Original Article

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Pharmacology Section

Pharmacological Evaluation of Metformin, Dapagliflozin alone and in Combination, in a Wistar Rat Model of Diabetic Neuropathy: An Experimental Study

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ABSTRACT

Introduction: Diabetic Neuropathy (DN) is a common and debilitating complication of Diabetes Mellitus (DM), involving the sensory, motor, and autonomic nervous systems, with limited disease-modifying treatment options. Emerging evidence suggests that commonly used antidiabetic agents may exert additional neuroprotective effects beyond glycaemic control.

Aim: To evaluate the individual and combined effects of metformin and dapagliflozin on the Wistar rat model of DN.

Materials and Methods: This experimental study was conducted in the Department of Pharmacology, KAHER's Jawaharlal Nehru Medical College, Belagavi, Karnataka, India from January 2022 to December 2022. Thirty-two male Wistar rats were used, of which six served as normal controls. Diabetes was induced in the remaining animals by a single intraperitoneal injection of Streptozotocin (STZ) (60 mg/kg). After confirmation of diabetes (fasting blood glucose >250 mg/dL), rats were randomly allocated to Diabetic Control (DC), metformin (180 mg/kg), dapagliflozin (0.9 mg/kg), or combination treatment groups and treated orally for eight weeks. Sensory neuropathy was assessed by measuring the Paw Withdrawal Latency (PWL) using Eddy's hot plate at 2, 4, 6, and 8 weeks. At the end of eight weeks, motor neuropathy was evaluated by measuring Motor Nerve Conduction Velocity (MNCV) of the sciatic nerve, and autonomic dysfunction was assessed by measuring gastrointestinal transit using the charcoal meal method. Histopathological changes in the sciatic nerve were examined for perineural inflammation using Haematoxylin and Eosin (H&E). The quantitative data were expressed as Mean±SEM and analysed using ANOVA followed by Tukey's post-hoc test, and p-value <0.05 was considered significant.

Results: A total of 32 rats were included (n=6 each in normal control and treatment groups; n=8 in DC). At eight weeks, DC rats exhibited significant neuropathic alterations compared with normal controls, including prolonged PWL (8.33±0.25 vs 3.58±0.33 s; p-value=0.0000011), reduced MNCV (11.48±1.24 vs 56.67±3.33 m/s; p-value=0.0001), and decreased intestinal transit (49.25±4.74% vs 85.69±0.97%; p-value=0.0000011). Metformin and dapagliflozin significantly improved thermal latency (5.42±0.24 s; p-value=0.000119 and 5.58±0.15 s; p-value=0.0000022), nerve conduction velocity (28.52±4.03 m/s; p-value=0.0004 and 27.67±2.49 m/s; p-value=0.0007), and intestinal transit (74.66±3.79%; p-value=0.000245 and 69.85±3.10%; p-value=0.01347) compared with DC. Combination therapy further improved PWL (5.17±0.21 s), MNCV (34.00±5.89 m/s), and intestinal transit (77.44±2.08%). Sciatic nerve inflammation score was significantly elevated in DC (2.50±0.22) and reduced with metformin (0.33±0.21), dapagliflozin (0.33±0.21), and combination therapy (0.00±0.00).

Conclusion: Metformin and dapagliflozin, alone and more effectively in combination, significantly ameliorated sensory, motor, and autonomic neuropathy in STZ-induced diabetic rats, which highlights the potential of combination antidiabetic therapy for DN, warranting further mechanistic and translational studies.

Keywords: Autonomic neuropathy, Motor nerve conduction, Sensory neuropathy

INTRODUCTION

The DN is among the most prevalent and disabling complications of DM, affecting up to 50% of diabetic individuals and contributing substantially to chronic pain, functional impairment, and diminished quality of life [1]. DN encompasses a spectrum of disorders involving both somatic and autonomic nervous systems and is commonly classified into sensory, motor, and autonomic neuropathies. Sensory neuropathy manifests as numbness, paresthesia, and neuropathic pain in the distal extremities. Motor neuropathy leads to muscle weakness and atrophy, and autonomic neuropathy affects gastrointestinal, cardiovascular, and genitourinary systems, resulting in significant systemic morbidity [2].

The development of DN is driven by chronic hyperglycaemia-induced metabolic and vascular disturbances, which promote oxidative stress, mitochondrial dysfunction, inflammation, and accumulation of Advanced Glycation End-products (AGEs), ultimately leading to progressive neuronal degeneration and microvascular damage [3]. Despite advances in glycaemic management, current therapeutic strategies remain largely symptomatic, offering limited protection against nerve degeneration and failing to modify the underlying disease course [4]. This highlights a substantial therapeutic gap, emphasising the need for antidiabetic agents that deliver dual metabolic control and disease-modifying neuroprotective benefits.

