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Glutamine or Glutamine Dipeptide Supplementation Improves Gluconeogenesis and Liver Glycogenosis in Type 1 Diabetic Rats

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Abstract: The effects of the supplementation with L-glutamine (GLN) or L-alanyl-L-glutamine (GDP) on the progression of the systemic and hepatic metabolic status of rats having untreated type 1 diabetes mellitus (T1DM) were investigated. Male Wistar diabetic rats (streptozotocin, 60 mg/kg) were allotted to four groups supplemented by gavage for thirty days as follows: control and diabetic receiving saline; diabetic receiving GLN (248 mg/kg); and diabetic receiving GDP (400 mg/kg). Body weight, plasmatic parameters and kidney function were analyzed. Isolated hepatocytes were used to assess gluconeogenic capacity. Liver and kidney were used for morphological analyses. T1DM decreased the number and increased the area of the hepatocytes, possibly because of the observed enlargement of glycogen stores. Kidney weight, glomerular area and proteinuria increased, and glomerular filtration rate decreased, in non-supplemented T1DM rats. Glomerular area and proteinuria were reversed by both supplementations. The T1DM hepatocytes released less glucose, which could have been diverted to glycogen synthesis and secondary glycogenosis observed in T1DM; this was partially reversed by the supplementations. The results point to a possible beneficial effect of glutamine on the metabolic and hepatic impairments of T1DM.

Key words: Type 1 diabetes mellitus, L-glutamine, glutamine dipeptide, gluconeogenesis, glycogenosis.

1. Introduction

The maintenance of normal blood glucose is essential for the survival of the Central Nervous System and precise glucoregulatory mechanisms modulate it at three levels: hepatic auto-regulation, autonomic nervous system activity and hormone action [1, 2]. The impairment or absence of these mechanisms predisposes to several diseases related to glucose homeostasis, such as diabetes mellitus [3].

Type 1 diabetes mellitus (T1DM) is a disease resulting from the immunogenetic and/or environmental destruction of the pancreatic beta cells that leads to the loss of the endogenous release of insulin. Among the many consequences of this event, there is a significant decrease in the plasma concentration of L-glutamine [4, 5].

Under normal metabolic situations, L-glutamine is the most abundant amino acid in blood [6]. However, in general states of metabolic stress, such as diabetes, there is an enhanced use of this amino acid by the

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tissues, resulting in a state of glutamine deficiency [7-9].

L-glutamine is considered a multi-functional amino acid with a role in many processes, among which ammonia transport from one tissue to another, donation of carbon skeletons to gluconeogenesis and glycogen metabolism [10, 11].

The clinical use of L-glutamine is gaining prominence because it decreases the oxidative stress of the diabetic patient [12] and the chronic complications of this disease [13, 14]. It is the precursor of L-glutamate, which can be used to reduce glutathione, a potent antioxidant [15]. Nevertheless, the use of L-glutamine in the clinical routine has limitations [16], especially related to its role in gluconeogenesis [17]. Such limitations are surpassed when the amino acid is used as dipeptide [16]. Studies of jejunal perfusion demonstrated that the competition between free amino acids during their uptake can be avoided or decreased if the amino acids are present as dipeptides [18].

Although in T1DM the intracellular and plasmatic levels of L-glutamine are decreased [8], so that supplementation would improve the patient's condition, L-glutamine could also favor hepatic gluconeogenesis, contributing to hyperglycemia and worsening the metabolic control. Based on these issues, this work had the purpose of comparing the effects of the oral supplementation for 30 days with free L-glutamine (GLN) or L-glutamine conjugated with L-alanine (glutamine dipeptide, GDP) on the progression of the systemic and hepatic metabolic profile in an experimental model of untreated T1DM.

2. Methods and Materials

Male Wistar rats were kept at constant temperature (23 ± 2 ºC) and light:dark cycles (12 h light:12 h dark) and were given standard rodent chow (Nuvital®-Nuvilab, Colombo, Brazil) and water *ad libitum*. The animals were allotted to the following experimental groups: normal (non-diabetic) rats receiving 0.9% saline (GC); diabetic rats receiving 0.9% saline (GD); diabetic rats receiving L-glutamine (GLN) at the dose of 248 mg/kg (GDGLN); diabetic rats receiving glutamine dipeptide (GDP) at the dose of 400 mg/kg (GDGDP). These solutions were given through gavage for 30 days.

