

Effect of Vitamin D₃ vs. Atorvastatin on the progress of Streptozotocin-induced diabetic peripheral neuropathy in rats

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Abstract: Background: Diabetic sensorimotor peripheral neuropathy (DSPN) is a common health problem that is a lifelong disease that affects quality of life. It is a chronic microvascular diabetic complication. The exact cause is not entirely understood. There are many medical therapies, however they have several drawbacks. **The aim of the study:** to evaluate the possible anti-inflammatory, antioxidant, anti-apoptotic effects of vitamin D₃ & atorvastatin each alone or combined in prevention of STZ induced-DSPN in albino rats. **Methods:** This experiment was performed on 50 male albino rats divided into 5 equal groups; (group 1) control group, (group 2) untreated STZ induced-DSPN group, (group 3) vitamin D₃ treated, (group 4) Atorvastatin treated, (group 5) vitamin D₃ & Atorvastatin treated. At the end of the experiment, serum glucose, CPK-MM, sciatic nerve MDA, catalase activity, NGF & IL-6 were assessed as well as histopathological examination and immunohistochemistry for Caspase-3 activity. **Results:** the untreated group showed significant increase in serum glucose, MDA, IL-6 & Caspase-3 and significant decrease in catalase activity & NGF, treatment with either vitamin D₃ or atorvastatin produce significant improvement in the most parameters when compared to untreated group, also their combination produces significant improvement in all parameters & produce better response than each drug alone. **Conclusion:** These findings suggest that each of vitamin D₃ and atorvastatin produce promising effects in prevention of STZ induced-DSPN, also combination of vitamin D₃ and atorvastatin exhibited additional effects superior to each monotherapy. It could be recommended to verify these results in further clinical studies.

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Keywords: DSPN, STZ, Vitamin D₃, Atorvastatin.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease in which hyperglycemia and insulin dysregulation occurs, mostly accompanied by chronic complications. Currently, DM affects more than 425 million people all over the world and this number is progressively increasing. Diabetic peripheral neuropathy (DPN) is a popular disabling complication with an estimated prevalence from 13% to 58% among uncontrolled diabetic patients (Pop-Busui et al., 2017). DPN can affect autonomic and sensorimotor peripheral nervous systems. The most common form is diabetic sensorimotor polyneuropathy (DSPN) (Zenker et al., 2013) and accounts for 75% among diabetic neuropathies. However, DSPN occurs in at least 20% of people as a complication of T1DM. DSPN definition is the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after exclusion of other causes (Pop-Busui et al., 2017). The pathogenesis of DSPN is multifactorial & the exact mechanism of this disease remains poorly understood. Hyperglycemia may be responsible for multiple biochemical changes in the nerve tissue (Stavniichuk et al., 2012). The involved pathological

mechanisms are inflammatory changes, oxidative stress, microvascular damage, metabolic disorders, advanced glycation end products & growth factor deficiency (Zochodne, 2007, Van Dam et al., 2013). Impaired insulin signaling and hyperglycemia are primary pathogenic events and multiple pathways involving protein glycation, loss of trophic support, mitochondrial dysfunction, oxidative stress and inflammation (Daugherty et al., 2018). Patients with DSPN usually experience positive sensory symptoms (pain, tingling and paraesthesia) and negative symptoms (allodynia, hyperalgesia) (Feldman et al., 2017).

In Europe, U.S and Canada, Pregabalin and duloxetine have been approved for treatment of DPN. Tapentadol is a novel FDA approved centrally acting opioid for the treatment of DPN pain. However, many non-approved drugs have been used in the treatment of DPN pain e.g. Amitriptyline, imipramine, tramadol & oxycodone (Pop-Busui et al., 2017).

