The Neurobehavioral Effect of Ketogenic Diet on Cognitive Functions in Chronic Unpredictable Stress Adult Male Rat Model

Lamis M. Asran¹, Samy Makary¹, Horeya E. Korayem², Eman Kolieb*¹

¹Department of Human Physiology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
²Department of Histology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

Abstract

Background: The ketogenic diet (KD) is a high-fat diet in which fatty acids become an obligatory energy source for the brain. Stressful events lead to structural and functional disturbances in the hippocampus and dentate cells responsible for memory. Aim: This work aimed to study how the KD modulates the effect of chronic unpredictable stress (CUS) on cognition via the modulation of the adult hippocampal and dentate neuronal cell neurogenesis in adult male rats. Materials and Methods: For this study; Twenty-four adult male Wistar albino rats, were divided into the Control group, normally fed palatable diet (NPD); KD group, fed on KD; CUS group, rat model fed on NPD, (CUS/NPD); CUS model group, fed on KD (CUS/KD) for 37 days. Barnes Maze (BM) assessed spatial memory and learning. Elevated Plus Maze (EPM) and open field test evaluated the anxiety-like behavior from CUS. To evaluate the KD effect, serum levels of ACTH, corticosterone, and brain homogenate of brain-derived neurotrophic factor (BDNF), dopamine, and acetylcholine (ACh). Results: CUS/KD group showed a reduction in the number of errors and latency time in BM. CUS/KD reduced the anxiety level compared to CUS. The same results were shown for brain neurotransmitters, antioxidant effect, and hormonal levels. For the hippocampus and dentate microstructure, CUS/KD showed an increase of neurogenesis doublecortin stain and restoration of the hippocampus and dentate neuronal cells. Conclusions: KD improved hippocampal neurogenesis and adverse stress effects on cognition, memory, and learning via decreasing anxiety and stress hormones and by increasing ACh, antioxidant effect, and BDNF.

Keywords: Ketogenic diet, stress, neurogenesis, spatial learning and memory, impairment.

Introduction

The ketogenic diet (KD) is a high-fat diet in which carbohydrates provide low glucose sources, with high fatty acids constituting the brain’s primary energy source. KD causes elevated levels of ketone bodies as the liver produces β-Hydroxybutyrate (β-HB). In the absence of glucose, the ketone body is the preferred energy source by the brain as fuel(1). The classic KD mimics the periods of fasting(2). The KD is used clinically for epilepsy(3,4). KD improved cognitive functions in normal states and different neurological disorders(4). Stressful events are associated with depression and anxiety(5). The biological consequences of stress on the brain and its neural basis are

*Corresponding Author: eman.kolieb@med.suez.edu.eg
still poorly understood\(^6\). Adults with chronic stress have reduced medial prefrontal cortex volume, hyperactivity of the hypothalamic-pituitary-adrenal axis, and a decline in cognitive functions. Weather animal models of stress can demonstrate a further step in exploring neuronal plasticity, learning, and cognition against stress needs further investigation\(^6\). The hippocampus has been found in animal models to show structural plasticity as remodeling of dendrites and synaptic connections and neurogenesis in response to acute changes in neurotransmitter and hormonal changes during stress, which is not the case in chronic conditions\(^7\). This difference in response needs further investigation. The chronic unpredictable stress (CUS) animal model has been used to study stress exposure consisting of random and unpredictable exposure to various stressors during several weeks \(^4\). Glucocorticoids are hormones released during the stress response. The basal or acutely elevated glucocorticoid levels increase synaptic plasticity and facilitate hippocampal-dependent cognition, which is not the case in chronic conditions\(^8\). Therefore, this study evaluated the KD effect. Adult neurogenesis is a dynamic modulation by various physiological stimuli\(^9\). The hippocampus and dentate gyrus (DG) in adult animals are the areas of neurogenesis\(^10\). CUS causes impairment of cognitive function and memory\(^11\). The current study investigated the effect of CUS in the presence and absence of KD. The response of the animal weight, cognition, depression, inflammatory cytokines, oxidative stress, and stress hormones, which could affect adult neurogenesis, were investigated. KD can modulate neurological disorders. Could KD modulate stress-induced adult neurogenesis and cognitive functions?

