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# Uncontrolled diabetes worsens cerebral ischemia-reperfusion injury in rats: insights into the involved mechanisms

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**Abstract**

Uncontrolled diabetes mellitus (DM) has been associated with poor clinical outcomes in patients with ischemic stroke. Limited knowledge is available regarding the molecular mechanisms and the etiological complexity of both pathologies. Hence, this study aimed to investigate the interplay between long-term DM and ischemic stroke. A comparative study was held between long-term DM-induced adult male Wistar rats which were subjected to sham operation or global cerebral ischemia/reperfusion (I/R) injury on one hand, and adult male Wistar rats which were subjected to sham operation or global cerebral I/R injury on the other hand. Results showed a disturbance in cerebral oxidative stress parameters (catalase, reduced glutathione and malondialdehyde) in diabetic rats compared to non-diabetic ones. Additionally, in comparison with sham diabetic group, diabetic I/R-injured rats showed up regulation of cerebral oxidative stress, caspase-3 and tumor necrosis factor- alpha (TNF-α) protein expression along with increase in carotid inducible nitric oxide synthase (iNOS) protein expression and down regulation of carotid endothelial nitric oxide synthase (eNOS) protein expression. However, no significant difference was observed in term of cerebral oxidative stress, cerebral caspase-3 and TNF-α protein expression upon comparing non-diabetic I/R-injured rats and diabetic rats subjected to cerebral I/R injury. Carotid vascular dysfunction is prominent in diabetic I/R-injured rats compared to non-diabetic rats with cerebral I/R injury.

This study supposed that long term diabetes could exacerbate brain damage following global cerebral I/R injury via promoting inflammation, oxidative stress and vascular dysfunction. Diabetes-mediated vascular dysfunction in ischemic stroke warrants further experimental and clinical investigations to be fully elucidated.

***Key words***

*uncontrolled diabetes , global cerebral ischemia , oxidative stress*

## 1. Introduction

Diabetes mellitus (DM) is one of the most common metabolic diseases associated with several vascular complications on long-term exposure. Stroke is one of the major complications associated with DM. The combination of diabetes and stroke leads to extensive brain damage and worsens the clinical outcomes of stroke [1].

The experimental model of global cerebral I/R via transient bilateral common carotid arteries occlusion (BCCAO) yields high-grade ischemia of forebrain structures [2, 3]. The reported neurological and behavioral defects in rats subjected to global I/R injury supported the brain atrophy associated with this model of ischemia [4, 5]. Cerebral ischemia rapidly enhances strong cerebral oxidative imbalance, inflammatory and apoptotic responses in the rodent brain which eventually leads to neuroinflammation and neuronal death [6 , 8].

The DM-stroke combination was shown to aggravate the infarct size and the brain injury in ischemic stroke subject. Uncontrolled DM associated hyperglycemia increases production of reactive oxygen species (ROS) causing neuronal death [9]. Moreover, hyperglycemia is usually associated with increased proinflammatory cytokines, which in turn increase inflammatory response [10, 11]. Moreover, modulation of vasculature and vasoactive mediators in diabetic subjects can have a significate role in decreasing cerebral blood flow (CBF) [12]. However, the role of DM-induced oxidative stress and vascular impairment in cerebral ischemia are not well identified, Furthermore, the mechanisms by which diabetes increases ischemic brain damage is still elusive.

The present study is a comparative study held between diabetic rats (either sham-operated or cerebral I/R-injured ones) against non-diabetic rats subjected to either sham operation or global I/R injury. The study aimed to investigate the interplay between long-term DM and ischemic stroke. Moreover, this study was designed to investigate the underling mechanisms involved in ischemia-induced neuronal death and role of uncontrolled DM in exaggeration of stroke pathology.

## 2. Materials and methods

### 2.1. Chemicals

Streptozotocin (STZ) and 2,3,5-Triphenyltetrazolium chloride, 2-thiobarbituric acid (TTC) stain were purchased from Sigma Aldrich (St. Louis, MO, USA). Polyclonal rabbit primary antibodies against caspase-3, tumor necrosis factor-alpha (TNF-α), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) were purchased from Thermo Fischer Scientific Inc. /Lab Vision (Fermont,CA,USA).