Metformin, the cornerstone first-line antihyperglycaemic therapy, has recently attracted attention for its potential neuroprotective properties beyond glucose-lowering. In addition to improving insulin sensitivity and suppressing hepatic gluconeogenesis, metformin exerts antioxidant, anti-inflammatory, and neuroprotective actions, primarily through activation of AMP-Activated Protein Kinase (AMPK) and subsequent attenuation of oxidative stress [5,6]. In diabetic rodent models, metformin has been shown to restore nociceptive thresholds, improve sensory and motor nerve conduction velocities, preserve axonal integrity, and reduce oxidative stress–mediated neuronal damage [7,8]. Further evidence suggests that metformin downregulates proinflammatory cytokines such as TNF-α and IL-6, inhibits NF-κB signalling [9],

and ameliorates the morphological changes in the sciatic nerve by downregulating autophagy via p-AMPK upregulation [10]. However, despite these promising findings, most existing studies have evaluated metformin in isolated neuropathic dimensions, [7,8] and there remains a notable scarcity of studies employing a single integrative model of sensory, motor, and autonomic dysfunction, a critical limitation in translational relevance.

Likewise, dapagliflozin, another drug commonly used in diabetes management, functions as a selective SGLT2 inhibitor that lowers plasma glucose through insulin-independent enhancement of urinary glucose excretion. In addition to glycaemic control, dapagliflozin has demonstrated antioxidant, anti-inflammatory, and endothelial protective effects [11,12]. Findings from a limited preclinical study have reported that dapagliflozin may attenuate diabetes-induced neural injury and improve peripheral nerve function through reductions in oxidative stress and inflammatory burden [12]. Most existing studies have primarily examined general neural protection or broad biochemical improvements, such as reductions in oxidative stress markers or inflammatory mediators, without providing detailed functional or structural assessments [13,14]. The data on newer antidiabetic agents such as SGLT2 inhibitors remain insufficient and inconclusive [12], emphasising the need for focused preclinical investigation to evaluate their specific neuroprotective potential. Against this backdrop, a notable gap exists in the literature on the combined effects of metformin and dapagliflozin on sensory, motor, and autonomic neuropathy in a single preclinical diabetic rodent model.

The present study aimed to evaluate the individual and combined effects of metformin and dapagliflozin in a single rat model of DN, including sensory, motor, and autonomic components.

MATERIALS AND METHODS

The present experimental study was conducted in the Department of Pharmacology, KAHER's Jawaharlal Nehru Medical College, Belagavi, Karnataka, India from January to December 2022. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) vide approval letter 627/PO/Re/S/02/CPCSEA.

The minimum number of animals required to obtain statistically significant results in preclinical studies was five per group [15]. Accordingly, six animals were included in the normal control and all treatment groups. Considering the expected increase in mortality, the sample size in the DC groups was increased to eight.

Inclusion criteria: Healthy male Wistar rats weighing 200±20 g, animals with confirmed diabetes, defined as fasting blood glucose >250 mg/dL after STZ induction, were included in the study.

Exclusion criteria: Animals not meeting the specified weight range or those with fasting blood glucose <250 mg/dL following STZ administration were excluded from the study.

Materials: Streptozotocin (Cayman Chemical, Catalogue No. 13104) was procured through Everon Lifesciences, New Delhi. Metformin and Dapagliflozin were purchased from the local market.

Animals: A total of 32 healthy adult male Wistar rats (200±20 g), procured from the Central Animal House, KAHER's Jawaharlal Nehru Medical College, Belagavi, were used in the study, with a minimum of six animals allocated per experimental group. Eight animals were included in the DC group to account for anticipated diabetes-related mortality. All animals were housed in polypropylene cages under controlled environmental conditions with a temperature of 22±2°C, relative humidity of 50-60%, and a 12-hour light/dark cycle.

Animals were acclimatised for 10 days prior to experimentation and were provided a standard laboratory pellet diet (*Amrut* laboratory animal chow, a cereal and plant protein based rodent diet containing approximately 18-20% protein and 4-6% fat, fortified with essential vitamins and minerals) and water ad libitum, consistent with the composition of standard laboratory chow diets used in experimental study [16]. Routine daily observation was performed to ensure normal activity and absence of overt illness. To minimise observer and analytical bias, the investigators involved in outcome assessment and data analysis were blinded to treatment allocation, while treatment administration was performed by a separate investigator.

Induction and confirmation of diabetes: Six rats were assigned to the normal control group received citrate buffer alone. Diabetes was induced in the remaining rats by a single intraperitoneal injection of STZ (60 mg/kg) in cold citrate buffer (pH 4.5). To prevent hypoglycaemic mortality, 5% glucose was provided for 24 hours post-injection. The STZ dose was selected based on established DN models [17,18].

After 72 hours of STZ administration, FBG was measured from tail-vein blood using a glucometer. Glucose levels were subsequently monitored once during the treatment period and again at the end of the treatment to assess glycaemic status. Rats with FBG >250 mg/dL were considered diabetic and included for further experimentation. Out of 26 diabetic rats, eight animals were included in diabetic control (DC) group that were treated with vehicle (0.5% carboxymethyl cellulose in distilled water) while, remaining diabetic animals were randomly picked up and allocated to different treatment groups with either metformin, dapagliflozin or combination of both with six animals in each as depicted in [Table/Fig-1]. The same vehicle was used consistently for all groups to ensure uniformity in administration.

