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Targeting Altered Mitochondrial Biogenesis in the Brain of Diabetic Rats: Potential Effect of Pioglitazone and Exendin-4

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Abstract

Background: Neuroprotective mechanisms triggered by peroxisome proliferator-activated receptor-gamma agonist: pioglitazone (PIO) and glucagon-like peptide 1 analog: exendin-4 (Ex-4) in neurological diseases were reported, but whether mitochondrial biogenesis is involved or not in their neuro-protective mechanisms in type 1 Diabetes Mellitus (T1DM); has not been studied before. To bridge this gap, we investigated the effect of PIO and Ex-4 on brain mitochondrial biogenesis in streptozotocin- induced diabetes in rats.

Methods: Seven weeks after induction of diabetes in rats, serum fasting glucose and insulin were measured in studied groups. The brain was removed for histological analysis and assessment of: mitochondrial complexes I and II, ATP, H_2O_2, brain derived neurotrophic factor (BDNF), cytochrome c and hemeoxygenase (HO)-1 activity, and relative gene expression of the nuclear factor; *Nrf2* and the apoptotic markers: *bax* & *bcl2* and mitochondrial biogenesis markers; peroxisome proliferator–activated receptor γ coactivator (*PGC) 1-α* and sirtuin 1 (*SIRT-1)* and AMP-activated protein kinase (AMPK) and c-Jun-N-terminal kinase (JNK) proteins.

Results: Brain in untreated rats showed neurodegeneration area and significantly rising H_2O_2 and JNK, up-regulation of *bax*, down-regulation of *bcl2*. These changes were paralleled with significant reduction in *Nrf2*, HO-1, BDNF, complex I, II and ATP and *SIRT-1/ PGC1-α* expression. PIO and Ex-4 significantly improved the reported changes. Combined modality showed better improvement relative to each drug alone.

Conclusions: PIO and Ex-4 may have neuroprotective effects in T1DM, via targeting altered mitochondrial biogenesis probably due to modulation of brain *SIRT-1* signaling, improvement of oxidative stress and equilibrating the balance between pro-apoptotic and anti-apoptotic mediators.

Keywords: Brain derived neurotrophic factor, Diabetic neurodegeneration, Exendin-4, oxidative stress, PGC1- α.

Introduction

Diabetics, especially those with poor glycemic control, had increased risks of brain complications (1). Previous studies suggested that, decreased antioxidant defenses in brain mitochondria of diabetic rats may play a role in the development of diabetic complications (2)

Mitochondrion has a central role in controlling the bioenergetic status of neurons; therefore, its dysfunction is considered a prime trigger of neurodegeneration. An important transcriptional coactivator driving mitochondrial metabolism and biogenesis at the molecular level is the peroxisome proliferator–activated receptor gamma coactivator (PGC)-1α. It appears to have a pivotal role in various brain diseases, Wang et al., (3) reported an altered expression of brain PGC-1α in Parkinson's

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and Alzheimer's diseases. However, its role in diabetes induced neurodegeneration is still unclear.

PGC-1α as a molecular switch in different mitochondrial metabolic pathways can potentiate the activity of mitochondrial sirtuins (SIRT) which deacetylates several key transcription factors leading to up-regulation of several genes of mitochondrial biogenesis (4).

Interestingly, Hull et al (5) described hemeoxygenase (HO)-1 role in regulating mitochondrial quality control. They showed that, HO-1 overexpression protecting the heart from oxidative injury via upregulating protein expression of PGC1α, thus promoting mitochondrial biogenesis (6).

Brain derived neurotrophic factor (BDNF), a well-established neurotrophic factor promotes growth, survival, differentiation and plasticity of neurons, can also regulate brain energy and glucose homeostasis (7). Several studies have recorded its neuroprotective effects in different mouse models, (8, 9) but its role on diabetic models with its underlying mechanisms still needs further investigations.

Recent studies have shown that thiazolidinedione, the peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist demonstrated protective effects in a Parkinson's disease model (10, 11). The protective mechanisms were shown to be attributed to anti-inflammatory (10) and antioxidative (11) effects, but the specific mechanism is not clear.

Accumulating evidence suggests that glucagon-like peptide 1 (GLP-1) analogs can cross the blood–brain barrier to influence several cellular pathways, such as neuro-inflammation, mitochondrial function, and cellular proliferation. Furthermore, GLP-1 receptor stimulation results in improvement in motor and non-motor deficits in Parkinson's disease models (12).

Wassef et al (13) demonstrated that PPAR-γ agonist and a GLP-1 analog, have been shown to exert glycemic control and improvement in mitochondrial functions in diabetic cardiomyopathy. However, their effects on mitochondrial function in the brain under diabetic condition have not been investigated before.

In this study we aimed to analyze mitochondrial biogenesis in the brain of streptozotocin (STZ) diabetic rats and the potential effect of PPAR-γ agonist and/or GLP-1 analog administration.

Materials and methods

Animals

This study included 30 adult male Wister rats of matched weight (120-170 gm). Animals were inbred in the experimental animal unit, Faculty of Medicine, Cairo University in the period from January 2018 to July 2018.

Ethics approval and consent to participate

Our research follows internationally recognized guidelines on animal welfare. The experiments undertaken guide for the care and use of laboratory animals published by the USA National Institute of Health (NIH, 1985), and approved by the local ethical and scientific committee (Faculty of Medicine- Cairo University).

Experimental design

After a week of stabilization, six rats were randomly selected as normal control group (group 1), which was received 0.5 ml of citrate buffer by I.P. injection, and then they were maintained on usual care for the next seven weeks. The remaining rats were used to establish STZ-induced Type 1 Diabetes Mellitus (T1DM) model according to Thulesen et al (14) The STZ powder was purchased from Sigma, it was dissolved in 0.1 M sodium citrate buffer (pH 4.5) and immediately administered I.P. at a dose of 60 mg/kg, as recommended by the National Institute of Diabetes Animal Models of Diabetic Complications Consortium.

