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Therapeutic effect of amino acid mixture on type 1 diabetes mellitus with impaired renal methionine reabsorption

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Abstract

Background: The main manifestation of Type 1 diabetes mellitus (T1DM) is insulin insufficiency which eventually leads to body weight loss and a diversity of organ dysfunctions. On the other hand, quercetin (QT) is an antioxidant and an insulin secretagogue. We hypothesize that the amino acid mixture (AAM) preparation and/or AAM+ quercetin(QT) probably could ameliorate these adverse effects.

Methods: The STZ-DM-Sprague Dawley rat model was carried out and respectively treated with QT, AAM, and AAM+QT. The relevant physiological and biochemical changes in serum and urinary parameters were examined.

Results: T1DM exhibited severe insulin insufficiency, body weight loss, increased kidney/body weight ratio, BUN, creatinine clearance, albuminuria and proteinuria, declined serum albumin and amino acid reabsorption involving methionine, leucine and isoleucine. The control showed severe serinuria (12.0±0.2 mg/mL) (p<0.001). AAM caused valinuria (3.6±0.2 mg/mL), argininuria (6.9±0.2 mg/mL) and histidinuria (6.5±0.1 mg/mL) (p<0.05). T1DM rats revealed hyperglycinuria (21.4±0.2 mg/mL) (p<0.01); AAM alleviated hyperglycinuria (13.7±0.2 mg/mL) (p<0.001) but evoked isoleucinuria (8.2±0.2 mg/mL) (p<0.05); QT elicited methioninuria (23.1±0.3 mg/mL) in T1DM and the control (p<0.001); AAM+QT alleviated the methioninuria with extra leucinuria (11.3±0.4 mg/mL) (p<0.05), isoleucinuria (11.4±0.4 mg/mL), tryptophanuria, argininuria and lysinuria (p<0.05).

Conclusion: T1DM exhibits severe insulin insufficiency, resulting in severe body weight loss and increased kidney/body weight ratio. T1DM and QT tend to induce methioninineuria. AAM+QT can rescue methioninuria at the expense of leucinuria, isoleucinuria, tryptophanuria, argininuria and lysinuria, implicating the use of AAM with extra supplement of insulin, leucine, isoleucine, tryptophan, arginine and lysine is feasible for alleviation of T1DM.

Keywords: Type 1DM, methioninuria, serinuria, leucinuria, amino acid therapy

Introduction

Diabetes mellitus (DM) is a progressive metabolic disorder affecting millions worldwide [1,2]. The SEARCH for Diabetes in Youth Study (www.searchfordiabetes.org/) has provided the first national data on prevalence of diabetes in youth: 1 of every 523 youth had physician diagnosed diabetes in 2001, including both type 1 (T1DM) and type 2 diabetes (T2DM), and about 15,000 youth are diagnosed with T1DM each year [3].

T1DM is a gene-linked disease that is characterized with insulin insufficiency, hyperglycemia, glucosuria and weight loss [4]. Scientists have identified nearly 50 genes or gene regions associated with T1DM. Scientists have identified a key gene region that contributes nearly half the increased risk of developing type 1 diabetes [3]. Individuals with this disease require medications to control their blood glucose [1,2].

Instead of burning the carbohydrates, triglycerides are broken down (lipolysis) and free fatty acids are beta-oxidized in liver of T1DM patients to compensate the insufficient energy, as consequence, leading to diabetic ketoacidosis. [5].

The complications with DM are divided into two groups, macrovascular and microvascular complications [6-8]. Microalbuminuria (urine albumin) occurs when the kidney

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leaks small amounts of albumin into the urine, in other words, when there is an abnormally high permeability for albumin in the renal glomerulus [6-8]. As a rule, plasma levels of total protein and albumin in DM patients are significantly lower than those in non DM patients [9]. Apart from this, in the kidney more than 95% of amino acids are reabsorbed in the proximal tubule [10-12]. The etiologic cause of aminoaciduria may involve the genetic and the environmental factors [13]. In view of the amino acid specificity, a diversity of inherited disordered amino acid reabsorption has been demonstrated, such as Hartnup disorder, cystinuria [14,15], argininuria [16], iminoglycinuria, dicarboxylic aminoaciduria [13,17], and lysinuric protein intolerance [10]. More relevantly, the alterations in plasma branched-chain amino acids (valine, isoleucine and leucine) and alanine have been described in patients with T1DM who had poor metabolic control [18]. On the other hand, quercetin (QT), a potent prooxidant as well as an antioxidant, exhibits rather potent insulin secretagogue bioactivity [19]. Considering that the supply of sufficient protein building stones could be beneficial to improve defected protein homeostasis as described by Nakamoto and Suzuki [9], we proposed that the amino acid mixture (AAM) preparation and/or AAM+ quercetin(QT) could be biochemically effective to ameliorate the severely lowered plasma levels of total protein and albumin in T1DM patients. The STZ-DM- Sprague Dawley rat model was carried out and treated with AAM alone and AAM plus QT treatments. The relevant physiological and biochemical changes in serum and urinary parameters were examined.

Methods

Chemicals

Streptozotocin (STZ) was a product of Ausmausco Pharma Co., Ltd. (China). Assay max mouse Insulin (Insulin ELISA Kit) was supplied by AssayPro (USA). The Moriamin-SN Injection® for peripheral i.v. was supplied by China Chemical And Pharmaceutical Co. Ltd. (Taiwan) (refer to **Supplementary Table S1**). N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (also known as TBDMS-MTFA or MTBSTFA): the amino acid derivating agent was obtained from Merck KGaA (Darmstadt, Germany). Other chemicals not indicated were provided by Wako Pure Chemical Co. (Osaka, Japan).

Animals

This experiment was proved by the Institutional Animal Care and Ethic Committee of The Taipei Medical University (Taipei, Taiwan) and performed in accordance with the ethical standards and Animal Welfare Act laid down in the 1964 Declaration of Helsinki and its later amendments. Sixty four male Sprague–Dawley rats, age week 6, weighing 265-287 g were purchased from BioLasCo Animal Centre (Taipei, Taiwan). These rats were randomly housed in animal room conditioned at 24±2°C, RH 70-75%, with a 12h/12h light/night cycle, fed on basic chows and water ad libitum, and acclimated in the animal room for the first week.

