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ORIGINAL ARTICLE

Nateglinide Exerts Neuroprotective Effects *via* Downregulation of HIF-1α/TIM-3 Inflammatory Pathway and Promotion of Caveolin-1 Expression in the Rat's Hippocampus Subjected to Focal Cerebral Ischemia/Reperfusion Injury

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Abstract— Ischemic stroke is a major cause of death and motor disabilities all over the world. It is a muti-factorial disorder associated with inflammatory, apoptotic, and oxidative responses. Nateglinide (NAT), an insulinotropic agent used for the treatment of type 2 diabetes mellitus, recently showed potential anti-inflammatory and anti-apoptotic effects. The aim of our study was to elucidate the unique neuroprotective role of NAT in the middle cerebral artery occlusion (MCAO)-induced stroke in rats. Fifty-six male rats were divided to 4 groups ($n = 14$ in each group): the sham-operated group, sham receiving NAT (50 mg/kg/day, p.o) group, ischemia/reperfusion (IR) group, and IR receiving NAT group (50 mg/kg/day, p.o). MCAO caused potent deficits in motor and behavioral functions of the rats. Significant increase in inflammatory and apoptotic biomarkers has been observed in rats' hippocampi. Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway was significantly stimulated causing activation of series inflammatory biomarkers ending up neuro-inflammatory milieu. Pretreatment with NAT preserved rats' normal behavioral and motor functions. Moreover, NAT opposed the expression of hypoxia-inducible factor-1α (HIF-1α) resulting in downregulation of more inflammatory mediators namely, NF-κB,

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tumor necrosis factor-β (TNF-β), and the anti-survival gene PMAIP-1. NAT stimulated caveolin-1 (Cav-1) which prevented expression of oxidative biomarkers, nitric oxide (NO), and myeloperoxidase (MPO) and hamper the activation of apoptotic biomarker caspase-3. In conclusion, our work postulated that NAT exhibited its neuroprotective effects in rats with ischemic stroke *via* attenuation of different unique oxidative, apoptotic, and inflammatory pathways.

KEY WORDS: nateglinide; inflammatory biomarkers; ischemia; oxidative pathway; neuroprotective.

INTRODUCTION

Stroke is fast becoming a substantial cause of death and disability in adults all over the world [1]. Ischemic stroke is caused by blockage of the cerebral artery as a result of thrombosis or embolism [2]. Consequently, a potent reduction in brain blood supply occurs leading to hindering the needed amount of nutrients and oxygen to reach the brain apparently resulting in neuronal dysfunction [3]. The mechanism of neuronal dysfunction is intricate and involves different oxidative, apoptotic, and inflammatory events which lead to functional disability and cognitive impairment [4, 5]. Recombinant tissue plasminogen activator (rTPA) is believed to be the only approved treatment for cerebral ischemic stroke which allows reperfusion of ischemic regions [6]. The reperfusion helps in recuperating some brain damage and in reversing ischemic brain injury. However, early blood reperfusion for ischemic tissue could negatively affect the prognosis of cerebral ischemia [7]. Following reperfusion, different apoptotic and inflammatory factors appear to be produced causing a series of deteriorating effects on the brain [8]. Therefore, there is an increasing interest to discover potential pathological pathways and providing protective measures against them in order to decrease the incidence of ischemic stroke. Under these ischemic conditions, the physiological response to hypoxia is primarily mediated by hypoxia-inducible factor-1α (HIF-1α) which is markedly activated to regulate cellular responses under such conditions [9]. Interestingly, HIF-1α also plays an important role in the pathogenesis of several inflammatory and ischemic-associated diseases [10, 11]. In addition, HIF-1α controls the expression of T cell immunoglobulin and mucin domain-3 (TIM-3) [12] which is an important molecular player in inflammation-associated brain injury under hypoxic conditions [13]. HIF-1α/TIM-3 axis consequently stimulates expression of series of inflammatory and apoptotic mediators, namely nuclear factor kappa-B (NFκB) [14], tumor necrosis factor-beta (TNF-β) [15], and the anti-survival gene (PMAIP-1) [16]. The modulation of this pathway can confer neuroprotection activity after ischemic events. The other potential pathway we aimed to target is the stimulation of the neuroprotective mediator, caveolin-1 (Cav-1) which casually switches off number of oxidative and apoptotic mediators, namely caspase-3, nitric oxide (NO), and myeloperoxidase (MPO) after ischemic events [17, 18]. In addition, Cav-1 appears to be a critical determinant of BBB permeability [17]. During focal cerebral ischemia, the expression of Cav-1 was downregulated in the brain and NO is highly produced as a result of the loss of Cav-1 [19]. High concentration of NO derived from inducible NO synthase (iNOS) can induce cell death [20], increase infarction volume, and induce BBB hyperpermeability [21]. Although the roles of NO in cerebral ischemia-reperfusion injury are intensively studied, the prevention of its expression prior to focal cerebral ischemia is not fully studied as a potential way for the protection against brain damage.

Nateglinide (NAT) is a d-phenylalanine derivative belonging to the meglitinide insulin secretagogue class usually used for the treatment of type 2 diabetic (DM) patients [22, 23]. It is a non-sulfonylurea β-cell activating agent which enhances insulin secretion by reducing K+ efflux *via* ATP-dependent K+ channels in pancreatic β-cells, hence depolarizing the cells and opening voltage-sensitive Ca2+ channels. The resultant increase in cytoplasmic Ca2+ triggers insulin release in a similar physiological pattern of insulin secretion [24, 25]. Additionally, NAT is believed to reduce glucose auto-oxidation, and hence reduce the generation of oxygen free radicals [26]. The safety margin of NAT is an added advantage over other meglitinide drugs. NAT showed no major hypoglycemia when used as a monotherapy in comparison with repaglinide which causes hypoglycemia in 7% of treated subjects [27].

Up to now, little attention has been paid to the investigation of the neuroprotective role of NAT and its impact

in prevention of ischemic stroke insults. Depending on the anti-inflammatory and anti-oxidative effects of NAT previously established [26, 28], the aim of the current work was to evaluate the potential neuroprotective effects of NAT in a rat middle cerebral artery occlusion (MCAO) model of focal cerebral ischemia and to clarify the possible underlying mechanisms. Apart from its hypoglycemic activity and the obvious effect of protecting against DM which is a serious risk factor of stroke, we examined the promising neuroprotective actions of NAT by investigating its capability to impede the neurodegenerative pathways (HIF-1α/TIM-3) and its downstream oxidative and inflammatory mediators that worsen the prognosis of the stroke and to examine its supportive role towards the neuroprotective pathway of Cav-1 previously mentioned.