Fasting blood glucose was above 250 mg/dl in all collected samples three days after the induction.

Those DM model rats were maintained untreated under usual care for 3 weeks, a duration supposed to be enough for developing the diabetic-associated brain changes (15), then they were randomly divided into four groups each include six as follows:

Group 2 (D): This group included the diabetic rats which were maintained on usual care and was left untreated for the next 4 weeks.

Group 3 (D+PPAR): diabetic rats were administered pioglitazone; a PPAR γ agonist, (10

mg/kg/day, orally) by oral gavage for the next 4 weeks (16).

Group 4 (D+EX): the diabetic rats received Ex-4 (Bio Basic Inc. Canada) I.P. daily at a low dose of 1 nmol/kg as modified from Liu et al, (17) for the next 4 weeks.

Group5 (D+PPAR+EX): diabetic rats received both pioglitazone and Ex-4, in dosage as in groups 3 and 4, respectively, for the next 4 weeks.

Study procedures

At the end of the experimental protocol, all rats were subjected to overnight fasting then we collected blood samples to measure fasting serum glucose and insulin. Also, changes in body weights in all groups were recorded at the end of the experiment.

Then all rats were sacrificed using lethal dose of ether. After which, the skull was broken using a bone cutter at the temporal regions, to expose the brain. Brain specimens were removed for histological and biochemical assessment of the Oxidation markers; H2O2 level, HO-1 activity and the nuclear factor erythroid-derived 2-like 2 (Nrf2) relative expression, Apoptotic markers; bax and bcl2 by relative expression and BDNF protein level.

Mitochondrial functions were estimated by the assessment of the mitochondrial complex (1& 2) activities, ATP content, cytochrome c, AMP-activated protein kinase (AMPK), c-Jun-N-terminal kinase (JNK) proteins relative expression by western blot and SIRT-1 and PGC-1α genes relative expressions by PCR.

Specimens from frontal lobe were embedded into paraffin wax. Coronal sections of 5 µm thickness were prepared and subjected to hematoxylin & eosin (H&E) staining. Histological studies were done in the Medical Histology and Cell Biology Department (Faculty of Medicine, Cairo University).

Isolation of mitochondrial fraction

Mitochondria were isolated from brain by differential centrifugation according to the method described by Gumustaz et al (18).

Biochemical assessments

Fasting serum glucose level was assessed by oxidase-peroxidase method. The test materials were supplied as kits by "Diamond Diagnostics". Fasting serum insulin level estimation was done by enzyme-linked immunosorbent assay kit procured from Monobind Inc. (Lake Forest, California, USA).

HO Enzyme Activity Assay: brain tissues were homogenized with 2.5 volume Tris-HCl buffer (10 mmol/L, pH 7.6) containing 250 mmol/L sucrose and 0.4 mmol/L phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 800 g for 10 minutes and then at 13,500 g for 20 minutes. The supernatant was withdrawn. The protein content was determined by the method of Lowry et al (19) The activity of HO in the supernatant was determined as previously described (20, 21).

Determination of the Mitochondrial NADH dehydrogenase activity (complex I): the enzyme specific activity was measured according to the method described by Birch-Machin et al (23). Complex I activity was expressed in nanomoles per minute per milligram of protein. Protein levels were detected according to Lowry et al (19).

Determination of the mitochondrial succinate dehydrogenase (SDH) activity (complex II): Complex II specific activity was measured following the reduction of 2,6 dichlorophenol indophenol at 600 nm using the method of Birch-Machin et al (22).

Measurement of ATP content: ATP level was detected as previously described (23) using ATP Assay Kit, Beyotime, Jiangsu, China. ATP content was determined by comparison to a concurrent standard curve and was then normalized by protein concentration and expressed as µmol/mg protein.

Estimation of Hydrogen Peroxide (H2O2): Its levels were measured by the method of Pick (24) Its standard curve was plotted by taking different concentrations of H2O2, ranging from 20 to 100 mmol in a total volume of 100 uL and processed in the same way. Results are expressed as mmol H2O2 formed/mL homogenate.

Estimation of BDNF and cytochrome c: they were assayed by commercially available ELISA kits (MyBiosource, USA) according to the manufacturer's instructions.

Detection of AMPK and JNK by Western Blot technique (using V3 Western Workflow™ Complete System, Bio-Rad® Hercules, CA, USA)

The protein was transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA)

with a Bio-Rad Trans-Blot system. Following blocking, the blots were developed using antibodies for AMPK, JNK and beta actin supplied by (Thermoscientific, Rockford, Illinois, USA). Band intensity was analyzed by ChemiDocTM imaging system with Image LabTM software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results were expressed as arbitrary units after normalization for β-actin protein expression.

Detection of gene expression of bax, bcl2, Nrf2, PGC1α & SIRT1 in brain tissue by Quantitative real time polymerase chain reaction (qRT-PCR)

Tissue samples of all the studied groups were lysed and total RNA was isolated with RNeasy purification reagent (Qiagen, Valencia, CA). The purity of total RNA was measured with a spectrophotometer and the wave length absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA was carried out with reverse transcription reaction (Superscript II, Gibco Life Technologies, Grand Island, NY, USA). Real-time PCR amplification and analysis were carried out using an Applied Biosystem with software version 3.1 (StepOne^{TM}, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs which were shown in Table 1, cDNA and nuclease-free water. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative cycle threshold method (25) All values were normalized to β-actin mRNA which was used as an internal control in all samples.