The GDP dose of 400 mg/kg is the most indicated as the maximal daily dose for humans, while the GLN dose of 248 mg/kg is the amount of glutamine present in 400 mg/kg of the dipeptide. All the experimental procedures were approved by the Ethics Commission on Animal Experimentation of the Institution.

2.1 Induction and Confirmation of the Diabetic State

Diabetes was induced by the intravenous injection of streptozotocin (STZ) at the dose of 60 mg/kg (Sigma®, St. Louis, EUA) dissolved in 10 mM citrate buffer pH 4.5. After seven days, the animals having fasting blood glucose ≥ 200 mg/dL were considered diabetic [19].

2.2 Proteinuria and Glomerular Filtration Rate (GFR)

One week before the collection of tissues, the animals were put into metabolic cages for 12 h, during which water ingestion and urinary volume were recorded. The GFR was calculated by urinary and plasmatic creatinine. Urine was also used to determine proteinuria. Protein and creatinine were determined by colorimetric methods.

2.3 Tissue Collection

After 15 h of fasting, the rats were anesthetized with thionembutal (120 mg/kg) plus lidocaine (5 mg/kg). After laparotomy, the liver and kidneys were collected and weighed. The kidney samples were destined to morphological analysis, while liver samples were destined to general morphological analysis and histochemical evaluation of glycogen content and lipid inclusions.

2.4 Processing and Histological Analyses of Liver and Kidneys

The liver samples were fixed in Bouin or in liquid

nitrogen. Bouin-fixed samples were subjected to routine histological procedures and semi-serial sections (6 μm) were made with Leica RM 2145 microtome. These sections were stained with Hematoxylin-Eosin (HE) for morphometric analysis and Schiff's periodic acid (PAS) for determination of glycogen content. The liver samples frozen in liquid nitrogen were sectioned (10 μm) with Leica® CM1850 cryostat and stained with Sudan III to determine lipid content.

The images for morphometric analyses were captured under a light microscope (Olympus BX41, Olympus America Inc., New York, USA) coupled to a high-resolution camera (Olympus Q Color 3 Olympus America Inc., New York, USA) under a 20× objective in the area of the central vein. Two hundred hepatocytes/animal were measured and the number of hepatocytes in 50 images/animal was counted. The histochemical analysis of glycogen percentage was calculated in 200 hepatocytes/image and the percentage of lipid inclusions in 50 images/animal under a light microscope (Olympus BX41, Olympus America Inc., New York, USA) coupled to a high-resolution camera (Olympus Q Color 3 Olympus America Inc., New York, USA) under a 40× objective in the area of the central vein.

The kidney samples were fixed in Metacarn and stained with Masson's trichrome after sectioning (5 μm semi-seriated sections). The images for morphometric analyses were captured under a light microscope (Olympus BX41) coupled to a high-resolution camera (Multicam-CAM1000 1.3 pixel) under a 20× objective in the equatorial portion of the organ. The analyses were made by measuring glomerular area [20]. Thirty glomeruli/animal were analyzed [21]. The analyses were performed using the program Image Pro Plus 4.5 (Media Cybernetics, Maryland, USA).

2.5 Hepatocyte Isolation

After 15 h of fasting, the rats were anesthetized with thionembutal (40 mg/kg) plus lidocaine (5 mg/kg). Laparotomy was followed by cannulation of the portal vein and inferior cava vein. The surgical details are described elsewhere [22]. Euthanasia was by hypovolemic chock. The perfused fluid consisted of Krebs-Henseleit (KH) buffer pH 7.4 warmed at 37 ºC and saturated with carbogenic mixture (O_2 95%/CO_2 5%). Initially, the liver was perfused with calcium-free KH for 15 min, then with KH containing collagenase (900 U/dL) for 9-12 min. Next, the cells were filtered and centrifuged three times (4 ºC, 530 rpm, in calcium-containing KH). Aliquots of 10^6 cells/mL with viability greater than 70% were incubated for 1 h with KH containing the test substance or KH alone in water bath under agitation and saturated with carbogenic mixture. The gluconeogenic substrates used were glycerol, lactate, alanine or glutamine (5 mM). The incubation mixture was centrifuged, and the supernatant used to determine the concentration of glucose, lactate, pyruvate, ammonia and urea through enzymatic-colorimetric methods. The difference of concentration between the substrate-containing flasks and those containing pure KH was the amount produced by the hepatocytes, which was expressed as μmol/10^6 cells/h.