There is a little data on investigating the ability of a neuroprotective agent in preventing the upregulation of HIF-1α/ TIM-3 and their subsequent pro-inflammatory mediators. Furthermore, the neuroprotective Cav-1 is a potential pathway that preserves normal neuronal status and prevents overexpression of oxidative and apoptotic mediators such as MPO and NO under ischemic conditions. Hence, we aim to evaluate the effect of NAT to positively affect both pathways and the reflection of these effects on preventing neuronal and brain damage.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 250–300 g were purchased from the National Scientific Research Centre (Giza, Egypt). The animals were housed in an air-conditioned room with 12-/12-h light/dark cycle, 25 ± 2 °C temperatures, and 60 ± 10% humidity with free access to food and water. All experiments were performed according to the protocol approved by the Ethics Committee of Faculty of Pharmacy Cairo University with approval number PT (2098). The study complies with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 85-23, revised 2011). All efforts were made to minimize animal suffering.

Drugs and Chemicals

NAT was obtained as a gift from the International Drug Agency for Pharmaceutical Industry company (Cairo, Egypt) and was suspended in 1% aqueous solution of carboxymethyl cellulose. NAT was administered to each animal in a dose 50 mg/kg/day, p.o. for 4 weeks. All other chemicals were of analytical grade and were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Experimental Design

As described in Fig. 1, fifty-six rats were randomly allocated into 4 groups ($n = 14$ per group) as follows:

Group (1) (CTRL): Control sham-operated rats that received 0.5 ml of 1% CMC solution, p.o. for 4 weeks [29]. On the last day, they underwent sham operation. Rats were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg; i.p) and xylazine (10 mg/kg; i.p.) and the carotid arteries were exposed for 45 min, but no ischemia was induced.

Group (2) (NAT): sham-operated rats that were pretreated with NAT (50 mg/kg/day, p.o. dissolved in 1% CMC solution) for 4 weeks [30]. On the last day, they underwent sham operation. Rats were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg; i.p) and xylazine (10 mg/kg; i.p.) and the carotid arteries were exposed for 45 min, but no ischemia was induced.

Group (3) (IR): Ischemia/reperfusion rats that received 0.5 ml of 1% CMC solution, p.o. for 4 weeks. On the last day, they were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg; i.p) and xylazine (10 mg/kg; i.p.) and underwent MCAO for 45 min followed by 24 h of reperfusion.

Group (4) (IR + NAT): Ischemia/reperfusion rats that were pretreated with NAT (50 mg/kg/day, p.o. dissolved in 1% CMC solution) for 4 weeks. On the last day, they were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg; i.p) and xylazine (10 mg/kg; i.p.) and underwent MCAO for 45 min followed by 24 h of reperfusion. On day 29, all animals underwent behavioral tests; the beam walk test, corner turn test and forelimb use asymmetry test with 2 to 3 h rest between tests. Behavioral tests were performed blindly in a half-lighted room (30 ± 5 lx) at 20–22 °C. At the end of the experiment, rats were euthanized with a pentobarbital overdose. Afterwards, the rats from each group were randomly divided into three sets and then brains were rapidly dissected. In the first set ($n = 2$), brains were fixed with 10% (v/v) formalin for 24 h to perform histopathological examination. In the second set ($n = 2$), brains were used to measure brain water content. Hippocampi from rats of the third set ($n = 10$) were promptly dissected and stored at − 80 °C to be used for biochemical analysis. Each hippocampus was divided into 2 hemispheres with a blade to ipsilateral (right) and contralateral sides (left) to MCAO. To evaluate the significant differences

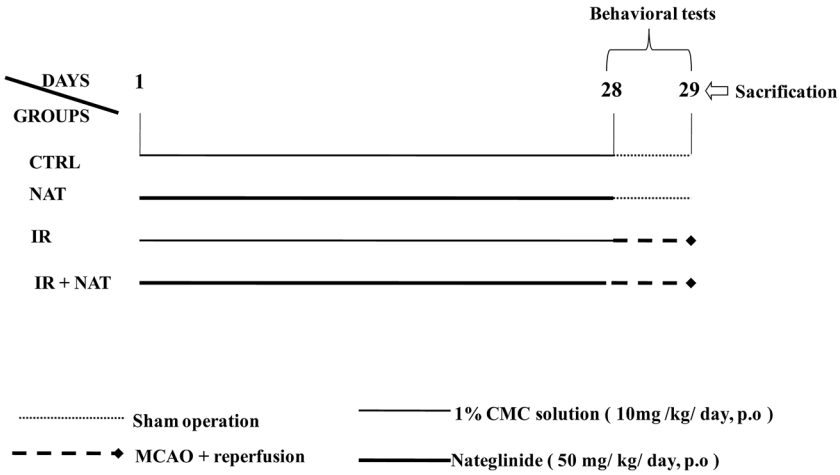

Fig. 1. Schematic diagram of the experimental design.

between the two sides and the action of the drug on both sides of the hippocampi, all the biochemical parameters are measured in both sides separately [12,31,32].

Methods

Induction of Focal Cerebral Ischemia

Focal cerebral ischemia was performed using the intraluminal monofilament occlusion of the right middle cerebral artery (MCA) for 45 min, according to the method previously described by Lee et al. (2005) [33]. The animals were anesthetized by a mixture of ketamine hydrochloride (50 mg/kg; i.p) and xylazine (10 mg/kg; i.p.). The ischemia was induced by occluding the right MCA *via* inserting a silicon-coated suture with a rounded tip (Doccol Corporation, Cat# 503856PK10) in the external carotid artery directly towards the internal carotid artery to block the origin of MCA. After 45 min of MCAO, reperfusion of the right MCA was initiated by removing the occlusive suture, and thus, the IR injury took place. The cerebral IR injury model was developed with 45 min of MCAO followed by 24 h reperfusion. In sham-operated rats, an incision was made over the MCA, but the artery was not occluded.

Neurobehavioral Tests

Different behavioral tests were carried out to evaluate the ameliorating actions of NAT on different behavioral and neurological dysfunctions in rats with IR injury. Scoring was done by an experimenter blinded to all experimental conditions.

Sensorimotor Testing. To estimate sensorimotor functions, the rats underwent beam walk tests 24 h after the IR insult. The test was performed strictly according to method previously illustrated by Goldstein et al. (2003) [34]. Rats were placed on a horizontal beam (60 cm long, 4.5 cm wide) for 60 s and assigned a score from 1 to 7 as follows: balances on the beam and crosses it without foot slips = 7, places the affected hind paw more than half times of the total steps = 6, places the affected hind paw more than one time = 5, places the affected hind paw on the beam once = 4, dragging the affected hind paw = 3, maintains balance for at least 5 s = 20, unable to place the affected hind paw on the beam and falls off the beam in less than 5 s = 1.