Table 1. Primers Sequence of studied genes

Gene	Sequence	Gene bank accession number
Bax	Forward primer:5′ CCCTGTGCACTAAAGTGCCC3′ Reverse primer: 5′-CTTCTTCACGATGGTGAGCG3′	XM_017603557.1
bcl2	Forward primer:5′ CGGGAGAACAGGGTATGA 3′ Reverse primer: 5′ CAGGCTGGAAGGAGAAGAT 3′	NM_016993.1
Nrf2	Forward primer: 5′-CCTCAACTATAGCGATGCTGAATCT-3′ Reverse primer: 5′-AGGAGTTGGGCATGAGTGAGTAG-3′	NM_001131722.1
PGC1 α	Forward primer:5′- AAACTTGCTAGCGGTCCTCA-3' Reverse primer:5′- TGGCTGGTGCCAGTAAGAG-3'	NM _00894
SIRT1	Forward primer:5′- GATCTCCCAGATCCTCAAGCC-3' Reverse primer:5′- CACCGAAGGAACTACCTGAT-3'	XM_001086493
Beta actin	Forward primer: 5' - GACGGCCAGGTCATCACTAT -3' Reverse primer: 5' - CTTCTGCATCCTGTCAGCAA -3'	NM_007393.5

Nrf2: Erythroid 2-related factor 2; ***PGC1 α***: Peroxisome proliferator-activated receptor gamma coactivator1-alpha; ***SIRT-1 :***sirtuin 1

Statistical methods

Data were coded and entered using the statistical package SPSS (version 24; IBM, New York, USA). Data were summarized using mean and standard deviation (SD). Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test were used. Correlations between quantitative variables were done using Spearman correlation coefficient**.** P-values less than 0.05 were considered as statistically significant.

Results

Assessment of fasting serum glucose, fasting serum insulin and body weight

The clinicopathological features of our CRC as shown in Table 2, there was a significant rise ($p<0.001$) in the mean fasting serum glucose levels in the non-treated diabetic group compared with the control group and the treated groups. No significant differences were found in the mean fasting serum glucose levels between treated groups with either PIO or Ex-4 but there was a

significant decrease (p<0.001) in them in the combined therapy treated-group compared to the single modality treated- groups.

There was a significant decrease (p<0.001) in the mean fasting serum insulin levels in the non-treated diabetic group compared with the control group. All treated groups showed significant improvement (p<0.001) in insulin levels compared with the non-treated diabetic group. No significant differences were found in the mean fasting serum insulin levels between the treated groups with either PIO or Ex-4. Interestingly, there was a significant increase (p<0.001) in the combined therapy treated group compared with the single modality treated groups, that was approaching the control level.

There was a significant decrease (p<0.001) in the mean levels of body weights in the non-treated diabetic group compared with the control group. No significant differences were found in the mean levels of body weight between the non-treated diabetic group and all other treated groups.

Table 2. Levels of Fasting serum glucose, Fasting serum insulin and body weight changes in the studied groups:

	Control (n=6)	Diabetic (n=6)	Diabetic + PPAR (n=6)	Diabetic + Exendin (n=6)	Diabetic + PPAR Exendin (n=6)
Fasting serum glucose (mg/dl)	93.50±5.244	271.17±17.325*****	179.33±5.854 ***#**	170.17±11/873 ***#**	123.67±12.160 ***#$%**
Fasting serum insulin (ng/ml)	2.015±0,174	0.51±0.11 *	1.03±0.15 *#	1.36±0.26 *#	1.71±0.195 **#$%**
Body weight (g)	182.8±0.14	119.6±20.4 *	142.5±19.35 *	113.5±8.06 *	123.3±20.6 *

*: Statistically significant as compared with control at P ≤ 0.05.
#: Statistically significant as compared with the diabetic group at P ≤ 0.05.
$: Statistically significant as compared with the diabetic+ PPAR group at P ≤ 0.05.
%: Statistically significant as compared with the diabetic+ exendin group at P ≤ 0.05.

Results of oxidative stress generation and inhibition

As observed in Figure 1, induction of diabetes showed a significant elevation of H_2O_2 levels (P<0.001) in association with significant reduction (P<0.001) of the protective oxidative enzyme HO-1 activity compared to control group, indicating its association with oxidative stress (Fig. 1). As HO-1 activity is regulated by Nrf2, *Nrf2* expression in brain tissue was assessed. Its level was shown in Figure 2 to be decreased significantly (P<0.001) in diabetic rats in relation to control (Fig. 2). Supporting to these results, HO-1 activity was positively correlated (r= 0.867, p< 0.001) with *Nrf2* expression in brain tissue. In addition, both HO-1 and *Nrf2* expression were negatively correlated with H_2O_2 level (r= -0.914, r = -0.876 respectively, P<0.001 for both). PIO administration protected diabetic rats from oxidative stress as indicated by decreased H_2O_2 levels significantly (P<0.001) as well as stimulating mitochondrial biogenesis confirmed by significant increase in HO-1 activity (P = 0.005) and *Nrf2* expression (P<0.001) in brain tissue. Diabetic rats' response to Ex-4 administration showed almost the same results. Combined PIO and Ex-4 administration showed insignificant improvement in H_2O_2 levels, HO-1 and *Nrf2* compared with either drug alone, on the other hand it reached a comparable level to that of the control values (p>0.05).

Results of mitochondrial function assessment in brain tissue

The activity of mitochondrial enzymes; complex I and complex II (Table 3) and ATP levels were assessed in the isolated mitochondria in all groups. Type I DM was associated with significant decrease in the activity of; complex I (p=0.006), complex II (p=0.001) and ATP (p<0.001) relative to controls. After administration of PIO and Ex-4, either separately or combined significant improvement was observed in complex II and ATP levels. Although non-significant improvement was noticed in complex 1 in treated groups, yet it appeared comparable with that of control group (p> 0.05).

Results of cytochrome c and pro-apoptotic: bax, and anti-apoptotic markers: bcl2

As shown in Table 4, diabetic group showed significant increase in cytochrome c ($p=0.003$) in association with increased bax gene ($p<0.001$), while antiapoptotic bcl2 gene was significantly decreased ($p<0.001$) compared to control group indicating stimulated apoptotic cascades in parallel with mitochondrial injury in the diabetic brain. All treated group showed partial protection against such changes compared with diabetic group. It is worth noting that the values of cytochrome c in the three treated groups showed no significant difference ($p > 0.05$) relative to control group. Correlation studies also revealed the positive correlation between HO-1 and *bcl2* ($r= 0.864$, $p < 0.001$), with the negative correlation between H_2O_2 and *bcl2* ($r= -0.917$, $p < 0.001$), emphasizing the role of HO-1 as antioxidative enzyme in improving mitochondrial injury, oxidative stress and apoptosis.

