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Antiseizure Effects of Ketogenic Diet on Seizures Induced with Pentylenetetrazole, 4-Aminopyridine and Strychnine in Wistar Rats

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Summary: The ketogenic diet (KD) is a cheap and effective alternative therapy for most epilepsy. There are paucity of experimental data in Nigeria on the usefulness of KD in epilepsy models. This is likely to be responsible for the poor clinical acceptability of the diet in the country. This study therefore aimed at providing experimental data on usefulness of KD on seizure models. The study used 64 Wistar rats that were divided into two dietary groups [normal diet (ND) and ketogenic diet (KD)]. Animal in each group were fed for 35days. Medium chain triglyceride ketogenic diet (MCT-KD) was used and it consisted of 15% carbohydrate in normal rat chow long with 5ml sunflower oil (25% (v/w). The normal diet was the usual rat chow. Seizures were induced with one of Pentelyntetrazole (PTZ), 4-Aminopyridine (AP) and Strychnine (STR). Fasting glucose, ketosis level and serum chemistry were determined and seizure parameters recorded. Serum ketosis was significantly higher in MCT-KD-fed rats (12.7 ±2.6) than ND-fed (5.17±0.86) rats [p=0.003]. Fasting blood glucose was higher in ND-fed rats (5.3±0.9mMol/l) than in MCT-KD fed rats (5.1±0.5mMol/l) with p=0.9. Seizure latency was significantly prolonged in ND-fed compared with MCT-KD fed rats after PTZ-induced seizures (61±9sec vs 570±34sec) and AP-induced seizures (49±11sec vs 483±41sec) p<0.05. The difference after Str-induced seizure (51±7 vs 62±8 sec) was not significant (p>0.05). The differences in seizure duration between ND-fed and MCT-KD fed rats with PTZ (4296±77sec vs 366±46sec) and with AP (5238±102sec vs 480±67sec) were significant (p<0.05), but not with STR (3841±94sec vs 3510±89sec) [p>0.05] respectively. The mean serum Na+ was significantly higher in MCT-KD fed (141.7±2.1mMol/l) than ND-fed rats (137±2.3mMol/l) with p<0.05. There was no significant difference in mean values of other serum electrolytes between the MCT-KD fed and ND-fed animals. MCT-KD caused increase resistance to PTZ-and AP-induced seizures, but has no effect on STR-induced seizures. This antiseizure property is probably mediated through GABAergic receptors (PTZ effect) and blockade of membrane bound K_{ATP} channels (AP effect) with some enhancement by serum ketosis.

Keywords*:* Ketogenic diet, Antiseizure, Pentylenetetrazole, Amino-Pyridine, Strychnine

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INTRODUCTION

Epilepsy causes a huge health burden on people of sub Saharan Africa like other developing countries. Unfortunately, the number of published experimental work on epilepsy from the region is very limited. Available evidences have shown that the level of seizure control is poor among people living with epilepsy (PLWE), especially in the sub-Saharan Africa (SSA) where sizeable numbers of PLWE resides (WHO, 2005). Part of the reasons for this observation include widespread poverty, unavailability and high cost of anti-epileptic drugs (AEDs), fake and substandard drugs and dearth of experimental findings on epilepsy models amongst others reasons (Mbuba *et al*.,2008; Sanya *et al*., 2013). The ketogenic diet (KD) is a well-established none surgical option for epilepsy, especially the pharmacoresistant and non-surgically correctable intractable epilepsy. The diet is basically a high-fat, adequate protein, and low-carbohydrate and various forms has been used in diet in Europe and America (Bailey *et al*.,2005; Freeman *et al*., 1998; Schwartz *et al*., 1989). Despite the established clinical effectiveness of KD, it is still believed that the potential of this diet has not been fully utilized. This is because knowledge on possible mode of action remains unclear (Huffman and Kossoff, 2006). To allow for better compliance and adaptation to different cultural diet, the medium chain triglyceride KD (MCT-KD) from corn oil was introduced as a suitable alternative to the traditional KD which is often unpalatable (Woody *et al*.,1988). While the traditional KD is calculated on a ratio, the MCT-KD is calculated based on percentages of the daily calorie from the MCT oil and it varies from 15% to 30% (Huffman and Kossoff, 2006). It has been reported to be more ketogenic than the traditional KD because octanoic

and decanoic acids contained in the MCT are more easily transported into the cell (Huttenlocher, 1976; Hartman, and Vining, 2007).

