Journal of Population Therapeutics & Clinical Pharmacology

Potential Neuroprotective Effect of Trimetazidine and Metformin on Autophagy in a Rat Model of Diabetic Peripheral Neuropathy: Role of LC3 and P62

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Abstract

Background: Diabetic peripheral neuropathy (DPN), a common complication of diabetes mellitus. Currently, no specific etiology treatment has been established promoting a keen investigation into the underlying mechanism and potential strategies in the medical field. This study tested the potential protective effect of trimetazidine or metformin each of them alone or combined on autophagy in a rat model of diabetic peripheral neuropathy.

Materials and methods: Forty-six adult male Sprague-Dawley rats (200-250g) were used in the present study. Type 1 diabetes was induced by a single intraperitoneal injection of 50 mg/kg STZ. After confirming hyperglycemia, diabetic rats were divided into four subgroups: Untreated diabetic, trimetazidine treated diabetic, metformin treated diabetic, combined trimetazidine and metformin treated diabetic groups; in addition to a control normal group. At the end of study, rats were subjected to hot plate test. Serum glucose, serum insulin, serum

nerve growth factor (NGF) levels were assessed and sciatic nerve affection was examined by histopathological assessment. Neuronal autophagy process was evaluated by study the immunohistochemistry of LC3 and P62 in addition to the electron microscope.

Results: Either metformin or trimetazidine caused improvement of pain threshold, biochemical changes. Also, each of them effectively prevented diabetes-associated nerve damage and caused significant induction of autophagy process in the diabetic rats' sciatic nerve.

Conclusion: Either metformin or trimetazidine therapy provides a favorable effect in a rat model of DPN due to induction of the autophagy process and secondary beneficial effects of improved hyperglycemia.

Keywords: Trimetazidine; metformin; autophagy; diabetic peripheral neuropathy.

1. Introduction

Diabetic peripheral neuropathy (DPN) is the most prevalent complication of diabetes mellitus affects approximately half of diabetic patients. A substantial proportion of diabetic patients develop DPN despite intensive glycemic control. Thus, new rational treatment is still in demand to prevent or reverse nerve injury of diabetic patients **(Q. Zhang et al., 2021).**

The development of DPN is attributed to multiple factors, such as increased advanced glycation end product, oxidative and inflammatory stress. These alterations are a part of a growing field of research that suggests that autophagy occurs as a cytoprotective response. Autophagy, a highly conserved self-digestion process, can degrade misfolded proteins and harmful organelles within the cell **(Yin et al., 2016)**

Trimetazidine, which is a first-line anti-anginal agent, selectively inhibits long-chain 3-ketoacyl coenzyme A thiolase (the last enzyme involved in β-oxidation) activity. The mechanism of action of trimetazidine can be attributed to its effect on energy metabolism. In addition to metabolic effects, some studies showed that trimetazidine has autophagic activity **(Shu et al., 2021)**.

Metformin, a classic and widely used anti-diabetic drug, has been proved to possess pleiotropic actions beyond glycemic control. Metformin, through activation of autophagy, can promote nerve repair and reduce toxic protein aggregates in neurological diseases **(Demaré et al., 2021).**

The present study aims to investigate the role of autophagy process changes in the development of diabetic peripheral neuropathy. Also, to evaluate the protective effect of trimetazidine or metformin each of them alone or combined on autophagy in a rat model of diabetic peripheral neuropathy.

2. Materials and Methods

2.1. Drugs and chemicals

Trimetazidine, Global Napi Pharmaceuticals company, Egypt. Metformin, chemical industries development (CID) company, Egypt. Streptozotocin (STZ), Sigma-Aldrich, USA.

2.2. Experimental animals

Forty-six adult male Sprague-Dawley rats (200-250 gm) obtained from Mansoura experimental Research Center (MERC), were utilized in this study. They were put in similar

optimum housing conditions with free access to food and water. Animals were kept in cages (4 rats/cage) at a room with a controlled temperature of 26°C and on a 12-h light-dark cycle. The study design and protocol were approved by Mansoura Faculty of Medicine, Institutional Research Board (IRB) under the code no: MDP.21.10.79.

2.3. Experimental design

2.3.1. Induction of type 1 DM model

Diabetes Type 1 was induced by a single intraperitoneal (i.p) injection of 50 mg/kg STZ after an overnight fast. Three days after STZ injection, induction of DM was confirmed by using GlucoDr™ super sensor glucometer (one touch technology, Allmedicus, Korea) for measuring tail vein blood-glucose level; rats with fasting blood glucose (FBG) levels greater than 200 mg/dl in two consecutive analyses were included in the study **(Malcangio and Tomlinson, 1998)**

2.3.2. Animal grouping and drug treatment:

Animals were classified and treated according to the following scheme: control normal group (9rats); rats received 1ml citrate buffer i.p. single dose then received saline, Untreated diabetic group (10rats); received saline, Trimetazidine treated diabetic group (9rats); treated with trimetazidine 30 mg/kg dissolved in saline **(Zhang et al., 2016),** Metformin treated diabetic group (9rats); treated with metformin (200 mg/kg) dissolved in saline **(Barragán-Iglesias et al., 2018),** Combined trimetazidine and metformin treated diabetic group (9rats)**;** treated with metformin (200 mg/kg) and Trimetazidine (30 mg/kg) each dissolved in saline. All drugs were administered by oral gavage after confirmation of T1DM daily for 8 weeks.

2.4. Reaction latency assessment.

Rats' heat pain sensitivity was evaluated using the Ugo Basile hot plate apparatus. Individual rats were placed on the heated plate fixed at a temperature of 55 ± 1 °C. The latency to withdraw or lick the hind paws or jump to avoid heat pain was recorded as hot plate reaction latency; with a cut-off time of 20 s **(Kamel et al., 2022).**

2.5. Biochemical assessment

From the heart, 5 mL of blood was withdrawn immediately after anesthesia. Sera were collected for measurement of serum glucose, insulin and nerve growth factor (NGF) levels. Serum glucose level was determined by enzymatic method according to **(Trinder, 1969)** using glucose kits by (**Bio Med-glucose Bio Med Co, Cairo, Egypt**). Insulin analysis was analyzed using ELISA kit for rat insulin Catalog Number CEA448R, Cloud-Clone Corp, USA. Measurement of serum NGF by Rat NGF ElISA kit, Catalog Number CSB-E04685r, Cusabio Co, China.

