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EFEITOS DA INSULINA DEGLUDECA E DA GLUTAMINA DIPEPTÍDEO SOBRE A HOMEOSTASE GLICÊMICA E O METABOLISMO HEPÁTICO DE CAMUNDONGOS DIABÉTICOS SUBMETIDOS À HIPOGLICEMIA INDUZIDA POR INSULINA

CAMILA BATAGLINI

Maringá
2020

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (área de concentração - Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Doutor em Ciências Biológicas.

Orientador: Dr. Jurandir Fernando Comar

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BANCA EXAMINADORA

_____
Dr. Jurandir Fernando Comar
Universidade Estadual de Maringá - UEM

_____
Dra. Sílvia Carla Santana Ferreira Azevedo
Universidade Estadual de Maringá - UEM

_____
Dra. Maria Raquel Marçal Natali
Universidade Estadual de Maringá - UEM

_____
Dr. Helenton Cristhian Barrena
Centro Universitário de Maringá - UNINGÁ

_____
Dra. Anacharis Babeto de Sá Nakanishi
Universidade Estadual de Maringá - UEM

BIOGRAFIA

Camila Bataglini nasceu em Maringá/PR em 15/11/1990. Em 2013 graduou-se em Ciências Biológicas pela Universidade Estadual de Maringá. Em 2014 iniciou o Programa de Pós-graduação em Ciências Biológicas (Área de concentração – Biologia Celular e Molecular) em nível de mestrado, na Universidade Estadual de Maringá (UEM), cuja conclusão ocorreu em 2016. Em 2016 iniciou o nível de doutorado no mesmo programa. Tem experiência na área de Fisiologia, Histologia, Biologia Celular e Bioquímica, atuando nos temas Metabolismo Hepático, Histologia Hepática, L-glutamina e diabetes mellitus tipo 1.

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APRESENTAÇÃO

Este é um trabalho realizado no Laboratório de Metabolismo Hepático e Radioisótopos do Departamento de Bioquímica, no Laboratório de Fisiologia do Departamento de Ciências Fisiológicas e no Laboratório de Histologia Animal do Departamento de Ciências Morfológicas da Universidade Estadual de Maringá, apresentado na forma de dois artigos científicos originais, em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas.

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RESUMO GERAL

INTRODUÇÃO: No diabetes mellitus tipo 1 (DM1) ocorre falência na produção de insulina, causando sérios desequilíbrios metabólicos, como hiperglicemia. Esta última resulta da menor captação periférica de glicose e da aumentada gliconeogênese hepática. A regulação da glicemia no DM1 requer um esquema rígido de insulinização baseado em múltiplas doses diárias de insulina prandial ou combinação de insulinas de ação longa e rápida. A mais utilizada é a insulina regular (ação rápida), mas insulinas de ação longa, como a degludeca, também são utilizadas. A maioria das terapias com insulina, entretanto, estão associadas a episódios de hipoglicemia, o maior efeito colateral da insulinização. Em indivíduos não diabéticos, a hipoglicemia induzida por insulina (HII) inibe a sua secreção e estimula à de hormônios contrarreguladores, como glucagon e cortisol, que ativam a produção hepática de glicose, elevando a glicemia. Mas no DM1, a secreção destes hormônios em resposta à HII é perdida com o tempo.

A L-glutamina é o aminoácido mais abundante do sangue e exerce papel importante em muitos processos fisiológicos, sobretudo como notável substrato para a gliconeogênese hepática, uma via crucial para manter a glicemia durante a HII. A glutamina tem sido mostrada inclusive melhorar o quadro clínico de indivíduos com DM1. Camundongos normoglicêmicos que oralmente recebem glutamina logo após a indução de HII apresentam maior gliconeogênese hepática e maior capacidade de recuperar a glicemia do àqueles que recebem alanina ou glicose. O mesmo não ocorre em camundongos DM1. No entanto, não se sabe se a administração de glutamina no longo prazo poderia atenuar a HII de camundongos DM1. Assim, este estudo investigou se a administração de glutamina dipeptídeo (GDP; L-alanina-L-glutamina), isolada ou combinadas com insulinas degludeca e regular, por 30 dias modifica a glicemia e o metabolismo hepático de camundongos DM1 durante a HII. O cortisol foi administrado logo após a indução da HII, a fim de verificar a sensibilidade dos animais a esse hormônio. O GDP é usado em substituição à glutamina porque este dipeptídeo está associado a uma maior biodisponibilidade de glutamina.

MÉTODOS: O DM1 foi induzido em camundongos Swiss com aloxana (180 mg/kg i.p.). Inicialmente, curvas glicêmicas de camundongos DM1 foram realizadas em quatro dias seguidos para definir a dose de insulina degludeca a ser usada no estudo. Insulina regular (1 U/kg i.p.) e insulina degludeca (5 U/kg

s.c.) foram usadas nos experimentos. Para isso, camundongos DM1 foram distribuídos em seis grupos: D e DG, que receberam salina e GDP, respectivamente; DIR e DIT, que receberam insulina regular e degludeca, respectivamente; DIR+G e DIT+G, que receberam GDP com insulina regular ou degludeca, respectivamente. Animais não diabéticos receberam salina e foram os controles. Os animais receberam por 30 dias GDP 1 g/Kg (gavagem) e, no mesmo período, insulina regular ou degludeca. Depois, os animais em jejum de 15 h receberam insulina regular para induzir a HII (tempo 0) e amostras de sangue foram coletadas da cauda em períodos regulares até 300 min para medir a glicemia. Esse mesmo protocolo foi realizado para avaliar a resposta dos animais ao cortisol (20 mg/kg s.c.), que foi administrado 15 min após indução da HII. O perfil lipídico e a atividade de AST e ALT no soro foram obtidos 60 min após indução da HII.

Seguindo o mesmo protocolo, 1 h após indução da HII, a perfusão de fígado foi conduzida para avaliar a glicólise, glicogenólise, gliconeogênese e ureagênese. Após 10 min iniciais de perfusão com tampão Krebs-Henseleit (KH), a perfusão do fígado foi conduzida da seguinte forma: 30 min com KH, 30 min com KH + glucagon 1,0 μM e 30 min com KH + L-alanina 5,0 mM (precursor gliconeogênico). O líquido de perfusão efluente foi coletado em intervalos de 5 min e analisado para o conteúdo de metabólitos. A morfologia e morfometria hepática foram avaliadas por histologia.

RESULTADOS E DISCUSSÃO: Os animais DM1 não tratados apresentaram glicemia de jejum elevada (tempo 0), menor ganho de peso corporal e aumento da atividade sérica de AST e ALT. A glicemia de jejum foi diminuída nos animais DM1 tratados com insulina degludeca, que também melhorou o ganho de peso corporal e a atividade de AST e ALT. Sem cortisol, os animais DM1 tratados com GDP (isolado ou combinado com as insulinas) apresentaram uma queda menor da glicemia aos 60 min após indução da HII, mas que não foi mantida aos 180-300 min. Sem cortisol, a glicemia de DIT aos 60 min foi ainda menor que do grupo D, mas foi menor e igual aos outros grupos quando os animais receberam cortisol. Este último também reduziu a queda da glicemia aos 300 min em DIR. Estes resultados mostram que o GDP e a insulina regular elevam ao menos infimamente a glicemia de camundongos DM1 na HII e o sistema contrarregulador é ao menos minimamente responsivo ao cortisol. Assim, os

experimentos seguintes foram conduzidos com cortisol e 60 min após indução de HII.

A produção hepática de glicose, lactato, piruvato, amônia e ureia foi calculada como área sob a curva nos três intervalos de perfusão: KH, KH + glucagon e KH + alanina. A partir desses valores, os fluxos glicogenolítico e glicolítico foram calculados nos dois primeiros intervalos de perfusão. O conteúdo de unidades glicosil liberado pela glicogenólise foi aumentado em D, DG e DIR+G, o que reflete um maior conteúdo hepático de glicogênio. A glicólise foi maior apenas no início para DIR. No intervalo gliconeogênico, a liberação hepática de glicose foi igual em todos os grupos, mas a desaminação da alanina, calculada como produção hepática de nitrogênio, diminuiu em DG, DIR, DIR+G, DIT e DIT+G. Isto resultou na queda proporcional da liberação hepática de unidades de 3 carbonos a partir da alanina, mas que não restringiu seu fluxo até glicose, pois o estímulo da gliconeogênese, calculado como incremento de glicose após introdução da alanina, aumentou igualmente em C, DIR e DIT. Em contraste, o estímulo gliconeogênico foi menor em D, DG, DIR+G e DIT+G e, portanto, a não reduzida liberação hepática de glicose pode estar ocorrendo às custas de aumentada glicogenólise. De fato, o nível de glicogênio é aumentado em ratos com DM1, mesmo após 24 h de jejum. A insulina regular e o GDP elevaram AST e ALT de animais DM1 e o GDP somado às duas insulinas elevou os níveis séricos de triglicerídeos. De fato, a glutamina é o aminoácido mais eficaz para síntese de glicogênio hepático e pode inclusive elevar o triglicerídeo sérico como efeito de maior provisão de substrato somado à sustentados níveis de insulina. Além disso, todos os grupos que receberam GDP apresentaram maior peso hepático, que pode ser o efeito do glicogênio aumentado, o qual tem sido associado com aumento de AST e ALT séricas. Não foi verificada relação entre o aumento do peso do fígado e a área ou número de hepatócitos.

CONCLUSÃO: O tratamento dos animais DM1 por 30 dias com degludeca, mas não insulina regular, melhora a glicemia de jejum, o ganho de peso corporal e a atividade sérica de AST e ALT. O GDP e as duas insulinas melhoram minimamente a HII de animais DM1. O GDP, inclusive, reduz a gliconeogênese hepática a partir da alanina, mas não modifica a liberação hepática de glicose como efeito da aumentada glicogenólise. O GDP somado às duas insulinas aumenta os triglicerídeos sérico e, o GDP e a insulina regular elevam a atividade sérica da AST e ALT, o que parece ser causado pelo elevado conteúdo hepático

de glicogênio. Assim, a administração GDP 1 g/Kg a longo prazo em indivíduos com DM1 deveria ser revista, especialmente se somada à insulina regular.

GENERAL ABSTRACT

BACKGROUND: In type 1 diabetes mellitus (T1DM) the body fails to produce insulin, a situation that leads to serious metabolic abnormalities, particularly hyperglycemia. The latter is the consequence of reduced peripheral glucose uptake and upregulation of gluconeogenesis in the diabetic liver. The efficient regulation of glycemia in T1DM requires a rigid schedule of insulinization based on multiple daily doses of prandial insulins and even the combination of long- and fast-acting insulins. The most commonly used is the short-acting regular insulin, but long-acting insulins, such as degludec, are also used in T1DM. The most insulin therapies, however, are associated with episodes of insulin-induced hypoglycemia (IIH), the main side effect of insulinization in T1DM. In healthy individuals, IIH inhibits the secretion of insulin and stimulates the secretion of counterregulatory hormones, such as glucagon and cortisol, which stimulate the hepatic production glucose, increasing the glycemia. In T1DM individuals, the secretion of counterregulatory hormones in response to IIH is lost over time.

L-Glutamine is the most abundant amino acid in the blood and plays important role in a number of physiological processes, particularly as a relevant substrate for hepatic gluconeogenesis, a crucial metabolic pathway to maintain the glycemia during IIH. The supplementation with glutamine has been reported to improve the clinical status in T1DM. Normoglycemic mice orally receiving glutamine shortly after IIH induction had higher hepatic gluconeogenesis and greater ability to recover the glycemia than mice receiving alanine or glucose. The same does not occur in T1DM mice. However, it is not known if the long-term supplementation with glutamine might attenuate the IIH in T1DM mice. This study then investigated whether 30-day supplementation with glutamine dipeptide (GDP; L-alanine-L-glutamine) alone or in combination with degludec and regular insulins modifies the glycemia and hepatic metabolism of T1DM mice during the IIH. Cortisol was given soon after IIH induction in order to verify the sensitivity of T1DM mice to this hormone. GDP is used in substitution of glutamine because this dipeptide is associated with higher bioavailability of glutamine.

METHODS: T1DM was induced in Swiss mice with alloxan (180 mg/Kg, i.p.). Firstly, glycemic curves of T1DM mice were performed on four consecutive days to define the dose of insulin degludec to be used in the study. Regular insulin (1

U/kg i.p.) and insulin degludec (5 U/kg s.c.) were used in the next experiments. For this, T1DM mice were distributed into six groups: D and DG, which received saline and GDP, respectively; DIR and DIT, which received regular and degludec insulins, respectively; DIR+G and DIT+G, which received GDP in combination with regular or degludec insulins, respectively. Non-diabetic mice received saline and served as control. Mice received for 30 days 1 g/Kg GDP (gavage) and, for the same period, regular or degludec insulins. After that, 15 h fasted mice received regular insulin to induce IIH (time 0) and blood samples were collected from the tail at regular times up to 300 min to measure glycemia. This same protocol was performed to verify the response of mice to cortisol (20 mg/kg; s.c.), which was given 15 min after IIH induction. Serum lipid profile and serum activity of AST and ALT were taken 60 min after IIH induction.

Following the same protocol, one hour after IIH, the liver perfusion was carried out to evaluate the gluconeogenesis, ureagenesis, glycogenolysis and glycolysis. After an initial perfusion period of 10 min with Krebs-Henseleit buffer (KH), liver perfusion was performed as follows: 30 min with KH, 30 min with KH + glucagon (1.0 μM) and 30 min with KH + 5.0 mM L-alanine (gluconeogenic precursor). Effluent perfusion fluid was sampled at five min intervals and analysed for the content of metabolites. Hepatic morphology and morphometry were also assessed by histology.