**Materials and Methods**

**Animals**
The study investigated twenty-four Wistar albino adult male rats, 4-5 months of age, with an average weight of 200 to 250 grams in the animal house, Faculty of Medicine, Suez Canal University (FOM/SCU). The rats were maintained on a day-light daily cycle with free access to food and water for one week for acclimatization before the start of the study. Then, the rats were divided into four groups, with six animals in each group. Ethics Committee, FOM/SCU Egypt approved the study protocol; code # 4515.

**Study groups**
The animal groups were as follows: Group 1 (control/NPD): Normal rats fed on the normal palatable diet (NPD); Group 2 (KD): Rats fed on KD; Group 3 (CUS/NPD): CUS model fed on NPD and Group 4 (CUS/KD): CUS model fed on KD.

**Experimental rat models**

**Chronic Unpredictable Stress (CUS) model**
The CUS model was induced in groups 3; (CUS/NPD) and 4; (CUS/KD). The model started from the 15\(^{th}\) day of the study’s beginning and continued till the end of the experiment. The CUS model was established according to a previous study with minor modifications. It consists of several unexpected stressors to prevent habituation, as described previously. Briefly, each stressor was given three times, and no same stressor was given continuously for two days. The stressors included a ten-minute tail pinch, physical restraint, moist bedding, reversed light/dark cycle, and hot air stream\(^4\).

**Ketogenic diet protocol**
All rats were fed the NPD, 15 g/rat/day, during the accommodation period for one week, but the control group continued until the end of the experiment. Rats in the KD, and CUS/KD groups, were fed a low
carbohydrate-high fat KD from the 8th day till the end of the experiment. The macronutrients percentages from the dry matter of the KD (29.18 MJ; energy) were fat 65, protein 20, carbohydrate 3, compared to where NPD (17.62 MJ; energy) was fat 8, protein 19, carbohydrate 66 compositions, as a percentage of dry matter (dm). On day 33, metabolic cages were used to collect urine for acetone to confirm ketosis and the serum β-HB level was measured.

Behavioral assessment

1-Assesment of spatial learning and memory: Barnes Maze time plan.

The Barnes maze is a dry land-based behavioral test developed to study rats' spatial memory and learning. Animals usually tend to escape from the open platform surface into a small dark recessed chamber under the platform called a "target box". The paradigm consists of a circular platform (100 cm in diameter) with 18 holes (hole diameter: 5 cm) along the perimeter. Rats were tested for the spatial location of the target box. Extra-maze cues were put all around the paradigm as reference cues to learn the position of the target hole (escape hole). On the habituation day (pre-training trial), the rat was placed in the middle of the maze in a black colored cylindrical start chamber for 10 seconds, then the chamber was lifted, and the rat was guided to the target hole and remained there for 2 mins. The apparatus and escape tunnel were cleaned with 70% ethanol after each rat to avoid odor cues. Then on acquisition training days, the animals were trained for four consecutive days to escape from the exposed platform surface to the dark recessed chamber (target hole), as previously described. Between the habituation and training phases, at least 1 hour should be allowed. Every day during the training phase (acquisition), the animals were placed in a black-colored cylindrical start chamber in the center of the platform. After 10s, the chamber was lifted, and the animals were allowed to explore the maze. The experimenter gently guided rats that did not enter the escape chamber within 3 minutes. On the 6th day, 24h after the last training, a 3-minute probe trial was conducted, and the number of errors and escape latency (time to reach the target box) was recorded.

