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RESEARCH ARTICLE

Neuroprotective Role of Dexpanthenol and Butafosfan-Vitamin B_{12} Against Brain Damage Induced by Circadian Rhythm Disorder

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Abstract

This study investigates the neuroprotective effects of dexpanthenol (DEX) and butafosfan-vitmain B_{12} combination (BUT) in a circadian rhythm disorder (CRD)-induced brain damage model in mice. Control, CRD, DEX, BUT, and BUT + DEX groups were subjected to a 19-day experimental period during which CRD was induced by repeated phase shifts in the light/dark cycle. The CRD group experienced circadian rhythm disorder, while the DEX and BUT groups received intraperitoneal DEX (1000 mg/kg/day) and subcutaneous BUT (200 mg/kg/day) treatments, respectively, for the same duration. Serum cortisol and *creatine kinase* (CK) levels were measured using ELISA to assess stress and tissue damage. Brain tissues were evaluated histopathologically using hematoxylin and eosin staining, and immunohistochemically for brain-derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP) expression using specific monoclonal antibodies. CRD significantly increased serum cortisol and CK levels compared to the control group ($P<0.001$). Both DEX and BUT treatments reduced these elevations, with the combination therapy showing the most pronounced effect ($P<0.001$). Histopathological examination revealed reduced neuronal degeneration, hyperemia, and hemorrhage in the treatment groups compared to the CRD group. Immunohistochemical analysis showed significantly increased BDNF and GFAP expression in the BUT + DEX group ($P<0.001$). These findings suggest that DEX and BUT, particularly in combination, exert neuroprotective effects against CRD-induced brain injury by modulating oxidative stress, inflammation, and neurotrophic signaling pathways.

Keywords: Butafosfan, Circadian rhythm, Dexpanthenol, Neuroprotective, Vitamin B_{12}

Introduction

The circadian system governs the timing of physiological processes and behaviors across a 24-h cycle, ensuring adaptation to environmental changes and internal coordination within the body. Central to this system is the suprachiasmatic nucleus (SCN) in the hypothalamus, which acts as the master pacemaker, orchestrating the timing of various biological rhythms. However, the circadian rhythm extends beyond the SCN, encompassing peripheral clocks present in diverse tissues, each contributing to the regulation of specific metabolic functions. Understanding the complex interplay between central and peripheral clocks is critical for clarifying the circadian system's role in metabolic health and disease [1]. The central clock in the SCN serves as the primary regulator of circadian rhythms, entraining the body to the external light-dark cycle. Through neural and hormonal signaling pathways, the SCN coordinates physiological processes such as sleep-wake cycles, hormone secretion, and metabolism. Notably, disruptions in SCN function, induced by factors like shift work or jet lag, can lead to desynchronization of internal clocks, contributing to metabolic disturbances and increased susceptibility to chronic diseases [2]. In addition to the central clock, peripheral clocks distributed throughout peripheral tissues play a crucial role in maintaining metabolic homeostasis. These clocks exhibit intrinsic rhythmicity and respond to both systemic signals from the SCN and local cues such as nutrient availability and physical activity. Peripheral clocks regulate tissue-specific metabolic processes, including glucose and lipid metabolism, insulin sensitivity, and energy expenditure. Given the detrimental impact of circadian rhythm disorder (CRD) on neuronal integrity and inflammatory balance, identifying agents that can

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counteract oxidative stress and promote cellular resilience is of paramount importance [3].