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### 2.2. Experimental procedure

#### 2.2.1. Study design

Thirty two male Wistar rats were randomly allocated into four groups, each group contains eight rats. Group (1), sham diabetic rats: DM-induced rats were subjected to the same surgical procedures as described below, except for BCCAO. Group (2), diabetic I/R group: diabetic rats were subjected to global cerebral ischemia via BCCAO followed by 24 h reperfusion. Group (3), sham operated group: non- diabetic rats underwent the same surgical procedures as described below, except for BCCAO. Group (4), I/R group: non-diabetic rats were subjected global I/R injury.

#### 2.2.2. DM induction

Animals groups (1, 2) were fed high fat diet (HFD) composed of 265 g powdered normal pellet diet, 313 g beef fat (suet), 250 g casein, 10 g cholesterol, 60 g vitamins and minerals mixture, 1 g yeast powder and 1 g sodium chloride per kg [13].

After 2 weeks of HFD, rats were injected intraperitoneally with STZ (35 mg/kg).Three days after STZ injection, diabetic rats exhibiting a blood glucose level of ≥300 mg/dl were selected for the experiment. Then, the selected rats were allowed to continue to feed on HFD for additional 4 weeks to induce a late stage of DM [14].

#### 2.2.3. Induction of cerebral I/R injury

Animal groups (2, 4) were subjected to anesthesia using i.p. injection of 50 mg/kg ketamine and 10 mg/kg xylazine. Then, anesthetized rats were subjected to cerebral I/R injury which was induced by transient BCCAO for 30 min followed by 24 h reperfusion [15].

All animal procedures were performed in accordance with Animal Care Community, Minia University, Egypt (Permit Number: MPH-12-015).

### 2.3. Behavioral and neurological outcome measures

After the end of reperfusion period, open field test was performed to measure spontaneous activity in rats. Briefly, each rat was placed in a wooden box whose bottom was divided equally into 16 squares. Rats were allowed to move freely for 3 min. Using a video camera, frequency of ambulation (the number of squares crossed with the four paws) and rearing (posture sustained with hind-paw) were recorded and counted manually [16]. Moreover, the neurological defect via Garcia score test was used to evaluate spontaneous activity, symmetry in the movement of the four limbs, forepaw outstretching, climbing, body proprioception and response to vibrissae touch. The summation of the neurological score ranges from 5 to 18 [17].

### 2.4. Cerebral and vascular tissue sampling

After scarifying the animals, both cerebral hemispheres of each rat were dissected, ice-cooled, and divided into two equally-sized sets. The first set was used for analysis of infarct volume. The other set was sagittally divided into two halves; one half was rapid frozen in liquid nitrogen and stored in -80 °C for biochemical and Western blot analysis.

Additionally, carotid artery segments were excised and deep frozen in liquid nitrogen, then stored for Western blot analysis.

### 2.5. Measuring of oxidative stress parameters

Brain tissues were homogenized in 0.05 M phosphate buffer saline (PBS) at PH 7.4. Cerebral homogenate was cool-centrifuged at 10,000 rpm for 10 min. The resulted supernatants were used for measuring brain malondialdehyde (MDA), reduced glutathione (GSH) contents and catalase activity using colorimetric kits according to instruction of manufacturer (Biodiagnostic, Egypt).

### 2.6. Western blots of caspase-3 and TNF-α in brain and protein expression of iNOS and eNOS in carotid arteries

Both brain tissues or carotid arteries were solubilized for protein extraction using RIBA lysis buffer (Bio BASIC INC, Ontario, Canada). 20 µg protein/lane were electrophoresed on 10% Sodium Dodecyl Sulfate Poly Acrylamide (SDS-PAGE) Gel. After electrophoresis, the separated proteins were transferred into cellulose membrane. Blocking the membrane with 5% skim dry milk was performed to prevent non-specific background binding. Membranes were incubated at 4 °C overnight with primary antibodies TNF-α (dilution, 1:3000) and caspase-3(dilution, 1:500) for brain tissues or iNOS, (dilution, 1:2000) and eNOS, (dilution, 1:1000) for carotid segments. Then, the membrane was incubated with a secondary antibody of Goat anti-rabbit IgG- (Novus Biologicals) for 1 h. Using chemiluminescent method, the blots were developed and signals were captured using a CCD camera-based imager. Image J software (freeware; *rsbweb.nih.gov/ij*) was used to determine the band intensity of the target proteins. The densities of the target protein bands were normalized to the corresponding density of β-actin band and presented as a ratio of the relative optical density.

