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ORIGINAL PAPER

Impact of caloric restriction on the *Wnt*/β-catenin pathway in the hippocampus and cortex of a Kindled rat model

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ABSTRACT

Introduction and aim. Epilepsy is a common neurological disorder, and despite numerous treatment options, approximately 30% of patients have drug-resistant epilepsy. This situation prompts the exploration of alternative treatments such as caloric restriction (CR), whose mechanisms of antiepileptic action need to be fully elucidated. One of the key overactivated pathways in epilepsy is the *Wnt*/β-catenin pathway.

Material and methods. To explore the potential regulatory effects of CR on this pathway, we conducted a study using twenty-eight male Wistar rats divided into four groups (7 animals each): Control, Sham (20% CR), kindling ad libitum (KAL), and kindling with CR (KCR). Caloric restriction rats received 80% of their daily food intake based on body weight, compared to those fed ad libitum. The kindling model was achieved by the introduction of an electrode in the basolateral nucleus of the amygdala. Immunofluorescence and Western blot techniques were used for the analysis of protein levels (*Wnt*, β-catenin, *GSK3*β, and cyclin D) in the frontal cortex and hippocampus.

Results. Electroencephalographically and behaviorally, the KCR group exhibited a shorter duration of seizures and an increased behavioral threshold compared to the KAL group. Protein analysis revealed an increase in *Wnt* pathway proteins (*Wnt*, β-catenin, and cyclin D) in the KAL group compared to the control group. In contrast, CR reduced protein levels in animals that were induced to kindling.

Conclusion. These findings suggest that CR may exert its antiepileptic effects through the regulation of the *Wnt* pathway by inhibiting its activity in the hippocampus and cortex of kindled rats.

Keywords. caloric restriction, drug-resistant epilepsy, epilepsy, *Wnt*/β-catenin

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Introduction

Epilepsy is defined as a neurological disorder marked by recurrent, unprovoked seizures due to abnormal electrical activity in the brain.[1] Epilepsy is associated with significant neurobiological, cognitive, psychological and social consequence.[1] Its etiology is multifactorial, involving structural, genetic, metabolic, immune, infectious, or sometimes unknown causes.[2] The cornerstone of epilepsy treatment is drug therapy with antiseizure medications (ASM) and lifestyle changes. However, at least one-third of people with epilepsy do not achieve seizure control despite the use of appropriate ASM or surgical interventions.[3]

In recent decades, dietary modifications, such as caloric restriction (CR), have been used in the treatment of drug-resistant epilepsy (DRE), demonstrating neuroprotective effects.[4] However, the mechanisms underlying these effects remain unknown. CR usually consists of a diet in which 20% of total calories are restricted, which simulates a fasting state and induces significant metabolic adaptations. These adaptations facilitate a shift from glucose to free fatty acids as the main cellular energy source.[5] CR-induced chronic ketosis activates several molecular mechanisms. It enhances neuronal hyperpolarization through ATP-sensitive potassium channels (K_{ATP}), increases the seizure threshold, and modulates gut microbiota and inflammation, notably reducing interleukin-1B and other cytokines in murine models.[5-8] In the CNS, CR is associated with increased mitochondrial biogenesis; enhanced antioxidant enzyme activity such as superoxide dismutase in the cerebral cortex and increased neuronal activity in the hippocampus of aged rats.[9-11]

The *Wnt* signaling pathway, integral to cell survival and apoptosis, includes a canonical pathway dependent on β-catenin and noncanonical pathways such as the Ca^{2+}-dependent and planar cell polarity pathways (Fig. 1).[12] In the canonical pathway, β-catenin is essential for the transmission of *Wnt* signals to the nucleus, allowing subsequent activation of *Wnt* target genes via TCF/β-catenin complexes. In the absence of *Wnt*, β-catenin is targeted for degradation. It is phosphorylated by GSK3β with the assistance of CK1α, AXIN, and APC, forming a destruction complex.[12-14] However, when *Wnt* ligands bind to Frizzled receptors (Fzd), it leads to the oligomerization of Dishevelled (Dvl) at the plasma membrane. This process repositions GSK3β and Axin, promoting the phosphorylation of the co-receptor LRP5/6. This sequence of events allows CK1γ to phosphorylate Dvl, allowing it to block *GSK3*β by binding to its binding protein (GBP) and to promote the degradation of Axin within the *Wnt*/Fz-LRP5/6 complex.[14-16] When these components are inhibited, the β-catenin degradation complex -catenin does not form, and β-catenin is free to travel to the nucleus. There, it binds to the TCF/LEF family of transcription factors, resulting in the release of Groucho, a transcriptional inhibitor, and facilitating gene transcription. In the nucleus, β-catenin also recruits other basal components of the transcriptional machinery, such as B-cell lymphoma 9 (Bcl-9), p300, and cyclic adenosine monophosphate (cAMP), which collectively activate the transcription of genes crucial for cellular functions, including those coding for cyclin D, the Myc family, and axin2.[12,14,15]

