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Melatonin ameliorates neuropathy in diabetic rats by abating mitochondrial dysfunction and metabolic derangements.

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ABSTRACT

Objectives: To investigate the impact of melatonin *per se* and in combination with gabapentin in a laboratory animal model of experimental diabetes and associated neuropathy in rats.

Methods: Rats from all the groups except the vehicle control group received a single dose of streptozotocin 45 mg/kg, *i.p.* and fructose 10% through drinking water for 8 weeks and subsequently administered with melatonin (25 and 50 mg/kg, *p.o.*) *per se* or along with gabapentin (100 mg/kg, *p.o.*) daily for 4 weeks. The impact of the treatments on blood glucose, serum lipid profile, nociceptive pain parameters, motor nerve conduction velocity was captured and analyzed. Further, the effect on histopathology of liver and sciatic nerve and mRNA expression of peroxisome proliferator-activated receptor-gamma coactivator 1-α (PGC1-α) and Mitochondrial transcription factor A (TFAM) in hepatic tissue were investigated.

Results: Rats administered with melatonin *per se* and in combination with gabapentin revealed significant improvement in glucose tolerance, lipid profile, hyperalgesia, nerve conduction velocity, and histopathological alteration in the liver and sciatic nerve when compared with the positive control group. Rats treated with melatonin expressed increased hepatic gene expression of PGC1-α and TFAM compared to positive control rats.

Conclusion: Melatonin *per se* positively modulated altered mitochondrial biogenesis by upregulating hepatic gene expression of PGC1-α and TFAM, which was not noted in the group administered with both melatonin and gabapentin. Thus, indicating an inverse relationship concerning the influence on mitochondrial biogenesis which needs to be explored further. In the current study melatonin in the higher dose shows improvement in the clinical outcomes in the model that inflicting hyperglycemia, glucose intolerance, dyslipidemia, and neuropathy in the rats.

1. Introduction

The prevalence of diabetes mellitus has been increasing globally and it was estimated to be 9.3% (463 million people) in 2019, expected to rise to 10.9 % (700 million people) by 2045 (Saeedi et al., 2019). There are two types of diabetic complications viz; microvascular (due to damage to small blood vessels) and macrovascular (due to damage to larger blood vessels). Microvascular complications include retinopathy, nephropathy, and neuropathy. Macrovascular complications include cardiovascular diseases. Amongst all these, neuropathy is encountered in 60% of diabetic patients. A major contributor to the reduced nerve function manifested as distal symmetrical polyneuropathy is due to hyperglycemia and dyslipidemia which is known to be driven by oxidative stress associated with diabetes mellitus (Akbar et al., 2016). Hallmarks of prolonged diabetes include derangements in mitochondrial function, increased production of reactive oxygen species, decreased nerve blood flow, reduced supply of trophic factors and slowed nerve conduction (Ling et al., 2014).

A functional decline in mitochondria is observed in diabetics (Sivitz and Yorek, 2010). The importance of coactivators and transcription factors, in particular, peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α); mitochondrial transcription factor A (TFAM) have been repeatedly emphasized by many researchers as being the major regulators of oxidative metabolism and mitochondrial content which are adversely impacted in diabetes (Choi et al., 2014; Handschin and Spiegelman, 2006; Kang et al., 2016; Shokouhi et al., 2015). Numerous studies have linked mitochondrial dysfunction with progressive diabetic neuropathy (Fernyhough, 2015; Lin et al., 2005; Sajic, 2014; Sifuentes-Franco et al., 2017). A high fructose diet is known to precipitate dyslipi-

Abbreviations: STZ, Streptozotocin; PGC1-α, Peroxisome proliferator-activated receptor gamma-coactivator 1-α; TFAM, Mitochondrial transcription factor A; VC, vehicle control; PC, positive control; GB, gabapentin; MT, melatonin; OGTT, oral glucose tolerance test; SGOT, Serum glutamic oxaloacetate transaminase; SGPT, Serum glutamate-pyruvate transaminase; Ct, Threshold cycle; MNCV, motor nerve conduction velocity.

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demia which in turn contributes to insulin resistance as well (Basciano et al., 2005).

As reported, the indoleamine hormone melatonin is capable of abating diabetic complications by neutralizing the unnecessary production of reactive oxygen species (ROS), protection of beta cells, as they possess low antioxidant potential to normalize the redox state in the cell (Afifi, 2013; Zephy and Ahmad, 2015). Melatonin has been documented to improve the mitochondrial electron transport chain and reduce electron leakage promoting protein synthesis subsequently (Areti et al., 2018; Guo et al., 2014). Further, gabapentin has proven to be effective in attenuating diabetic neuropathy and in alleviating allodynia and spontaneous pain behaviors as witnessed in numerous clinical and preclinical studies and thus was selected as the standard drug (Câmara et al., 2015; Wiffen et al., 2017; Roeska et al., 2008).

The primary objective of this study was to evaluate the therapeutic potential of melatonin *per se* and in combination with gabapentin in a laboratory animal model of experimental diabetic neuropathy, wherein we attempted to evaluate its efficacy in ameliorating the neuropathic state. Experimental diabetes mellitus can be induced by most commonly employed diabetogenic agents, i.e. alloxan and streptozotocin. Moreover, streptozotocin-induced diabetes mellitus has been used as a model for hyperalgesia (Bishnoi et al., 2011; Mehta et al., 2017; Shaikh and Somani, 2010; Wilson and Islam, 2012). Therefore, in this study, a diabetic neuropathy model was employed involving a single injection of streptozotocin (45 mg/kg) along with fructose (10%) through drinking water in male rats. While there is a previously established link between mitochondrial dysfunction and diabetes and its complications, we specifically sought to assess whether melatonin *per se* and in combination with gabapentin was associated with impacting the hepatic expression of key mitochondrial regulators PGC-1α and TFAM.

2. Material and methods

2.1. Materials

Streptozotocin was procured from Sisco Research Laboratories Pvt. Ltd., India. Gabapentin was obtained as a gift sample from Sun Pharmaceuticals Ltd., India. Melatonin was obtained as a gift sample from Swati Spentose Pvt. Ltd. India. All other chemicals and reagents obtained from local suppliers were of analytical grade.

