Research Article

Ketogenic diet-fed rats have increased fat mass and phosphoenolpyruvate carboxykinase activity

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The ketogenic diet (KD), characterized by high fat and low carbohydrate and protein contents, has been proposed to be beneficial in children with epilepsy disorders not helped by conventional anti-epileptic drug treatment. Weight loss and inadequate growth is an important drawback of this diet and metabolic causes are not well characterized. The aim of this study was to examine body weight variation during KD feeding for 6 wk of Wistar rats; fat mass and adipocyte cytosolic phosphoenolpyruvate carboxykinase (PEPCK) activity were also observed. PEPCK activity was determined based on the [$H^{14}CO_3^-$]-oxaloacetate exchange reaction. KD-fed rats gained weight at a less rapid rate than normal-fed rats, but with a significant increment in fat mass. The fat mass/body weight ratio already differed between ketogenic and control rats after the first week of treatment, and was 2.4 × higher in ketogenic rats. The visceral lipogenesis was supported by an increment in adipocyte PEPCK, aiming to provide glycerol 3-phosphate to triacylglycerol synthesis and this fat accumulation was accompanied by glucose intolerance. These data contribute to our understanding of the metabolic effects of the KD in adipose tissue and liver and suggest some potential risks of this diet, particularly visceral fat accumulation.

Keywords: Epilepsy / Glucose tolerance / Ketogenic diet / Lipidemia / PEPCK

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1 Introduction

The ketogenic diet (KD), characterized by high fat and low carbohydrate and protein contents, has been proposed as beneficial mainly in patients with epilepsy disorders in children [1], diabetes type 2 [2], bipolar illness [3], and astrocytomas [4]. In support, a recent meta-analysis showed clinical efficacy of the KD in pediatric epileptic patients [5]. The results indicate that children with generalized seizures who respond with >50% seizure reduction within 3 months tend to remain on the diet longer. The diet discontinuation occurred by failure in seizure reduction, diet restrictiveness, and incurrent illness or diet side effects.

Despite this clinical efficacy of KD in seizure disorders the underlying anticonvulsant mechanism is still unclear. Moreover, the understanding of metabolic changes of this diet in liver and other tissues is incomplete and, as yet, unknown biochemical alterations underlie the side effects of this diet. The low carbohydrate intake leads to reduction of serum insulin, which in turn downregulated several glycolytic and lipogenetic enzymes [6]. Besides low insulin, it is known that high-fat diets (including KD) upregulate the nuclear hormone receptor family peroxisome proliferator-activated receptors (PPARs), particularly types alpha and gamma, which would contribute directly to fatty acid (FA) trafficking and oxidation, and indirectly to ketone bodies production [7]

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Abbreviations: FA, fatty acid; **FFA,** free fatty acid; **Gly-3P,** glycerol 3-phosphate; **KD,** ketogenic diet; **PEPCK,** phosphoenolpyruvate carboxykinase; **TAG,** triacylglycerol

The metabolic side effects of KD that contribute to the diet discontinuation in children include hypoglycemia, hyperlipemia, weight loss, and inadequate growth [8]. KD efficacy has been evaluated in experimental animal models of induced seizure with kainic acid [9] and pentylenetetrazole [10, 11]. In Wistar rats we also observed a decrease in weight during the first weeks of this diet, even when this diet is administered *ad libitum* [12]. Considering that this diet involves dramatic changes in metabolism of lipids and proteins, the investigation of this finding can help us to understand the long-term KD-induced changes.

Considering the increased hepatic gluconeogenesis, our working hypothesis was that adipose glyceroneogenesis also would be altered in rats fed with KD and this alteration could be involved in the loss of weight compared to normal-fed rats observed during the first weeks of this diet. The anticonvulsant effect of the KD in young rats apparently depends on long-term changes (4–8 wk) induced by this diet [13]. Thus, this study had as aim to examine: body weight variation during KD feeding of Wistar young rats for 6 wk, evaluating at the end of this term the epididymal and perirenal fat mass; the activity of rate-limiting enzyme of the glyceroneogenesis pathway in adipose tissue (phosphoenolpyruvate carboxykinase (PEPCK), EC 4.1.1.32); liver PEPCK; glucose tolerance; glycemia; lipidemia; and proteinemia.