Presently, there are limited studies from Nigeria that had looked at the usefulness and role of KD in epilepsy care either with animal or human models. And unless there are sufficient evidence-based experimental data which had used local dietary formulation, it might be difficult to convince clinicians on the need to accept this effective diet as a suitable alternative for epilepsy, especially the difficult to treat forms. This, therefore, necessitated this study to use sunflower oil (as a form of MCT-KD) which is readily available in Nigeria to determine the effectiveness of the diet on drug-induced seizures in rats.

MATERIALS AND METHODS

Animals

The study used 64 male Wister rats weighing 210 ± 25 kg which were bred in the animal house of the faculty of Veterinary Medicine, University of Ilorin. They were divided into 2 dietary groups which consisted of 32 animals each. The groups are normal diet (ND) and ketogenic diet (KD). The animals were kept for 5 weeks in the animal house of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Ilorin. They were maintained under standard environmental conditions (room temperature, good ventilation and they had unrestricted access to water).

Diets

The ND is the standard rat chow obtained from animal feeds store. The form of KD consisted of 15% of normal carbohydrate content in rat chow with 5 ml sunflower oil added at 25% (v/w) to provide medium chain triglyceride fatty acid (MCT-KD). The diet dose for both groups was 20gm per rat/day (National institute of Health publication, 1985).

Animal weight

The rats were weighed at the commencement of study and subsequently once a week before they were fed in the morning using a standard animal weighing scale. The measurements were taken to the nearest gram.

Seizure induction and Animal grouping

The 64 animals were divided into 2 diet groups containing 32 rats per group. Animals in each group were divided into five subgroups (A-E). Animals in subgroups A, B and C [(experimental groups (8 rats each)] received one of 3 pro-convulsant drugs. Namely: Pentylenetetrazole (Sigma, St. Louis, USA), 4-Aminopyridine (AP, Merck-Sohuchard Germany) or Strychnine (Sigma, St. Louis, USA). Animals in groups D (control group -4 rats) were administered equal volume of normal saline to match the administered pro-convulsant medication (controls). The animals in group E (observatory group - 4rats) received no medication. The calculated drug dosages were: Pentylenetetrazole (PTZ) 25mg/kg, 4-Aminopyridien (AP) -10mg/kg and strychnine (STR) -0.25mg/kg. The route of administration for all drugs was the intraperitoneal (IP) route. Corresponding volume of normal saline /kg body weight was administered to the control rats and via IP route. Animals were observed within 2 hours of drug administration and the induced seizures noted in animals and graded using the method *by* Ono *et al* (Ono *et el.*, 1990). The staging includes: grade 0 - no change in activity i.e. no response (scored 0); grade 1- front or hind limb pawing or staring >5s (scored 1); grade II - stage I plus rearing, nodding or bilateral pawing (scored 2); grade III - jumping, wobbing or falling (scored -3); and stage IV - status epilepticus or death (scored 4).

Blood glucose and ketone level

On the morning of the experiment before animals were fed, blood was obtained from the rat tail vein to determine level of serum ketosis using blood ketone strips and ketone meter (Abbot, Diabetic care. UK). Also, drop of tail blood sample was used to determine blood glucose level before animal were fed using blood glucostix (Bayer Corp, Diagnostics. NY, USA).

Blood collection and analysis

At the end of the experimental period, the rats were sacrificed by cervical dislocation. About 6 to 8 ml of blood was obtained intracardially from each animal. The blood samples for serum chemistry were collected into heparin anticoagulated bottles and centrifuged at 3000rpm for 10 minutes to separate plasma from cells. The plasma was separated into clean plastic container before analysis. Plasma sodium and potassium levels were determined by the method of flame photometry (Amrutkar *et al*., (2013). The plasma concentrations of magnesium, zinc, calcium, urea and creatinine were determined by colorimetric methods using assay kits (Heinegård and Tiderström, 1973; Bishop *et al*., (2005).

Statistical Analysis

Data were computed and analyzed using SPSS (Statistical Package for Social Sciences) version 16.0 Chicago, IL. Categorical variables were expressed as proportions and continuous variables as mean ±standard error of mean (±S.E.M). Comparisons between continuous variables were analyzed using the analysis of variance (ANOVA). Differences were considered significant at p values of less than 5%.