Dexpanthenol (DEX), also known as D-pantothenol, is an alcoholic analog of pantothenic acid (PA), a precursor of coenzyme A (CoA) and an essential component of cellular energy metabolism. DEX and its derivatives have been extensively studied for their ability to enhance intracellular ATP synthesis and increase concentrations of reduced glutathione (GSH), thereby exerting antioxidant and cytoprotective effects. Moreover, DEX has been shown to play a crucial role in cellular defense and repair mechanisms, making it a promising candidate for the treatment of various oxidative stress-related disorders. Animal studies have demonstrated the potent antioxidant properties of DEX in conditions such as lung fibrosis, necrotizing enterocolitis, ischemia-reperfusion injury, and testicular damage [4-7]. Additionally, DEX exhibits anti-inflammatory effects and contributes to tissue repair processes [8]. Furthermore, emerging evidence from human studies suggests that DEX plays a significant role in brain function and health, with brain levels reported to be significantly higher than plasma levels [9,10]. DEX is implicated in the synthesis of multiple neurotransmitters, underscoring its importance in neuronal communication and synaptic transmission [11]. Studies have shown that DEX therapy can attenuate oxidative stress and enhance the levels of amino acid neurotransmitters associated with brain damage, potentially mitigating the neurological consequences of oxidative insults [12].

In addition to redox-based neuroprotection, metabolic regulators like butafosfan may offer complementary benefits by modulating cellular energy metabolism and stress-induced immune responses. Butafosfan, a phosphonic acid derivative, has attracted scientific interest due to its proposed immunomodulatory properties and potential roles in promoting systemic physiological resilience. Although primarily used in veterinary medicine, particularly in combination with vitamin B_{12}, recent studies have explored its broader therapeutic potential beyond its role as an energy enhancer. Understanding the immunomodulatory mechanisms of butafosfan is crucial for elucidating its therapeutic effects and exploring its potential applications in human health [13,14]. The immunomodulatory effects of butafosfan have not been extensively characterized; however, available studies indicate its potential to enhance immune function. In an experimental study on pregnant ewes, subcutaneous administration of butafosfan and cyanocobalamin significantly improved metabolic markers, reduced oxidative stress indices, and effectively reduced the incidence of subclinical pregnancy toxemia [15]. However, the precise cellular mechanisms underlying its immunomodulatory effects remain unclear and warrant further investigation [16]. Moreover, research in various animal species, including cattle, horses, pigs, chickens, and mice, has demonstrated the beneficial effects of butafosfan and vitamin B_{12} combination (BUT) supplementation on overall health. These effects include increased feed intake, improved immune function, enhanced liver and muscle function, and improved hemostasis. Furthermore, BUT supplementation has been shown to support erythropoiesis and alleviate stress responses in animals, further highlighting its potential health benefits [17].

The circadian system regulates various physiological processes, including sleep-wake cycles, hormone secretion, and metabolism. Disruptions in circadian rhythms can have profound effects on health, including cognitive impairment and metabolic disorders. Dexpanthenol and BUT have been implicated in cellular metabolism and immune function, but their effects on the circadian system and brain remain poorly understood.

This study investigates the neuroprotective potential of the DEX and BUT combination against CRD induced brain injury by modulating stress-related biochemical changes and neurotrophic responses.

Material and Methods

Ethical Statement

This study was approved by the Animal Experiments Local Ethics Committee of Burdur Mehmet Akif Ersoy University (Approval No. 14.02.2025/1273).

Animals

A total of 35 male C57BL/6 mice, aged 10-12 weeks and weighing approximately 20-30 g, were obtained from the Burdur Mehmet Akif Ersoy University Laboratory Animal Production and Experimental Research Center. The mice were housed in a facility with maintained at room temperature 21-23°C, and humidity levels ranging from 55% to 65%. *Ad libitum* access to water and food was provided to the animals.

Circadian Rhythm Disorder Model

Baseline data collection started 1 week after the mice were transferred to plastic cages. The experimental protocol began with a 1-week baseline period with lights on at 09.00-21.00, 13 weeks of 12-h phase shifts in the light/dark cycle two times per week, and 10 days of recovery period with lights on at 09.00-2100. Light/dark cycle shifts always involved a 24-h period of lights on *(Fig. 1)*. The control mice were maintained on the same lighting schedule with lights on at 09.00-21.00 throughout the experimental period except that lights-on periods were extended to 24 h every Monday during the light/dark cycle shift period to maintain an equal amount of exposure to light

for both the control mice and the experimental mice.