Streptozotocin (STZ) is an antimicrobial agent and chemotherapeutic alkylating agent (Lenzen, 2008, Furman, 2015). STZ is used to induce DM in experimental animals as it causes necrosis of β -cells of the pancreas through oxidative stress & liberation of

nitric oxide (NO) (Ghasemi et al., 2014) leading to pathological changes that mimic human type I DM. Due to its low cost, it became an attractive model for DM (Furman, 2015).

Vitamin D is an essential fat soluble vitamin; it is formed under the skin during exposure to ultra violet rays or obtained from diet (Sadat-Ali et al., 2014). Nowadays it has been shown to play a role as anti-inflammatory, antioxidant and immunomodulator in primary immune mediated peripheral neuropathies, multiple sclerosis (MS), Alzheimer & Parkinsonism disease (Elf et al., 2014, Wimalawansa, 2016).

Atorvastatin is an antihyperlipidemic drug that acts by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme. Recently it has been shown to have antioxidant action (neuroprotective) in Alzheimer (Barone et al., 2011) and anti-inflammatory action in experimental autoimmune encephalomyelitis & relapsing-remitting MS (Youssef et al., 2002, Aktas et al., 2003).

The present study was designed to evaluate and compare some possible antioxidant, antiapoptotic and anti-inflammatory effects of vitamin D3 and atorvastatin when used either alone or as combined therapy for prevention of STZ induced-DSPN in rats.

2. Material and Methods

Drugs & chemicals:

Atorvastatin tablets 10 mg purchased from Egyptian International Pharmaceutical Industries co. E.I.P.I.CO, Egypt. It was dissolved in saline and administered orally by oral gavage in a dose of 10 mg/kg/day (Li et al., 2014, AKALIN ÇİFTÇİ et al., 2015).

Pure white powder of water soluble form of cholecalciferol from Suprem Pharmaceuticals Mysore Pvt. Ltd. India purchased from October pharma, Egypt. It was dissolved in distilled water and administered orally by oral gavage in a dose of 0.03 µgm/kg/day (El-Hafiz et al., 2018).

STZ is pure yellow powder purchased from Sigma Aldrich, USA. It was used to induce DM in rats by dissolving the drug in 0.1M sodium citrate buffer (pH4.6). The drug was then injected once i.p. in a dose of 60 mg/kg to induce type I diabetes; the blood glucose level was checked 3 days later using a blood sample from the tail to detect diabetes (Lee et al., 2003, Malardé, 2012).

Experimental design:

This experiment was carried out using 50 male Wistar Albino rats weighting 150-200 gm., obtained from Tanta university animal house. Animals were acclimatized in the animal house of Pharmacology department of faculty of medicine Tanta university for 14 days before induction of DM, the animals then were kept in plastic cages; they were fed standard

food and water *ad libitum* through the whole period of the experiment. The animals were divided into 5 equal groups (10 rats each) as following:

Group1: Control normal rats each received 1ml citrate buffer i.p. single dose.

Group2: Diabetic rats injected by single injection of 60 mg/kg STZ i.p. to induce T1DM & received saline orally before & after induction of T1DM for 4 weeks.

Group3: Diabetic rats treated with vitamin D3 orally in a dose of 0.03 µgm/kg/day before & after induction of T1DM for 4 weeks.

Group4: Diabetic rats treated with atorvastatin orally in a dose of 10 mg/kg/day before & after induction of T1DM for 4 weeks.

Group5: Diabetic rats treated with both vitamin D3 and atorvastatin orally in a dose of 0.03 µgm/kg/day & 10 mg/kg/day respectively before & after induction of T1DM for 4 weeks.

After 3 days from induction of STZ, blood glucose level was checked via rat tail sample in all rats to exclude non-diabetic rats (blood glucose level < 200 mg/dl) (El-Hafiz et al., 2018).

After 4 weeks, the **Tail immersion test** was done at the end of the experiment as following: the tail of the rat was immersed at warm water (46° C) until tail withdrawal or signs of struggle were observed (cut off time: 15 sec.). A minimum 10 minutes interval was maintained between each measurement. The test was repeated 2-3 times to obtain a mean value (Courteix et al., 1993, Kamboj et al., 2010).