RESULTS AND DISCUSSION: Non-treated diabetic mice presented a high fasting glycemia (time 0), lower body weight gain and increased serum activity of AST and ALT. Fasting glycemia was lower in T1DM mice treated with insulin degludec, which also improved the body weight gain and serum activity of AST and ALT. Without cortisol, T1DM mice treated with GDP (alone or in combination with both insulins) had a lower blood glucose drop at 60 min after IIH induction, which was not sustained at 180-300 min. Without cortisol, the blood glucose of DIT at 60 min was even lower than group D, but this drop of glycemia at 60 min was lower and equal to the other groups when mice received cortisol. The latter also reduced the drop of glycemia at 300 min for DIR. These results show that the glycemia of T1DM mice after IIH is at least minimally improved by GDP or regular insulin and the cortisol-linked counterregulatory system remains at least minimally responsible to the exogenous hormone. Given the latter results, the next experiments were carried with cortisol and at 60 min after IIH induction.

The liver production of glucose, lactate, pyruvate, ammonia and urea were calculated as area under the curve at the three intervals of perfusion: KH, KH + glucagon and KH + alanine. From these values the glycogenolytic and glycolytic flows were calculated at the first two intervals of liver perfusion. The content of glucosyl units released by glycogenolysis was increased in D, DG and DIR+G, a situation that reflects an increased liver content of glycogen. Glycolysis was increased only initially for DIR. In the gluconeogenic interval, the liver output of glucose was not different for all groups, but the alanine deamination, calculated as liver output of nitrogen, was decreased for DG, DIR, DIR+G, DIT and DIT+G. This resulted in proportional reduction of the liver output of 3-carbon units from alanine, but that did not restrict its flow to glucose, because the stimulus of gluconeogenesis, calculated as the increment of glucose after alanine introduction, was equally increased for C, DIR and DIT. In contrast, the stimulus of gluconeogenesis was lower in the liver of D, DG, DIR+G and DIT+G and therefore the unmodified liver glucose output may be occurring at the expense of increased liver glycogenolysis. In fact, previous study showed that glycogen storages is increased in T1DM rats even after a 24 h fasting period. Regular insulin and GDP increased AST and ALT of T1DM mice and GDP in combination with both insulins increased the serum levels of triglycerides. In this regard, glutamine is the most effective amino acid for enhancing the liver glycogen storage and it might be including increasing the serum levels of triglycerides as consequence of increased availability of energy substrate associated with sustained levels of exogenous insulin. In addition, all groups receiving GDP presented higher liver weight, what might be the consequence of glycogen overload that in time has been reported to increase the serum activity of AST and ALT. No relationship was found between the increased liver weight and hepatocytes area or number.

CONCLUSION: the 30-days treatment of T1DM mice with insulin degludec, but not with regular insulin, improves the fasting glycemia, body weight gain and serum activity of AST and ALT. GDP and both insulins at least minimally improve the IIH of T1DM mice. GDP including decrease the liver gluconeogenesis from alanine, but without modifying the liver output of glucose as consequence of increased glycogenolysis. GDP in combination with both insulins was associated with increases in the serum triglycerides and regular insulin and GDP increased the serum activity of AST and ALT, what seems to be the consequence of hepatic

glycogen overload. Thus, the long-term administration of 1 g/Kg GDP in T1DM individuals should be reviewed, especially if associated with regular insulin.

Sumário

Effects of insulin degludec and glutamine dipeptide on glycemic homeostasis of type I diabetic mice undergoing insulin-induced hypoglycemia

Camila Bataglini[1] et al.

[1] Department of Biochemistry, State University of Maringa, PR, Brazil

Address for correspondence:

* Camila Bataglini

Department of Biochemistry

University of Maringá

87020900 Maringá, Brazil

Email: camilabataglini@hotmail.com

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ABSTRACT

Rapid- and long-acting insulins are used alone or in combination to regulate the glycemia in type 1 diabetes (T1DM), however, the insulin-induced hypoglycemia (IIH) is a very common problem related to insulinization. Glutamine dipeptide (GDP) has been reported to improve clinical status of T1DM individuals. Therefore, the present study investigated whether the 30-day co-treatment with GDP and regular (rapid acting) or degludec (long acting) insulins modifies the glycemic homeostasis of alloxan-induced T1DM mice undergoing IIH. Glycemic curves were performed in 15 h fasted mice after IIH induced with 1 U/Kg regular insulin. One hour after IIH, the lipid profile and AST and ALT activity was taken in the serum. Non-treated T1DM mice presented a very high fasting glycemia, lower body weight gain and increased serum activity of AST and ALT. Fasting glycemia was lower in T1DM mice treated with insulin degludec, which also improved the body weight gain and serum activity of AST and ALT. T1DM mice treated with GDP (alone or in combination with both insulins) had a lower blood glucose drop at 60 min after IIH induction. The drop of glycemia at 60 min for T1DM mice treated with degludec insulin was improved when mice received cortisol. Regular insulin and GDP increased AST and ALT in the serum of T1DM mice and GDP in combination with both insulins increased the serum levels of triglycerides. In addition, all groups receiving GDP presented higher liver weight. The results show that the 30-days treatment of T1DM mice with insulin degludec, but not with regular insulin, improves the fasting glycemia, body weight gain and serum activity of AST and ALT. GDP and both insulins at least minimally improve the IIH of T1DM mice. However, regular insulin and GDP increased the serum activity of aminotransferases, what seems to be the consequence of hepatic glycogen overload due to increased availability of energy substrate associated with sustained levels of exogenous insulin. Thus, the long-term administration of 1 g/Kg GDP in T1DM individuals should be reviewed, especially if associated with regular insulin.

Keywords*: insulin degludec, glutamine dipeptide, liver metabolism, type I diabetes, gluconeogenesis, ureagenesis.*

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that results from the loss of β-pancreatic cells and consequent failure to produce insulin [American Diabetes Association, 2014]. The disease leads to serious metabolic abnormalities, particularly hyperglycemia, which occurs mainly as consequence of decreased peripheral glucose uptake [Sriram et al, 2014]. Daily replacement of insulin is mandatory for regulating the glycemic homeostasis in T1DM and there are currently available many types of insulin, which differ mainly in how long they are active in the body [Silver et al., 2018]. The most commonly used is the regular insulin, a short-acting insulin with bolus activity and effects lasting 6-8 h [American Diabetes Association, 2019]. A shortcoming of regular insulin is that it can lead to alternating bouts of hypo and hyperglycemia due to periods of hyperinsulinemia and lack of insulin, respectively.

Long-acting or basal insulins, on the other hand, have lower plasma peaks and a longer acting. Among them, the ultralong-acting insulin degludec (25 h half-life) has stood out for maintaining a more constant level of serum insulin and a lower day-to-day glycemic variability [Heise et al., 2012]. Degludec is an insulin analogue containing one molecule of hexadecandioic acid attached to lysine B29 by means of a gamma-L-glutamyl spacer [Steensgaard et al., 2013]. This modification allows insulin degludec to complex with zinc and, after injection, to form a soluble depot of multihexamers in the subcutaneous tissue with slow release of insulin into the systemic circulation and an ultralong glucose-lowering effect [Jonassen et al., 2012].

The efficient regulation of glycemia in T1DM requires a rigid schedule of insulinization based on multiple daily doses of prandial insulins or continuous subcutaneous insulin infusion, or even the combination of basal and fast-acting insulins [American Diabetes Association, 2016]. Nevertheless, most insulin therapies are usually associated with episodes of insulin-induced hypoglycemia (IIH), the main side effect of insulinization in T1DM [Abraham et al., 2016]. In healthy individuals, IIH inhibits the secretion of endogenous insulin and stimulates the secretion of counterregulatory hormones, particularly glucagon, catecholamines and cortisol, which in turn stimulate the hepatic production and release of glucose and reduce its peripheral utilization, increasing the glycemia [Cryer, 2008]. In T1DM individuals, the exogenous insulin does not decrease as

glycemia decreases and, furthermore, the secretion of glucagon is reduced with the disease progression, in other words, the primary physiological defenses against hypoglycemia are lost over time [McCrimmon & Sherwin, 2010].

L-Glutamine is the most abundant free amino acid in the human blood and plays significant role in a number of physiological processes, such as blood ammonia transport, blood pH regulation and biosynthetic pathways associated with cellular integrity and function [Cruzat et al., 2018]. This amino acid is also associated with the regulation, via cellular swelling, of the expression of proteins linked to energy metabolism and in addition has been reported to have anti-inflammatory and immunomodulatory activities [Cruzat et al., 2018]. In fact, L-glutamine supplementation improves the clinical condition of individuals with chronic diseases related to loss of cell mass, including T1DM [Darmaun et al., 2019; Hartmann et al., 2017]. Normoglycemic mice, but not T1DM mice, orally receiving glutamine shortly after the IIH induction had a greater ability to recover the glycemia than mice receiving alanine or even glucose [Santiago et al., 2013; Bataglini et al., 2017]. However, it is not known if the long-term supplementation with glutamine attenuates an episode of IIH in T1DM mice. Aiming at filling this gap, this study investigated whether 30-day supplementation with glutamine dipeptide (GDP) alone or in combination with insulin degludec or regular insulin therapy modifies the glycemic homeostasis of T1DM mice during the insulin-induced hypoglycemia (IIH). The dipeptide L-alanine-L-glutamine, known as glutamine dipeptide, has been widely used in clinical practice in substitution of free glutamine because this dipeptide is associated with a higher bioavailability of glutamine in the blood, given the high consumption of free glutamine by intestinal cells [Raizel & Tirapegui, 2018]. In addition, cortisol was administered soon after the hypoglycemia induction in order to evaluate the sensitivity of T1DM mice to this counterregulatory hormone.

MATERIAL AND METHODS

Chemicals

Glutamine dipeptide and alloxan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial kits for glucose, total cholesterol, triglycerides, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from Gold Analisa Diagnóstica Ltda (Belo Horizonte, MG, Brazil). Insulin degludec Tresiba® FlexTouch® (Novo Nordisk®, Bagsværd, Denmark) and regular insulin Humulin® R (Eli Lilly, Indianapolis, EUA) were purchased from commercial pharmacies. All other chemicals were of analytical grade.

Animals and induction of diabetes

Male *Swiss* mice weighing around 25g (30 days old adult animals) were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and maintained under standard laboratory conditions at a temperature of 24±2ºC and a regulated 12h light/dark cycle. The animals were housed in conventional steel cages (1 mice/cage) and were fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil). After 3 days for acclimatization, animals fasted for 15 h were diabetized with alloxan (180 mg/Kg, i.p.). Four days after, fed and fasting glycemia was measured in the blood collected by tail incision (glucometer and glycophyte Optium Xceed®, Abbott, Brazil). Animals with fasting and postprandial glycemia higher than 300 mg/dL were considered T1DM. All procedures were performed as recommended by the Brazilian Council for the Control of Animal Experimentation (CONCEA) and were approved by the Ethics Committee for Animal Use (CEUA) of the State University of Maringá (protocol number 1334110116).

Experimental design and treatment

Part of the animals (n = 30) was initially used to define the dose of insulin degludec to be used in the study. For this, glycemic curves of T1DM mice were performed on four consecutive days with regular insulin (fixed at 1 U/kg i.p.) [Santiago et al., 2013] and insulin degludec at doses of 1 and 5 U/kg (s.c.). Blood samples were collected by tail incision. The dose of insulin degludec

chosen was 5 U/kg and the criterion was the dose that promoted the best glycemic control (Fig. 1).

Sixty diabetic mice were subsequently distributed into six groups (n=10 per group): D, which received saline; DG, which received glutamine dipeptide (GDP); DIR, which received regular insulin; DIR+G, which received regular insulin and GDP; DIT, which received insulin degludec; and DIT+G, which received insulin degludec and GDP. Ten non-diabetic mice received saline and served as control (C). The animals received daily for 30 days glutamine dipeptide (GDP; 1 g/kg, oral gavage) or saline (gavage) and, for the same period, saline (s.c.) or regular insulin (1 U/kg, ip) or insulin degludec (5 U/kg; sc, via infusion pump). This treatment protocol was repeated three times to determine all proposed parameters.

Glycemic curves

After treatment for 30 days, T1DM animals fasted for 15 h received regular insulin (1 U/Kg) to induce IIH at 8 a.m. (time 0). Blood samples were collected from the tail at times 0, 15, 30, 60, 120, 180, 240 and 300 min to measure glycemia. This procedure aims to characterize the period of lower glycemia and that of blood glucose recovery. This same protocol was performed to evaluate the response of T1DM animals during IIH to counterregulatory hormones glucagon (0.02 mg/kg; s.c.) or cortisol (20 mg/kg; s.c.) or adrenaline (1 mg/kg; s.c.). For this, these hormones were administered 15 min after IIH induction. Only cortisol showed a minimum response during IIH and was chosen as counterregulatory hormone in the next procedures.

Tissue collection and processing

After treatment for 30 days, animals fasted for 15 h received regular insulin to induce IIH and cortisol 15 min later. One hour after IIH, animals were weighed and intraperitoneally anesthetized with Thionembutal® (30 mg/Kg) and lidocaine (10 mg/kg). Blood was then collected by cardiac puncture, centrifuged (3,000 rpm/10 min) and the serum separated to measure total cholesterol and triglyceride levels, and the activity of ALT and AST enzymes. Next, a vertical laparotomy was performed and the liver removed for morphometric analysis.