Group	Treatment	Dose
Group I (Normal control -NC)	Vehicle	1mL
Group II (Diabetic Control - DC)	Diabetic group treated with vehicle	1mL
Group III (MF)	Diabetic group treated with therapeutic dose of Metformin	180mg/kg of rat
Group IV (DP)	Diabetic group treated with therapeutic dose of Dapagliflozin	0.9 mg/kg of rat
Group V (M+D)	Diabetic group treated with a combination of metformin and dapagliflozin	180 mg/kg of rat of MF +0.9 mg/Kg of rat of DP

[Table/Fig-1]: Treatment protocol.

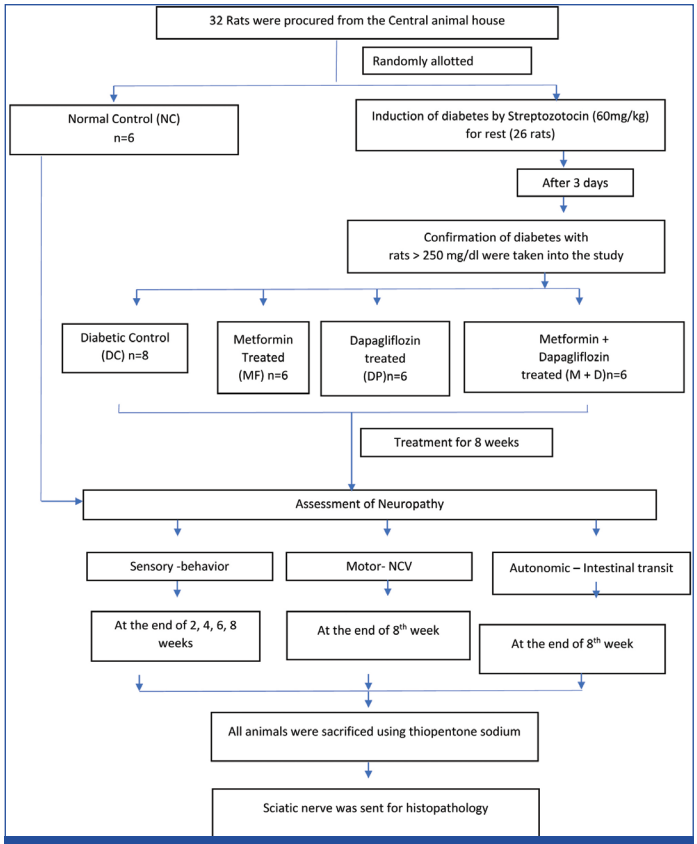
All drugs were administered orally once daily for eight weeks. The doses of metformin and dapagliflozin were derived from the maximum recommended human therapeutic doses and converted to equivalent rat doses using the Paget and Barnes dose conversion method [19], corresponding to human doses of 2 g/day for metformin and 10 mg/day for dapagliflozin [20]. Dosing was calculated according to the initial body weight of the animals and was maintained constant throughout the experimental period without adjustment for weekly body weight variations.

Outcome measures: Body weight and FBG levels were measured at baseline and at the end of four and eight weeks.

Following confirmation of diabetes, all animals underwent a structured evaluation of sensory, motor, autonomic, and histological parameters of DN.

The experimental design and timeline for induction, treatment, and assessment of DN in Wistar rats outline the sequence of diabetes induction, treatment administration, and evaluation at specific time points [Table/Fig-2].

a. **Assessment of sensory neuropathy - Behavioural Assessment:** The effect of treatments on sensory neuropathy was evaluated using the thermal nociception test with Eddy's hot plate maintained at 55±0.5°C. Rats were acclimatised for 30 minutes before testing, then placed individually on the hot plate, and PWL, the time to hind paw withdrawal, flicking, or



[Table/Fig-2]: Experimental design and timeline for induction, treatment, and assessment of Diabetic Neuropathy (DN) in Wistar rats.

licking was recorded, with a cut-off time of 20 seconds imposed to prevent tissue damage [21]. Assessments were performed at 2, 4, 6, and 8 weeks, and PWL among the various treatment groups were compared.

b. **Assessment of motor neuropathy -Motor Nerve Conduction Velocity (MNCV):** At the end of eight weeks, MNCV of the sciatic nerve was measured under thiopentone anaesthesia using a non invasive electrophysiological method as an end-point assessment [22]. After aseptic preparation of the shaved right hind limb, bipolar stimulation electrodes were placed proximally at the sciatic notch and distally at the ankle, with surface recording electrodes over the tibialis anterior. Square-wave pulses were delivered to evoke Compound Muscle Action Potentials (CMAPs), and latencies were recorded using a Biopac MP150 system, USA. MNCV (m/s) was calculated as:

MNCV= Distance between stimulation sites (mm)/Distal latency – Proximal latency (ms)

Although baseline MNCV measurements were not recorded, the observed MNCV values in various treatment groups were compared against the age-matched normal control group, which served as a physiological baseline.

c. **Assessment of autonomic dysfunction:** Autonomic gastrointestinal motility was assessed at the end of eight weeks using the charcoal meal method to evaluate diabetic gastric autonomic dysfunction [23]. Following overnight fasting, rats received 2 mL of 10% activated charcoal in 1% gum acacia by oral gavage. After 30 minutes, animals were euthanised, the small intestines were excised, and the distance travelled by charcoal relative to total intestinal length was measured. Gastrointestinal transit was thus assessed as a terminal endpoint parameter and calculated and expressed as a percentage of the total length of the small intestine as:

GI Transit (%) = Distance travelled by charcoal/Total length of small intestine x 100

d. **Histopathological evaluation of sciatic nerve:** At study completion, sciatic nerves were bilaterally dissected during euthanasia with thiopentone overdose, fixed in 10% neutral buffered formalin, processed, and paraffin-embedded. Longitudinal and transverse sections were stained with H&E. Perineural inflammation was assessed using a semiquantitative scoring system under light microscopy by a blinded pathologist and scored as:

No inflammatory cells or oedema;

1. Focal mild oedema and sparse inflammatory infiltrates;
2. Locally extensive moderate oedema and inflammatory infiltrate;
3. Widespread diffuse oedema with marked inflammatory cell infiltration [24].