2.6. Histopathological assessment

2.6.1 Hematoxylin and Eosin stain

The rat sciatic nerves were prepared to be stained with hematoxylin–eosin (HE). The extent of sciatic nerve fiber degeneration, Schwann cell loss, and inflammatory cells infiltration was used for grading the severity of the pathologic changes in the HE-stained sections. A 4-point scoring scale was used with 0, 1, 2, and 3 indicating no (0%), mild (1–25%), moderate (26–50%), and severe (>50%) pathological changes, respectively **(Abdelkader et al., 2022).**

2.6.2. Immunohistochemistry of LC3 and P62:

5-µm thick sections were used to assess efficiency of new autophagosome biogenesis, using polyclonal LC3 (purified 10; cat. no GB13431, diluted at 1:300-1200, Servicebio Co, china) according to the manufacturer's instructions; P62 immunohistochemistry, to detect somatic P62 aggregates, using P62 antibody (anti Rabbit polyclonal SQTM1/P62 polyclonal antibody (purified 10; cat. no A11247, diluted at 1:50-200) according to the manufacturer's instructions. The deparaffinized sections were incubated with the primary antibody for the time recommended by the manufacturer. Unbound primary antibody was washed out, and slides were then incubated with the secondary antibody, counter-stained with hematoxylin and visualized using diaminobenzidine. Negative control sections, done after omitting primary antibodies, showed no specific labeling **(Satoh et al., 2014)**. Assessment was done by measurement of optical density by image analysis and also by microscopic examination that graded the severity of brown staining into: mild (light brown), moderate (brown) and severe (dark brown). Assessment of the optical density (OD) was done by ImageJ v2. 35 (NIH) to identify the extent of the LC3 and P62 expression in the images and for data collection. The intensity numbers were converted into OD through the following formula:

$$OD = \log\left(\frac{\text{max intensity}}{\text{mean intensity}}\right)$$

where the max intensity = 250; mean intensity = mean gray value **(Varghese et al., 2014).**

2.7. Transmission electron microscope:

The left sciatic nerve of each rat was prepared for electron microscope. The sciatic nerves were pre-fixed in a solution of 2.5% glutaraldehyde and 1% osmium tetroxide, post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer pH 7.2 then dehydrated in an escalating sequence of alcohols before being embedded in epoxy resin. Uranyl acetate and lead citrate were used to stain ultrathin sections, which were then viewed under a transmission electron microscope **(H-600; HITACHI, Tokyo, Japan) (Amelinckx et al., 2008).**

2.8. Statistical Analysis

The results were statistically analyzed using the Statistical Package for Social Science (SPSS) program version 23. The parametric results were expressed as Mean ± SD. One-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparisons was used for statistical analysis between groups. Kruskal Wallis test was used for comparison of means of more than two different groups of non-parametric data followed by post-hoc Dunne's test, data were presented as median & range (minimum-maximum). For all above mentioned statistical tests done, the threshold of significance is fixed at 5% level (p-value) or less.

3. Results

3.1 Effect of oral administration of trimetazidine, metformin alone or in combination on reaction latency using hot plate test

Induction of diabetic peripheral neuropathy produced a significant shortening in reaction latency compared to control normal rats ($P< 0.001$). Treatment with trimetazidine or metformin showed significant prolongation of reaction latency compared to both untreated diabetic rats ($P < 0.001$, $P < 0.01$respectively). There was no significant difference in reaction latency between trimetazidine or metformin. Combination of metformin and trimetazidine caused significant prolongation of reaction latency compared to untreated diabetic rats, metformin treated rats or trimetazidine treated rats ($P < 0.001$, $P < 0.05$, $P < 0.05$ respectively) (Table 1).

3.2. Effect of oral administration of trimetazidine, metformin alone or in combination on the serum glucose level and serum insulin level

Untreated diabetic rats showed significant elevation in the serum glucose level and a significant reduction in the serum insulin level compared to the control normal rats ($P< 0.001$, $P< 0.001$ respectively). Trimetazidine or metformin showed a significant reduction in serum glucose level, as well as significant elevation of serum insulin levels when compared to untreated diabetic rats ($P< 0.001$, $P< 0.01$ respectively) ($P< 0.001$, $P< 0.001$ respectively). Serum glucose level of metformin treated diabetic rats was significantly less than trimetazidine treated diabetic rats ($P< 0.05$). However, serum insulin level of metformin treated diabetic rats is non-significantly different compared to trimetazidine treated diabetic rats. Combined group showed significant reduction in serum glucose level and significant elevation of serum insulin level when compared to both diabetic untreated rats and trimetazidine treated diabetic rats ($P< 0.001$, $P< 0.01$; $P< 0.001$, $P< 0.01$ respectively). However, serum glucose level and serum insulin level were non-significantly different when compared to metformin treated diabetic rats (Table 1).

3.3. Effect of oral administration of trimetazidine, metformin alone or in combination on the serum level of nerve growth factor

A significant reduction was noted in the serum level of NGF in the untreated diabetic group compared to the control normal group ($P< 0.001$). Trimetazidine or metformin caused a significant increase in NGF as compared to untreated diabetic rats ($P< 0.001$, $P< 0.001$ respectively). Combined group showed significant increase in NGF as compared to untreated diabetic rats, trimetazidine treated diabetic rats or metformin treated diabetic rats ($P< 0.001$, $P< 0.001$, $P< 0.01$ respectively) no significant difference as compared to control normal rats (Table 1).

3.4. Effect of oral administration of trimetazidine, metformin alone or in combination on the sciatic nerve histopathology

Sciatic nerve sections of untreated diabetic rats showed Schwan cells loss, hemorrhage, edema with severe axon loss and a significant increase in histopathological score compared to control normal rats ($P< 0.001$). Trimetazidine or metformin caused partial improvement in pathology and histopathological score compared to untreated diabetic rats ($P< 0.05$, $P< 0.05$ respectively). Combination of trimetazidine and metformin resulted in restoration of nerve axons, improvement of histopathological score compared to untreated diabetic rats, trimetazidine treated diabetic rats, metformin treated diabetic rats ($P< 0.001$, $P< 0.05$, $P< 0.05$ respectively) (Figure 1) (Table 1).