2.6 Statistical Analysis

The data were subjected to the Kolmogorov-Smirnov normality test. Parametric data were compared through analysis of variance (ANOVA) followed by Tukey's post-test or unpaired *t* test. The results were presented as mean ± standard error (SE). The statistical data were analyzed using GraphPad Prism program (GraphPad Software, version 5.0, San Diego, USA) and considered significant at $p < 0.05$.

3. Results and Analysis

The kidney analyses are in Table 1. Diabetes increased kidney relative weight, proteinuria and glomerular area. The supplementations did not change kidney weight, but decreased glomerular area and proteinuria when compared with group GD. On the other hand, glomerular filtration rate, which was decreased

Table 1 Renal parameters of control (GC), type 1 diabetic rats (GD) and GD rats supplemented with GLN (GDGLN) or GDP (GDGDP).

	GC	GD	GDGLN	GDGDP
Kidney weight (g/100 g)	0.77 ± 0.03a	1.23 ± 0.03b	1.18 ± 0.04b	1.37 ± 0.09b
Glomerular area (μm^2)	7370 ± 64.34a	9429 ± 93.45b	8376 ± 184.90c	8789 ± 115.10c
Glomerular filtration rate (mL/min/100 g)	0.52 ± 0.02a	0.18 ± 0.01b	0.22 ± 0.02b	0.29 ± 0.03b
Proteinuria (mg/24 h)	2.500 ± 0.15a	3.154 ± 0.22b	2.362 ± 0.07a	2.250 ± 0.07a

Results shown as mean ± SE; $n = 5$ per group.

Different letters in the same row represent significant difference ($p < 0.05$); ANOVA-Tukey.

Table 2 Liver weight, number and area of hepatocytes of control (GC), type 1 diabetic rats (GD) and GD rats supplemented with GLN (GDGLN) or GDP (GDGDP).

	GC	GD	GDGLN	GDGDP
Liver weight (g/100 g)	3.318 ± 0.17a	5.527 ± 0.15b	5.872 ± 0.40b	4.894 ± 0.60b
Hepatocyte number	933.20 ± 30.77a	760.50 ± 18.78b	746.70 ± 21.30b	819.60 ± 12.07b
Hepatocyte area (μm^2)	219.00 ± 2.25a	253.30 ± 10.81b	247.10 ± 4.69b	250.00 ± 6.02b

Results shown as mean ± SE; $n = 5$ per group.

Different letters in the same row represent significant difference ($p < 0.05$); ANOVA-Tukey.

by the disease, was not restored by any of the supplementations.

Microscopically, the histological organization of the liver was maintained in all the groups, with radial lines of hepatocytes surrounded by sinusoids converging to the central vein. Table 2 brings liver weight, number and area of hepatocytes. Liver weight was higher in all diabetic groups. The morphometric analyses indicated that diabetes decreased the number of hepatocytes compared with group GC. However, the area of these cells increased in the diabetic groups. These alterations were not changed further by the supplementations.

The hepatocyte glycogen content in Fig. 1A indicated an increase caused by diabetes (GD) when compared with group GC. The supplementation with GLN or GDP decreased this percentage, although not to the values of the control group. In contrast, the disease significantly decreased the lipid infiltrations in the liver (Fig. 1B). In addition, although the GLN supplementation did not change this parameter, the GDP supplementation increased these infiltrations, reaching values similar to the control.

The liver response to each gluconeogenic substrate is presented in Figs. 2 and 3. Fig. 2 gives the hepatocyte release of glucose, pyruvate, lactate, ammonia and urea of groups GC and GD. There was a detectable release of pyruvate only from lactate, however this release was decreased in group GD. Lactate release was detected only in group GC in the presence of alanine. There was a decreased release of urea from alanine, but both ammonia and urea from glutamine were increased in group GD. As for glucose release, there was a decrease from glycerol, alanine and glutamine in group GD, while groups GC and GD did not differ in their glucose release from lactate.

