

Article

Tirzepatide Failed to Improve Neuropathy in a Murine Nonobese Streptozotocin-Induced Type 1 Diabetes Model, Despite Alleviating Systemic Inflammation

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Abstract

Background/Objectives: This study aimed to assess the effect of tirzepatide on systemic inflammation in a diabetic neuropathy (DN) model in mice. **Methods:** Type 1 diabetes was induced with streptozotocin 180 mg/kg i.p. One month later, we injected two doses of tirzepatide (5 or 15 mcg/kg BW s.c. e3d) over the next 4 weeks, then performed a battery of neurological tests and measured blood cytokines with the Simple Plex[®] platform CE-IVD version. Data were compared with naïve controls with or without diabetes induction. **Results:** Tirzepatide treatment failed to improve both glycemia and neuropathic behavior post-streptozotocin treatment. Higher-dose tirzepatide-treated mice had significantly decreased grip strength ($p = 0.013$) and higher cold ($p = 0.04$) and mechanical ($p < 0.001$) sensitivity, despite substantially lower systemic inflammation, as assessed by TNF- α and IL-6. An exploratory composite inflammatory index (ECII) derived from IL-6 and TNF- α showed statistically significantly lower systemic inflammation in the group treated with a higher tirzepatide dose vs. the treatment-naïve control ($p < 0.05$), the diabetic group ($p < 0.05$), and the diabetic group treated with lower-dose tirzepatide ($p < 0.05$). **Conclusions:** Tirzepatide failed to improve DN in a streptozotocin-induced murine nonobese model of type 1 diabetes, although it substantially decreased systemic inflammation. Testing tirzepatide for potential protection against DN in non-streptozotocin-based DN animal models is warranted.

Keywords: tirzepatide; diabetic neuropathy; systemic inflammation; diabetes type 1; mice



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1. Introduction

Of the adult population worldwide, one in nine is living with diabetes, accounting for as many as 589 million. Forty percent of those people are unaware they are affected. Living longer with diabetes is complicated by micro- and macrovasculopathy; peripheral and/or autonomic neuropathy; kidney and eye damage; diabetic foot, potentially ending in amputation; and progressively worsening quality of life [1], in addition to high CNS involvement, including cognitive and mood dysfunction. Diabetes-related neuropathy (DN) is the most common complication of both type 1 and type 2 diabetes, affecting 75% [1]

to 88% [2] of some patient populations, depending on diagnostic tools. The incidence of DN in type 2 diabetes (6100 per 100,000) is twice as high as in type 1 (2800 per 100,000), suggesting different pathological players [3]. In both conditions, however, long-standing hyperglycemia, dyslipidemia, aging, and genetics interplay with environmental factors.

Diabetic peripheral neuropathy is a length-dependent, predominantly axonal, sensorimotor neuropathy with distal-to-proximal progression, presenting as numbness, weakness, or sensory changes; it is most often observed with the “stocking-glove” pattern, although other presentations also exist [4]. Degeneration of small nerve fibers, along with ectopic discharge activity and central and peripheral sensitization, underlies hyperalgesia and allodynia. The disease progresses to affect larger myelinated nerve fibers, resulting in sensory ataxia, loss of ankle reflexes, and decreased proprioception [5].

Hyperglycemia and free fatty acids fuel inflammation by stimulating glucose utilization and altering oxidative phosphorylation [6]. Hotamisligil first discussed chronic inflammation in the context of insulin resistance and type 2 diabetes [7]. Now, it is well accepted that insulin resistance is also present in type 1 diabetes. Recent studies have highlighted the β -cell-macrophage crosstalk pathway as a crucial factor in chronic low-grade inflammation and glucose homeostasis imbalance in both type 1 and type 2 diabetes. Abnormal macrophage function contributes to the occurrence and development of DM and its complications [8]. Similarly, in the brain, diabetes induces neuroinflammation, with microglial activation, cytokine production, and increased blood-brain barrier permeability [9].

Inflammation promotes peripheral neuronal sensitization, and low-grade systemic inflammation is associated with DN severity [10]. Diabetic rodents showed increased TNF- α and a marked decrease in IL-2 compared with controls [11]. Inactivating TNF- α , either genetically or with infliximab, prevented or ameliorated diabetic neuropathy in mice [12]. Similarly, patients with DN have increased plasma levels of TNF- α or IL-6. However, in the dorsal root ganglia (DRG) of diabetic animals, IL-6, TNF- α , and IFN- γ were diminished, contributing to sensory neuropathy [13]. IFN- γ is a pleiotropic cytokine balancing hyperalgesic activity and neuroprotection [14]. It is also a crucial modulator of the central and peripheral immune responses. IFN- γ receptor signaling is an essential driver of neuropathic pain [15] and also stimulates neuroprotection.