Treatment

The rats were divided into eight groups, 8 in each: Group 1 was the normal control. Group 2, AAM ip treated control with Moriamin 1.6 mL/rat-day. Group 3, QT only ip treated control (70 mg/kg-day, suspended in PBS). Group 4, ip AAM+QT. Group 5, DM group. Group 6, DM treated with ip AAM. Group 7, DM treated with ip QT. Group 8, DM treated with ip AAM+QT. The animals were separated and caged, 2 rats in each cage. To induce DM model, STZ at 65 mg/kg was ip applied at week 2. The DM induction period took 2 weeks (from week 2 to week 4). The blood sugar levels of rats were taken at week 4 and at week 9. The body weight was taken every week. The QT and AMM therapy started at the end of week 4 until week 9. In the whole duration of experiment, the rats were not allowed to access any extra outside-cage exercise.

Urine collection and analysis

Rats were moved to the metabolic cage two days before the end of week. The urine was collected from 8:00 am to 8:00 am of the following day. The total volume of urine excreted per day by each individual was taken. The urinary samples were analyzed fresh for urinary protein, urinary urea nitrogen, creatinine, or immediately stored in the freezer at 0-4°C when not in use. The urinary urea nitrogen (N_{UU}) (the equivalent of BUN in blood), glucose, and creatinine were measured by reagents supplied by Siemens (Bakersfield, CA, USA) and the automatic analyzer (Type Ciba-Corning Express Plus was purchased from Ciba-Corning (USA). The urinary protein concentration was measured using ELISA reader.

Blood collection and analysis

The blood samples were withdrawn from the abdominal aorta under i.p ketamine and xylasine anesthesia. The sample blood was centrifuged at 3000g to separate the serum, which was measured for parameters including insulin, glucose, albumin, BUN, and creatinine adopting reagents supplied by Siemens (Bakersfield, CA, USA) and the automatic analyzer manufactured by Ciba-Corning Express Plus) (Ciba-Corning, USA). After the final blood collection, the rats were euthanized by i.p ketamine and xylasine anesthesia.

Estimated glomerular filtration rate (eGFR)

The eGFR is typically recorded in units of volume per time, e.g., mL/min as suggested by Stevens et al., [20]. The following formula were used to calculate the estimated glomerular filtration rate (eGFR):

$$eGFR = (C_{ur} \times F_{ur})/C_{pl} \ldots\ldots \text{Eq 1}$$

where C_{ur} is the urine concentration F_{ur} is the urine flow rate, C_{pl} is the plasma concentration. For clinical use, e-GFR usually is expressed in creatinine clearance, C_{Cr}. Briefly, 24 h urine was collected to determine the amount of creatinine that was removed from the blood over 24 h interval. The creatinine

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clearance (C_{cr}, mL/min) is calculated from the creatinine concentration in the collected urine sample (Cr_{ur}, mg/dL), urine flow rate (F, mL/min), and the plasma concentration (Cr_{pl}, mg/dL) (Eq. 2). Since the product of urine concentration and urine flow rate yields creatinine excretion rate, which is the rate of removal from the blood, creatinine clearance is calculated as removal rate per min ($Cr_{ur} \times \upsilon$) divided by the plasma creatinine concentration (Cr_{pl}). (National Kidney Foundation, 2002) [21], which mathematically is expressed as

$$C_{cr} = (Cr_{ur} \times F)/Cr_{pl}\,(mL/min) \ldots\ldots Eq\ 2$$

Organ collection

After euthanized, the kidneys were immediately excised, rinsed twice with PBS, and the adhering PBS was sucked off using the face-tissues. The kidney weights were taken.

Amino acid analysis

Derivatization of amino acids

Method of Deng et al., [22]. was adopted with slight modification to conduct the amino acid analysis. This method has the advantages to successfully detect glutamine and asparagine. Briefly, the urine samples were lyophilized. To each lyophilized residue, norleucine (to serve the internal reference standard) 0.4 mg and 200 mL pyridine was added. The mixture was agitated to facilitate the dissolution. The reaction mixture was transferred into a 2 mL reaction vessel. The derivation of amino acids were conducted using the derivatization reagent (N-methyl-N-(tert-butyldimethylsilyl) -trifluoroacetamide (MTBSTFA) in pyridine. MTBSTFA 100 mL was added, agitated and well mixed. The reaction vessels were placed onto a derivation heating plate held at 100°C for 50 min. The reaction vessels were agitated every 10 min while undergoing reactions. After the reaction was finished, the reaction mixture was transferred into sample vials. The volume of solution was reduced by the nitrogen blowing until a minimum soluble phase was achieved (note: avoid any crystallization to occur). The concentrated sample solution was subjected to GC-FID analysis. The authentic amino acid samples, each 0.3 mg, were similarly treated.

GC/FID instrument and analysis

The GC/MS chromatography (Angilent 6890, Wilmington, DE, USA) installed with an FID detector and a column HP-5MS (ℓ×id =30m×0.25 mm, film thickness =0.25 μm) was used for GC/FID analysis. The flow rate of carrier gas nitrogen was operated at 0.8 mL/min. The temperature of the detector FID and the injection port was held at 305 and 300°C, respectively. The elution temperature was programmed initially at 50°C for 1 min, then at an elevation rate 10 °C/min up to 300°C and held at which for 6.5 min. The reference retention times for each amino acid are listed in **Table 1**. The contents of glutamine (Gln) and asparagine (Asn) were calculated according to the following equations.

1. To calculate glutamine (Gln) content:

$$W_{Gln} = (A_{Gln}/A_{nol}) \times 0.4 \times 7.4459$$

Where W_{Gln} is the total amount of glutamine under glutamine peak.

A_{Gln} is the total area under glutamine peak.

A_{nol} is the area under peak exhibited by the internal standard norleucine (0.4 mg). And 7.4459 is the reaction coefficient of glutamine.

2. To calculate asparagine (Asn) content:

$$W_{Asn} = (A_{Asn}/A_{nol}) \times 0.4 \times 4.3429$$

Where W_{Asn} is the total amount of asparagine under asparagine peak.

A_{Asn} is the total area under asparagine peak

A_{nol} is the area under peak exhibited by the internal standard norleucine (0.4 mg). And 4.3429 is the reaction coefficient of asparagine.