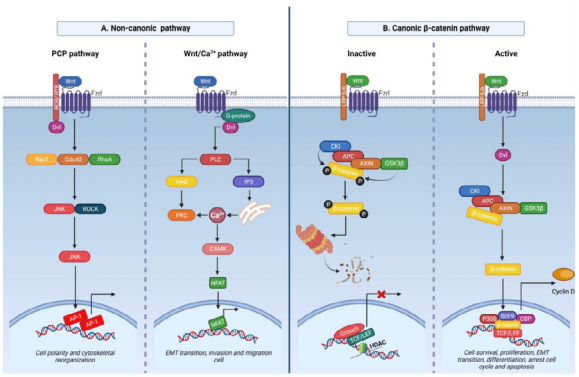

Fig. 1. A: Two non-canonical *Wnt* pathways have been described, a polar cell pathway that mainly regulates the reorganization of the cytoskeleton and cell polarity, and another intracellular Ca^{2+}-dependent pathway that regulates cell migration and invasion, as well as epithelial-mesenchymal transition. B: The canonical pathway will depend on whether it is activated or inactivated by Wnt family ligands. When the pathway is inactive, the β-catenin protein is phosphorylated by the destruction complex and destroyed by the ubiquitin proteasome system; this will result in blocking gene transcription in the nucleus. On the other hand, when the pathway is activated, the destruction complex is inhibited so that β-catenin remains free in the cytosol and translocates to the nucleus where it will promote the transcription of genes such as those of the Myc family, cyclin D, among others. The canonical pathway regulates cell survival, proliferation and differentiation, cell arrest, and apoptosis, created with https://www.biorender.com

Previous research suggests that CR can reduce seizures by stabilizing neuronal membranes through K_{ATP} channel-mediated hyperpolarization and inhibiting the mTOR signaling cascade via GSK3β. Understanding how CR affects the *Wnt*/β-catenin pathway, which is critical for central nervous system homeostasis and is involved in epilepsy and other neural diseases, is essential.[12-17] This could provide the foundation for new therapies for refractory epilepsy, offering new strategies for patients who are resistant to existing treatments.

Aim

Therefore, the aim of this study was to evaluate the impact of caloric restriction on the *Wnt*/β-catenin pathway in the hippocampus and cortex of a kindled rat model.

Material and methods

Experimental subjects

Twenty-eight male Wistar rats (120-130 g) were obtained from the Neurological Diseases Medical Research Unit of the Specialty Hospital CMN S. XXI. Our handling and treatment of the animals adhered to institutional protocols, complying with national regulations (NOM-062-ZOO-1999) and international ethical standards (Council for International Organizations of Medical Sciences, CIOMS) study approval number 100/17. The rats were housed individually in transparent cages with corn-cob bedding, maintained at 23±1°C with a 12-hour light-dark cycle (lights on at 07:00 h). The cages were cleaned regularly and kept dry. The sample size was determined using a G-Power analysis[18], with the aim of minimal animal use while achieving statistical strength; this analysis specified a sample of n=28 with an error value of 0.75.