Recently, tirzepatide was approved for the treatment of type 2 diabetes, obesity, and moderate-to-severe obstructive sleep apnea [16]. Tirzepatide is named twincretin, indicating its synergistic activity on the incretin system. Tirzepatide binds to the glucose-dependent insulinotropic polypeptide (GIP) receptor with an affinity equal to that of native GIP. In contrast, its binding with the glucagon-like peptide-1 (GLP-1) receptor is ~5-fold weaker than that of native GLP-1 [17]. Co-stimulation of GIP and GLP-1 receptors in neurons suggests synergistic pro-proliferative neuronal progenitor activity, particularly in relation to memory and appetite [18]. GLP-1 and GIP receptors are expressed in sensory, motor, and enteric neurons in both humans and mice, making incretin-mimetic drugs a promising new treatment option for DN [19]. In a high-fat-diet-fed mouse model with low-dose streptozotocin, dual incretin receptor activation demonstrated neuroprotective potential, with reduced oxidative stress, improved memory, and neurogenesis [20]. Neuroprotection was demonstrated in a Parkinson's disease model [21]. Multiple mechanisms have been suggested for tirzepatide's neuroprotective activity, e.g., through the brain-derived neurotrophic factor (BDNF) pathway and by overcoming hyperglycemia and neuronal insulin resistance [22]; however, to our knowledge, its role in systemic inflammation in diabetic neuropathy has never been reported. Studies in rodent models of DN are scarce. We aimed to study the effect of tirzepatide on markers of systemic inflammation in a streptozotocin-induced type 1 diabetes neuropathy model.

2. Materials and Methods

2.1. Chemicals

We obtained streptozotocin, tirzepatide, ketamine, xylazine, and citrate buffer 4.5 from Sigma-Aldrich (Merck, Darmstadt, Germany).

2.2. Animals

2.2.1. Predefinition of the Sample Size

Following the guidelines for testing substances in a model of diabetic neuropathy in rodents [23], we used a 4-group design with $n = 12$ per group. The following group labeling was used: 1-C—non-diabetic controls; 2-D—diabetic controls; 3-L—low-dose tirzepatide-treated diabetic mice; 4-H—high-dose tirzepatide-treated diabetic mice.

2.2.2. Diabetes Induction

Based on previous experience, we anticipated a 10–20% attrition rate during streptozotocin injection, so we ordered 56 male BALB/c mice (10–12 weeks old) from the Vivarium of the Medical University of Sofia. After several days of acclimatization, we assigned animals to groups using the formula = RAND() * 4 in Excel 2016. We converted the continuous output to group labels as follows: 0–1 converted to group 1-C, 1–2 to group 2-D, 2–3 to group 3-L, and more than 3 to group 4-H. A technician unrelated to the experiment performed blinded randomization. At week 0, we checked variations in BWs among groups using one-way ANOVA and found no statistically significant differences between groups.

We housed 6 mice per cage. We bedded the cages with sawdust and cleaned them at least 4 times a week. Rearing conditions met the requirements of EU Directive 2010/63/EU: 24–25 °C, aeration, 50–60% humidity, and a 12/12 light/dark cycle. Standard laboratory chow and fresh water were available ad libitum.

Diabetes was induced by intraperitoneal injection of a single high dose of streptozotocin, 180 mg/kg, within 20 min of dissolution in citrate buffer with pH 4.0–4.5. We replaced the drinking water in the cages for 24 h with a 10% glucose solution to prevent initial hypoglycemia. Control mice received only citrate buffer i.p. Diabetes was confirmed the next week using a glucose test strip on the Wellion CALLA glucometer (MED TRUST GmbH, Ottendorf-Okrilla, Germany) from the tail vein (cut-off 14 mmol/L) [24]. All animals that entered the study had a blood glucose level above the cut-off.

Six streptozotocin-injected mice died during the pretreatment period. They were replaced with mice from the preceding randomization, so all 48 mice underwent the same streptozotocin induction protocol and the same four-week pretreatment period, and were allowed to elapse for 4 weeks to allow neuropathy to develop.

We measured body weight (BW) weekly throughout the experiment.

2.2.3. Tirzepatide Administration

Two doses of tirzepatide were administered: a low dose (5 mcg/kg s.c. every 3rd day), and a high dose (15 mcg/kg s.c. every 3rd day)—Figure 1.

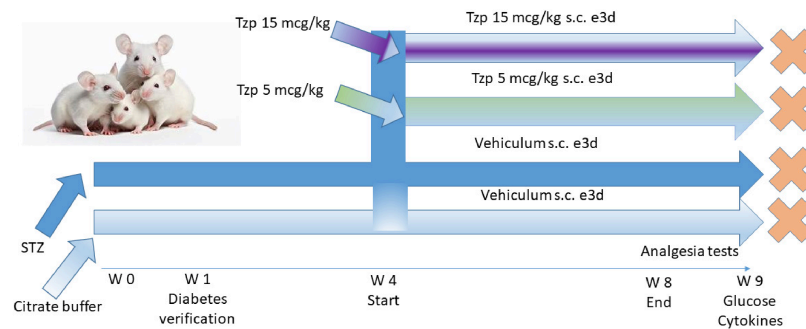


Figure 1. The experimental design. STZ—streptozotocin, Tzp—tirzepatide, W—week. The 4 weeks before treatment initiation began with STZ administration. —end of trial.