2 Materials and methods

2.1 Animal research and diets

The experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Male 30 days-old Wistar rats came from the local breeding colony (ICBS-UFRGS). Animals were weight matched and divided into two groups: control rats that received regular laboratory chow (Nuvilab-CR1, from Nuvital, Brazil) and treated rats that received KD (Table 1) for 6 wk [12]. Animals were maintained in a ventilated room at 21°C, with free access to food (except where stated otherwise) and water on a 12 h light/dark cycle.

Table 1. Composition of the control and KDs

Control diet[a)]	(g/kg)	KD	(g/kg)
Total fat	110	Lard	690
		Sunflower oil	5
Protein	220	Protein[b)]	240
Fiber	30	Fiber	10
Ash	60	Ash[c)]	40
Vitamin	20	Vitamin[d)]	15
Carbohydrates	520	Carbohydrates	0

a) Commercial nonpurified diet, Nuvilab-CR1 (Curitiba, Brazil).
b) Casein, purity 87% (from Herzog, Porto Alegre, Brazil) supplemented with 0.15% L-methionine (from Merck, Rio de Janeiro, Brazil).
c) Mineral mixture (from Roche, São Paulo, Brazil), 100 mg/g of ration: NaCl, 557; KI, 3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4 \cdot 7H_2O$, 108; $MnSO_4 \cdot H_2O$, 16; $ZnSO_4 \cdot 7H_2O$, 2.2; $CuSO_4 \cdot 5H_2O$, 1.9; $CoCl_2 \cdot 6H_2O$, 0.09.
d) Vitamin mixture (from Roche), 100 mg/g of ration: Vitamin A, 4; Vitamin D, 0.5; Vitamin E, 10; Menadione, 0.5; Choline, 200; PABA 10; Inositol 10 mg; Niacin, 4; Pantothenic acid, 4; Riboflavin, 0.8; Thiamin, 0.5; Pyridoxine, 0.5; Folic acid, 0.2; Biotin, 0.04; Vitamin B 12, 0.003.

2.2 Body and WAT weight measurement

Control and KD-fed rats were weighed at weekly intervals. Each week a group of animals from both groups were sacrificed by decapitation. Epididymal plus perirenal WAT were dissected out and weighted by only one researcher (LCR, nutritionist).

2.3 PEPCK activity assay

Determination of PEPCK activity was carried out as described previously [14]. Briefly, WAT and liver were homogenized in ice-cold 0.25 mM sucrose (1:9 w/v) and 1 mM PMSF with a Teflon pestle homogenizer. All steps were carried out at 0–3°C. The homogenate was centrifuged for 10 min at $600 \times g$. The supernatant fluid (below a thick lipid phase, in WAT sample) was recentrifuged at $10\,000 \times g$ for 60 min. The sediment of this second centrifugation was washed twice and resuspended to the original volume in 0.25 M sucrose. Since, in rats, the activity of PEPCK is predominantly (90%) cytosolic [15] we decided to use the whole fraction containing both cytosolic and mitochondrial PEPCK. PEPCK activity was determined by the method based on the $[H^{14}CO_3^-]$-oxaloacetate exchange reaction. PEPCK catalyzes the nucleotide-dependent decarboxylation of oxaloacetic acid to produce phosphoenolpyruvate. Inosine triphosphate (instead of GTP) was used as nucleotide [16]. The reaction was stopped by the addition of 5% trichloroacetic acid. After centrifuging, the solution was gassed for 10 min with CO_2 (removing residual $^{14}CO_2$) and aliquots were immediately counted in a liquid scintillation counter in toluene-triton X-100 (2:1 v/v) – PPO – POPOP. Blanks in which inosine triphosphate was omitted were used and the values obtained were subtracted from all assay measurements. The values of PEPCK activity are given as nmol of $H^{14}CO_3^-$ incorporated *per* mg of protein *per* minute. Results are expressed as mean ± SEM. Protein content was measured following Lowry's method [17]

2.4 Glucose tolerance assay

Awake overnight-starved rats (control and KD groups) were given an intraperitoneal injection of glucose (2 mg/g of

body weight). Blood samples were taken from the tail vein of the same animal at 0, 30, 60, and 120 min after injection. Values of glycemia (in mmol/L), measured by a kit from Merck, are means ± SEM of 5 – 6 animals for each group.