For histological processing, the liver samples were weighed and fixed in 10% Bouin solution, dehydrated in ethanol gradient, cleared in xylol and embedded in paraffin blocks. Semi-serial 6mm thick cross-sections were prepared with a rotary microtome (Leica RM2245), mounted on slides and stained with hematoxylin-eosin to determine morphology and morphometry.

Histological analyses

Morphological and morphometric analysis were done with images of the liver parenchyma in the region near the central vein. Images were captured from an optical microscope (Olympus BX41®, Japan) with a QColor3® camera (Olympus American INC, Canada), coupled to the software Q-Capture®. Hepatocytes number and area were evaluated using the program Image-Pro Plus® 4.5 (Media Cybernetics). For quantifying the hepatocytes areas, 200 hepatocytes per animal were examined, totaling 1000 hepatocytes per group (μm^2). For the number of hepatocytes, 30 images per animal were counted, totaling 150 images per group. The results of this analysis were expressed as mean ± SEM.

Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of the GraphPad Prism Software (version 5.0). Data were firstly submitted to Kolmogorov-Smirnov (K-S) test to verify the normality. The statistical significance of the normalized data was analyzed by means of ANOVA ONE-WAY and the Tukey post-hoc test was applied with the 5% level of significance ($p<0.05$).

RESULTS

Glycemic curves: standardization of the dose of insulin degludec

The first experiments were done to define the dose of insulin degludec to be used in the study. For this, the glycemic curves of T1DM mice were performed on four consecutive days with the regular insulin 1 U/kg and insulin degludec at the doses of 1 and 5 U/kg. On the first day, the fed animals received the dose of insulin to be tested at 8 am (8h) and blood glucose was measured at 8, 9, 10, 11, 12, 15 and 17h; on the second and third day, the animals also fed received insulin at time 8h and blood glucose was measured at 8, 12 and 17h; On the fourth day, the animals fasted for 15h received insulin at 8 h and blood glucose was measured at 8 and 12h. The results are shown in Fig. 1. For regular insulin, blood glucose at 8h (before insulin administration) was lower on the third (22%) and fourth (28%) days; and insulin administration decreased blood glucose on the third day at 12h (17%) and 17h (26%), and on the fourth day at 12h (19%) when compared to the same times as the first day (Fig. 1A). For 1 U/kg insulin degludec, blood glucose was lower at 17h on the second (17%) and third (25%) days and at 12h on the fourth day (22%; Fig. 1B). For 5 U/kg insulin degludec, blood glucose at 8h was lower on the third (22%) and fourth (19%) days and the insulin administration decreased blood glucose at 12h on the third (35%) and fourth (29 %) days (Fig. 1C).

These results show that the better glycemic control was achieved with insulin degludec at the dose of 5 U/Kg, since the drop of blood glucose already occurs before the insulin administration (8h) on the third and fourth days. In addition, the drop of blood glucose was more pronounced for the dose of 5 U/kg when compared to the dose of 1 U/kg (35% vs 22% at 12h on the fourth day; and 33% vs 26% when 12h on the fourth day is compared to 8h on the same day). Thus, 5 U/kg insulin degludec was chosen for the treatment of mice.

Effects of glutamine dipeptide (GDP) and insulin on glycemic curves during IIH

After treatment for 30 days, the glycemic curves were performed with the animals fasted for 15h using regular insulin to induce IIH and, after 15 min, glucagon or adrenaline or cortisol. This protocol allows to assess whether the glycemic response to counterregulatory hormones was improved by treatment.

The results are shown in Fig. 2. The values obtained with glucagon and adrenaline were omitted because they decreased even more the glycemia when compared to the group that received only insulin (D), i.e., there was loss of the counterregulatory response triggered by these hormones. Only cortisol prevented the drop in blood glucose to values below those of the group D. This phenomenon shows that this model of IIH appropriately simulates the uncontrolled T1DM with impairment of the counterregulatory system.

Blood glucose was measured at times 0, 15, 30, 60, 120, 180, 240, and 300 min after IIH induction, however, times 15, 30, 120, and 240 min were omitted from Fig. 2. It is important to note that the blood glucose of diabetic animals (D) at 60 min is still high, but it is a hypoglycemic situation, since the blood glucose reaches less than 50% of the value before IIH (time 0). In this situation signs of hypoglycemia, particularly lethargy, were observed in the animals. Fasting blood glucose (time 0) was lower in T1DM animals treated with insulin degludec, regardless of GDP (Fig. 2A and Table 1). In IIH without cortisol (Fig. 2A), animals treated with GDP or regular insulin had a lower blood glucose drop at 60 min (compared to group D). This drop in blood glucose was even smaller at 60 min for DIR+G, which received both substances. Without cortisol, the blood glucose of DIT at 60 min was even 40% lower than group D, however, with cortisol, this drop in blood glucose at 60 min was lower and equal to the other groups (Fig. 2B). These results show that, at 60 min and with cortisol, treatments maintained part of the counterregulatory response, but did not hold at 300 min, except for the group DIR (Fig. 2C). Given the latter results, the next experiments were carried with cortisol and at 60 min after IIH induction.

Effects of glutamine dipeptide (GDP) and insulin on serum lipid profile and AST and ALT activities during IIH

Table 1 shows in addition the body weight gain and the serum levels of total cholesterol and triglycerides in mice following IIH. The body weight gain during the 30-day treatment was 36% lower in the T1DM groups and even lower (64%) in T1DM mice treated only with glutamine. The levels of serum triglycerides were increased by approximately 80% in T1DM only when they were treated simultaneously with GDP and insulins (DIR+G and DIT+G). The levels of total cholesterol were practically not modified. The serum activity of

AST and ALT were measured to evaluate if the treatments are associated with liver damage. The results are shown in the lower part of Fig. 3. The activity of AST was increased by 240, 480, 600 and 390%, respectively for groups D, DG, DIR and DIR+G when compared to the controls. The activity of ALT was similarly increased in the same groups and the activity of both was not modified in T1DM mice treated with insulin degludec (DIT and DIT+G).

Effects of GDP and insulins on the liver morphology during IIH

The liver weight was not different for C, D, DIR and DIT, but as shown in the lower part of Fig.3, it was approximately 30% higher in T1DM animals treated with GDP (DG, DIR+G and DIT+G). For this reason, morphological and morphometric analyses were performed in the liver of all groups. Fig.3 depicts the morphology of liver sections stained with hematoxylin-eosin. Control mice (C) showed a normal histological structure as noted by typical central vein and portal space, and hepatocytes arranged in cords with central nucleus and preserved cytoplasm. All groups of T1DM mice showed only a slight modification in the parenchyma characterized by the presence of small vacuoles in the hepatocyte cytoplasm. The morphometric analysis was additionally performed and the results are shown in the lower part of Fig.3. The area of hepatocytes was not different for all groups, however, the number of hepatocytes was approximately 25% higher in T1DM animals (D), DG, DIT and DIT+G when compared with the controls. The increase of the number of hepatocytes in animals receiving regular insulin was lower for DIR (+12%) and non-existent for DIR+G.

DISCUSSION

In T1DM individuals, recurrent episodes of hyperglycemia and hypoglycemia take place as consequence of inadequate insulin therapy or even non-balanced diet. Particularly the recurrent hypoglycemia has devastating effect on the glycemic control and on the short and the long-term well-being of individuals. Experimental models of T1DM in rats and mice are very used to evaluate this condition considering the serious implications that the hypoglycemia may cause in humans. This study used the alloxan-induced T1DM in Swiss mice subjected to 30-day supplementation with glutamine dipeptide (GDP) in combination with insulin degludec or regular insulin. The results show that the blood glucose of 15 h fasted untreated T1DM mice drops sharply 60 min after the administration of insulin to induce hypoglycemia (IIH) and reaches even lower levels after 180 min, which are not modified even after 300 min. Similar phenomenon has also been verified in patient and rodent models of type 2 diabetes and T1DM during IIH [Chakera et al., 2018; Bataglini et al., 2017]. The blood glucose of fasted normoglycemic mice also drops sharply 60 min after IHH but it is recovered almost completely at 300 min [Santiago et al., 2013]. In the present study, only a very poor response to hypoglycemia was observed 300 min after IIH induction in untreated T1DM mice receiving cortisol, but not glucagon or epinephrine. Therefore, the model of IIH of this study is similar to T1DM patients with very low or no response to the hypoglycemic counterregulatory system [McCrimmon & Sherwin, 2010]. Untreated T1DM mice had in addition lower body weight gain and increased serum activity of aminotransferases. The latter has also been reported for T1DM patients and it is related to a poorer glycemic control and even liver failure [Stadler et al., 2017].

T1DM mice treated with insulin degludec for 30 days had lower fasting glycemia and higher body weight gain when compared to those treated with regular insulin. However, T1DM mice treated with insulin degludec had a more severe hypoglycemia at 60 min after induction of IIH, which was minimally reversed when mice received cortisol, although at 180 or 300 min both insulins did not improve IIH. In other words, mice treated with insulin degludec may be minimally responding to the cortisol-linked counterregulatory system. Another difference related to these insulins was the serum activity of aminotransferases. While insulin degludec decreased the serum activity of AST and ALT in T1DM

mice, regular insulin markedly increased the activity of both enzymes. In this regard, a transient elevation of serum aminotransferases after starting insulin therapy has been reported for T1DM patients with ketosis or ketoacidosis and might imply severe dysfunction of glucose metabolism in the liver [Takaike et al., 2004; Takaike et al., 2008]. The causes of these alterations are not fully comprehended and might be even transient for T1DM mice treated with regular insulin, however, ketosis and ketoacidosis are associated with a poorly controlled T1DM. In fact, hyperglycemia and sustained levels of exogenous insulin are associated with hepatic glycogen overload and consequent liver damage with marked elevation in the serum aminotransferases in T1DM [Giordano et al., 2014; Rajas et al., 2013]. In any case, insulin degludec provided better results than regular insulin in relation to parameters not modified by IIH, such as fasting glycemia, body weight gain and serum activity of aminotransferases.

Glutamine is the most abundant endogenous gluconeogenic precursor and the supplementation with this amino acid could relieve the episodes of hypoglycemia [Cruzat et al., 2018]. Glutamine was orally administered as a dipeptide (GDP), which is not metabolized by the gastrointestinal tract [Rosa et al., 2015]. After absorption by the enterocytes, it first passes through the liver and might modify the hepatic response and contribute to glycemic recovery. GDP at the dose of 0.4 g/Kg is most commonly used for hospital treatment of catabolic individuals, however, under other nutritional conditions, the doses may be as high as 1.0 g/kg [Oguz et al., 2007]. This study employed the dose of 1 g/kg, as previously used in T1DM mice [Bataglini et al., 2017]. This dose of GDP is equivalent to the dose of 0.6 g/Kg of glutamine, which has been reported to cause no adverse effects in humans [Poindexter et al., 2003; Garlick, 2001]. The long-term supplementation of T1DM mice with GDP did not improve the fasting glycemia or body weight gain, but it slightly attenuated the IIH at 60 min, a phenomenon that was independent of cortisol and not maintained at 180 and 300 min. In this regard, glutamine orally administered at a dose of 0.1 g/Kg in normoglycemic mice shortly after the IIH induction has been associated with greater ability to recover glycemia [Santiago et al., 2013]. In addition, T1DM mice receiving glutamine (1 g/Kg) shortly after IIH induction had a smaller fall of glycemia at 60 min but it was not maintained at 180-300 min and only occurred when associated with cortisol [Bataglini et al., 2017].

The supplementation of T1DM mice with GDP was accompanied by a considerable increase in the serum activity of AST and ALT and organ weight, phenomena that may be associated with liver glycogen overload. Glutamine is not only the most effective amino acid for enhancing glycogen formation, but it has been also associated with the stimulation of glycogen synthetase as consequence of hepatocyte swelling after rapid uptake of glutamine [Cruzat et al., 2018; Stumvoll et al., 1999]. In addition, T1DM mice receiving GDP in combination with both insulins presented higher levels of serum triglycerides. One possible justification would be the increased availability of energy substrate as consequence of GDP supplementation associated with sustained levels of exogenous insulin.

Some results found in the present study are difficult to understand, for example, the relationship between liver weight and hepatocyte area or number of hepatocytes. Glutamine is known to cause hepatocyte swelling that in time should increase the hepatocyte area. The latter was not found in this study, however, all groups receiving glutamine presented higher liver weight, what might be the consequence of the higher glycogen content. In addition, T1DM treated with GDP in combination with insulin degludec had a higher liver weight, and serum aminotransferases were not increased.

In summary, the 30-days treatment of T1DM mice with insulin degludec, but not with regular insulin, improved the fasting glycemia, body weight gain and serum activity of AST and ALT. GDP and both insulins at least minimally improved the IIH of T1DM mice. GDP in combination with both insulins was associated with increases in the serum triglycerides and regular insulin and GDP increased the serum activity of AST and ALT, what seems to be the consequence of hepatic glycogen overload. However, the liver glycogen content and possibly the liver metabolism should be evaluated to clarify these effects. Finally, the recommendation of long-term supplementation with 1 g/Kg GDP for T1DM individuals should be viewed with caution, especially if associated with regular insulin.

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List of abbreviations

Abbreviations: T1DM, type 1 diabetes; IIH, insulin-induced hypoglycemia; GDP, glutamine dipeptide; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Competing interests

The authors declare that no competing interest exists and that all approved the final manuscript.

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Table 1. Body weight gain, blood glucose and serum lipids mice fasted for 15h after 30-days treatment with GDP and regular insulin or insulin degludec. After Control (C), T1DM (D), T1DM + GDP (DG), T1DM + regular insulin (DIR), T1DM + insulin degludec (DIT), T1DM + regular insulin + GDP (DIR+G) and T1DM + insulin degludec + GDP (DIT+G). Values are the mean ± SEM of 5 mice.