2.2. Animals

All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), protocol no. CPCSEA-BCP/2018-01/06 and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Male Sprague Dawley rats (180-220 g) were acquired from the National Institute of Biosciences, Pune were used for the study. The animals were maintained under standard conditions of temperature (25°C ± 2°C), relative humidity (65 ± 5%), and 12-h light-dark cycle. The animals were housed in standard polypropylene cages with a wire mesh top and corn cob as bedding. Animals were fed with commercially available rodent food pellets and water *ad libitum*.

2.3. Experimental design

After a week of acclimatization, rats were randomized into six groups having eight rats each. The rats in group I were fed with commercially available rodent food pellets and normal drinking water *ad libitum* throughout the experimental period. The rats from the other five groups were administered with a single dose of streptozotocin 45 mg/kg *i.p.* on day 15 and fed with rodent food pellets and 10 % fructose-containing drinking water *ad libitum* up to week 8, further they received normal drinking water until week 12. Due to the alleged instability of streptozotocin, fresh solutions were prepared at the time of administration (Motyl and McCabe, 2009). A 10% fructose solution was prepared regularly in freshwater. The energy provided by rat chow was 3020 kcal/kg whereas that provided by fructose was 1300 kcal/kg. At the end of 8th week, oral glucose tolerance test was performed to confirm the induction of diabetes. Rats with blood glucose level greater than 280 mg/dL were considered diabetic and were randomized for treatment as follows: Group II, positive control; Group III, Gabapentin 100 mg/kg, *p.o.*; Group IV, Melatonin 25 mg/kg, *p.o.*; Group V, Melatonin 50 mg/kg, *p.o.*; Group VI, Gabapentin 100 mg/kg+Melatonin 50 mg/kg, *p.o.* daily for four weeks (Jahnke et al., 1999; Kalmykova et al., 2017; Mehrzadi et al., 2018; Miki et al., 2001).

2.4. Behavioural parameters

2.4.1. Cold Water Tail Immersion Test

In week 12, a cold water tail immersion test was performed. The tail was immersed in water maintained at a temperature of around 5°C±5. The flicking of the tail was considered a positive response. To prevent tissue damage, the maximum time for which the tail to be immersed was considered as 15 seconds. For each rat, tail-flick latency was measured three times and the mean was reported. Between every test, rats were left free for half an hour. (Anjaneyulu and Chopra, 2004; Sewell and Spencer, 1976; Soliman et al., 2020)

2.4.2. Hot Water Tail Immersion Test

In week 12, a hot water tail immersion test was performed. The tail was immersed in water maintained at a temperature of around 55°C±5. The flicking of the tail was considered a positive response. To prevent tissue damage, the maximum time for which the tail to be immersed was considered as 15 seconds. For each rat, tail-flick latency was measured three times and the mean was reported. Between every test, rats were left free for half an hour. (Anjaneyulu and Chopra, 2004; Sewell and Spencer, 1976; Soliman et al., 2020)

2.4.3. Electrophysiology of Nerve

After termination of the treatment in week 12, motor nerve conduction velocity was assessed as an index of the speed of conduction of an electrical impulse through a nerve by using student physiograph (AD Instruments South Asia (India) Pvt Ltd). Rats were anesthetized using Ketamine: Xylazine cocktail, intraperitoneal injection (91 mg: 9.1 mg/kg), and motor nerve conduction velocity was measured by stimulating the sciatic nerve (proximal to sciatic notch) using MLA0320 Animal Nerve Stimulating Electrode with a supra-maximal stimulus, i.e., 0.5 V, single stimulus and square-wave pulses with a duration of 0.2 ms. Ground, reference, and recording electrodes were inserted percutaneously at a distance of 1 cm at the dorsum of the hind paw. The stimulating electrode was placed over the sciatic nerve. During the study, the body temperature of rats was maintained at 37°C. The recordings due to stimulation of motor fibers were recorded by student physiograph software (LabChart 7.3.7). Latency (from the point of stimulation to the onset of the response) was measured in milliseconds.

The conduction velocity of the action potential was determined by measuring the distance travelled (length of the nerve in m) and dividing by the time (sec) taken to complete the reflex arc, also called the latency. The measurement of the distance was carried out using tape and the time taken to complete the reflex arc was provided by the software.

Motor nerve conduction velocity (MNCV) = Distance between the recording electrode and the stimulation site (m)/Latency (s).

2.5. Biochemical estimations in serum

OGTT was performed in week 8 and week 12 of the experimental period. After an overnight fast (12 h), rats were orally administered with D-glucose solution (2.0 g/kg b.w.) and blood glucose concentrations were

Table 1
Primer sequences for qRT-PCR.

Primer	Forward primer	Reverse primer
PGC-1α	5'TGACTGGCGTCATTCAGGAG3'	5'AGTGCTAAGACCGCTGCATT3'
TFAM	5'ATCTCATCCGTCGCAGTGTG3'	5'CTTCACAAACCCGCACGAAA3'
β-Actin	5'AGGCTGTGTTGTCCCTGTATG3'	5'AACCGCTCATTGCCGATAGT3'

subsequently measured at 0 (just prior to oral glucose dosing), 30, 60, 120, and180 min after the oral dosing of glucose by withdrawing blood through the tail vein. The glucometer (Dr. Morepen® glucose monitor) was utilized to determine the glucose concentration. On the 85th day, rats were anaesthetized using dry CO_2; blood was collected from the retro-orbital plexus, and rats were euthanized in CO_2 Chamber. Blood samples were then centrifuged at 3000 × g for 15 min at 4°C. Serum was aliquoted and stored at −20°C for various biochemical assays. Quantitative estimation of serum total cholesterol, triglyceride, creatinine, Serum glutamate pyruvate transaminase (SGPT), Serum glutamate oxaloacetate transaminase (SGOT) was done using commercial diagnostic kits (Coral Clinical Systems, India).