2.5 Blood sampling and analysis

Blood samples from awake overnight-starved rats (sixth hour) were incubated at 37°C for 10 min and centrifuged at 800 × *g* for 10 min (Eppendorf 5402; Hamburg, Germany). Serum was stored at 8°C for 24 h. Biochemical analysis was carried out in a Multitest Analyzer (Mega; Merck, Darmstadt, Germany), using specific kits supplied by Merck as follows: total protein (protein-SMT, 1.19703.0001, biuret method); glucose (GLUC-DH 1.07116.0001); triacylglycerol (TAG) (SMT-triglyceride, 1.19706.0001, GPO-PAP method); cholesterol (cholesterol-SMT, 1.19738.0001, CHOD-PAP method). HDL cholesterol was determined using a kit (HDL cholesterol direct FS) from DiaSys (Diagnostic Systems International, Holzheim, Germany).

2.6 Statistical analysis

All analyses were performed using the SPSS program, Version 12.0 (SPSS, Chicago, IL, USA). Data are reported as mean ± standard error mean and were analyzed by Student's *t*-test (when two groups were considered) or by one-way ANOVA followed by post-test Tukey. Values of $p < 0.05$ were considered significant.

3 Results

3.1 Body weight and fat mass changes

Control and ketogenic rats and WAT were weighed during 6 wk (Fig. 1A). Both groups gained weight during this period (during fourth and tenth postnatal week). However, KD-fed rats exhibited a significantly lower body weight, compared to controls, from the second week of KD on. Epididymal plus perirenal fat mass also increased in both groups in this period (Fig. 1B). The fat mass/body weight ratio already differed between the ketogenic and control rats after the first week of treatment (Fig. 1C), being about 2.4 × higher in ketogenic rats. In both groups a positive correlation was observed between this ratio and age in ketogenic and control rats ($R^2 = 0.90$ and $R^2 = 0.83$, respectively).

3.2 White adipose tissue and liver PEPCK activity

A strong increase of PEPCK activity was observed in adipose tissue in ketogenic rats when compared to the adipose tissue of control rats at the sixth week of treatment (Fig. 2). On the other hand, a significant decrease in liver PEPCK was observed at the same time.

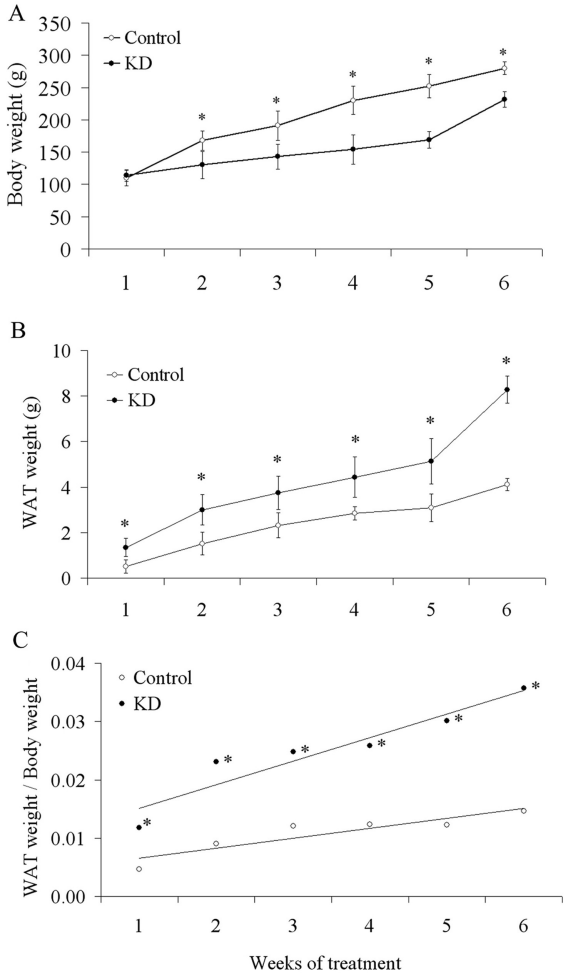

Figure 1. Changes in body and WAT weight during KD feeding during 6 wk. Weeks are indicated in the *X*-axis. Panel A, body weight (express in grams); Panel B, epididymal plus perirenal adipose tissue weight (express in grams); Panel C, ratio between WAT and body weight. Each point is the mean of 6 – 8 rats. * Statistically significant between groups at each time was determined by Student's *t*-test, with the level of significance set at $p < 0.05$.