Then, the rats were anesthetized using ether then blood samples were collected by intracardiac puncture in plain tubes to be centrifuged and serum was separated and stored to be used in detection of serum glucose and creatine phosphokinase (CPK-MM). After that, the rats were sacrificed, and sciatic nerves were dissected. The right one was kept in buffered formalin for histopathological examination & Immunohistochemical assay of caspase-3, and the left one was homogenized and centrifuged, then the supernatant was stored and used for detection of malondialdehyde (MDA), catalase activity, nerve growth factor (NGF), interleukin-6 (IL-6).

Measured parameters:

Serum glucose level (mg/dl): Blood glucose levels were measured in the serum using kit from Biodiagnostic Company according to the technique described by (Trinder, 1969).

Serum creatine phosphokinase (CPK-MM) (pg/dl): CPK-MM was measured in serum by kits obtained from Chongqing Biospes Co., Ltd Company, China, No.: BYEK2098 according to the technique described by (Goldblatt, 1969).

Tissue catalase (U/g): It was measured in the tissue using kit from Biodiagnostic Company, No.CA

25 17 according to the technique described by (Aebi, 1984).

Lipid Peroxides [Malondialdehyde (MDA)] (nmol/g): It was measured in the tissue homogenate using kit from Biodiagnostic Company, No. MD 25 29 according to the technique described by (Kei, 1978, Ohkawa et al., 1979).

Nerve Growth Factor (NGF) (pg/mg): NGF was measured in tissue homogenate by kits obtained from Chongqing Biospes Co., Ltd Company, China, catalog No.: BEK1171 according to the technique described by (Levi-Montalcini, 1987, Fiore et al., 2009).

Interleukin-6 (IL-6) (pg/mg): IL-6 was measured in tissue homogenate by kits obtained from Chongqing Biospes Co., Ltd Company, China, catalog No.: BEK1110 according to the technique described by (Banks et al., 1994).

Histopathological examination: The full thickness sciatic nerve samples were collected and immediately fixed in 10% formalin. Paraffin sections were done (5 micrometer) and stained with heamatoxyllin and eosin (H & E) and examined by light microscope for histopathological changes.

Immunohistochemical staining of Caspase-3: Immunohistochemical study was done to assess the reactivity of the sciatic nerve tissue to the antibody of caspase-3. The caspase protein is a member of the cysteine-aspartic acid protease (caspase) family. This method is according to (Buchwalow and Böcker, 2010). Evaluation of the percent of antigen-positive cells was conducted using a light microscope. Percentage of apoptotic cells per section was scored according to the method described by (BODEY et al., 2004).

Statistical analysis:

Results were analyzed statistically using software Statistical Package for the Social Science (SPSS) for windows, version 23 (SPSS Inc., USA). The level of significance was established as 5% by **One Way ANOVA** followed by **Tukey’s** post-Hoc test. Descriptive statistics were presented as Mean + Standard Error of Mean (SEM). **Kruskal-Wallis test** is a non-parametric test for statistical analysis of the clinical score followed by **Mann-Whitney U** test to test the difference between groups.