Forelimb Use Asymmetry Test. Rats were placed in a transparent cylinder (30 cm in height and 20 in cm diameter) for 5 to 10 min as described previously by Hua et al. (2002) [35]. Forelimb use was assessed by videotaping the rats in the cylinder and the number of times to use the ipsilateral and contralateral forelimb was counted. The score was assigned as follows: the occasions when the contralateral forelimb was used as a percentage of total number of limb use observations on the wall (C); the occasions when the ipsilateral forelimb was used as a percentage of total number of limb use observations on the wall (I); and the occasions when both forelimbs were used simultaneously as a percentage of total number of limb use observations on the wall (B). Limb use asymmetry score was calculated as follows: Limb use asymmetry score = $\frac{I}{I+C+B} - \frac{C}{I+C+B.}$

Corner Turn Test. Rats were subjected to the corner turn test using the method described by Zhang et al. (2002)

[36]. The rat was placed in an apparatus consisting of two vertical boards with an angle of 30° corner; each rat was allowed to move towards the corner. To exit the corner, the rat could turn either to the right or the left side, and the number of each turn was recorded. Only turns involving full rearing along either wall were included. The rat was allowed to repeat this process 10 times, with 30 s between trials, and then the percentage of right turns to the total attempts was calculated. The non-ischemic rat turns either left or right, but the ischemic rat preferentially turns towards the non-impaired, ipsilateral (right) side.

Evaluating Brain Water Content

Percent of brain water content was calculated according to the previously mentioned method by Hua et al. (2002) [35]. The brains were removed and the brain slice was divided into 2 hemispheres along the midline with a blade to ipsilateral and contralateral sides. Brain samples were immediately weighed on an electronic analytical balance (model AE 100, Mettler Instrument Co) to obtain the wet weight of the brain. The brain samples were then dried in an incubator for 24 h at 100 °C to get the dry weight. The % brain water content was calculated as follows:

$$\%\ \text{brain water content} = \frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} \times 100$$

Biochemical Measurements

Enzyme-Linked Immunosorbent Assay. Levels of PMAIP-1, MPO, HIF-1α, Caspase-3, TNF-β, NF-κB, and NO in hippocampal tissue homogenate were measured using Rat ELISA kits (R&D Systems Inc., Minneapolis, USA) according to the manufacturer's instructions.

Real-time Quantitative Reverse Transcription Polymerase Chain Reaction Analysis. Total RNA was prepared using Trizol reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions, and 1-mg RNA samples were used for cDNA synthesis. First-strand cDNA synthesis was primed with random hexamers and conducted according to the manufacturer's specifications (RT- PCR kit; Roche, Mannheim, Germany). cDNA equivalent to 200 ng of total RNA was subjected to PCR using the manufacturer's protocol (PCR core kit; Roche). The sense and antisense primers were used for the analysis of rat CXCL-1, Cav-1, and TIM-3. The primer sequences used in this study are represented in Table 1. The relative expression of target gene was obtained using the $2^{-\Delta\Delta CT}$ formula and β-actin as a housekeeping gene.

Western Blot Analysis. Rats' hippocampi tissues were subjected to protein expression analysis. The hippocampus tissues were homogenized, washed with phosphate buffered saline (PBS), and incubated in lysis buffer. Protein extraction kits were used to isolate cytosolic and nuclear protein fractions. After centrifugation at 12,000×*g* for 10 min, the proteins were collected from the supernatant, and their concentrations were determined using a BCA protein assay kit obtained for Pierce Biotechnology (Rockford, IL). The samples were loaded and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then transferred onto a poly-vinyl-idene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk and then incubated at 4 °C overnight with primary antibodies that recognize JAK2, STAT3, P-TYR Y1007-JAK2, P-TYR705 STAT3, and β-actin (Cell Signaling Technology, Inc., Beverly, MA, USA). After washing with Tris-buffered saline-tween 20 for three times, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1.5 h at room temperature. Finally, the quantitative protein band density was detected and assayed by the Quantity One system (Bio-Rad, CA, USA). The results are expressed after normalization to levels of the beta-actin protein.

Histological Examination

Brains samples were and fixed in 10% (v/v) neutral buffered formalin for 72 h. Samples were processed in serial grades of ethanol, cleared in xylene, and infiltrated and embedded with paraplast tissue embedding media into blocks. Five-micrometer-thick sagittal tissue sections were cut out from each sample and mounted on glass slides for evaluation of hippocampal regions. Tissue sections were stained with. The sections were stained with hematoxylin and eosin (H&E) staining Kit (Beyotime, China), then examined under a light electric microscope [37].

Statistical Analysis

Data were presented as the mean ± S.D. Statistical analysis was performed by 2 way ANOVA followed by Tukey's post hoc test to identify the difference in levels of biochemical parameters between different groups. Sidak's multiple comparisons test was used to assess differences in

Table 1. The Primer Sequences Used for Real-time Polymerase Chain Reaction (RT-PCR)

Gene	Primer sequence
CXCL1	F:5'CCAAACCGAAGTCATAGCCA3'
	R:5'CACCCTTTAGCATCTTTTGGA3'
Cav-1	F:5'CAGACGAGGTGAATGAGAAG3'
	R:5'TGCCGAAGATGGTAGACAG3'
TIM -3	F:5'GAACCTGCAACTGGAGAACC3'
	R:5'CTTCGTAAGCAGGTGCATCA3'

biochemical parameters between the ipsilateral and contralateral sides of the same group. One way ANOVA test was used to assess the difference in behavioral tests between different groups. The criterion for statistical significance was set at the $p < 0.05$ level. Statistical analysis and graphs were performed and created using GraphPad Prism software (version 6; GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

NAT Ameliorates MCAO-Induced Motor and Behavioral Impairments

Induction of IR in rats significantly dropped scores of beam walk test as compared to normal ones by 65.94% (Fig. 2). NAT managed to significantly preserve high score of the test when compared to IR by 138.09%. Marked higher scores have been documented in IR rats when compared to normal groups under the corner and forelimb asymmetry tests by 49.03% and 836.91%, respectively. NAT failed to markedly decrease the scores of corner turn test and forelimb asymmetry test in IR group although a little drop in their scores is observed.

NAT's Effects on the JAK2/STAT3 Signaling Pathway

As shown in Fig. 3, a significant increase in P-JAK2 and P-STAT3 levels, and the ratios of P-JAK2/JAK2 and P-STAT3/STAT3 in the ipsilateral side of the hippocampus of IR rats occurred as compared to the contralateral side to 105.9%, 123.48%, 118.11%, and 151.36%, respectively. The results of the IR group also showed a significant strike in both P-JAK2 and P-STAT3 levels by more than 5-folds, and their ratios P-JAK2/JAK2 and P-SATA3/STAT3 by 119.94% and 80.12%, respectively when compared to normal sham groups.

However, there is a significant decrease in both P-JAK2 and P-STAT3 to almost 37.6% and 32.56% of their values respectively, and for P-JAK2/JAK2 and P-STAT3/STAT3 ratios by almost half of their values in NAT + IR group when compared to IR values. Normal rats treated with NAT did not show any significant change in JAK/STAT signaling pathway as compared to normal rats.