3.3 Serum content of glucose and triacylglycerol (TAG)

No differences were observed in the serum content of glucose, TAG, cholesterol and protein of control and KD-fed rats for 6 wk (Table 2).

3.4 Glucose tolerance

Glycemia was measured 30, 60, and 120 min after intraperitoneal infusion of glucose (2 mg/g of body weight) to investigate a possible change in glucose tolerance (Fig. 3). A very different profile of glycemia was observed between ketogenic and control rats. The peak observed at 30 min was much higher in ketogenic rats, this being significant

Table 2. Serum biochemistry of rats fed on control and KDs for 6 wk

Parameters	Control rats	KD rats
Total protein (g/L)	62.9 ± 1.8	61.2 ± 1.7
Glucose (mmol/L)	6.90 ± 0.77	6.17 ± 0.66
TAG (mmol/L)	0.67 ± 0.07	0.68 ± 0.07
Total/HDL cholesterol (mmol/L)	3.1 ± 0.1	2.6 ± 0.5

Values are means ± SEM, $n = 5-6$.

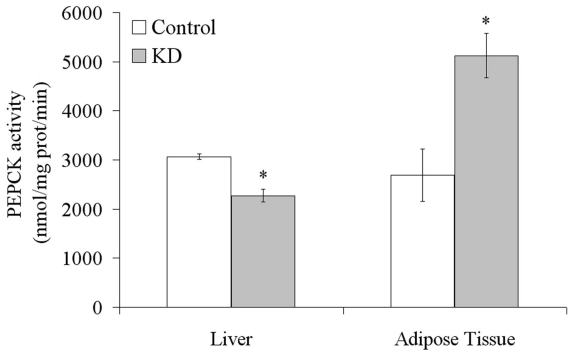

Figure 2. Effect of KD feeding for 6 wk on white adipose tissue and liver PEPCK activity. PEPCK activity was determined by the method based on the $[H^{14}CO_3^-]$ – oxaloacetate exchange reaction after 6 wk of treatment. Each value is the mean of 5 – 6 rats. * The statistical significance was determined by Student's *t*-test, with the level of significance set at $p < 0.05$.

considering that this group received a glucose infusion that was about 20% lower. At 60 min, glycemia dropped in control rats, but remained elevated in ketogenic rats. At 120 min, glycemia was close to the basal level in control rats and it was still elevated in ketogenic rats.

4 Discussion

Confirming previous results, we observed that rats fed with KD gained weight but at less rapid rate than normal-fed rats during the first 6 wk [12]. A decreased body weight was observed in KD group compared to normal-fed group from the second week on and a more than 30% reduction was observed between the fourth and fifth weeks of this diet.

Changes in body weight have also been observed in children on KD [1, 18, 19]. This weight reduction cannot be due to glycogen and associated bound water depletion observed at the beginning of starving or high-fat diets [20]. In fact, poor weight gain is observed in young humans and rodents under KD, controlling caloric intake or *ad libitum* administration, but the metabolic long-term reasons for this finding are still unclear [1, 12, 21, 22]. A careful monitoring of growth of children under KD is recommended, making adjustments in caloric intake and nutritional supplementation [1].

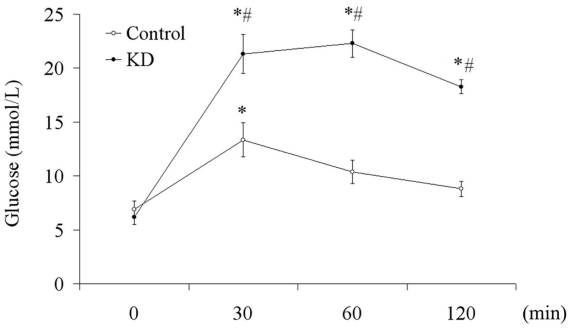

Figure 3. Glucose tolerance assay. Awake overnight-starved control and KD-fed rats (6 wk) were given an intraperitoneal injection of glucose (2 mg/g of body weight). Blood samples were taken at the indicated times from the tail vein of the same animal. Values of glycemia (in mmol/L) are means ± SEM of 5 – 6 animals in each group. * Significant difference among the treatment groups by one-way ANOVA with post-test Tukey. # Significant difference from respective control at each time, analyzed by Student's *t*-test ($p < 0.05$).