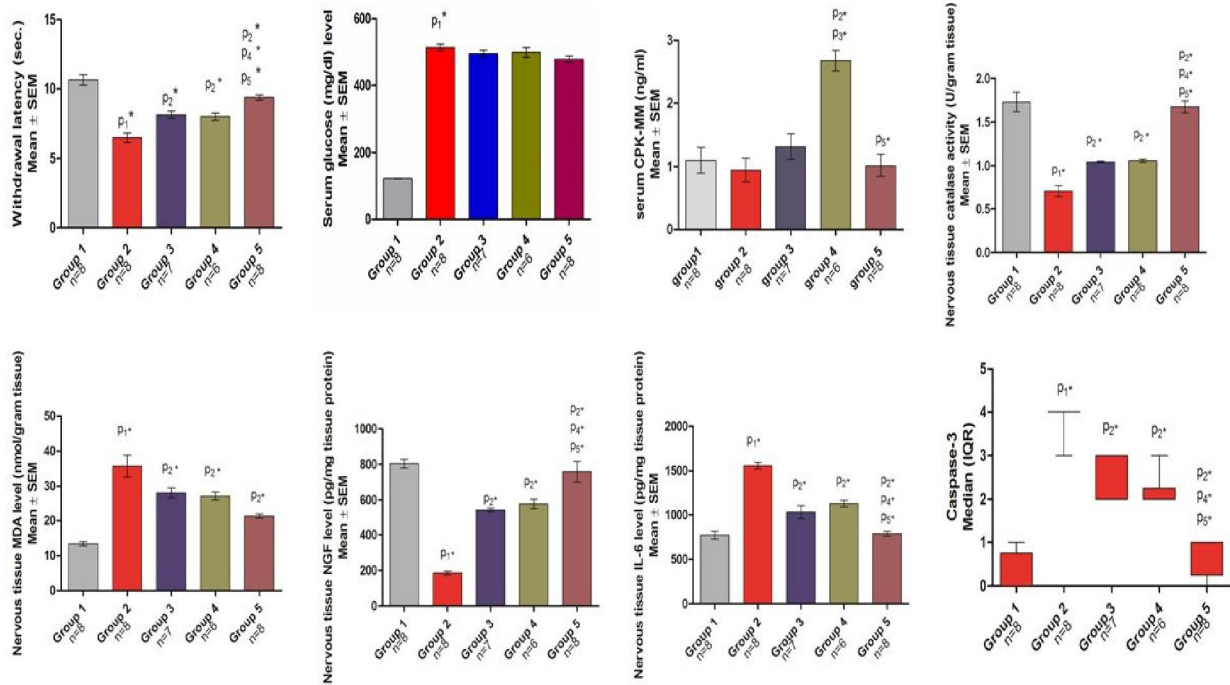
3. Results

Table (1): Collective table for comparison of all parameters in different studied groups.

Parameter	Group 1 (n=8)	Group 2 (n=8)	Group 3 (n=7)	Group 4 (n=6)	Group 5 (n=8)	*One-way ANOVA F value (P value) #Kruskal-Wallis Test X ² value (P value)
Withdrawal latency (sec.)	10.63 ± 0.375	6.5 ± 0.3273 P ₁ <0.001*	8.143±0.261 P ₂ <0.01*	8.00 ± 0.2582 P ₂ <0.05* P ₃ >0.05	9.375 ± 0.183 P ₂ <0.001* P ₄ <0.05* P ₅ <0.05*	29.69 (p<0.001)*
Serum glucose level (mg/dl)	121 ± 0.655	514.1 ± 10.29 P ₁ <0.001*	495.7±10.18 P ₂ >0.05	499.8 ± 14.55 P ₂ >0.05 P ₃ > 0.05	478.9 ± 10.57 P ₂ >0.05 P ₄ > 0.05 P ₅ >0.05	311.3 (P<0.001)*
Serum CPK-MM levels (pg/mg tissue protein)	1.098 ± 0.21	0.944 ± 0.19 P ₁ >0.05	1.314 ± 0.204 P ₂ >0.05	2.675 ± 0.168 P ₂ <0.001* P ₃ <0.001*	1.015 ± 0.179 P ₂ >0.05 P ₄ >0.05 P ₅ <0.001*	11.99 (p<0.001)*
Nerve tissue catalase activity (U/g tissue protein)	1.729 ± 0.112	0.703 ± 0.062 P ₁ <0.001*	1.043 ± 0.01 P ₂ <0.05*	1.057 ± 0.019 P ₂ <0.05* P ₃ > 0.05	1.673 ± 0.069 P ₂ < 0.001* P ₄ <0.001* P ₅ <0.001*	41.06 (P<0.001)*
Nerve tissue MDA activity (nmol/g tissue protein)	13.36 ± 0.587	35.75 ± 3.117 P ₁ <0.001*	28.10 ± 1.462 P ₂ <0.05*	27.21 ± 1.130 P ₂ <0.05* P ₃ > 0.05	21.30 ± 0.615 P ₂ < 0.001* P ₄ >0.05 P ₅ >0.05	24.86 (p<0.001)*
Nerve tissue NGF levels (pg/mg tissue protein)	804.8 ± 25.04	186.1 ± 10.36 P ₁ <0.001*	543.5 ± 11.80 P ₂ <0.001*	577.9 ± 26.28 P ₂ <0.001* P ₃ > 0.05	759.4 ± 57.89 P ₂ < 0.001* P ₄ <0.001* P ₅ <0.01*	59.86 (p<0.001)*
Nervous tissue IL-6 levels (pg/mg tissue protein)	772.1 ± 42.82	1559 ± 35.85 P ₁ <0.001*	1035 ± 75.52 P ₂ <0.001*	1133 ± 37.22 P ₂ <0.001* P ₃ > 0.05	789.2 ± 26.75 P ₂ < 0.001* P ₄ <0.01* P ₅ <0.001*	52.94 (p<0.001)*
Caspase-3 immuno-staining	0 (0.75)	4 (0.00) P ₁ <0.001*	3 (1) P ₂ <0.01*	2 (0.25) P ₂ <0.01* P ₃ > 0.05	1 (0.75) P ₂ < 0.001* P ₄ <0.01* P ₅ <0.01*	27.383 (P<0.001)*