Experimental procedure

Rats were randomly assigned to one of four groups: a control group (n=7) that received standard dietary pellets and ad libitum; a sham group (n=7) under caloric restriction without stimulation; and two groups that underwent a standardized amygdala kindling stimulation protocol, one with caloric restriction (KCR, n=7) and one with an ad libitum diet (KAL, n=7). CR was implemented by providing rats with 80% of the daily amount of a standard diet (Harlan Standard Commercial Diet No. 2018S Teklad Global 18% Protein, Harlan Laboratories), adjusted daily based on recorded body weight. This regimen was compared to a control group of rats allowed to feed ad libitum. The diet restriction protocol was established based on previous studies and accumulated experience.[19,20] Sixteen rats, four from each group, were used for Western blot analysis and twelve, three from each group, for immunohistochemistry. Caloric restriction was established at 20% below the individual weight to prevent hypoglycemia, which could confound the results by triggering seizures. This restriction began upon receiving the rats and continued until they reached 250-300 g. For those subjected to kindling, the restriction was maintained during the stimulation period.

Amygdala Kindling model

The Kindling model involves administering a subthreshold electrical stimulus that triggers electroencephalographic and behavioral changes, culminating in generalized seizures.[21] For this study, stereotaxic surgery was performed in rats to implant an electrode in the basolateral nucleus of the amygdala. They were anesthetized with sodium pentobarbital (50 mg/kg i.p., Pfizer Laboratories, Mexico City, Mexico), and once anesthesia was administered, their heads were fixed in a stereotaxic frame to introduce the electrode into the amygdala (Ap=-6.2 mm, L=5 mm relative to Bregma suture; H=1.5 mm interaural, according to the Paxinos and Watson Atlas). The electrode was then secured with dental acrylic. Post-surgery, rats were monitored and administered Gentamicin (Aurofarma) at a dose of 4-5 mg/kg b.w. intramuscularly every 24 hours for 10 days after surgery. Stimulation was carried out using a Grass S88 Stimulator at 50 microvolts of amplification. Rats received daily stimulation at a frequency of 60 Hz, with a pulse duration of 1.0 ms and an intensity of 400 μA. Behavioral changes were assessed using Racine's stages, and the duration and intensity of the ictal and postictal states were recorded. The Kindling stimulation protocol was deemed complete when a rat experienced 10 generalized tonic-clonic seizures, reaching Racine's stage 5. All rats were sacrificed on postnatal day 85, the day following their final stimulation, after undergoing a total of 64 days of caloric restriction (CR).

Immunofluorescence

At the end of the experimental phase, rats were anesthetized with sodium pentobarbital and transcardially perfused with saline, followed by 4% paraformaldehyde. The brains were then removed and embedded in paraffin and sagittal sections (7 μm) were prepared. These sections were deparaffinized, hydrated with graded alcohols and blocked with bovine serum albumin (Sigma, St. Louis, MO, USA). They were incubated overnight with primary antibodies including Wnt-3, β-catenin, GSK3β, actin, and Cyclin-D (all mouse monoclonal, diluted 1:100, Santa Cruz). Following three washes with PBS, secondary antibodies were applied: Rhodamine Red for *GSK3*β and Cyclin-D and Fluorescein IsoThioCyanate for Wnt-3 and β-catenin, producing red and green fluorescence, respectively. The sections were mounted with Vectashield containing DAPI (ab104139) for nuclear staining. The imaging was performed with an Olympus IX81-F3 microscope equipped with a Q-Imaging digital camera, using a 40X objective in a field of 520 μm^2. Areas imaged included the CA3 zone and the apical area of the frontal cortex because they constitute the brain structures most susceptible to epileptogenesis. Protein densities were quantified using ImageJ software (Rasband, 1997-2016, version 1.45).

Western blot

For Western blot analysis, tissue samples from the hippocampus, dentate gyrus, subiculum, and frontal cortex of the rats were homogenized. Protein concentrations were quantified using the Lowry method.[19] Fifty micrograms (μg) of protein were separated by SDS-PAGE and subsequently transferred to PVDF membranes (BioRad, Hercules, CA, USA). The mem-

branes were blocked with 5% milk in PBS containing 0.01% Tween-20 for two hours. Santa Cruz Biotechnology primary antibodies, including those against β-actin (1:3000), Wnt-3 (1:2000), β-catenin (1:3000), cyclin D (1:500) and *GSK3*β (1:3000), were incubated overnight. The following day, the membranes were washed and incubated with horseradish peroxidase conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) at a dilution of 1:15,000. Protein bands were visualized using the UVP ChemiDoc Imaging System (Upland, CA), and band density was quantified with ImageJ software (Rasband, 1997-2016, version 1.45).