RESULTS

Weight gain

The mean weight of the animals fed on ND increased from 240.5±6.5gm at commencement of study to 303.7±9.1gm after 5 weeks with percentage weight gain of 23%. The mean weight in animal fed on MCT-KD increased from 241.4±8.2gm at commencement to

296.7±5.5gm at the end of the study and the percentage weight was 21%. The differences in weight gain among the two groups of animals did not attain any statistical significant (p>0.05). Table 1

Blood electrolytes, glucose and ketosis

The value of serum electrolytes among the animal fed with ND and MCT- KD is as shown in table 2. The ND-fed rats had significantly lower mean serum Na^+ of 137.5±2.25 (mMol/l) compared with MCT- KD-fed rats with 141.7±21 (mMol/l) with p>0.05. Although the mean value of serum Zn^{2+} was lower in ND (138±34.5 mMol/l) compared with MCT-KD (143.5±22.1 mMol/l) the difference did not attain statistical significance (p>0.05). The ND-fed animals had higher serum glucose (5.3±0.9 mMol/l) compared with MCT-KD (4.9±0.5 mMol/l) but the difference was not significance (p>0.05). The mean value of blood ketosis was significantly higher with MCT-KD (12.7±2.6 mMol/l) compared with ND (5.1±0.8 mMol/l) and p-value was 0.003. Details are as shown in table 2.

Seizure characteristics

Among the 24 MCT-KD fed animals, 3 of the 8 rats given PTZ developed convulsion resulting in 37.5% response rate; 3 out of 8 rats that received AP developed convulsed with 37.5% response rate, while 6 out of the 8 that received STR developed convulsion with 75% response rate. No convulsion was observed among the rats that received normal saline and animals in observatory group. Of the 24 ND-fed animals, 7 out of the 8 rats that received PTZ developed convulsion given 87.5% response rate, and 7 out of the 8 rats that received AP developed convulsion given 87.5% response rate. All the 8 rats that were given STR developed convulsion giving 100% response. One of the rats given STR died resulting in 12.5% fatality rate. No convulsion was observed among the rats in the control and observatory groups. The values of seizure latency among rats that developed convulsion following PTZ injection vary from 456 - 627 second with a mean of 570 ±39 seconds. The seizure latency amongst the AP group vary from 412 - 495 seconds with a mean of 483±45 seconds. Among the STR group the seizure latency vary from 41 - 72seconds with a mean of 62 ± 12seconds.

In the ND-fed animals, PTZ-induced seizure group had mean seizure latency of 61±09 seconds (range of 44 to 65 seconds). The AP-induced seizure had mean latency of 49±11 seconds (range of 39 to 51 seconds). The mean seizure latency was 57±07 seconds with a range of 51 to 72 seconds in STR-induced seizure.

Table 1: Effect of Ketogenic diet on pattern of weight gain in rats

	Normal diet	Ketogenic diet
Initial weight (g)	211.3±7.3	213.4±6.5
Final weight (g)	274.5±8.1	269.5±11.1
Weight difference (g)	63.3±6.6	56.7±6.2
Percentage weight gain (%)	23	21

Table 2: Effect of ketogenic diet on blood electrolytes, glucose and degree of ketosis.

Variables	Normal diet	Ketogenic diet
Na^+ (mMol/l)	137.5±2.3	141.7±2.1*
K^+ (mMol/l)	5.0±.06	5.0±0.3
Mg^{2+} (mMol/l)	2.3±0.3	2.2±0.2
Zn^{2+} (mMol/l)	138±34.5	143.5±22.1
Ca^{2+} (mMol/l)	11.1±1.0	11.1±0.7
Glucose (mMol/l)	5.3 ±0.9	5.1 ±0.5
Ketosis (mMol/l)	5.1±0.8	12.7 ±2.6*

* = P<0.01

DISCUSSION

The finding of this study is that MCT-KD increased animal resistance to PTZ-and-AP- induced seizures. This was evident by significantly prolonged seizure latency, shorter duration of seizures and reduction in number of MCT- KD fed rats that developed convulsion after these two pro-convulsant drugs compared to the ND-fed rats. However, MCT- KD had little protection against STR-induced seizures because of the similarity in seizure latency, duration of the induced seizures and similar number of animals in the MCT- KD fed and ND-fed group following its administration. This result is consistent with those of earlier studies that had demonstrated effectiveness of ketogenic diets against most type of epilepsy in animal models (Appleton and Devivo, 1974; Luttjohann *et al*., 2009; Payne *et al*., 2011).