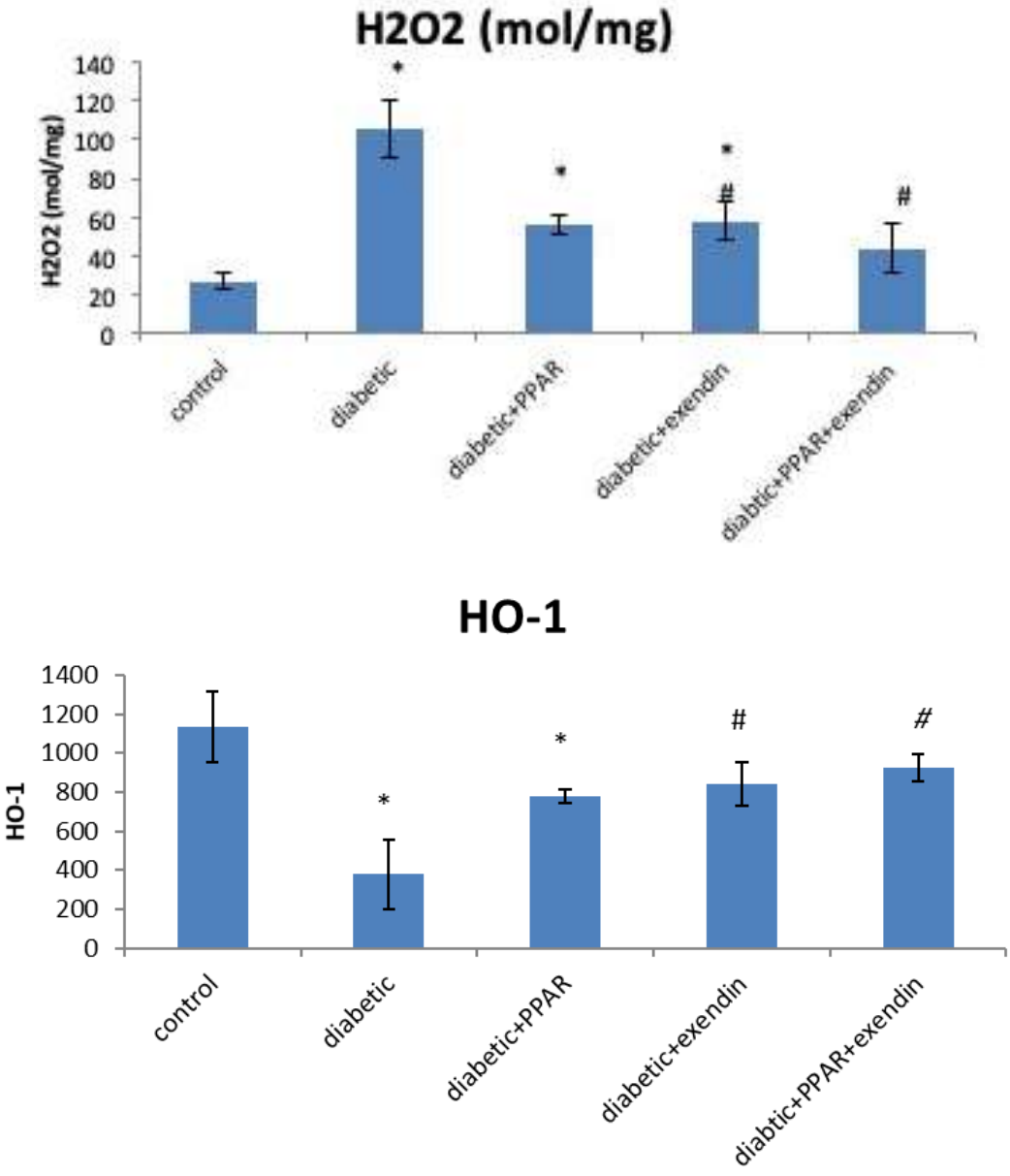

Fig. 1. Levels of: hydrogen peroxide (H_2O_2); heme oxygenase-1 activity (HO-1) in all studied groups. Values are represented as mean ± S.D, (n = 6), *Statistically significant as compared with control at $P \leq 0.05$, # Statistically significant as compared with the diabetic group at $P \leq 0.05$.

Table 3. Levels of brain mitochondrial complexes 1 and 2 and ATP in the studied groups.

	Control (n=6)	Diabetic (n=6)	Diabetic + PPAR (n=6)	Diabetic + Exendin (n=6)	Diabetic + PPAR +Exendin (n=6)
Complex 1 (ng/mg)	5.17± 1.72	1.54± 1.05 *	3.50± 0.88	3.23± 1.04	3.70± 1.07
Complex 2 (ng/mg)	8.78± 2.00	2.25± 0.58 *	4.85± 0.72 *#	4.40± 0.56 *	6.00± 0.55 *#
ATP (umol/mg protein)	0.91± 0.04	0.35± 0.14 *	0.65± 0.06 *#	0.68± 0.07 #	0.77± 0.14 #

Values are presented as mean ±SD

*: statistically significant compared to corresponding value in control group (P<0.05).

#: statistically significant compared to corresponding value in diabetic group (P<0.05).

Table 4. Levels of Cytochrome c (ng/mg) and relative expression *of Bax* and *Bcl2* in the studied groups.

