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Research Article

Effects of riboflavin on hyperalgesia and serum glutamine-to-glutamate ratio in rats with painful diabetic neuropathy

Milen Hristov[1], Zafer Sabit[2], Tsvetomir Kirilov[1], Dimitar Bakalov[2], Rumiana Tzoneva[3], Sonia Apostolova[3], Irina Georgieva[3], Pavlina Andreeva-Gateva[1]

1 *Department of Pharmacology and Toxicology, Faculty of Medicine, Medical University of Sofia, Sofia, Bulgaria*
2 *Department of Physiology and Pathophysiology, Faculty of Medicine, Medical University of Sofia, Sofia, Bulgaria*
3 *Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Acad. G. Bonchev Str, Bl. 21, Sofia 1113, Bulgaria*

Corresponding author: Milen Hristov (milen_hristov@abv.bg)

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Abstract

Previous studies have explored the antinociceptive effects of riboflavin (vitamin B2) across various experimental models. However, there remains a gap in the literature regarding its potential to alleviate neuropathic pain in diabetes. This study aims to investigate the effects of riboflavin on hyperalgesia and serum glutamine-to-glutamate ratio in rats with painful diabetic neuropathy. In fasted rats, a model of painful diabetic neuropathy was induced through intraperitoneal injection of streptozotocin. In the fifth week post-injection, diabetic rats experiencing neuropathic pain were administered daily doses of riboflavin (25 or 50 mg), dissolved in their drinking water, for a duration of two weeks. Results demonstrate that riboflavin significantly reduced mechanical and cold-induced hyperalgesia in diabetic rats compared to controls. Formalin-induced hyperalgesia was alleviated by riboflavin in the second phase. Additionally, riboflavin supplementation increased the serum glutamine-to-glutamate ratio in these animals. These findings highlight the therapeutic potential of riboflavin in managing neuropathic pain associated with diabetes.

Keywords

diabetes, glutamine, glutamate, painful neuropathy, riboflavin

Introduction

Diabetic neuropathy is the most common complication of diabetes, which is characterized by loss of sensory function starting distally in lower limbs, pain, and considerable morbidity. Diabetic neuropathy arises from both diffuse and focal damage to the nervous system, and it is estimated that over time, at least 50% of individuals diagnosed with diabetes will experience this complication. Neuropathic pain develops in approximately 30–50% of patients with diabetic neuropathy and manifests as a spontaneous burning pain in the legs, brush-evoked allodynia, and paresthesias. It is considered that various changes in peripheral and central neurons are involved in the pathophysiological mechanisms of neuropathic pain in diabetic neuropathy (Feldman et al. 2019).

Previous studies have indicated that abnormal glutamate-glutamine homeostasis may contribute to the

pathogenesis of diabetes (Hristov et al. 2024). Specifically, patients with diabetic neuropathy were found to have significantly higher levels of glutamate/glutamine in the posterior insula compared to controls (Petrou et al. 2012). Also, there was a significant increase in basal glutamatergic transmission of anterior cingulate cortex neurons in rats with diabetic neuropathic pain (Li et al. 2014). The ratio of glutamine to glutamate in the soleus muscle was found to be reduced in male Wister rats with diabetes induced by streptozotocin (Lambertucci et al. 2012). Additionally, studies have shown that a higher plasma glutamine-to-glutamate ratio is associated with a lower risk of developing diabetes mellitus (Liu et al. 2019).

Riboflavin, also known as vitamin B2, serves as a precursor for flavin adenine dinucleotide and flavin mononucleotide, which are coenzymes essential for the functioning of enzymes involved in various biochemical reactions. These reactions span a range of processes, including mitochondrial bioenergetics, redox status regulation, basic protein disulfide maturation, neurotransmitter catabolism, cellular methylation, amine catabolism, and DNA replication (Penberthy 2013). Previously, it has been demonstrated that systemic administration of riboflavin in mice induced an analgesic effect in the chemically induced nociception models (França et al. 2001). Another study reported that oral riboflavin produced antinociceptive, antihyperalgesic, and anti-inflammatory effects in carrageenan and formalin-induced hyperalgesia models in rats (Granados-Soto et al. 2004). Furthermore, it has been found that daily oral administration of riboflavin significantly reduced migraine frequency (Boehnke et al. 2004). However, there is a lack of literature data demonstrating whether riboflavin can alleviate neuropathic pain during diabetes. Therefore, the current study was devised to investigate the potential advantageous impact of riboflavin on hyperalgesia in rats with painful diabetic neuropathy. Additionally, given that vitamin B2 intake can alter plasma levels of amino acids (Jacques et al. 2001), we also examined the effect of riboflavin on the serum glutamine-to-glutamate ratio in these rats.