Statistical analysis

Data were presented as mean±SEM and analyzed by one-way analysis of variance with Tukey multiple *t*-test to compare between treatment groups using GraphPad Prism v4 (GraphPad Software, La Jolla, CA, USA). The CL level $p<0.05$ was considered statistically significant for the indicated n per group.

Results

Obviously, the T1DM rats were suffering from severe body weight loss, insulin insufficiency (**Table 1**), hyperglycemia and glucosuria (**Table 2**). An apparent positive correlation between the body weight gain and the serum insulin levels was perceived in all groups (**Table 1**). The rats exhibited a body weight range 265-287g and insulin levels within 1.4±0.5 μg/mL when initially caged. The insulin levels at week four were 1.4±0.4, 1.3±0.4, 1.4±0.2, and 1.4±0.3 μg/mL respectively for the control, AAM, QT, and AAM+QT rats. Contrasting to the DM-associated rats, the serum insulin levels were significantly lowered to 0.7±0.3, 0.8±0.2, 0.7±0.4, and 0.9±0.2 μg/mL in the DM, DM+AAM, DM+QT, and DM+AAM+QT rats ($p<0.001$) (**Table 1**). Similar trend was found at week six and week nine (**Table 1**). At week four the difference in body weight gain between the DM-associated and the normal groups became 290±9, 290±5g vs. 324±8 - 326±7 g (**Table 1**). As seen, the body weight of the control groups increased steadily from 324-325 g at week four to 350±8g - 363±7g at week nine. Worth note, the QT alone administered group showed significantly lowered body weight. While the body weights in the DM-associated rats were almost retarded all the way through the same period to 318±6g, 292±5g, 309±9g, and 292±6g respectively in the DM, DM+AAM, DM+QT, and DM+AAM+QT rats ($p<0.001$) (**Table 1**). Similarly, the body weight gain in the DM+AAM+QT as well as the DM+AAM rats was severely retarded (**Table 1**). The urinary glucose was entirely undetected in all controls compared to that raised to 80±6, 52±7, 43±6, and 45±5 μg/L in the DM, DM+AAM, DM+QT, and DM+AAM+QT rats ($p<0.001$) (**Table 2**). The blood glucose level of the normal, AA and QT controls were very comparably to maintain at 185±15 mg/

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Table 1. Correlation of serum insulin levels with body weight variation*,†,‡.

Group	week/body weight (g)/insulin level (μg/L)							
	1	2	3	4	5	6	8	9
control	265-287 1.4±0.5	299±5 --	312±6 --	325±5 1.4±0.4	335±6 --	345±5 1.4±0.5	357±6 --	363±7 1.4±0.5
AAM	265-287 1.4±0.5	300±5 --	313±6 --	326±7 1.3±0.4	336±5 --	347±7 1.3±0.6	355±6 --	362±6 1.3±0.4
QT	265-287 1.4±0.5	298±7 --	316±6 --	324±8 1.4±0.2	332±8 --	344±8 1.4±0.3	357±7 --	362±8 1.4±0.4
AAM+QT	265-287 1.4±0.5	299±8 --	317±7 --	324±7 1.4±0.3	333±8 --	340±7 1.4±0.3	347±6 --	350±8$^{\&}$ 1.4±0.3
DM	265-287 1.4±0.5	290±6$^{\&}$ --	300±6$^{\&}$ --	305±6$^{\&}$ 0.7±0.3$^{\&}$	310±8$^{\&}$ --	314±5$^{\&}$ 0.7±0.2$^{\&}$	316±7$^{\&}$ --	318±6$^{\&}$ 0.7±0.3$^{\&}$
DM+AAM	265-287 1.4±0.5	288±7$^{\&}$ --	287±6$^{\&}$ --	290±5$^{\&\partial}$ 0.8±0.2$^{\&}$	291±5$^{\&}$ --	291±6$^{\&\partial}$ 0.7±0.3$^{\&}$	291±7$^{\&}$ --	292±5$^{\&\partial}$ 0.7±0.3$^{\&}$
DM+QT	265-287 1.4±0.5	287±7$^{\&}$ --	288±8$^{\&}$ --	290±9$^{\&\partial}$ 0.7±0.4$^{\&}$	303±9$^{\&}$ --	307±8$^{\&}$ 0.7±0.2$^{\&}$	308±6$^{\&}$ --	309±9$^{\&\partial}$ 0.7±0.4$^{\&}$
DM+AAM+QT	265-287 1.4±0.5	288±6$^{\&}$ --	289±6$^{\&}$ --	290±7$^{\&\partial}$ 0.9±0.2$^{\&}$	290±8$^{\&}$ --	291±7$^{\&\partial}$ 0.8±0.2$^{\&}$	291±8$^{\&}$ --	292±6$^{\&\partial}$ 0.8±0.1$^{\&}$

*week 1 for acclimation; week 2-week 3 for STD induction; week 4-week 9: treated with ip Moriamin 1.6 mL/rat-day (AAM) or quercetin (QT) ip (70 mg/kg-day, suspended in PBS).
†Data expressed in mean±SD (n=8).
‡The symbol "&" in the same column indicate significant difference from the normal control (p<0.05). The symbol "∂" in the same column indicates significant difference among the DM-related groups taking the DM rats as the reference.

Table 2. Characteristic physiological parameters associated with the Type 1 diabetes mellitus*,†,‡.

