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The Ameliorating Effect of Erythropoietin on Diabetic Neurodegeneration by Modulating the Antioxidant-Oxidant Imbalance and Apoptosis in Diabetic Male Rats

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Abstract

Diabetes mellitus (DM) is a complex syndrome which leads to multiple dysfunctions including neuropathy. Thus, the present study is aimed at assessing the effect of erythropoietin on neurodegeneration and oxidative stress in STZ diabetic rats. Group 1, control; group 2, diabetic; group 3, diabetic+erythropoietin; group 4, erythropoietin. The present results showed a significant increase in fasting blood sugar and insulin resistance while, insulin, body weight and EPO levels were decreased in the diabetic group. The protein expression of BDNF revealed a down regulation in the brain regions. Moreover, AchE was significantly decreased in the diabetic rats compared with the control ones. The oxidative status of diabetic rats was documented by an elevation in MDA and a reduction in antioxidant enzyme activities (SOD, GPx and CAT). In addition, excessive activation of PKCβ and caspase-6 were observed in the diabetic rats. This study confirms the protective effect of eerythropoietin treatment in relieving the different alterations in the diabetic rats by scavenging reactive oxygen species and modulating the PKCβ and caspase-6. EPO has been demonstrated to be a cytoprotective and block neurodegeneration by inhibiting caspase activity and apoptosis.

Keywords: Diabetes mellitus, EPO, Oxidative stress, BDNF, PKCβ and caspase-6

1. Introduction

Diabetes can lead to serious complications if it is not properly managed. Most of these complications are related to complications arising from microvascular (e.g., nephropathy, neuropathy, and retinopathy) and macrovascular (e.g., coronary artery disease, CAD; peripheral artery disease, PAD, and cerebrovascular disease) (Badran and Laher, 2012; Gupta *et al*., 2015).

It has been suggested that the term "diabetes-associated cognitive decline" (DACD) describes a state of mild to moderate cognitive impairment, in particular psychomotor slowing and reduced mental flexibility, not attributable to other causes. In addition, it is now clear that diabetes increases the risk of Alzheimer's disease (AD), vascular dementia and any other type of dementia (Chawala *et al*., 2016).

Erythropoietin (EPO), a glycoprotein with a molecular weight of 34 kDa, was identified as a cytokine responsible for the production of erythrocytes (Broxmeyer, 2013; Palis, 2014). EPO has various effects such as modulation of inflammation, slowing down of apoptosis, stimulation of angiogenesis and limitation of reactive oxygen species production. Therefore, EPO can protect neurons with a combination of these effects (Genc *et al*., 2004). Previous studies have reported that there may be an association between EPO levels and hypoglycemia, which suggests a potential protective effect of EPO in the treatment of diabetes (Choi *et al*., 2011; Zhang *et al*., 2014; Chen *et al*., 2015).

The present study is aimed to assess the effect of erythropoietin on neurodegeneration and oxidative stress in STZ-induced diabetes in male rats.

2. Materials and Methods

2.1. Chemicals

Streptozotocin (STZ) and Erythropoietin (Epo) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Animals

Male *Wistar* rats (200-250 g) were obtained from the Faculty of Medicine of Alexandria University in Egypt. They were housed under controlled conditions (25±1°C constant temperature, 55% relative humidity, 12 h lighting cycle) for two weeks prior to the experiment for acclimation and received standard diet and water *ad libitum.* All animal procedures and the experimental protocols were carried out according to the guidelines of the National Institutes of Health (NIH).

2.3. Experimental Design

Forty male Wistar rats were randomly divided as the following:

Control group: The rats of this group were treated intraperitoneally citrate buffer (pH 4.5).

Diabetic group: The rats of this group were treated intraperitoneally with a single injection of streptozotocin (STZ) at a dose of 50 mg/kg body weight dissolved in citrate buffer (Hongying *et al*., 2013).

Diabetic and erythropoietin treated group: Diabetic rats were treated intraperitoneally with erythropoietin injection at a dose of 40 µg/kg body weight three times per week for five weeks after diabetes induction (Bianchi *et al*., 2003).

Erythropoietin treated group: The rats of this group were treated intraperitoneally with erythropoietin only at a dose of 40 µg/kg body weight three times per week for five weeks

The body weight records were measured at the end of the experiment. At the end of the experimental period all rats were anesthetized with ether. Blood samples were collected by cardiopuncture and plasma samples were separated.