2.3. Behavioral Testing

Three days after the last injection, the behavioral experiments began. They began at 10 a.m. and ran for 3 consecutive days. The same experienced scientists, blinded to treatment allocation, conducted the experiments and used animals in random order. We used the average of three measurements for further statistical analysis.

2.3.1. Grip Strength Test

We assessed neuromuscular function using a grip strength meter (product code 47200, Ugo Basile, Gemonio (VA), Italy) as follows. We placed the animal's front legs on a horizontal lever and pulled its tail; the device recorded the maximum force exerted on the lever, determining the maximum grip force. Measurements included strength (g), peak strength (s), and duration (s).

2.3.2. Cold Plate Test

We tested thermal sensitivity with the cold plate test (product code 35300, Ugo Basile, Gemonio (VA), Italy) at 6 °C. We recorded the time to the appearance of shaking or to aversive behavior (licking, paw withdrawal) in seconds. We removed mice from the plate no later than the 60th second if no antinociceptive behavior was observed.

2.3.3. Von Frey Test

Tactile sensitivity was assessed by a Dynamic Plantar Aesthesiometer (product code 37550, Ugo Basile, Gemonio (VA), Italy). Mice were placed in individual cages of the apparatus for half an hour to habituate. During the test, a needle (monofilament) was mechanically passed through the mesh floor of those cages, applying different forces to the hind paw surface. Paw withdrawal was measured in response to different forces (g) and stimulus durations (s).

2.4. Biochemistry Testing

Mice were anesthetized with ketamine/xylazine (100/10 mg/kg). Blood was drawn with cardiopuncture in Vacutainer tubes with EDTA. We measured whole-blood glucose using the test described above. We then centrifuged samples at 2000 rpm for 15 min at 4 °C and stored the supernatant at −80 °C until evaluation.

We measured blood cytokines IFN- γ , IL-2, IL-6, and TNF- α using the Ella analyzer and the SimplePlex[®] platform (Ella, Bio-Techne, Minneapolis, MN, USA) with SimplePlex Explorer 4.1.0.22, which enables automatic processing, calibration, and visualization of results with high accuracy and reproducibility.

To capture the synergistic effect of two key proinflammatory mediators, we constructed an exploratory composite inflammatory index (ECII) as the average raw product ($\text{TNF-}\alpha \times \text{IL-6}$).

2.5. Statistics

We checked data for normality and equality of variances before using parametric tests. Otherwise, nonparametric testing was used. To analyze BW changes during the experiment, we used a mixed ANOVA with group (4 levels) as a between-subjects factor and time (9 levels) as a within-subjects factor, with Bonferroni correction. We applied the Greenhouse–Geisser correction for sphericity. The effect size was assessed by partial eta-squared $\eta^2p = \text{SS-effect}/(\text{SS-effect} + \text{SS-error})$. Interpretation: small effect < 0.01 ; medium effect—0.06 to 0.14; large effect > 0.14 .

One-way ANOVA with a post hoc Tukey–Kramer test for all pairwise comparisons, or a Kruskal–Wallis test with a post hoc Dunn test, was used to compare the 4 groups. We assessed the effect size in the Kruskal–Wallis test as $\epsilon^2 = H/(n - 1)$. The interpretation is the same as above.

We accepted $p < 0.05$ as statistically significant. We analyzed the data with MedCalc software (MedCalc software Ltd., Ostend, Belgium) v.23.3.7.

3. Results

3.1. Metabolic Effects

Analysis of BW changes during the experiment revealed a significant group $\times$ time interaction, $F(9.97, 146.28) = 2.44$, $p = 0.01$, $\eta^2p = 0.06$ (medium effect). Simple effects analysis showed that the outcome changed significantly over time in the control group (1-C), but not in the diabetic groups (2-D, 3-L, and 4-H). Between-group comparisons at each time point (Bonferroni-corrected, $\alpha = 0.0056$), however, revealed no significant differences among groups (Figure 2A).