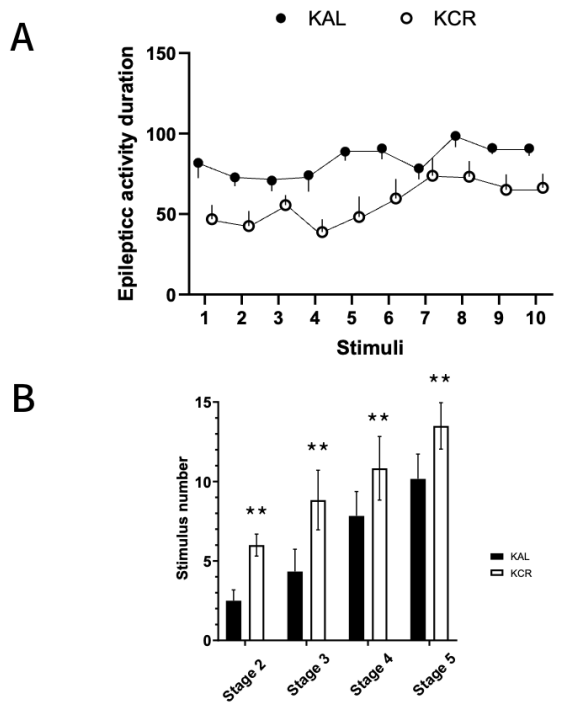

Fig. 2. Kindling model measurements in the KAL and KCR groups. Two bar graphs showing the result of what was observed and recorded during the application of the Kindling model. A: Graph showing the duration as a function of the stimuli given to the rat (x-axis). The solid line represents the KAL group and the dotted line the KCR group; note the differences in duration along the 10 stimuli. (F=45.75 p<0.0001). B: Graph showing the number of stimuli required to reach each of the Racine stages. The white bar represents the KAL group, and the black bar represents the KCR group. The KAL group needed a smaller number of stimuli to reach each stage compared to KCR (F=11.79 p<0.0014). *p<0.05, **p<0.01 and ***p<0.001 compared to KAL

Statistical analysis

Data were presented as mean values with standard deviations (mean±SD). The normality of the data distribution was evaluated using IBM SPSS Statistics v29.02.0 (Armonk, NY, USA). For datasets following a normal distribution, a two-way analysis of variance (ANOVA) was employed to investigate the effects of caloric restriction on the *Wnt* pathway. Alternatively, for nonnormally distributed data, a one-way of variance ANOVA was performed. Tukey's post hoc test was applied to analyze the kindling data, whereas Duncan's post hoc test was used for the other datasets. Statistical significance was established at $p<0.05$.

Results

Statistical analysis using a two-way ANOVA revealed significant differences in the durations after discharge between the KCR and the KAL ($F=45.75$, $p<0.0001$). Specifically, the KCR group exhibited shorter after discharge durations, not exceeding 75 seconds, even after ten stimuli, while durations in the KAL group surpassed 90 seconds at the final stimulus (Fig. 2). Similarly, significant differences were observed in the number of stimuli required to progress through Racine's stages ($F=11.79$, $p<0.0014$). The KCR group required a higher number of stimuli to reach Racine stage 5 compared to the KAL group, indicating a mitigated progression of seizure severity under dietary restriction.

Immunofluorescence

Using immunofluorescence, thus providing dual validation of the findings through two methodologies. In the hippocampus (Fig. 3A), all proteins except *GSK3*β exhibited higher concentrations in the KAL group compared to other groups, notably β-catenin ($p<0.001$ $F=4.57$), *Wnt* ($p<0.015$ $F=6.52$), and cyclin D ($p<0.030$, $F=5.020$). Further analysis revealed that the Sham group had the lowest protein concentrations relative to all other groups, with values that closely matched those of the control group. *GSK3β* was significantly increased in the KCR group ($p<0.002$, $F=12.79$) in comparison to the KAL group. A subsequent Duncan post hoc test identified significant differences across all groups, particularly from Sham to all other groups without exception, and between KAL and KCR for all proteins analyzed.