Materials and methods

Experimental animals

Male Wistar rats, (2 months of age, body mass range: 190±15 g) were used in the experiment. They were provided with unrestricted access to standard chow pellets and water. Animals were housed in a temperature-controlled room (20–22 °C) on a 12:12-h light-dark cycle (07:00 to 19:00 h). The experiment was carried out in compliance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, regarding the protection of animals used for scientific purposes. The Ethical Council of the Bulgarian Food Safety Agency provided approval for all experimental procedures (№330/01.06.2022).

Drugs

Riboflavin (#10438870) was purchased from Thermo Scientific Chemicals, Sweden. Streptozocin (#18883-66-4) was purchased from Sigma-Aldrich, Germany.

Experimental protocol

Painful diabetic neuropathy was induced by an intraperitoneal injection of streptozotocin (50 mg/kg, dissolved in citrate buffer, pH=4.5) in fasted rats. The normal control rats were administered the same volume of saline per kilogram. At the fifth week after the injection of streptozotocin, the rats were characterized by typical manifestations of type 1 diabetes. They showed hyperglycemia, polydipsia, polyuria, polyphagia, loss of fur, and difficulty in gaining body mass (Ma et al. 2015). Their average body mass at week five was 220±30 g. During the fifth week, diabetic rats received daily riboflavin (25 or 50 mg) dissolved in 600 ml of drinking water for two weeks. The diabetic animals were divided into 3 groups (n=8 animals per group): the diabetic group (Diabetic), the diabetic group receiving 25 mg riboflavin every day (Diabetic + Rf 25 mg) and the diabetic group receiving 50 mg riboflavin every day (Diabetic + Rf 50 mg). Riboflavin concentration in drinking water of "Diabetic + Rf 25 mg" group was 0.042 mg/ml and of "Diabetic + Rf 50 mg" group was 0.083 mg/ml. After these 2 weeks, the animals underwent tests to evaluate pain behaviors. The paw pressure test (Randall-Selitto Test) was administered on the first day, followed by the cold plate test on the second day, and the formalin test on the third day. On the fourth day, the animals were anesthetized with a combination of xylazine (10 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.), and blood was collected through cardiac puncture. The blood serum was separated through centrifugation, and the samples were then stored at -20 °C until they were needed for further analysis.

Randall-Selitto test

The Randall-Selitto or paw pressure test was devised as a method for evaluating response thresholds to mechanical pressure stimulation, commonly regarded as an indicator of mechanical hyperalgesia (Deuis et al. 2017). The test was conducted using an Analgesy-Meter (Ugo Basile, Italy). This procedure involved applying increasing mechanical force to the dorsal surface of the hind paw until withdrawal or vocalization occurred. Each animal was gently immobilized in a soft cotton towel. The test was performed in triplicate for each animal, ensuring that no more than 200 g of force was applied to avoid potential paw damage.

Cold plate test

This test was employed to assess cold-induced hyperalgesia. Rats were positioned inside a transparent cylinder, and the plate (Hot/Cold Plate, Ugo Basile, Italy) was set to a temperature of 5 °C (Allchorne et al. 2005). The time taken for

paw lifting was recorded, with a cut-off time set at 60 seconds. The test was performed three times for each animal.

Formalin test

A 0.5% formalin concentration is suitable for inducing hyperalgesia in rats with diabetic neuropathy (Calcutt et al. 1995). An intraplantar injection of 50 µL of 0.5% formalin was administered, and rats were subsequently placed in individual transparent chambers for observation. An observer recorded the time spent flinching, licking, and/or biting the injected paw. The durations of the first phase (0–5 min) and the second post-injection phase (20–30 min) were reported in seconds. Studies have elucidated the distinct mechanisms underlying the first and second phases of the formalin test. The initial phase (the first phase) primarily results from activation of sensory nerve endings in the skin by peripheral stimuli, while the subsequent phase (the second phase) is associated with a combination of peripheral tissue inflammation and functional alterations in the dorsal horn of the spinal cord (Tjølsen et al. 1992).

Determination of serum glutamine-to-glutamate ratio

The serum glutamine-to-glutamate ratio was calculated based on the measurement of serum glutamate and glutamine levels. These measurements were performed using the Glutamine and Glutamate Determination Kit (#GLN1, Sigma-Aldrich, Germany) in accordance with the manufacturer's instructions.