Parameter	**C**	**D**	**DG**	**DIR**	**DIR+G**	**DIT**	**DIT+G**
Body weight gain (g)	22 ± 1^{a}	14 ± 2^{b}	8 ± 1^{c}	12 ± 1^{b}	13 ± 1^{b}	18 ± 1^{a}	12 ± 1^{b}
Fasting glycemia ($mg \cdot dL^{-1}$)	106 ± 2^{a}	439 ± 10^{b}	487 ± 13^{b}	480 ± 10^{b}	490 ± 15^{b}	395 ± 4^{c}	390 ± 4^{c}
Triglycerides ($mg \cdot dL^{-1}$)	45 ± 2^{a}	49 ± 3^{a}	49 ± 2^{a}	61 ± 5^{a}	90 ± 4^{c}	47 ± 3^{a}	80 ± 4^{b}
Total cholesterol ($mg \cdot dL^{-1}$)	$94 \pm 1{,}6^{a}$	103 ± 1^{a}	109 ± 6^{a}	67 ± 2^{b}	107 ± 3^{a}	103 ± 2^{a}	72 ± 4^{b}

Values with different superscript letters in the same line are different ($p<0.05$)

Figure captions

Fig. 1. Glycemic curves after administration of regular insulin **(A)** and insulin degludec at doses of 1 U/kg **(B)** and 5 U/kg **(C)** in T1DM mice. The glycemic curves were performed on four consecutive days. On the first, second and third day the curve was performed with the animals fed and on the fourth day with the animals fasted for 15 h (see *Experimental design and treatment*). On each day, insulin was administered at time 8 h (8 am) and blood glucose was measured at the times indicated on the x axis. Values are mean ± SEM of 5 animals per group. ■ $p \leq 0.05$ vs 8 h of the 1st day; ● $p \leq 0.05$ vs 12 h of the 1st day; ◆ $p \leq 0.05$ vs 17h of the 1st day.

Fig. 2. Effects of glutamine dipeptide (GDP; 1 g/kg) and regular insulin (1 U/kg) or insulin degludec (5 U/kg) on the glycemic curves of T1DM mice during IIH **(A)** and IIH + cortisol **(B)**. Animals received for 30 days saline or GDP (p.o.) and, for the same period, regular insulin (i.p.) or insulin degludec (s.c.). The IIH was induced in mice fasted for 15 h with regular insulin (time 0). Part of the animals received cortisol (20 mg/kg) 15 min after insulin. Panel A and B are compared in **(C)**. D, saline; DG, GDP; DIR, regular insulin; DIT, insulin degludec; DIR+G, regular insulin + GDP; DIT+G, insulin degludec + GDP. Values are the mean ± SEM of 5 animals. $^{a}p \leq 0.05$ for 0 min x 0 min of D; $^{b}p \leq 0.05$ for 60 min x 60 min of D; $^{\star}p \leq 0.05$ for 300 min x 60 min intragroup. $^{\#}p \leq 0.05$ for 300 min of IIH + cortisol x 300 min of IIH.

Fig. 3. Photomicrographs of liver sections (above) and morphometric analysis and serum transaminases activity (below) of control and T1DM mice. Animals were treated during 30 days with GDP (1 g/Kg, p.o.) and regular insulin (1 U/Kg, i.p.) or insulin degludec (5 U/Kg, s.c.). Animals fasted for 15 h received regular insulin (1 U/Kg) to induce IIH and cortisol (20 mg/kg), respectively, 1 h and 45 min prior to euthanasia. Control (C), T1DM (D), T1DM + GDP (DG), T1DM + regular insulin (DIR), T1DM + insulin degludec (DIT), T1DM + regular insulin + GDP (DIR+G) and T1DM + insulin degludec + GDP (DIT+G). Hematoxylin eosin staining (x330). Scale = 100 μm. Number of hepatocytes per mm^2. Data represent the mean ± SEM (n = 5). Data were evaluated by analysis of variance (ANOVA) with Tukey post-test. V indicates the central lobular vein.

Fig.1.

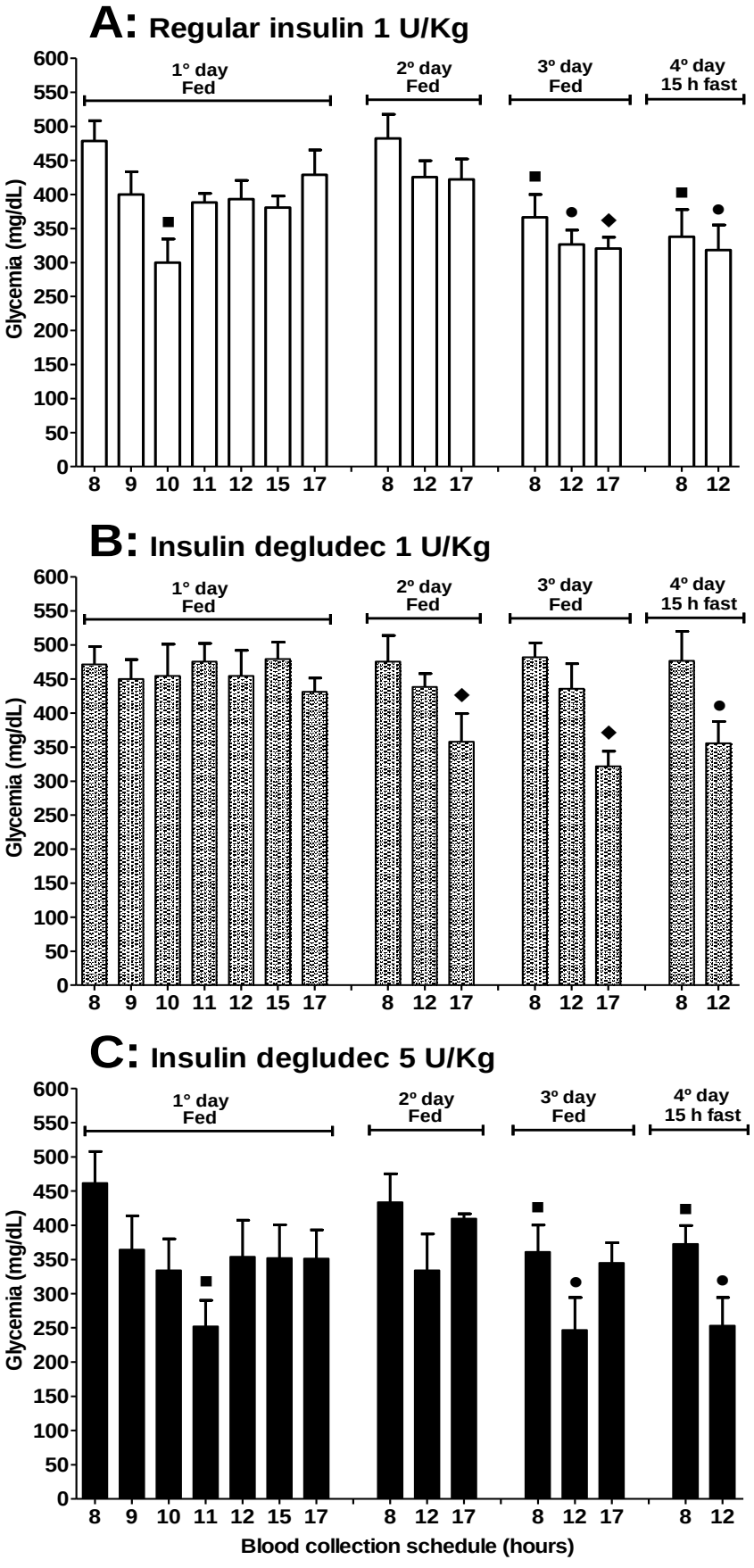

A: Regular insulin 1 U/Kg
B: Insulin degludec 1 U/Kg
C: Insulin degludec 5 U/Kg
1° day Fed
2° day Fed
3° day Fed
4° day 15 h fast
Glycemia (mg/dL)
0
50
100
150
200
250
300
350
400
450
500
550
600
8 9 10 11 12 15 17 8 12 17 8 12 17 8 12
Blood collection schedule (hours)

Fig.2.

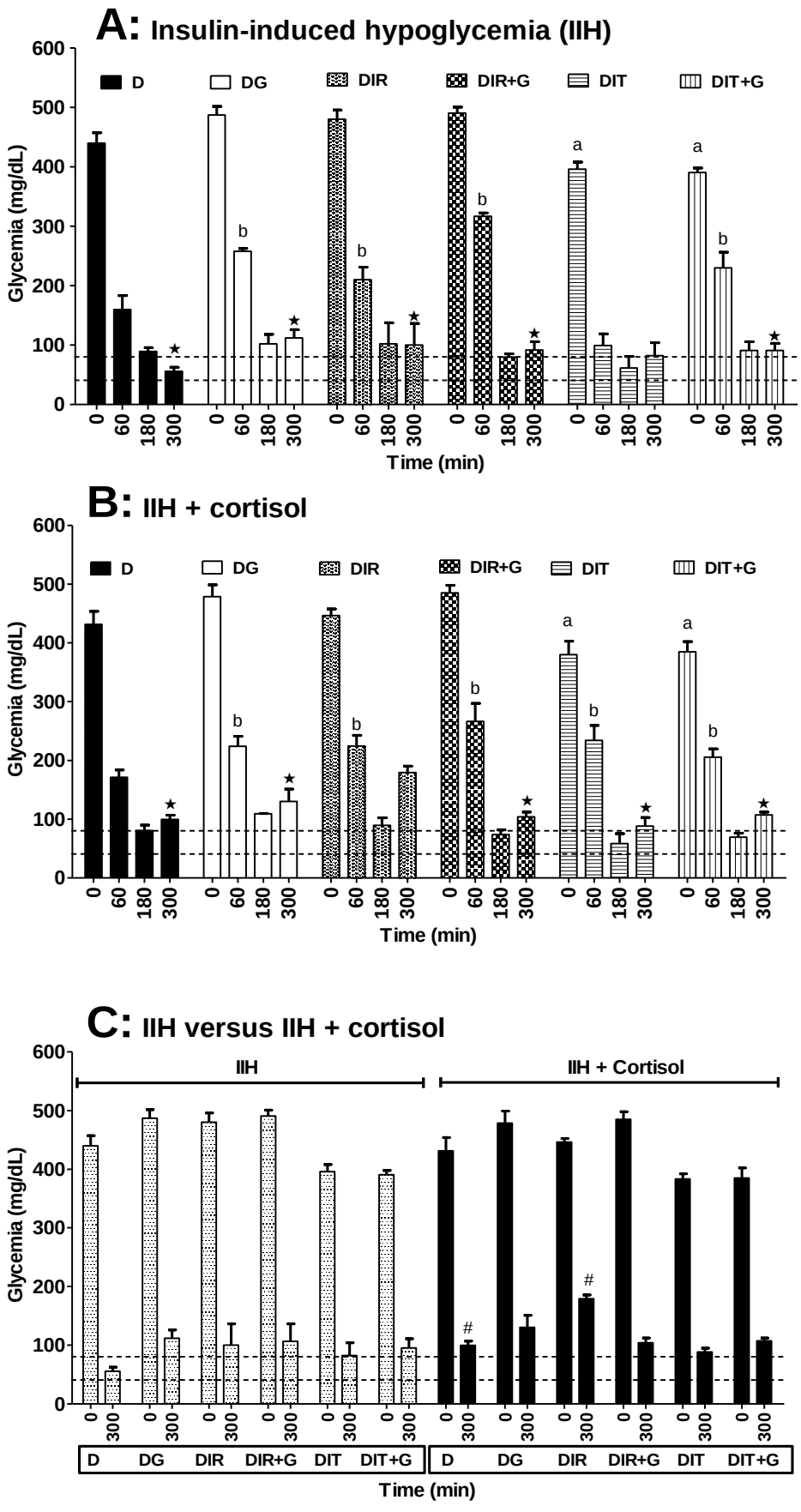
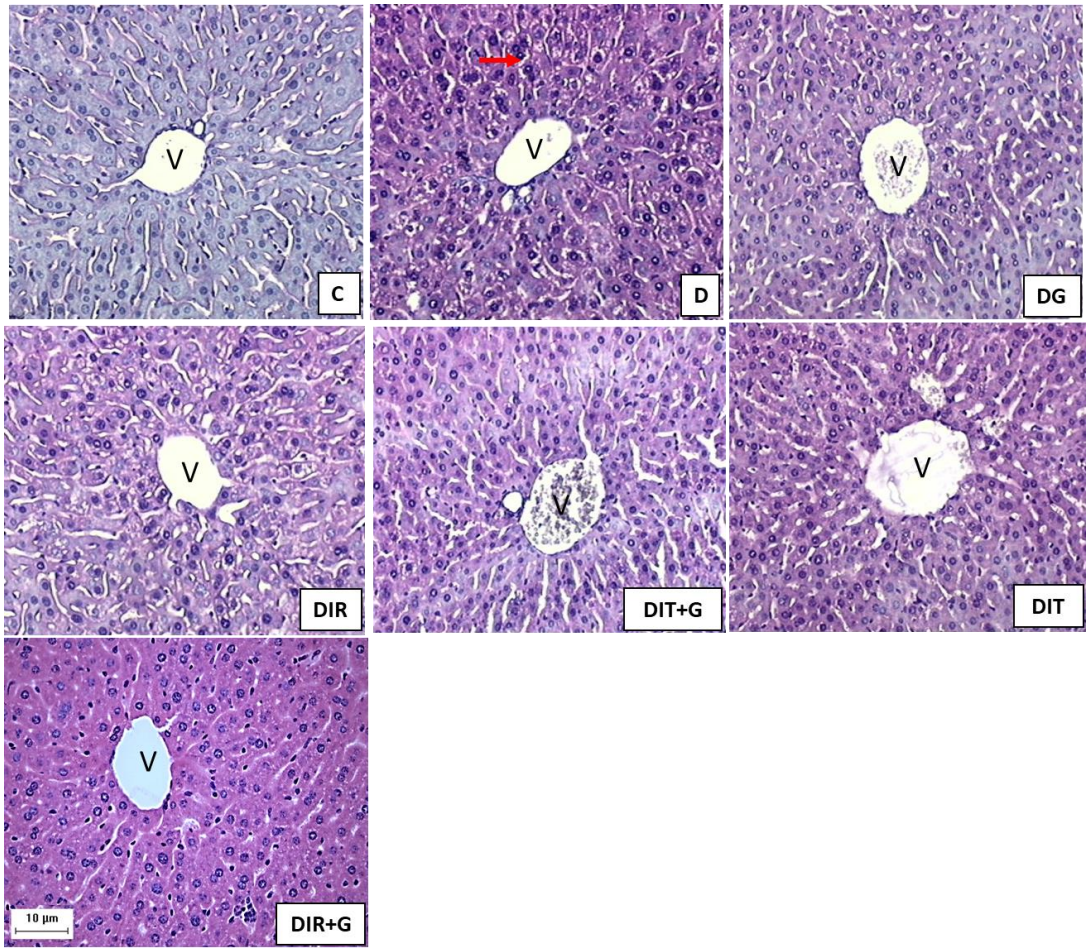

A: Insulin-induced hypoglycemia (IIH)
D
DG
DIR
DIR+G
DIT
DIT+G
Glycemia (mg/dL)
Time (min)
B: IIH + cortisol
C: IIH versus IIH + cortisol
IIH
IIH + Cortisol

Fig.3.