3.5. Effect of oral administration of trimetazidine, metformin alone or in combination on the expression of autophagy markers (LC3 and P62)

A statistically significant decline in the expression of LC3 in the sciatic nerve of untreated diabetic rats was observed compared to control normal rats ($P< 0.001$). Either trimetazidine or metformin enhanced LC3 expression in sciatic nerve as compared to untreated diabetic rats ($P< 0.05$, $P< 0.05$ respectively). There is no significant difference between trimetazidine and metformin. Combination of drugs cause significant increase of LC3 expression compared to untreated diabetic rats and control normal rats ($P< 0.001$, $P< 0.001$) but no significant difference compared to each drug alone (Figure 2).

Untreated diabetic rats showed significant rise in the the expression of P62 compared to control normal rats ($P< 0.001$). Trimetazidine or metformin treated diabetic group showed significant decline in P62 expression in sciatic nerve compared to untreated diabetic rats ($P<0.001$, $P<0.001$ respectively). Combined group showed significant decrease in the the

expression of P62 compared to both untreated diabetic rats and trimetazidine treated diabetic rats (P<0.001, P<0.05 respectively). There was no significant difference in optical density of P62 compared to metformin treated diabetic rats or control normal group (Figure 3).

3.6. Effect of oral administration of trimetazidine metformin alone or in combination for on the ultrastructural changes of sciatic nerve

TEM micrograph of sciatic nerve sections from untreated diabetic rats showed disorganized, separated and swollen demyelinated nerve fiber with abundant vacuolation and autophagosomes. TEM micrograph of sciatic nerve of trimetazidine or metformin treated diabetic group showed partial improvement with a number of autophagic vacuoles in Schwan cell cytoplasm. The diabetic changes in the sciatic nerve were markedly mitigated by combination of metformin and trimetazidine (Figure 4)**.**

3.7. Percentage of mortality of different groups after 8 weeks of experimental period

As shown in (Table 2) the control normal group showed mortality rate throughout 8weeks period (0%) while untreated diabetic group had mortality rate (30%). Treatment of diabetic rats with trimetazidine produced decrease in mortality rate (22%) while treatment with metformin produced mortality rate (11%). Combined group had mortality rate (11%).

4. Discussion

Diabetic peripheral neuropathy (DPN) is the most common microvascular complication which progressively leads to neuronal degeneration. The present work provided evidence that administration of trimetazidine or metformin had a favorable metabolic and neuroprotective effects in experimental DPN. A rat model of DPN was conducted using a single dose STZ (50 mg/kg). STZ causes pathological changes that mimic human T1DM with chronic pancreatic islet inflammation, insulitis, and insulin deficiency **(Furman, 2015)**. Rats were used as rodents, especially rats, are most sensitive to STZ **(Rees and Alcolado, 2005)**.

The choice of trimetazidine (TMZ) in this study may stem from its availability, its safety profile and in its potential role in modulation of autophagy in some previous studies other than DPN **(Ferraro et al., 2013;Y. Yang et al., 2019).** The 30 mg/kg/day dose of TMZ is the dose that was previously shown to exert autophagy induction in rats with diabetic cardiomyopathy **(Zhang et al., 2016).** Considering drug safety and efficiency, this dose was recommended in rat model of exercise-induced myocardial injury compared to 60 mg/kg/day **(Zhang et al., 2019)**. The choice of metformin in our study was likely influenced by its safety profile, widespread clinical use, and existing evidence of its pleotropic effects on cellular pathways relevant to diabetes and neuropathy beyond glycemic control including autophagy modulation **(Lu et al., 2021)**. The choice of metformin dose (200mg/kg) was based on its neuroprotective effect in diabetic rats **(Barragán-Iglesias et al., 2018)** and its potential role in autophagy regulation in a rat model of transient forebrain ischemia **(Sarkaki et al., 2015)**.

We assessed thermal hyperalgesia as an indicator of pain sensation in the rats by hot plate test. Measurement of serum glucose and insulin level were done to confirm the diabetic status of rats and to evaluate the metabolic effect of tested drugs. In addition we assessed the effect of diabetic induction on serum level of NGF that maintain the health of neurons. Histological analysis of sciatic nerve was done to show strong morphological evidence with a relatively accurate data. Moreover, we performed the immunohistochemistry of LC3 (autophagosome marker**)** and P62 (marker of autophagic degradation activity) to assess

autophagy process changes. Furthermore, utilization of an electron microscopic evaluation in this study may have been able to provide stronger additional evidence of diabetic changes development in sciatic nerve and to demonstrate the autophagic process in the different groups.

The mortality of diabetic rats could be attributed to hyperglycemia that can lead to organ damage and failure, affecting vital systems. Moreover, Diabetes disrupts metabolic processes, impacting various organs and systems, including the cardiovascular, renal, and nervous systems **(Deeds et al., 2011).** Treatment of diabetic rats with either trimetazidine or metformin alone or combined produced decrease in mortality rate due to improvement of these factors.

Untreated diabetic rats showed thermal hyperalgesia in comparison to the control normal group. A similar finding was reported by **Alkhudhayri et al. (2020) and Rossi et al. (2011).** Thermal hyperalgesia may be caused by damage to nociceptors or peripheral nerves. It has been studied in STZ induced animals with short-term (2–8 weeks) diabetes **(Gao and Zheng, 2014).** STZ induces a direct effect on neurons through increase the expression and the function of the Transient receptor potential vanilloid 1 (TRPV1) channel in sensory neurons and increased levels of inflammatory mediators (TNF-α, IL-6, and IL-1β). TRPV1 is a chemical irritant receptor that mediates the detection of noxious environmental stimuli. **(Bishnoi et al., 2011).**

STZ injection produced marked hyperglycemia and hypoinsulinemia compared to the control normal rats. Our results are in agreement with previous studies **(Fatani et al., 2015;Archana et al., 2022)**. STZ induced T1DM is associated with the destruction of a large number of endogenous β-cells, which leads to a reduced production of endogenous insulin, followed by development of hyperglycemia **(Rees and Alcolado, 2005).** A significant reduction of the serum level of NGF was observed in untreated-diabetic rats. This result also was observed by **Lane (2014)** and **Zhang et al. (2022)**. One of the mechanisms of development of DPN is a deficiency of growth factors that may leave nerves susceptible to damage and death. One of these deficient growth factors is NGF that is essential for the development and maintenance of neurons in the peripheral nervous system. Histopathological examinations of sciatic nerve of untreated diabetic group showed several changes consistent with previous reports **(El-Sawaf et al., 2021 and Abdelkader et al., 2022).**