Chemicals

DEX was obtained from dexpanthenol (Bepanthen®; 500 mg/2 mL, Bayer Corp., Istanbul, Türkiye), and the BUT was obtained from butafosfan and vitamin B_{12} (Catosal® 10%; 100 mg butafosfan/0.05 mg vitamin B_{12}, Bayer Animal Health GmbH, Leverkusen, Germany).

Experimental Design

The study was conducted using a total of 35 C57BL/6 mice, which were divided into five groups (n = 7). Prior to the experiment, all animals were housed under standard laboratory conditions with a 12:12-h light-dark cycle for a 10-day adaptation period [18]. To induce CRD, animals in the CRD, DEX, BUT, and BUT + DEX groups were housed for 19 consecutive days in specialized chambers with programmable light-dark cycles [19]. All animals in these groups were provided standard diet and water *ad libitum*. During this period, the DEX group received intraperitoneal dexpanthenol (1000 mg/kg/day), the BUT group received subcutaneous butafosfan + vitamin B_{12} (200 mg/kg/day), and the BUT + DEX group received both treatments simultaneously at the same dosages [16,19,20]. The control group remained under a normal 12:12-h light-dark cycle throughout the study and did not receive any pharmacological treatment. The CRD induction protocol is illustrated in *Fig. 1*, and the overall experimental design is summarized in *Fig. 2*.

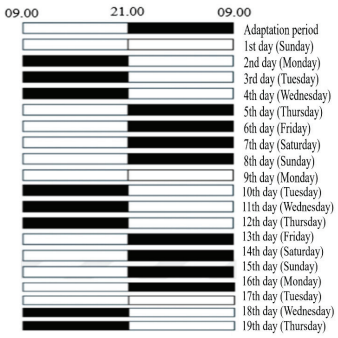

Fig 1. The program applied to create circadian rhythm disorder

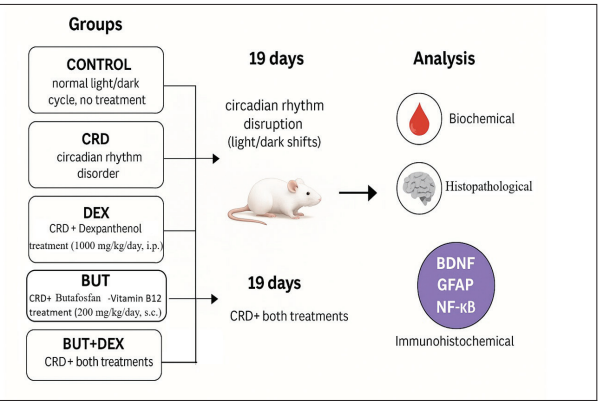

Fig 2. Schematic illustration of the experimental design

On day 20, all animals were anesthetized with ketamine HCl (90 mg/kg, i.p.) and xylazine HCl (10 mg/kg, i.p.), and cardiac blood samples were collected. Euthanasia was performed via cervical dislocation under deep anesthesia. Brain tissues were harvested and divided into two portions: one was homogenized for the biochemical analysis of cortisol and creatine kinase (CK) levels, while the other was fixed in 10% buffered formalin for histopathological and immunohistochemical examinations.

Biochemical Analyses

The blood samples were taken directly into tubes without anticoagulant and centrifuged at 4000 rpm for 10 min to separate the serum. The tissues were initially washed with 0.9% saline solution. Subsequently, the tissues were homogenized with 0.01 M pH 7.4 phosphate buffer solution (1 g tissue/9 mL PBS) as specified in the kits. The homogenate was then centrifuged at 15.000 rpm for 45 min at 4°C to obtain supernatants.

Determination of Creatine Kinase Levels

CK levels of serum and tissue homogenate samples were determined according to the procedure described in the commercial ELISA kit (BT-LAB E0609Mo) and measured at 450 nm with ELx800-Biotek. Total protein concentrations of brain homogenates were measured according to the Biuret method [21]. The results were expressed in mIU/mL tissue based on the prepared standard curve.

