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THE THERAPEUTIC TREATMENT OF PEPITEM INCREASES THE PROTEIN EXPRESSION OF SIRT1 IN A MOUSE MODEL OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) AS A MODEL FOR HUMAN MS

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Abstract

Objective: To investigate the effect of the prophylactic and therapeutic treatment of the immunopeptide PEPITEM on the protein expression of SIRT1 in a mouse model of experimental autoimmune encephalomyelitis (EAE) as a model for human MS.

Methods: Using C57BL/6 female mice, we dosed the PEPITEM in the EAE model via intraperitoneal injections either prophylactically or therapeutically. The disease was induced using MOG35-55 and complete Freund's adjuvants augmented with pertussis toxin. The EAE

score was recorded daily until the end of the experiment (21 days). A Western blot analysis was performed on the brain lysate to measure the protein concentration of SIRT1.

Results: The therapeutic treatment with PEPITEM increased the protein expressions of SIRT1 on the EAE mice whereas the prophylactic injections did not affect the protein expression of SIRT1.

Conclusion: Collectively, the therapeutic treatment with PEPITEM suggesting anti-inflammatory effect of PEPITEM on the brain damage in EAE mice which offers a novel and safe strategy for drug therapy in MS, opening new avenues for research and treatment.

Keywords

PEPITEM, Experimental Autoimmune Encephalomyelitis, SIRT1, Anti-Inflammatory

Introduction

Multiple sclerosis (MS) is a persistent autoimmune condition that affects the central nervous system, encompassing the brain, spinal cord, and optic nerves. In MS, there is harm inflicted upon the myelin sheath, which is a protective covering enveloping nerve fibres and facilitating the transmission of signals. When this myelin sheath sustains damage, it leads to a disruption in communication between the brain and the rest of the body.

Approximately 2.8 million individuals worldwide are living with multiple sclerosis (MS), which translates to a prevalence rate of 35.9 cases per 100,000 people [1]. The global prevalence of MS has shown an overall increase, although there are still areas where gaps exist in accurately estimating its prevalence. Multiple sclerosis (MS) is a debilitating neurological condition that impacts the central nervous system (CNS) [2]. The primary pathological characteristics of MS involve proinflammatory cytokines, demyelination, and subsequent axonal degeneration. Nevertheless, the effectiveness of addressing these factors therapeutically remains uncertain.

PEPITEM, known as The Peptide Inhibitor of Trans-Endothelial Migration, is a naturally occurring peptide. It was initially discovered in 2015 by scientists affiliated with the University of Birmingham in the United Kingdom. PEPITEM plays a pivotal role in the adiponectin-PEPITEM pathway, which serves as a regulator of immune responses and inflammation within the body [3]. Recent research has indicated that PEPITEM may hold promise for addressing conditions related to obesity and type 2 diabetes [4]. This is because PEPITEM has the ability to reduce inflammation and enhance glucose metabolism. Furthermore, PEPITEM demonstrates efficacy in curtailing the recruitment of T cells into inflamed tissues, contributing to its potential therapeutic value in various health conditions such as peritonitis, hepatic ischemia-reperfusion injury, Salmonella infection, uveitis, and Sjögren's syndrome [3]

Silent information regulator 2 homolog 1 SIRT1 is a protein that belongs to sirtuins family. One of the well-known functions of SIRT1 is its role in regulating gene expression by deacetylating histone proteins [5]. Histones are proteins that help package and organize DNA in the cell nucleus, and acetylation of histones can influence gene expression. SIRT1 can remove acetyl groups from histones, which can lead to changes in gene expression patterns. The available evidence indicates that SIRT1, a histone deacetylase enzyme, plays a role in extending lifespan through calorie restriction. However, it is noteworthy that SIRT1 may have

the paradoxical effect of potentially elevating the risk of cancer [6-9] Nonetheless, the function of SIRT1 as a facilitator of tumour growth or as a suppressor of tumours is still controversial due to conflicting studies [10]. Regarding its association with inflammation, SIRT1 protects the body against various oxidative and inflammatory injuries by promoting the activation of antioxidant defence pathways and mitigating the inflammatory response [11, 12]. Certain research findings have shown that SIRT1 can suppress the expression of pro-inflammatory cytokines, as it plays a role in moderating the onset and advancement of inflammatory processes such as deacetylating nuclear factor kappa B (NF- κ B)) [13, 14].