2.6. Gene expression studies

Total RNA was extracted from liver tissue using the PureLink® RNA mini kit (Invitrogen,) as per the manufacturer's guidelines. Qubit® 3.0 Fluorometer (Invitrogen) was used to analyze total RNA. Verso SYBR® Green 1-Step qRT-PCR ROX Mix (Thermo Scientific) was used to carry out the amplification of RNA. To quantitate the relative mRNA levels Real-time PCR using gene-specific primers was used. Standard thermal cycling conditions were used for all genes, amplification was performed at 95°C for 15 min for thermostat activation, followed by 40 cycles of 95°C for 15 s, 50-55°C for 30 s and 72°C for 30s. Samples were analyzed using the Applied Biosystems 7500 Real-Time PCR System. The threshold cycle (Ct) values of each sample gene were parentized with Ct values of β-actin to perform relative quantitation. The results were expressed as fold changes of the threshold cycle (Ct) value relative to the controls by the 2-ΔΔCt method. (Bustin et al., 2009; Kang et al., 2016; Livak and Schmittgen, 2001) The sequences of the primers used are shown in Table 1.

2.7. Histopathological Examination

Tissue specimens of the liver and sciatic nerve were collected from animals belonging to different treatment groups. After collection, the tissue specimens were dehydrated using ascending strengths of ethanol and later cleared in benzene. They were then embedded in paraffin blocks. Sections of 5 μm thickness were taken and stained with regular haematoxylin and eosin stain (H&E) and observed under the microscope at 400X magnification.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 software (Graph Pad Software, San Diego, CA, USA). The differences among the experimental groups were analyzed with one-way analysis of variance, followed by *Dunnett's* multiple comparison test as a *post-hoc* analysis. For OGTT data, two- way analysis of variance (with time and treatment as variables) followed by *Tukey's* multiple comparison test as a *post-hoc* analysis was used. All the values were expressed as mean±SEM. Statistical significance was considered at $^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ and $^{\#\#\#\#}P < 0.0001$; $^{\#\#\#}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ when compared with positive control and vehicle control groups respectively.

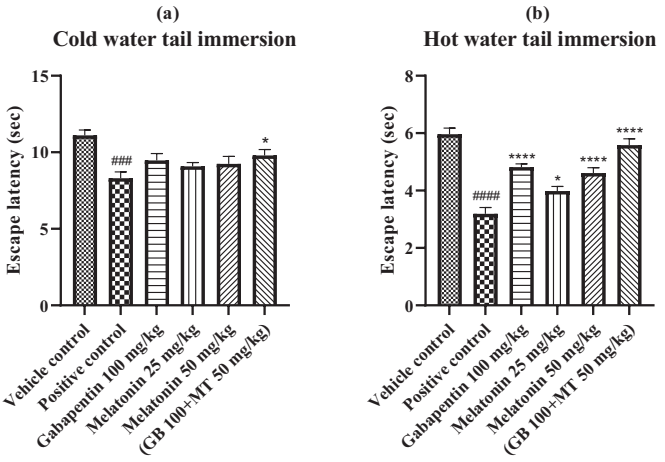

Fig. 1. Effect of melatonin, gabapentin *per se* and in combination on nociceptive pain parameters (a) Cold water tail immersion and (b) Hot water tail immersion. Values are expressed as mean±SEM (n=6 for all groups). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test as a post-hoc analysis to identify significant differences among the groups. $^{\#\#\#\#}P < 0.0001$; $^{\#\#\#}P < 0.001$ when compared to the vehicle control group. $^{****}P < 0.0001$; $^{*}P < 0.05$ when compared with positive control group.

3. Results

3.1. Effect of melatonin per se and in combination on nociceptive pain parameters

Thermal allodynia was assessed with cold water and hot water tail immersion tests. Decreased escape latency is suggestive of neuropathic state. It showed a significant difference between vehicle control and positive control rats. In the cold water tail immersion test, the escape latency of combination treatment (gabapentin 100 mg/kg+melatonin 50 mg/kg) rats was 9.78±0.38 seconds, significantly higher than positive control rats, which was 8.30±0.41 seconds (Fig. 1).

3.2. Effect of melatonin per se and in combination on sciatic nerve conduction velocity

Slowed nerve conduction velocity, which is a characteristic of neuropathy, was observed to be pronounced in positive control rats i.e. 42.20±0.343 m/s, as compared to 75.77±0.72 m/s in the vehicle control rats, which may be due to neuronal degeneration, oxidative stress, and infiltration by MNCs. Treatment with melatonin *per se* and in combination with gabapentin resulted in statistically significant improvement in sciatic nerve conduction velocity as 63.64±0.64 m/s in group III rats; 53.58±1.89 m/s in group IV rats; 59.87±0.87 m/s in group V rats; 70.36±2.18 m/s in group VI rats (Fig. 2).

3.3. Effect of melatonin per se and in combination on blood glucose levels during the oral glucose tolerance test

Fasting blood glucose levels as recorded after 48 hr. post streptozotocin 45 mg/kg *i.p.* injection on the 15th day were above 280 mg/dL and were consistent throughout the experimental period. OGTT carried before initiating treatment (in week 8) revealed impaired glucose tolerance in rats with all the groups when compared to vehicle control groups ($p<0.0001$). Interpretation of OGTT data suggests that there was impaired glucose tolerance, which is a hallmark of type-2 diabetes. OGTT carried after the termination of treatment (in week 12) revealed that after 180 min post glucose load, the blood glucose concentration of positive control rats was 367.66±11.83 mg/dL. This indicated impaired glucose tolerance, which is a forerunner to type 2

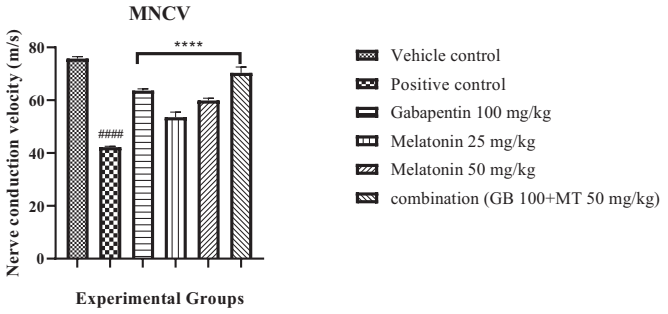

Fig. 2. Effect of melatonin, gabapentin *per se* and in combination on sciatic motor nerve conduction velocity (MNCV). Values are expressed as mean±SEM (n=6 for all groups). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test as a post-hoc analysis to identify significant differences among the groups. $^{\#\#\#\#}P < 0.0001$ when compared to the vehicle control group. $^{****}P < 0.0001$ when compared with positive control group.

diabetes. Group III and group IV rats showed improvement in glucose tolerance as with the blood glucose levels post 180 min of glucose load were 133.66±11.86 mg/dL and 141.66±65.66 mg/dL respectively ($p<0.001$) whereas, group V and group VI rats had shown pronounced improvement in glucose tolerance with blood glucose levels 125.33±19.74 mg/dL and 87±7.93 mg/dL respectively ($p<0.0001$). The AUC glucose (0-180 min) was the highest in the positive control group (80420 mg/dL. min180) which was indicative of insulin resistance. The total AUC glucose (0-180 min) post-treatment was decreased in group III (52685 mg/dL. min180), group IV (49445 mg/dL. min180), group V (45295 mg/dL.min180), group VI (35655 mg/dL.min180) (Fig. 3).