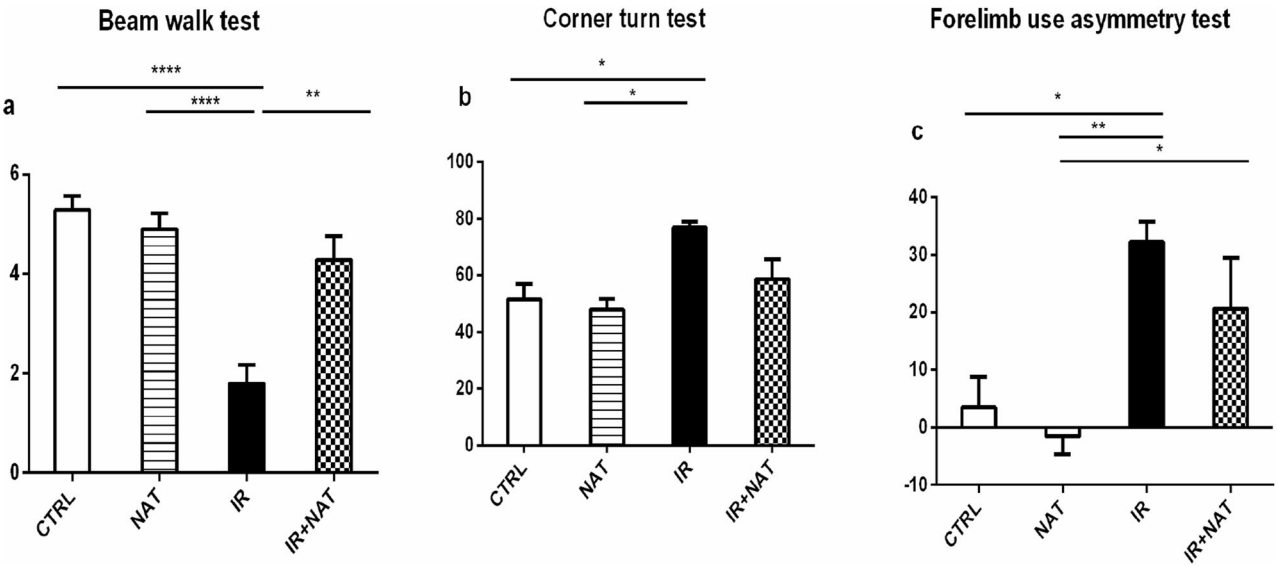

Fig. 2. Effects of NAT on IR- induced behavioral changes: **a** beam walk, **b** corner turn, and **c** forelimb use asymmetry test. Each bar with a vertical line represents mean ± S.D. Statistical analysis was performed by 1 way ANOVA followed by Tukey's post hoc test; the criterion for statistical significance was set at * $p < .05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ levels. CTRL, control sham; NAT, nateglinide; IR, ischemia/reperfusion.

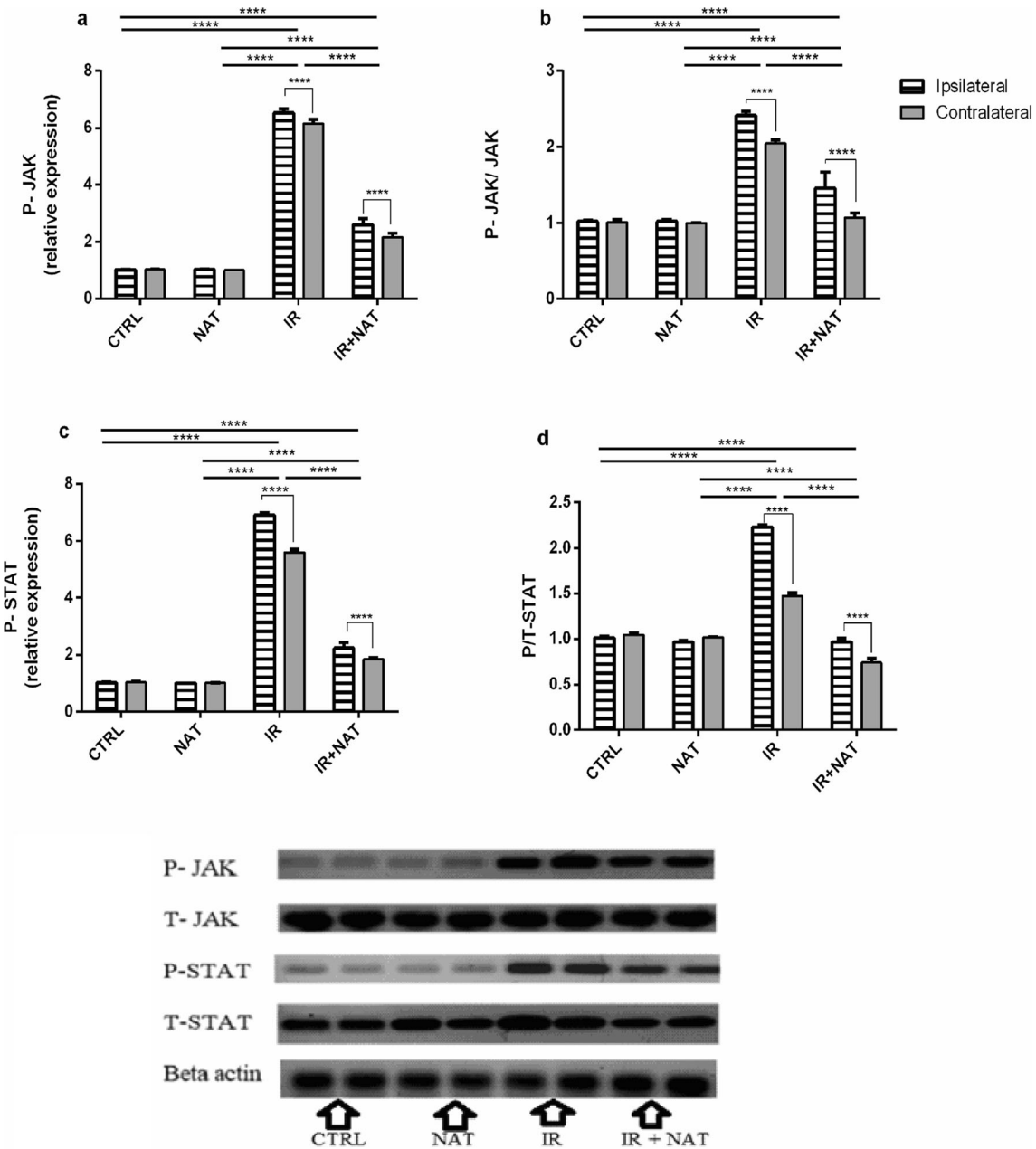

Fig. 3. NAT's effects on JAK2/STAT3 signaling pathway: **a** P-JAK2, **b** P-JAK2/JAK2, and **c** P-STAT3 and **d** P-STAT3/STAT3. Each stripped bar represents the mean ± S.D. of the ipsilateral side and each gray bar represents mean ± S.D. of the contralateral side of the hippocampus. Western blot analysis and quantification of P-JAK2, T-JAK2, P-STAT-3, T-STAT-3, and beta actin in sham and IR groups with and without NAT treatment. Statistical analysis was performed by 1 way ANOVA followed by Tukey's post hoc test; the criterion for statistical significance was set at * $p < .05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ levels. CTRL, control sham; NAT, nateglinide; IR, ischemia/reperfusion.