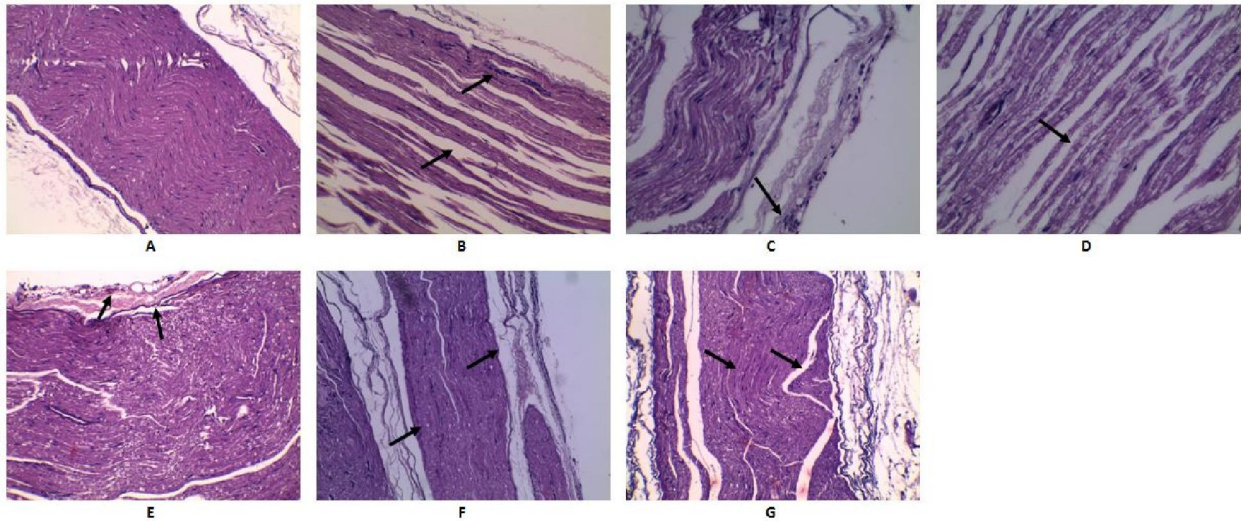
*Significant at P value (< 0.05); values expressed as mean ± SEM. #Significant at P value (< 0.05); values expressed as median (IQR).