### 2.7. Assessment of cerebral infarct volume

Whole brains were removed carefully and frozen for 1-h, and then each brain was sliced into serial coronal sections of 2 mm thickness. The sectioned slices were stained with 2% TTC stain for 15 min at 37 °C then fixed by 10% formaldehyde solution. The percentage of infarct volume was calculated by summation of the infarct area in each slice multiplied by the thickness of the slice [5].

### 2.8. Statistical analysis

Data are represented as Mean ± SEM. One way analysis of variance (one-way ANOVA test) was used to detect statistical significance. A post analysis test of Tukey-Kramer was used for comparing groups. Statistical significance was presented at $p < 0.05$. Analysis was performed using GraphPad Prism® software (Version 5.0).

## 3. Results

### 3.1. Effect on neurobehavioral outcomes

Cerebral I/R-injured diabetic rats showed a significant deterioration in the spontaneous activity and neurological function of rats evident in getting low scores in both open field and Garcia tests compared to sham diabetic group. In addition, diabetic I/R-injured rats showed a significant decrease in test scores of both open field and Garcia tests compared to non-diabetic I/R rats (**Figure 1**).

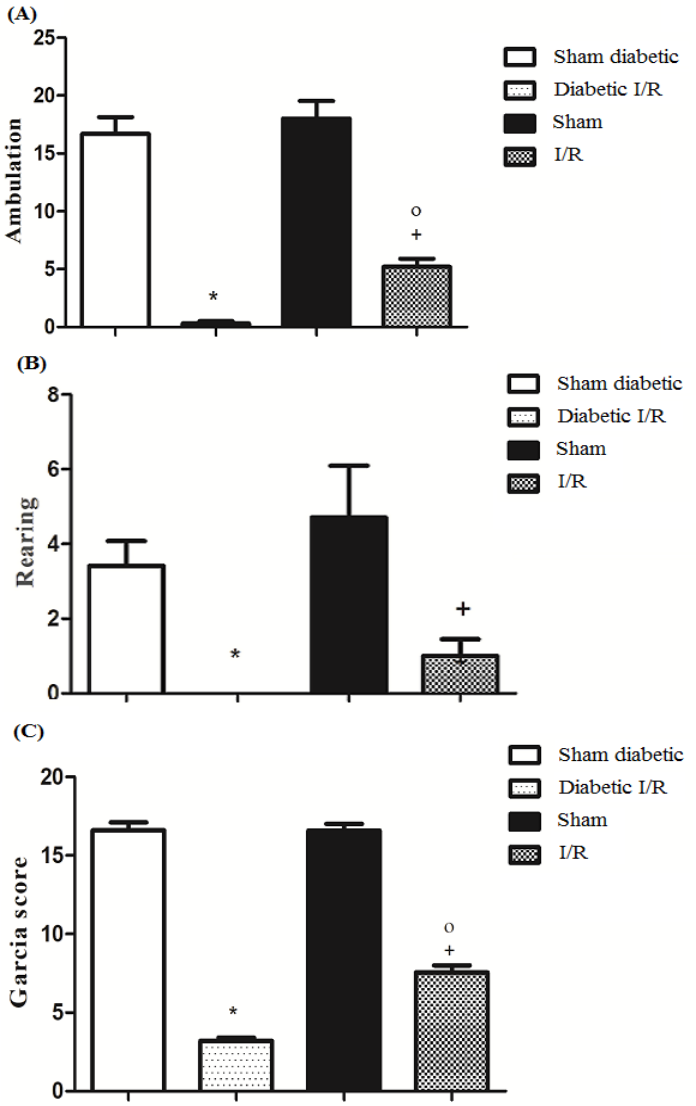


**Figure (1):** Effect of diabetes induction on ambulation frequency **(A)** and raring frequency **(B)** as a part of open field behavior test and effect on neurological deficiency **(C)** as a part of Garcia neurobehavior test in global cerebral I/R model. Data are represented as mean ± S.E.M. of 8 rats per group. (*, + , °) are significant difference from sham diabetic, sham and diabetic I/R groups, respectively.