NAT's Effects on HIF-1α/TIM-3 Pathway

Results presented in Fig. 4 revealed a marked increase in levels of HIF-1α and TNF-β in ipsilateral sides of hippocampi of IR group to 126.64% and 107.5% as compared to their levels in contralateral sides, respectively. No significant difference appeared in TIM-3, PMAIP-1, CXCL-1, or NF-κB levels in ipsilateral side and contralateral sides.

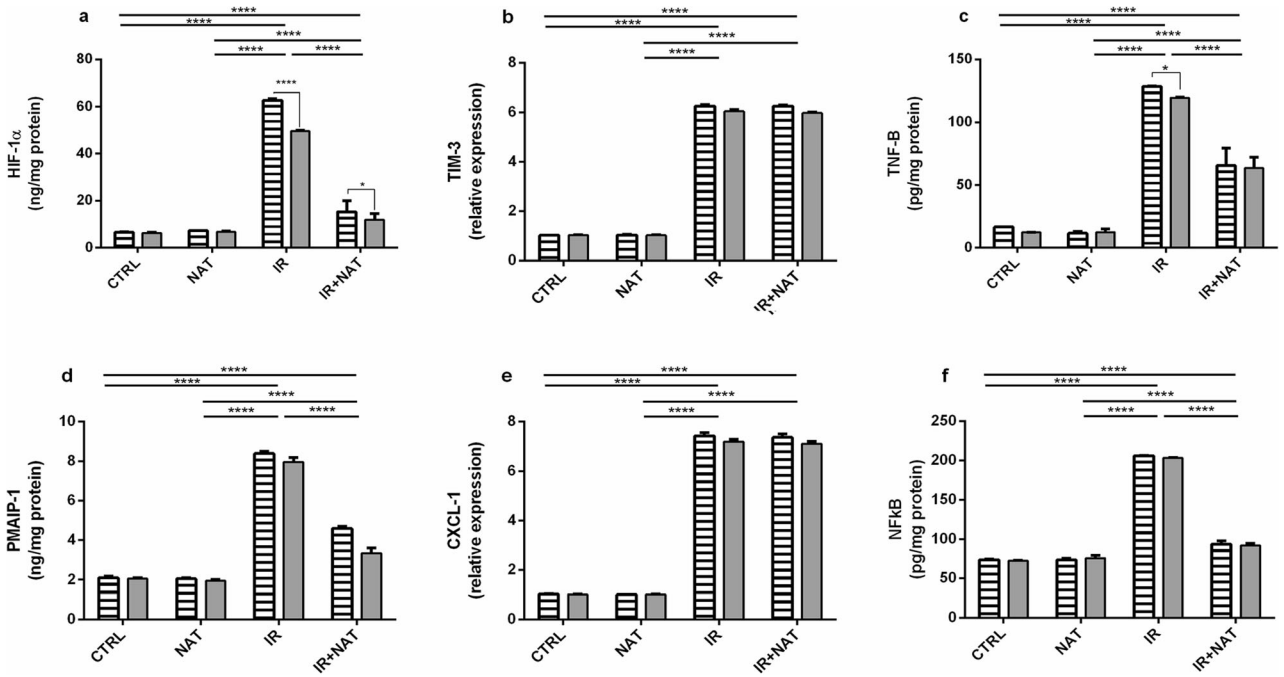

Fig. 4. Nateglinide's effects on HIF-1α/TIM-3 pathway: **a** HIF-1α, **b** TIM-3, **c** TNF-β, **d** PMAIP-1, **e** CXCL-1, and **f** NF-κB. Each stripped bar represents the mean ± S.D. of the ipsilateral side and each gray bar represents mean ± S.D. of the contralateral side of the hippocampus. Statistical analysis was performed by 1 way ANOVA followed by Tukey's post hoc test; the criterion for statistical significance was set at * $p < .05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ levels. CTRL, control sham; NAT, nateglinide; IR, ischemia/reperfusion.

NAT treatment produced a non-significant change in the levels of TNF-β in ipsilateral sides as compared with the contralateral side's values. On the other hand, HIF-1α levels showed a significant elevation in ipsilateral side than that of contralateral sides by 21.71% even after treatment with NAT.

The results also showed a major uplift in HIF-1α, TIM-3, TNF-β, PMAIP-1, CXCL-1, and NF-κB levels in IR group by approximately 8.7, 6, 8, 4, 7, and 2-folds from their values in normal groups, respectively. Administration of NAT to IR rats showed a significant drop in HIF-1α, TNF-β, PMAIP-1, and NF-κB levels as compared to IR group by 75.9%, 48%, and 51.5% and 54.8%, respectively.

NAT's Effects on Cav-1 Pathway

Figure 5 showed that there is a marked rise in the level of NO, MPO, and caspase-3 and percent of water content in ipsilateral sides of rats' hippocampi to reach 141.9%, 106.12%, and 119.11% and 115.17% of their levels in contralateral sides, respectively. Cav-1 showed a magnificent downregulation in the ipsilateral side by 31.75% when compared to contralateral side of hippocampi. Interestingly, administration of NAT to IR rats diminished the difference between ipsilateral and contralateral of caspase-3 and MPO levels, unlike the NO level and percent of water content which were found to be significantly higher in ipsilateral side by 16.4% and 12.35%, respectively. NAT failed to improve Cav-1 level in ipsilateral side, and there is an obvious difference between there levels as compared to contralateral sides by 18.6%.

NO, MPO, caspase-3, levels, and percent of water content showed a remarkable rise in IR groups by 453.66%, 235.39%, 417.29%, and 280.49%of their normal control levels, respectively. However, a marked inhibition of Cav-1 in IR group was observed by 76.5% of its normal value. Administration of NAT had a considerable effect in reducing NO, MPO, and caspase-3 levels to 32.23%, 90.35%, and 32.18% of their values in IR group and caused also a significant upregulation of Cav-1 levels when compared to that of IR group by almost 3-folds. NAT failed to produce a significant effect on MPO levels and percent of brain water content as compared to IR groups.

Histopathological Examination

Histopathological Examination of Hippocampal CA1 Region from Different Groups' Samples

Sections from CTRL and NAT groups demonstrated normal histological features of CA1 hippocampal layers