Determination of Cortizol Levels

Serum cortisol levels were determined using a commercial ELISA kit (BT-LAB E0609Mo) and measured at 450 nm with an ELx800-Biotek instrument. Cortisol levels are expressed as ng/ml.

Histopathological Examination

At the conclusion of the experimental phase, all groups of mice were humanely sacrificed. The brains were carefully extracted from the skull to prevent any damage to the delicate brain tissue during necropsy. Following removal, all brain samples underwent a meticulous gross examination. Brain tissues were fixed in 10% buffered formalin for 48 h, followed by routine paraffin embedding using an automated tissue processor. Serial sections, each with a thickness of 5 µm, were obtained using an automated rotary microtome. These sections were then stained with routine hematoxylin and eosin (HE) staining to facilitate microscopic examination. Histopathological evaluation of the brain tissue focused on identifying key lesions, including hyperemia, hemorrhage, and degenerative changes. A semi-quantitative ordinal grading system was

employed to assess these alterations. The severity of lesions was categorized as follows: normal (score = 0), mild (score = 1), moderate (score = 2), and severe (score = 3). The stained sections were examined under a light microscope, and the histological findings were systematically recorded.

Immunohistochemical Examination

Sections mounted on poly-L-lysine-coated slides were subjected to streptavidin-biotin peroxidase immunohistochemical staining. Immunohistochemical analysis was performed on brain sections using primary antibodies targeting brain-derived neurotrophic factor (BDNF) (Anti-BDNF antibody [EPR1292], ab108319), glial fibrillary acidic protein (GFAP) (Anti-GFAP antibody, ab7260), and nuclear factor kappa B (NF-κB) (Anti-NFκB p100/p52 antibody, ab227078). All primary antibodies were obtained from Abcam and diluted at a ratio of 1:100 using antibody dilution solutions. The immunohistochemical procedure was carried out following the manufacturer's instructions. For secondary detection, the Mouse and Rabbit Specific HRP/DAB Detection Kit - Micropolymer (ab236466) from Abcam (Cambridge, UK) was used. All sections underwent identical staining procedures. Negative controls were processed by replacing the primary antibodies with antibody dilution buffer to verify staining specificity. Immunohistochemical evaluation was performed by quantifying the percentage of positively stained cells in regions associated with circadian rhythm regulation. For each animal, 100 cells were evaluated per brain region, by analyzing 20 randomly selected cells from each of five non-overlapping fields under a 40X objective lens. ImageJ 1.46r software (National Institutes of Health, Bethesda, MD) was employed to determine the number of immunopositive cells. Microscopic imaging was conducted using an Olympus CX41 microscope, and the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan) was utilized for microphotography.

Statistical Analysis

The data obtained from the study were analyzed using SPSS version 22.0 for Windows (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard error (SE). Initially, the data were analyzed for normality using the Shapiro-Wilk test. It was determined that the histopathological scoring data exhibited a nonparametric distribution, while all other data exhibited a parametric distribution. Statistical analysis of biochemical parameters, antioxidant-oxidative stress markers, anti-inflammatory parameters, inflammatory parameters, transcription factor levels, and percentages of immunohistochemically positive cells was performed using One-Way ANOVA, and differences between groups were determined using the Tukey test. Values of $P<0.05$ were considered significant. Since histopathological scores did not show a normal distribution, the Kruskal-Wallis test and the Mann-Whitney U test with Bonferroni correction were used, with values of $P<0.05$ considered statistically significant.

Results

Effects of DEX and BUT on Blood and Brain Function

Both serum and brain tissue levels of CK and cortisol were significantly elevated in the CRD group compared to the control ($P<0.001$). Treatment with DEX, BUT, and their combination (BUT + DEX) led to a significant reduction in these parameters compared to the CRD group ($P<0.001$). Among the treatment groups, the BUT group showed the most pronounced decrease in serum cortisol and CK levels ($P<0.001$), while brain CK levels were lowest in the DEX group ($P<0.001$). These findings indicate tissue-specific effects of DEX and BUT on stress and damage markers *(Fig. 3)*.