	Control (n=6)	Diabetic (n=6)	Diabetic+ PPAR (n=6)	Diabetic + Exendin (n=6)	Diabetic+ PPAR + Exendin (n=6)
Cytochrome c	0.58±0.22	2.80±1.41*	0.82±0.21 #	0.76±0.26 #	0.72±0.25 #
Bax	1.00±0.01	6.00±1.89*	3.61±1.06 *#	3.30±0.37 #	2.32±0.48 #
Bcl2	1.00±0.01	0.26±.15 *	0.66±0.10 *#	0.62±.09 *#	0.73±0.10 *#

Values are represented as mean ± S.D., (n = 6),

*: Statistically significant as compared with control at $P \leq 0.05$;

#: Statistically significant as compared with the diabetic group at $P \leq 0.05$.

Regulation of AMPK and JNK signaling pathway mediated by oxidative stress and apoptosis:

Induction of diabetes caused significant activation in both AMPK ($p < 0.001$) and JNK ($p = 0.003$) pathways in relation to control. Up-regulation of JNK pathway was positively correlated with H_2O_2 level and *bax* ($r= 0.747$ and $r= 0.771$ respectively, $p < 0.001$) and negatively correlated with *bcl2* ($r= - 0.812$, $p < 0.001$) (Figs. 3A and 3B).

Ex-4 treatment showed significant reduction in AMPK ($p = 0.006$) compared with non-treated diabetics, however, combined treatment showed more reduction in AMPK expression ($p < 0.001$) and approaching the control values. All treated groups showed insignificant reduction in JNK expression compared to diabetics.

Relative expression of PGC-1 and SIRT1 in brain tissue

The expression levels of *PGC-1α* markedly decreased ($p < 0.001$) in diabetic group when compared with the control group. Following combined treatment, *PGC-1α* expression was significantly increased ($p = 0.04$) relative to diabetic group and approaching the control values ($p > 0.05$) (Fig. 3B).

SIRT-1 expression levels were markedly decreased ($p < 0.001$) in non-treated diabetic group compared to control group. Ex-4 treatment an combined treatment caused significant improving effect ($p < 0.05$) on *SIRT-1* expression levels compared to untreated diabetics.

Since PGC-1α is an important transcriptional coactivator, correlation studies were done between *PGC-1α* and *SIRT-1*, JNK & AMPK. It was positively correlated with *SIRT-1* ($r= 0.792$, $p < 0.001$), HO-1 ($r= 0.770$, $p < 0.001$), and its regulator *Nrf2* gene expression ($r= 0.884$, $p < 0.001$) and negatively correlated with *JNK* ($r= -0.554$, $p = 0.01$) and *AMPK* ($r= -0.879$, $p < 0.001$).

Regulation of BDNF protein in the brain tissue

As shown in Figure 3, BDNF was markedly depressed ($p < 0.001$) in diabetic group relative to control group. These values improved significantly ($p < 0.001$) in all treated groups compared to diabetic group with no significant difference ($p > 0.05$) in-between.

The role of BDNF in this study was supported by its positive correlations with signaling pathways *SIRT-1/PGC-1α* ($r= 0.750$, $r= 0.709$ respectively, $p < 0.001$), with antioxidative markers (HO-1 and *Nrf2*) ($r= 0.881$, $r= 0.861$ respectively, $p < 0.001$), with complex I ($r = 0.605$, $p = 0.005$), complex II ($r = 0.728$, $p < 0.001$) and ATP ($r = 0.856$, $p <0.001$). We reported negative correlations between BDNF and the pro-apoptotic markers: cytochrome c and *bax* ($r= -0.731$, $r= -0.807$ respectively, $p < 0.001$).