Untreated diabetic rats revealed decreased LC3 and increased P62 expression level in sciatic nerve of the rats indicating impaired autophagy process. Previous researchers found that autophagy in DM was reduced, which might contribute to DPN as **Liu et al. (2018) and X. Zhang et al. (2021).** Whereas other studies reported enhanced autophagy in diabetic rats indicating that autophagy overactivity causes self-neuronal death **(Wang et al., 2021).** These results indicated that the changes of autophagy in diabetic animal models is complicated and needs further investigation. Autophagy is an important metabolic pathway in eukaryotic cells that keeps the balance between the synthesis and degradation of intracellular organelles and/or macromolecules **(Elshenawy et al., 2022)**. Reduced autophagy in DM may weaken the capacity for self-repair and cause diabetic complications including DPN **(Qu et al., 2016).** Autophagy process is initiated by the formation of autophagosomes that involves processing of LC3. The closed mature autophagosomes then traffic to lysosomes where the autophagic cargo and cargo adaptor, P62, are degraded **(Lieberman et al., 2020).**

Electron micrograph of sciatic nerve sections from untreated diabetic rats showed several neural alternations and with abundant vacuolation and autophagosomes. Similar changes were reported by **Shi et al., 2013** and **Zhang et al., 2022**. **Choi et al., 2014** who noted that increased autophagic vacuoles and empty vacuolar structures in dorsal root ganglion neuron of PGC-1α (−/−) phenotype diabetic mice that is consistent with peripheral neuropathy. This may be due to their retarded maturation and fusion of autophagosome to form autolysosomes.

TMZ administration resulted in reduced hyperalgesia in the diabetic rats that agree with **Nasser et al. (2022)** who stated that trimetazidine alleviated thermal hyperalgesia in a paclitaxel induced neuropathy model due to promotion of axonal regeneration and up regulation of neuronal progranulin which is an endogenous adaptive pain defense following nerve injury. Diabetic rats treated with trimetazidine (30 mg/kg) showed significant reduction in serum glucose levels and significant increase in insulin level. Our results are in accordance with **Ramezani-Aliakbari et al. (2020)**. The activation of AMP-activated protein kinase (AMPK) pathway associated with TMZ administration is one of the main mechanisms of TMZ action that might cause blood glucose improvement. However, **Yang et al., (2020)** reported that treatment of diabetic rats with TMZ (5mg/kg) of did not change blood glucose level. This may be due to using lower dose of TMZ than used in our study.

Trimetazidine significantly increased serum level of NGF that was in line with **Karahan et al. (2019)** suggesting TMZ role in nerve regeneration. Trimetazidine caused significant improvement of microscopic changes compared to the pathological group. This result agrees with previous result of **Scaricamazza et al. (2022).** Trimetazidine caused a significant increase in LC3 expression and a significant reduction in P62 expression indicating autophagy activation. Previous study showed that trimetazidine enhanced autophagy in the myocardium of diabetic rats as it restored AMPK activity and dissociated the interaction between Bcl-2 and Beclin1 **(Zhang et al., 2016).** Regarding the ultrastructure study, electron micrograph of sciatic nerve of trimetazidine treated diabetic group showed improvement of diabetic changes.

Metformin caused alleviation of hyperalgesia in diabetic rats. This is matched with **Ma et al. (2022)** who suggested that metformin plays enhance recovery of heat sensation of diabetic rats with transection injury of the sciatic nerve due to promotion of nerve regeneration. **Augusto et al. (2019)** stated that metformin antinociceptive activity in his study was thought to be due to partially activation of opioidergic pathways as AMPK has also been shown to play a role in nociceptive processing. Activation of this enzyme negatively regulates many signalling events via phosphorylation of key molecules involved in the nociceptive processing both in the central and peripheral nervous systems**.** Diabetic rats treated with metformin showed a significant reduction in serum glucose levels and a significant increase in the insulin level that came in accordance with **Han et al. (2017)** and **Nna et al. (2018)**. It is well established that antihyperglycemic effects of metformin are mediated through AMPK stimulation. AMPK activation decreases the blood glucose level through attenuation of glucose uptake and inhibition of gluconeogenesis **(Cho et al., 2015).** Bedside the improvement of insulin sensitivity by metformin, it also exerts direct beneficial effects on β cell function such as insulin release, transcriptional regulation in pancreatic islets, and islet cell viability **(Hashemitabar et al., 2015).**

Metformin caused an increase in serum level of NGF that agree with **Houshmand et al., (2019) and Lós et al. (2019.)** Metformin has improved the histopathological changes in sciatic nerve and reduced nerve degeneration compared to the pathological group. Our results agree with **(Lós et al. 2019 and Javadi et al., 2022).**

Metformin caused autophagy activation as metformin treated diabetic rats showed significant increase in LC3 expression and significant reduction in P62 expression. Metformin enhanced the expression of autophagy markers LC3 and beclin1, while it abrogated the abundance of p62 in the spinal cord after spinal cord ligation. This indicates that metformin may contribute to restoring the autophagy-lysosome pathway **(Weng et al., 2019)**. Metformin regulates autophagy through AMPK pathway activation. Metformin induces AMPK activity through direct LKB1-mediated phosphorylation or, indirectly, through the reduction of mitochondrial production of ATP. Active AMPK stimulates autophagosomes formation **(Thellung et al., 2019).** The diabetic changes in the sciatic nerve and autophagosome accumulation that assessed by EM was reduced by treatment with metformin that agree with study of metformin activity by **Ma et al., (2022)** and **Bhattacharya et al., (2020)**

The combination of metformin and trimetazidine produced more attenuated thermal hyperalgesia, a significant improvement of serum level of NGF in addition to a significant improvement of the histopathological and ultrastructural changes in sciatic nerve compared to each drug alone. This is appeared to be due to synergistic effect. In addition, the combination appeared to have a synergistic effect on reducing serum glucose levels and elevating serum insulin levels compared to trimetazidine alone. The non-significant difference in insulin levels between the combined group and the metformin treated group suggests that metformin is more potent in its antidiabetic effects compared to trimetazidine.