2-Assessment of anxiety-like behavior

The EPM was used to assess the anxiety responses of rodents. It consists of 4 arms in a cross shape with a central zone in the middle; it consists of 2 open arms and 2 closed arms placed approximately 45 cm above the ground. The test took 10 minutes. Stressed animals usually tend to avoid open places. The frequency and time spent in the open arms were recorded. As mentioned before, the open field test was also used to assess spontaneous locomotion and freezing time as an indicator of anxiety behavior in rats. Time spent in the dark box in the Light/dark box and immobility time in the tail suspension test were also used to assess anxiety-like behavior in rats.

Urine, blood, and tissue samples

The Blood samples were withdrawn from retro-orbital rat sinus, for the pre-post test, according to Menyawi et al[4]. The collected blood was placed into tubes. The serum samples collected were centrifuged at 2000g for 15 min and stored at −20 °C for hormonal assays. At the end of the experiment (Day 37), blood samples were collected from the heart under anesthesia for hormonal assay at scarification. Then, all rats were euthanized with 100 mg/kg of ketamine and 10 mg/kg of xylazine, then sacrificed by aortic exsanguination as done previously[4]. Blood was kept on ice for 1 hour, and serum was separated by centrifugation at 2000 rpm for 15 min. Samples
were subsequently stored at -80°C until chemical analysis.

**Beta-hydroxy butyrate and acetone assessment for ketosis**
The β-HB level and acetone were measured in the serum\(^{(13)}\). An automatic biochemistry analyzer analyzed the serum, and chemicals were purchased from MyBioSource; San Diego, CA, USA. Serum adrenocorticotropic hormone (ACTH, cat# MBS453311) and corticosterone (cat# MBS2608983)\(^{(19-21)}\) are assessed after (day 37) of the CUS protocol to detect the impact of stress on corticosterone level and the impact of corticosterone level on ACTH by enzyme-linked immuno-sorbent assay.

**Brain tissue samples**
The brain (Hippocampus) was divided into two halves; one half was stored in -80 for molecular examination, and the other was stored in formaldehyde for histological examination as described previously\(^{(4)}\). For ELISA assays, the hippocampus was homogenized in 4 vol. of 10 mM phosphate-buffered saline (PBS; pH 7.4) containing 0.1% sodium dodecyl sulfate, 0.1mM phenylmethylsulfonyl fluoride, cooled) to 4°C with the Teflon homogenizer. The homogenate was centrifuged for 1 hour, and the resulting fractions were used\(^{(4)}\).

**Stress-induced hippocampus molecular changes analyzed by enzyme-linked immuno-sorbent assay (ELISA)**
All the biomarkers were purchased from MyBioSource, Inc. San Diego, CA, USA. Malondialdehyde (MDA) was purchased from Lifespan Biosciences Inc., Seattle, Washington, USA. 1. Detection of neurogenesis by measuring Brain-derived neurotrophic factor (BDNF): Brain-derived neurotrophic factor supports neurogenesis\(^{(4,22)}\). ELISA test was used to analyze the hippocampus BDNF tissue level (BDNF, cat# MBS824814).

2. Detection of oxidative stress and inflammatory cytokines: Stress-induced oxidative stress and the release of inflammatory cytokines in hippocampus tissue\(^{(4,23)}\). ELISA test was used to detect the hippocampus Malondialdehyde (MDA) tissue level (cat# LS-F28018), total Antioxidant capacity (TAC, cat# MBS1600693), tissue necrosis factor alpha (TNFa, cat# MBS355371), nuclear factor-κB (NF-κB, cat# MBS722386).

3. Detection of neurotransmitters changes
Stress also induces changes in hippocampus neurotransmitter release, affecting learning and memory. The neurotransmitters evaluated in this study were acetylcholine\(^{(24,25)}\) (Ach, cat# MBS766203) and dopamine\(^{(26)}\) (cat# MBS761192).

Stress-induced changed histological hippocampus microstructures: Brain (hippocampus complex, hippocampus, and dentate gyrus) tissues collected and for histological studies were fixed in formalin and embedded in paraffin as described previously\(^{(4)}\). Sagittal sections were cut with a microtome (Leica RM 2025, Germany) at 5 µm thicknesses, mounted on glass slides, and stained with cresyl fast violet (Nissl) staining to confirm the protective effect of KD of cornu ammonis region 1 and 3 (CA1, CA3) of the hippocampus and DG regions\(^{(27)}\).