P₁= (group 2) versus (group 1), P₂= (group 3), (group 4), (group 5) versus (group 2), P₃= (group 4) versus (group 3), P₄= (group 5) versus (group 3), P₅= (group 5) versus (group 4). n= number.



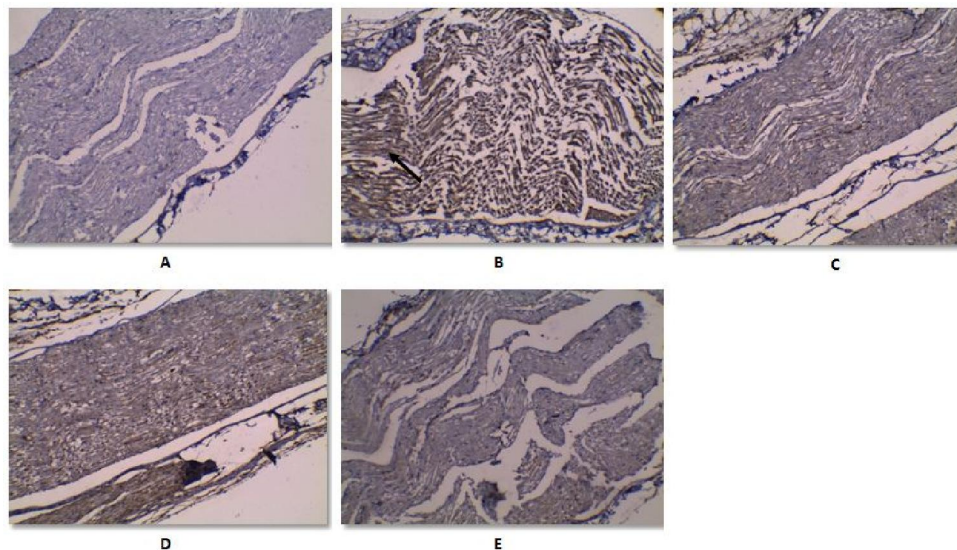
*Significant at P value (< 0.05); P₁= (group 2) versus (group 1), P₂= (group 3), (group 4), (group 5) versus (group 2), P₃= (group 4) versus (group 3), P₄= (group 5) versus (group 3), P₅= (group 5) versus (group 4). n= number

Figure (1): collective statistics of the measured parameters.



A: showed normally arranged sciatic nerve fibers in group1 ($\times 200$), **B:** showed edema & inflammation of sciatic nerve fibers in group2 ($\times 400$), **C:** showed inflammation of the perineurium of sciatic nerve in group2 with inflammatory cellular infiltrate ($\times 200$), **D:** sciatic nerve section of group 2 showing multiple vacuoles of the cytoplasm ($\times 400$), **E:** sciatic nerve section in group 3 showed milder edema & minimal inflammatory cellular infiltrate ($\times 200$), **F:** sciatic nerve section in group 4 showing mild edema & few cytoplasmic vacuoles ($\times 200$), **G:** sciatic nerve section in group 5 showing apparently normal nerve fibers with marked decrease of edema & inflammatory cellular infiltrate ($\times 200$).

Figure (2): histopathological findings in sciatic nerve samples.



A: sciatic nerve fibers in group1 showing normal negativity staining($\times 200$), **B:** sciatic nerve section in group 2 showing strong positively staining of Caspase-3 ($\times 400$), **C:** sciatic nerve section in group3 showing mildly positive staining of caspase-3 ($\times 200$), **D:** sciatic nerve section of group 4 showing mildly positive staining of caspase-3 ($\times 200$), **E:** sciatic nerve section in group 5 showing apparently negative staining of Caspase-3 in the nerve fibers ($\times 200$).

Figure (3): Immunohistochemical staining of caspase-3 in sciatic nerve fibers.