As regard the effect on autophagy process, the combined group showed non significant increase in LC3 expression as compared to each drug alone and a significant decrease in P62 expression as compared to trimetazidine. Therefore, this combination may have a specific effect on p62 accumulation and degradation, which is not reflected in the LC3 measurements.

5. Conclusion:

Trimetazidine represents as potentially useful drug for DPN especially group of patients who have concomitant diabetic cardiomyopathy. Metformin also is suggested to be a potential neuroprotective drug against DPN especially for patients already use it for diabetic control. In addition, combination therapy of these drugs was more effective in improvement of DPN.

6. Limitation of the study

Neuronal functional parameters were only assessed by hot plate test and histopathological measurements; future studies using nerve conduction test are indicated. Additional mechanistic studies are needed to further elucidate the molecular pathways by which trimetazidine and/or metformin may mediate the enhanced autophagic activity observed in this study. Measurement of LC3I/LC3-II ratio that represents more indicator of autophagy process.

7. Statements

Funding sources: No specific funding was received from any public bodies and commercial or not-for-profit sectors to conduct the work described in this article.

Conflict of interest: The authors declare no relevant financial or nonfinancial interests.

Ethical approval: All procedures were in full compliance with local, national, ethical, and regulatory principles, and local licensing regulations. The animal ethics committee of the Institutional Research Board, Faculty of Medicine, Mansoura University, approved all procedures (MDP.21.10.79).

8. Acknowledgment:

The authors gratefully acknowledge Dr. Ahmed Mohamed Yousef; Lecturer of Community Medicine, Faculty of Medicine, Al- Alazhar University, for his help in the analysis of data using the SPSS program. In addition, great thanks to staff of Electron microscope unit, Faculty of Agriculture, Mansoura University, for samples' preparation and processing.

9. Author contributions

Abdel-Motaal M. Fouda, Doaa Genedy, and Ahlam Elmasry Substantial contributions to study conception and design**; Abdel-Motaal M. Fouda, Doaa Genedy, Ahmed Abdel-monem Elmetwally, Heba Hany, Ahlam and Elmasry** Substantial contributions to acquisition of data; and Substantial contributions to analysis and interpretation of data**. Samah fouad**, help in the handling of the animals and experimenting**.**

Abdel-Motaal M. Fouda, Doaa Genedy, Ahmed Abdel-monem Elmetwally, Heba Hany, Ahlam Elmasry Drafting the article or revising it critically for important intellectual content. **Abdel-Motaal M. Fouda, Doaa Genedy, Ahmed Abdel-monem Elmetwally, Heba Hany, Ahlam Elmasry** Final approval of the version of the article to be published

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Figure legends:

Fig.1: **Histological finding of different groups A) Control normal group:** longitudinal section of sciatic nerve showing normally arranged nerve fibers (arrow) with scattered Schwan cells (curved arrow). (H & E X 200). **B) Untreated diabetic group**: longitudinal section of sciatic nerve showing Schwan cells loss (curved arrow), hemorrhage (star), axon loss (arrow) and edema (double head arrow). (H & E X 200). **C) Trimetazidine treated diabetic group**: longitudinal section of sciatic nerve showing no hemorrhage, no Schwan cells loss (curved arrow), moderate inflammatory cell infiltrate (thick arrow) and edema (double head arrow**).** (H & E X 200). **D) Metformin treated diabetic group:** longitudinal section of sciatic nerve showing edema (double head arrow), no hemorrhage, no Schwan cells loss (curved arrow), and mild inflammatory cell infiltrate (thick arrow). (H & E X 200). **E) Combined trimetazidine and metformin treated diabetic group**: longitudinal section of sciatic nerve showing edema (double head arrow), no hemorrhage, no Schwan cells loss (curved arrow) and no inflammatory cell infiltrate. (H & E X 200). **F) Control normal group:** transverse section of sciatic nerve showing multiple axons (arrow) with surrounding myelin sheath and scattered Schwan cells (curved arrow). (H & E X 400). **G) Untreated diabetic group**: transverse section of sciatic nerve showing severe axon loss (arrow). (H & E X 400). **H) Trimetazidine treated diabetic group**: transverse section of sciatic nerve showing minimal axon loss (arrow). (H & E X 400). **I) Metformin treated diabetic group:** transverse section of sciatic nerve showing minimal axon loss (arrow) (H & E X 400). **J) Combined trimetazidine and metformin treated diabetic group**: transverse section of sciatic nerve showing no axon loss (arrow). (H & E X 400).

Fig.2: Immunohistochemical image of sciatic nerve immunolabeled with LC3 antibody: punctuated LC3 expression indicated by brown granules (x400). A) Control normal group shows severe expression of LC3. **B)** Untreated diabetic group shows mild expression of LC3. **C)** Trimetazidine treated diabetic group shows moderate expression of LC3. **D)** Metformin treated diabetic group shows moderate expression of LC3. **E)** Combined trimetazidine and metformin treated diabetic group shows moderate expression of LC3. F) Effect of oral administration of different drugs on optical density of LC3: Data are presented as mean± SD. Multiple comparisons between groups was done by one-way ANOVA followed by Tukey *post-hoc* test.Significance levels: * $P < 0.05$ † $P < 0.01$ ‡ $P < 0.001$ a Significance versus the control normal group. b Significance versus the diabetic control group.

Fig.3.: Immunohistochemical image of sciatic nerve immunolabeled with P62 antibody: punctuated P62 expression indicated by brown granules (x400). A) Control normal group shows mild expression of P62. **B)** Untreated diabetic group shows severe expression of P62. **C)** Trimetazidine treated diabetic group shows moderate expression of P62. **D)** Metformin treated diabetic group shows mild expression of P62. **E)** Combined trimetazidine and metformin treated diabetic group shows mild expression of P62**. F)** Effect of oral administration of different drugs on optical density of P62. Data are presented mean± SD. Multiple comparisons between groups was done by one-way ANOVA followed by Tukey *post-hoc* test. Significance levels: * $P < 0.05$ † $P < 0.01$ ‡ $P < 0.001$. a Significance versus the control normal group. b Significance versus the diabetic control group. c Significance versus the trimetazidine treated diabetic group.