We observed a normal proteinemia at the end of 6 wk of KD feeding, which does not support the possibility of inadequate protein intake to explain weight loss. It is important to mention that preliminary experiments using a KD with 20% protein (as used in children) caused undernutrition of the rats as shown by a significant loss of weight and hair (data not shown). For this reason we used 24% protein, equivalent to that used in controls. This result may be related to the Wistar strain of rats used in this study, because Sprague-Dawley rats did not show signs of undernutrition when fed a KD containing 10% protein, even when the diet was started on the 22nd postnatal day [11].

In contrast to this decreased body weight gain, we observed an increment in WAT weight from the first week on. WAT weight to body weight ratio in KD-fed rats was 2.4 × higher and constant during all 6 wk. Despite the fact that weight loss and inadequate growth have been described in children under KD [1, 8, 18, 19], fat accumulation (visceral or other) has not been reported in these children, to our knowledge.

In order to investigate WAT accumulation in rats, we measured PEPCK, the rate-limiting enzyme for glyceroneogenesis [23, 24]. This pathway is an abbreviated version of gluconeogenesis, in which glycerol 3-phosphate (Gly-3P) is produced from glucose precursors and used for the re-esterification of free fatty acids (FFAs) in WAT. A schematic representation of this pathway is shown in Fig. 4. Gly-3P during KD feeding (or fasting) cannot be synthesized from glucose (preferentially used by brain tissue). In addition, Gly-3P can scarcely originate from glycerol since glycerol kinase has a very low activity in WAT when compared with liver [24]. Notice that the fraction of FFAs during lipolysis,

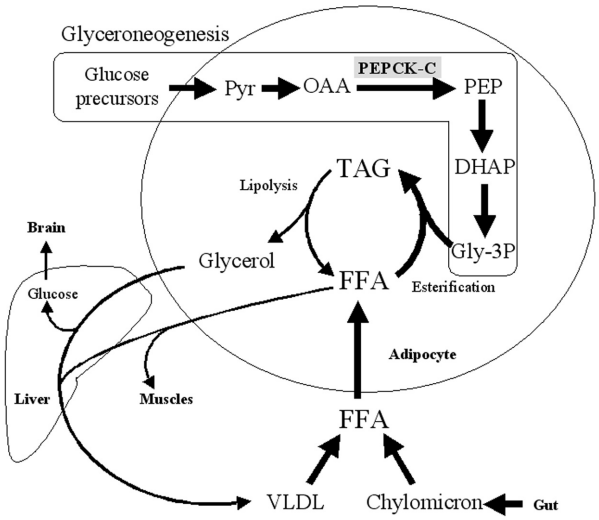

Figure 4. Relationship between the pathway of glyceroneogenesis in white adipose tissue and other tissues in KD-fed rats. Under high-fat/no-carbohydrate diet, the rate of FFA released from adipocyte is regulated by both lipolysis and re-esterification (lipogenesis). As glucose is not present from diet, Gly-3P for lipogenesis is generated from glucose precursors by glyceroneogenesis pathway regulated by PEPCK-C. Glycerol from lipolysis practically is not phosphorylated in adipocytes because there is a negligible level of glycerol kinase activity in these cells. Therefore, glycerol is released into the blood for re-esterification of FFA to TAG and for gluconeogenesis in the liver. Part of the FFA pool from lipolysis is consumed by muscles. PEPCK-C, cytosolic phosphoenolpyruvate carboxykinase isozyme; pyr, pyruvate; OAA, oxaloacetate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; FFA, free fatty acid; Gly-3P, glycerol 3-phosphate; TAG, triacylglycerol; VLDL, very low-density lipoprotein.

which is recycled back to TAG (re-esterification), represents about 30%. Thus, the visceral lipogenesis found in KD-fed rats would be supported by an increment in PEPCK activity. Muscle amino acids are consumed to maintain glycaemia and also to support glyceroneogenesis, providing Gly-3P for the re-esterification of FAs in WAT [23].

Interestingly, liver PEPCK was lower in KD-fed rats. This result is in agreement with the measurement of gene expression of liver PEPCK in KD-fed mice for 5 or 9 wk [22]. In addition, this previous report found low levels of insulin in the KD group (at 9 wk), which would support a reduced glucose tolerance. Another interesting and common finding, that is still not explained, is the reduced blood content of urea in KD-fed animals [12, 22].