Statistical analysis

Statistical analysis utilized SigmaPlot 12.5 software (Systat Software GmbH, Erkrath, Germany). The Shapiro-Wilk test was employed for normality testing. Parametric data underwent analysis through a one-way ANOVA, followed by a Student-Newman-Keuls multiple comparison test (specifically, for the Randall-Selitto Test and Cold Plate Test). Non-parametric data were analyzed with a Kruskal-Wallis test, followed by a Student-Newman-Keuls multiple comparison test (for the Formalin test) or a Dunnett's multiple comparison test (for the Determination of serum glutamine-to-glutamate ratio). A significance threshold of $p < 0.05$ was employed to determine statistical significance.

Results

Effects of riboflavin supplementation on mechanical hyperalgesia in diabetic rats with neuropathic pain

Supplementation with Vitamin B2 effectively mitigated mechanical hyperalgesia, as evidenced in Fig. 1 (one-way ANOVA: $F_{3,28} = 4.606$, $p = 0.01$, power of the test with alpha = 0.050: 0.734). As depicted in Fig. 1, the mechanical threshold in vehicle-treated diabetic rats was significantly lower than that in normal rats (p=0.04), indicating the development of mechanical hyperalgesia. Notably, post hoc analysis revealed a statistically significant increase in the paw withdrawal threshold only in the group receiving 25 mg of riboflavin in the water when compared to the vehicle-treated diabetic group. No statistically significant difference in paw withdrawal threshold was observed between the group receiving 50 mg of riboflavin in water and both the control and vehicle-treated diabetic groups.

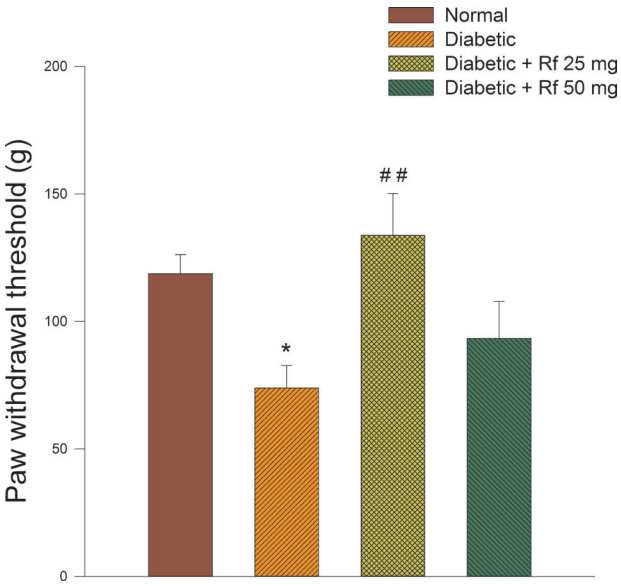

Figure 1. Effects of riboflavin on changes in pain threshold were investigated using the paw pressure test. Streptozotocin administration significantly induced mechanical hyperalgesia by reducing the mechanical threshold and enhancing paw withdrawal responses, while supplementation with Vitamin B2 effectively mitigated mechanical hyperalgesia. Significant differences compared to the control group (Normal) are denoted as $^{*}P < 0.05$, while significant differences compared to the diabetic group (Diabetic) are denoted as $^{\#\#}P < 0.01$. n=8 animals per group.

Effects of riboflavin supplementation on cold-induced hyperalgesia in diabetic rats with neuropathic pain

Administration of streptozotocin resulted in notable cold-induced hyperalgesia, evidenced by a reduction in paw withdrawal latency to cold stimuli. The addition of riboflavin into the drinking water demonstrated a notable alleviation of cold-induced hyperalgesia, as indicated in Fig. 2. The statistical analysis conducted through a one-way ANOVA yielded a significant result ($F_{3,28} = 71.378$, $p < 0.001$), with a robust test power of 1.000 at a significance level of 0.050. Subsequent post-hoc analysis revealed that rats administered with either 25 mg or 50 mg of vitamin B2 in their drinking water exhibited a statistically significant improvement compared to the vehicle-treated diabetic group.

Effects of riboflavin supplementation on formalin-induced hyperalgesia in diabetic rats with neuropathic pain

During phase 1 of the formalin test, the vehicle-treated diabetic group did not show any statistically significant difference in nociceptive behavior compared to the control group. The introduction of riboflavin into the drinking water did not change the nociceptive behavior of the diabetic animals, as indicated by Fig. 3 (Kruskal-Wallis test: H = 5.511, df = 3, p = 0.138).