Another important finding of this study is that there was no significant difference in the weight gained between the MCT-KD fed and ND-fed rats; although, the percentage weight gain was smaller in the MCT-KD fed rats compared to the ND-fed rats. In existing literature, there are inconsistent findings on pattern of weight changes after MCT-KD usage both in humans and in animal studies (Bough and Eagles, 1999; Ribeiro *et al*., 2008). While some studies documented weight loss after a minimum of 5 weeks (Peres *et al.,*

Table 3: Seizure characteristic between the different diets and pro-convulsant drugs

	KETOGENIC DIET (n=32)					NORMAL DIET (n=32)				
Drug (number)	PTZ (8)	STR (8)	AP (8)	NS (4)	None (4)	PTZ (8)	STR (8)	AP (8)	NS (4)	None (4)
No. that convulsed (%)	3 (37.5)*	6 (75)	3 (37.5)	0	0	7 (87.5)	8 (100)	7 (87.5)	0	0
Seizure latency (sec)	570±34*	62±8	483±41**	-	-	61±9	51±7	49±11	-	-
Seizure duration (sec)	366±46*	3510±89	480±67**	-	-	4296±77	3841±94	5238±102	-	-

* = P<0.01, PTZ= Pentylenetetrazole, STR= Strychnine AP = 4-Aminopyridien, NS= Normal Saline.

2013, Frommelt *et al*., 2013), others reported that the diet was associated with weight gain (Frommelt *et al*., 2013; Al-Khalifa *et al*., 2009). Although, some earlier works have suggested that the weight loss might be an essential component of the mechanism of action of KD (Alaei *et al*., 2010), later studies have refuted this claim which support the finding of this study

Another important finding of this study is that the MCT- KD fed rats had significantly higher blood ketosis than the ND-fed rats. This observation is in tandem with earlier reports (Schwartz *et al.*, 1989, Kossoff, 2004) and it is possible that the antiseizure effect of MCT-KD may be related to the blood ketosis because the MCT-KD fed rats had increased seizure latency and shorter duration of convulsion (Payne *et al*., 2011; Kossoff, 2004). There are several clinical reports that seems to support this view since the antiseizure effect of KD is usually lost abruptly whenever the degree of ketosis is broken, either through ingestion of carbohydrate or glucose ingestion (Huttenlocher., 1976). This undoubtedly implicates serum ketosis as demonstrated to be linked with the antiseizure property of MCT-KD. Our study used blood ketosis level rather than urine ketosis, since only blood rather urine ketosis has been documented to have significant correlation with the anticonvulsant effect of MCT-KD (33). This study did not find any significant difference in fasting glucose level among KD-fed rats and the ND-fed rats. This shows that hypoglycemia may not be path of mechanism of action of KD. Our result also reinforces the view that MCT-KD is unlikely to cause hypoglycemia that has been variously reported with the use of the traditional KD (34). Similarly, MCT-KD does not seem to jeopardize blood ketosis (Woody *et al*., 1988; Nakamura *et al*., 1994).

The result of our study also showed that MCT- KD resulted in significant change in level of serum Na^+ but did not affect the serum level of other electrolytes. This is because the MCT-KD-fed rats had significantly elevated serum Na^+ than the ND-fed animals. There had been postulation that MCT-KD could cause increase activity of sodium potassium ATPase, the enzyme responsible for movement of ions across the membrane (Fernandes *et al*, 1996). It is plausible that the observed increase in serum Na^+ in this study is due to increase in extracellularly Na^+ ion movement by the N^+/K^+ enzyme ATPase.

It is likely that MCT-KD produced increased in resistance to PTZ-induced seizure through increase in GABA inhibition. This is because PTZ-induce seizure in animal occurs through the inhibition of GABA transmission (Ribeiro *et al*., 2008). Our result showed that MCT-KD was effective in increasing resistance AP-induced seizures. Earlier studies have shown AP-induced seizure in animals to occur through blockade of membrane bound K_{ATP} channels (Armand *et al*., 1999). Therefore, it is most likely that MCT-KD increase resistance to seizures through facilitation of both GABAergic activity and also causes increase in activity of the membrane bound K_{ATP} channels. Because KD did not protect against Strychnine-induced seizure, which occur through antagonism of glycine receptor like kainic acid (Gupta (2009); It is likely that KD has no influence on glycine receptor activity. To reinforce our postulate that KD possibly acting through enhancement GABA activity, earlier studies has shown that sodium valproate, a broad-spectrum AED which acts through enhancement of GABAergic activity acts similarly to KD and both medications have synergistic antiseizure effect when administered together to epilepsy patient (Loscher *et al*., 1991; Payne *et al*., 2011).

This study showed that MCT-KD has antiseizure, hence antiepileptic property which resulted in increased resistance to PTZ-and AP-induced seizures, but not STR-induced seizures. It is possible that the elevation in blood ketosis have some relationship with the antiseizure protection effect MCT-KD. Loss of weight may not be an essential component of the mechanism of action of MCT-KD in causing seizure resistance.

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