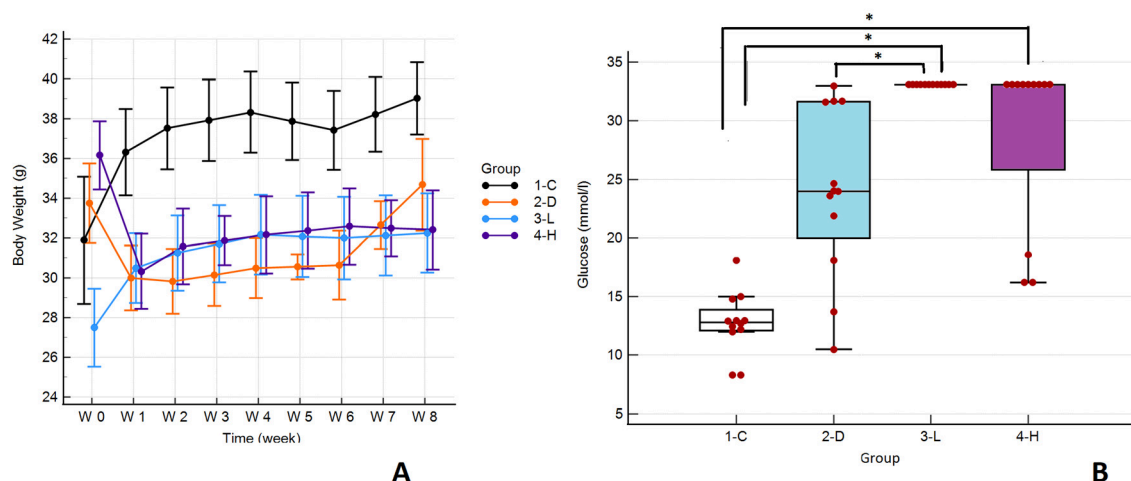


Figure 2. (A). BW changed significantly over time in the control group (1-C), but not in the diabetic groups (2-D, 3-L, and 4-H). Mixed ANOVA, pairwise comparisons with Bonferroni correction. Data are expressed as mean values and standard error of mean. (B). Blood glucose at the end of the treatment—box-and-whisker plot. Kruskal–Wallis test with Dunn post hoc analysis. $* p < 0.05$. Data are expressed as median values and interquartile ranges. Red dots represent individual values. 1-C—control; 2-D—diabetes; 3-L—low-dose tirzepatide (5 mcg/kg); 4-H—high-dose tirzepatide (15 mcg/kg). W—week of the treatment.

At the end of the experiment, all three streptozotocin-treated groups had very high blood glucose levels, regardless of tirzepatide dose (H-group, L-group, or D-group)

(Figure 2B). We used the nonparametric Kruskal–Wallis test because pain ratings violated the normality assumption. We found statistically significant differences, $H(3) = 36.50$ corrected for ties, $p < 0.000001$, $\epsilon^2 = 0.78$ (a large effect size, indicating that group membership explains 78% of the variance in ranks).

3.2. Neurologic Effects

We assessed diabetic neuropathy using three tests: grip strength, the cold plate (a test of cold perception), and the von Frey test (for mechanical sensitivity).

3.2.1. Grip Strength Test

The treatment significantly affected the animal's muscle strength, $F(3, 47) = 4.05$, $p = 0.013$, $\eta^2 p = 0.17$ (large effect indicating that 17% of the variance in ranks is explained by group membership). Post hoc analysis revealed that the 2-D and 4-H groups had significantly lower muscle strength than the control 1-C group ($p < 0.05$) (Figure 3A).

