake for glycogen synthesis in the liver by activating glycogen synthase. On the other hand, insulin increases the uptake of glucose into muscles and adipose tissues by inducing the translocation of glucose transporter 4 (GLUT-4) to cell surface [15]. Excess glucose will be stored as glycogen in the muscle. Meanwhile, glucose will be converted to fatty acids and stored as triglyceride in adipose tissues. Besides glucose, insulin also promotes the uptake of amino acids and free fatty acid into the muscle and adipose tissues respectively [15]. When there is insufficient insulin, the tissues is unable to take up glucose and utilize it for energy production. Prolonged energy starvation will stimulate proteolysis in the muscle and lipolysis in adipose tissues [16]. Both muscles breakdown and depletion of triglyceride stores contribute to weight loss [17]. Hence, the weight gain across time for rats in Group B is significantly lesser than rats in Groups A and C.

### Chronic hyperglycemia induces high blood pressure

Increased blood glucose level accelerates the early glycation process, increasing the formation of  $\alpha$ -oxoaldehydes such as glyoxal, methylglyoxal and 3-deoxyglucosone [18]. These reactive glucose metabolites are the precursors for the formation of advanced glycation end products (AGEs) and are thought to activate the renin-angiotensin system (RAS) thereby elevating systolic blood pressure.

RAS activation is initiated by the secretion of renin from the kidney. As the rate-limiting enzyme, renin catalyzes the conversion



**Figure 4:** Relative expression ratios ( $\pm$  SEM) of RAGE of (A) Group B to A (B) Group B to C (C) Group A to C in base log 2 scale for different tissues with BAC as the reference gene. The positive values imply up-regulation and negative values imply down-regulation. (\* indicates  $p \leq 0.05$  when compared between groups) L: liver; K: kidney; H: heart; AM: abdominal muscle; SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue.

of angiotensinogen to angiotensin I (AngI). Angiotensin I is further hydrolyzed to angiotensin II (AngII) by angiotensin-converting enzyme (ACE). Angiotensin II actively stimulates the sympathetic activity, increases sodium reabsorption, promotes arteriole vasoconstriction and increases anti-diuretic hormone. All these activities raise the systolic blood pressure [19,20]. Dhar et al. [21] showed that excessive methylglyoxal production activated the transcription factor nuclear factor kappa B (NF- $\kappa$ B) via a receptor for advanced glycation end products (RAGE) with subsequent activation of RAS.

Apart from the RAS, activation of the protein kinase C (PKC) is also contributing to the systolic blood pressure elevation. Increased generation of the second messenger diacylglycerol (DAG) during hyperglycemia activates PKC. Activated PKC suppresses the production of endothelial nitric oxide (NO) and reduces NO-mediated vasodilation in microvasculature [22-25]. In type 1 diabetes, development of diabetic nephropathy is often the major cause of hypertension [26]. Activation of PKC- $\beta$  isoforms was observed in the renal glomeruli of diabetic rats and the use of specific PKC- $\beta$  inhibitor improved the glomerular filtration rate and albumin excretion rate [27-29]. Since the use of PKC inhibitor had been shown to restore vascular function, the systolic blood pressure can be lowered following the occurrence of vasodilation [30]. This is observed towards the end of our study when rats in Group B had lowered systolic blood pressure as a result of improved glucose metabolism. The rats in Group A were given higher insulin dosage to lower the blood glucose to near normal level. A better glycemic control with the use of high-dose insulin reduced the activation of RAS and

PKC [27,31]. Therefore, there was no significant difference in the systolic blood pressure between Group C (non-diabetic control) and Group A.

#### Low-dose insulin treatment reduces glucose level whereas high-dose insulin treatment is deduced to cause insulin resistance

Our results suggest that low-dose insulin treatment helped to reduce the mean fasting blood glucose concentration to normal level. Meanwhile, high-dose insulin treatment increased the fasting blood glucose concentration of rats. The high-dose insulin treatment given is speculated to promote insulin resistance. A short term hyperinsulinization promotes the translocation of glucose transporter to the plasma membrane of adipose tissue thereby enhancing the insulin-stimulated glucose uptake [32]. However, long term exposure to high concentration of insulin may suppress the glucose transporter 4 (GLUT-4) gene expression [33]. The development of insulin resistance in hyperinsulinized subjects may cause disruption at different branch points of the insulin-mediated phosphatidylinositol 3-kinase/ protein kinase B (PI-3kinase/Akt) pathway [34]. These include reduction in tyrosine autophosphorylation of insulin receptor (IR), phosphorylation of glycogen synthase kinase (GSK-3), translocation of GLUT-4 to the plasma membrane of muscle tissue as well as deregulated FOXO1 transcription factor [34,35]. This may explain the reason for the high fasting blood glucose recorded in Group A rats treated with high-dose insulin.

