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The influence of high fat diets with different ketogenic ratios on the hippocampal accumulation of creatine – FTIR microspectroscopy study

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ABSTRACT

The main purpose of this study was the determination and comparison of anomalies in creatine (Cr) accumulation occurring within CA3 and DG areas of hippocampal formation as a result of two high-fat, carbohydrate-restricted ketogenic diets (KD) with different ketogenic ratio (KR). To reach this goal, Fourier transformed infrared microspectroscopy with synchrotron radiation source (SRFTIR microspectroscopy) was applied for chemical mapping of creatine absorption bands, occurring around 1304, 1398 and 2800 cm^{-1}. The samples were taken from three groups of experimental animals: control group (N) fed with standard laboratory diet, KD1 and KD2 groups fed with high-fat diets with KR 5:1 and 9:1 respectively. Additionally, the possible influence on the phosphocreatine (PhCr, the high energetic form of creatine) content was evaluated by comparative analysis of chemical maps obtained for creatine and for compounds containing phosphate groups which manifest in the spectra at the wavenumbers of around 1240 and 1080 cm^{-1}. Our results showed that KD2 strongly modifies the frequency of Cr inclusions in both analyzed hippocampal areas. Statistical analysis, performed with Mann-Whitney *U* test revealed increased accumulation of Cr within CA3 and DG areas of KD2 fed rats compared to both normal rats and KD1 experimental group. Moreover, KD2 diet may modify the frequency of PhCr deposits as well as the PhCr to Cr ratio.

1. Introduction

Epilepsy is one of the most common neurological diseases, characterized by spontaneously recurrent seizures [1]. Without proper medical treatment it may lead to physical and cognitive dysfunction as well as chronic changes in personality and social isolation [2]. According to the latest WHO report nearly 50 million people worldwide suffers from epilepsy and around 30% of them do not respond to drug therapy. Seizures may appear as a result of genetic defects or brain damage caused by e.g. stroke, mechanical damage, tumor. However, in 6 out of 10 cases the causes of seizures still remain unknown [3].

The ketogenic diet (KD) is an alternative therapy used to control seizures, especially in the case of pharmacoresistant status epilepticus (SE) [4]. It is known as epilepsy treatment method since 1920s and shows great effectiveness particularly in children [5]. Characteristic feature of KD is a high-fat, moderate-protein and low-carbohydrate content with most commonly used fat to combined protein and carbohydrate ratio (ketogenic ratio, KR) of 4:1 or 3:1 [4,5]. The use of KD leads to imitate biochemistry of fasting state which makes brain more resistant to seizures. The exact mechanism of KD action is still unexplained but it is presumed to be multifactorial [5,6]. Clinical practice shows that KDs with higher ketogenic ratio have greater effectiveness in epilepsy treatment but also stronger adverse effects [7]. Therefore, clinical and experimental researches focus on uncovering which of the diet components are critical for KD effectiveness.

Ketogenic diet results in ketosis, which leads to increased level of ketone bodies in the organism. Ketone bodies (β-hydroxybutyrate, acetoacetate and acetone) are products of fatty acids metabolism in liver. They are transported through the blood and cross blood-brain barrier by monocarboxylic acid transporters [6]. Ketone bodies are more efficient source of energy than glucose [8]. Therefore, their elevated level leads to increase in ATP/ADP ratio and energy reserves in brain [9]. Moreover, some reports suggest that KD results in increased mitochondrial biogenesis within nerve cells, which is an important factor contributing to the replenishment of cerebral energy stores [10].

Transport of energy, in the form of high energy phosphate groups, from mitochondria (site of production) to the cytoplasm (site of consumption) requires the presence of the specific transporter – creatine (Cr). Creatine (*N*-aminoiminomethyl-*N*-methylglycine) is guanidine compound synthesized from the amino acids arginine, glycine and methionine. The compound is provided exogenously with diets containing fish or meat as well as by endogenous synthesis in liver, kidney and

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pancreas. Cr is continuously transported through the blood and taken up in tissues with high energetic requirement such as brain [11,12]. Transport of Cr through blood-brain barrier (BBB) requires presence of specific Cr transporter namely CRT1 [13]. Cr and its phosphorylated form pohosphocreatine (PhCr) are involved in Cr/PhCr/CK system that serves as temporal and spatial energy buffer by connecting the mitochondrial spots of energy production with cytosolic sites of its utilization. ATP and ADP can diffuse through mitochondrial membrane in very limited amounts. Creatine and phosphocreatine are much smaller than ATP and ADP therefore can easily pass through the mitochondrial walls and accumulate to high concentrations within cells cytoplasm [11,14]. Once creatine is transported into cells, creatine kinase (CK) catalyzes the reversible transphosphorylation of creatine via ATP to enhance the phosphocreatine energy pool [11]. In turn, large pool of PhCr within cells enable continuous and efficient replenishment of ATP [13,15]. Existing reports demonstrate 46% increase in the density of mitochondrial profiles in the DG area as well as elevated PhCr/Cr ratio within hippocampal formation of KD-fed rats in case of high-fat diet with KR of 4:1. Such results, together with the improved resistance to low glucose level indicate increased capacity to sustain ATP regeneration in hippocampus in face of increased physiological need [10,16,17]. The elevated energy reserves, occurring as a result of KD treatment, may compensate the interictal metabolic deficits that take place i.e. within epileptic foci [17,18]. Therefore, one of the objectives of this study is to determine whether and how the severity of this phenomenon depends on the KR of KDs.