Fig. 3 gives the hepatocyte release of glucose, pyruvate, lactate, ammonia and urea of groups GD (diabetic without supplementation), GDGLN and GDGDP (diabetic supplemented with glutamine or glutamine dipeptide, respectively). Pyruvate release in the presence of lactate was increased by both supplementations compared with group GD. Pyruvate and lactate were undetected in the presence of alanine. Urea production from alanine was unchanged, but ammonia release was decreased in group GDGLN and increased in group GDGDP. In the presence of glutamine, there was a decrease in ammonia and urea production after GLN supplementation. GDP supplementation increased urea release in the presence of glutamine. Ammonia from alanine and urea from glutamine were increased in group DGDP when compared with group GDGLN. Glucose production was

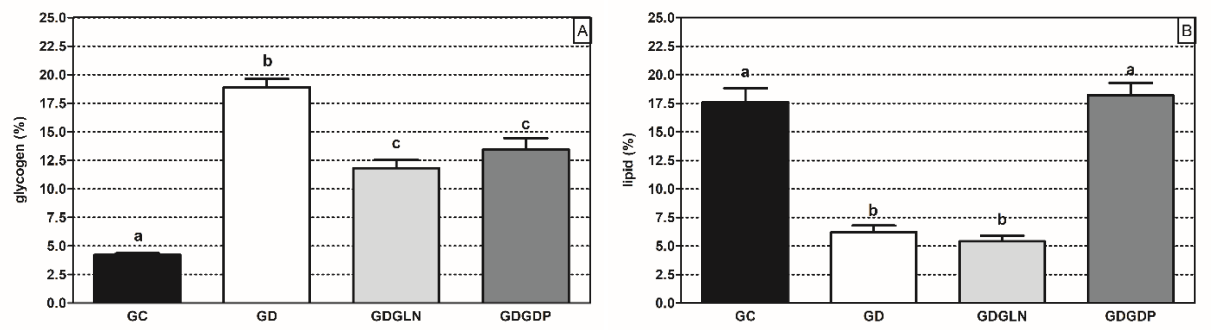

Fig. 1 Percentage of glycogen (A) and lipids (B) in hepatocytes of control rats (GC), type 1 diabetic rats (GD) and GD rats supplemented with GLN (GDGLN) or GDP (GDGDP).

Results shown as mean ± SE; $n = 5$ per group.

Different letters on top of the columns represent significant difference ($p < 0.05$); ANOVA-Tukey.