In the cerebellar cortex (Fig. 4A), the concentration of β-catenin protein ($p<0.01$ $F=9.87$) was found to be higher in the KAL group than in the other groups. Duncan's post hoc test showed a significant difference between the Sham group and the control group, as well as differences between KCR and KAL. *The Wnt* protein ($p<0.001$ $F=7.897$) and cyclin D ($p<0.037$ $F=4.615$) showed higher values in the KAL group compared to the rest of the groups, Duncan's post hoc test shows significant differences in *Wnt* in the KAL group compared to Sham and KCR. Similarly for cyclin D we found the same differences between the groups, as well as a difference between KAL and the control.

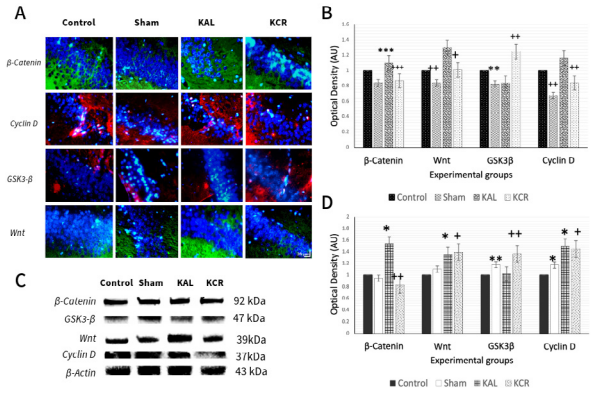

Fig. 3. Effect of caloric restriction and the amygdala Kindling model on *Wnt* pathway proteins. A: *Wnt* pathway proteins by immunofluorescence technique in the hippocampus, apical area with a 40X objective in a 520 mm2 field (Scale Bar 20 µm). Cell nuclei (DAPI) in blue and proteins analyzed in red for cyclin D and *GSK3*β, and green for *Wnt* and β-catenin and B: analysis of proteins of the *Wnt* pathway in each of the experimental groups. C: Representative images in the hippocampus and D: Densitometric analysis of β-catenin, *Wnt,* GSK3β and cyclin D expression levels. Mean±SEM graphed, using one-way ANOVA followed by a post hoc Duncan's test. *p<0.05, **p<0.01 and ***p<0.001 compared to the control group, $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 compared with the KAL group

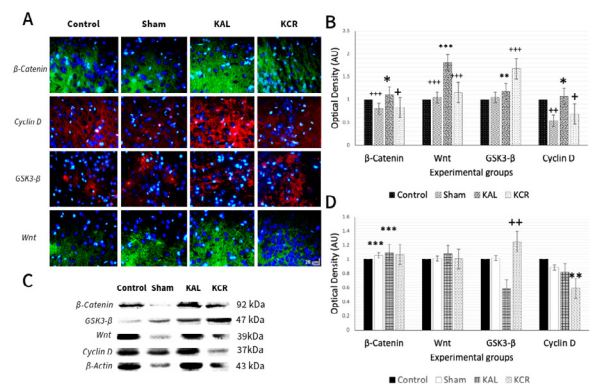

Fig. 4. A: *Wnt* pathway proteins by immunofluorescence technique in the cerebral frontal cortex, apical area with a 40X objective in a 520 mm2 field (Scale Bar 20 µm). Cell nuclei (DAPI) in blue and proteins analyzed in red for cyclin D and *GSK3*β, and green for *Wn*t and β-catenin and B: analysis of proteins of the *Wnt* pathway in each of the experimental groups. C: Representative images in the cerebral cortex and D: Densitometric analysis of β-catenin, *Wnt, GSK3*β and cyclin D expression levels. Mean±SEM graphed, using one-way ANOVA followed by a post hoc Duncan's test. *p<0.05, **p<0.01 and ***p<0.001 compared to the control group, $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 compared with the KAL group