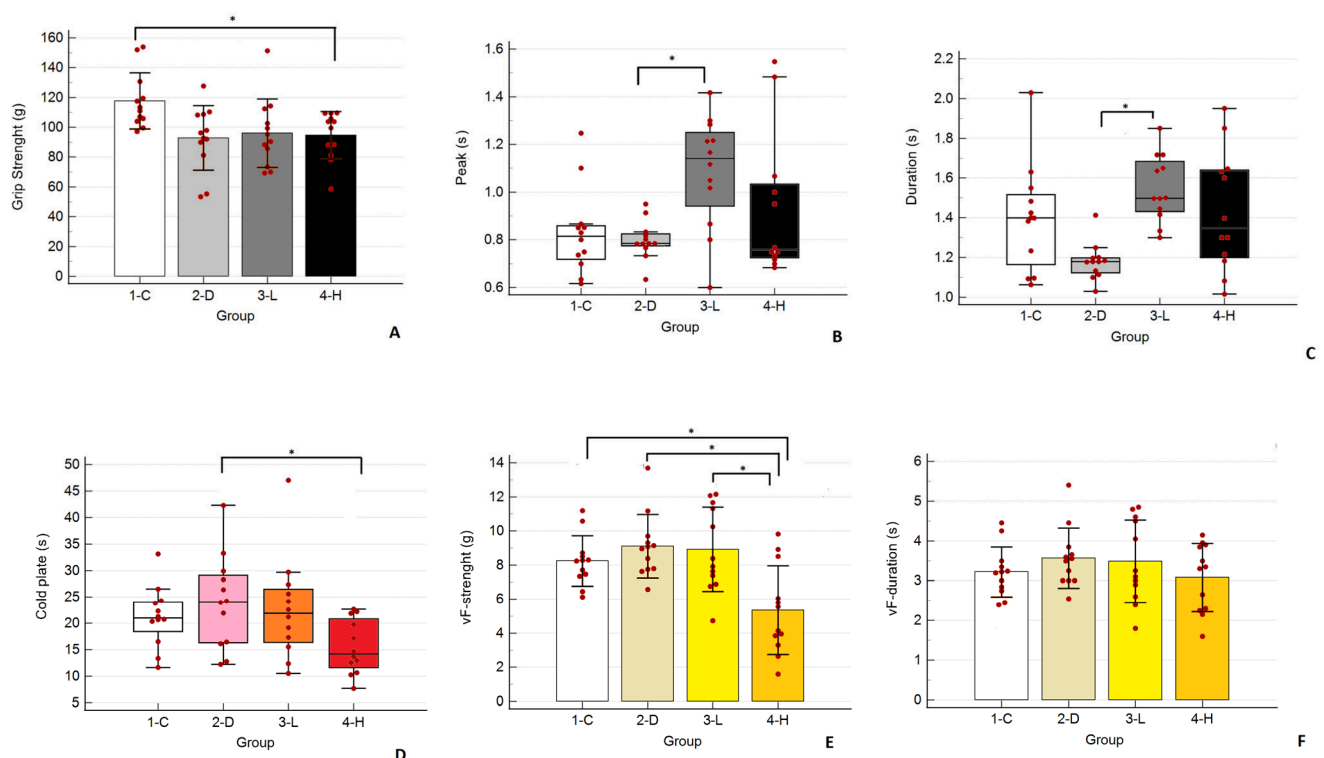


Figure 3. Neurologic effects. (A). Grip strength (g). (B). Peak (s) of the grip strength. (C). Duration of gripping (s). (D). Cold plate test (s). (E). von Frey test stimulus strength (g). (F). von Frey test duration (s). Data from mean values, standard deviations, one-way ANOVA and Tukey–Kramer post hoc—for (A,E,F). Data from median values, interquartile ranges, Kruskal–Wallis test and Dunn post hoc—for panels (B–D). * $p < 0.05$. Red dots represent individual values. 1-C—control; 2-D—diabetes; 3-L—low-dose tirzepatide (5 mcg/kg); 4-H—high-dose tirzepatide (15 mcg/kg).

The peak (s) also differed significantly among groups, with $H(3) = 9.2$; $p = 0.027$; $\epsilon^2 = 0.2$ (large effect). A statistical difference was observed with a higher peak in group 3-L vs. 2-D ($p < 0.05$) (Figure 3B).

Treatment also influenced gripping duration (s) ($H(3) = 15.63$; $p = 0.001$; $\epsilon^2 = 0.33$; a large effect size, indicating that group membership explains 33% of the variance in ranks). The 2-D vs. 3-L group showed a statistically higher value ($p < 0.05$) (Figure 3C).

3.2.2. Cold Plate Test

The cold plate test showed a significant effect of group treatment: $H(3) = 8.32$, $p = 0.04$, $\varepsilon^2 = 0.18$ (large effect). The statistical significance was mainly due to the difference between the 2-D and 4-H groups ($p < 0.05$) (Figure 3D).

3.2.3. Von Frey Test

The strength of the stimulus causing an aversive reaction was influenced by the mouse treatment groups, $F(3, 47) = 36.51$, $p < 0.001$, $\eta^2 p = 0.30$ (a large effect, indicating that 30% of the variation is due to the treatment group). Tukey's test showed a statistically significant lower stimulus-induced behavioral reaction in the 4-H group than in 1-C, 2-D, and 3-L ($p < 0.05$; Figure 3E).

The duration of the von Frey test stimulus (Figure 3F) was similar across all tested mice, regardless of group, $F(3, 47) = 0.90$, $p = 0.45$.

3.3. Cytokine Effects

Treatment group significantly influenced blood TNF- α levels (Figure 4A), $H(3) = 14.83$, $p = 0.002$, $\varepsilon^2 = 0.32$ (large effect). The group receiving a higher dose of tirzepatide (4-H) had statistically significantly lower values than the group with diabetes not treated with tirzepatide (2-D) ($p < 0.05$).