Items/Normal range	Group							
	1	2	3	4	5	6	7	8
	CTL	AAM	QT	AAM+ QT	DM	DM+AAM	DM+QT	DM+AAM+ QT
Insulin, (μg/L) (0.5-2.0)	1.4±0.5	1.3±0.4	1.4±0.4	1.4±0.3	0.7±0.3$^{\&}$	0.7±0.3$^{\&}$	0.7±0.4$^{\&}$	0.8±0.1$^{\&}$
Blood sugar mg/dL	185±15	175±10	160±5	225±40	320±45	400±93	421±80	520±57
Urine glucose (mg/dL)	0.0	0.0	0.0	0.0	80±6$^{\&}$	52±7$^{\&\partial}$	43±6$^{\&\partial}$	45±5$^{\&\partial}$
BW, g KW, g	363±7 2.9±0.1	362±6 2.9±0.1	362±8 2.9±0.1	350±8 2.8±0.1	318±6$^{\&}$ 3.5±0.2$^{\&}$	292±5$^{\&}$ 3.5±0.2$^{\&}$	309±9$^{\&}$ 3.4±0.2$^{\&}$	292±6$^{\&}$ 3.8±0.2$^{\&}$
KW/BW (%)	0.8±0.1	0.8±0.4	0.7±0.1$^{\&}$	0.8±0.1	1.1±0.3$^{\&}$	1.2±0.4$^{\&}$	1.1±0.1$^{\&}$	1.3±0.2$^{\&}$

*Measured on urine collected the first day after different treatments.
†Data expressed in mean±SD (n=8).
‡The symbol "&" in the same row indicate significant difference from the normal control (p<0.05). The symbol "∂" in the same row indicates significant difference among the DM-related groups taking the DM rats as the reference.
Dose: ip Moriamin 1.6 mL/rat-day. QT ip treated control (70 mg/kg-day, suspended in PBS).

dL, 175±10 mg/dL, and 160±5 mg/dL, respectively (**Table 2**). A slightly raised level 225±40 mg/dL was found for AA+QT control (p<0.001) (**Table 2**). To compare, the blood glucose level in DM rats was raised to 320±45 mg/dL. Further highly raised levels were seen in groups DM+AAM, DM+QT, and DM+AAM+QT to 400±93 mg/dL, 421±80 mg/dL and 520±57 mg/dL, respectively (p<0.01) (**Table 2**). Apparently, neither QT alone nor the cotherapy AMM+QT was effective to suppress the hyperglycemic state of T1DM (**Table 2**).

Conversely, the kidney weight (KW) in the DM associated rats were all increased due to inflammatory edema (**Table 2**). As seen, the kidney weight in the DM-related groups ranged within 3.5±0.2g -3.8±0.2g and the percent ratio KW/BW ranged within 1.1±0.3–1.3±0.2 (p<0.05) (**Table 2**). The kidney weights of experimental control rats were rather comparable (i.e., 2.8±0.1g- 2.9±0.1g) and the percent KW/BW ratio yielded a range within 0.7±0.1 -0.8±0.4 (p<0.05) (**Table 2**). As a consequence of compromization, the percent ratio KW/BW in DM groups was significantly raised (**Table 2**). QT alone suppressed the ratio to 0.7, while in DM rats the ratio was

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slightly raised to 0.9, underlying QT to be slightly effective for prevention of renal edema and inflammation (**Table 2**). The serum albumin levels in the four controls all fell into the normal range 3.8-5.4 g/dL (**Table 3**). T1DM rats exhibited severely lowered serum albumin level, 3.2±0.1 g/dL ($p<0.05$) (**Table 3**). However, both QT and AAM+QT therapies seemed to be entirely ineffective and the final serum albumin levels still remained at 3.0±0.1 g/dL and 3.3±0.1 g/dL, respectively ($p<0.05$) (**Table 3**).

Substantially increased levels of the blood urea nitrogen (BUN) and the urinary urea nitrogen (N_{uu}) associated with intensive serum albumin declination were perceived in all DM-associated groups. Healthy rats, when administered AAM alone or AAM+QT, yielded a slightly higher BUN than the normal and QT controls (**Table 3**). The T1DM rats revealed highest level of BUN, 30±5 mg/dL ($p<0.05$), and AAM, QT, or the cotherapy AAM+QT showed only slight suppressive effect (**Table 3**). Contrast to BUN, the N_{uu} in all DM-related groups was more prominently elevated. The N_{uu} levels were prominently elevated to 147±9 mg/dL and 158±8 mg/dL in T1DM and DM+AAM groups. Administration of QT or AAM+QT alleviated the N_{uu} level to 131±4 mg/dL and 121±6 mg/dL, respectively ($p<0.05$) (**Table 3**). Similar to N_{uu}, the urinary proteins were severely raised to 189±6 mg/dL in the T1DM rats (**Table 3**). AAM therapy further increased the level to 256±8 mg/dL, QT and coadministration of AAM<+QT effectively suppressed the urinary protein levels to 137±8 mg/dL and 121±8 mg/dL, respectively, yet still not completely recovered to the normal ranges 24±6 mg/dL, 88±5 mg/dL, 36±5 mg/dL, and 78±7 mg/dL respectively for the healthy control, AAM, QT, and AAM+QT controls ($p<0.05$) (**Table 3**).

The normal range of triglyceride (TG) for SD rats is 26–145 mg/dL [**23**]. Level of TG in T1DM and AAM+DM did not show any significant difference from that of the healthy control 75±15 mg/dL (**Table 3**). Amazingly, QT raised the TG level of DM to 255±45 mg/dL, and further elevated to 553±75 mg/dL after treated with AA+QT ($p<0.05$) (**Table 3**).

Moreover, compared to the normal range 0.6±0.1 mg/dL, 45±4 mL/24 h, 1.88 mL/h and 33±6 mg/dL, the increase in serum creatinine (Cr_{sr}), the 24h-urine volume (V_{ur}), the urine flow rate (υ_h) and the urinary creatinine (Cr_{ur}) levels were highly elevated in all DM-associated groups (**Table 4**). The serum creatinine levels were highly raised by T1DM, DM+AAM, DM+QT, and DM+AAM+QT respectively to 1.6±0.2 mg/dL, 1.8±0.2 mg/dL, 1.4±0.2 mg/dL, and 1.5±0.2 mg/dL compared to 1.1±0.1 mg/dL, 0.7±0.1 mg/dL, and 0.8±0.1 mg/dL for AAM, QT and AAM+QT controls (**Table 4**). Accordingly, the creatinine clearance was substantially affected by T1DM, yielding respectively 11.0±0.6 mL/min, 13.7±0.4 mL/min, 10.8±0.4 mL/min, and 8.6±0.3 mL/min in DM, DM+AAM, DM+QT, and DM+AAM+QT rats (**Table 4**), which were far excess in concentration when compared to 1.7±0.1 mL/min, 3.8±0.1 mL/m 3.7±0.2 mL/min, and 7.0±0.3 mL/min in the normal, AAM, QT, and AAM+QT control groups (**Table 4**).