Parameter	C	D	DG	DIR	DIR+G	DIT	DIT+G
Number of hepatocytes $(mm^2)^{-1}$	228 ± 10^a	282 ± 7^b	284 ± 10^b	256 ± 7^c	235 ± 6^a	297 ± 4^b	286 ± 7^b
Area of hepatocytes (μm^2)	329 ± 8^a	384 ± 13^b	330 ± 8^a	370 ± 10^b	364 ± 7^b	328 ± 14^a	332 ± 7^a
Liver weight (g)	1.9 ± 0.1^a	2.2 ± 0.1^a	2.5 ± 0.1^b	1.7 ± 0.1^a	2.8 ± 0.1^c	1.9 ± 0.1^a	2.7 ± 0.1^c
AST $(U \cdot L^{-1})$	53 ± 4^a	179 ± 7^b	308 ± 13^c	381 ± 10^d	261 ± 4^e	34 ± 6^a	35 ± 2^a
ALT $(U \cdot L^{-1})$	12 ± 1^a	128 ± 3^b	178 ± 9^c	173 ± 5^c	153 ± 7^c	24 ± 1^a	12 ± 1^a

Effects of insulin degludec and glutamine dipeptide on liver metabolism of type I diabetic mice undergoing insulin-induced hypoglycemia.

Camila Bataglini[1] et al.

[1] Department of Biochemistry, State University of Maringa, PR, Brazil

Address for correspondence:

* Camila Bataglini

Department of Biochemistry

University of Maringá

87020900 Maringá, Brazil

Email: camilabataglini@hotmail.com

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ABSTRACT

Rapid- and long-acting insulins are used alone or in combination to regulate the glycemia in type 1 diabetes (T1DM), however, the insulin-induced hypoglycemia (IIH) is a very common problem related to insulinization. Normoglycemic mice, but not T1DM mice, orally receiving glutamine shortly after the IIH induction had a higher hepatic gluconeogenesis and greater ability to recover the glycemia than mice receiving glucose. On the other hand, T1DM mice receiving 1 g/Kg glutamine dipeptide (GDP) for 30 days in combination with regular or degludec insulin minimally improved the IIH of T1DM mice, however, both GDP and regular insulin caused considerable increases in the serum activity of hepatic aminotransferases, which is normally associated with liver damage and modifications of liver metabolism. Therefore, this study investigated whether the 30-day co-treatment with GDP and regular (rapid acting) or degludec (long acting) insulins modifies the glucose metabolism in the perfused liver of alloxan-induced Swiss T1DM mice undergoing IIH. Treated and untreated mice fasted for 15 h received regular insulin to induce IIH and one hour after the liver perfusion procedure was performed to evaluate the glycogen metabolism, gluconeogenesis and ureagenesis. Glycogenolysis was increased in T1DM mice receiving GDP alone or in combination with regular insulin, a situation that reflects an increased liver content of glycogen. In the gluconeogenic interval, the liver output of glucose was not different for all groups, but the alanine deamination, calculated as liver output of nitrogen, was decreased for all groups receiving GDP or insulins. In contrast, the stimulus of gluconeogenesis was lower only in the liver of T1DM mice receiving GDP alone or in combination with both insulins. In this case, the unmodified liver glucose output may be occurring at the expense of increased liver glycogenolysis and possibly a hepatic glycogen overloaded.

Keywords*: insulin degludec, glutamine dipeptide, liver metabolism, type I diabetes, gluconeogenesis, ureagenesis.*

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that results from the loss of β-pancreatic cells and consequent failure to produce insulin [American Diabetes Association, 2014]. The disease leads to serious metabolic abnormalities, particularly hyperglycemia, which occurs mainly as consequence of decreased peripheral glucose uptake and upregulation of gluconeogenesis in the diabetic liver [Sriram et al, 2014]. Rapid and long-acting insulins are used alone or in combination to regulate the glycemia in type 1 diabetes (T1DM however, the insulin-induced hypoglycemia (IIH) is a very common problem related to insulinization [Abraham et al., 2016]. The most commonly short-acting insulin used is the regular insulin, with have a bolus activity and effects lasting 6-8 h [American Diabetes Association, 2019]. Long-acting or basal insulins, on the other hand, have no plasma peaks and a longer acting. Among them, the ultralong-acting insulin degludec (25 h half-life) has stood out for maintaining a more constant level of serum insulin and a lower day-to-day glycemic variability [Heise et al., 2012]. The regulation of glycemia in T1DM requires multiple daily doses of prandial insulins or continuous subcutaneous insulin infusion, or even the combination of basal and fast-acting insulins [American Diabetes Association, 2016]. Nevertheless, the insulin therapies are usually associated with episodes of insulin-induced hypoglycemia (IIH) [Abraham et al., 2016].

L-Glutamine is the most abundant free amino acid in the human blood and plays significant role in a number of physiological processes, such as blood ammonia transport, blood pH regulation and biosynthetic pathways associated with cellular integrity and function [Cruzat et al., 2018]. This amino acid is also associated with the regulation, via cellular swelling, of the expression of proteins linked to energy metabolism and in addition has been reported to have anti-inflammatory and immunomodulatory activities [Cruzat et al., 2018]. In fact, L-glutamine supplementation improves the clinical condition of individuals with chronic diseases related to loss of cell mass, including T1DM [Darmaun et al., 2019; Hartmann et al., 2017].

Dietary nutrients absorbed by the enterocytes first pass through the liver before reaching the systemic circulation. This strategic position of the liver, between the gastrointestinal tract and peripheral circulation, gives it a pivotal

role in the metabolic homeostasis so that is the site of numerous pathways linked to intermediary metabolism, including those related to glucose metabolism, such as glycolysis and gluconeogenesis [Sharabi et al., 2015]. Therefore, the liver is no wonder to be engaged in metabolic diseases such as diabetes. Glutamine is also a relevant substrate for hepatic gluconeogenesis, a crucial metabolic pathway to maintain the glycemia after glycogen depletion during fasting and hypoglycemic episodes [Cruzat et al., 2018]. In this regard, normoglycemic mice, but not T1DM mice, orally receiving glutamine shortly after the IIH induction had a higher hepatic gluconeogenesis from alanine and greater ability to recover the glycemia than mice receiving alanine or even glucose [Santiago et al., 2013; Bataglini et al., 2017]. T1DM rats orally receiving 1 g/Kg glutamine dipeptide (GDP) shortly after IIH induction show even a lower gluconeogenesis from alanine plus glutamine (each 5 mM) [Bataglini et al., 2017]. On the other hand, T1DM mice receiving 1 g/Kg GDP for 30 days in combination with regular or degludec insulin had minimally improved the IIH, however, both GDP and regular insulin caused considerable increases in the serum activity of hepatic aminotransferases, which is normally associated with liver damage and modifications of liver metabolism [Bataglini et al., 2020]. Therefore, this study investigated whether 30-day supplementation with glutamine dipeptide (GDP) alone or in combination with insulin degludec or regular insulin therapy modifies the glucose metabolism in the liver of T1DM mice during the insulin-induced hypoglycemia (IIH).

The dipeptide L-alanine-L-glutamine, known as glutamine dipeptide, has been widely used in clinical practice in substitution of free glutamine because this dipeptide is associated with a higher bioavailability of glutamine in the blood, given the high consumption of free glutamine by intestinal cells [Raizel & Tirapegui, 2018].

MATERIAL AND METHODS

Chemicals

Glutamine dipeptide, alloxan, lactate dehydrogenase and L-glutamic dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial kits for glucose, urea, ammonia, pyruvate and lactate were purchased from Gold Analisa Diagnóstica Ltda (Belo Horizonte, MG, Brazil). Insulin degludec Tresiba® FlexTouch® (Novo Nordisk®, Bagsværd, Denmark) and regular insulin Humulin® R (Eli Lilly, Indianapolis, EUA) were purchased from commercial pharmacies. All other chemicals were of analytical grade.

Animals and induction of diabetes

Male *Swiss* mice weighing around 25g (30 days old adult animals) were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and maintained under standard laboratory conditions at a temperature of 24±2ºC and a regulated 12h light/dark cycle. The animals were housed in conventional steel cages (1 mice/cage) and were fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil). After 3 days for acclimatization, animals fasted for 15 h were diabetized with alloxan (180 mg/Kg, i.p.). Four days after, fed and fasting glycemia was measured in the blood collected by tail incision (glucometer and glycophyte Optium Xceed®, Abbott, Brazil). Animals with fasting and postprandial glycemia higher than 300 mg/dL were considered T1DM. All procedures were performed as recommended by the Brazilian Council for the Control of Animal Experimentation (CONCEA) and were approved by the Ethics Committee for Animal Use (CEUA) of the State University of Maringá (protocol number 1334110116).

Experimental design and treatment

Sixty diabetic mice were subsequently distributed into six groups (n=10 per group): D, which received saline; DG, which received glutamine dipeptide (GDP); DIR, which received regular insulin; DIR+G, which received regular insulin and GDP; DIT, which received insulin degludec; and DIT+G, which

received insulin degludec and GDP. Ten non-diabetic mice received saline and served as control (C). The animals received daily for 30 days glutamine dipeptide (GDP; 1 g/kg, oral gavage) or saline (gavage) and, for the same period, saline (s.c.) or regular insulin (1 U/kg, i.p.) or insulin degludec (5 U/kg; s.c., via infusion pump). This treatment protocol was repeated two times to determine all proposed parameters, totaling 140 animals.

Liver perfusion

After treatment for 30 days, control and T1DM mice fasted for 15 h received regular insulin (1 U/Kg) to induce IIH and cortisol 15 min later. One hour after IIH, the non-recirculating liver perfusion was performed as previously described [Santiago et al., 2013]. For this, animals were deeply anesthetized with Thionembutal® (30 mg/Kg) and lidocaine (10 mg/kg) and submitted to midline laparotomy to liver exposure. After cannulation of the portal and cava veins, the liver was perfused *in situ* with Krebs/Henseleit-bicarbonate buffer (KH buffer; pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment to 37ºC. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and it was adjusted to approximately 4 mL/min. After an initial period of 10 min of perfusion to stabilize the preparation, samples of the effluent perfusion fluid were collected at five minutes intervals and analyzed for their metabolites content. During this period, liver perfusion was performed as follows: 30 min with KH buffer, 30 min with KH buffer plus glucagon (1.0 µM), 30 min with KH buffer plus the gluconeogenic precursor L-alanine (5.0 mM) and another 30 min with KH buffer. Fig. 1 illustrates the perfusion protocol used in the present study. Glucose, lactate, pyruvate, ammonia and urea were measured by enzymatic procedures [Bergmeyer, 1974].

Glycolytic and glycogenolytic fluxes were calculated from the values (area under the curve; AUC) of glucose, lactate and pyruvate released in the effluent perfusate when the liver was perfused with KH buffer (0 to 30 min in Fig. 1) and with KH buffer plus glucagon (30-60 min). At steady state, glycolysis = (pyruvate + lactate)/2 and glycogenolysis = glucose + [(lactate + pyruvate)/2] [Castro-Ghizoni et al., 2017]. Gluconeogenesis and ureagenesis correspond

respectively to glucose and urea production (AUC) when the liver was perfused with KH buffer plus L-alanine.

Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of the GraphPad Prism Software (version 5.0). Data were firstly submitted to Kolmogorov-Smirnov (K-S) test to verify the normality. The statistical significance of the normalized data was analyzed by means of ANOVA ONE-WAY and the Tukey post-hoc test was applied with the 5% level of significance ($p<0.05$).