In phase 2 of the formalin test, the vehicle-treated diabetic group showed a significant increase in nociceptive behavior compared to the control group. The addition of 25 mg or 50 mg of vitamin B2 to the drinking water resulted in a notable alleviation of formalin-induced hyperalgesia, as depicted in Fig. 3 (Kruskal-Wallis test: H = 13.493, df = 3, p = 0.004).

Effects of riboflavin supplementation on the serum glutamine-to-glutamate ratio in diabetic rats with neuropathic pain

Riboflavin supplementation in the drinking water significantly affected the serum glutamine-to-glutamate ratio in diabetic rats experiencing neuropathic pain. A statistically significant difference was observed (refer to Fig. 4; Kruskal-Wallis test: H = 6.180, df = 2, p = 0.046). Subsequent multiple comparisons against the vehicle-treated diabetic group using Dunnett's Method revealed a significant difference only between the "Diabetic" group and the "Diabetic + Rf 50 mg" group (see Fig. 4).

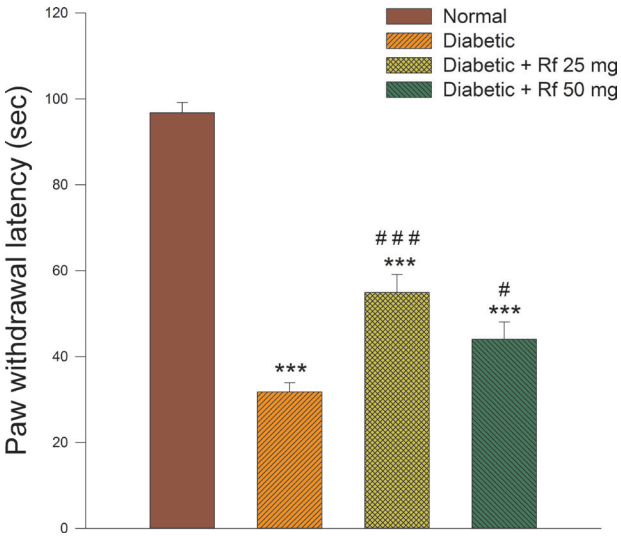

Figure 2. Effects of riboflavin on changes in pain threshold were investigated using the cold plate. Streptozotocin administration induced significant cold-induced hyperalgesia, alleviated notably by riboflavin in drinking water. Significant differences compared to the control group (Normal) are indicated as ***P < 0.001. Significant differences compared to the diabetic group (Diabetic) are indicated as #P < 0.05 and ###P < 0.001. n=8 animals per group.