3.4. *Effect of melatonin* per se *and in combination on serum lipid profile, SGOT, SGPT and creatinine*

Dyslipidemia is the hallmark of metabolic imbalance and abnormal increase in serum triglycerides, cholesterol acts as cues for it. We encountered a significant increase in serum triglycerides and serum cholesterol levels in positive control rats. Serum SGPT levels were found to be increased which was reversed by subsequent treatment and in contrast with this serum SGOT levels were found to be decreased which was also reversed by subsequent treatments. Serum creatinine levels were increased in positive control rats, which was indicative of progressive renal damage and metabolic derangements. Treatment with melatonin 50 mg/kg and combination (Gabapentin 100 mg/kg+Melatonin 50 mg/kg) resulted in significant decrease in serum creatinine levels (Fig. 4).

3.5. *Effect of melatonin* per se *and in combination on hepatic mRNA expression of PGC-1α and TFAM*

Hepatic mRNA expression levels were traced for two genes participating in the cascade of mitochondrial biogenesis, viz. Peroxisome proliferator-activated receptor-gamma coactivator 1-α (PGC-1α) and mitochondrial transcription factor A (TFAM). Hepatic gene expression relative to the positive control group was observed and quantified. Expression fold change revealed that rats treated with melatonin 25 mg/kg and melatonin 50 mg/kg showed fold change greater than 1, which was suggestive of gene upregulation. Other groups treated with gabapentin 100 mg/kg and combination (gabapentin 100 mg/kg+melatonin 50 mg/kg) didn't show significant changes in gene expression levels of PGC-1α and TFAM (Fig. 5).

3.6. *Effect of melatonin* per se *and in combination on histology of liver tissue*

Severe vacuolar degeneration of hepatocytes along with mild periportal infiltration of inflammatory cells was suggestive of severe liver damage in contrast to the vehicle control architecture. The positive control rats showed severe degeneration of hepatocytes and there was mild focal infiltration of MNCs. Rats treated with gabapentin 100 mg/kg, melatonin 25 mg/kg, melatonin 50 mg/kg, combination (gabapentin 100 mg/kg+melatonin 50 mg/kg) showed improvement in liver tissue architecture with few infiltrations of leukocytes and mild degenerations (Fig. 6).

3.7. *Effect of melatonin* per se *and in combination on histology of sciatic nerve*

Moderate neuronal degeneration of the sciatic nerve along with mild infiltration of inflammatory cells was suggestive of moderate nerve damage in contrast to the vehicle control architecture. The positive control rats showed moderate degeneration of the sciatic nerve and there was mild focal infiltration of MNCs. Rats treated with gabapentin 100 mg/kg, melatonin 50 mg/kg and combination (gabapentin 100 mg/kg + melatonin 50 mg/kg) showed improvement in sciatic nerve damage with few infiltrations of leukocytes and mild degenerations (Fig. 7).

4. Discussion

Diabetic neuropathy is a common and serious complication stemming from metabolic abnormalities causing lesions of the peripheral and/or central nervous system leading to sensory signs and symptoms (Haythornthwaite et al., 2003). A clinical study in type-2 diabetic patients has revealed that the circadian rhythm of melatonin secretion was found to be blunted in the subjects (Tütüncü et al., 2005). While mitochondria have been enmeshed, the extent of the mitochondrial decline in diabetes and how it exacerbates neuropathy remains controversial; however, a recently published review article discussed the findings and addressed the potential mechanisms that could serve as the liaison between mitochondrial malfunction and insulin resistance (Sangwung et al., 2020).

Consistent with previous studies, our results confirmed induction of diabetic neuropathy in the model adopted as evident by resultant degenerative changes (Barrière et al., 2018; Filho and Fazan, 2006; Mehta et al., 2017; Wilson and Islam, 2012; Wongchitrat et al., 2016). OGTT at baseline before treatment initiation showed impaired glucose tolerance characterized by increased area under the curve from 0-180 min; a hallmark of diabetes mellitus. Additionally, data obtained from the evaluation of nociceptive pain parameters defined the progression of the disease. Slowed sciatic nerve conduction velocity confirmed the induction of neuropathic state. Collectively these results point to an association between persistent hyperglycemia, oxidative damage, mitochondrial malfunction, and insulin resistance altogether worsening neuropathy. Treatment with melatonin *per se* and in combination improved the impaired glucose tolerance, thermal allodynia, and nerve conduction velocity providing supporting potential of melatonin to be employed in the treatment of diabetic neuropathy (Ma et al., 2015; Yerra and Kumar, 2017; Zangiabadi, 2011). The increase in ectopic fat deposition has been proven to be associated with insulin resistance (Garcia et al., 2004; Krssak et al., 1999). In the present study increased serum cholesterol and triglycerides confirmed dyslipidemia as was expected from high fructose administration, which was subsequently ameliorated by treatment with melatonin alone and along with gabapentin (Suman et al., 2016; Wilson and Islam, 2012). Though not in line with previous studies, the attenuation of the dyslipidemia post-treatment with gabapentin can be attributed to its effect on oxidative stress. Gabapentin has been proven to be effective in attenuating oxidative stress in the diabetic rat retina