RESULTS

In order to evaluate the liver metabolism during IIH, after treatment for 30 days, the mice fasted for 15h received regular insulin and, 15 min latter, cortisol. One hour after the insulin administration, the liver perfusion was performed. The hepatic production of glucose, lactate, pyruvate, urea and ammonia as a function of perfusion time is shown in Fig. 1, which also presents the experimental protocol. It is noted that glucose production in groups D, DG and DIR+G is high at the beginning of the liver perfusion with Krebs-Henseleit buffer (KH) and declines continuously until time 30 min. This phenomenon is associated with glucose release from the glycogen storages, which is high in diabetic rats even after a 24 h fasting period [Comar et al., 2016]. With the intention of completely depleting the glycogen storage, glucagon was then simultaneously infused in the liver by additional 30 min (until time 60 min). There was no considerable modification in the production of metabolites during this perfusion period, except by an increase of glucose production in DIR+G. After the introduction of alanine (time 60 min), there were different degrees of stimulation in the production of glucose, lactate, pyruvate, ammonia and urea. With the purpose to compare the metabolites production, the production of these metabolites for all groups was calculated as area under the curve (AUC) at the three intervals of perfusion: KH (0 to 30 min), KH + glucagon (30 to 60 min) and KH + alanine (60 to 90 min). From these values the glycogenolytic and glycolytic flow were calculated at the first two intervals. The results are shown in Fig. 2. Similarly, the production of these metabolites in the perfusion period following alanine introduction (60 to 90 min) is shown in Fig. 3.

The content of glucosyl units released by glycogenolysis and those metabolized through glycolysis are shown in Fig. 2. In the first perfusion period (KH: AUC 0-30 min), glycogenolysis was 230, 470 and 140% higher than the controls for D, DG and DIR+G, respectively. In the presence of glucagon (KH + Glucagon: AUC 30-60 min), glycogenolysis was not modified for C, DIR, DIT and DIT+G, but it was lower for D, DG and DIR+G (compared to the first interval; $p<0.05$, Student t test), although the values were still 130 and 50% higher for DG and DIR+G when compared to the controls in the same perfusion period. Glycolysis was increased at the first perfusion period for DG (150%) and decreased for DIT (40%) and DIT+G (50%) when compared to the controls. In

the presence of glucagon, there was no modification of glycolysis for all groups (compared to the first interval; $p<0.05$, Student t test), except by a reduction of 40% for DG.

In the liver alanine is first converted to pyruvate by the enzyme alanine aminotransferase (ALT). This reaction transfers the amino group from alanine to alpha-ketoglutarate generating glutamate, which can be deaminated by the enzyme glutamate dehydrogenase releasing ammonia and regenerating the alpha-ketoglutarate. The pyruvate is then mainly converted to lactate by the enzyme lactate dehydrogenase (LDH) or glucose through gluconeogenesis, while ammonia is converted to urea through the urea cycle. Fig. 3 shows the content of glucose, lactate, pyruvate, ammonia and urea produced in the perfusion period following the alanine introduction (KH + Alanine: AUC 60-90 min). The values of the previous perfusion period (KH + Glucagon: AUC 30-60 min) are also shown for the purpose of comparison and they are those used to calculate glycolysis and glycogenolysis in Fig. 2. Following the introduction of alanine, the production of glucose was higher for all groups (compared to the previous interval; $p<0.05$, Student t test), except for DG and DIR+G, to which the production of glucose was already increased in the previous interval. In absolute values, the liver output of glucose in the perfusion period following the alanine infusion was the same for all groups, except for DIR+G (Fig. 3A). However, the increment of the glucose content due to alanine infusion was different, as shown in Table 1. For C, DIR and DIT the increment of the glucose content doubled when compared to the previous interval, while this increment was much smaller for D and DIT+G, non-existent for DIR+G and even negative for DG. In other words, T1DM mice had a lower increment in the glucose production from alanine and the treatment with both insulins in the absence of GDP prevented this drop, while a further fall occurred when they received GDP.

The production of lactate due to alanine infusion was lower in diabetic animals (D) when compared to the controls and much lower or not increased in the other groups (Fig. 3 and Table 1). The production of pyruvate after alanine introduction was 52% higher for D and 50% lower for DIR+G and DIT+G (compared to controls in the same interval). Table 1 also shows the increments normalized as 3-carbon units produced from alanine and transformed into glucose, lactate or pyruvate. The increment in the content of 3-carbon units was not different for C and D, but it was 40% lower in T1DM mice treated with both

insulins and even much lower when treated with GDP. The ratio of lactate to pyruvate was calculated in the interval following alanine introduction and it is an indicative of the cytosolic ratio of NADH to NAD^+. When compared to the controls (C), this ratio was greatly decreased in all T1DM animals, excepted for D+G (Table 1).

The production of ammonia and urea in the interval before alanine infusion (KH + Glucagon: AUC 30-60 min) was low and relatively equal for all groups (Fig. 3D and 3E). Following alanine infusion, the production of ammonia was greatly stimulated in the perfused liver. This stimulus was the same for C and D, but approximately 20% lower for DIR and DIT and 50% lower for DG, DIR+G and DIT+G. The production of urea was greatly stimulated after alanine infusion and it was equal for C, D and DG, but approximately 40% lower for DIR, DIT, DIR+G and DIT+G. This condition shows that the treatment with both insulins decreases the urea production of T1DM mice. Table 1 also presents the increment normalized as nitrogen units produced from alanine, which was not decreased in diabetic animals (D), but it was approximately 30% lower for T1DM animals receiving GDP or insulin (DG, DIR and DIT) and even much lower in T1DM animals receiving insulin plus GDP (DIR+G and DIT+G).

DISCUSSION

In T1DM individuals, recurrent episodes of hyper and hypoglycemia take place as consequence of inadequate insulin therapy or even non-balanced diet. Particularly the recurrent hypoglycemia has devastating effect on the glycemic control and on the short and the long-term well-being of individuals. The blood glucose of fasted normoglycemic mice drops sharply 60 min after IIH but it is recovered almost completely at 300 min [Santiago et al., 2013]. The blood glucose of fasted T1DM mice drops sharply 60 min after the administration of insulin to induce IIH and it is not modified even after 300 min [Bataglini et al., 2020]. Similar phenomenon has also been verified in patient and rodent models of type 2 diabetes and T1DM during IIH [Chakera et al., 2018; Bataglini et al., 2017]. T1DM mice present in addition lower body weight gain and increased serum activity of aminotransferases [Bataglini et al., 2020]. The latter has also been reported for T1DM patients and it is related to a poorer glycemic control and even liver failure [Stadler et al., 2017]. This study used the alloxan-induced T1DM in Swiss mice subjected to 30-day supplementation with GDP in combination with insulin degludec or regular insulin. The model of IIH of this study is similar to T1DM patients with very low or no response to the hypoglycemic counterregulatory system [McCrimmon & Sherwin, 2010].

The increment of gluconeogenesis from alanine during IIH was lower in the liver of untreated T1DM mice. In fact, glucose production from alanine has been reported absent in perfused liver of T1DM rats and even normoglycemic rats present no additional stimulus in the hepatic glucose production from alanine (5 mM) during IIH [Gazola et al., 2007; Ferraz et al., 1997]. However, the liver glucose output was not decreased because it was already increased as consequence of the higher glycogenolysis in the livers of T1DM mice. Similar phenomenon also occurs when livers of T1DM rats are perfused with glutamine [Comar et al., 2016]. This has been attributed to high hepatic glycogen content as consequence of high glycogen synthase to glycogen phosphorylase activity ratio [Gannon et al., 1997]. By the way, the liver glucose output following alanine infusion was not decreased for all groups. The lactate output due to alanine was decreased in the liver of T1DM mice, but pyruvate production was higher, a situation that led to a lower ratio of lactate to pyruvate, indicating a

lower cytosolic ratio of NADH to NAD^+. On the other hand, the production of ammonia and urea were not modified in the liver of these animals.

The stimulus of hepatic gluconeogenesis from alanine during IIH was similar to that in normoglycemic mice when T1DM animals were treated with both regular and degludec insulins, even with a low cytosolic ratio of NADH to NAD^+. The latter condition shows that the reduced stimulus of hepatic gluconeogenesis of T1DM mice is probably not associated with liver failure or oxidized environment. On the other hand, both insulins decreased the hepatic production of ammonia and urea in T1DM mice, a situation that was not observed even in untreated T1DM mice. This resulted in proportional reduction in the liver output of nitrogen units and 3-carbon units in the interval following alanine introduction, a phenomenon probably associated with a reduction in the hepatic deamination this amino acid. The reduction of 3-carbon units, however, did not restrict its flow to produce glucose nor occurred at the expense of decreased production of pyruvate, but as consequence of a drop in the production of lactate (Table 1).

Glutamine is the most abundant endogenous gluconeogenic precursor and the supplementation with this amino acid could relieve the episodes of hypoglycemia [Cruzat et al., 2018]. Glutamine was orally administered as a dipeptide (GDP), which is not metabolized by the gastrointestinal tract [Rosa et al., 2015]. After absorption by the enterocytes, it first passes through the liver and might modify the hepatic response and contribute to glycemic recovery. This study employed the dose of 1 g/kg, as previously used in T1DM mice [Bataglini et al., 2020; Bataglini et al., 2017] and it is equivalent to the dose of 0.6 g/Kg of glutamine, which has been reported to cause no adverse effects in humans [Poindexter et al., 2003; Garlick, 2001]. The long-term supplementation of T1DM mice with GDP does not improve the fasting glycemia or body weight gain and it only slightly attenuates the IIH [Bataglini at al., 2020; Bataglini et al., 2017]. In the present study the supplementation of T1DM mice with GDP did not modify the glucose output due to alanine infusion in the perfused livers, but it significantly decreased the stimulus of hepatic gluconeogenesis and the increase of lactate production during IIH, phenomenon that also resulted in a marked reduction in the production of 3-carbon units from alanine (Table 1). Considering that the treatment decreased the ammonia production without modifying ureagenesis, it is possible to say that alanine deamination was decreased, a

situation that provided less pyruvate and lactate for hepatic gluconeogenesis. Thus, the unmodified liver output of T1DM mice treated with glutamine might be occurring at the expense of increased hepatic glycogenolysis. However, the simple subtraction of the high basal rates after infusion of alanine is a risky procedure that might lead to erroneous interpretations of the gluconeogenesis [Oliveira et al., 2007; Akimoto et al., 2000]. For discriminating gluconeogenesis from glycogenolysis in this case, it will be necessary to use radioactive alanine, which allow to measure specifically the newly formed glucose. Otherwise, glycogen levels and gluconeogenesis flow can also be simultaneously measured so that the adequate corrections can be done.

The higher glycogenolysis in the liver of T1DM mice receiving GDP alone or in combination with regular insulin is probably associated with a high hepatic glycogen content, i.e., a hepatic glycogen overloaded, which can result in increased serum activity of AST and ALT as consequence of liver damage. In this regard, glutamine is not only the most effective amino acid for enhancing glycogen formation, but it has been also associated with the stimulation of glycogen synthetase as consequence of hepatocyte swelling after rapid uptake of glutamine [Cruzat et al., 2018; Stumvoll et al., 1999]. One possible justification would be the increased availability of energy substrate as consequence of GDP supplementation associated with sustained levels of exogenous insulin.

In summary, the 30-days treatment of T1DM mice with GDP decreases the liver gluconeogenesis from alanine, but without modifying the liver output of glucose as consequence of increased glycogenolysis. The latter seems be the consequence of hepatic glycogen overload, which also increased the serum activity of AST and ALT, a situation that can be associated with liver damage. Thus, the long-term administration of 1 g/Kg GDP in T1DM individuals should be reviewed, especially if associated with regular insulin.

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List of abbreviations

Abbreviations: T1DM, type 1 diabetes; IIH, insulin-induced hypoglycemia; GDP, glutamine dipeptide; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Competing interests

The authors declare that no competing interest exists and that all approved the final manuscript.

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Table 1. Increment in the production of glucose, lactate, 3-carbon units and nitrogen units and lactate to pyruvate ratio due to alanine infusion in perfused liver. The increments (Δ) for all metabolites were calculated by subtracting AUC of perfusion period before alanine infusion (KH + Glucagon: 30-60 min) from those of AUC in the perfusion period following alanine infusion (KH + alanine: 60-90 min) in Fig. 3. The increment in glucose and lactate production is expressed as percentage (%). The 3-carbon units increment (AUC) was calculated as lactate + pyruvate + 2 x glucose. The increment of nitrogen units (AUC) was calculated as ammonia + 2 x urea. Control (C), T1DM (D), T1DM + GDP (DG), T1DM + regular insulin (DIR), T1DM + insulin degludec (DIT), T1DM + regular insulin + GDP (DIR+G) and T1DM + insulin degludec + GDP (DIT+G). Values are the mean ± SEM of 5 mice. Values are the mean ± SEM of 5 animals.