Western blot

In the Western transfer technique, the analysis of the same proteins confirmed the results obtained by immunofluorescence, statistically significant results were observed. The group subjected to the amygdala kindling model was expected to result in overexpression of *Wnt* pathway proteins, as demonstrated in previous studies.[22] Therefore, a one-way ANOVA was performed to investigate the decrease in *Wnt* pathway proteins in the hippocampus and cortex in groups subjected to caloric restriction compared to the other groups. At the hippocampal (Fig. 3B) level, β-catenin concentrations were higher in the KAL group (p<0.005 F=9.476), and lower in the KCR group. This indicates an increase in β-catenin expression where the kindling amygdala model was applied and a decrease in groups undergoing caloric restriction. Given β-catenin's role in signal transmission to the nucleus and transcription activation, an elevated concentration of cyclin D was anticipated. Our findings reveal an increase (p<0.035 F=3.98) in both groups subjected to the kindling model with a more pronounced expression in the KAL group One of the most significant results pertains to GSK3β, a component of the β-catenin destruction complex. Its expression was expected to decrease in groups without Wnt stimulation. Indeed, *GSK3*β levels were reduced in the KAL group (p<0.023 F=4.62) and increased in the KCR group due to reflex inhibition by *GSK3*β. The control group exhibited the lowest protein concentrations across all groups. Compared to the Sham group, the differences were not statistically significant, except for cyclin D concentrations, which did not vary significantly between the Sham and Kindling caloric restriction groups. A post hoc test revealed significant differences in β-catenin concentrations in the hippocampus between the KAL group and the remaining groups, mainly with the control group and KCR. In contrast, *GSK3β* protein had significant differences between the Sham group and the control group and KCR with respect to KAL. *Wnt* had differences in the KAL group compared to the Control group, as did KCR compared to KAL. In the cyclin D protein we found differences in all groups compared to the control, and differences between KCR compared to KAL.

In the cerebral cortex (Fig. 4B), the findings followed trends similar to those observed in the hippocampus, although the differences between groups were less pronounced for each protein, particularly for β-catenin and *Wnt*. β-catenin displayed higher concentrations in the KAL group (p<0.001 F=14.72), as did *Wnt*, although *Wnt* concentrations were lower in the KCR group. In the cortex, there was a significant decrease in *GSK3*β levels in the KAL group compared to all other groups (p<0.030 F=4.196), with a notable increase in the KCR group. To validate these findings, a Duncan's test was performed, which confirmed significant differenc-

es between the groups. At the level of the cerebral cortex, we did not find significant differences for the *Wnt* protein.

Discussion

The primary objective of this study was to explore the efficacy of CR as an alternative therapy for DRE and to elucidate the molecular mechanisms involving the *Wnt*/β-catenin pathway and its potential modulation.

It has been suggested that molecular changes may be associated with *Wnt*/β-catenin signaling as many components of this pathway are acutely elevated after seizures.[23] Two decades ago, it was confirmed that seizures induced by electroconvulsive events lead to positive up-regulation of β-catenin and *Wnt*2 ligand in neurons of the rat dentate gyrus.[24] Similarly, elevated expression of *Wnt*3a, β-catenin, and Cyclin D1 was observed in the rat hippocampus, peaking 14 days post-status epilepticus.[25] Additionally, in astrocytes, seizures induced by pentylenetetrazole were linked to β-catenin overexpression and increased susceptibility to seizures.[26]

Our findings are consistent with these observations, confirming a close relationship between the overexpression of these proteins in an established epilepsy model. Differential protein concentrations were observed in both the hippocampus and the cerebral cortex between the experimental groups. In the KAL group, increased concentrations of *Wnt* proteins, β-catenin, and Cyclin D were found, along with decreased *GSK3*β levels of GSK3 compared to the KCR group and the other groups. This suggests that there is indeed an overexpression of this pathway and its components in this epilepsy model. On the other hand, all *Wnt*/β-catenin pathway proteins, except for *GSK3*β, exhibited lower optical densities in the KCR group compared to the KAL group. It should be noted that despite the reductions caused by caloric restriction in the KRC group, it was not possible to reach the concentration levels of the control group, as this group had the lowest values compared to the other groups in the hippocampus in the Western blot. On the contrary, the cerebral cortex showed similar findings, with lower concentrations in the simulated group compared to the other groups. Among the pathway components, β-catenin and *GSK*3β showed the most significant statistical differences in both regions of the brain. Although the *Wnt* protein showed significant statistical values in the hippocampus, it did not show significant differences in the cerebral cortex. Furthermore, the Sham group, where caloric restriction was applied without the kindling epilepsy model, showed lower protein concentrations compared to the other groups in both brain structures in immunohistochemistry. The rest of the immunofluorescence findings were similar for the other groups, mainly for KAL and KCR.