Fig.4: TEM micrograph of sciatic nerve of different groups **A)**Control normal group shows that sciatic nerve consisted of myelinated axon (my) formed of multilayered myelin sheath has anchoring particles and wrapping neurofilaments (ne) in addition to numerous mitochondria (m) beside a remark bundle (re) that consisted of C (C) nonmyelinated fibers. **B)** Untreated diabetic group shows disorganized, separated and swollen demyelinated nerve fiber (thick arrow) with abundant vacuolation and autophagosomes (thin arrows). **C)** Trimetazidine treated diabetic group shows partially affected nerve fibers with a number of autophagic vacuoles (thin arrow) in Schwan cell cytoplasm, some axon showing a clumped, myelin debris (thick arrow) and some appeared completely normal (arrow head). **D)** Metformin treated diabetic group shows partially irregular arranged multilayered myelin sheath wrapping a numerous neurofilaments (ne) and mitochondria (m) in addition to autophagic vacuole (thin arrow). **E)** Combined trimetazidine and metformin treated diabetic group shows normally arranged myelinated axon wrapping neurofilaments (ne).

Table (1): Reaction latency, serum glucose, serum insulin, serum NGF and histopathological score in different OA groups. Data are presented as means ± SD, except the histopathological score presented as median (range). Significance levels: * $P < 0.05$ † $P < 0.01$ ‡ $P < 0.001$.

a Significance versus the control normal group.
b Significance versus the diabetic control group.
c Significance versus the trimetazidine treated diabetic group.
d Significance versus the metformin treated diabetic group.

Animal groups	**Reaction latency** (seconds)	**Serum insulin level (pg/ml)**	**Serum gluc level (mg/dl)**	**Serum NGF** (pg/ml)	**Histopathological score**
Group 1 Control normal group (n=9)	8.7 ± 1	278.6 ± 25.2	95.1 ±16	6.4 ± 1.1	0 (0-0)
Group 2 Diabetic control	3.6 ± 0.9 ‡ a	102.9 ± 20.2 ‡ a	425.3 ± 63.6 ‡ a	1.3 ± 0.4 ‡ a	3 (2-3) ‡ a

group (n=7)					
Group 3 Trimetazidine treated diabetic group (n=7)	6 ± 0.8 ‡ *a,†b*	161.9 ± 34.9 ‡ *a,† b*	251.3 ± 68.8 ‡ *a,‡ b*	3.2 ± 0.6 ‡ *a,‡ b*	1 (0-2) *†a, *b*
Group 4 Metformin trea diabetic group (n=8)	6.5 ± 1.5 *†a, ‡ b*	190.8 ± 35.1‡ *a,‡ b*	182.5 ± 29.7 *†a,‡ b,*c*	3.8 ± 0.8 *‡ a,‡ b*	1(1-2) *† a, *b*
Group 5 Combined trimetazidine metformin trea diabetic group (n=8)	8.6 ± 1.2 ‡ *b, *c, *d*	212.5 ± .6 *†a, ‡ b, † c*	153.3 ± 33.6 ‡ *b, † c*	5.5 ± 0.7 *‡ b,‡c ,† d*	0 (0-1) *‡ b, *c, *d*

Table 2: Percentage of mortality of different groups after 8 weeks of experimental period

Animal groups	Number of animal taken	Number of animals died	Percentage of mortality (%)
Group 1 Control normal group	9	0	0
Group 2 Untreated diabetic group	10	3	30
Group 3 Trimetazidine treated diabetic group	9	2	22
Group 4 Metformin treated diabetic group	9	1	11
Group 5 Combined trimetazidine and metformin treated diabetic group	9	1	11