On the other hand, GC/FID analysis for urinary amino acid excretion revealed the retention times were relatively long for all amino acids. The overall retention time covered a span from 13.291 min for alanine to 27.132 min for cysteine (**Table 5**).

More amazingly, the normal SD rats prominently showed serinuria (12.0±0.2 mg/mL urine). When AAM was applied, the serine reabsorption was completely effected, instead the reabsorption of valine, arginine and histidine were suppressed (**Table 6**). QT alone severely inhibited the methionine reabsorption in the healthy rats, leading to extremely severe methioninuria up to 35.4±0.3 mg/mL ($p<0.001$) (**Table 6**). Although AAM+QT was able to completely alleviate the urinary loss of methionine, severe leucinuria (11.3±0.2 mg/mL) and phenylalaninuria (7.9±0.2 mg/mL) occurred instead (**Table 6**). In T1DM rats severe glycinuria (21.4±0.2 mg/mL) and leucinuria (6.6±0.2 mg/mL) were apparently perceived.

Table 3. Serum and urinary biochemical parameters*,†,‡.

Items/Normal range	Group							
	1	2	3	4	5	6	7	8
	CTL	AAM	QT	AAM+ QT	DM	DM+AAM	DM+QT	DM+AAM+QT
Serum Ab(g/dL) (3.8-5.4)	4.5±0.2	5.1±0.3$^{\&}$	4.4±0.1	4.7±0.2	3.2±0.1$^{\&}$	3.8±0.1$^{\&\partial}$	3.0±0.1$^{\&}$	3.3±0.1$^{\&}$
TG mg/dL	75±15	75±15	81±5	75±5	120±20	103±17	255±45	553±75
BUN (mg/dL)(15-21)	16±2	20±3$^{\&}$	18±2	22±3$^{\&}$	30±5$^{\&}$	28±3$^{\&}$	27±2$^{\&}$	29±4$^{\&}$
N_{UU} (mg/dL)	55±6	77±6$^{\&}$	60±8	80±7$^{\&}$	147±9$^{\&}$	158±8$^{\&}$	131±4$^{\&\partial}$	121±6$^{\&\partial}$
PT_{ur} (mg/dL) (<100)	24±6	88±5$^{\&}$	36±5$^{\&}$	78±7$^{\&}$	189±6$^{\&}$	256±8$^{\&\partial}$	137±8$^{\&\partial}$	121±8$^{\&\partial}$

*Measured on urine collected the first day after different treatments.
†Data expressed in mean±SD (n=8).
‡The symbol "&" in the same row indicate significant difference from the normal control ($p<0.05$). The symbol "∂" in the same row indicates significant difference among the DM-related groups taking the DM rats as the reference.
Dose: ip Moriamin 1.6 mL/rat-day. QT ip treated control (70 mg/kg-day, suspended in PBS).

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Table 4. Parameters used to estimate the creatinine clearance*,†,‡.

Items/Normal range	Group							
	1	2	3	4	5	6	7	8
	CTL	AAM	QT	AAM+ QT	DM	DM+AAM	DM+QT	DM+AAM+ QT
Cr_{sr} (mg/dL) (0.2-0.8)	0.6±0.1	1.1±0.1$^{\&}$	0.7±0.1	0.8±0.1$^{\&}$	1.6±0.2$^{\&}$	1.8±0.2$^{\&\partial}$	1.4±0.2$^{\&\partial}$	1.5±0.2$^{\&}$
V_{ur} (mL/24 h)	45±4	90±6§	54±7	105±6§	197±7$^{\&}$	171±8$^{\&\partial}$	187±7$^{\&}$	180±9$^{\&\partial}$
υ_h (mL/h)	1.88	3.75	2.25	4.38	8.21	7.13	7.79	7.50
υ_{min}, $\times 10^2$ (mL/min)	3.1	6.3	3.8	7.3	13.7	11.9	13.0	12.5
Cr_{ur} (mg/dL)	33±6	67±6$^{\&}$	68±7$^{\&}$	77±8$^{\&}$	128±7$^{\&}$	131±5$^{\&}$	116±4$^{\&\partial}$	103±3$^{\&\partial}$
C_{Cr} (mL/min)	1.7±0.1	3.8±0.1$^{\&}$	3.7±0.2$^{\&}$	7.0±0.3$^{\&}$	11.0±0.6$^{\&}$	13.7±0.4$^{\&\partial}$	10.8±0.4$^{\&\partial}$	8.6±0.3$^{\&\partial}$

*Measured on urine collected the first day after different treatments.
†Data expressed in mean±SD (n=8). The superscript symbol "&" in the same row indicate significant difference from the normal control ($p<0.05$). The superscript symbol "∂" in the same row indicates significant difference among the DM-related groups taking the DM rats as the reference. Dose: ip Moriamin 1.6 mL/rat-day. QT ip treated control (70 mg/kg-day, suspended in PBS).
‡The creatinine clearance is calculated by the equation: $Ccr = (Cr_{ur} X \upsilon_{min})/Cr_{pl}$.

Table 5. Retention times of each individual amino acid in GC/FID analysis*.

Amino acid	Retention time (min)
Alanine	13.291
Glycine	13.496
Valine	14.629
Leucine	15.340
Isoleucine	15.554
Norleucine (the internal standard)	15.730
Proline	15.934
Methionine	17.835
Serine	18.123
Threonine	18.381-18.477
Phenylalanine	19.007
Aspartic acid	19.627
Hydroxy Proline	19.816
Glutamic acid	20.566
Asparagine	21.042
Lysine	21.450
Glutamine	21.926
Tryptophan	21.983-22.113
Arginine	22.255
Histidine	23.132
Tyrosine	23.535
Cysteine	27.132

*Derivation of amino acids were conducted derived with the derivatization reagent MTBSTFA (N-Methyl-N-(Tert-Butyldimethylsilyl)trifluoroacetamide in pyridine .