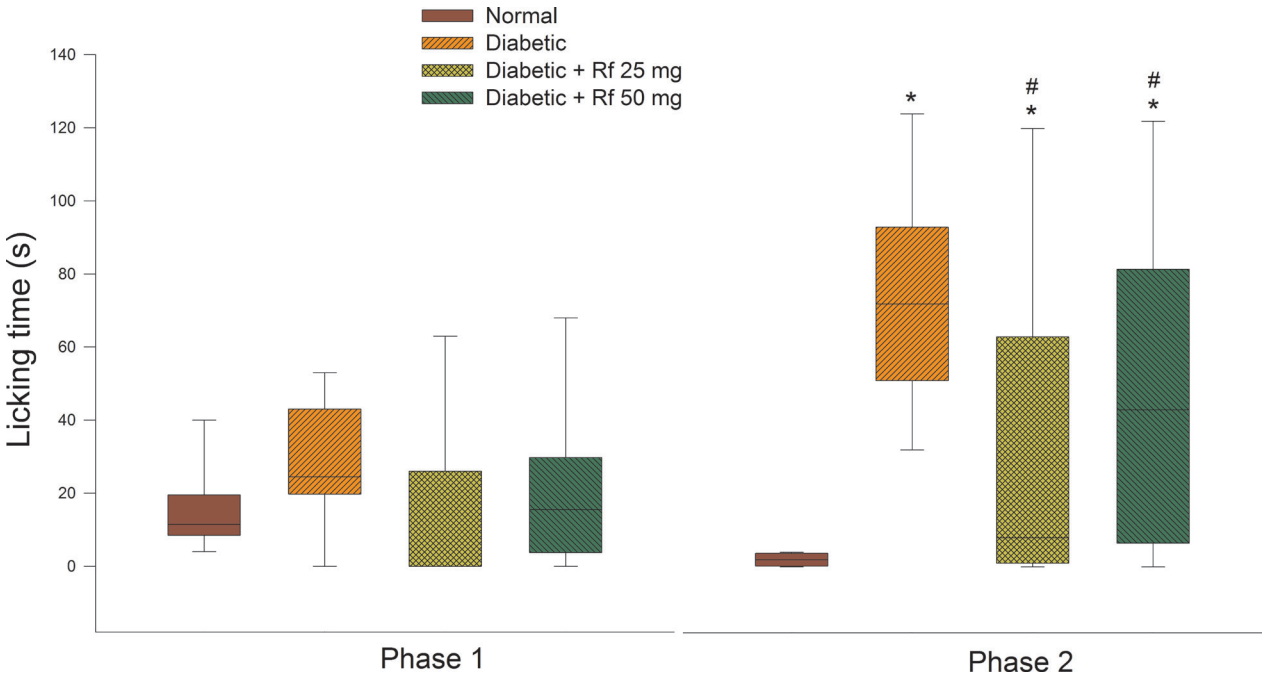

Figure 3. Effects of riboflavin on changes in pain threshold were investigated using the formalin test. The ends of the boxes depict the 25th and 75th percentiles, with a line at the median, while error bars extend to the 10th and 90th percentiles. During phase 1 of the formalin test, neither the vehicle-treated diabetic group nor the introduction of riboflavin showed significant changes in nociceptive behavior compared to the control group. In phase 2, the vehicle-treated diabetic group exhibited increased nociceptive behavior, while the addition of 25 mg or 50 mg of vitamin B2 significantly alleviated formalin-induced hyperalgesia. Significant differences compared to the control group (Normal) are indicated as *P < 0.05. Significant differences compared to the diabetic group (Diabetic) are indicated as #P < 0.05. n=8 animals per group.

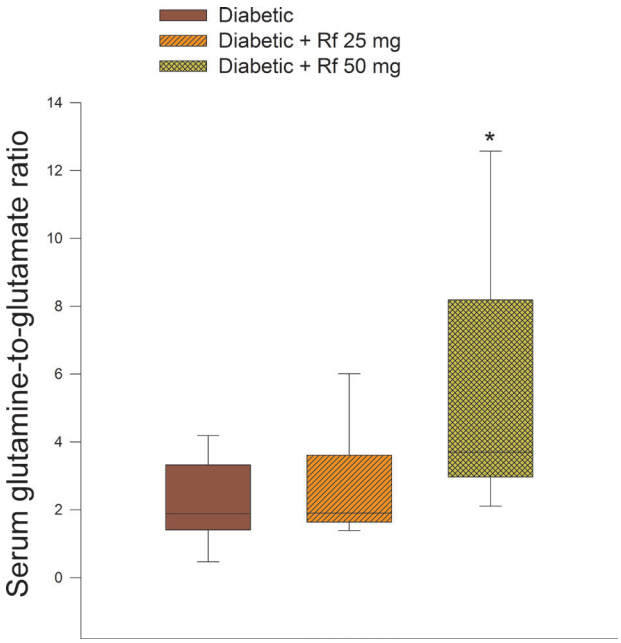

Figure 4. Box plot illustrating the effect of riboflavin supplementation on the serum glutamine-to-glutamate ratio in diabetic rats. The ends of the boxes depict the 25th and 75th percentiles, with a line at the median, while error bars extend to the 10th and 90th percentiles. Riboflavin supplementation in the drinking water significantly altered the serum glutamine-to-glutamate ratio in diabetic rats with neuropathic pain, with a significant difference found only between the "Diabetic" and "Diabetic + Rf 50 mg" groups. Significant differences compared to the diabetic group (Diabetic) are indicated as *$p < 0.05$. n=8 animals per group.

Discussion

A previous study demonstrated that a 3-month treatment with a combination of riboflavin, inosine, succinic acid, and nicotinamide helps reduce numbness, burning, and paresthesia in patients with diabetic polyneuropathy (Kharitonova et al. 2022). However, there is a lack of data in the literature regarding whether riboflavin alone has a beneficial effect on diabetic neuropathy. In our research, we found that supplementing drinking water with riboflavin effectively mitigated mechanical, cold-induced, and formalin-induced hyperalgesia in rats experiencing diabetic neuropathic pain. The favorable impact of vitamin B2 on the behavioral manifestations in diabetic rats implies its potential utility in the treatment of painful diabetic neuropathy. Consistent with the present findings, Bertollo et al. (2006) previously reported on the analgesic and anti-inflammatory effects of riboflavin in various experimental models. The authors showed that riboflavin, administered at doses of 50 or 100 mg/kg i.p., inhibited the second phase of the painful response induced by formalin in mice, while only the highest dose showed inhibition in the first phase and hot-plate model. The vitamin demonstrated antiedema and antinociceptive effects when administered immediately and 2 hours after carrageenan injection. Additionally, riboflavin inhibited fever induced by lipopolysaccharide in rats and suppressed fibrovascular tissue formation caused by a cotton pellet implant, suggesting its potential as an antinociceptive and anti-inflammatory agent (Bertollo et al. 2006).