2.4. Induction of Diabetes

Diabetes was induced chemically by intraperitoneal injection of streptozotocin (STZ) at a dose of 50 mg/kg body weight (Hongying *et al.,* 2013). The fasting blood glucose level was measured 72 h after the STZ treatment, using reagent strips (Accu-ChekW, Roche) with a drop of blood obtained by tail-vein puncture. Animals were considered diabetic if blood glucose values were higher than 200 mg/dl.

2.5. Preparation of Serum

The heparinized blood samples were centrifuged at 3000 xg for fifteen min. Serum was separated and then stored at -20 °C until the biochemical analysis.

2.6. Preparation of Brain Homogenate

Whole tissues of the brain were obtained by dissection, cleaned from the adhering matters, and washed with physiological saline. Then the brain tissues from each rat was minced and homogenized in phosphate buffer (pH 7.4). Homogenates were centrifuged at 10000 xg for twenty minutes at 4°C and the clear supernatants were separated for lipid peroxidation and antioxidant enzyme determination.

2.7. The Biochemical Parameters

Fasting blood sugar and insulin levels were determined according to the methods of Bergmeyer and Berndt (1974) and Valverde *et al*. (1988), respectively. The insulin resistance was estimated by Matthews *et al*. (1985) HOMA score = fasting insulin (µIU/L) x fasting glucose (mmol/L)/22.5. Sandwich enzyme-linked Immune-Sorbent assay technology was used for erythropoietin (EPO) determination (Haroon *et al*., 2003). The brain derived neurotrophic factor (BDNF) activity was determined by using sandwich enzyme immunoassay (EIA) (Elfving *et al*., 2010). Determination of acetylcholinesterase (AchE) activity (EC 3.1.1.7) was assayed according to Srikumar *et al*. (2004). Malondialdehyde (MDA) (EC 1.2.1.3) (Ohkawa *et al*., 1979), superoxide dismutase (SOD) (EC 1.15.1.1) (Nishikimi *et al*., 1972), glutathione peroxidase (GPx) (EC 1.11.1.9) (Paglia and Valentine, 1967), catalase (CAT) (EC 1.11.1.6) (Aebi, 1984) were estimated. Protein kinase c (PKC) (EC 2.7.11.13) was measured by the method of Keranen and Dutil (1995). Caspase 6 was assayed by using commercial kits (www. MyBioSource.com) (Cat. No. MBS721980).

2.8. Statistical Analysis

Statistical analyses were performed using the SPSS package for Windows version 22.0. Data were expressed as mean±SE. One-way ANOVA was used to analyze differences among groups. Differences among groups were considered statistically significant at $P \leq 0.05$.

3. Results

3.1. The Effect of Erythropoietin (EPO) on Fasting Blood Sugar, Insulin and Insulin Resistance Levels in the Different Studied Groups:

Table 1 illustrates that the fasting blood sugar levels and insulin resistance were significantly ($P \leq 0.05$) increased in the diabetic group compared to the control group. In contrast, insulin levels and body weight were decreased in the diabetic group. Meanwhile, the treatment of diabetic rats with EPO caused a significant ($P \leq 0.05$) decrease in the fasting blood sugar levels and insulin resistance and an increase in insulin levels and body weight compared to the untreated diabetic group. In the EPO group, the fasting blood sugar and insulin levels were more or less similar to the control values.

Table 1. The effect of erythropoietin (EPO) on fasting blood sugar, insulin levels, insulin resistance and body weight in the different studied groups.

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+ EPO	EPO
Fasting blood sugar (mg/dL)	96.333[a] ±2.422	300.167[b] ±32.307	176.429[c] ±8.772	93.833[a] ±3.251
Insulin level (µlU/mL)	5.794[a] ±0.046	3.577[b] ±0.368	5.113[a] ±0.251	5.650[a] ±0.143
Insulin resistance	26.954[a] ±2.997	47.953[b] ±3.894	38.338[c] ±2.631	23.416[d] ±2.773
Body weight (g)	225[a] ±11.452	178.367[b] ±12.684	190.252[c] ±12.541	220.250[a] ±13.351

Values are expressed as means±S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different ($P \leq 0.05$)

Table 2. EPO, BDNF and AchE levels in the different studied groups.

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+EPO	EPO
EPO level (pg/ml)	112.974[a] ±1.803	98.581[b] ±1.292	114.806[a] ±1.928	110.769[a] ±3.724
BDNF level (ng/ml)	0.018[a] ±0.001	0.013[b] ±0.001	0.015[c] ±0.002	0.019[a] ±0.001
AchE level (ng/ml)	0.869[a] ±0.063	0.529[b] ±0.061	0.659[c] ±0.097	0.846[a] ±0.066

Values are expressed as means ± S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b) were significantly different ($P \leq 0.05$)

3.2. The Effect of EPO on MDA, SOD, GPx and CAT Levels in the Different Studied Groups:

Table 3 shows that MDA was significantly ($P \leq 0.05$) increased in the diabetic group compared to the control ones. On the other hand, the levels of antioxidant enzyme activities (SOD, GPx and CAT) were significantly ($P \leq 0.05$) decreased in the diabetic group compared to the control. EPO administration significantly improved the MDA, SOD, GPx and CAT levels in comparison with the untreated diabetic rats.