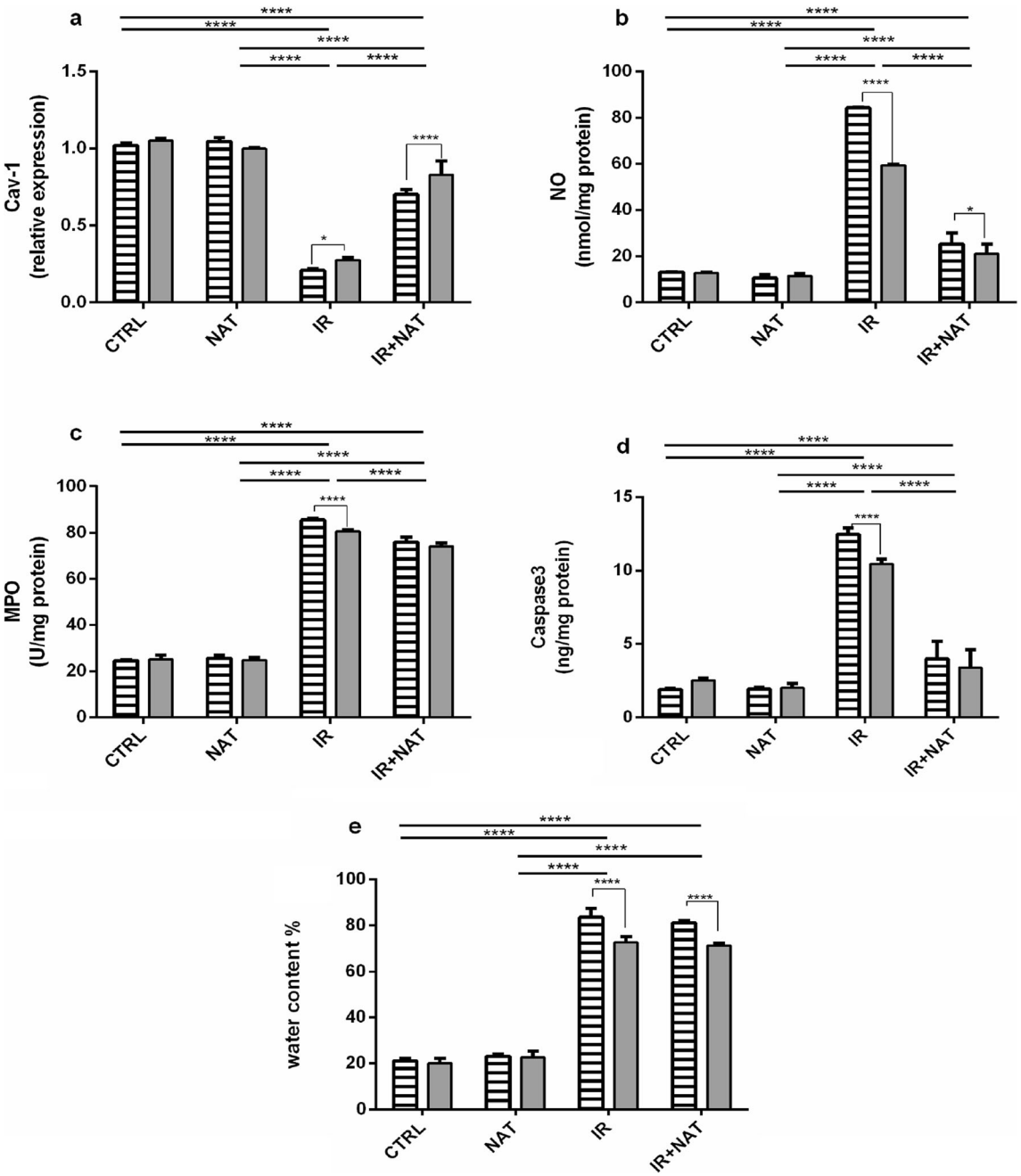

Fig. 5. Effects Nateglinide's effects on Cav-1 pathway: **a** Cav-1, **b** NO, **c** MPO, **d** caspase-3, and **e** brain water content. Each stripped bar represents the mean ± S.D. of the ipsilateral side and each gray bar represents mean ± S.D. of the contralateral side of the hippocampus. Statistical analysis was performed by 1 way ANOVA followed by Tukey's post hoc test; the criterion for statistical significance was set at * $p < .05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ levels. CTRL, control sham; NAT, nateglinide; IR, ischemia/reperfusion.

with apparent intact pyramidal neurons (arrows), intact cellular details, and minimal glial cells infiltrate as shown in Fig. 6. IR group showed severe neuronal loss with many necrotic shrunken neuronal cell bodies (red arrow), moderate perineuronal edema, and mild glial cells infiltrate (arrow head). IR + NAT group showed partially protected pyramidal neurons with variable mixture of shrunken, damaged darkly pyknotic cells (red arrow), apparent intact

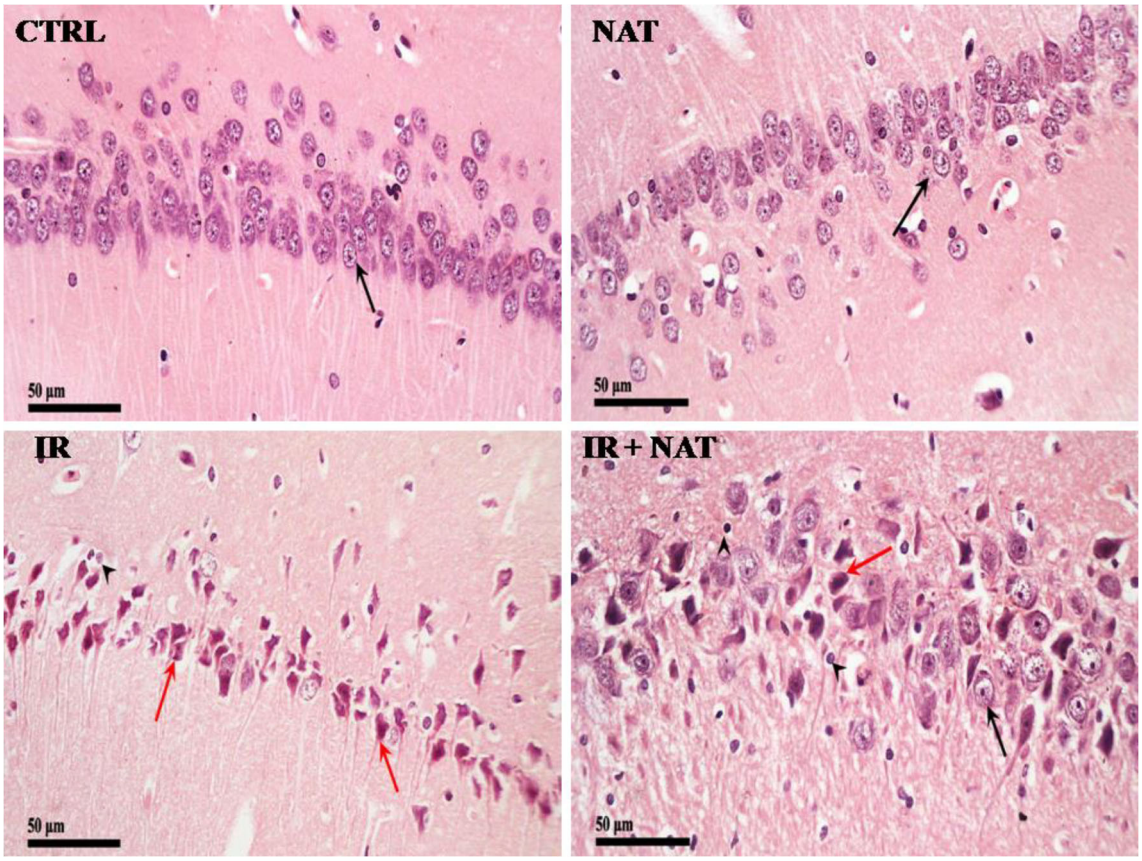

Fig. 6. Histological sections in the CA1 region of hippocampi of the experimental groups: control sham-operated group (CTRL), sham-operated group treated with nateglinide (NAT), control ischemia/reperfusion group (IR), ischemia/reperfusion group treated with nateglinide (IR + NAT).

neurons (black arrow), perineuronal edema, and mild higher glial cells infiltrates (arrow head). A significant increase in IR rats' neuronal degeneration has been shown when compared to sham control group. In addition, IR + NAT group showed magnificent reduction in degeneration score when compared to IR group (Table 2).