STATISTICAL ANALYSIS

The quantitative data were expressed as mean±standard error of mean and analysed using statistical software GraphPad Prism Version 8.0. To assess the differences between the groups, One-way ANOVA was performed, followed by Tukey's post-hoc test for multiple comparisons. A p-value of <0.05 was considered significant. Histopathological inflammation of the sciatic nerve was graded uniformly within each group, and data were analysed by the Kruskal-Wallis test.

RESULTS

Effect of Treatments on Body Weight

Body weight changes across experimental groups are depicted in [Table/Fig-3].

Groups	Baseline	End of 4th week	End of 8th week
Normal control	197.83±2.09	257.67±2.43	295.83±3.54
Diabetic Control (DC)	195.63±2.09	185.75±1.98 *p=0.000001	176.13±2.79 *p=0.000001
Metformin	200.83±2.46	241.67±3.04 *p=0.000001 #p=0.000001 @ p=0.087	258.17±2.90 *p=0.000001 #p=0.000001 @ (p=0.064)
Dapagliflozin	196.83±2.64	226.33±3.41 *p=0.000001 #p=0.000001 $ p=0.087)	252.83±2.97 *p=0.000001 #p=0.000001 $ p=0.064
Dapagliflozin+ Metformin	201.83±1.49	242.50±2.09 *p=0.000001 #p=0.000001 @p=0.0018	284.67±2.71 *p=0.000001 #p=0.000001 @p<0.001 $p<0.001

[Table/Fig-3]: Effect on body weight (in grams).
Values are expressed as mean±SEM. Data analysed using One-way ANOVA followed by Tukey's post-hoc test. *indicates comparison with normal control group; #indicates comparison with Diabetic Control (DC) group; $indicates comparison with Metformin group; @indicates comparison with Dapagliflozin group

At baseline, body weight did not differ significantly among groups (p-value=0.9645).

At the end of the 4th week, One-way ANOVA revealed a significant difference among groups (p-value <0.0001). DC rats showed a marked reduction in body weight (185.75±1.98 g) compared with normal controls (257.67±2.43 g; p-value=0.000001). Metformin (241.67±3.04 g), dapagliflozin (226.33±3.41 g), and combination therapy (242.50±2.09 g) significantly improved body weight compared with DC (all p-value=0.000001). Combination therapy produced greater improvement than dapagliflozin (p-value=0.0018), while the difference between metformin and dapagliflozin was not significant (p-value= 0.087).

At the end of the 8th week, ANOVA remained significant (p-value <0.0001). DC animals exhibited further weight reduction (176.13±2.79 g) compared with normal controls (295.83±3.54 g; p-value=0.000001). Metformin (258.17±2.90 g), dapagliflozin (252.83±2.97 g), and combination therapy (284.67±2.71 g) significantly restored body weight compared with DC (all p-value=0.000001). Combination

therapy showed superior improvement compared with dapagliflozin (p-value <0.001), whereas the difference between metformin and dapagliflozin remained non-significant (p-value=0.064).

Effect of Treatments on Fasting Blood Glucose (FBG)

Fasting blood glucose levels at different time points are shown in [Table/Fig-4].

Groups	Baseline	After 72 hours	End of 4th week	End of 8th week
Normal control	92.33±1.82	91.16±1.30	92.50±1.34	95.33±1.94
Diabetic control (DC)	91.12±1.82	304.63±4.10 *p=0.000001	376.38±4.69 *p=0.000001	389.75±3.16 *p=0.000001
Metformin	94.17±1.33	304.50±4.86 *p=0.000001 #p=1.000 (ns)	287.67±5.36 *p=0.000001 #p=0.000001	232.50±7.86 *p=0.000001 #p=0.000001
Dapagliflozin	91.67±1.82	309.33±3.78 *p=0.000001 #p=0.8840 (ns)	278.50±2.36 *p=0.000001 #p=0.000001	218.50±2.75 *p=0.000001 #p=0.000001
Metformin + Dapagliflozin	92.33±2.40	311.50±4.19 *p=0.000001 #p=0.6616 (ns)	254.67±6.09 *p=0.000001 #p=0.000001 @p=0.0087 $p=0.0002	194.33±4.42 *p=0.000001 #p=0.000001 @p=0.0067 $p=0.000001

[Table/Fig-4]: Effect on FBG (mg/dL) at various time intervals.
Values are expressed as mean±SEM. Data analysed using One-way ANOVA followed by Tukey's post-hoc test. *indicates comparison with Normal Control group; #indicates comparison with Diabetic Control (DC) group; $indicates comparison with Metformin group; @indicates comparison with Dapagliflozin group; ns indicates non-significant

At baseline, FBG levels were comparable among all groups (p-value=0.9827).