### 3.2. Effect on oxidative stress markers

DM induction caused an increase in brain MDA level compared to non-diabetic rats (5.30 ± 0.30 vs. 2.25 ± 0.20 nmol/g protein). Moreover, induction of I/R injury in diabetic rats caused a significant increase ($p<0.05$) in cerebral MDA compared to sham diabetic group.

In addition, cerebral I/R injury resulted in a significant increase ($p<0.05$) in brain MDA levels compared with sham-operated group (10.31 ± 0.90 vs. 2.25 ± 0.20nmol/g protein). No significant difference in cerebral MDA level was observed in diabetic I/R-injured group and non-diabetic group subjected to I/R injury.

The antioxidant content of both GSH level and catalase activity were significantly decreased in brain tissues upon induction of cerebral I/R injury in both diabetic and non-diabetic groups compared to sham operated diabetic or non-diabetic animals, respectively .

Sham diabetic groups showed low levels of catalase and GSH compared to sham non-diabetic animals. However, no significant difference was observed in the antioxidant enzymes between diabetic I/R-injured group and non-diabetic group subjected to I/R injury (**Table 1**).

### 3.3. Effects on the cerebral protein expressions of caspase-3 and TNF-α

The protein expression of caspase-3 and TNF-α were significantly ($p<0.05$) increased in ischemic hemispheres of both diabetic and non-diabetic rats compared to sham-operated diabetic and non-diabetic rats, respectively. In brain tissues of diabetic rats subjected to I/R injury, no significant difference was observed in term of protein expressions of both caspase-3 and TNF-α compared to I/R-injured non-diabetic group (**Figure 2**).

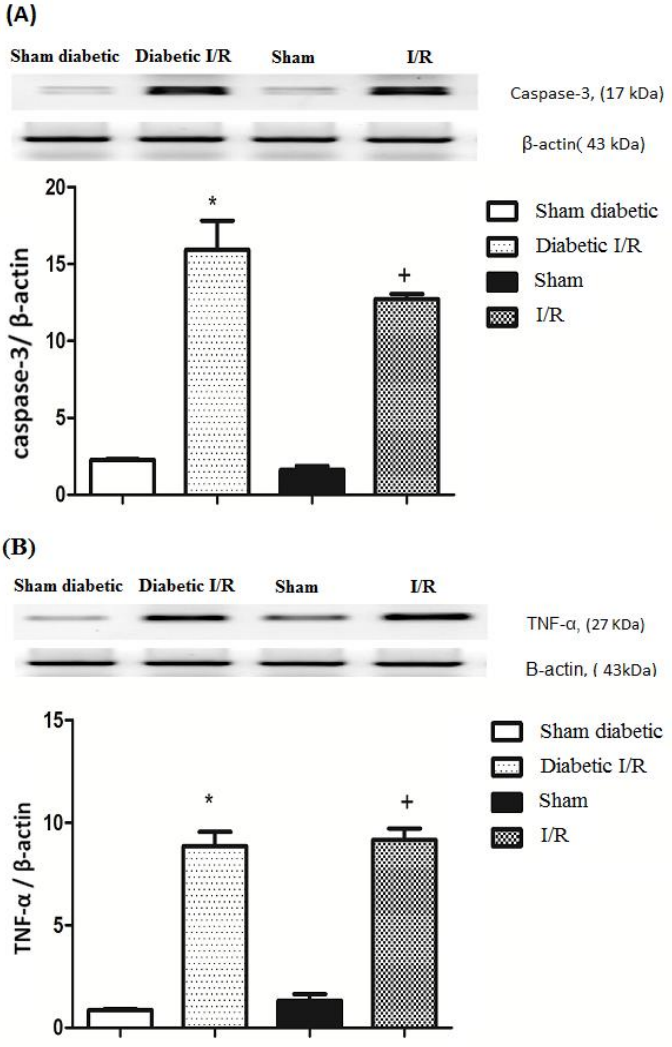


**Figure 2:** Effect of diabetes induction on cerebral caspase-3 (A), and TNF-α (B) protein expression in global cerebral I/R model.
Data are represented as mean ± S.E.M. of 8 rats per group. (*, +) are significant difference from sham diabetic and sham groups, respectively.