Histopathological Findings

The control group exhibited normal brain histology without any pathological alterations. The CRD group exhibited a statistically significant increase in histopathological scores compared to the control group ($P<0.001$ for both) *(Fig. 4)*. On average, scores increased from approximately 0.4 in the control group to 3.1 in the CRD group, representing a **7.75**-fold increase, indicating marked neuronal degeneration, hyperemia, and mild perivascular hemorrhage. Treatment with BUT and DEX reduced histopathological scores by approximately 2.4-fold and 2.6-fold, respectively, compared to the CRD group. The combined BUT + DEX treatment provided the most prominent improvement, with an approximately 5.3-fold reduction in histopathological damage relative to the CRD group *(Fig. 5)*.

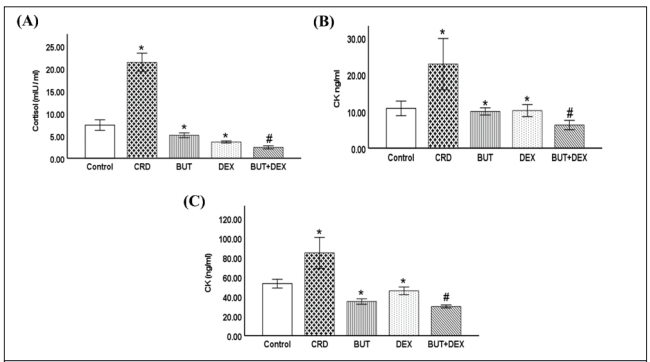

Fig 3. (A) Cortisol levels in serum, (B) creatine kinase levels in serum and (C) brain tissue control, CRD, BUT, DEX and BUT+DEX groups. All values are expressed as mean ± SEM (n = 7). *P<0.001 when compared to the control group. # P< 0.001 when compared to the CRD group

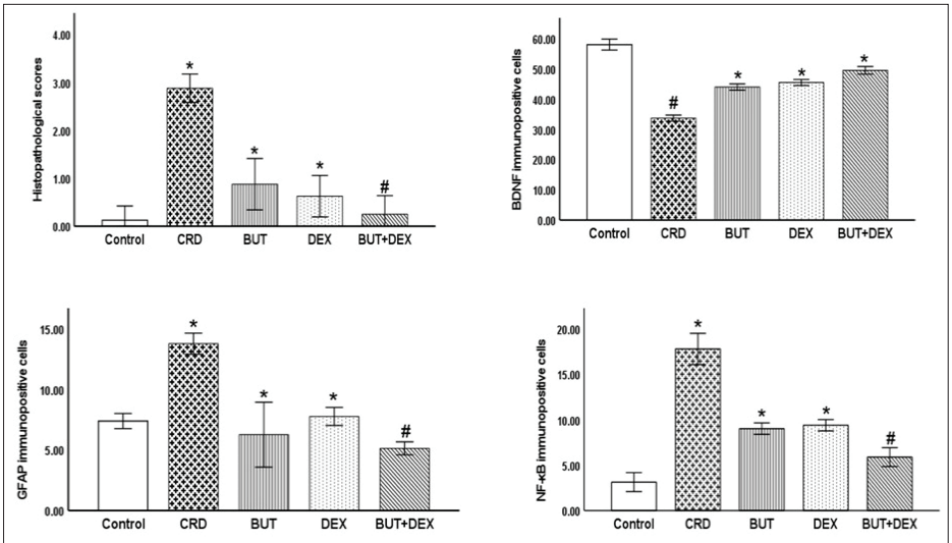

Fig 4. Histopathological scores and BDNF, GFAP, and NF-κB immunopositive cells between the control, CRD, BUT, DEX and BUT+DEX groups. All values are expressed as mean ± SEM (n = 7). *P<0.001 when compared to the control group. # P<0.001 when compared to the CRD group