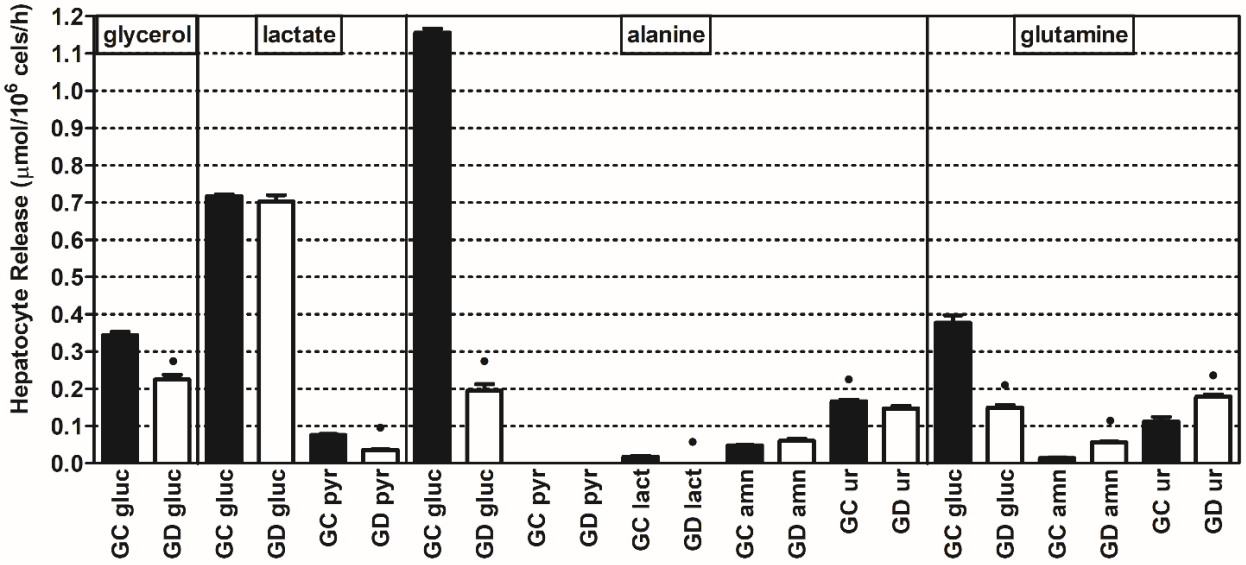

Fig. 2 Hepatocyte release of glucose (gluc), pyruvate (pyr), lactate (lact), ammonia (amn) and urea (ur) from the substrates glycerol, lactate, alanine and glutamine of control (GC) and type 1 diabetic rats (GD).

Results shown as mean ± SE; $n = 5$ per group. ● $p < 0.05$ GC VS GD, unpaired t test.

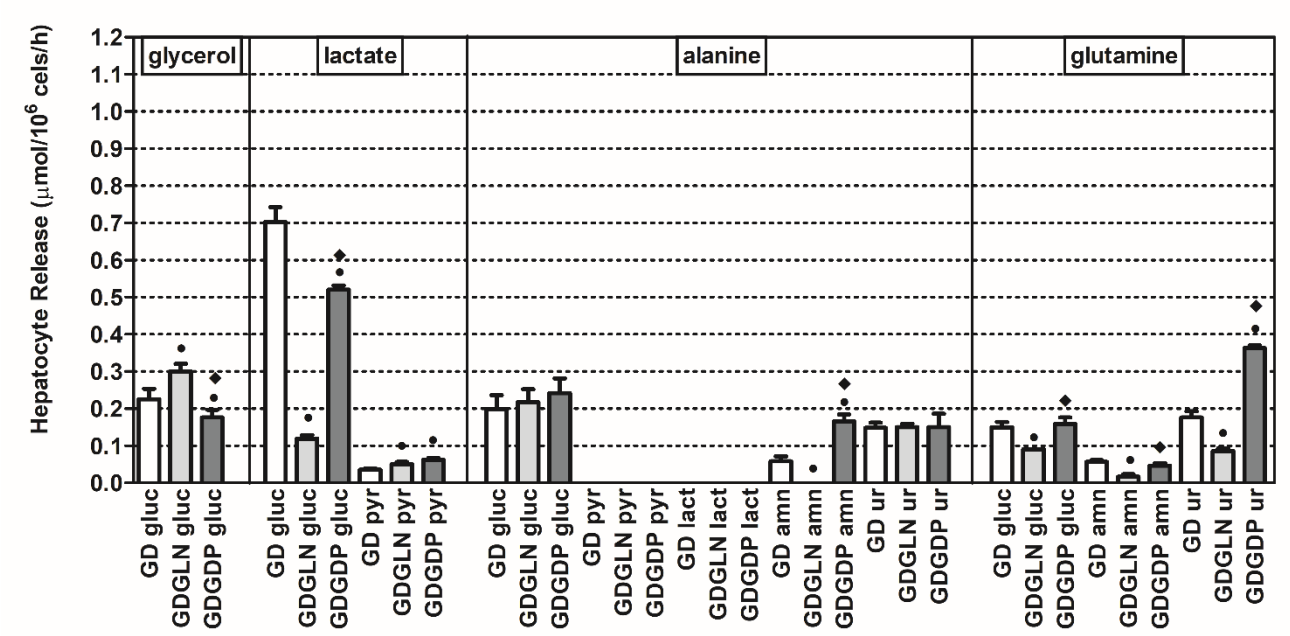

Fig. 3 Hepatocyte release of glucose (gluc), pyruvate (pyr), lactate (lact), ammonia (amn) and urea (ur) from the substrates glycerol, lactate, alanine and glutamine of type 1 diabetic rats (GD) and GD rats supplemented with GLN (GDGLN) or GDP (GDGDP).