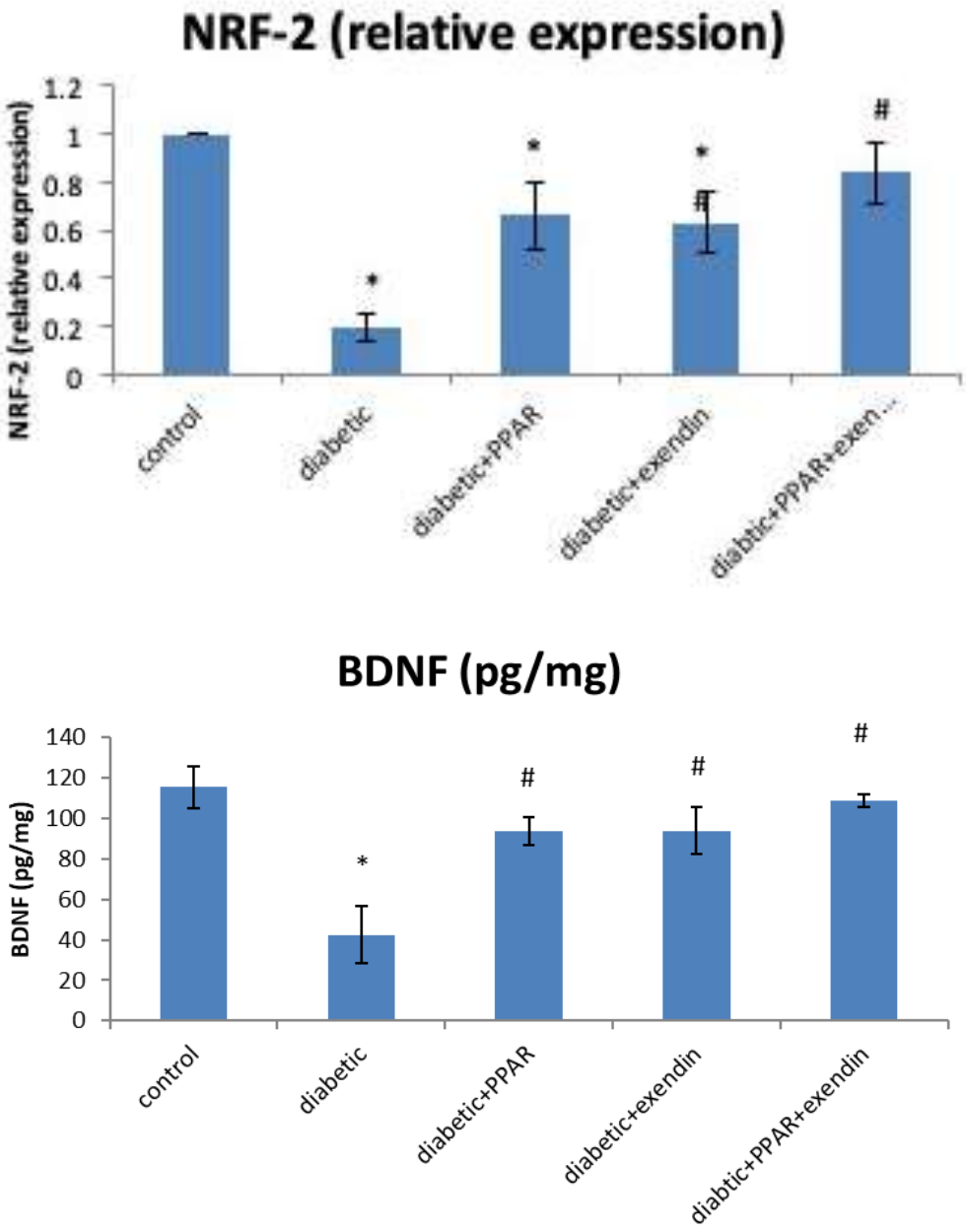

Fig. 2. Levels of BDNF protein and brain relative expression of NF-E2 related factor (Nrf) 2; level in brain tissue in all studied groups. Values are represented as mean ± SD, (n = 6), *Statistically significant as compared with control at $P \leq 0.05$; #Statistically significant as compared with the diabetic group at $P \leq 0.05$.

Results of histological examination

Low power examination (×200) of H&E stained cerebral sections of external pyramidal layer (EPL) and high-power examination (×400) of H&E stained cerebral sections of internal pyramidal layer (IPL) in all studied groups (Fig. 4). Diabetic rats, EPL and IPL exhibited acidophilic neurons and deformed neurons with absence of normal pyramidal neurons. Treated diabetic rats with either Ex-4 or with PPAR, showed fewer deformed neurons and fewer acidophilic neurons in the EPL and the IPL than that were seen in untreated diabetic groups with appearance of some preserved normal pyramidal neurons. In diabetic rats treated with combined modality, fewer deformed neurons and fewer acidophilic neurons in the EPL and the IPL than that were seen in the treated diabetic group with each drug alone with appearance of more preserved normal pyramidal neurons.

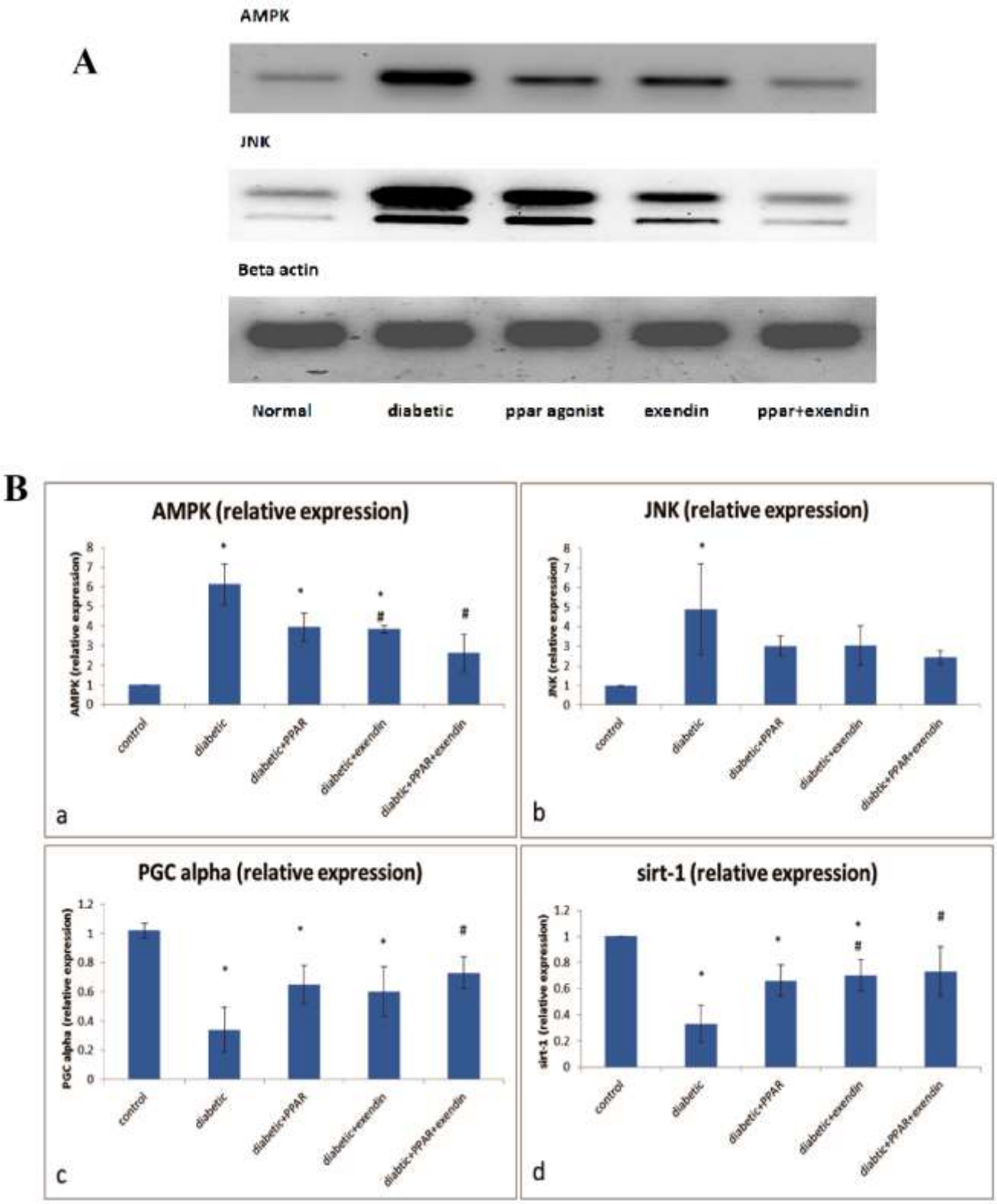

Fig. 3. Relative gene expression of studied genes and the levels of AMPK and JNK proteins

A: Western blot of AMPK and JNK proteins.