Parameter	**C**	**D**	**DG**	**DIR**	**DIR+G**	**DIT**	**DIT+G**
Δ Glucose (%)	+92	+67	-31	+95	0	+110	+42
Δ Lactate (%)	+210	+128	+43	0	0	+67	0
Δ 3-carbon units (µmol·g^{-1})	18.5 ± 2,1^{a}	19.4 ± 1.2^{a}	7.5 ± 0.7^{b}	11.2 ± 0.3^{c}	6.0 ± 0.2^{b}	11.5 ± 0.6^{c}	5.6 ± 0.1^{b}
Lactate to pyruvate ratio	1.7 ± 0.1^{a}	0.7 ± 0.1^{b}	1.8 ± 0.3^{a}	0.6 ± 0.1^{b}	0.5 ± 0.2^{b}	0.9 ± 0.2^{b}	0.4 ± 0.1^{b}
Δ Nitrogen units (µmol·g^{-1})	208 ± 4^{a}	198 ± 5^{a}	142 ± 4^{b}	146 ± 4^{b}	99 ± 4^{c}	133 ± 2^{b}	114 ± 5^{c}

Values with different superscript letters in the same line are different ($p<0.05$)

Figure captions

Fig. 1. Time courses of glucose, lactate, pyruvate, ammonia and urea in the perfused liver of T1DM mice during IIH. Animals were treated during 30 days with GDP (1 g/Kg, p.o.) and regular insulin (1 U/Kg, i.p.) or insulin degludec (5 U/Kg, s.c.). Animals fasted for 15 h received regular insulin (1 U/Kg) to induce IIH and cortisol (20 mg/kg), respectively, 1 h and 45 min prior to the liver perfusion procedure. Glucagon (1.0 μM) and L-alanine (5.0 mM) were infused in combination with Krebs-Henseleit bicarbonate buffer (KH) as shown by the horizontal bars. Control (C), T1DM (D), T1DM + GDP (DG), T1DM + regular insulin (DIR), T1DM + insulin degludec (DIT), T1DM + regular insulin + GDP (DIR+G) and T1DM + insulin degludec + GDP (DIT+G). Values are the mean ± SEM of 5 animals for each group.

Fig. 2. Glycogenolysis and glycolysis in the perfused liver of T1DM mice during IIH. KH (AUC: 30-60): area under the curve (AUC) corresponding to the interval of time 0 to 30 in Fig. 1; KH + Glucagon: AUC corresponding to the interval of time 30 to 60 min in Fig. 1. Glycolysis = (pyruvate + lactate)/2 and glycogenolysis = glucose + [(lactate + pyruvate)/2]. Control (C), T1DM (D), T1DM + GDP (DG), T1DM + regular insulin (DIR), T1DM + insulin degludec (DIT), T1DM + regular insulin + GDP (DIR+G) and T1DM + insulin degludec + GDP (DIT+G). Values are the mean ± SEM of 5 animals for each group. Values with different letters in the same protocol are different ($p \leq 0.05$ C). Data were evaluated by analysis of variance (ANOVA) with Tukey post-test.

Fig. 3. The production of glucose, lactate, pyruvate, ammonia and urea from alanine in the liver of T1DM mice during IIH. KH + Glucagon: corresponds to the area under the curve (AUC) for each metabolite in the interval of time 30 to 60 min in Fig. 1. KH + alanine: corresponds to AUC for each metabolite in the interval of time 60 to 90 min in Fig. 1. Control (C), T1DM (D), T1DM + GDP (DG), T1DM + regular insulin (DIR), T1DM + insulin degludec (DIT), T1DM + regular insulin + GDP (DIR+G) and T1DM + insulin degludec + GDP (DIT+G). Values are the mean ± SEM of 5 animals for each group. Values with different letters in the same protocol are different ($p \leq 0.05$ C). Data were evaluated by analysis of variance (ANOVA) with Tukey post-test.

Fig. 1.

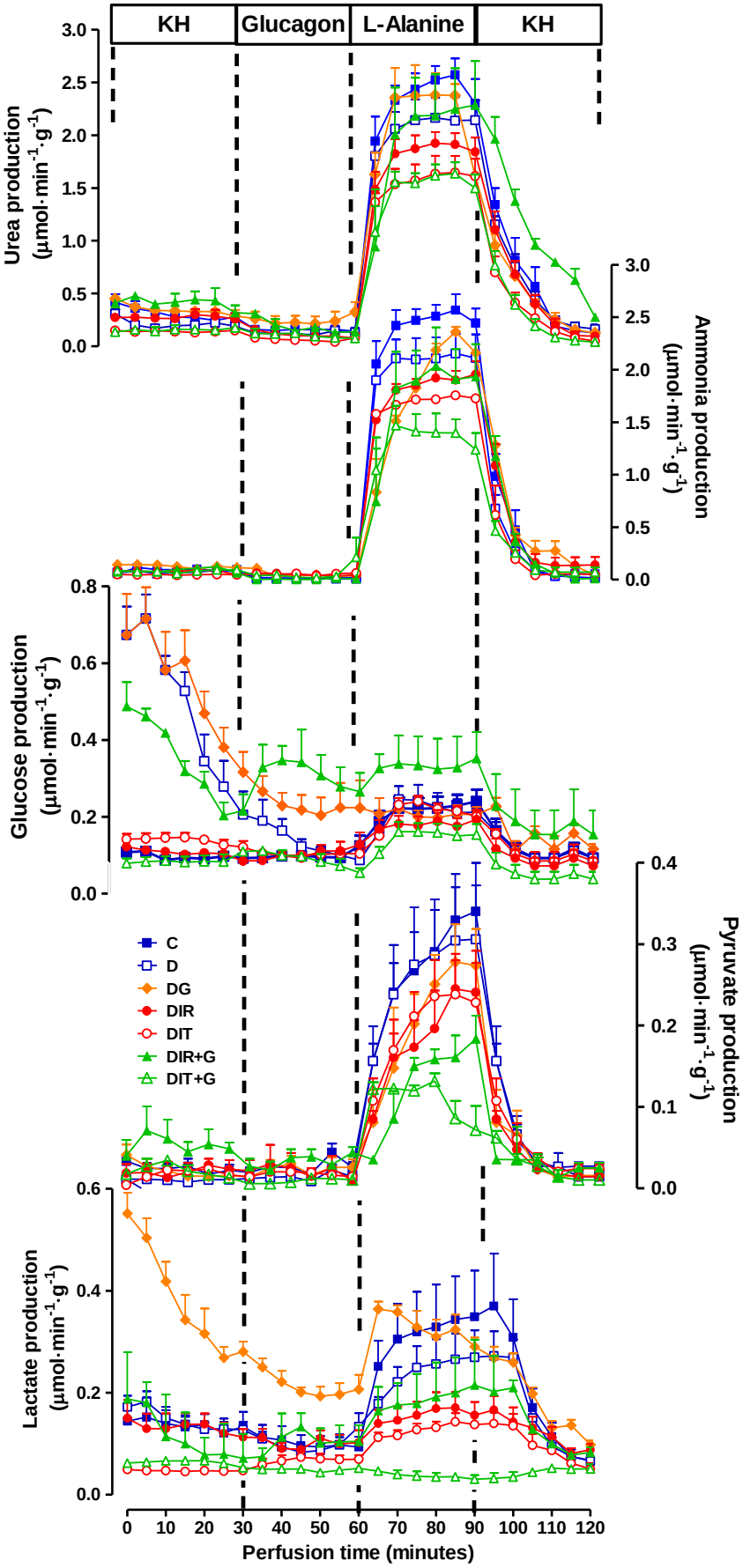

KH
Glucagon
L-Alanine
KH
Urea production (µmol·min-1·g-1)
Ammonia production (µmol·min-1·g-1)
Glucose production (µmol·min-1·g-1)
Pyruvate production (µmol·min-1·g-1)
Lactate production (µmol·min-1·g-1)
C
D
DG
DIR
DIT
DIR+G
DIT+G
Perfusion time (minutes)

Fig. 2.

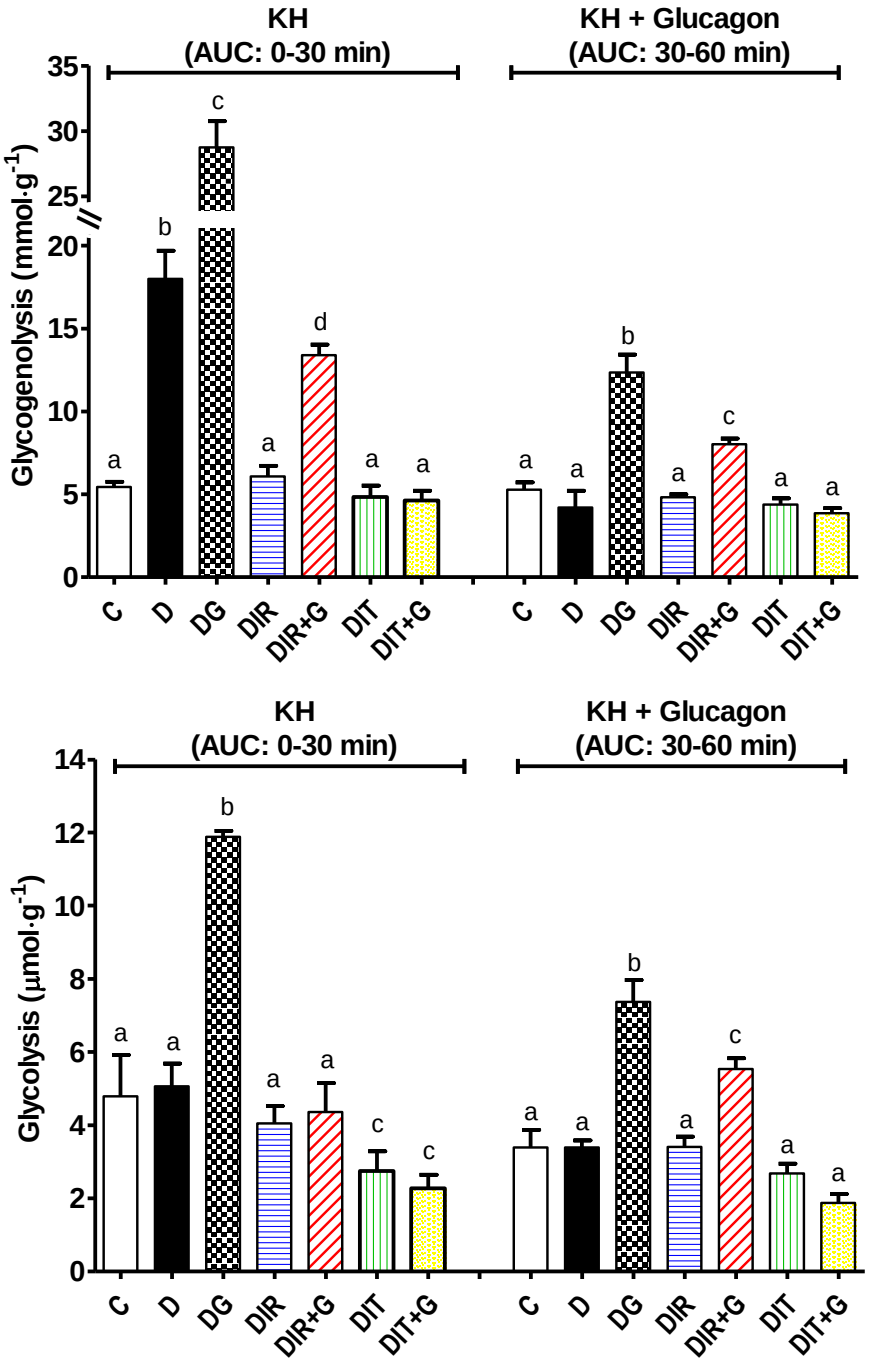
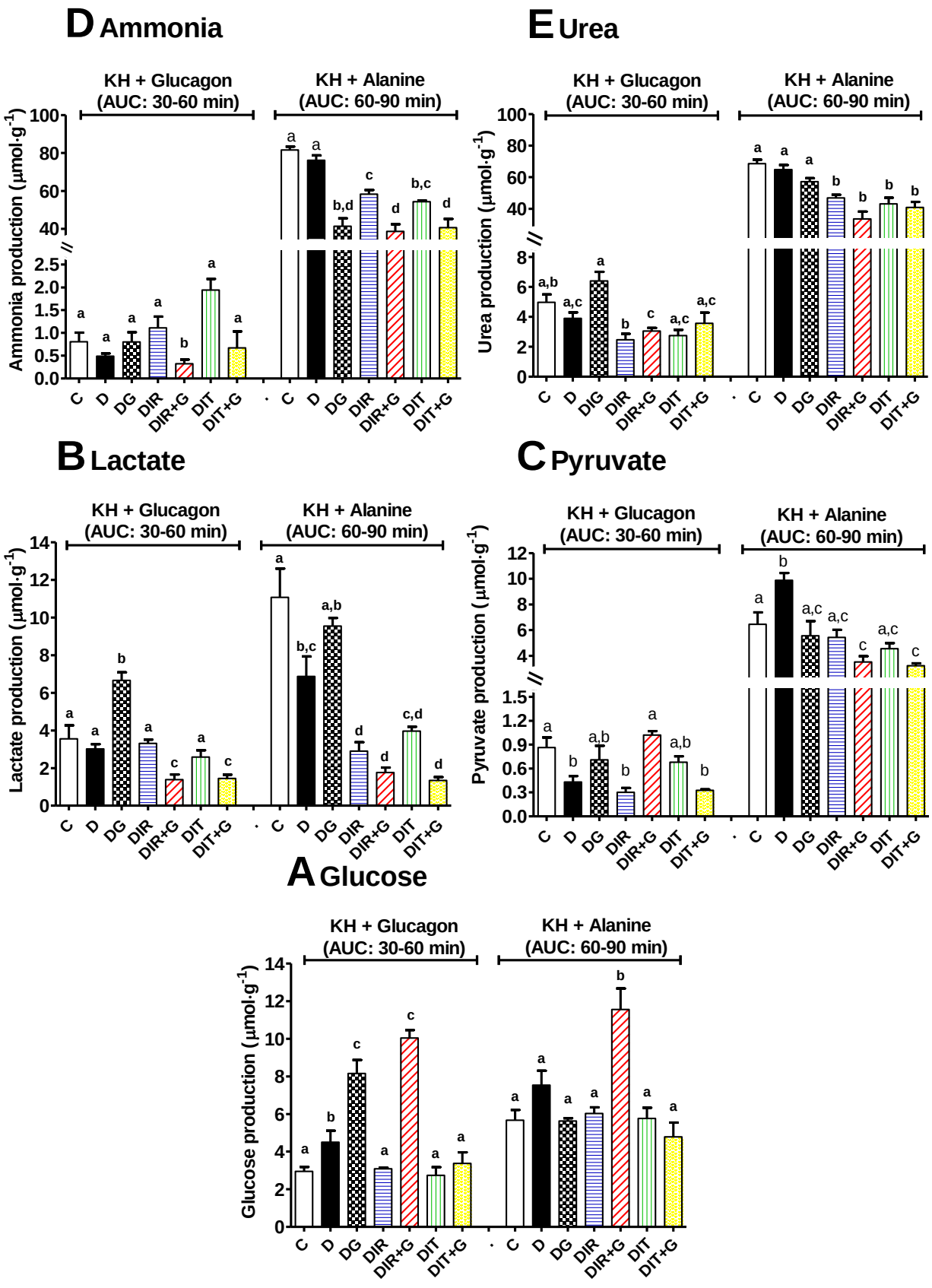

KH
(AUC: 0-30 min)
KH + Glucagon
(AUC: 30-60 min)
Glycogenolysis (mmol·g-1)
35
30
25
20
15
10
5
0
a
b
c
a
d
a
a
a
a
b
a
c
a
a
C
D
DG
DIR
DIR+G
DIT
DIT+G
KH
(AUC: 0-30 min)
KH + Glucagon
(AUC: 30-60 min)
Glycolysis (µmol·g-1)
14
12
10
8
6
4
2
0
a
a
b
a
a
c
c
a
a
b
a
c
a
a
C
D
DG
DIR
DIR+G
DIT
DIT+G

Fig. 3.