Table 3. The effect of EPO on MDA, SOD, GPx and CAT levels in the different studied groups.

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+EPO	EPO
MDA (nmol/mL)	7.98[a] 0.91	38.16[b] 0.68	14.6[c] 1.14	7.90[a] 0.53
SOD (U/mL)	75.40[a] ±4.547	26.250[b] ±4.678	46.95[c] ±4.883	73.90[a] ±6.656
GPx (nmol/min/mL)	64.30[a] ±6.165	21.85[b] ±2.943	45.55[c] ±3.940	64.75[a] ±5.220
CAT (nmol/min/mL)	46.650[a] ±4.626	16.60[b] ±2.583	31.05[c] ±3.268	45.70[a] ±4.497

Values are expressed as means ± S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different ($P \leq 0.05$)

3.3. The Effect of EPO on PKC and Caspase 6 in the Different Studied Groups.

Table 4 shows that PKC and Caspase 6 levels were significantly ($P \leq 0.05$) enhanced in the diabetic group compared to the control group. The treatment of diabetic group with EPO caused a significant ($P \leq 0.05$) decrease in the PKC and Caspase 6 levels compared to the untreated diabetic group.

Table 4. The effect of EPO on PKC and Caspase 6 in the different studied groups**.**

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+EPO	EPO
PKC (ng/mL)	6.94[a] ±0.54	32.84[b] ±2.45	12.8[c] ±0.91	6.12[a] ±0.45
Caspase 6 (ng/mL)	20.0[a] ±1.58	59.8[b] ±1.92	36.2[c] ±2.39	20.8[a] ±1.79

Values are expressed as means ± S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different ($P \leq 0.05$)

4. Discussion

Type 2 diabetes (T2D) is one of the modifiable risk factors for Alzheimer's disease (AD) and has a 1.5 to 2 fold increased risk to develop cognitive impairment (Yael *et al*., 2010). AD is characterized by a progressive decline in the cognitive function (Revett *et al*., 2013; Honjo *et al*., 2012). Insulin plays a major role in the memory and brain function in patients with AD by regulating neuronal functions, neurogenesis and neurotransmitter regulation (Myint *et al*., 2013).

The present hyperglycemia status was attributed to STZ-induced depletion in the secretion of insulin by partial destroying pancreatic β-cells, reduction in insulin production, resulting in enhancement of glucose level that in turn causes protein glycosylation (Forde and Medeiros, 2008) and poor glucose utilization by tissues (Eliza *et al.*, 2009). These results came in accordance with Selvan *et al.* (2008) and Dewanjee *et al.* (2009). This glucose overloading can activate many metabolic or signaling pathways that not only attempt to dispose excessive glucose, but also generate more reactive oxygen species, leading to oxidative stress and β-cell failure together with the evidence of a low-level antioxidant capacity in β-cells which are thought to be responsible for secondary diabetic β cell failure (Wu and Yan, 2015). Similar results were reported by another researcher who found that the fasting blood glucose level in diabetic rats was significantly higher than that in the control rats in the first week (Hwang *et al.*, 2017). Hyperglycemia is also associated with the consequences of hyperinsulinemia, insulin resistance and glucose intolerance (Nasry *et al.*, 2013). EPO is under investigation for the treatment of a variety of diseases, and appears to be especially suited for the treatment of disorders of metabolism including diabetes mellitus (DM). In addition to EPO utility for the treatment of anemia, EPO can improve cardiac function, reduce fatigue, improve cognition in patients with DM, and regulate cellular energy metabolism, obesity, tissue repair and regeneration, apoptosis, and autophagy in experimental models of DM (Maiese, 2015). Therapeutic strategies that can specifically target and control EPO and its signaling pathways hold great promise for the development of new and effective clinical treatments for DM and the complications of this disorder.

However, EPO supplementation significantly lowered the fasting blood glucose compared with the untreated diabetic group. Katz *et al.* (2005) reported that after one

week of treatment, all EPO-treated mice had lower blood glucose levels which were further reduced during the second week of treatment.