### **HbA1c value weighted proportionally towards recent glycation levels reflecting value similar to fasting blood glucose**

Theoretically, HbA1c and mean plasma glucose should demonstrate a linear relationship by providing an average blood glucose for the preceding two to three months. By analysing the trend, our HbA1c results seem to reflect the fasting blood glucose results obtained at the end of the experiment. Studies on the kinetic between glucose and haemoglobin with consideration of the age of RBC and glucose concentration, revealed that HbA1c level will weigh heavily towards recent glycation levels rather than presenting the exact average blood glucose for the past three months [36]. This means that 50% of the glycation will occur in the current month (phase 3) prior to the test and the month before (phase 2) accounts for another 25%. The remaining 25% is formed in the earlier stage (phase 1) when the RBC is newly produced [37]. Although the rats in Group B had poor blood glucose control during the first five weeks of treatment, their HbA1c levels were the lowest among all the groups. Since HbA1c is a reversible glycated product, enzymatic deglycation catalyzed by fructosamine-3-kinase (FN3K) can reverse the glycation of glucose to haemoglobin [38]. The enzymatic deglycation is a defense mechanism to safeguard against cellular damage caused by the glycated product. However, the enzymatic deglycation mechanism is challenged by extreme hyperglycemia [38]. Our treatment period might be too short that the enzymatic deglycating system is still able to reverse the glycation of haemoglobin [38]. Diabetic rats from both the Groups A and B had lower HbA1c level than non-diabetic rats from Group C. This may due to the effects of insulin as insulin has shown to alter the ionic composition of RBC [39]. This possibly affects the glycation process. Apart from that, the blood glucose level in Groups A and B fluctuated from day to day compared to Group C in which the rats had stable blood glucose level throughout the study. Thus, the observed significantly higher HbA1c value in Group C might be due to the glycemic instability in both Groups A and B [40].

### **Multiple insulin injections given in high-dose insulin treatment reduce excessive fluctuation of blood glucose thereby preventing sustained activation of NF- $\kappa$ B**

Multiple insulin injections per day received by rats in Group A had shown to prevent excessive fluctuation of blood glucose thereby preventing the sustained activation of NF- $\kappa$ B. A constant high glucose level promotes intracellular glucose oxidation. In this metabolic pathway, the increased production of reactive oxygen species (ROS) as induced by high glucose initiates the synthesis of diacylglycerol (DAG) from phosphatidylcholine (PC). High DAG level further increases the activation of protein kinase C (PKC) followed by the activation of NF- $\kappa$ B [41]. Aside from the activation of PKC, oxidative stress stimulation is another route for NF- $\kappa$ B activation [42]. In spite of the negative impact, NF- $\kappa$ B activation is essential as part of the positive regulatory circuit for glucose-stimulated insulin secretion (GSIS) [43]. Calcium influx in GSIS has been shown to trigger the activation of NF- $\kappa$ B via mitogenactivated protein (MAP)/ERK (extracellular signal-related kinase) (MEK)/MAP kinase (MAPK) to ensure high GLUT-2 expression. Suppressed NF- $\kappa$ B is shown to impair GSIS [43]. This may explain the NF- $\kappa$ B activation observed in the control group.

### **Short term glucose overload and exogenous insulin decrease the rate of AGE accumulation**

Methylglyoxal (MG) is an intermediate metabolite in the AGEs formation pathway. Increased rate of MG accumulation as a

consequence of high glucose level, leads to a build-up of dicarbonyl stress. Dicarbonyl stress build-up during the non-enzymatic glycation might have induced over-expression of the glyoxalase system, which is comprised of enzymes responsible for the degradation of MG [44]. Hence, with less amount of MG, subsequent formation of AGE will be reduced as well. Increased expression of glyoxalase 1 enzyme (Glo1) inhibits posttranslational modification of proteins by MG thereby preventing the formation of AGEs and other stable glycated products [44]. Apart from this, increased exposure to insulin can promote elimination of AGEs. Insulin acts via the PI-3K signalling pathway to regulate macrophage scavenger receptor (MSR)-mediated endocytic uptake and degradation of AGEs [45]. However, the exact mechanism of this still remains unclear and needs to be explored further. The expected increase in serum AGE level in Groups A and B might be offset due to the effects of exogenous insulin.

### **RAGE gene in rats undergoes various alternative splicing and other RAGE isoforms may be prevalent in diabetic state**

RAGE protein can exist in different isoforms as a result of alternative splicing of the RAGE gene [46]. The splice variants produced may have different amino acid sequences that give distinct ligand binding domain and possibly with transmembrane region excluded, generating non-membrane-bound receptors in secreted form [46]. In murine model, various splice variants had been identified to be uniquely distributed in different tissues. Kalea et al. [47] comparing the changes in splice variant for age-matched diabetic and non-diabetic mice showed that the production of the soluble RAGE (sRAGE) splice variants increased in diabetic condition compared to normal physiological condition. This may explain the non-significant changes in RAGE expression on tissues studied because the RAGE protein could be expressed in other isoforms such as sRAGE. In addition to that, AGE-mediated up-regulation of RAGE expression is dependent on the exposure duration and concentration of AGE [48]. Since there was no significant difference in AGE level among the groups in the present study, AGE-mediated increase in RAGE expression was not significant as well. A longer duration would be needed to investigate whether significant AGE accumulation could be detected in the diabetic rats from Groups A and B.

By comparing the relative expression ratio of Group B to Group A, there is a significant down-regulation of RAGE gene in the heart tissue. Besides binding to pathological ligand (AGE), RAGE binds to physiological ligands such as amphoterin (HMGB1) and S100/calgranulins [49]. Cellular stress caused by glucose overload may stimulate the production of these pro-inflammatory ligands thereby up-regulating the expression of RAGE [50]. Improved glucose metabolism as shown in Group B rats that were given low-dose insulin treatment had a comparably lower RAGE gene expression than Group A rats. This may be possibly due to minimal production of the pro-inflammatory ligands.

### **Conclusion**

In conclusion, low-dose insulin treatment was shown to be a better therapeutic intervention for type 1 diabetes as compared to high-dose insulin treatment. Our study suggested that low-dose insulin improved glucose metabolism in diabetic rats. Future work can focus on the underlying mechanism of the hormone action and metabolism.

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