Seventy-two hours after STZ administration, DC rats showed a significant rise in FBG compared with normal controls (p-value <0.0001). At this time point, metformin, dapagliflozin, and combination groups did not differ significantly from DCs (p-value=1.000, p-value=0.8840, and p-value=0.6616, respectively).

At the end of the 4th week, FBG remained significantly elevated in DC rats versus normal controls (p-value <0.0001). All treatment groups demonstrated significant reductions compared with DC (p-value <0.0001). Combination therapy showed greater reduction than metformin (p-value=≤0.001) and dapagliflozin (p-value=0.0087).

At the end of the 8th week, DC rats continued to exhibit markedly elevated FBG compared with normal controls (p-value <0.0001). Metformin, dapagliflozin, and combination therapy significantly reduced FBG compared with DC (p-value <0.0001). Combination therapy demonstrated superior glycaemic control compared with metformin (p-value <0.0001) and dapagliflozin (p-value=0.0067).

Assessment of Sensory Neuropathy

Sensory nerve function was assessed at 2, 4, 6, and 8 weeks using the hot plate latency test. One-way analysis of variance (ANOVA) revealed a significant overall difference in PWL among the experimental groups at all evaluated time points. At two weeks, ANOVA demonstrated a significant group effect (p-value=0.0207). Similarly, highly significant differences were observed at four weeks (p-value=0.0000011), six weeks (p-value=0.0000011), and eight weeks (p-value=0.0000011). Subsequent Tukey's post-hoc analysis showed that treatment with metformin, dapagliflozin, and their combination significantly improved sensory response compared with the DC group at the corresponding time points (p-value <0.05; [Table/Fig-5]).

At two weeks, all the treatments demonstrated a significant reduction in latency (p-value <0.05) as compared with DC; the latencies were comparable among the various treatment groups.

Significant reductions (p-value <0.05) were observed in the metformin (4.08±0.20 s), dapagliflozin (4.58±0.15s), and combination groups (4.33±0.17 s) as compared with DC while, the latencies were comparable among the treatment groups.

Time point	2 weeks	4 weeks	6 weeks	8 weeks
Normal control	3.58±0.30	3.42±0.24	3.50±0.22	3.58±0.33
Diabetic Control (DC)	4.67±0.25 *p=0.0207	6.25±0.17 *p=0.0000011	7.00±0.22 *p=0.0000011	8.33±0.25 *p=0.0000011
Metformin	4.00±0.13 *p=0.0207 #p=0.2079 ns	4.08±0.20 *p=0.0000011 #p=0.000245	4.83±0.28 *p=0.0000011 #p=0.000119	5.42±0.24 *p=0.0000011 #p=0.000119
Dapagliflozin	4.25±0.17 *p=0.0207 #p=0.5027ns	4.58±0.15 *p=0.0000011 #p=0.01347	5.42±0.15 *p=0.0000011 #p= 0.0000022	5.58±0.15 *p=0.0000011 #p=0.0000022
Dapagliflozin + Metformin	4.00±0.18 *p=0.0207 #p=0.2079ns	4.33±0.17 *p=0.0000011 #p=0.000029	4.83±0.17 *p=0.0000011 #p=0.000119 @p=0.000119	5.17±0.21 *p=0.0000011 #p=0.000029 @p= 0.000029

[Table/Fig-5]: Effect on thermal hyperalgesia (in seconds) at different time intervals.
Values are expressed as mean±SEM. Data analysed using One-way ANOVA followed by Tukey's post-hoc test. *indicates comparison with Normal Control group; #indicates comparison with Diabetic Control (DC) group; $indicates comparison with Metformin group; @indicates comparison with Dapagliflozin group; ns indicates non-significant

At six weeks, the DC group showed progressive increase in latency period (7.00±0.21 s) as compared with NC (3.50±0.22 s; p-value <0.05). Metformin (4.83±0.29 s), dapagliflozin (5.42±0.17 s), and combination therapy (4.83±0.1 7 s) significantly decreased the latency periods (p-value <0.05) as compared with DC; these values were comparable among the treatment groups.

At 8 weeks, in comparison to NC (3.58±0.33 s), latency was markedly prolonged (p-value <0.05) in DC rats (8.33±0.25 s). As compared with the DC group, significant improvements (p-value <0.05) were observed in the metformin (5.42±0.24 s), dapagliflozin (5.58±0.15s), and combination groups (5.17±0.21 s). These values of the combination group were comparable to those of metformin but significantly lower as compared with dapagliflozin (p-value <0.05).

Assessment of Motor Neuropathy (MNCV)

At the end of the 8th week, One-way ANOVA revealed a significant overall group difference in MNCV (p-value=0.000001). DC rats exhibited a marked reduction in MNCV (11.48±1.24 m/s) compared with normal controls (56.67±3.33 m/s; p-value=0.0001), confirming the development of DN. Treatment with metformin (28.52±4.03 m/s) and dapagliflozin (27.67±2.49 m/s) significantly improved MNCV compared with DCs (p-value=0.0004 and p-value=0.0007, respectively). Combination therapy (34.00±5.89 m/s) produced a significantly greater improvement compared with DCs (p-value=0.0048), metformin (p-value=0.0071), and dapagliflozin (p-value=0.0063) [Table/Fig-6].