In this research, our objective was to assess the impact of PEPITEM, a newly developed peptide inhibitor of proinflammatory cytokines, on the disease's severity in the EAE mouse model. This model mirrors numerous aspects of human MS. We used western blot analysis to assess the impact of PEPITEM on the protein expression of SIRT1 on EAE mice.

Methodology:

1. Materials and methods

1.1 Animals

Hooke Laboratories supplied the immunization peptides and toxin required for inducing EAE in the mouse model. Female C57BL/6 mice, obtained from the animal facility at King Saud University's Experimental Surgery Center (KSU), were utilized in the study. The mice were divided randomly into five experimental groups, each containing 10 female mice aged between 9 to 13 weeks, with an average weight of 21 ± 3 grams. These mice were housed collectively in standard laboratory cages equipped with bedding and nesting materials. The animal facility was maintained at a temperature of 22 ± 2 °C and a relative humidity of $50 \pm 10\%$. The mice were subjected to a 12-hour light and 12-hour dark cycle, with lights turning on at 7:00 a.m. and turning off at 7:00 p.m. They had unrestricted access to water, water gel provided in the cage, and food. Veterinary professionals monitored and weighed the mice daily. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at King Abdullah International Medical Research Center (KAIMRC) under the reference number RC-19/084/R and was also approved by the Research Ethics Committee at KSU with the reference number KSU-SE-19-10.

1.2 Experimental design

Five experimental groups of mice were randomly assigned to the normal control group (G1), the EAE-induced group receiving the scrambled peptide from day 10 $\pm$ 1 (G2), and the EAE-induced group receiving PEPITEM treatment from day 10 $\pm$ 1 (G3), the EAE-induced group receiving prophylactic scrambled peptide from day 0 (G4), and the EAE-induced group receiving prophylactic PEPITEM from day 0 (G5).

Any mice from G2, G3, G4, or G5 who did not develop the EAE signs after 10 $\pm$ 1 days from the start of the experiment were excluded from the study and euthanized.

1.3 Reagents

We obtained EAE induction kits from Hooke Laboratories, which consisted of pre-filled syringes containing an emulsion of myelin oligodendrocyte glycoprotein 35-55 (MOG35-55/mL) with complete Freund's adjuvant, as well as vials containing pertussis toxin (PTX) in glycerol buffer (Hooke Laboratories, LLC, USA). Thermo Fisher Scientific (USA) synthesized both the PEPITEM and scrambled peptides. We procured recombinant antibodies against myelin basic proteins (MBP), microtubule-associated protein-2 (MAP-2), N Cadherin, and GAPDH Cadherin, as well as the Quant-TI protein assays kit from Abcam (UK). For our experimental needs, we acquired acrylamide/bis solution and thick blot filter paper from BioRad (Bio-Rad Laboratories, USA). Additionally, we purchased Mouse Interleukin 17 (IL17) and Mouse Forkhead Box Protein P3 (FOXP3) ELISA kits (Catalog numbers: MBS455642 and MBS452652) from MyBioSource, Inc. (USA).

1.1 Induction of EAE animal model

To induce the disease, we utilized EAE kits sourced from Hooke Laboratories. These kits were freshly prepared, used within their designated expiration date, and stored in a refrigerator at a temperature range of 2-8 degrees Celsius. Following the manufacturer's guidelines, we administered immunization to the mice using an emulsion containing MOG35-55 in complete Freund's adjuvant (CFA). All mice received two injections of pertussis toxin in PBS on both day 0 and day 1.