Results shown as mean ± SE; n = 5 per group. ● $p < 0.05$ GDGLN and GDGDP VS GD, ◆ $p < 0.05$ GDGDP VS GDGLN; ANOVA-Tukey.

not different between groups in the presence of alanine. In group DGLN, there was a decrease in glucose production from lactate and glutamine, while there was an increase from glycerol. In group GDGDP glucose release decreased in the presence of glycerol and lactate and increased from glutamine.

4. Discussion

On the long-term, consequences of the increased ingestion of nitrogenous compounds are the renal alterations [23, 24]. The diabetes model of this work increased proteinuria; according to Gross et al. [25], this is generally related to glomerular damage or excess plasma proteins, which would explain the enlarged glomerular area in the diabetic group because glomerular damage can lead to increased urinary protein [26]. In addition, as the supplementations decreased both proteinuria and glomerular area, a link between the two was shown.

If on one hand the increased kidney weight in the diabetic group—almost twice that of the control group—can be an indicative of renal overload, on the other hand glomerular filtration rate was decreased by half, regardless of supplementation. This could be as such exactly because of renal impairment.

Experiments carried out by Gulle et al. [27] with diabetic animals have shown hepatocyte vacuolization with sinusoidal dilatation and increased periportal connective tissue. This was not seen after four weeks in this study or by Ruan et al. [28], as the liver tissue did not exhibit morphological alterations, suggesting a possible correlation between duration of the disease and the appearance of such alterations.

Despite that, the liver weight was considerably higher in the diabetic animals, which is typical of hepatomegaly secondary to excessive glycogen content or non-alcoholic steatosis [29]. Although there was not a positive correlation between liver weight and lipid inclusions, the glycogen content could be the responsible for this inflammatory process [30]. Excessive liver glycogen content is considered a diabetic complication [31] related to adaptive changes

to insulin absence, hyperglycemia and enhanced gluconeogenesis [5]. This complication can be corrected by glycemic control [32, 33].

In response to impaired increase in glucose uptake, hepatocytes synthesize glycogen even in the absence of insulin. This increased storage becomes evident as increased cellular area in an attempt of the hepatocytes of coping with the excess glucose, making the cell larger than normal. This is not unusual and can be associated with degenerative and pathological process such as diabetes, as demonstrated by Silva et al. [34]. In addition, maybe because of the increased cellular area, there is a decrease in the number of hepatocytes.

Studies carried out by Chen et al. [35] reported glycogen accumulation in diabetic animals after 90 days, and in 80% of patients with uncontrolled diabetes [36, 37]. Although the supplementation with GLN or GDP did not change the number and area of the hepatocytes, the amount of glycogen was decreased by 35% in the supplemented groups. Experiments from Rosa et al. [19] show the capacity of glutamine in decreasing the blood concentrations of AST and ALT that are increased by diabetes. In addition, there is a correlation between the increased plasmatic values of AST and ALT and the glycogen content of hepatocytes [38]. Therefore, the ability of the supplementation in attenuating this condition independently of glycemic control and in decreasing AST and ALT is in favor of GLN supplementation on these parameters. Factors other than glycogen content may be increasing hepatocyte area, such as the catabolic state and the metabolic flux typical of diabetic animals.

Dyslipidemias are common in diabetic individuals, as experimentally demonstrated by Rosa et al. [19] and Tronchini et al. [39]. These alterations can favor the appearance of lipid infiltrations in the hepatic tissue [40], a condition known as hepatic steatosis. However, the fact that the disease decreases the normal lipid content of the liver can be due to the enhanced peripheral use, which would result in increased VLDL, as also observed by Rosa et al. [19]. Although GLN did not change this parameter, GDP increased these infiltrations to levels similar to group GC. The higher availability of glutamine in group GDGDP and/or some direct effect of GDP on the adipose and/or hepatic tissue may have favored those infiltrations. Nevertheless, this effect of GDP needs further investigations.