Normal control	Diabetic Control (DC)	Metformin	Dapagliflozin	Dapagliflozin + Metformin
56.67±3.33	11.48±1.24 *p=0.0001	28.52±4.03 *p=0.0001 #p=0.0004	27.67±2.49 *p=0.0001 #p=0.0007 $p=0.9999 ns	34.00±5.89 *p=0.0001 #p=0.0048 @p=0.0063 $p=0.0071

[Table/Fig-6]: Effect on Motor Nerve Conduction Velocity (MNCV) (in m/sec).
Values are expressed as mean±SEM. Data analysed using One-way ANOVA followed by Tukey's post-hoc test. *indicates comparison with Normal Control group; #indicates comparison with Diabetic Control (DC) group; $indicates comparison with Metformin group; @indicates comparison with Dapagliflozin group; ns indicates non-significant

The NC values were considered as baseline readings for subsequent MNCV analysis.

Compared with the DC group, treatment with metformin and dapagliflozin resulted in significant improvement in MNCV (p-value=0.0004 and p-value=0.0007, respectively), with values of 28.52±4.03 m/s and 27.67±2.49 m/s.

Combination therapy (34.00±5.89 m/s) showed a significant improvement compared to the DC (p-value=0.0001), metformin (p-value=0.0048), and dapagliflozin (p-value=0.0063) groups.

There was no significant difference between the metformin and dapagliflozin groups (p-value=0.9999).

Assessment of Autonomic Neuropathy: Effect on Intestinal Transit

Intestinal transit was significantly reduced in the DC group (49.25±4.74) compared with NC (85.69±0.97) (p-value=0.0000011), indicating marked gastrointestinal autonomic dysfunction. Metformin, dapagliflozin and combination therapy exhibited a significant increase (p-value <0.05) in the intestinal transit as compared with DC, with values of 74.66±3.79, 69.85±3.10 and 77.44±2.08, respectively. The combination group exhibited a significant difference (p-value <0.05) as compared with dapagliflozin [Table/Fig-7].

Group	Percentage intestinal transit
Normal control	85.69±0.97
Diabetic Control (DC)	49.25±4.74 *p=0.0000011
Metformin	74.66±3.79 *p=0.0000011 #p=0.000245
Dapagliflozin	69.85±3.10 *p=0.0000011 #p=0.01347 $p=0.9999 ns
Metformin+Dapagliflozin	77.44±2.08 *p=0.0000011 #p=0.000029 @p=0.000029 $p=0.000029

[Table/Fig-7]: Effect on intestinal transit (in percentage).
Values are expressed as mean±SEM. Data analysed using One-way ANOVA followed by Tukey's post-hoc test. *indicates comparison with Normal Control group; #indicates comparison with Diabetic Control (DC) group; $indicates comparison with Metformin group; @indicates comparison with Dapagliflozin group

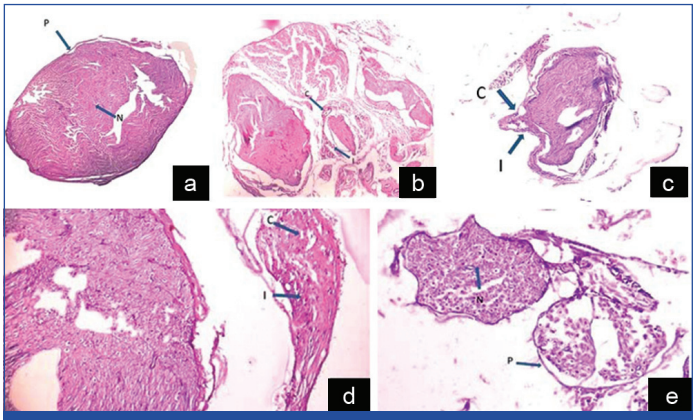
Histopathological Assessment of Sciatic Nerve

Histopathological evaluation of the sciatic nerve was performed using a semiquantitative inflammation scoring system on H&E- stained sections. No inflammatory changes were observed in the normal control group (mean score: 0.00±0.00). The Kruskal-Wallis test showed significant differences in sciatic nerve inflammation among groups. The DC group exhibited markedly higher inflammation scores compared with the normal control group (p-value=0.0001). Treatment with metformin and dapagliflozin significantly reduced inflammation compared with DC (p-value=0.0004 and p-value=0.0007, respectively). Combination therapy also demonstrated a significant reduction (p-value=0.0059) and resulted in complete normalisation of histology. Notably, combination therapy with metformin and dapagliflozin resulted in complete normalisation of sciatic nerve histology, with a mean inflammation score of 0.00±0.00, comparable to the normal control group [Table/Fig-8].

Qualitative histopathological examination further supported these findings with NC demonstrating intact nerve architecture without inflammation, while DC demonstrated pronounced perineural lymphocytic infiltration and capillary congestion. Metformin and dapagliflozin produced partial histological improvement with reduced inflammation, while combination therapy resulted in near-complete restoration of normal sciatic nerve structure [Table/Fig-9a-e].