In brief, we employed a mouse restraint cage to subcutaneously deliver the antigen emulsion at the midline of the upper and lower back, each site receiving 0.1 mL for a total of 0.2 mL per mouse. Following a two-hour interval, we freshly prepared the PTX toxin and

diluted it according to the manufacturer's instructions. This toxin was administered intraperitoneally (IP) at a dosage of 0.1 mL per dose on both day 0 and day 1. For the G1 mice, we administered injections of normal saline."

1.2 PEPITEM Administration

Since the study was therapeutic and prophylactic, the daily injections started on day 0 for the G4 and G5 groups, whereas; the daily injections started on day 10 $\pm$ 1 post-induction for G2 and G3, when the first sign of inflammation appeared until the end of the experiment on day 21. Daily PEPITEM IP injections (concentration of 100mg/ml; 200ul total volume per injection) were given accordingly to our previous work [3].

1.3 Animal Observations

The mice were subject to daily monitoring, covering aspects such as their weight, EAE score, behavior, incidents, and survival rates. We adopted the EAE scoring system outlined in the Hooke Laboratories induction kit manual, and these scores were documented on a daily basis by both the animal care team and our research staff.

The scoring system was as follows:

- A score of 0 indicated the absence of symptoms.
- A score of 0.5 represented a slight limp at the tip of the tail.
- A score of 1 denoted complete tail flaccidity.
- A score of 1.5 indicated tail flaccidity along with an unsteady gait.
- A score of 2 signified tail flaccidity paired with hind leg impairment.
- A score of 2.5 corresponded to tail flaccidity and weakness in the hind legs.
- A score of 3 meant complete tail and hind leg paralysis.
- A score of 3.5, in addition to complete tail and hind leg paralysis, indicated that a mouse was unable to return to a normal position when placed on its side.
- A score of 4 encompassed complete tail and hind leg paralysis, along with partial impairment of the front limbs.
- Finally, a score of 5 indicated severe, complete paralysis or the unfortunate circumstance of finding a deceased mouse, at which point euthanasia was recommended.

1.4 Western-Blot Analysis

We initiated the process by subjecting 1 µg of mouse brain homogenate to separation through a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique. Subsequently, the separated components were transferred to a PVDF blotting membrane (GE Healthcare, Germany, Catalog No. 10600021). The membrane was then blocked with 5% skimmed dried milk for a duration of 60 minutes. Following this, the membrane underwent an incubation phase with primary antibodies including anti-beta Actin (at a 1:2000 dilution), SIRT1 Antibody (at a 1:1000 dilution). This incubation occurred in a blocking solution and took place overnight at 4°C. All of the antibodies employed in this process were sourced from Abcam in Cambridge, United Kingdom.

Subsequent to three washes of 5 minutes each, the membranes were incubated with secondary antibodies goat anti-mouse at a 1: 10,000 dilutions, following four additional washes of 5 minutes each, the membranes underwent incubation in an ECL western blotting substrate kit from Abcam for a duration of 2 minutes.

The resulting bands on the membranes were then detected utilizing a ChemiDoc Touch imaging system from Bio-Rad in Hercules, California, United States. To analyze the data semi-quantitatively, Image Lab software was employed."

1. Statistical analysis

The outcomes of the quantitative experiments were presented as the mean value accompanied by the standard error of the mean (SEM). Data analysis was performed using GraphPad Prism software (GraphPad Prism 8 software Inc., La Jolla, CA, USA). To assess and compare the mean values among three or more groups, we employed a one-way analysis of variance (ANOVA) test, followed by the Tuki Kramer multiple comparisons test as a post-hoc analysis. A significance threshold of $p < 0.05$ was utilized to ascertain the statistical significance of the results.

Results:

Here, we wanted to assess the effect of PEPITEM administration on the protein expression of SIRT1 in the brain of EAE mice using Western-Blot (WB). The Western-Blot analysis revealed that the protein expression of SIRT1 in PEPITEM treatment group (G3) significantly increased compared to G2. However; there was no changes in the protein expression of SIRT1 in PEPITEM prophylactic group (G5) compared to G4.