The gluconeogenic capacity of the liver was changed by diabetes and the supplementations. Although alanine and lactate are pyruvate precursors, this product was detected only from lactate, which may indicate that the capacity of the deaminases and/or transaminases is lower than that of lactate dehydrogenase (LDH). In addition, the degradation of alanine begins with a step energetically more expensive, which is the formation of ammonia [41], while lactate, by means of LDH, generates reducing equivalents [42] that are energetically more advantageous to the cells.

Alanine and glutamine can be used in protein turnover and cell growth, but the excess nitrogen has to be excreted [43]. The liver production of urea and ammonia from alanine was not affected by diabetes, but both were increased from glutamine, as this amino acid generates more nitrogenous residues. Both supplementations (GLN and GDP) changed these aspects; however, GDP increased ammonia and urea production from both amino acids. This could be related to its increased availability favoring ureagenesis from these amino acids [44].

One of the major features of diabetes, apart from hyperglycemia, is the increased gluconeogenesis due to the lack of insulin signaling in the liver, leading to the continuous glucose production even in the absence of energy deficit and in the fed state [45]. However, under the experimental conditions used in this study, gluconeogenesis was decreased from glycerol, alanine and glutamine and preserved from lactate in the diabetic animals, when compared with the control (GC) group. The fact that lactate generates cytosolic reducing equivalents that can feed the gluconeogenic

pathway may be an explanation for this difference.

On the other hand, insulin absence and enhanced peripheral catabolism [46] maximize the release and/or effects of glucagon and enhance liver gluconeogenesis; however, during uncompensated diabetes, the loss of insulin release and actions together with hyperglycemia impair the counterregulatory system of which glucagon is the major element [1]. In this way, the decreased gluconeogenesis observed suggests that glucagon release and/or signaling may be impaired. The glycogen content could be the result of the combination of higher glycemia with inappropriate glucagon signaling [1]. The higher glucose and glucose-6-phosphate concentrations trigger a substrate-mediated, insulin-independent mechanism, that stimulates the dephosphorylation of phosphorylase a via stimulation of phosphorylase phosphatase and inhibition of phosphorylase kinase. In addition, glucose-6-phosphatase directly activates glycogen synthase phosphatase; the dephosphorylation of glycogen synthase b (inactive) to the active form (a) promotes glycogen synthesis by substrate flux [47-50]. This characterizes a hepatic auto-regulatory mechanism triggered by hyperglycemia in an attempt to bring glycemia back to normal. In this way, if glucagon release and/or signaling are compromised, gluconeogenesis may be reduced and glycogen synthase activation by substrate flux and glycogen synthesis may be favored.

Differently, GLN supplementation increased gluconeogenesis from glycerol, which was decreased by diabetes, in group GDGLN. This shift of glycerol to glucose synthesis could be responsible for the decreased plasmatic TGL and VLDL observed by Rosa et al. [19]. This was not observed in the group supplemented by GDP, and despite plasmatic TGL and VLDL not being changed by GDP supplementation [19], the rats of this study had an increased lipid infiltration in the hepatic parenchyma that could result from the enhanced esterification of TGL and production of VLDL. As the decrease of glucose production by GDP supplementation was of 28% and the increase of lipid infiltration of 240%, the activation of hepatic lipogenesis by this supplementation cannot be discarded.

If in fact the impaired glucagon release and/or signaling is reducing gluconeogenesis, this could explain the reduced glycogen store in the supplemented groups if some of it was caused by the indirect pathway (gluconeogenesis-derived) of glycogen synthesis.

5. Conclusions

It seems possible that in the fed state the gluconeogenesis induced by substrate flux from the intestine and the high blood glucose *per se* can induce direct and indirect glucogenogenesis. Indirect glucogenogenesis, from the gluconeogenesis-derived glucose, would be responsible for the reduced glucose release from lactate and glutamine in the diabetic group supplemented with GLN, and from lactate and glycerol in the rats supplemented with GDP.

This glucogenogenic state could promote a condition of secondary glycogenosis and progressive injury of the hepatocytes, which could decrease their number and compromise the liver during the course of the disease. In addition, as GLN and GDP decreased liver glycogen content, kidney glomerular area and glomerular filtration rate to control values, a positive effect of these supplementations on the control of catabolic diseases such as diabetes is suggested.

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