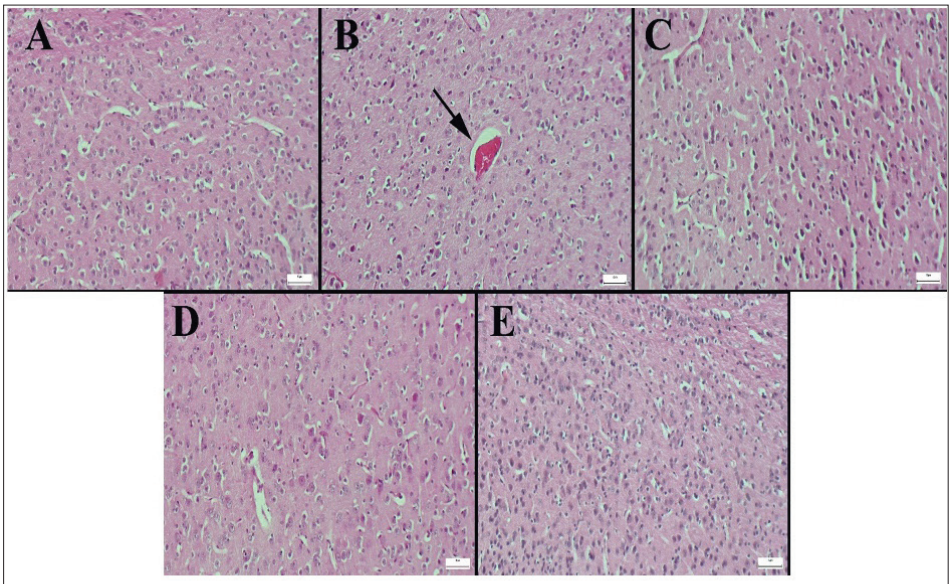

Fig 5. Histopathological comparison of brain tissues across experimental groups. (A) Normal brain tissue architecture in the control group. (B) The CRD group exhibits mild hyperemia and perivascular edema (arrow). (C) Decreased pathological alterations in the BUT-treated group. (D) Reduced pathological alterations in the DEX-treated group. (E) Markedly diminished pathological changes in the BUT+DEX-treated group. HE staining, scale bars = 50 µm

Immunohistochemical Findings

Immunohistochemical analysis revealed that the control group exhibited marked BDNF expression alongside slight to negative GFAP and NF-κB levels. In contrast, CRD group showed decreased BDNF and increased GFAP and NF-κB levels. Treatment with BUT and DEX led to a significant increase in BDNF expression while reducing GFAP and NF-κB levels in the treated groups. BDNF-positive cell counts increased by approximately 50% in the BUT + DEX group compared to CRD (P<0.001), while NF-κB and GFAP expression decreased by 65% and 55%, respectively *(Fig. 4)*. Notably, the combined therapy demonstrated greater effectiveness compared to single treatments, showing a more pronounced enhancement in BDNF and a stronger reduction in GFAP and NF-κB expression *(Fig. 6)*.

Discussion

This study investigates the impact of CRD on neuro-inflammation, glial activation, and neuroplasticity, highlighting the therapeutic potential of BUT and DEX treatments. Our results show that circadian misalignment