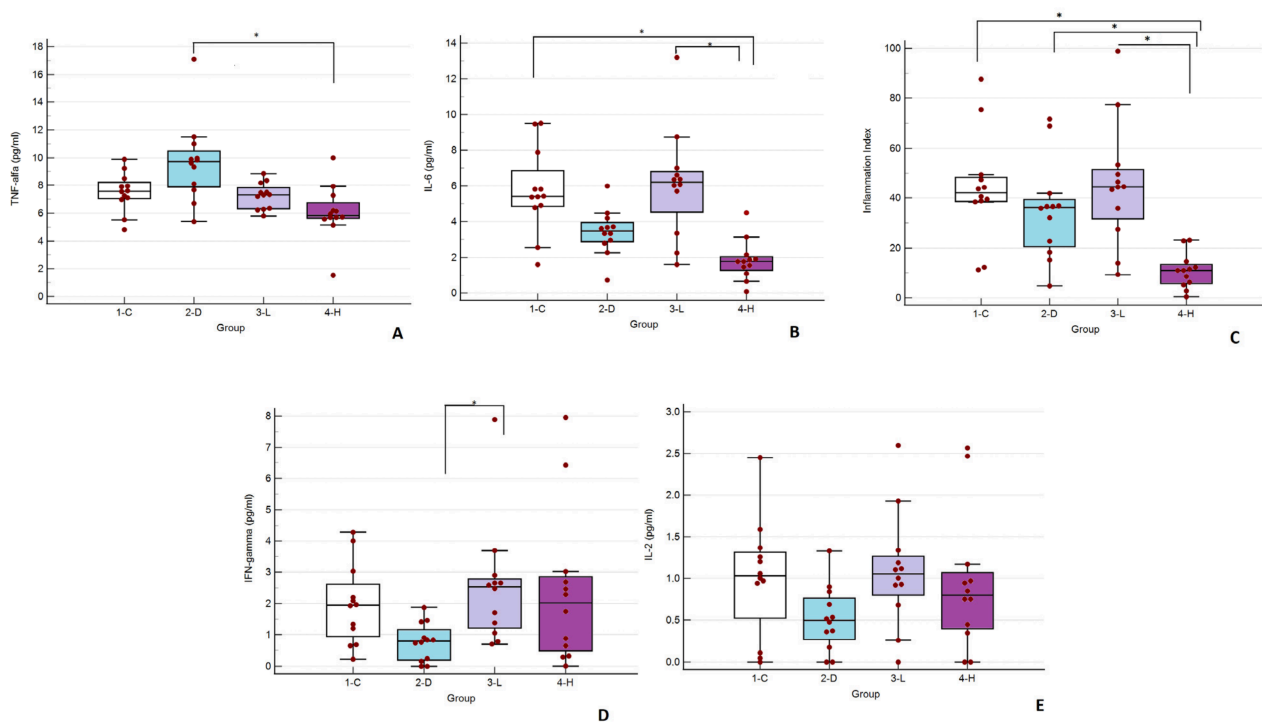


Figure 4. Cytokine effects. (A). Blood level of TNF- α . (B). Blood level of IL-6. (C). ECII—an exploratory composite inflammatory index = TNF- α $\times$ IL-6. (D). Blood level of IFN- γ . (E). Blood level of IL-2. Data from Kruskal–Wallis test and Dunn post hoc analysis. * $p < 0.05$. The IL-2 values did not differ significantly among groups. Data are expressed as median values and interquartile ranges. Red dots represent individual values. 1-C—control; 2-D—diabetes; 3-L—low-dose tirzepatide (5 mcg/kg); 4-H—high-dose tirzepatide (15 mcg/kg).

The blood level of IL-6 (Figure 4B) was also dependent on treatment ($H(3) = 23.20$, $p = 0.00004$, $\varepsilon^2 = 0.60$; large effect). Post hoc analysis revealed that mice treated with the higher dose of tirzepatide (4-H group) had significantly lower values than groups 1-C ($p < 0.05$) and 3-L ($p < 0.05$).

We calculated the inflammation index as the average raw product ($\text{TNF-}\alpha \times \text{IL-6}$) to more precisely characterize systemic inflammation. The treatment group significantly influenced the inflammation index ($H(3) = 20.98$, $p = 0.0001$, $\epsilon^2 = 0.45$; a large effect, indicating that 45% of the variation was due to treatment group affiliation). The 4-H group had a statistically significantly lower inflammation index compared with the 1-C, 2-D, and 3-L groups ($p < 0.05$).

Group treatment influenced IFN- γ blood levels (Figure 4D), $H(3) = 9.72$, $p = 0.02$, $\epsilon^2 = 0.21$ (large effect). The 2-D vs. 3-L group showed a statistically significant difference ($p < 0.05$).

Treatment did not significantly affect IL-2 levels (Figure 4E), $H(3) = 6.98$, $p = 0.07$.

4. Discussion

The main finding of this trial is that tirzepatide substantially reduces systemic inflammatory markers but fails to improve neuropathic functional outcomes in this streptozotocin-induced type 1 diabetes mouse model and in some tests is associated with worse outcomes. We could not find similar publications about testing tirzepatide in a rodent DN model.

4.1. Tirzepatide in Nonobese Type 1 Diabetes Model

To our knowledge, results from testing tirzepatide in murine type 1 diabetes models are lacking. Notably, type 1 diabetes is not an indication for tirzepatide, as its mechanism of action involves insulin storage and secretion from beta cells. However, DN can complicate both type 1 and type 2 diabetes, so we decided to test tirzepatide in a type 1 diabetes model. The American Diabetes Association (ADA) recommends screening for DN in all people living with diabetes starting from diagnosis of type 2 and five years after the diagnosis of type 1 [25]. In off-label use [26] and in some clinical trials [27], tirzepatide successfully reduced body weight and improved carbohydrate metabolism in adults with type 1 diabetes and obesity. Identifying DN-modifying antidiabetic drugs is urgent, so the search focused on incretin mimetics.