Previously conducted studies, concerning elemental changes in the hippocampal formation following two different formulas of ketogenic diet, showed that stronger anomalies occur for ketogenic diet with higher KR [19]. Moreover, the obtained results exhibit that the long-term influence of KD on the nervous tissue produces the elemental abnormalities similar to those occurring as a result of epileptic discharges in the pilocarpine model of seizures [20]. This may suggest that KD prepares the brain for epileptic seizures by modification of biochemical properties of nervous tissue so that it is no longer able to develop a complete seizure response after administration of the chemoconvulsant. Such results can be explained by preconditioning phenomenon, which was described in numerous reports [21,22]. Thus, research carried out in the current experiment additionally aimed at verifying whether the stronger effects will also be seen in the case of Cr incidence within hippocampal formations of animals fed with higher KR diet.

Our earlier studies suggest that KD with lower KR = 5:1 may lead to some alterations in Cr hippocampal frequency which are similar to those following seizures [23–25]. However, such trend was found based on the results obtained for limited number of experimental animals and tissue areas. Therefore, the purpose of the present paper is to evaluate and compare the influence of two KDs with different ketogenic ratios on the hippocampal accumulation of creatine for number of cases and size of areas allowing to obtain a reliable result.

To achieve the purposes of the paper, the hippocampal frequency of creatine inclusions in the KDs treated rats was evaluated and compared with analogous data obtained for the animals fed with standard laboratory diet. The examined KDs had KR of 5:1 (KD1) and 9:1 (KD2). Two hippocampal areas, namely sector 3 of Ammon's horn (CA3) and dentate gyrus (DG) were selected for this study. The following regions were chosen based on our previous studies, which exhibited that, although some seizure-related biochemical changes were present in whole hippocampal formation, the anomalies in biomolecular content and structure were the most intensified within CA3 and DG regions [24,25]. The principal cellular layer of CA3 are pyramidal cells whilst in DG – granular cells. Both granular and pyramidal cells are of glutamatergic excitatory and therefore are often the source of epileptic discharges [26]. Moreover, seizure induced neuronal injury may lead to mossy fiber sprouting. The phenomenon means the formation of new axon collaterals of DG granular cells, which form connections with other granule cells whose proximal dendrites are in the inner molecular layer and hilus, as well as stratum oriens in CA3. Mossy fiber sprouting, occurring after status epilepticus and during the subsequent epileptogenesis, results in creation of recurrent excitatory circuits among dentate granule cells [27–30]. Moreover, hippocampal mossy fibers contain large amounts of zinc which acts as neuromodulator. Reuptake of synaptically released zinc into the neuron cells requires large amount of energy and Cr and PhCr play and important role in the transport and storage of energy [31].

Fourier-transform infrared microspectroscopy with synchrotron source of infrared radiation (SRFTIR) was used for detection of creatine in the nervous tissue. This technique is combination of two methods – light microscopy and infrared spectroscopy. The first one allows to localizing microscopic details in the analyzed brain section, whilst the second one provides information about its chemical composition [32]. SRFTIR, due to the great brilliance of synchrotron source of infrared radiation, provides high spatial resolution images at or close to the diffraction limit [33]. Since in biomedical research micrometer spatial resolution is often of great significance, SRFTIR microspectroscopy is becoming more and more desired analytical tool in the investigation of different biological systems [34–37]. It was also applied in the present study for detection of creatine inclusions which typically have sizes varying from a few to dozens of micrometers.

2. Materials and Methods

2.1. Animals

Adult male Wistar rats came from the animal colony of the Department of Neuroanatomy (Institute of Zoology, Jagiellonian University). The animal-manipulation procedures were approved by the Bioethical Commission of the Jagiellonian University and were in accordance with international standards (European Commision Directive 2010/63/EU). Three groups of animals were examined in the study and their characteristic is presented in the Table 1.

The animals were maintained under conditions of controlled temperature (20 ± 2 °C) and illumination (12-h light and 12-h dark cycle). On the 30th day of postnatal development animals were divided into three groups which were subsequently fed with one of the KDs (KD1 or KD2 groups) or with standard laboratory diet (N group). The content of the main nutrients in applied diets is compared in Table 2.

2.2. Sample Preparation

On the 60th day of postnatal development, the animals were perfused intracardially with physiological saline solution. The brains were removed, frozen in liquid nitrogen and sectioned with a cryomicrotome into 12-μm-thick slices. Sections containing the dorsal part of the hippocampal formation were placed on MirrIR low-e microscopic slides and stored in −70 °C till the measurements were taken.

2.3. IR Data Acquisition

The experiment was carried out at the SMIS beamline of SOLEIL synchrotron (Saint Aubin, France). SRFTIR microspectroscopy was used for biochemical analysis of the rat brain samples. The measurements were

Table 1
The characteristic of animal experimental groups.

Experimental group	n[a]	Ketogenic diet 1[b]	Ketogenic diet 2[b]	Standard diet	Perfusion[c]
N	5			+	+
KD1	6	+			+
KD2	6		+		+

[a] Number of animals in a group.
[b] Both KDs were introduced to rats on the 30th day of postnatal life.
[c] Perfusion with physiological saline was done on the 60th day of rat postnatal life.