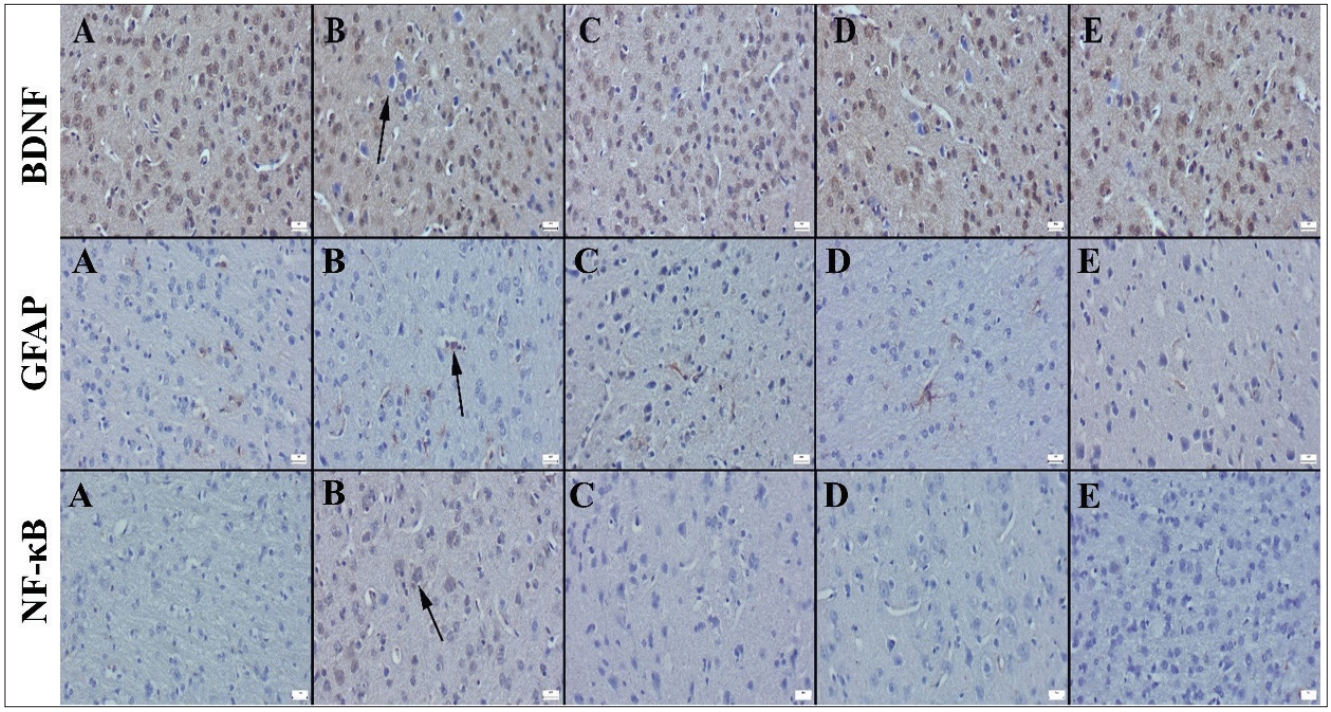

Fig 6. Immunohistochemical expression of BDNF (top row), GFAP (middle row), and NF-κB (bottom row) in brain tissues across experimental groups. (A) The control group shows strong BDNF expression with minimal to negative GFAP and NF-κB expression. (B) The CRD group exhibits decreased BDNF expression and significantly increased GFAP and NF-κB levels. (C) The BUT-treated group shows increased BDNF expression and reduced GFAP and NF-κB levels. (D) The DEX-treated group similarly demonstrates increased BDNF and decreased GFAP and NF-κB expression. (E) The BUT+DEX combination group exhibits the most pronounced increase in BDNF expression and the greatest

leads to elevated GFAP and NF-κB expression, alongside a significant reduction in BDNF levels ($P<0.001$ for all). These pathological changes were significantly attenuated by treatment, with the combination of BUT and DEX showing the most pronounced effect: BDNF levels increased by ~50%, NF-κB expression decreased by ~65%, and histopathological scores were reduced by ~81% compared to the CRD group.