**Immunohistochemical analysis of double-cortin**: Doublecortin (DCX) of the dentate gyrus is a marker for immature neurons (indicated by brown coloration) as evidence of neurogenesis\(^{(4,28)}\). Immunostaining by DCX antibody [cat# ab18723, Abcam Biomedical, Cambridge, UK] in concentration 1:400.

**Statistical Analysis**
Data were analyzed using SPSS, version 28, for Windows (Statistical Package for Social Sciences) (IBM Corporation, Armonk, New York, USA).
Ketogenic Diet and Cognitive Functions in Chronic Unpredictable Stress

York, USA). Data were displayed as mean ± standard deviation (SD). Significant differences between the 4 study groups were calculated using One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests. Paired T-test was done to compare pre-post experiments. Calculated regression analysis was done to prove tidiness between data, and R² and P values were estimated. The P-value of less than 0.05 was considered significant.

Results

The ketogenic diet and CUS modulate food intake, body weight, and ketosis

The average food intake (Fig. 1A) of the CUS/NPD group decreased compared to the control/NPD group, 124.3±6.3 vs. 163.3±24.27, respectively; P<0.05). The CUS/NPD also showed a significant decrease in body weight compared to the control/NPD and KD groups P<0.05 (Fig 1B). The KD group β-HB level was higher than the control/NPD (β-HB: 85.3±10.46 vs. 54.66±7.86 µmol/l, respectively; P<0.05; Fig. 1C). The CUS/KD value was higher also than the control/NPD and CUS/NPD groups; P<0.05.

Behavioral tests assessments

1. The Ketogenic diet decreases stress-induced anxiety behavior: Anxiety-like behavior was assessed initially by the open field test. The CUS/NPD showed more frequency number of freezing behavior in comparison with the Control/NPD (P<0.05; Fig.2A). The CUS/KD improved this behavior in the CUS/NPD (P<0.05; Fig. 2A). The CUS/NPD group was observed to stay less time in the light box in light/ dark box test compared with CUS/KD group which improved this behavior (P<0.05; Fig. 2B). Other experiments which showed the improvement of anxiety were the elevated plus maze and the tail suspension test. The CUS/KD improved the anxiety-like behavior by increasing the time spent in the open arm in EPM and reducing immobility time in the tail suspension test compared to the CUS/NPD group (P<0.05; Fig. 2 C-D).
Effect of diets and stress on battery behavioral assessment examine the anxiety like behavior. A, freezing in open field test; B, time spent in light box; C, time spent in open arm in Elevated Plus maze (EPM) test and D, immobility time in tail suspension test. Values are mean ± SD (n = 6/group), analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test. *,#, $P < 0.05; *compared with NPD control group, #compared with KD control group, $CUS compared with CUS/KD group.

2. Ketogenic diet improved cognition under stress conditions.
Spatial memory and learning were assessed using the Barnes Maze test (Fig. 3). The effect of the CUS/NPD in the probe trial showed a significant increase in the latency time compared to the Control/NPD group (154 ± 44.45 vs. 31.17 ± 10.66 (sec.); P <0.05; Fig. 3A). On the other hand, the CUS/NPD was significantly higher in the number of errors than Control/NPD group. The CUS/KD group reduced the number of errors committed by the animals compared to the CUS/NPD group (4 ± 1.41 vs. 7.67 ± 2.50 ; P <0.05; Fig. 3B) and there was no significant difference between CUS/KD and Control/NPD groups. There was a significant difference in the CUS/NPD and CUS/KD groups between pre- and post-test implementation (P<0.05; Fig. 3). This is a direct relationship between emotion/KD and cognitive functions.