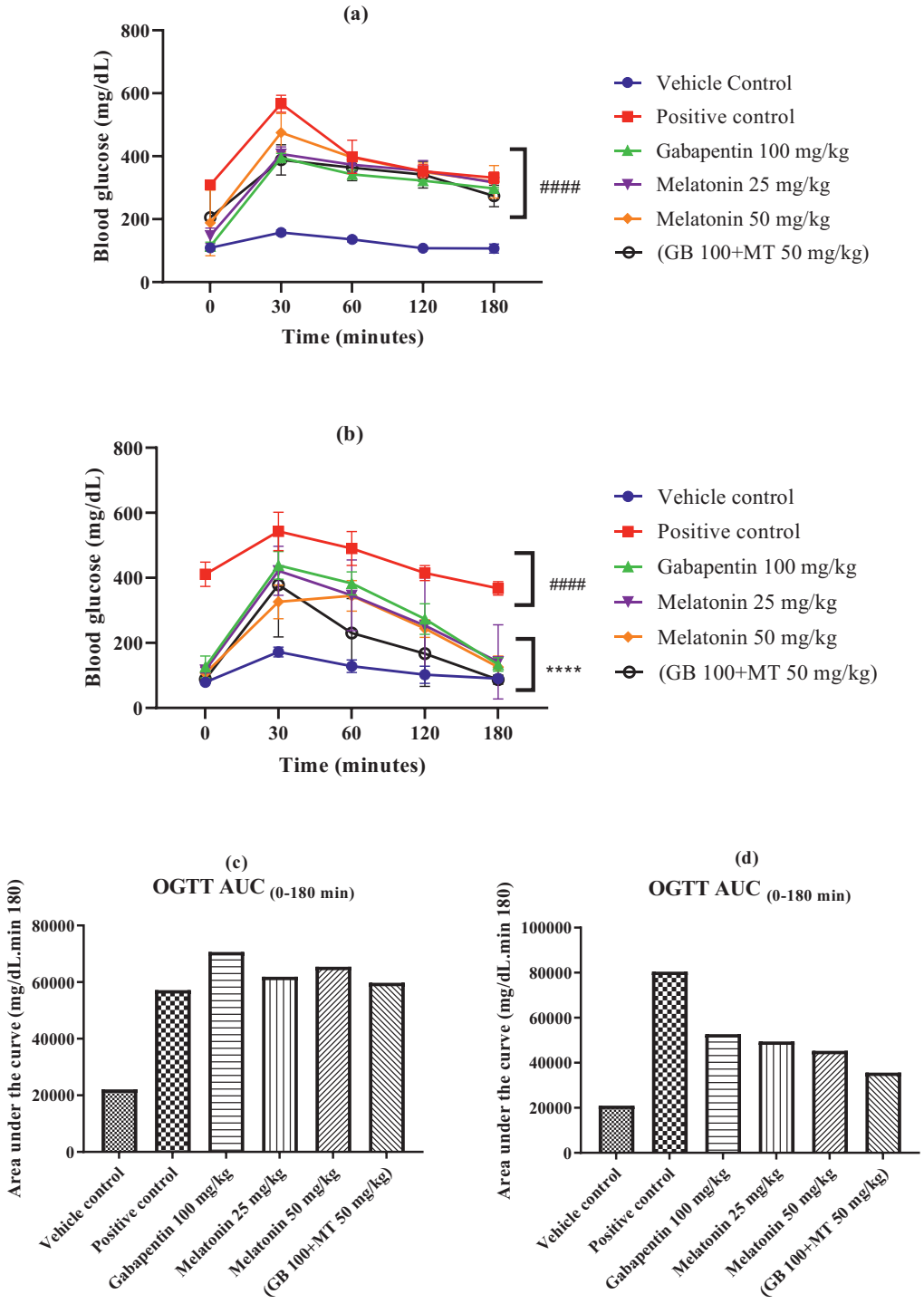

Fig. 3. Effect of melatonin, gabapentin *per se* and in combination on serum glucose concentration during oral glucose tolerance test (OGTT) before treatment and after treatment (a) OGTT before treatment and (b) OGTT after treatment; Values are expressed as mean±SEM (n=6 for all groups). Statistical analysis was performed by using two-way analysis of variance (with time and treatment as variables), followed by Tukey's multiple comparison test as a post-hoc analysis to identify significant differences among the groups. (c) OGTT AUC $_{(0\text{-}180\ min)}$ before treatment and (d) OGTT AUC$_{(0\text{-}180\ min)}$ after treatment. Values are expressed as mean. Statistical analysis was performed using one-way analysis of variance. $^{####}P < 0.0001$ when compared to the vehicle control group. $^{****}P < 0.0001$ when compared with positive control group.

(Ola et al., 2019). A previous report in humans suggests that, oxidative stress is an early event in the evolution of hyperlipidemia, and appropriate support for enhancing antioxidant supply in higher lipid subjects may help prevent the course of the disease (Yang et al., 2008). Thus, the nexus between oxidative stress and progressive dyslipidemia can be accounted for the attenuation of dyslipidemia by gabapentin treatment. Serum SGOT levels strangely deflected abnormally wherein serum SGOT was found to be decreased in positive control rats as compared to vehicle control which was subsequently increased post-treatment with melatonin *per se* and in combination, unlike previous reports (Marella et al., 2015; Ramachandran et al., 2012). Serum SGPT levels were increased following induction of the disease which eventually decreased