B: The relative gene expression of AMPK; JNK; *SIRT-1*; *PGC* 1-α in all studied groups. AMPK: AMP-activated protein kinase; JNK: c-Jun-N-terminal kinase; SIRT-1: sirtuin 1; PGC1 α: Peroxisome proliferator-activated receptor gamma coactivator1-alpha. Values are represented as mean ± S.D, (n = 6), *Statistically significant as compared with control at $P \leq 0.05$; #Statistically significant as compared

with the diabetic group at P ≤ 0.05.

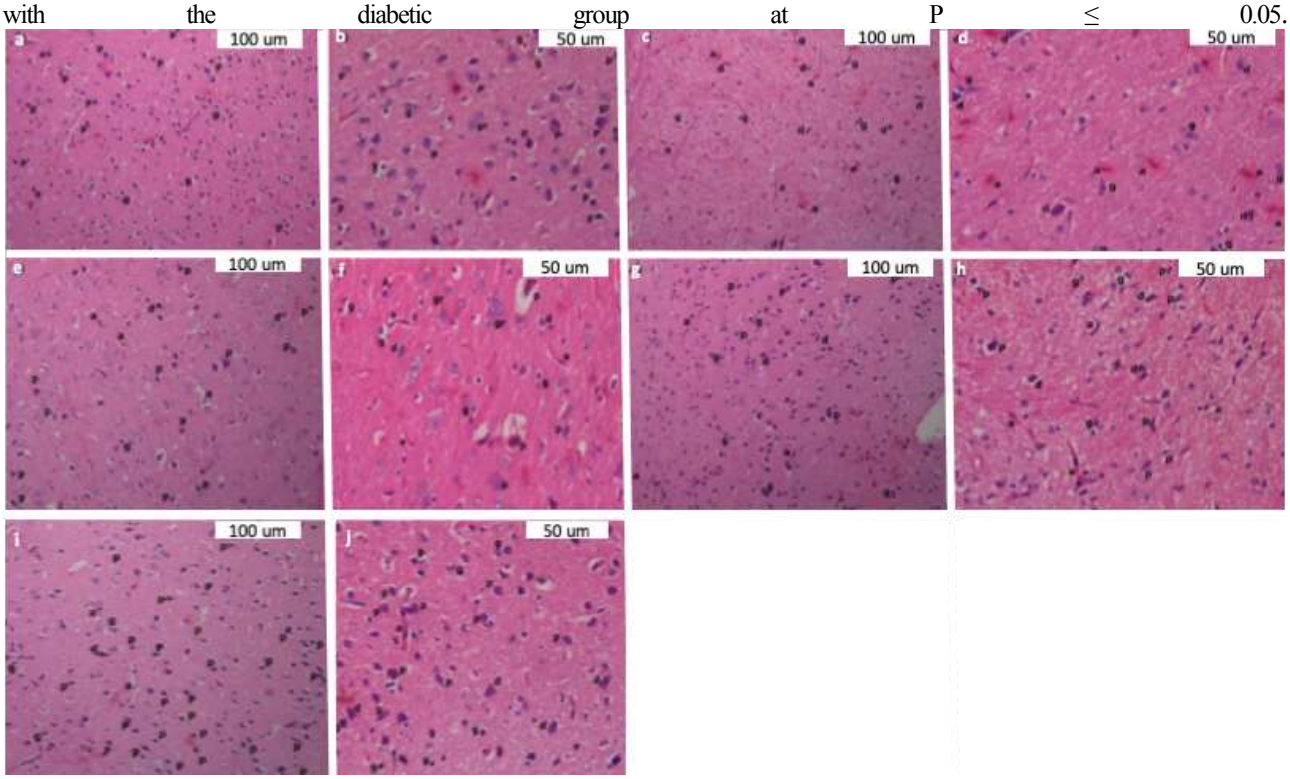

Fig. 4. Sections in the frontal cerebral cortex of all studied groups (H&E ×200) for EPL (H&E ×400) for IPL: a) EPL in the control group, b) IPL in the control group, c) EPL in the diabetic rats, d) IPL in the diabetic rats, e) EPL with Ex-4 treatment, f) IPL with Ex-4 treatment, g) EPL with PIO treatment, h) IPL with PIO treatment, i) EPL with combined Ex-4 +PIO treatment, j) IPL with combined Ex-4 +PIO treatment.
H&E: Hematoxylin & Eosin, EPL; external pyramidal layer, IPL; internal pyramidal layer, Ex-4; Exendin-4, PIO; pioglitazone, p; pyramidal cells, g; glial cells, s; stellate cells, gr; granule cells, d; deformed neurons, a; acidophilic neurons with dark nuclei.

Discussion

Indeed, T1D patients treated with exogenous insulin, experience fluctuations in their blood glucose levels that result in neuronal dysfunction (26) so, scientists research about other therapeutic modalities to support neuronal functions in T1D.

Mitochondrial dysfunction has been proposed to mediate development of diabetes complications in many tissues including neurons (27) So, we hypothesize that mitochondria could act as a potential drug target for both prophylaxis and treatment of diabetic encephalopathy beyond adequate glucose metabolic control.

Consistent with excess ROS production in diabetes, we demonstrated increased brain H2O2 in non-treated diabetic rats, which may result in activation of mitochondrial cascade of apoptosis (28).

The observed oxidative stress was associated with increase c-Jun N-terminal kinase (JNK) protein level. This agrees with the work of Chambers and LoGrasso, (29) who found that superoxide production was the source of JNK-induced ROS amplification indicating an association between JNK and H_2O_2, which goes in line with their correlations which demonstrated in the current study.