KD improved the harmful effects of the CUS on neurotransmitters and stress hormones. Stress-induced significant decrease in the hippocampus complex ACh in the CUS/NPD group compared to the Control/NPD group (9.35 ± 1.73 vs. 41.87 ± 7.19 ng/g ; P <0.05; Fig. 4A). The CUS/KD showed significant improvement in levels of ACh neurotransmitters compared to the CUS/NPD (P<0.05; Fig. 4A). The same pattern was observed with the brain dopamine and serotonin with the improvement of the CUS/KD group compared with CUS/NPD group (P<0.05; Fig. 4B-C). The KD in the rats exposed to CUS mitigated the stress effect.
Ketogenic Diet and Cognitive Functions in Chronic Unpredictable Stress

This modulating effect could influence the tested cognition and anxiety behaviors (Figs 2 and 3). The CUS/NPD group showed a significant increase in the pituitary secretion of ACTH level compared to the Control/NPD group (173.17 ± 39.15 vs. 19.5 ± 4.14 pg/ml; P <0.05; Fig. 4D). As a result CUS/NPD showed an increase in the post-stress corticosterone level compared to the Control/NPD group (7.32 ± 0.78 vs. ± 2.02 ± 0.66 pg/ml ; P <0.05; Fig. 4E). The results show the modulating effect of KD on the stress-induced brain neurotransmitters and the secreted stress hormones.

**Figure 4. KD modulated CUS effect on neurotransmitters and stress hormones.**

A, acetylcholine (Ach); B, dopamine; C, serotonin brain tissue levels. The neurotransmitters of brain hippocampus complex homogenate were assessed in response to change to stress and KD. D, pituitary ACTH level and E, corticosterone levels. Values are mean ± SD (n = 6/group), analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test. *,#; $P < 0.05; *compared with NPD control, #compared with KD control, $CUS compared with CUS/KD group.

KD decreased stress-induced brain hippocampus inflammatory cytokines and oxidative stress.

The CUS/NPD group showed a significant increase in levels of all inflammatory cytokines compared to the Control/NPD group (P <0.05; Fig 5). KD in the CUS/KD group showed significantly reduced inflammatory markers levels compared to the CUS/NPD group for TNFα and NF-κβ (P
KD was associated with a decrease in stress-induced brain inflammation. A similar observation was detected with oxidative stress where KD improved the CUS/KD significantly increased MDA level compared to Control/NPD group (42.37 ± 4.86 vs. 13.3 ± 2.88 ng/g; P < 0.05; Fig 5C).

While it significantly reduced the TAC compared to the Control/NPD group (16.3 ± 3.35 vs. 65.22 ± 5.17 U/g; P < 0.05; Fig. 5D). KD showed significant improvement in stress-induced inflammatory cytokines and oxidative stress. The photographs of the CA3 area were stained with Cresyl fast violet (Fig. 6, left) and doublecortin (DCX, right). Left, Control/NPD, and KD groups, Cresyl violet stain purple Nissl granules in the perikarya of the large pyramidal cells. CUS/NPD showed a decrease in the perikaryal stain, which moderately improved in the CUS/KD group. Right, the DCX stain shows mild brownish cytoplasmic immunoreaction of immature neurons in the granular layers of the dentate gyrus. These results indicate that the KD keeps and restores new and old cells in a vital state and can resist inflammation and oxidation stress.

BDNF supports neurogenesis and cell vitality and improves cognitive function. Cresyl violet stain is indicative of vital cells, and cognitive tests are indicative of hippocampus functions. The regression model between hippocampus BDNF level and Nissl’s granules showed significant correlation and predictive effects (P<0.05; Table
1). The same was observed between the DCX, indicative of neurogenesis, and the time to reach the target box in the BM test. In both examples, KD of the CUS/KD group showed strong predictive values (P<0.05; Table 1). These results indicate that the BDNF preserves the newly formed cells by neurogenesis under stressful conditions.