It is important to mention that recent results (measuring PEPCK content) indicate that the decrease in liver PEPCK is not necessarily accompanied by an equivalent decrease in gluconeogenesis [25]. This helps to explain normal levels of glucose that are found KD-fed rats [12] or even lower levels in mice [22].

Cytosolic PEPCK is the rate-limiting enzyme in this pathway and its activity is controlled by the rate of transcription of its gene, which readily changes in response to various hormonal and nutritional conditions, including upregulation of PPARs and low levels of insulin [26–28]. There is no known post-translational regulation of this enzyme and thus changes in PEPCK activity directly reflect modifications of PEPCK synthesis and/or degradation [29]. We found elevated activity of this enzyme in adipose tissue of KD-fed rats. This high activity provides enough Gly-3P for the synthesis of TAG in adipose tissue, smoothing the putative elevated lipidemia during KD feeding. In addition, an increment of this enzyme was also found in adipose tissue of animals fed with a high-protein and carbohydrate-free diet [30].

Confirming the "TAG saving activity" of the visceral adipose tissue, circulating lipids in KD-fed rats were normal, in agreement with other studies in human and rodents [18–20]. Whether high fat intake is accompanied by normal lipidemia it may be possible that a very efficient system for lipid storage and/or expenditure is taking place. Moreover, high-fat fed mice for 3 months did not presented abnormal lipid storage in muscles [31]. As such, some benefits of the KD have been suggested to be healthy for the human heart [32] and diabetes type 2 [2].

Considering the WAT increment as a novel feature for investigation in KD, we investigated whether this adipose tissue accumulation could affect the resistance to insulin, that it has been suggested to be a key element in understanding many problems resulting from visceral obesity [33]. We observed a clear change in the glucose tolerance in animals fed with KD, possibly resulting from insulin resistance. Insulin resistance is the hallmark of the metabolic syndrome, which is associated with an elevated risk of type-2 diabetes and cardiovascular diseases [34–36]. Elevated circulating FFAs have been strongly associated with insulin resistance [37, 38].

Transgenic mice overexpressing PEPCK exhibited an increase in hepatic glucose production, which in turn contributes to insulin resistance independent of the plasma FFA levels [39]. Therefore, a specific decrease in liver PEPCK (induced by KD) would not contribute to the glucose intolerance observed. However, circulating FFA and increased adipose PEPCK would be involved [38, 40].

Some authors (*e.g.*, [37]) suggest that increasing fat storage, which avoids the toxic effects of excessive circulating FFAs, may protect against insulin resistance. In KD-fed animals, an elevated flux of FFAs must occur [41], which is not necessarily accompanied by the dyslipidemia observed in metabolic syndrome, particularly characterized by elevated TAG levels [36].

It is important to mention some limitations and perspectives of this work. Firstly, the normal lipidemia observed does not necessarily reflect a normal storage or distribution. Moreover, most of the data available regarding PEPCK regulation are in rodents, thus elevated adipose glyceroneogenesis and its consequences deserve further investigation in

humans. Secondly, the visceral lipogenesis was accompanied by glucose intolerance. This apparent insulin resistance also has been reported in KD-fed rats subjected to insulin-induced hypoglycemia [42]. On the other hand, an increase in insulin sensitivity has been described in children during KD [43]. Thirdly, it is important to mention that KD is not prescribed *ad libitum* to children refractory to conventional anti-epileptic drug treatment. In fact, some authors have proposed KD with caloric restriction to investigate and to obtain the anti-epileptic effect of this diet [9]. In our study, rats received *ad libitum* ketogenic or regular diet. Caloric restriction *per se* appears to induce an increment of hepatic PEPCK activity [44]. Possibly KD with caloric restriction also increases adipose PEPCK activity. However, this issue also deserves further investigation.

In summary, our data show, for first time to our knowledge, an elevated visceral fat accumulation accompanied by glucose intolerance in rats fed on a KD, despite a normal lipidemia reported here and in a number of other studies. These data contribute to understanding the metabolic effect of this diet in adipose tissue and liver of rats and suggest some caution with their potential risks, particularly visceral fat accumulation.

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The authors have declared no conflict of interest.

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