Normal control	Diabetic Control (DC)	Metformin	Dapagliflozin	Dapagliflozin+ Metformin
0.00±0.00	2.50±0.22 *p=0.0001	0.33±0.21 *p=0.0001 #p=0.0004	0.33±0.21 *p=0.0001 #p=0.0007 $p=1	0.00±0.00 *p=0.0001 #p=0.0059 @p=0.0059 $p=0.0059

[Table/Fig-8]: Effect on histopathological scoring for inflammation in sciatic nerve.
Values are expressed as mean±SEM. Data were analysed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Kruskal–Wallis statistic: H(4)=21.67, p=0.0002. * indicates comparison with Normal Control group; # indicates comparison with DC group; $ indicates comparison with Metformin group; @ indicates comparison with Dapagliflozin group; ns indicates non-significant. p-values were derived using Dunn's multiple comparisons post-hoc test following Kruskal–Wallis analysis (non-parametric), and are not from Tukey's test.



[Table/Fig-9]: Representative photomicrographs of sciatic nerve histology stained with Haematoxylin and Eosin (H&E) showing morphological alterations across experimental groups.
Panel a (10x) depicts a normal nerve with well-preserved nerve fibres (N) and an intact perineurium (P). Panels b (20x) and c (10x) show marked perineural inflammation (I) characterised by lymphocytic infiltration and congested capillaries (C). Panel d (20x) demonstrates reduced perineural inflammation with partial restoration of nerve architecture following treatment. Panel e (20x) shows near-normal sciatic nerve morphology with clearly identifiable nerve fibres (N) and intact perineurium (P), without evidence of inflammatory changes.

DISCUSSION

A single intraperitoneal dose of STZ (60 mg/kg) is a well-established method for inducing persistent hyperglycaemia in rodents, reliably producing metabolic alterations that lead to diabetic peripheral neuropathy via selective pancreatic β-cell destruction through DNA alkylation and oxidative stress. The resulting sustained hyperglycaemia promotes progressive sensory, motor, and structural nerve deficits characteristic of DN [25]. Normal control animals consistently maintained stable sensory, motor, autonomic, and histological parameters throughout the study, confirming the absence of spontaneous neuropathic changes and validating their use as a physiological reference group. The present study demonstrated that STZ-induced diabetes resulted in consistent functional and structural alterations across multiple neuropathic domains, thereby providing a suitable experimental framework to assess pharmacological interventions targeting DN. Accordingly, this model was employed to evaluate the effects of metformin, dapagliflozin, and their combination on sensory, motor, and gastric autonomic neuropathy.

Diabetic sensory neuropathy, a well-recognised consequence of chronic hyperglycaemia, is reliably reproduced in STZ-induced models, which cause progressive degeneration of peripheral nociceptive fibres and reduced pain sensitivity [25]. In the present study, this model was confirmed by the consistently elevated paw withdrawal latencies in thermal hyperalgesia test in DC rats across 2, 4, 6, and 8 weeks, reflecting the characteristic hypoalgesia associated with sensory neuropathy. At two weeks, the DC group showed a significant increase in latency compared with the normal control group (p-value <0.05). Although the metformin, dapagliflozin, and combination groups exhibited lower latency values compared to DC, these differences were not statistically significant (p-value >0.05), and values were comparable among the treatment groups. However, at 4, 6 and 8 weeks, these groups demonstrated reduced PWL compared with DCs, indicating progressive and sustained sensory recovery, with values approaching normal control levels. Combination therapy showed comparable or slightly greater benefits than monotherapy. The findings of the present study are consistent with previous reports evaluating metformin [10] and dapagliflozin [26] on diabetic sensory neuropathy. However, this is the first study to report the effect of combination of both these two drugs.

Diabetic peripheral neuropathy involves significant motor dysfunction, reflected by reduced MNCV due to axonal degeneration, demyelination, and Schwann cell impairment [27]. In this study, the DC group showed a marked decline in MNCV compared with normal

controls, confirming motor neuropathy, consistent with reports linking chronic hyperglycaemia to oxidative stress and microvascular damage [28]. Treatment with metformin, dapagliflozin, and their combination significantly improved MNCV, indicating restoration of motor nerve function. Although baseline MNCV measurements were not performed, as the experimental design prioritised an end-point electrophysiological assessment to minimise repeated anaesthetic exposure and stress-related variability, the inclusion of age-matched normal control values served as a physiological reference; nevertheless, future studies incorporating serial baseline and follow-up MNCV recordings would further strengthen the interpretability and robustness of the findings.

The improvement in MNCV observed in this study was in line with previous reports demonstrating similar neuroprotective effects of metformin [10] and dapagliflozin [26] in diabetic animal models. However, effect of combination therapy with metformin and dapagliflozin has not been reported.

Gastrointestinal dysmotility is a well-recognised but often underreported complication of diabetes, arising from autonomic neuropathy, altered smooth muscle function, and reduced enteric neuronal activity [29]. In agreement with these established mechanisms, the DC group showed a significant reduction in intestinal transit distance compared with NC, reflecting impaired gut motility. This slowing of intestinal transit is typically attributed to hyperglycaemia-induced oxidative stress, decreased nitric oxide availability, and degeneration of enteric neurons that regulate peristalsis [29].