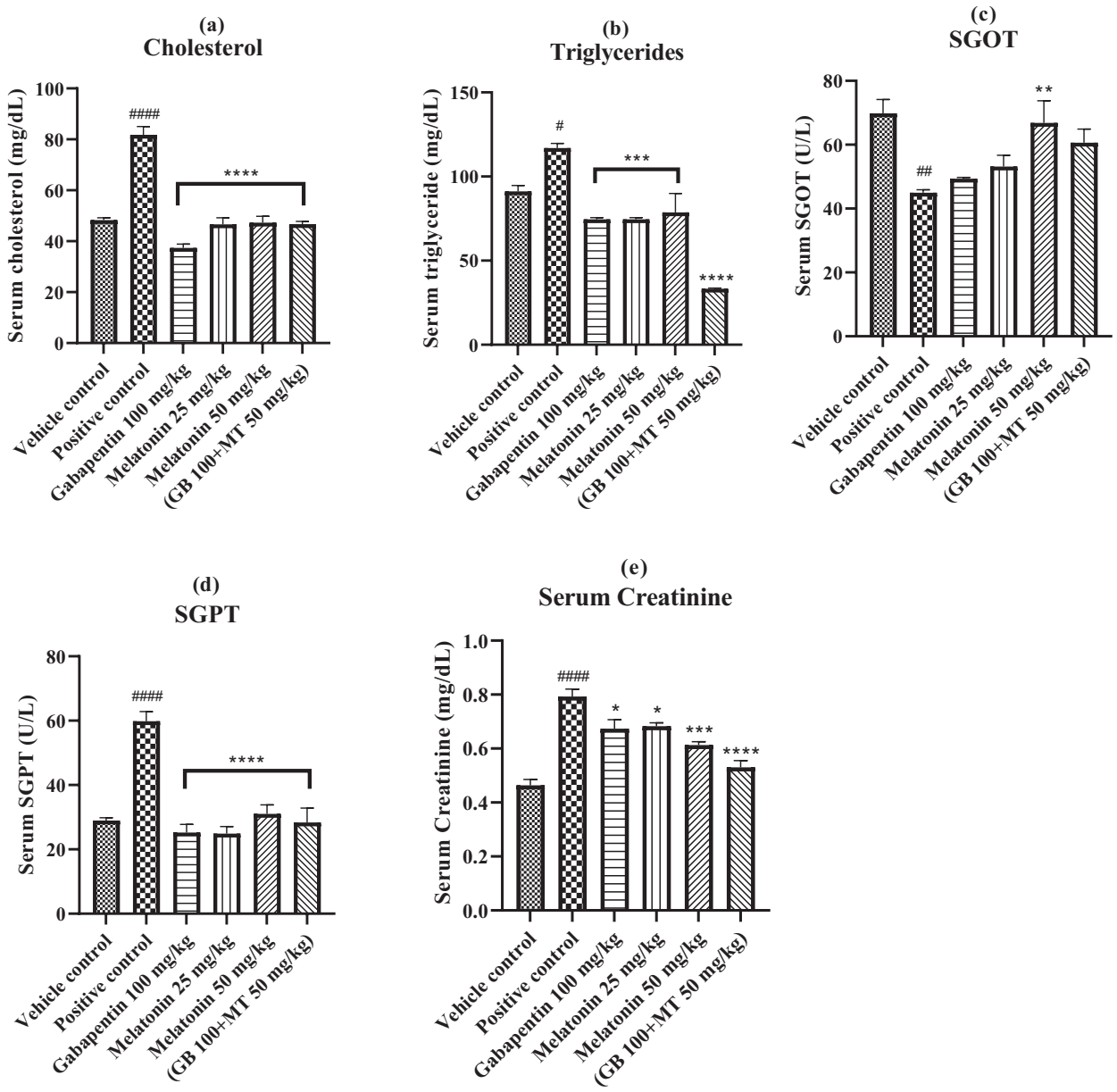

Fig. 4. Effect of melatonin, gabapentin *per se* and in combination on serum (a) Cholesterol; (b) Triglycerides; (c) SGOT; (d) SGPT and (e) Creatinine. Values are expressed as mean±SEM (n=6 for all groups). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test as a post-hoc analysis to identify significant differences among the groups. $^{\#\#\#\#}P < 0.0001$; $^{\#\#}P < 0.01$ when compared to the vehicle control group. $^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ when compared with positive control group.

post-treatments, consistent with the previous results. Raised serum creatinine levels, which were indicative of progressive renal damage and metabolic derangements, were combated subsequent by the treatment with melatonin *per se* and along with gabapentin. This improvement in serum creatinine levels could be attributed to the restoration of renal tissue architecture which might have been damaged due to persistent hyperglycemia (Ebaid et al., 2020). Collectively, all these results were consistent with the recently published study wherein co-administration of melatonin and pregabalin was assessed for synergism in improving vincristine induced neuropathy (Soliman et al., 2020).

The state of persistent hyperglycemia leads to an increase in the production of cytosolic and mitochondrial ROS (Sifuentes-Franco et al., 2017). Recent insights from *in vivo and ex vivo* metabolic studies in rodents and humans have supported the hypothesis that altered mitochondrial function in tissues such as liver, skeletal muscle, and white adipose tissue, which are prominent insulin responsive tissues, is irretrievably accompanied with a tissue or whole-body insulin resistance (Asmann et al., 2006; Chem, 1999; Rudich et al., 1998; Stump et al., 2003). PGC1-α levels determine the relative ratio of insulin receptor substrate 1 (IRS1) and IRS2 in hepatocytes, impacting insulin receptor signalling via protein kinase B/AKT (AKT) (Sifuentes-Franco et al., 2017). PGC1-α is mainly expressed in tissues with high energy oxidative capacity such as the liver. Metabolic switching studies in the liver have shown that the activity of AMPK and PGC1-α signalling axis declines in diabetic conditions (Fernyhough, 2015). Owing to such substantial evidence pointing towards the nexus of altered hepatic gene expression status and progression of the disease, we specifically sought to assess gene expression in liver tissue. A case-control study of an Iranian population revealed an association between PGC1-α gene polymorphism and risk of type 2 diabetes and further complications (Shokouhi et al., 2015).

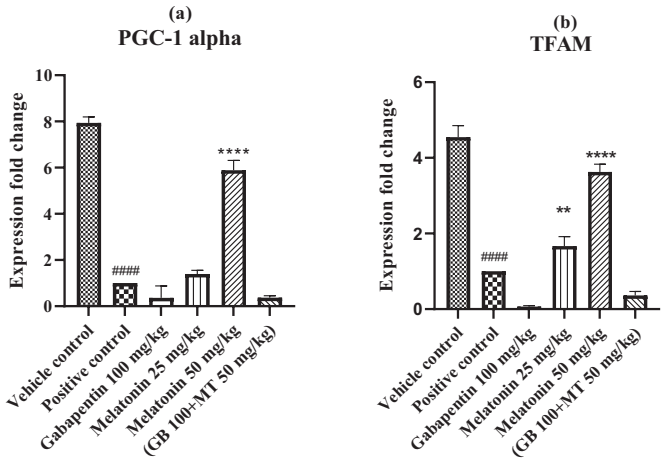

Fig. 5. Effect of Melatonin, Gabapentin *per se* and in Combination on hepatic mRNA Expression fold change for: (a) Peroxisome proliferator-activated receptor-gamma coactivator 1-α (PGC1-α); (b) Mitochondrial transcription factor A (TFAM); Values are expressed as mean±SEM (n=6 for all groups). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test as a post-hoc analysis to identify significant differences among the groups. $^{\#\#\#\#}P < 0.0001$ when compared to the vehicle control group. $^{****}P < 0.0001$; $^{**}P < 0.01$ when compared with positive control group.