DM+AAM attenuated the glycine loss to 13.7±0.2 mg/mL with concomitantly increased urinary loss of isoleucine (8.2±0.2 mg/mL) (**Table 6**). To our astonishment, treating T1DM with QT elicited huge amount of methionine loss (23.1±0.3 mg/mL) (**Table 6**). However, in the meanwhile, we recognized that the combined therapy AAM+QT restored methionine reabsorption (3.6±0.1 mg/mL in urine) at the expense of severe leucinuria (11.3±0.4 mg/mL in urine) and isoleucinuria (11.4±0.4 mg/mL in urine) which were accompanied with moderate lysine and tryptophan loss (**Table 6**). Although the sum of amino acid excretion was very comparable among all groups, the total amino acid (TAA) loss was found to be extremely high, in particular in groups T1DM and DM+AAM+QT rats, showing losses of 8491 mg/day and 8910 mg/day (**Table 6**). Treatment with AAM alone and QT alone moderately suppressed the level to 6584 mg/d and 6545 mg/d, respectively (**Table 6**).

Discussion

Consistent with Cooke and Plotnick [**4**], we showed the STZ induced DM rats revealed symptoms of hyperglycemia, insulin insufficiency, glucosuria and weight loss (**Tables 1** and **2**). In the meanwhile, the urinary glucose level was severely raised (**Table 2**), implicating the characteristic T1DM, a result similar to Cooke et al., [**4**].

Apparently, the body weight loss in the DM-associated rats was closely related with the serum insulin levels (**Table1**). Although QT is a potent prooxidant and acts as the insulin secretagogue [**19**], QT in this case failed to suppress the states of hyperglycemia (**Figure 1**) and hypertriglyceridemia (**Table 3**), underlying the typical T1DM resulting from the complete destruction of islets in pancreatic b cells by STZ. Moreover, attention must be paid to the fact that both QT and AAM were unable to stimulate either insulin biosynthesis or secretion in the T1DM rats (**Table 1**), hence the DM+AAM and DM+AAM+QT rats showed the least weight gain (**Table 1**). Insulin helps cells in the liver, skeletal muscles, and fat tissue uptake glucose from blood. Action of insulin is mediated by tyrosine kinase

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Table 6. The absolute amount of amino acids appearing in the first day urine after different treatments*,†,‡.

AA	†Group No. /mg amino acid /mL urine							
	1	2	3	4	5	6	7	8
	Control	AAM	QT	AAM+QT	DM	DM+AAM	DM+QT	DM+AAM +QT
Gly	1.4±0.1	4.1±0.1$^{\&}$	0.2±0.1$^{\&}$	4.6±0.2$^{\&}$	21.4±0.2$^{\&\&}$	13.7±0.2$^{\&\&\partial}$	3.9±0.2$^{\&\partial}$	4.2±0.1$^{\&\partial}$
Val	0.7±0.1	3.6±0.2$^{\&}$	0.2±0.1$^{\&}$	ND	2.6±0.1$^{\&}$	2.5±0.2$^{\&}$	2.7±0.1$^{\&}$	3.1±0.2$^{\&\partial}$
Leu	4.4±0.2	6.0±0.2$^{\&}$	0.3±0.0$^{\&}$	11.3±0.2$^{\&}$	6.6±0.2$^{\&}$	6.2±0.2$^{\&}$	2.7±0.1$^{\&\partial}$	11.3±0.4$^{\&\partial}$
Ile	0.7±0.1	ND	0.3±0.0$^{\&}$	1.2±0.3$^{\&}$	0.5±0.1$^{\&}$	8.2±0.2$^{\&\partial}$	1.3±0.0$^{\&\partial}$	11.4±0.4$^{\&\partial}$
Ser	12.0±0.2	ND	ND	ND	ND	ND	ND	ND
Phe	3.9±0.1	2.4±0.1$^{\&}$	0.2±0.0$^{\&}$	7.9±0.2$^{\&}$	2.0±0.1$^{\&}$	1.5±0.1$^{\&}$	1.7±0.1$^{\&\#}$	2.9±0.1$^{\&\partial}$
Met	6.2±0.2	3.9±0.1$^{\&}$	35.4±0.3$^{\&\&\&}$	2.5±0.2$^{\&}$	5.9±0.1	2.1±0.1$^{\&}$	23.1±0.3$^{\&\&}$	3.6±0.1$^{\&\partial}$
Trp	1.2±0.2	1.7±0.1$^{\&}$	0.1±0.0$^{\&\&}$	1.8±0.1$^{\&}$	2.1±0.1$^{\&}$	1.7±0.1$^{\&}$	0.2±0.1$^{\&\partial}$	3.4±0.1$^{\&\partial}$
Arg	0.6±0.2	6.9±0.2$^{\&}$	0.1±0.0$^{\&}$	2.4±0.0$^{\&}$	ND	0.8±0.1	0.1±0.0$^{\&\partial}$	1.9±0.1$^{\&\partial}$
Lys	3.3±0.1	1.0±0.0$^{\&}$	0.3±0.1$^{\&}$	4.4±0.1$^{\&}$	2.9±4$^{\&}$	1.2±0.1$^{\&\partial}$	0.3±0.0$^{\&\partial}$	5.8±0.2$^{\&\partial}$
His	0.7±0.1	6.5±0.1$^{\&}$	ND	ND	ND	0.6±0.1	0.1±0.0$^{\&}$	1.9±0.0$^{\&}$
Sum	35.1	36.1	37.1	36.1	43.1	38.5	35.0	49.5
TAA (mg/d)	1580	3249	2003	3791	8491	6584	6545	8910

*Measured on urine collected the first day after different treatments.
†Data expressed in mean±SD (n=8). The superscript symbol "&" in the same row indicate significant difference from the normal control. "&" for $p<0.05$; "&&" for $p<0.01$; "&&&" for $p<0.001$. The superscript symbol "∂" in the same row indicates significant difference among the DM-related groups taking the DM rats as the reference ($p<0.05$). Dose: ip Moriamin 1.6 mL/rat-day. QT ip treated control (70 mg/kg-day, suspended in PBS).
‡Sum: sum of amino acid in per mL (mg/mL).
TAA: total amino acid excreted per day (mg/24 h)
ND: not detected