As noted previously, the *GSK3*β protein is a crucial component of the β-catenin destruction complex, which facilitates transcription within the nucleus.[27] Given its role in an inhibitory complex, it is reasonable to expect higher concentrations of *GSK3β* in the KCR group compared to those in KAL, as a mechanistic approach to counteracting pathway deregulation. However, it appears that elevated levels of *GSK3*β do not reach overexpression but can instead contribute to other molecular changes. It is plausible that the observed *GSK*3β levels in the KCR group facilitate proper ubiquitination of β-catenin, thus preventing the complete deregulation of the *Wnt* pathway. Several studies have established that inhibition of *GSK3*β exhibits anticonvulsant effects, similar to protective effects against hippocampal neuronal damage observed with lithium treatment after seizures.[28,29] However, previous research also suggests that excessive inhibition of this protein could adversely affect neuronal plasticity and cognitive dysfunctions.[30-32] In light of these complexities, our study identifies the need for future research to determine optimal levels of *GSK3*β for proper regulation of the *Wnt*/β-catenin pathway. Such research could significantly enhance our understanding and improve treatments that target this pathway.

Our results indicate significant modulation of the *Wnt*/β-catenin pathway by CR, as evidenced by the differential expression of key proteins. We propose a bidirectional relationship, starting from the assumption that an increase in the expression of the components of the *Wnt* pathway, which ultimately allow for the translation of signals to the cellular nucleus (*Wnt* and β-catenin), is directly related to seizure activity (postictal activity). On the other hand, the decrease in protein concentrations allows for regulation of seizure activity, minimizing postictal activity, as reflected by the effects of caloric restriction, as observed in the KCR group findings. Increase in GSK3β through caloric restriction leads to inhibition of the pathway via β-catenin ubiquitination, allowing a decrease in signal translation and an increase in the expression of proteins such as Cyclin D, and consequently seizure activity. These molecular changes, along with increased seizure thresholds and decreased duration observed in the KCR group, are likely to persist and be reinforced over time, highlighting the potential of CR to influence neuronal signaling and alter epileptic activity.

Based on the data presented, our objective is to propose a model to elucidate the relationship between CR, the *Wnt* pathway, and epilepsy, as shown in Figure 5. This model suggests that the inhibitory effects of CR on epilepsy could be mediated through several mechanisms within the canonical *Wnt* pathway. These include mTOR inhibition, reduced glucose absorption due to decreased affinity for GLUT3, electrochemical stabilization of the membrane through the opening of K_{ATP} channels, and enhanced mitochondrial biogenesis and stabilization.

Collectively, these mechanisms contribute to the overall inhibitory influence of CR on epileptogenic activity.

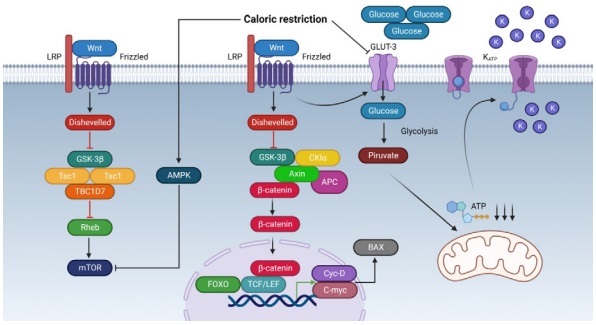

Fig. 5. Molecular mechanisms of the antiepileptic effect of caloric restriction through the regulation of the *Wnt*/β-catenin pathway, created with https://www.biorender.com

Mutations that lead to mTOR hyperactivation are significantly implicated in the pathophysiology of epilepsy, contributing to the production of reactive oxygen species and subsequent neuronal damage.[33] The *Wnt*/β-catenin pathway intersects with mTOR signaling, which is a likely target of the inhibitory actions of CR. Specifically, activation of the canonical *Wnt* pathway results in inhibition of *GSK3β*, a component of the β-catenin destruction complex. This inhibition triggers the activation of the tuberin protein complex, encoded by the tuberous sclerosis genes TSC1 and TSC2.[34] Activation of TSC1 and TSC2 subsequently inhibits Ras homologous enriched brain protein (Rheb), leading to an indirect inhibition of mTORC1, since Rheb is a direct activator of mTORC1.[35]