4. Discussions

The present study was designed to evaluate and compare some possible antioxidant, antiapoptotic and anti-inflammatory effects of vitamin D₃ and atorvastatin when used either alone or as combined therapy for prevention of STZ induced-DSPN in rats.

We chose hot tail immersion test in this study as an indicator of pain sensation in the rats. The test measures withdrawal latency in seconds in response to noxious stimulus (Ramabadran et al., 1989). The present study revealed significant decrease of withdrawal latency in the untreated diabetic group in comparison to the control group which agrees with Courteix et al. (1993) who found that the rats that injected by STZ showed signs of hyperalgesia 4 weeks after injection that appeared as diminished withdrawal latency. However, the present study showed significant enhancement of withdrawal latency in vitamin D₃, atorvastatin and combined treated groups in comparison to the untreated diabetic group which agrees with Lee and Chen (2008) who stated that vitamin D supplementation can reduce neuropathic pain in T2DM patients and Pathak et al. (2014) also stated that atorvastatin can enhance withdrawal latency and attenuate neuropathic pain in rat model of neuropathy. However, combined treatment by both vitamin D₃ & atorvastatin showed significant enhancement of withdrawal latency in comparison to monotherapy either by vitamin D₃ or atorvastatin which may be due to additional effect between vitamin D₃ & atorvastatin.

In the present study, the results of untreated STZ induced DSPN group when compared to control group showed significant increase in serum glucose level and this result is in agreement with (Furman, 2015) and (Wu and Yan, 2015).

As regarding to serum CPK-MM, there is significant increase of serum CPK-MM in atorvastatin treated group in comparison to the untreated diabetic group and with vitamin D₃ treated diabetic group which agree with Soinen et al. (2006) & Parker and Thompson (2012) who stated that serum CPK-MM may be elevated with atorvastatin administration which is an indicator of muscle damage. The study showed significant decrease in the combined treated diabetic group in comparison to the atorvastatin treated diabetic group that agree with Glueck et al. (2011) who found that vitamin D supplementation can reverse the atorvastatin induced-muscle adverse effects.

As regarding to the sciatic nerve catalase activity, the present study showed significant decrease in catalase activity in the untreated STZ-induced DSPN in comparison to the control group which agree with Brownlee (2005) who reported that DM can induce DSPN through oxidative stress via ROS. However, treatment either by vitamin D₃, atorvastatin or both showed significant elevation of sciatic nerve catalase activity in comparison to the untreated diabetic group that agree with Eyles et al. (2007) who found that vitamin D₃ has antioxidant activity through different ways including increase expression of catalase enzyme, Chen et al. (2012) also stated that

atorvastatin also increase catalase activity in bronchogenic carcinoma, but disagree with Sezer et al. (2011) who found that atorvastatin has non-significant effect on catalase activity in atherosclerosis. Nevertheless, the present study showed that the combined therapy showed significant increase in sciatic nerve catalase activity in comparison to each alone that may be due to synergistic effect.

Several studies reported the importance of oxidative stress in the pathogenesis of DSPN. The present study measured MDA as a marker for lipid peroxidation. We found that there was a significant increase of sciatic nerve MDA level in the untreated diabetic group in comparison to the control group which agrees with Kumar et al. (2007) who pointed out the important role of MDA in the pathogenesis of DSPN. Moreover, we found significant decrease in MDA levels in the treated diabetic groups either by vitamin D₃, atorvastatin or both in comparison to the untreated diabetic group; this agree with Tarcin et al. (2009) who reported the effect of vitamin D₃ replacement in lowering MDA levels in asymptomatic vitamin D deficient patients on their endothelial functions due to antioxidant effect. Meanwhile, Khodayar et al. (2014) found significant decrease in MDA levels in paraquat-induced pulmonary fibrosis in rats due to atorvastatin antioxidant activity.