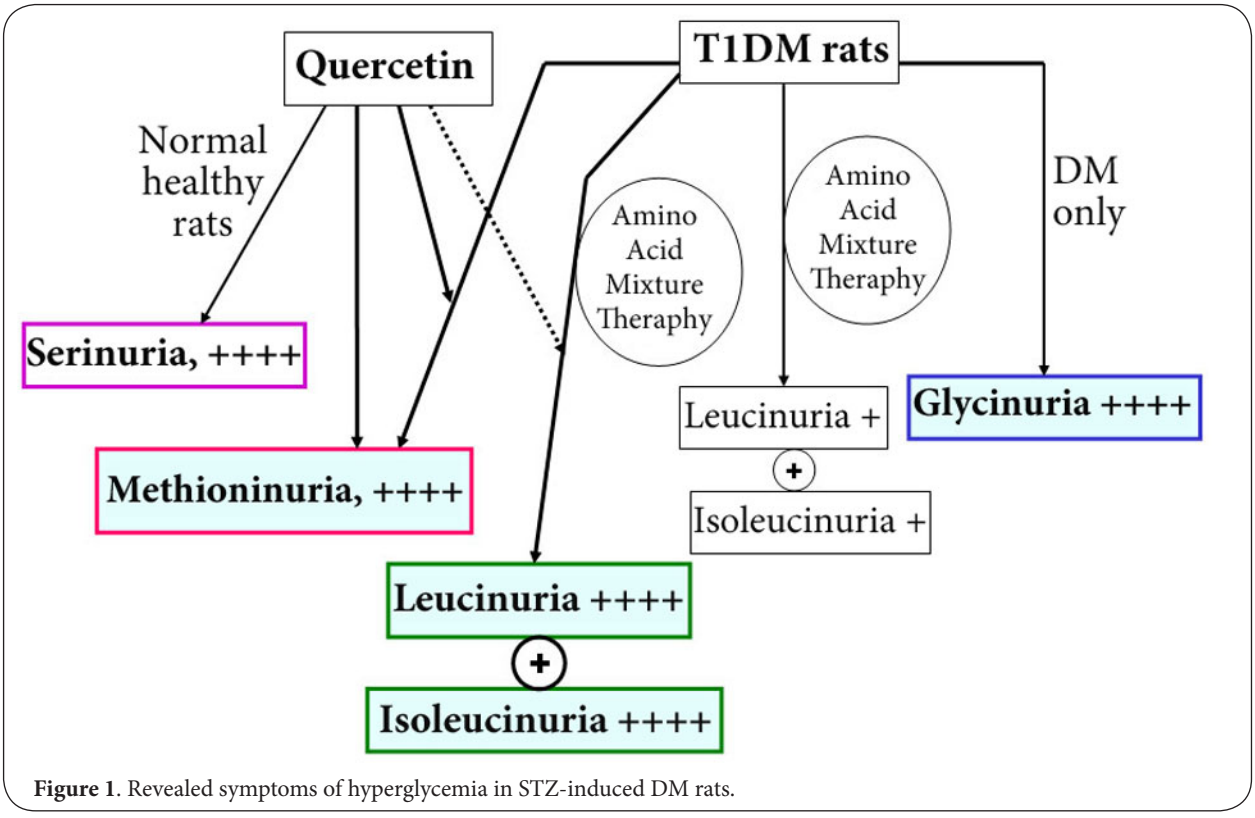

Figure 1. Revealed symptoms of hyperglycemia in STZ-induced DM rats.

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enzyme, which is part of the insulin receptor, and the tyrosine kinase triggers a phosphorylation cascade in the cytoplasm to influence intracellular processes [8]. Without the insulin biosynthesis, QT totally failed to affect the blood glucose level, a phenomenon entirely different from that for type 2 DM [19].

Taken together, the severe body weight loss was ascribed to insulin insufficiency (**Tables 1**and **2**), declined serum albumin level (**Table 3**) [9], increased urinary albumin excretion (albuminuria) [9], proteinuria (**Table 3**), elevated BUN (**Table 3**), increased creatinine clearance (**Table 4**), and amino acid loss due to failure of reabsorption (**Table 6**). In addition, the urinary amino acid profile might give certain degree of information about the body weight loss. In the DM-associated groups, more apparent loss of essential amino acids was perceived, like loss of leucine and methionine in the T1DM rats; loss of leucine and isoleucine in DM+AAM rats, the methionine loss in the DM+QT rats, and loss of leucine, isoleucine and lysine in the DM+AAM+QT group (**Table 6**). Protein helps maintain muscle mass, is used for enzymes involved in liver detoxification, and is required for immune function. Albuminuria and reduced GFR have been demonstrated to be risk factors for progressive kidney failure and cardiovascular disease [24]. The increase of KW/BW ratio in reality was a compromised consequence, i.e., the severely reduced body weight and the enlarged kidney due to renal edema and inflammation [4].

Literature indicated that amino acid infusions can increase the renal blood flow (RBF), glomerular filtration rate (GFR) and stimulate tubular reabsorption in adults [25], the plasma amino acid levels accordingly can be increased up to 20-fold (p<0.005) with GFR increased by 50±8% (p< 0.001) [24]. Under such circumstance, the reabsorption threshold could be easily exceeded.

On the other hand, QT inhibited the renal catechol O-methyltransferase, only O-methylated estradiols could be normally excreted into urine [19,25,26], suggesting the possibility that quercetin may injure kidney by inhibiting the methylation of certain estrogenic nucleus.

In renal, the colocalization of exclusive reabsorption and metabolism of amino acids makes the pars recta the tubule site for the recycling of the carbon structure of D-amino acids [27]. Nonetheless, the great excess of serinuria (12.0±0.2 mg/mL) by normal SD rats (**Table 6**) was really astonishing. The reason is still unclear. AAM supplementary therapy completely abolished the serinuria (**Table 6**). Conversely, in DM rats serinuria was entirely undetected. Instead, glycinuria predominated (**Table 6**), implicating QT being able to play the role in remodeling the amino acid (or protein) metabolism. Biochemically the rates of homocysteine remethylation and serine synthesis were inversely correlated (r = -0.89, p< 0.001) [28], suggesting the recovered serine reabsorption (**Table 6**) could decrease the demand for homocysteine remethylation.

In addition, biochemical and clinical vitamin B_{12} deficiency have been demonstrated to be highly prevalent among patients with T1DM and T2DM [29,30]. B_{12}–deficiency could elicit impaired conversion of 5-MTHF to THF, which in turn inhibits the methionine biosynthesis from homocysteine [29,30] (see **Supplement Figure S1**). Alternatively, serine is required for conversion of THF into 5,10-methylene-THF that acts a methyl donor for conversion of dUMP into dTMP (See **Supplement Figure S1**). Wagner et al., had demonstrated the complex interactions between the different subunits of heterodimeric amino acid transporters [31]. Whether quercetin could remodel these different subunits remains to further investigation.