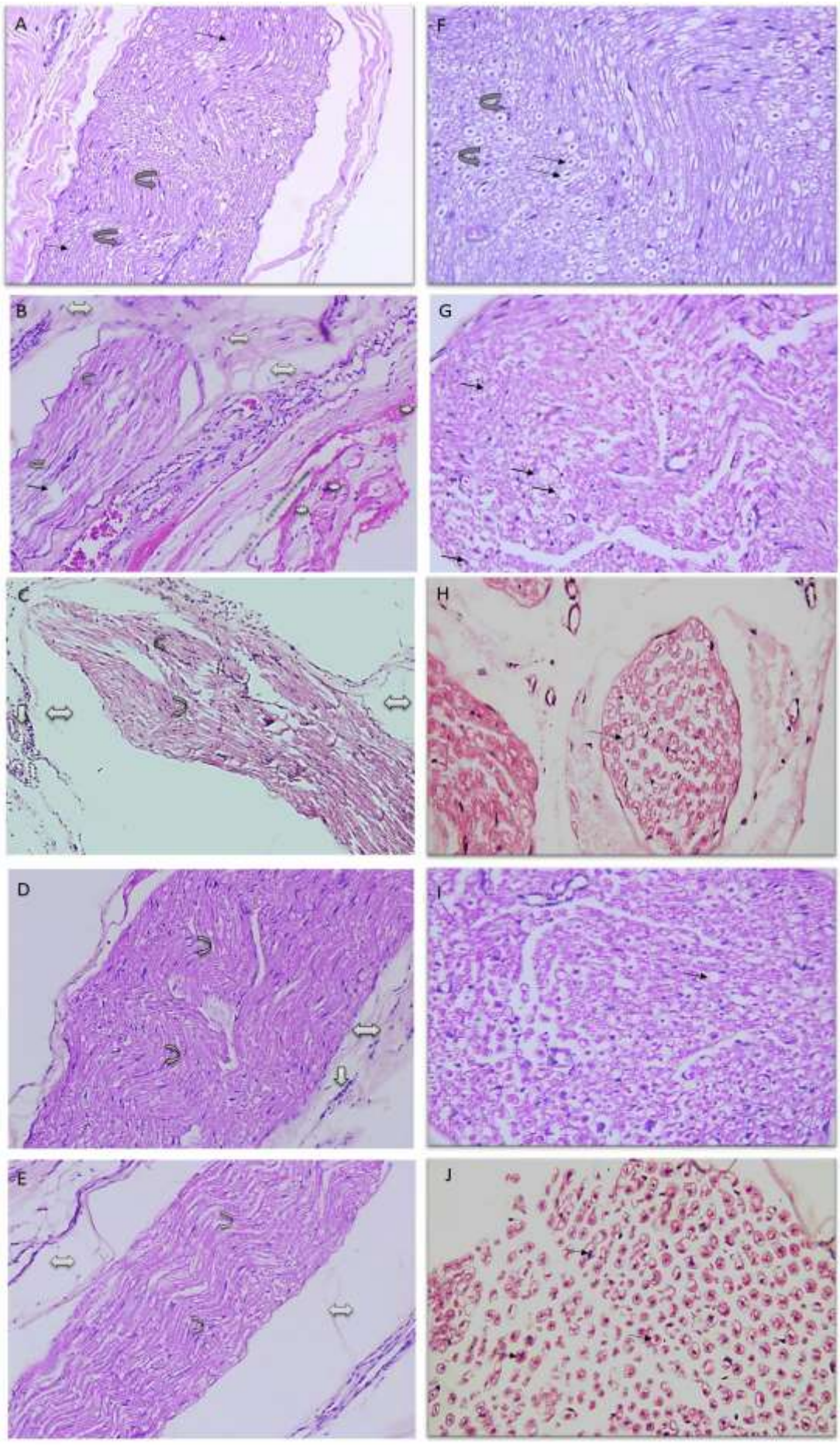

Figure 1

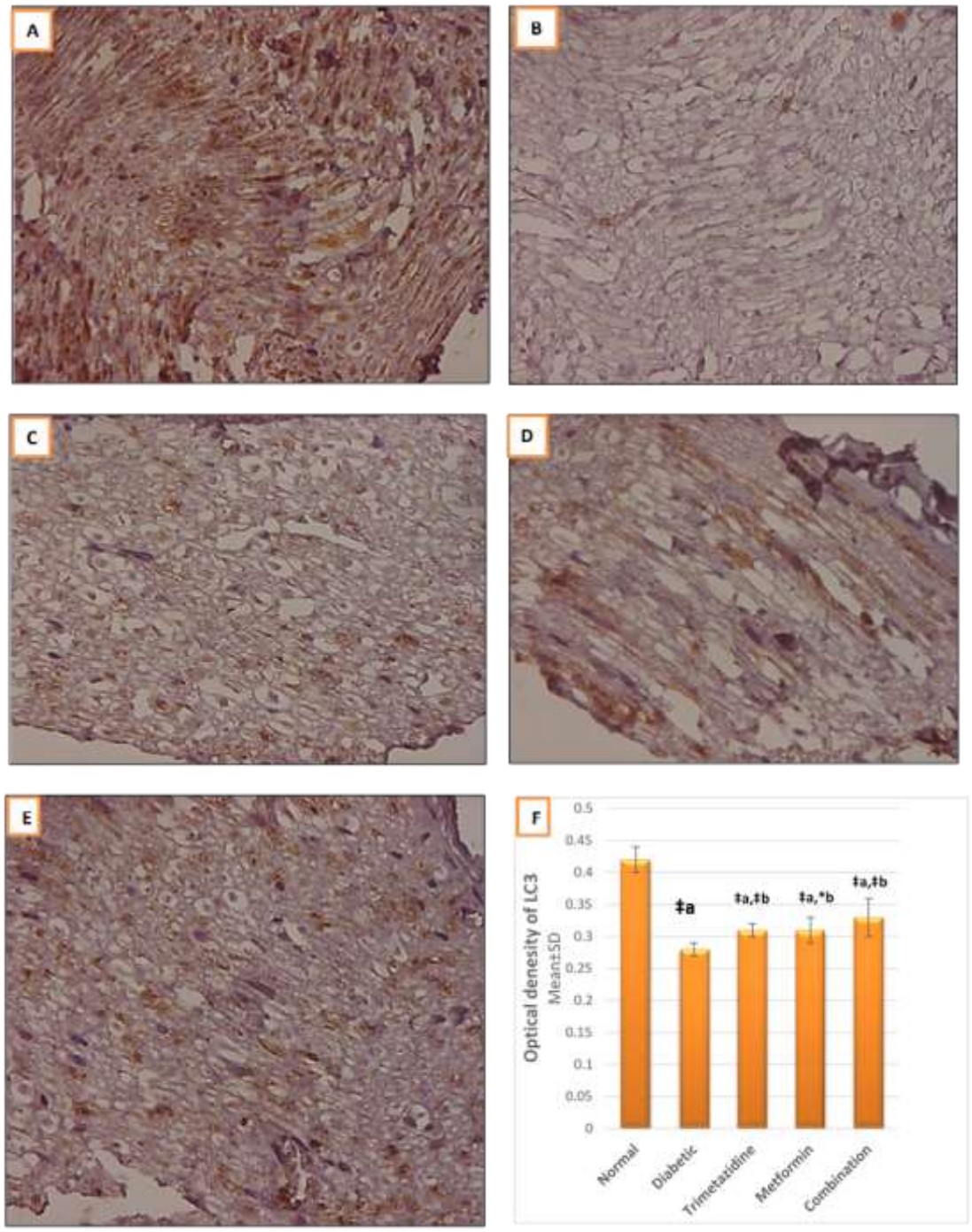
A
B
C
D
E
F
Optical denesity of LC3
Mean±SD
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
‡a
‡a,‡b
‡a,*b
‡a,‡b
Normal
Diabetic
Trimetazidine
Metformin
Combination