Regarding the possible mechanism(s) for EPO-induced attenuation of hyperglycemia, it was found that EPO increases the glucose utilization that seems to be responsible for the reduction of hyperglycemia in the diabetic rats (Caillaud *et al*., 2015). Moreover, a pleiotropic action of EPO is not associated with endogenous insulin, indicating an insulinotropic action of EPO. EPO has been demonstrated to show antioxidant-like activity. Also, reduction of hyperglycemia induced by a bolus injection of EPO seems to be related to the antioxidant-like action. In addition, EPO enhanced the insulin sensitivity in diabetic rats. This result is reasonable because EPO has been linked to many signals that occur after activation of receptors (Si *et al*., 2013).

Several previous studies have demonstrated that treatment with EPO improves insulin sensitivity and reduces insulin resistance in dialysis patients, obesity and metabolic syndrome mice and 3T3L1 adipocytes, via EPO receptors in insulin-responsive tissues, including the muscle and liver, or indirectly through the correction of anemia (Pan *et al*., 2013; Zhang *et al*., 2014). Chen *et al.* (2015) demonstrated that treatment with EPO reduced fasting blood glucose (FBG), ameliorated glucose tolerance and insulin sensitivity, and lowered glyconeogenesis in experimental diabetic rats. A previous randomized placebo controlled trial (Benstoem *et al*., 2015) demonstrated that insulin sensitivity is improved following treatment with EPO in healthy humans. He *et al.* (2010) suggested that EPO can protect neonatal islet cells in the porcine model by upregulating β-cell lymphoma-2 and down-regulating β-cell-associated X protein and caspase 3.

The body weight decline in diabetic rats may be attributed to the association of diabetes with increased glycogenolysis, lipolysis, and gluconeogenesis. These biochemical activities result in muscle wasting and loss of tissue protein (Ewenighi, 2015). Treatment of diabetic rats with rHuEPO produced significant increase in both body weight and food intake (Khalaf *et al*., 2015).

Brain derived neurotrophic factor BDNF is an important neurotrophic factor in the nervous system that can promote nerve regeneration and maintain normal neuronal function. BDNF may play a role through binding with receptor P75 and TrkB on nerve cells and activating various signaling pathways (Bathina and Undurti, 2015). The protein expression studies of BDNF revealed a down regulation in the brain regions of the diabetic rats. The results of the present study were in agreement with the report of Yang and Gao (2017) who showed a decrease in the expression of this neurotrophin in diabetes.

The increased oxidative stress which occurred as a result the pre-existing diabetes in the rats could cause a reduction in the expression BDNF, which could have contributed to the memory impairments observed in the diabetic rats. A positive correlation between brain BDNF concentration and cognitive performance has been described, while decreasing BDNF production has been proposed as one possible pathogenetic factor for Alzheimer's disease and major depression (Zoladz and Pilc, 2010). Interestingly, BDNF levels are decreased in patients with diabetes mellitus type 2 (DM2), and have been inversely correlated with plasma glucose and insulin resistance as assessed by a homeostatic model assessment (Ortíz *et al*., 2016).

EPO has been shown to protect primary hippocampal neurons by increasing the expression of brain-derived neurotrophic factor (Viviani *et al.,* 2005). Wang and Xia (2015) demonstrated that EPO can upregulate BDNF expression in the diabetic rats. Promising research is emerging around–BDNF, in cognitive dysfunction associated neurological disorders. In the brain, BDNF promotes the optimum communication between neurons by enhancing "plasticity" at the synapse. BDNF plays an important role in the survival of neurons, their growth (axons and dendrites), and the formation and function of the synapse (Blurton-Jonesa *et al.,* 2009). Without sufficient BDNF and other neurotrophic factors, neurons die. Cholinergic transmission in the brain cortical and hippocampal regions plays a fundamental role in memory (Zanardini *et al*., 2016).

Under normal conditions, AChE activity is a specific marker of cholinergic neurons in the cerebral cortex and hippocampus. It plays a critical role in the modulation of the cholinergic pathway (Mao *et al*., 2014). The reduction in AchE in the diabetic group may be attributed to the insufficient activity of AChE as a contributing factor in the development of diabetic neuropathy (Baquer *et al.,* 2011). AChE is one of the important membrane bound enzymes in the brain that influence the acetylcholine levels. Several earlier studies reported a decrease in the AChE level in diabetic male rats with a significant increase in lipid peroxidation in the brain tissue (Kumar *et al.*, 2015).

Elevated MDA is regarded as a specific indicator of lipid peroxidation during oxidative impairment. In addition, oxidative injury could also destroy the antioxidant defense system, such SOD, GPx and CAT. In fact, it was previously found that oxidative brain damage caused by oxidative stress contributed to the serious impairment of learning and memory deficits during aging in rats (Wu *et al*., 2013; Mao *et al.*, 2014).