**Table 1:** Effect of diabetes induction on cerebral catalase activity and levels of reduced glutathione (GSH), malondaldehyde (MDA) in global cerebral ischemia-reperfusion model.
Data are represented as mean ± S.E.M. of 8 rats per group. (*, +) are significant difference from sham diabetic and sham groups, respectively.

| Groups | Catalase (U/mg tissue protein) | GSH (nmole/mg tissue protein) | MDA (nmole/mg tissue protein) |
|---|---|---|---|
| **Sham diabetic** | 6.37±0.60 | 28.20±2.10 | 5.30±0.30 |
| **Diabetic I/R** | 1.40±0.50* | 13.70±1.10* | 11.60 ±0.80* |
| **Sham** | 9.27±0.50* | 41.60±0.80* | 2.25±0.20* |
| **I/R** | 2.20±0.40+ | 20.00±1.60+ | 10.31±0.90+ |

### 3.4. Effect on carotid iNOS and eNOS protein expressions

Compared to sham-operated groups (diabetic and non-diabetic), induction of I/R injury showed a marked increase in iNOS protein expression with a substantial decrease in the expression of eNOS. Diabetic I/R group showed a significant difference ($p<0.05$) in carotid iNOS protein over expression and low expression of carotid eNOS compared to non-diabetic I/R-induced group (**Figure 3**).

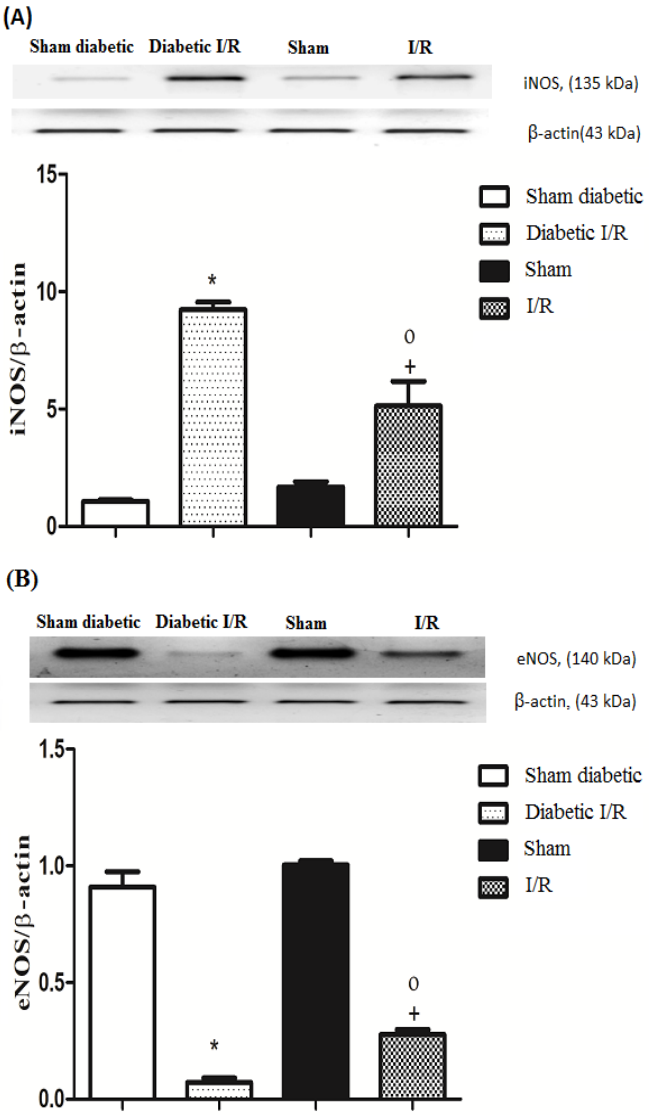


**Figure 3:** Effect of diabetes induction on carotid iNOS (A), and eNOS (B) protein expression in global cerebral I/R model.
Data are represented as mean ± S.E.M. of 8 rats per group. (*, + , °) are significant difference from sham diabetic, sham and diabetic I/R groups, respectively.