In the context of CR, reducing carbohydrate intake alters the dynamics of cellular energy and confers a state of cellular stabilization. Activation of the Wnt pathway has been specifically linked to increased affinity of GLUT3 channels for glucose in neurons, rather than to changes in their expression or functional activity. This increase in glucose uptake, particularly observed in the cortex, is stimulated by ligands such as Wnt3a, which enhance glucose uptake through this enhanced affinity mechanism.[36] Associating these dynamics with our findings, where a decrease in *Wnt* pathway signaling was observed in the KCR group, suggests that CR can exert homeostatic control over glucose metabolism through inhibition of the *Wnt* pathway. This inhibition likely results in a reduced affinity of GLUT3 channels for glucose and impacts the enzymes responsible for stimulating glycolysis. Consequently, this reduction in enzyme activity would decrease the energy supply and metabolic capacity of neurons necessary to propagate synaptic signals, conferring cellular stability.

Although this study provides important information on the effects of CR on the *Wnt*/β-catenin pathway and its potential implications for the treatment of DRE, some limitations must be acknowledged. First, reliance on animal models, although necessary, may not fully replicate the complexities of human epilepsy, which could limit the generalization of our findings. Therefore, it is crucial to advance clinical research with controlled clinical trials and specifically apply caloric restriction as a dietary therapy to patients with refractory epilepsy. Additionally, the study's focus on specific proteins within the *Wnt*/β-catenin pathway, while providing significant data, does not encompass all possible molecular interactions and pathways affected by CR. Similarly, only Cyclin D was measured as a from signal transduction result of this pathway, excluding C-*myc* and other proteins that are part of the final outcome of this pathway, which will be considered in future work.

Another limitation is the exclusion of the cerebellum from our analyses. Taking into account the documented involvement of the cerebellum in epilepsy and its association with the *Wnt*/β-catenin pathway in epileptic animal models, including this region could have potentially provided more comprehensive information on the systemic effects of CR.[37-39] Furthermore, the biochemical assays used, though precise, do not capture dynamic interactions in vivo that may influence the behavior of these proteins under different physiological conditions. Future research should aim to address these limitations by incorporating more diverse biological models, including cerebellar evaluations, a broader range of molecular evaluations, and dynamic in vivo analyzes to better understand the therapeutic potential of CR in human epilepsy.

Although we observed promising results indicating potential regulation of pathway overexpression, these findings are limited to the duration of our study and do not encompass chronic periods. Recognizing this limitation, we strongly advocate for future research to extend these investigations. We suggest implementing caloric restriction in longer-term studies to fully assess its effects on the *Wnt*/β-catenin pathway and its implications for epilepsy treatment.

Conclusion

Epilepsy is one of the most prevalent neurological disorders, with up to 30% of patients showing resistance to conventional treatments. This study explored CR as adjuvant therapy, particularly its impact on the canonical *Wnt*/β-catenin pathway, known to be involved in the pathophysiology of epilepsy. The results revealed that CR reduces key proteins in the amygdala initiation model, with a compensatory increase in *GSK3*β. These results suggest that CR can exert anticonvulsant effects through mechanisms such as neuronal membrane stabilization, ROS reduction, mTOR inhibition, and modulation of inflammatory signaling. This highlights the

potential as a therapeutic modifier in epilepsy, which merits further investigation.

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Declarations

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Author contributions

Conceptualization, C.R. and M.R.O.; Methodology, E.U. and E.O.; Software, H.R.P.; Validation, C.R., M.R.O. and H.R.P.; Formal Analysis, A.L.L.; Investigation, A.L.L. an D.V.; Data Curation, H.R.P.; Writing – Original Draft Preparation, A.L.L.; Writing – Review & Editing, C.R.; Visualization, D.V.; Supervision, L.M.A.C. and G.G.G.; Project Administration, C.R.

Conflicts of interest

We declare that there are no conflicts of interest on the part of any of the authors in this study.

Data availability

All clinical and statistical data and materials are available for the benefit of science.

Ethics approval

Our handling and treatment of the animals adhered to institutional protocols, complying with national regulations (NOM-062-ZOO-1999) and international ethical standards (Council for International Organizations of Medical Sciences, CIOMS) study approval number 100/17.

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