Histopathologic Examination of Hippocampal CA3 Region from Different Groups' Samples

Figure 7 illustrated that sections from CTRL and NAT groups showed normal histological features of hippocampal layers with apparent intact pyramidal neurons (arrows), intact cellular details, and minimal glial cells infiltrate in CA3 region of hippocampus. IR group showed severe neuronal loss, many necrotic shrunken neuronal cell bodies (red arrow), and moderate perineuronal edema. Treatment with NAT showed partially protected pyramidal neurons with variable mixture of shrunken, damaged darkly pyknotic cells (red arrow), and apparent intact neurons (black arrow), along with persistence of perineuronal edema.

Statistical analysis showed a significant increase in IR rats' neuronal degeneration when compared to sham control group. Treatment with NAT showed slight non-significance improvement in degeneration score when compared to IR group (Table 2).

DISCUSSION

In the present study, we aimed to investigate the possible ameliorative effects of NAT against focal cerebral ischemia *via* novel inflammatory and anti-apoptotic pathways and to examine the subsequent improvement in different motor functions. The results of our study advocated that behavioral and motor functions of the rats showed magnificent deficits after induction of ischemia. Our results

Table 2. Scoring System of Different Neurological Deficits of A1 and A3 Regions of Hippocampi of the Experimental Groups; Control Sham-Operated Group (CTRL), Sham-Operated Group Treated with Nateglinide (NAT), Control Ischemia/Reperfusion Group (IR), Ischemia/Reperfusion Group Treated with Nateglinide (IR + NAT)

	CTRL		NAT		IR + NAT		IR	
	CA1	CA3	CA1	CA3	CA1	CA3	CA1	CA3
Neuronal damage	–	–	–	–	+++	+++	++	++
Edema	–	–	–	–	++	++	++	++
Glial cells infiltration	–	–	–	–	+	+	+	+

–, Null; +, Mild; ++, Moderate; +++, Severe

demonstrated that apart from its neuroprotective actions and its role in downregulating the expression of neurodegenerative biochemical parameters, NAT has a positive protective effect against some of the behavioral deficit induced by ischemia. Although NAT could not manage to significantly improve scores of forelimb asymmetry and corner turn tests, it has an obvious useful effect on motor balance as evidenced by the results of the beam walk test. Interestingly, the positive effects of NAT towards motor functions could be attributed to the supportive role that NAT played to maintain normal level of Cav-1 and prevent its reduction during ischemia. This suggestion is based on a recent study which documented that overexpression of Cav-1 is neuroprotective against brain injury and plays an important role in maintaining neurobehavioral and motor functions [38].

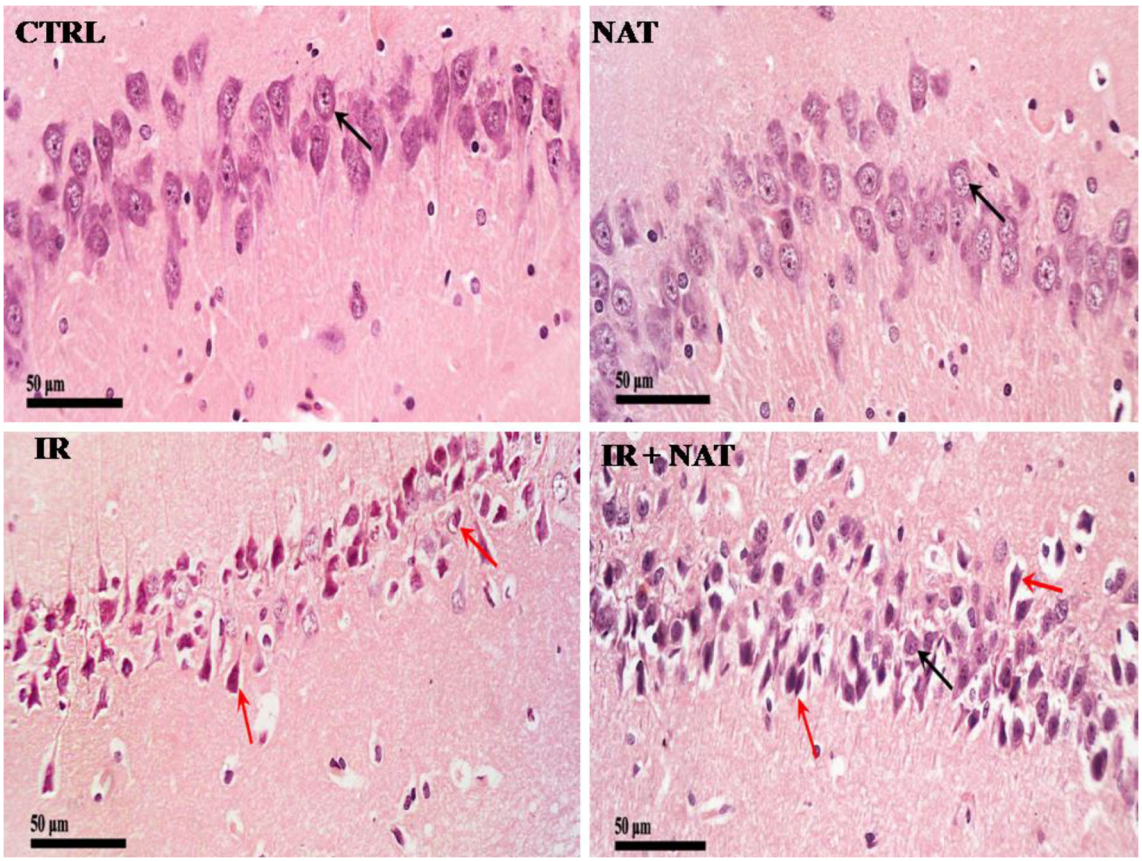

Fig. 7. Histological sections in the CA3 region of hippocampi of the experimental groups: control sham-operated group (CTRL), sham-operated group treated with nateglinide (NAT), control ischemia/reperfusion group (IR), ischemia/reperfusion group treated with nateglinide (IR+ NAT).