It is well documented that MDA is a stable end product of free radicals induced by lipid peroxidation. Thus, MDA serves as a reliable marker for the assessment of free radical induced damage to tissues. In diabetic patients, a major factor that is responsible for enhanced free radical generation is hyperglycemia through auto-oxidation of glucose (Kangralkar *et al*., 2010) protein glycation, lipid peroxidation, and low activities of antioxidant enzymes. The mechanism of the enhancing of the oxidative stress might be due to protein glycation and inhibition of antioxidant enzyme activities (SOD, GPx) (Sheweita *et al*., 2015). In addition, oxidative injury could also destroy the anti-oxidant defense system, such SOD, GPx and CAT.

On the other hand, erythropoietin has an antioxidative effect as indicated by decreasing the level of MDA, resulting in decreasing the amount of oxidative stress and subsequent lipid peroxidation (Ahmadias *et al.,* 2013). In the Chen *et al*. (2015) study, administration of EPO decreased the level of MDA, and increased the activity of antioxidant enzymes, including SOD and GPx, compared with the diabetic model group. EPO increases antioxidant enzyme activities by increasing levels of nuclear factor erythroid 2-related factor 2 (Nrf-2) to the nucleus where it

binds and activates the antioxidant response element (Jin *et al.*, 2014; Genc *et al.*, 2010). EPO also causes the increased activity of the antioxidant protein glutathione peroxidase *in vivo* (Kumral *et al*., 2005), superoxide dismutase (Barichello *et al*., 2014).

Increased activation of PKC isozymes has been observed in cancer (Toton *et al*., 2011), diabetes, ischemic heart disease, acute and chronic heart disease (Palaniyandi *et al.*, 2009) and in a number of neurological diseases, including stroke, Parkinson's disease (Burguillos *et al.*, 2011), dementia, Alzheimer's disease, pain and even in psychiatric diseases, including bipolar disease (Davari *et al*., 2013).

Preclinical research suggests that hyperglycemia leads to the activation of PKCβ, which may play an important role in mediating the microvascular disease complications of retinopathy, nephropathy, and neuropathy. Hyperglycemia leads to the chronic activation of PKCβ, causing aberrant signaling and a variety of pathologies such as cytokine activation and inhibition, vascular alterations, cell cycle and transcriptional factor mis-regulation and abnormal angiogenesis (Geraldes and King, 2010).

The present study showed a remarkable elevation in caspase-6 activity in the diabetic rats. Some data have suggested that elevated glucose level may increase caspase-6 expression levels and induce oxidative stress, leading to apoptosis (Maedler *et al*., 2009). Similarly, caspase activation has been noted before the development of neurofibrillary tangles of Tau in the brain of tau transgenic mice (De Calignon *et al*., 2010). Caspase-6 activation is one of the main characteristics of many neurodegenerative diseases, including diabetes-associated cognitive deficits and AD (O'Brien and Wong 2011; Zawada *et al.*, 2015 and Sadeghi *et al*., 2016).

Wang *et al.* (2017) reported that EPO treatment significantly decreased cell apoptosis. This result may be associated with an EPO-induced decrease in the levels of caspase-6 and oxidative stress. EPO also blocks Apaf-1 activation, and prevents the early activation of several caspases (Park *et al*., 2011; Shang *et al*., 2012).

Neuroprotective and neurotrophic effects of EPO have been shown in different experimental brain damages. Kumar *et al.* (2010) proposed that the beneficial effect of EPO mediated in STZ dementia may be attributed to its multiple effects, including antioxidative (Wang *et al*, 2009), anti-inflammatory and neuroprotective actions (Granic *et al.*, 2009; Salminen *et al*., 2009).

EPO was effective in preventing neuronal apoptosis in many types of neurodegenerative conditions in the brain and spinal cord (Maiese *et al.*, 2012). Treatment with EPO decreases cellular damage caused by ROS/RNS, including lipid peroxidation, protein carbonylation (Barichello *et al*., 2014), and protein nitrosylation (Lu *et al*., 2012), thus, preventing downstream damaging effects on cells that lead to apoptosis.

5. Conclusion

Hence, it may be concluded that EPO has shown ameliorative effects on neurodegenerative and brain oxidative stress. Further studies should be directed towards knowing new biomarkers for AD, enabling improved understanding, prediction and prevention of this major clinical problem of diabetes complications

Conflict of interest

The authors declare that they have no conflict of interest.

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