The NGF plays a great role in neurite growth & modulating neuropathic pain (Khan and Smith, 2015). This study showed significant decrease in sciatic nerve NGF levels in the untreated diabetic group in comparison to the control group which agree with (Gao et al., 2017). In addition to that, we found that there was significant increase of sciatic nerve NGF level in treated diabetic groups either by vitamin D₃, atorvastatin or both in comparison to the untreated diabetic group which agree with Brown et al. (2003) who stated that vitamin D induces NGF synthesis and promotes neurite outgrowth. Ali et al. (2011) also proved that atorvastatin can increase NGF levels in cases of diabetic retinopathy via restoring the balance between pro-NGF & NGF. The present study showed significant increase in sciatic nerve NGF levels in combined treatment by both vitamin D₃ and atorvastatin in comparison to each alone which may be due additional effect.

ILs are group of cytokines named for their ability to communicate between leukocytes. They are either pro-inflammatory e.g. IL-1, IL-6 & IL-8 or anti-inflammatory e.g. IL-4 & IL-10 (Dewanjee et al., 2018). As the inflammatory process has a key role in the pathogenesis of DSPN, IL-6 was chosen as a marker for inflammation in the present study. It showed significant increase in sciatic nerve IL-6 level in the untreated diabetic group in comparison to the

control group which agree with Navarro and Mora (2005) who found increased IL-6 levels in experimental DM. The present study showed significant decrease in sciatic nerve IL-6 levels in the diabetic treated groups either by vitamin D₃, atorvastatin or both in comparison to the untreated diabetic group that agree with Gopal et al. (2018) who pointed out the anti-inflammatory effect of vitamin D via lowering IL-6 levels in rheumatoid arthritis. Moreover, Barsante et al. (2005) found that atorvastatin exhibit anti-inflammatory and analgesic effects in rat model of arthritis via local inhibitory effect on IL-6 expression. In addition to that, the present study showed that combined therapy by both vitamin D₃ and atorvastatin showed significant decrease in sciatic nerve IL-6 levels in comparison to monotherapy by each alone which may be due to additional effect.

According to Tolkovsky (2002), apoptosis plays a key role in the pathophysiology of DSPN. One of the hallmarks of apoptosis is condensation and fragmentation of DNA via cleavage and activation of caspase-3 (Sahara et al., 1999). The present study showed significant increased in caspase-3 expression in sciatic nerve fibers in the untreated diabetic group in comparison to the control group which agrees with Cheng and Zochodne (2003) who found increased immunoreactivity of caspase-3 in sciatic and tibial nerves in diabetic rats. In addition to that, the study showed significant decrease of caspase-3 expression in treated diabetic groups by vitamin D₃, atorvastatin and both in comparison to the untreated diabetic group which agrees with Riachy et al. (2002) who documented that vitamin D protects human islet cells via activation of antiapoptotic protein A20. Also, Jazi et al. (2017) reported the antiapoptotic effect of atorvastatin in myocardial infarction in rats. Moreover, there was significant reduction of caspase-3 expression in combined treated group in comparison to monotherapy by each which may be due to additional effect between vitamin D₃ and atorvastatin.

Many of the drugs used in the treatment of DSPN have side effects hindered their use for long periods, it is logical to search for and choose other safe drugs for long-term prevention.

Conclusion:

These findings suggest that each of vitamin D₃ and atorvastatin produces promising effects in prevention of STZ induced-DSPN as each of them decreases inflammation, apoptosis, and oxidative stress and improve histopathological picture that's all reflected as amelioration in withdrawal latency. However, combination of vitamin D₃ and atorvastatin provided significant additional amelioration on the disease activity when compared to each monotherapy

either by vitamin D₃ or atorvastatin as this combination exhibited additional effect superior to each monotherapy in regard to improvement of inflammation, anti apoptotic and antioxidant effect.

As a conclusion; either vitamin D₃ or atorvastatin or their combination can be used as preventive therapy to decrease the incidence of DSPN.

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