Additionally, both the major cellular cytoprotectant, HO-1 activity together with *Nrf2* gene expression; the master regulator of the antioxidant response, were found to be reduced significantly in non-treated diabetic rats, thus amplifying the brain oxidative stress state, which explain their negative correlation with H_2O_2 level in our work.

In the current study a significant improvement in HO-1 activity was shown in the treated compared with non-treated diabetic rats, which further aids in mitochondrial bioenergetics. This agrees with Bindu et al (30) who found that, the mitochondrial translocation of HO-1 resulted in the prevention of NSAID-induced mitochondrial dysfunction and oxidative stress.

Also, we showed that *PGC-1α* expression was reduced following DM induction, an effect seen to be rescued significantly with the intervening combined treatment of PIO and Ex-4. PGC-1α

down-regulation is probably the leading factor to decreased oxidative stress observed in our work in the brain of diabetic rats. This was consistent with reports demonstrating mitochondrial dysfunction in diabetes models (27, 31, 32).

In contrast to previous works in different models, (3,33) *PGC-1α* expression was not significantly improved with either drug alone, an effect which could be related to different dosage regimen which will need further clarification in next studies.

PGC-1α transcription and activity are enhanced by two important enzymes AMPK and SIRT1 through phosphorylation or deacetylation, respectively which regulate cellular energy homoeostasis in a coordinated way [34]. In our study, AMPK protein appears elevated at this early stage of DM, contrary to the long-term diabetic models of Roy Chowdhury et al., (35) which showed depressed AMPK expression. Thus, we provide evidence the down regulation of *SIRT-1* could explain the ineffective PGC-1α activity in short term DM.

We demonstrated the improvement in *PGC-1α* activity under either PIO or Ex-4 is most probably associated with up-regulation of *SIRT-1*, an observation which agrees with Zhang et al (33) study, who reported the up-regulation of *SIRT 1* expression in the retina of diabetic rats reducing ROS generation.

Also, Santos-Lozano et al (36) described the neuro-protective role of HO-1 in Alzheimer's disease, through suppressing aberrant neuronal apoptosis and increasing the expression of *SIRT-1/PGC-1α* signaling. Others showed that its over-expression promotes mitochondrial biogenesis by up-regulating protein expression of PGC1α (5).

In relation to the respiratory chain; complex I, which plays a pivotal role in the control of ATP synthesis; its deficiencies have been linked to neuropathic disorders (37). Additionally, the complex II mutations cause a variety of diseases and the variations in its dependent respiration rates may improve the motor performance, and the total number of cortical neurons (38, 39).

The present study demonstrated decreased mitochondrial I & II complexes as early as seven weeks of DM. Although, PIO and/ or Ex-4 treatment, produced significant improvement in complex II activity.

The diabetes induced alterations in oxidative phosphorylation, was also observed in other tissues such as cardiac muscle, liver, and kidney (40-42).

Our previous results demonstrated antioxidant effects of PIO and Ex-4. These results were consistent with previous works (43, 44). Furthermore, Ex-4 was reported to mitigate the neuronal oxidative stress in several neurological disorders (45, 46).

We showed activation of apoptotic state in non-treated diabetic rats. In line with our findings, previous studies (47) demonstrated increased cytochrome c release to the cytosol and translocation of the pro-apoptotic protein BAX to mitochondria in retinal capillary cells exposed to hyperglycemia. Also, we showed down regulation in *bax* and up-regulation of *bcl2* in both PIO and Ex-4 treatment, which indicate their antiapoptotic role that adding to their neuroprotective mechanisms. Their antiapoptotic role was shown previously in other models [10-12]. Up-regulation of the protective *bcl-2* may aid in the neuroprotective mechanisms especially those associated with mitochondria, according to Bonneau et al. (48).

A significant reduction in the BDNF was also observed in non-treated diabetic rats, in the current work. Abdelwahed et al. (49). showed that alterations in the activity BDNF can produce detrimental changes to cortical function and synaptic transmission resulting in neuronal dysfunction and brings about diabetic encephalopathy and they showed BDNF significant positive correlations with *SIRT-1/PGC 1-α*.

Thus, PIO and Ex-4 by raising BDNF protein level can preserve the neuronal mass together with their antioxidant and antiapoptotic mechanisms. Our results were in consistent with that of Liao et al (50) and Abdelwahed et al. (49), respectively. Liao et al (50) showed that the improving effect of PIO in depression model was involving up-regulation of the cAMP response-element-binding protein/brain derived neurotrophic factor (CREB/BDNF) pathway.

We suggested that BDNF beneficial effect on mitochondrial function was mediated via complexes I and II, as evidenced from its positive correlation with them. This finding was also supported by the study of Markham et al (51) Furthermore, BDNF was reported to up-regulate

PGC-1α, be involved in increased ATP and NAD+, formation and maintenance of synapses (52).

Indeed, Larsen et al (53) demonstrated synergistic effects of the combined therapy of PIO with Ex-4, in glycemic control of in T2DM model, but no results available about the efficacy of this combination therapy in T1DM. We demonstrated that the combined treatment was more efficacious in glycemic control and keeping normal healthy neuronal cells in our histological assessment. Other biochemical parameters mostly approaching the control values with combined PIO / Ex-4 modality, but still insignificant with either drug separately. We hope that further trials with different dosage might obtain more optimal combinations.

The current study presents evidence that the brain antioxidant and anti-apoptotic effects triggered by PPAR-γ agonist and/or GLP1 analog in T1DM were associated with increasing brain BDNF, HO-1 and *Nrf2*. These molecules are shown to maintain mitochondrial bioenergetic homeostasis and metabolic adaptation. Up on our findings, that PIO and/ or Ex-4 are involved in neuroprotective mechanism in T1DM, we suggest employing either PIO or Ex-4 as adjuvant in T1DM to support neuronal survival and prevent the development of diabetic encephalopathy. These findings need to be confirmed by functional assessment of improvement of cognitive performance.

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