Tirzepatide is a synthetic peptide with dual GLP-1R and GIPR agonistic activity with impressive effectiveness regarding glycemic control and body weight reduction. Its molecule consists of 39 amino acid residues with an unsaturated fatty acid conjugated at Lys20, which determines about 99% binding to human plasma albumin and hence a plasma half-life of about 5 days, allowing once-weekly dosing [28]. Binding with murine albumin, however, is weaker. This is a well-known issue with fatty-acid-acylated peptides: the albumin-binding moiety is often optimized for human albumin, so rodent albumin binding is reduced, and the plasma half-life is about 6–7 h [29]. Most chronic-treatment murine obesity studies report a dose of 10 nmol/kg once daily [30] every other day [31], every third day [32,33], or once weekly [32]. However, based on the pharmacokinetic study, we administered the tested doses every 3rd day [29]. The total treatment duration was 4 weeks.

The 10 nmol/kg dose is within the established effective weight-control range for murine models. Other investigators used the minimal effective dose of tirzepatide (0.5 nmol/kg/day) as a positive control to evaluate small-molecule targeting of energy and lipid metabolism to treat obesity and related metabolic disorders [30].

To our knowledge, tirzepatide has not been tested in murine DN, so neither the duration nor the effective dose has been reported. Our study is a feasibility test using two doses above the minimal effective dose for obese mice.

Our model consisted of nonobese male mice with glycemia confirmed at weeks 1 and 9. We demonstrated no statistically significant differences among groups. Tirzepatide did not reduce glycemia or the BW of male mice with type 1 diabetes.

4.2. Tirzepatide Failed to Improve Neuropathic Symptomatology in a Nonobese Type 1 Diabetes Model

Here, we report significantly decreased grip strength and increased sensitivity to cold and mechanical stimuli (allodynia) in a mouse model of nonobese streptozotocin-induced type 1 diabetes treated with tirzepatide. A recent meta-analysis of tirzepatide's neuroprotective potential included five in vitro reports; nine in vivo reports in models of Parkinsonism, Alzheimer-type transgenic models, metabolic-cognitive impairment, diabetes-related cognitive deficit, and drug-induced neurotoxicity; and three retrospective propensity score-matched cohort studies using the TriNetX database. Overall, tirzepatide showed biologically plausible neuroprotective mechanisms preclinically, requiring clinical trial confirmation [33]. Data from preclinical tirzepatide studies informed an ongoing clinical trial of tirzepatide in DN (e.g., NCT07706088); however, one report noted an increased incidence of DN with tirzepatide, attributed to rapid HbA1c reduction, with an unknown mechanism [34]. Peripheral neuropathy is listed as a rare adverse event of tirzepatide in the US FDA adverse event reporting system (FAERS) [35] and post-marketing reports [36]. This indicates that further mechanistic and outcome trials are needed.

Our negative results on tirzepatide in DN better align with those obtained in a cisplatin-induced murine neuropathy model [37]. We cannot exclude an additive or synergistic effect of the tirzepatide-streptozotocin combination, which warrants further investigation. Our results must be further confirmed or rejected in other non-streptozotocin-based models.

One can consider either a direct or indirect effect of tirzepatide. Direct tirzepatide-specific peripheral nerve studies remain very limited, despite identification of the peripheral nerve localization of GIP/GLP-1 receptors. The indirect tirzepatide effect may result from a rapid change in BW or glycemia. For example, peroneal nerve palsy was attributed to rapid weight loss and an extremely rapid decrease in glycemia causes the so-called treatment-induced neuropathy of diabetes (TIND) [38]. In our experiment, we did not observe excessively rapid body weight loss. However, we cannot exclude a drop in glycemia, as we did not measure blood glucose continuously.

Regarding glycemia, we note that at the end of treatment, we measured very high glucose levels in all streptozotocin-treated groups. However, signs and symptoms of neuropathy were recorded mainly in the 4-H group treated with higher doses of tirzepatide, so we cannot exclude a synergistic or additive effect of the glucose and tirzepatide, interfering with DN. Further experiments are needed to elucidate the underlying mechanisms.

4.3. Tirzepatide Decreases Systemic Inflammation in a Nonobese Type 1 Diabetes Model

Here, we report a substantial reduction in systemic inflammation, consistent with observations in obese mouse models [32,33]. In our study, we assessed TNF- α , IL-6, IFN- γ , and IL-2 in a murine model of DN and the effects of tirzepatide treatment.

TNF- α is a proinflammatory cytokine often elevated in insulin resistance. However, the effects of GLP-1 receptor agonists, including tirzepatide, on blood TNF- α were inconsistent (it either decreased or increased), according to a recent meta-analysis [39]. Here, we report a significant suppression of high-dose tirzepatide (4-H) vs. the diabetic group (2-D).

IL-6 is a pleiotropic cytokine involved in systemic inflammation. High blood levels of IL-6 were reported both in type 2 diabetes and in young patients with type 1 diabetes. A recently published meta-analysis reported a significant positive association between higher IL-6 levels and DN in both painless patients and those with pain [40]. Our study also aligns with another meta-analysis showing a significant reduction in systemic inflammation across different tirzepatide populations and treatment regimens [41].