Furthermore, contrary to Vannini et al., [18], we found severe glycinuria instead of alaninuria in both DM and DM+AAM rats (**Table 6**). Either QT or AAM+QT alleviated such abnormal excretion (**Table 6**).

Based on functional studies in kidney and intestine and the amino acid profile in the urine of individuals with different aminoacidurias, five transport activities have been proposed, the "neutral system" or "methionine preferring system" is the one that transports all neutral amino acids [32]. The mechanistic cause in impairing renal methionine reabsorption is still unclear. Presumably methionine, leucine and isoleucine compete at this transporter. While in treating rats with DM+AAM+QT, it seemed such an inhibition apparently was shifted to transporters of leucine, isoleucine, valine, tryptophan, arginine, lysine, and histidine (**Table 6**).

Impaired arginine transport contributes to low renal NO bioavailability and may evoke renal type hypertension [33]. Tryptophan glycoconjugate, 2-(a-mannopyranosyl)-l-tryptophan (MPT), has been shown to be a novel marker of renal function [34]. Lysine has been considered to be a major constituent of amino acid parenteral nutrition solutions which was shown to increase the severity of various types of acute renal failure in the rat [35]. High-dose lysine alone is capable of causing acute renal failure [35]. Recent literature revealed the action mechanism to be due to the formation of carboxymethyllysine (CML), an advanced glycation end product (AGE). We showed T1DM also exhibited hypertriglyceridemia (**Table 3**). Hypereglycemia and hyperlipidemia can trigger AGE-production [19]. AGEs are associated with impaired renal function in diabetes and in uremia [36]. Plasma CML is independently associated with chronic kidney disease (CKD) and is an independent predictor of decline in renal function in older community-dwelling adults [36]. In pregnant women, expression of histidine decarboxylase is upregulated in superficial cortical nephrons [37]. The amino acid L-arginine has been shown to be a reducer of cross-linking in aging collage type IV and is strongly associated with a reduction of collagen accumulation of N-epsilon-(carboxymethyl)lysine (an AGE) [38] in aging mice and in diabetic mice [36], enhancing glomerular filtration rate [39], decresed the formation of AGE-proteins in the vascular mesenteric bed and in the lens of Golden Syrian hamsters stimulated by hyperlipidemia and hyperglycemia [40]. The plasma methionine, leucine, and isoleucine levels of normal SD rats are 60.1±7.4 μmol/L (11.50

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mg/mL), 173.9±23.2 μmol/L (7.54 mg/mL), and 99.2±12.3 μmol/L (13.01 mg/mL) respectively (**Table 7**). We found huge amount of methionine excreted into urine by groups QT and DM+QT (**Table 6**), underlying the inherent inhibitory effect of QT on the renal methionine transporter. Methionine loss may impair DNA methylation, resulting in birth defects, muscle weakness as well as body weight loss [19].

Table 7. Comparison of the plasma amino acid levels between normal human and Sprague Dawley rats.

Amino acid	Plasma level (μmol/L)	
	Human*	SD rats†
Glycine	--	--
Childre	110-240	230.4±19.5
Adults	170-330	--
Serine	--	--
Children	93-150	169.3±8.0
Adults	56-140	--
Valine	--	--
Children	160-350	205.3±25.4
Adults	150-310	--
Isoleucine	--	--
Children	37-140	99.2±12.3
Adults	42-100	--
Leucine	--	--
Children	70-170	173.9±23.2
Adults	66-170	--
Methionine	--	--
Children	13-30	60.1±7.4
Adults	16-30	--
Lysine	--	403.4±95.9
Children	120-290	--
Adults	150-220	--

*Human data are depicted from Health Encyclopedia.
†Muratsubaki and Yamaki (2011).

Pathologically, the distribution profile among the plasma amino acid concentrations varies with the stage of T1DM [41] and the plasma concentrations of valine, leucine, isoleucine, as well as the total branched chain amino acids, alanine, citrulline and proline in T1DM are significantly higher than the normal [42]. Failure in intestinal uptake and renal reabsorption of amino acids can elicit protein malnutrition-related diabetes mellitus [42].

Conclusions

Taken together, the severe body weight loss and reduced KW/BW ratio can be ascribed to insulin insufficiency, declined serum albumin level, increased albuminuria, proteinuria, elevated BUN, increased creatinine clearance, and amino acid loss due to failure of reabsorption and the urinary amino acid profile. Normal rats exhibits serinuria and methioninuria. AAM therapy decreases the glycine excretion, instead evokes isoleucinuria. QT alone therapy can provoke severe methioninuria in both the healthy and DM+QT groups. AAM+QT can rescue methioninuria of T1DM at the expense of leucinuria, isoleucinuria and reabsorption failure for tryptophan, arginine and lysine, implicating the use of AAM with extra supplement of insulin, leucne, isoleucine, tryptophan, arginine and lysine is feasible for alleviation of T1DM.

Additional files

Supplement Table S1
Supplement figure S1

List of abbreviations

AAM: Amino acid mixture treated with Moriamin 1.6 mL/rat-day.
eGFR: Estimated glomerular filtration rate
BW: Body weight
KW: Kidney weight
CCr: Creatinine clearance
NUU: Urinary urea nitrogen
CNS: Central nervous system
PTur: Urinary protein level
Crpl: Plasma creatinine level
QT: Quercetin
Crur: Urinary creatinine level
STZ: Streptozotocin
CTL: Control
T1DM: Type 1 diabetes mellitus
CV: Cardiovascular Vur 24 h-urine volume
DM: Diabetes mellitus

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	CP	YK	CH	CKH	KC	RYP
Research concept and design	✓	--	--	--	✓	--
Collection and/or assembly of data	--	✓	✓	--	--	--
Data analysis and interpretation	✓	✓	--	--	✓	✓
Writing the article	✓	--	--	--	✓	✓
Critical revision of the article	--	--	--	--	✓	✓
Final approval of article	✓	--	--	--	--	--
Statistical analysis	--	--	--	✓	--	--

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