Upregulation of genes involved in mitochondrial biogenesis has been proven to be beneficial in combating diabetic complications (Liu et al., 2019; Sabahi et al., 2020; Xue et al., 2019). Furthermore, melatonin has been proven to have a significant impact on mitochondrial biogenesis (Jiang et al., 2019; Lee et al., 2020). Based on all this evidences we specifically sought to assess whether treatment with melatonin *per se* and in combination with gabapentin could improve the altered mitochondrial function through upregulating hepatic gene expression of PGC1-α and TFAM. Our results showed decreased expression of these two key mitochondrial regulators in positive control rats. Treatment with melatonin significantly upregulated hepatic expression of PGC1-α and TFAM which is expected to improve mitochondrial biogenesis. Surprisingly, rats treated with both gabapentin 100 mg/kg and melatonin 50 mg/kg didn't show any significant impact on mitochondrial biogenesis. These findings can be accounted for the previous report which stated that GABA inhibits melatonin production *in vitro* via interaction with GABA type A receptor sites (Rosenstein et al., 1989). Moreover, gabapentin is known to inhibit mitochondrial aminotransferase isoenzyme (Goto et al., 2005). An antiepileptic drug such as gabapentin exhibits adverse as well as beneficial effects on mitochondria by interfering with mitochondrial biogenesis, carrier proteins, membrane potential, antioxidative defense mechanism, and with mitochondrial DNA (Finsterer and Scorza, 2017). This is the first study to report the beneficial effects of melatonin on hepatic mitochondrial biogenesis and appealing for exploring its potential use in leveraging the nexus of altered mitochondrial function and insulin resistance in diabetic patients in clinical settings. In the current study, we observed that gabapentin downregulates the genes PGC1-α and TFAM, involved in mitochondrial biogenesis in hepatic tissue while melatonin upregulated them.

Histopathological assessment of liver and sciatic nerve of positive control rats revealed severe tissue damage and altered architecture with vacuolar degeneration of hepatocytes alongside the periportal infiltration of inflammatory cells. Melatonin *per se* and in combination significantly abated detrimental histological effects restoring the normal architecture. The improvement in serum SGPT levels, sciatic nerve conduction velocity can be speculated to be associated with improvement in the histological architecture of these tissues post-treatment with melatonin *per se* and in combination.

The limitations of this study were the lack of capturing all the parameters associated with pain and altered nerve conduction showcasing neuropathy. Assessment of renal morphology could have also been done post termination. Herein, we examined the ambient effects of fructose diet in concert with streptozotocin in inducing a hyperglycemic state

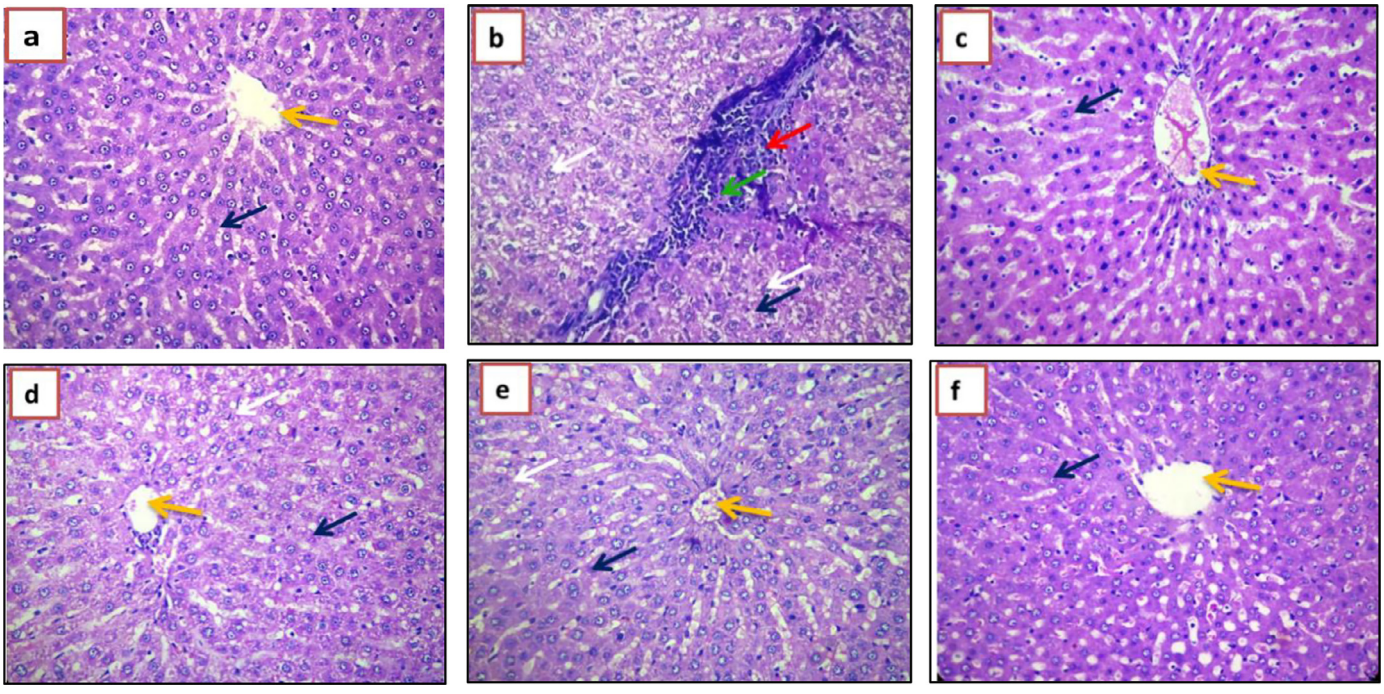

Fig. 6. Effect of melatonin, gabapentin *per se* and in combination on histopathological features of liver tissue. Representative photomicrographs of Haematoxylin and Eosin (H and E) stained sections of hepatic tissue showing vacuolar degeneration of hepatocytes along with mild periportal infiltration of inflammatory cells per high-power field in (a) Vehicle control; (b) Positive control; (c) Gabapentin (100 mg/kg, p.o.); (d) Melatonin (25 mg/kg, p.o.); (e) Melatonin (50 mg/kg, p.o.); (f) Gabapentin+Melatonin (100 mg/kg:50 mg/kg, p.o.). [H and E × 400]. Red arrow- Multifocal infiltration; Blue arrow- Hepatocytes; Green arrow- Portal triad; Yellow arrow- Central vein; White arrow- degenerative changes.