Inflammatory processes play a crucial role in mediating brain injury under stress-related conditions. Agents with both antioxidant and anti-inflammatory properties offer a dual mechanism of neuroprotection. For instance, DEX and B-complex vitamins have been reported to exert beneficial effects in mitigating neuroinflammation, enhancing cellular repair mechanisms, and preserving blood-brain barrier integrity. The therapeutic efficacy of such compounds in experimental animal models further support their potential clinical applications in neurological disorders [22,23]. GFAP is a marker for astrocytic activation and gliosis, commonly associated with injury or inflammation [24]. The observed increase in GFAP expression in the CRD group is consistent with previous studies, such as those by Moriya et al.[25], which showed elevated GFAP levels following chronic CRD, indicating heightened astrocyte reactivity. Zimmermann et al.[26] further reported glial cell density reduction in the suprachiasmatic nucleus, further supporting the link between CRD and glial changes. NF-κB, a key regulator of neuroinflammation, was also upregulated in the CRD group. This finding aligns with Chen et al.[27], who demonstrated melatonin's role in modulating NF-κB activity to control neuroinflammation. Additionally, Başak et al.[28] observed oxidative stress-induced GFAP upregulation and increased NF-κB activity in pinealectomized rats, reinforcing the impact of CRD on neuroinflammatory processes. The reduction in NF-κB expression following DEX and BUT treatment underscores the potential of these therapies to counteract neuroinflammation. BDNF is crucial for neuroplasticity and cognitive function, and its significant reduction following CRD is consistent with findings by Liang et al.[29], Pang et al.[30], and Dingding et al.[31], who reported impaired BDNF signaling due to CRD or neurodegenerative conditions such as Parkinson's disease. Together, they may act to restore neurotrophic support and mitigate glial activation. This interpretation is supported by increased BDNF expression and reduced NF-κB and GFAP levels in the combination group, suggesting that targeting multiple pathological pathways enhances neuroprotection. Notably, DEX is known to enhance antioxidant enzyme activity, particularly via glutathione pathway modulation, while BUT influences energy metabolism and reduces proinflammatory cytokine release. Moreover, BUT, especially in combination with

vitamin B_{12}, may downregulate NF-κB and promote BDNF expression, providing a multifaceted approach to neuroprotection [32].

Additionally, we explored the effects of CRD on cortisol and CK levels. Disruptions in sleep patterns are known to alter cortisol rhythms [33,34], but the effects of BUT and DEX on cortisol and CK levels remain unclear and warrant further investigation. In conclusion, CRD significantly impacts neuroinflammation, glial activation, and neuroplasticity. The combined treatment of BUT and DEX effectively mitigated these effects, offering therapeutic potential. Future studies should investigate the long-term efficacy and mechanisms of these treatments, particularly their impact on cortisol and CK levels in human models, to inform potential therapies for CRD and stress-related conditions.

As highlighted by Nair and Jacob [35], dose extrapolation from animal models to humans is inherently complex and requires careful consideration of species-specific differences in metabolism and pharmacokinetics, typically using body surface area as a reference. Although the doses of DEX (1000 mg/kg/day) and BUT (200 mg/kg/day) used in this study are consistent with previous animal studies, translating these doses to human equivalents would require significant adjustment. Therefore, their translational relevance to human therapy remains to be determined. In light of the rising prevalence of CRD among shift workers, adolescents, and the elderly [36], the present findings may contribute to the development of adjunctive therapeutic strategies involving DEX and BUT to attenuate cognitive and neurological impairments. However, comprehensive clinical trials are warranted to assess the safety, optimal dosing, and therapeutic efficacy of these agents in human populations. The present study focused on acute effects following a 3-week CRD protocol. Chronic exposure studies are also needed to assess tolerance, toxicity, and behavioral outcomes.

This study demonstrates that CRD causes significant biochemical, histological, and immunohistochemical alterations in mouse brain tissue, indicative of cellular stress and damage. Treatment with DEX and BUT, particularly in combination, provided significant neuroprotection by reducing cortisol and CK levels, alleviating tissue damage, suppressing inflammatory NF-κB and astrocytic GFAP expression, and preserving BDNF expression. These findings suggest a potential therapeutic approach using antioxidant and metabolic support compounds in conditions related to CRD.

Declarations

Availability of Data and Materials: The data used in this article will be provided by correspondin author (S.G.) upon request.

Ethical Statement

This study was approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee dated 14.02.2025 and numbered 1273.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The tables and figures used in this article were not created by artificial intelligence.

Author Contributions: Forming the hypothesis and planning the study: S.G.; Carrying out the experimental phase: S.G., Ö. Ö. & Ş.T.; Obtaining data and writing the article: S.G. & Ö.Ö.

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