The mechanism underlying the beneficial effects of riboflavin in rats with diabetic neuropathy remains unclear. However, Alam et al. (2015) provided insightful findings, demonstrating that riboflavin supplementation played a protective role against oxidative stress, improved metabolic parameters, and mitigated histological damage in the livers and kidneys of diabetic mice. Their study, revealing a 4-week treatment regimen of riboflavin in diabetic mice significantly impeding disease progression, highlights the potential therapeutic impact of riboflavin. Moreover, riboflavin supplementation reinstated the activity of crucial antioxidant enzymes, including glutathione reductase, superoxide dismutase and catalase, essential for combating diabetes-associated oxidative stress. Additionally, riboflavin treatment significantly reduced the formation of lipid peroxides and protein carbonyls, signifying a notable decrease in oxidative damage to lipids and proteins. Furthermore, dose-dependent reductions in fasting blood glucose levels were observed with riboflavin treatment, accompanied by increased calcium levels and enhanced GLUT-4 expression. These outcomes suggest promising benefits for enhancing insulin secretion, sensitivity, and glucose uptake in response to insulin (Alam et al. 2015). In another study, riboflavin was found to significantly reduce histamine-induced scratching behaviors and nerve discharges in mice (Lee et al. 2021). In cultured dorsal root ganglion neurons, riboflavin demonstrated inhibitory effects on currents induced by histamine and capsaicin. Flavin mononucleotide also demonstrated similar inhibitory effects, while flavin adenine dinucleotide did not. Moreover, riboflavin exerted antipruritic effects through modulation of transient receptor potential vanilloid 1 (TRPV1) activity (Lee et al. 2021). Interestingly, previous reports indicate that diabetic thermal hyperalgesia is mediated by the up-regulation of TRPV1 function, suggesting that targeting TRPV1 might offer a promising strategy for relieving pain linked to diabetic peripheral neuropathy (Pabbidi and Premkumar 2017). It has also been shown that oral riboflavin administration demonstrates analgesic effects in a mouse model of neuropathic pain induced by paclitaxel. This analgesic activity is attributed to the inhibition of tumor necrosis factor-alpha and chemokine (C-X-C motif) ligand 1 production in the dorsal root ganglia and thalamus, along with the activation of ATP-sensitive potassium channels (Braga et al. 2020).

In our study, we observed that administering riboflavin through drinking water increased the serum glutamine-to-glutamate ratio in diabetic rats with neuropathic pain. Elevated levels of blood glutamate are associated with diabetes development, while glutamine is thought to confer beneficial effects on diabetes (Hristov et al. 2024). We hypothesize that the mechanism through which vitamin B2 increases the serum glutamine-to-glutamate ratio may involve an impact on the gut microbiota. Previous research has shown that vitamin B2 supplementation significantly

alters the microbiota in the caeca of broiler chickens, with the highest dosage demonstrating the most pronounced effect in promoting the abundance of beneficial bacterial groups such as *Bifidobacterium*. This, in turn, enhances the production of butyrate, a widely recognized health-promoting metabolite, within the caecal environment (Biagi et al. 2020). Notably, the gut microbiota can influence the blood concentration of amino acids. For instance, studies have indicated that the androgen-induced modulation of the glutamine-to-glutamate ratio is influenced in part by the gut microbiota (Gao et al. 2021). Recently, researchers discovered that transferring microbiota from healthy mice to those with streptozotocin-induced diabetes restored serum glutamine levels to normal (Zhao et al. 2023).

Conclusion

In conclusion, our research demonstrates that riboflavin effectively alleviates mechanical, cold-induced, and formalin-induced hyperalgesia in rats with diabetic neuropathic pain. Additionally, administration of riboflavin via drinking water was associated with an increase in the serum glutamine-to-glutamate ratio in these animals. These findings suggest that riboflavin supplementation may offer beneficial effects in the management of painful diabetic neuropathy.

Competing interests

The authors have declared that no competing interests exist.

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