Consistent with the previous studies, DC group demonstrated marked inflammatory and structural changes in the sciatic nerve, as evidenced by elevated histopathological inflammation scores, prominent perineural lymphocytic infiltration, and vascular congestion. These pathological features are characteristic of diabetic peripheral neuropathy and are widely attributed to chronic hyperglycaemia-driven mechanisms, including oxidative stress, inflammatory activation, and microvascular dysfunction [26,28]. Treatment with metformin and dapagliflozin significantly attenuated sciatic nerve inflammation, indicating partial restoration of nerve architecture as evident from improved inflammatory scores and histopathological findings with decreased perineurial inflammation. Notably, combination therapy resulted in near-complete normalisation of histopathological features, with inflammation scores comparable to those of normal control animals providing structural evidence of enhanced neuroprotection.

In the present study, STZ-induced diabetes reproduced marked pathological and functional features of sensory, motor, and autonomic neuropathy. DC rats exhibited significant thermal hyperalgesia, reduced MNCV, impaired intestinal transit and elevated sciatic nerve inflammation scores. These findings are consistent with previous experimental studies demonstrating that STZ-induced diabetes leads to sensory hypoalgesia, reduced nerve conduction velocity, autonomic dysfunction, and structural nerve damage mediated by oxidative stress and inflammatory activation [25,27,29]. Similar mechanisms of hyperglycaemia-induced axonal degeneration and demyelination have been widely reported in experimental models of DN.

The improvements observed following metformin treatment across sensory, motor, and autonomic parameters, including histological findings, may be attributed to its multifaceted actions on metabolic and cellular pathways implicated in DN. Metformin activates AMPK, which enhances antioxidant defences, improves mitochondrial efficiency, and suppresses proinflammatory signalling processes that play a central role in diabetes-induced nerve damage and axonal dysfunction [10,30]. In addition to its effects on peripheral nerves, metformin-mediated improvements in gastrointestinal motility may be related to better glycaemic control, partial restoration of enteric neuronal function, and attenuation of local inflammatory responses within the gastrointestinal tract [31,32]. Collectively, these mechanisms may explain the broad neuroprotective and prokinetic effects of metformin observed in the present study.

Dapagliflozin treatment was associated with improvements across multiple parameters of DN, encompassing sensory, motor, and autonomic function. These effects can be attributed to the reduction in glucotoxicity by virtue of interference with renal reabsorption of glucose by inhibiting SGLT2, thereby attenuating oxidative stress, and improving metabolic homeostasis, hence creating a more favourable milieu for nerve function and repair [33]. Improvements in sensory function and motor nerve conduction may further reflect dapagliflozin's ability to reduce inflammatory signaling and alleviate endoneurial microvascular dysfunction, mechanisms that are central to the progression of DN [33,34]. In addition, the observed enhancement of gastrointestinal transit suggests a beneficial influence on autonomic nerve function, potentially arising from reduced oxidative burden and improved microvascular support of enteric neurons [35]. These findings are supported by previous studies that have reported dapagliflozin to exert significant neuroprotective effects in experimental diabetic peripheral neuropathy by reducing oxidative stress, inflammation, fibrosis and maintaining the structural and functional integrity of peripheral nerves [26,33].

The combination therapy showed a significantly greater improvement in PWL than dapagliflozin monotherapy at later points of time, though the differences compared with metformin were not consistently significant, indicating a partial additive benefit rather than uniform superiority. Although combination treatment demonstrated numerically higher MNCV values than either monotherapy, statistically significant superiority over both individual agents was not observed, suggesting that improved metabolic stabilisation rather than true synergistic neuroprotection may account for the functional gains [34]. Notably, however, combination therapy achieved complete normalisation of histopathological inflammation scores, which were significantly lower than those observed with either monotherapy, providing structural evidence of enhanced neuroprotection. These findings highlight the strong rationale for combining SGLT2 inhibitors with metformin in the management of type 2 DM. Metformin primarily reduces hepatic glucose production and improves insulin sensitivity, while SGLT2 inhibitors lower plasma glucose through insulin-independent urinary glucose excretion. As these agents act via complementary and non-overlapping mechanisms, their combination provides additive glycaemic control with a low risk of hypoglycaemia [35].

Overall, the present study demonstrates that metformin and dapagliflozin administered either alone or in combination ameliorate sensory, motor, autonomic, and histopathological manifestations of DN in a single experimental model. These findings support the rationale for further exploration of combination antidiabetic therapy as a potential adjunctive strategy in the management of DN.

Limitation(s)

While the acute STZ model does not fully replicate the chronic progression of human DN, the consistent functional and structural improvements observed suggest potential translational relevance. Future studies incorporating molecular pathway analysis and chronic models of diabetes are warranted to better elucidate mechanistic interactions.

CONCLUSION(S)

Across all evaluated parameters, DC animals consistently showed significant deterioration compared with normal controls, while pharmacological treatment resulted in substantial recovery toward normal values. The selected doses were based on established human-equivalent dosing strategies and reflect clinically relevant exposure levels. While combination therapy showed clear advantages over monotherapy for glycaemic control, body weight restoration, autonomic function, and histopathological recovery, its benefits for sensory and motor parameters were largely comparable to individual agents, underscoring the importance of outcome-specific interpretation.

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