In diabetic mice, TNF- α and IL-6 regulate each other to drive key features of diabetic complications, particularly endothelial dysfunction and insulin resistance via increased

oxidative stress. In type 2 diabetic (db/db) mouse hearts, studies have shown a reciprocal regulation between the two cytokines: genetic deletion of TNF- α reduced IL-6 expression, and neutralizing IL-6 suppressed TNF- α expression [42]. To better characterize inflammation, we introduced an exploratory composite inflammatory index (ECII) derived from the average raw product of TNF- α and IL-6. This captures their synergistic interaction. This approach was informed by Varadhan et al., who demonstrated that a simple additive index of IL-6 and a soluble TNF- α receptor were the strongest predictors of mortality among 15 inflammatory markers [43]. Although ECII is not validated in either rodent models of diabetes or in humans, researchers have attempted to demonstrate the predictive value of an index composed of TNF- α and IL-6 in humans [44]. Our finding that the composite index, rather than the individual cytokines, showed a significant treatment effect as compared with controls (group 1-C) and the diabetes group (2-D) underscores the advantage of using aggregate measures. This approach is consistent with other works demonstrating the risk-predictive value of other simple composite indices for diabetes complications [45,46].

IFN- γ is another pleiotropic cytokine that contributes to islet cell inflammation and control of T-cell proliferation [47]. Here, we report a statistically significant increase in blood IFN- γ with tirzepatide treatment (the 2-D group had the lowest levels among all groups).

IL-2 is a classical anti-inflammatory cytokine that favors regulatory T-cell proliferation and is involved in the onset and progression of type 1 diabetes; it also promotes beta-cell proliferation [48]. In our study, we could not find a statistical difference between diabetic and control groups or under treatment with tirzepatide.

A new chronic inflammation- and immune dysregulation-centered paradigm has recently challenged the classical paradigm that hyperglycemia drives diabetes complications [49]. Both type 1 and type 2 diabetes are characterized by local pancreatic islet inflammation, while systemic low-grade inflammation is more consistently reported in relation to obesity and type 2 diabetes [50]. Type 1 and type 2 diabetes seem to display different systemic inflammation trajectories; however, the progression of complications parallels the rise in inflammatory cytokines in blood in both cases [51].

Recently, a proinflammatory subpopulation, named “metabolically activated macrophages,” was discovered to induce a different proinflammatory phenotype in a medium with high concentrations of insulin, glucose, and palmitate [52]. Its primary niche is the adipose tissue, where it promotes the clearance of dead adipocytes [53].

In our trial, the beneficial decrease in systemic inflammation under tirzepatide treatment seemed unrelated to neuropathy. Recent findings show a complex effect of tirzepatide on adipose inflammation, suppressing classically rather than metabolically activated macrophages [31]. The putative involvement of metabolically activated macrophages in systemic inflammation and tirzepatide’s effect on this macrophage subpopulation need further confirmation.

4.4. Limitations

This study has several limitations. 1. Neuropathy was diagnosed by analyzing three behavioral tests. These tests are well validated and widely accepted in animal testing; however, further investigations are needed to identify specific molecular mechanisms in peripheral nerves and determine how tirzepatide influences neuropathy. 2. The behavioral tests were performed only after treatment was completed. Therefore, it is not possible to directly demonstrate that the diabetic groups had comparable functional neuropathy at the start of tirzepatide treatment. This does not invalidate the final between-group findings, but it limits the strength of statements suggesting that tirzepatide worsened pre-existing neuropathy. 3. We checked glucose levels twice, at weeks 1 and 9. Although we did not expect diabetes to resolve at week 4, we cannot determine whether the diabetic groups

had comparable glycemic status at treatment initiation. 4. The randomization was done in Excel, which is not validated for animal trials.

5. Conclusions

Tirzepatide failed to improve DN behavioral symptoms in a model of nonobese streptozotocin-induced type 1 diabetes. Although it substantially reduces systemic inflammation in diabetes, this effect appears unrelated to neuropathic complications. Based on existing in vitro and in vivo evidence, we cannot rule out positive DN outcomes of tirzepatide in type 2 diabetes and obesity. Tirzepatide's potential protection against DN would be better tested in non-streptozotocin-based animal models.

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Abbreviations

The following abbreviations are used in this manuscript:

ADA	American Diabetes Association
ANOVA	Analysis of Variance
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
DN	Diabetic Neuropathy
FAERS	FDA Adverse Events Reporting System
FDA	Food and Drug Administration
GIP	Glucose-Dependent Insulinotropic Polypeptide
GLP-1	Glucagon-Like Peptide 1
IFN- γ	Interferon- γ
PNS	Peripheral Nerve Society
TIND	Treatment-Induced Neuropathy of Diabetes
TNF- α	Tumor Necrosis Factor- α

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