Discussion

PEPITEM has an anti-inflammatory effect of many of T cell diseases such as MS, and the objective of this project is to evaluate its effect of SIRT1 protein expression. Using a mouse model of experimental autoimmune encephalomyelitis (EAE) as a model for human MS and WB analysis, we showed that the PEPITEM treatment significantly increases the expression of SIRT1 in the brain of EAE. This interesting finding is consistent with the literature which suggests that the increase of SIRT1 is associated with suppression of inflammation as it plays a role in moderating the onset and progression of inflammatory processes such as deacetylating nuclear factor kappa B (NF- κ B)) [14, 15]. Moreover, SIRT1 is associated with the suppression of activator protein 1 (AP-1) which is known to be inflammatory transcription factor [16]. Furthermore, body of evidence suggest a correlation between the reduction of SIRT1 level and many inflammatory diseases [16-18].

The PEPITEM prophylactic administration did not show the same finding seen on the treatment group; There were no changes in the protein expression of SIRT1 in PEPITEM prophylactic group (G5) compared to the control group (G4). The aim of the PEPITEM prophylactic administration was to prevent the disease induction, however; the symptoms were observed in both groups and the disease was induced. This data suggest that PEPITEM does not inhibit the induction of the disease unlike what has been suggested by another study where PEPITEM was able to stop the induction the onset of collagen induced arthritis (CIA) in mouse models [19].

Conclusion:

Collectively, the therapeutic treatment with PEPITEM suggesting anti-inflammatory effect of PEPITEM on the brain damage in EAE mice which offers a novel and safe strategy for drug therapy in MS, opening new avenues for research and treatment.

Future work:

Comparing the protein expression of SIRT1 in the spinal cord of EAE mice is recommended since the spinal cord is the most common site of EAE lesions in this model. Moreover; using increased dose of PEPITEM might stop the induction of the disease as seen on the collagen induced arthritis (CIA) in mouse models.

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Data Availability Statement

The data presented in this study are available on a reasonable request from the corresponding author.

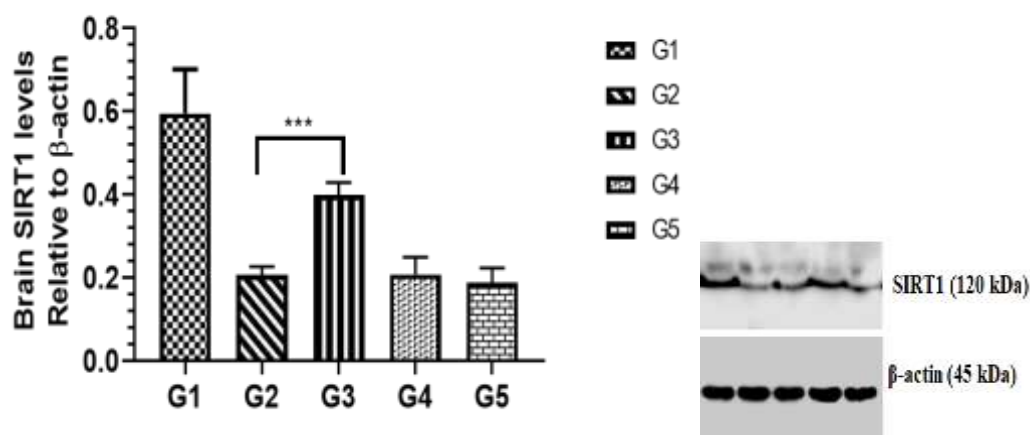


Figure 1: Protein levels of SIRT1 in the brain of all groups of EAE mice as detected by western blotting and relatively expressed to β -actin. SIRT1 protein expression in the brain of EAE mice on day 21. EAE mice received a daily IP injection of scramble peptide (G2) and (G4) or PEPITEM (G3) and (G5) until day 21 post EAE induction. The protein expression was evaluated by western blot. Mean $\pm$ SEM are depicted (n=4 per group in triplicate). Note: *** P < 0.0001.

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