D Ammonia
E Urea
B Lactate
C Pyruvate
A Glucose
KH + Glucagon (AUC: 30-60 min)
KH + Alanine (AUC: 60-90 min)
Ammonia production (µmol·g-1)
Urea production (µmol·g-1)
Lactate production (µmol·g-1)
Pyruvate production (µmol·g-1)
Glucose production (µmol·g-1)
C
D
DG
DIR
DIR+G
DIT
DIT+G

ANEXO I: Parecer da Comissão de Ética no Uso de Animais – UEM

Comissão de Ética no Uso de Animais
da
Universidade Estadual de Maringá

CERTIFICADO

Certificamos que o Projeto intitulado "AVALIAÇÃO DO ESTRESSE OXIDATIVO E DO METABOLISMO DE HEPATÓCITOS ISOLADOS DE CAMUNDONGOS SWISS DIABÉTICOS TIPO 1 SUPLEMENTADOS COM GLUTAMINA DIPEPTÍDEO E SUBMETIDOS A DIFERENTES PADRÕES DE INSULINIZAÇÃO.", protocolado sob o CEUA nº 1334110116, sob a responsabilidade de **Vilma Aparecida Ferreira De Godoi e equipe; *Camila Bataglin; Cristian Lima Borrasca; Elizete Rosa Dos Santos Silva; Juliana Nunes De Lima Martins; Jurandir Fernando Comar; Marcia Fabricio; Nayra Thais Delatorre Branquinho ; Silvia C S F Azevedo; Vilma Aparecida Ferreira De Godoi*** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei 11.794, de 8 de outubro de 2008, com o Decreto 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA/UEM) em reunião de 26/01/2016.

We certify that the proposal "ASSESSMENT OXIDATIVE STRESS AND ISOLATED HEPATOCYTES METABOLISM OF MICE SWISS TYPE 1 DIABETES SUPPLEMENTED WITH GLUTAMINE DIPEPTIDE AND SUBJECT TO DIFFERENT INSULINIZATION STANDARDS.", utilizing 432 Heterogenics mice (432 males), protocol number CEUA 1334110116, under the responsibility of **Vilma Aparecida Ferreira De Godoi *and team; Camila Bataglin; Cristian Lima Borrasca; Elizete Rosa Dos Santos Silva; Juliana Nunes De Lima Martins; Jurandir Fernando Comar; Marcia Fabricio; Nayra Thais Delatorre Branquinho ; Silvia C S F Azevedo; Vilma Aparecida Ferreira De Godoi*** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes (or teaching) - it's in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the State University of Maringá (CEUA/UEM) in the meeting of 01/26/2016.

Vigência da Proposta: de 03/2016 a 03/2019 Laboratório: Dfisbiofísica

Procedência: Biotério Central da UEM

Espécie:	Camundongo heterogênico	Gênero: Machos	Idade:	50 dias	N: 432
Linhagem:	Swiss		Peso:	30 g	

Resumo: O metabolismo hepático é fundamental para manutenção da glicemia. É regulado pelos hormônios insulina e os seus antagonistas glucagon, adrenalina, cortisol e hormônio do crescimento. A ausência da insulina promove o Diabetes Mellitus tipo 1 (DM1), cujo tratamento consiste da administração de insulina. No entanto, a insulina-terapia intensiva também aumenta a incidência de hipoglicemia induzida por insulina (HII) e representa o principal obstáculo à implementação deste tratamento. Existe uma associação entre a progressão das complicações crônicas do DM1 com o excesso de radicais livres (ROS) e os produtos de glicação avançada (AGE) que contribuem com o desenvolvimento das complicações associadas ao aumento do estresse oxidativo. Dentro desse contexto, a L-glutamina, um aminoácido classificado como condicionalmente essencial no DM1, tem ganhado destaque científico por ser uma substância que pode reduzir o estresse oxidativo diabético e as complicações crônicas associadas, abrindo possibilidades terapêuticas coadjuvantes para sua suplementação. A Insulina Regular, mais comum e acessível, apresenta picos elevados e ação é rápida (3–6 h), criando períodos de hiperinsulinemia e de ausência basal de insulina. A insulina basal degluteca, não provoca picos e tem longa duração (de 24–42 h) promovendo melhor controle metabólico. Em humanos, a falência do sistema contrarregulador surge após decorridos dez anos do diagnóstico da doença. Neste contexto, a avaliação do potencial terapêutico da L-glutamina como coadjuvante no tratamento de estados catabólicos como o DM1, em associação com insulinoterapias promovidas por insulinas basais como a degluteca torna-se relevante. Portanto, este projeto visa avaliar, após um período de trinta dias, os efeitos da suplementação oral com L-glutamina, na forma de GDP (glutamina dipeptídeo) em camundongos Swiss DM1 submetidos à insulinoterapia, com Insulina regular ou Insulina degluteca (Tresiba®), sobre o perfil plasmático, a resposta contrarreguladora, a morfofisiologia e histoquímica hepática e o estresse oxidativo.

Maringá, 27 de janeiro de 2016

Comissão de Ética no Uso de Animais
da
Universidade Estadual de Maringá

Prof. Dr. Alexandre Ribas de Paulo
Coordenador da Comissão de Ética no Uso de Animais
Universidade Estadual de Maringá

ANEXO II: Normas da revista "Journal of Cellular Biochemistry"

1. SUBMISSION

Authors should kindly note that submission of a paper will be held to imply that it is unpublished work which is not being considered for publication elsewhere. If accepted, it is expected that the paper will not be published in another journal or book in either the same or another format or language.

4. PREPARING THE SUBMISSION

Cover Letters

Submissions should include a cover letter. The cover letter must state that the manuscript has not been submitted or published at any other journal, the researchers' compliance with local, state and national regulations for use of animal or human subjects, and anything else the author wishes for the editor to know. This could be specific questions the author wishes for reviewers to address, or suggestions for reviewers.

Parts of the Manuscript

The manuscript should be submitted in separate files: main text file; figures.

Main Text File

The text file should be presented in the following order:

- A short informative title containing the major key words. The title should not contain abbreviations (see Wiley's best practice SEO tips);
- A short running title of less than 40 characters;
- The full names of the authors;
- The author's institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;
- Acknowledgments;
- Abstract and keywords;
- Main text;
- References;
- Tables (each table complete with title and footnotes);
- Figure legends;
- Appendices (if relevant)

Figures and supporting information should be supplied as separate files.

Authorship

Please refer to The *Journal of Cellular Biochemistry*'s Authorship policy in the Editorial Policies and Ethical Considerations section for details on author listing eligibility.

Acknowledgements

Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section. Financial and material support should also be mentioned. Thanks to anonymous reviewers are not appropriate.

Conflict of Interest Statement

Authors will be asked to provide a conflict of interest statement during the submission process. For details on what to include in this section, see the 'Conflict of Interest' section in the Editorial Policies and Ethical Considerations section below. Submitting authors should ensure they liaise with all co-authors to confirm agreement with the final statement.

Abstract

The Abstract should be clearly written in 300 words or less and should succinctly state the objectives of the study, experimental design, major observations and conclusions, and their major significance. The abstract should be intelligible to neuroscientists in general and should

thus be free of specialized jargon and abbreviations. References should generally not be cited in the abstract, but if they are, the complete citation should be given. Please provide main keywords.

Keywords

Please provide up to 5-7 keywords. Keywords should be taken from those recommended by the US National Library of Medicine's Medical Subject Headings (MeSH) browser list at **www.nlm.nih.gov/mesh**.

Main Text

- The *Journal of Cellular Biochemistry* uses British/US spelling; however, authors may submit using either option, as spelling of accepted papers is converted during the production process.
- Footnotes to the text are not allowed and any such material should be incorporated into the text as parenthetical matter.

References

References should be prepared according to the Publication Manual of the American Psychological Association (6th edition). This means in text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). The complete reference list should appear alphabetically by name at the end of the paper. References should be limited to 50. (A maximum of 25 table and figure footnotes is allowed if necessary).

Examples of APA references are listed below. Please note that a DOI should be provided for all references where available. For more information about APA referencing style, please refer to the **APA FAQ**. Please note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

Endnotes

Endnotes should be placed as a list at the end of the paper only, not at the foot of each page. They should be numbered in the list and referred to in the text with consecutive, superscript Arabic numerals. Keep endnotes brief; they should contain only short comments tangential to the main argument of the paper.

Footnotes

Footnotes should be placed as a list at the end of the paper only, not at the foot of each page. They should be numbered in the list and referred to in the text with consecutive, superscript Arabic numerals. Keep footnotes brief; they should contain only short comments tangential to the main argument of the paper and should not include references.

Figure Legends

Legends should be concise but comprehensive—the figure and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

Tables

Tables should be self-contained and complement, not duplicate, information contained in the text. They should be supplied as editable files, not pasted as images. Legends should be concise but comprehensive – the table, legend, and footnotes must be understandable without reference to the text. All abbreviations must be defined in footnotes. Footnote symbols: †, ‡, §, ¶, should be used (in that order) and *, **, *** should be reserved for P-values. Statistical measures such as SD or SEM should be identified in the headings.

Figures

Although authors are encouraged to send the highest-quality figures possible, for peer-review purposes, a wide variety of formats, sizes, and resolutions are accepted. Click **here** for the basic figure requirements for figures submitted with manuscripts for initial peer review, as well as the more detailed post-acceptance figure requirements.

No more than 8 figures may be presented, approximately equivalent ot 3 pages-worth total.

Color Figures

Figures submitted in color may be reproduced in color online free of charge. Please note, however, that it is preferable that line figures (e.g. graphs and charts) are supplied in black and white so that they are legible if printed by a reader in black and white. If an author would prefer to have figures printed in color in hard copies of the journal, a fee will be charged by the Publisher.

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Supporting information is information that is not essential to the article, but provides greater depth and background. It is hosted online and appears without editing or typesetting. It may include tables, figures, videos, datasets, etc.

Click **here** for Wiley's FAQs on supporting information.

Note: if data, scripts, or other artefacts used to generate the analyses presented in the paper are available via a publicly available data repository, authors should include a reference to the location of the material within their paper.

General Style Points

The following points provide general advice on formatting and style.

- **Abbreviations:** In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Initially, use the word in full, followed by the abbreviation in parentheses. Thereafter use the abbreviation only.
- **Units of measurement:** Measurements should be given in SI or SI-derived units. Visit the Bureau International des Poids et Mesures (BIPM) website for more information about SI units.
- **Numbers:** Numbers under 10 are spelt out, except for: measurements with a unit (8mmol/l); age (6 weeks old), or lists with other numbers (11 dogs, 9 cats, 4 gerbils).
- **Trade Names:** Chemical substances should be referred to by the generic name only. Trade names should not be used. Drugs should be referred to by their generic names. If proprietary drugs have been used in the study, refer to these by their generic name, mentioning the proprietary name and the name and location of the manufacturer in parentheses.

Resource Identification Initiative

The *Journal of Cellular Biochemistry* supports the **Resource Identification Initiative**, which aims to promote research resource identification, discovery, and reuse. This initiative, led by the **Neuroscience Information Framework** and the **Oregon Health & Science University Library**, provides unique identifiers for antibodies, model organisms, cell lines, and tools including software and databases. These IDs, called Research Resource Identifiers (RRIDs), are machine-readable and can be used to search for all papers where a particular resource was used and to increase access to critical data to help researchers identify suitable reagents and tools.

Authors are asked to use RRIDs to cite the resources used in their research where applicable in the text, similar to a regular citation or Genbank Accession number. For antibodies, authors should include in the citation the vendor, catalogue number, and RRID both in the text upon first mention in the Methods section. For software tools and databases, please provide the name of the resource followed by the resource website, if available, and the RRID. For model organisms, the RRID alone is sufficient.

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ANEXO III: Normas da resvista "Cell Biochemistry and Function"

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- v. Acknowledgments;
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- vii. Main text;
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- ix. Tables (each table complete with title and footnotes);
- x. Figure legends;
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Figures and supporting information should be supplied as separate files.

Acknowledgments

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