Accumulating evidence indicates that inflammation, apoptotic, and oxidative stress are implicated in the pathogenesis of ischemic stroke [39]. The current study revealed that induction of focal cerebral ischemia by MCAO results in activation of different inflammatory and apoptotic pathways. JAK2/STAT3 is considered one of the classic inflammatory pathways which is highly activated during ischemic stroke and negatively affects the neurological and vascular functions. However, there are conflicting reports on the effects of blocking this pathway. Some studies have reported worsened neurological recovery when blocking this pathway [40], while others have demonstrated advantageous effects [41]. NAT showed substantial prevention of JAK2/STAT3 phosphorylation and activation. This recorded decline could be attributed to the anti-inflammatory effect of NAT previously reported in different studies [28, 42]. Moreover, NAT stimulated glucagon-like peptide-1 (GLP-1) secretion *via* a SUR1/KATP channel-independent mechanism [30]. Significant neuroprotection effects gained *via* GLP-1 receptor activation have been shown in several animal neurodegenerative models of stroke and Alzheimer's disease [43, 44]. The beneficial effects of these substances are mediated in the brain, often independently from the regulation of glycaemia [45]. This can be an explanation of the ability of NAT to downregulate serious inflammatory biomarkers in the brain prior to stroke induction. In addition, phosphorylated STAT3 can stimulate number of ischemic and inflammatory mediators namely HIF-1α to which STAT-3 binds and promote its stability during ischemia [46]. Under the hypoxic conditions of cerebral ischemia, HIF-1α acts as the key biological mediator and plays a pivotal role in mediating vascular responses and preventing vascular damage in a hypoxic environment, therefore it is highly upregulated in cerebral ischemia [47] and after MCAO [48]. Although the expression of HIF-1α plays crucial role in hypoxic conditions, it may cause neurodegenerative actions and affects negatively on vascular and neurological levels following ischemic state [49]. HIF-1α activates a sequence of pro-inflammatory and apoptotic biomarkers that affects the intima of blood vessels and deteriorate the neurological functions. NF-κB is the first inflammatory biomarker triggered by HIF-1α [50]. HIF-1α binds directly to NF-κB enhancing its stability and activity [51]. NF-κB is a key player in induction of inflammatory responses and acts also as an activator of various pro-inflammatory cytokines and is reported to be one of the most deteriorating pro-inflammatory mediators in stroke [52]. It contributes in the activation of TNF-β, T cell immunoglobulin, and mucin domain protein (TIM-3) gene and CXCL-1, the important chemokines that play a vital role in neutrophils recruitment during stroke giving more chance for excessive deteriorating and inflammatory lesion [15, 53, 54]. In addition, the present study also revealed the obvious rush of anti-survival gene PMAIP-1. This can be justified depending on the role of HIF-1α in aggravating PMAIP-1 expression under hypoxic conditions with subsequent worsening of the ischemic insult [55, 56]. The ability of NAT to reticence phosphorylation of STAT-3, which is important for HIF-1α stabilization, can directly lead to subsequent prevention of HIF-1α stimulation and stabilization. Preventing HIF-1α stimulation allows NAT to stop expression of the HIF-1α-dependent inflammatory and apoptotic mediators, namely, PMAIP-1, NF-κB, and its subsequent pro-inflammatory biomarkers TNF-β. However, NAT did not show a significant protection against TIM-3 and CXCL-1 overexpression. The detailed mechanisms underlying this process are still obscure.

Another promising avenue of our investigation was that the efficiency of NAT to notably promote the defensive molecular signaling pathway that is mastered normally by Cav-1 which is a scaffolding plasma protein domains that plays a vital helpful function in the process of endothelial cell differentiation [57]. Our results showed the ability of NAT to offer potent activation and preservation of Cav-1 prior induction of ischemia as compared with MCAO group. The deficiency of Cav-1 can endorse the activation of number of deteriorating inflammatory, apoptotic, and oxidative stress biomarkers such as NO and MPO that can end up with extensive neurological injuries following the ischemic stroke [58, 59]. MPO is a pro-inflammatory mediator profusely secreted after stroke and highly expressed in absence or deficiency of Cav-1 [60]. The increased MPO activity leads to neutrophils recruitment which is the major source of reactive oxygen species causing serious oxidative stress [61]. In a border context, the reduction of Cav-1 expression, occurring in focal cerebral ischemia, can consequently enhance endothelial nitric oxide synthase (eNOS) activation which in turn stimulate NO production in endothelial cells and leads to marked microvascular hyperpermeability with a subsequent elevation in percent of brain water content [62]. In particular, the high level Cav-1 inhibits NO signaling by binding and inhibiting eNOS [63]. One of the protective roles of Cav-1 is that it significantly lower expression of the apoptotic biomarkers including caspase-3 [64]. Consistently, [65] showed that expression of cleaved caspase-3 was also higher in the Cav-1 restrained cells ensuring the defensive function of Cav-1 against neuronal apoptosis. These compelling data may help us to understand the findings of the current study

which advocated significant upregulation of MPO, NO, and caspase-3 levels and the percent of brain water content side by side with the suppression of Cav-1 level following the MCAO. Based on these data, the ability of NAT to counteract the decline in Cav-1 expression may, therefore, give the answer to the mechanism of action by which NAT can significantly hinder the levels of MPO, NO, caspase-3, and brain water content as postulated in the current results.

Histological evaluations revealed that the MCAO model caused neuronal degeneration and glial cell infiltration in hippocampal tissues. Many reports have already confirmed histological degeneration of hippocampus after IR insult [66]. Pretreatment with NAT effectively prevented these histological alterations and preserved normal tissue morphology in IR rats. In harmony with this finding, previous study postulated that NAT can effectively prevent hippocampal CA3 region neuronal death [67]. The prevention of inflammatory and apoptotic mediator's activation caused by NAT administration may provide a reasonable explanation of the neuroprotective action showed in the histological examination.

In conclusion, the most obvious findings to emerge from this study are that (i) NAT is able to oppose some motor activity deterioration and histological alteration in IR group. This improvement in the motor function and histological morphology is hypothesized to be attributed to the positive role that NAT played to maintain high level of Cav-1. (ii) NAT firmly showed an anti-inflammatory effect which appeared by hindering number of pivotal inflammatory controllers, namely JAK2/STAT3 as well as HIF-1α/TIM-3 pathways. (iii) NAT switched off the expression of apoptotic and oxidative biomarkers, NO and MPO, due to its ability to preserve normal level of Cav-1 after incidence of stroke. (iv) As a result of the different neuroprotective actions gained by NAT, the histopathological examination showed a marked improvement in rats treated with rats as compared to those non-treated ones.

These promising findings provide some support for the conceptual premise that the importance of NAT as a protective agent against stroke is not restricted upon its ability to manage type 2 DM, but also appears to be contributed to its ability to act directly on the brain and to hinder different inflammatory and anti-survival pathways, and thus, we suggest that further research should be undertaken to investigate the possibility of using NAT as a prophylactic strategy for patients who are highly suspicious to be affected by stroke or any other neurodegenerative disease.

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COMPLIANCE WITH ETHICAL STANDARDS

All experiments were performed according to the protocol approved by the Ethics Committee of Faculty of Pharmacy Cairo University with approval number PT (2098). The study complies with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 85-23, revised 2011). All efforts were made to minimize animal suffering.

Conflict of Interest. The authors declare that they have no conflicts of interest.

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