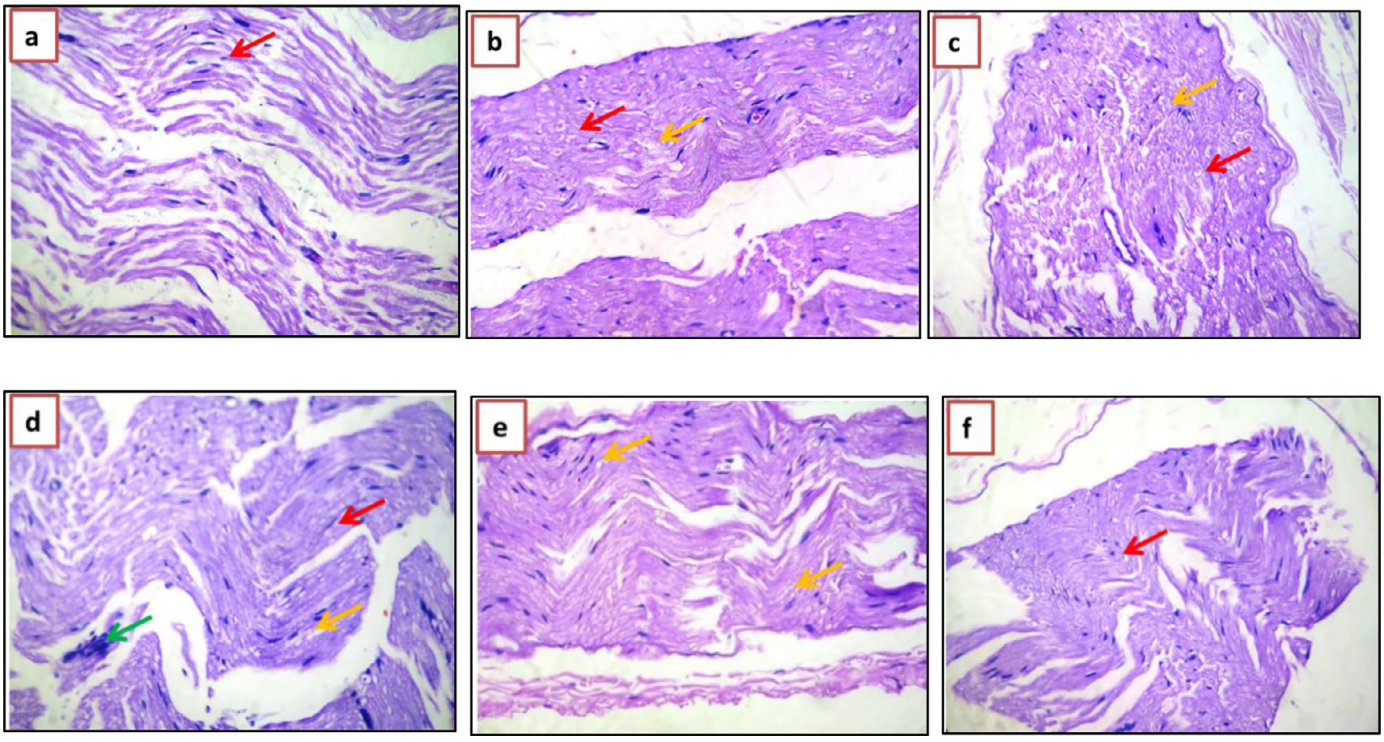

Fig. 7. Effect of melatonin, gabapentin *per se* and in combination on histopathological features of the sciatic nerve. Representative photomicrographs of Haematoxylin and Eosin (H and E) stained sections of sciatic nerve tissue showing neuronal degeneration of sciatic nerve along with mild infiltration of inflammatory cells per high-power field in (a) Vehicle control; (b) Positive control; (c) Gabapentin (100 mg/kg, p.o.); (d) Melatonin (25 mg/kg, p.o.); (e) Melatonin (50 mg/kg, p.o.); (f) Gabapentin+Melatonin (100 mg/kg:50 mg/kg, p.o.). [H and E × 400]. Red arrow- Nerve axon; Green arrow- Multifocal infiltration; Yellow arrow- Neuronal degeneration.

with neuropathy and explored the efficacy of treatment with melatonin alone and with gabapentin to circumvent the outcomes. Hence our insight helped to surmise that melatonin as well as its combination with gabapentin may be a viable alternative for treating neuropathy, a deleterious consequence of chronic hyperglycemia. Melatonin alone should be considered as a promising molecule to treat diabetic neuropathy which needs to be substantiated by empirical clinical studies in humans.

5. Conclusions

In conclusion, the current study highlighted that STZ administration and exposure to 10% fructose in drinking water induced hyperglycemia, glucose intolerance & neuropathic state in male rats. Further, we observed that melatonin *per se* and in combination with gabapentin had a more pronounced positive potential in abrogating the detrimental histological and biochemical changes that were triggered in this model of diabetes and diabetic neuropathy. Thus, melatonin can be a value add in the treatment of diabetic neuropathy with gabapentin. However, they show an inverse influence on markers of mitochondrial biogenesis. In the current study melatonin in the higher dose shows noteworthy merit in the model of hyperglycemia, glucose intolerance, dyslipidemia, and neuropathy. Melatonin needs to be further explored in clinical setting on patients of diabetic neuropathy.

Authors' contribution

Ajit Magar: Conceptualization, Investigation, Writing- Original draft preparation, Reviewing and editing, statistical application. Karan Devasani: Conceptualization, Visualization, software, reviewing and editing, statistical validation. Anuradha Majumdar: Conceptualization, supervision, reviewing and editing, validation.

Compliance with ethical standards

The study was performed according the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India after the approval of Institutional Animal Ethics Committee (IAEC), protocol no. (IAEC NO: CPCSEA-BCP/2018-01/06).

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest to disclose.

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Peer Review Summary

Peer Review Summary associated with this article can be found in the online version, at doi:10.1016/j.endmts.2020.100067.

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