### 3.5. Effect on cerebral infarct volume

Brain tissues of diabetic I/R-injured rats showed a significant ($p<0.05$) enlargement in infarct volume compared to brains of sham-operated diabetic rats. Similarly, brain tissues of non-diabetic I/R-injured rats showed a significant ($p<0.05$) enlargement in infarct volume compared to brains of sham-operated non-diabetic rats. Diabetic I/R group exhibited a significant increase in infarct volume compared with I/R non-diabetic rats (12.18 ± 0.80% vs. 8.00 ± 0.50 %, respectively). Representative images of TTC staining are shown in( **Figure 4**).


**Figure 4:** Effect of diabetes induction on cerebral infarct volume in global cerebral I/R model. (A) Representative coronal diabetic brain sections showed % of brain infarction in sham group. (B) Quantitative changes in brain infarction are represented as % of infarct volume.
Data are represented as mean ± S.E.M. of 8 rats per group. (*, + , °) are significant difference from sham diabetic, sham and diabetic I/R groups, respectively.

## 4. Discussion

Stroke and cerebral ischemia are typical DM complications due to the impairments in cerebral vascular supply and neuronal toxicity associated with hyperglycemia [12]. It has been reported that diabetic patients experience stroke more than non-diabetic population [18]

DM has been associated with structural brain changes and many studies reported brain atrophy in late diabetic patients [18, 19]. Hyperglycemia increases the production of ROS through a protein kinase C-mediated pathway and enhances production of nicotinamide adenine dinucleotide phosphate (NADPH), which can lead to neuronal death [10]. Consistent with these data, we reported that induction of DM caused an increase in oxidative stress parameters compared to non-diabetic rats.

The up regulation of oxidative stress is also an important pathological pathway for ischemic stroke. After transient brain ischemia, there is a burst of free radical generation that begins within minutes of reperfusion and resolves within minutes to hours [20, 21]. The oxidative stress due to I/R injury serves as activators of transcription factors leading to induction of gene expression of pro-inflammatory cytokines like TNF-α. Moreover, apoptosis also could be mediated by free radicals and I/R-induced oxidative stress [3, 22, 23]. As shown in previous results, we showed that induction of I/R injury via transient BCCAO causes an increase in oxidative stress and cerebral protein expression of TNF-α and caspase-3. These results were exhibited in both non-diabetic and diabetic groups. However, we noticed intense oxidative stress, inflammatory and apoptotic response in the setting of hyperglycemia-associated DM oxidative stress and these results were in line with previous studies [24, 25]

Previous studies have shown abnormal CBF in diabetic patients [19, 26]. DM elicited complications at the micro vascular level including low perfusion rates, thickening of capillary walls and abnormal proliferation of endothelial cells with increased vascular permeability [18]. DM-associated vascular injury could be explained by various mechanisms [27]. Endothelial dysfunction in DM subjects evident by suppression of vascular eNOS activity and up regulation of iNOS subsequently leads to production of cytotoxic peroxynitrite (ONOO-) which aggravates vascular injury [28, 29]. Disturbance in CBF after transient BCCAO may exaggerate endothelial dysfunction and vascular impairment, which eventually leads to increased stroke severity and delayed recovery **[**30]. In the current study, we observed that carotid arteries of diabetic rats, subjected to cerebral I/R injury, showed a significant reduction in eNOS protein expression along with an increase in protein expression of iNOS compared to non-diabetic rats with cerebral I/R-induced injury.

Interestingly, we reported no significant difference in term of cerebral oxidative stress and protein expression of TNF-α and caspase-3 in brain tissues of diabetic and non-diabetic rats groups subjected to cerebral I/R injury. These results could be explained by the effect of DM on CBF. Vascular impairment associated with DM decreases CBF and prevents full reperfusion to the ischemic brain. Free radical abrupt and subsequent increase in inflammatory and apoptotic responses would be blunted by DM-induced CBF reduction.

In conclusion, the present study showed that DM caused an exaggeration of ischemic stroke in rats subjected to transient BCCAO. The observed deleterious effect of DM in cerebral I/R injured-rats could be related to vascular impairment and neurotoxicity produced as a result of increased oxidative stress, inflammatory and apoptotic responses.

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