Figure2

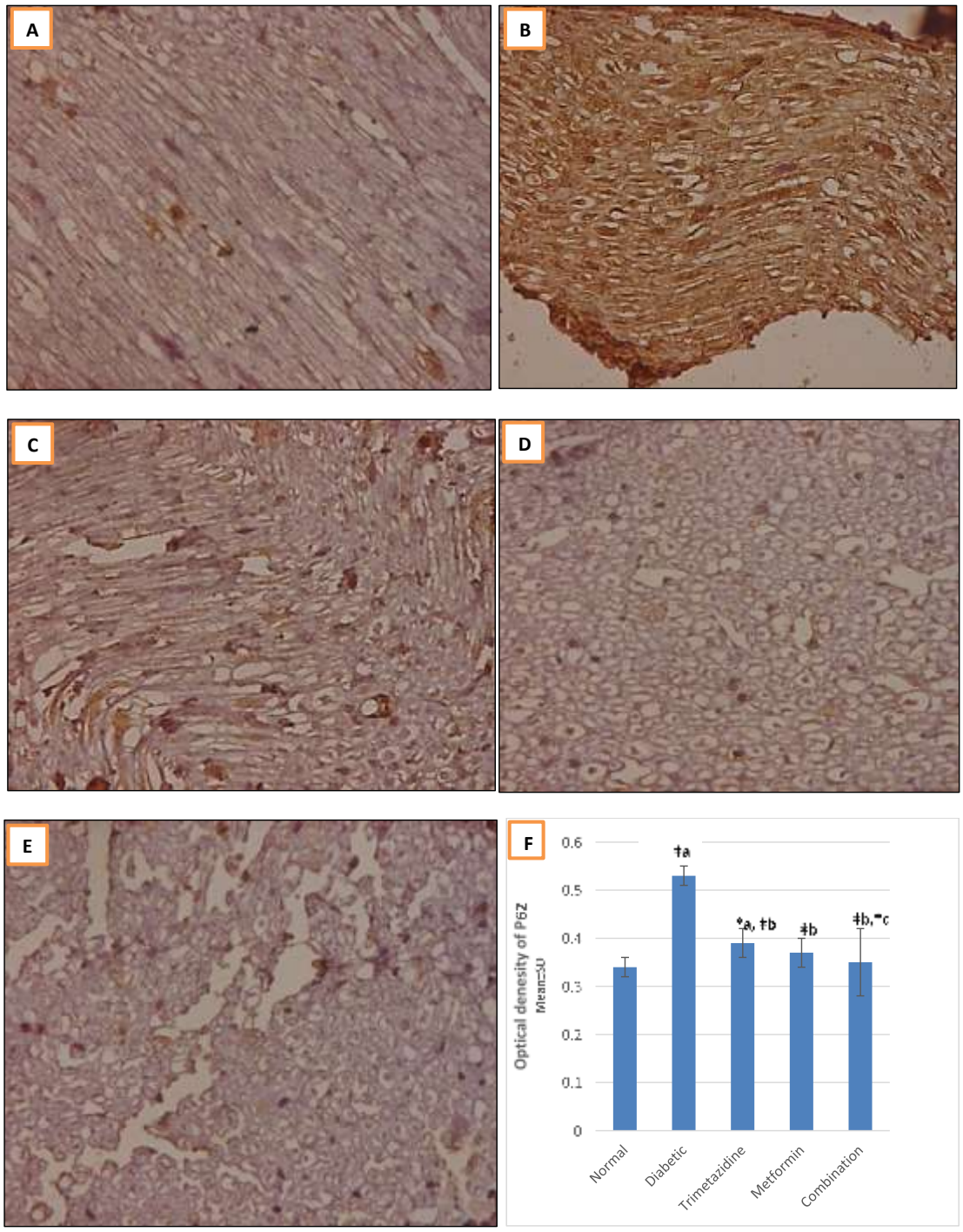
A
B
C
D
E
F
Optical denesity of P62
Mean±SD
0.6
0.5
0.4
0.3
0.2
0.1
0
†a
*a, †b
†b
†b,*c
Normal
Diabetic
Trimetazidine
Metformin
Combination

Figure 3

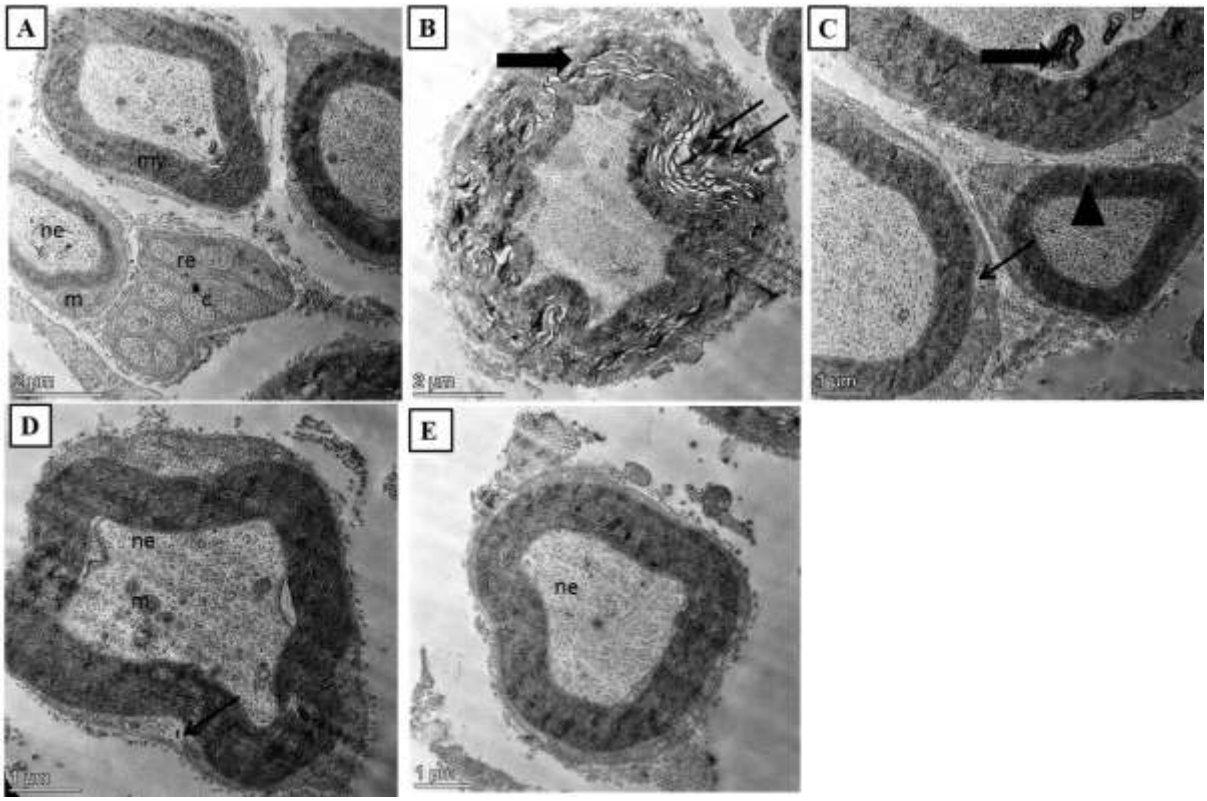

Figure 4