![Photomicrographs of CA3 sections stained with Cresyl fast violet and dentate gyrus with double-cortin stain (X400) for morphometric analysis.](image)

**Figure 6.** Photomicrographs of CA3 sections stained with Cresyl fast violet and dentate gyrus with double-cortin stain (X400) for morphometric analysis. Groups are A, control/NPD; B, KD; C, CUS/NPD and D, CUS/KD. CA3, Cornu Ammonis region 3; DCX, doublecortin. Light microscope [Leica, Model: DM 1000] was used.

<table>
<thead>
<tr>
<th>Table 1: BDNF protective effect on neurogenesis and cognitive function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CA3/BDNF</td>
</tr>
<tr>
<td>DCX/BM time</td>
</tr>
</tbody>
</table>

CA3, Cornu Ammonis 3; BM time, time the animal spent to reach the target; BDNF, Brain-derived neurotrophic factor; Normal palatable diet, NPD; ketogenic diet, KD; chronic unpredictable stress, CUS; r², regression value; P value

**Discussion**

This present study investigated the protective effect of the KD against the deleterious effects of the CUS on cognition and learning. On microstructure, KD protected against the stress-damaging effect on the hippocampus and dentate gyrus neurogenesis. The effect of KD against stress came in the context of preserving stressed animal weight, stress hormones, and brain inflammatory cytokines, oxidative stress...
and BDNF level. The current study showed that KD reduced animal weight and increased the β-HB level. This result was explained previously as an effect of the low carbohydrate content of the high-fat diet, but the study showed less food intake in the animals fed the KD. The increase of the β-HB is the base of the neuroprotective effect, as proved by Sahagun et al. The current results showed CUS increasing anxiety behavior in rats. Staying more time in the closed arm of the EPM was an indicator of stress to the rats exposed to the CUS model. Other stress and anxiety indices were open field freezing, the tendency to stay in the dark box, and tolerating the tail suspension test. CUS induces anxiety-like behavior and increases the anxiety index. In the current study, the KD effect agreed with previous studies to improve the stress effect and decrease stress-induced anxiety. In the rodent model, previous studies showed the negative impact of stress on cognitive functions and learning similar to the current study observations. The stress-impaired memory could be explained partially by the depressing effect of the stress effect on the animal. The relieving effect of the KD on cognitive functions was explained as a dual effect on the neurons and the whole cerebral functions. Neuronal and glial cell uptake of ketone bodies works efficiently as a fuel in stress. Ketone bodies improve cerebral energy metabolism by increasing mitochondrial efficacy and biogenesis. Therefore, KD decreases anxiety and improves cerebral energy, resists stress-induced decrease in cognitive functions such as learning and memory. The current CUS animal model showed an increase in the hormonal level of ACTH and corticosterone, which were improved by KD as observed previously and described as stress hormones. Stress also induces an inflammatory brain state with increased inflammatory cytokines and oxidative stress, as observed in the current study and previous studies. This inflammatory state improved under ketosis. As observed in the current study, part of the protective effect of the KD, against stress was shown due to the effect on BDNF, which supports neurogenesis. The disease-modifying properties of the KD work via decreasing stress hormones, increasing BDNF, antioxidant effect, and improving programmed cell death. KD diet carries a neuroprotective effect; therefore, it supports learning and memory under stressful conditions. Besides KD neuroprotective effect, the current results showed it increases hippocampus Ach level, which regulates memory and learning. Current results also showed a supporting effect of KD to neurogenesis in stressful conditions. This experiment aimed to illuminate the pathophysiological effect of the CUS and the molecular changes of the KD protective effects. Although the anxiety-like behavior is associated with the CUS on learning and memory, the KD increased resilience to this stress effect. Perhaps these observations would further test other contributing factors such as age, gender response to stress, and KD. They were also testing the low carbohydrate-high fat diet to see if it could affect mood disorders.

Conclusions

KD modulated the effects of CUS on the hippocampus and neurogenesis. These processes were achieved by decreasing the CUS-associated hippocampus inflammation and oxidative stress. Allowing these factors improved cognitive functions and neurogenesis in the rats fed on KD and exposed to CUS.

Funding: There are no financial resources.
Conflict of interest: There is no conflict of interest.

References