Bull. Egypt. Soc. Physiol. Sci. *Vol. (45), Issue (2)*,206-218

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

The Prophylactic Role of Amlodipine in Cerebral Ischemia via PGC1α/Nrf2/TFAM Pathway Activation

Amlodipine activates PGC1α/Nrf2/TFAM in Cerebral ischemia

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Submit Date : 15 Oct. 2024
Revised Date : 25 Nov. 2024
Accept Date: 26 Nov. 2024

Keywords

- Amlodipine
- Cerebral ischemia
- PGC1α
- Nrf2
- TFAM

Abstract

Ischemic stroke is a leading cause of permanent disability and death. Mitochondrial dynamics is a novel target strategy for management of cerebral ischemia. Amlodipine was recently reported to target mitochondria in renal ischemia suggesting it as a possible mechanism in cerebral ischemia. 30 adult male rats were divided into 3 groups: 1-Sham-operated group: with no ischemia, received normal saline. 2-Ischemic Group: with Middle Cerebral Artery Occlusion (MCAO) model & 3-Amlodipine group: received Amlodipine (10 mg/kg) via gavage twice, 10 minutes before and 24 hours after developing MCAO model 72 hours after MCAO, rotarod test was performed. At the end of the experiment serum oxidative DNA damage marker (8-hydroxy-2'-deoxyguanosine level) was assayed. Additionally, oxidative stress markers (Reactive Oxygen Species, malondialdehyde and superoxide dismutase), inflammatory marker (tumor necrosis factor-alpha), mitochondrial (Peroxisome proliferator-activated receptor gamma coactivator (PGC1α), Mitochondrial transcription factor A (TFAM)) levels were measured in brain tissue as well as the gene expression of nuclear factor erythroid 2-related factor 2 (NRF2). Histopathological and caspase-3 immunohistochemistry were also performed. Our results showed that amlodipine significantly protects against the ischemic brain injury as evident by the upregulation of the PGC1α/Nrf2/TFAM which restored normal mitochondrial function. Also, the ischemia-induced oxidative stress, inflammatory stress, oxidative DNA damage and apoptosis were mitigated by amlodipine with subsequent decrease of the neuronal damage. Accordingly, amlodipine use in protection against ischemic stroke is favored due to its mitochondrial targeting mechanism. Nonetheless, more research is needed to fully understand its additional effects.

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Introduction

Ischemic stroke represents about 85% of all stroke cases, making it the leading cause of permanent disability and death.(1)

It is established that enhanced mitochondrial function results in increased intracellular ATP level and mitigates neuronal injury (2, 3). Since mitochondria is the main regulator of cell energy, ATP production, Ca2+ homeostasis, lipid peroxidation, ROS production and antioxidant defense signals especially in high energy cells such as brain cells, any disruption of mitochondrial dynamics causes severe cell damage and apoptosis. Thus, targeting mitochondrial dynamics can provide an alternative whenever common treatment is limited or failed(4) .

Mitochondrial dynamics refers to the maintenance of integrity, distribution, and size of mitochondria in response to external stimuli through the continuous cycle of mitochondrial fission, fusion, degradation, and biogenesis (5).

Peroxisome proliferator-activated receptor gamma coactivator (PGC-1α) signaling pathway stimulation induces mitochondrial biogenesis. Therefore, drugs targeting this pathway are promising new strategy for brain ischemia treatment or prophylaxis (6)

Mitochondrial transcription factor A (TFAM) is downstream of PGC-1α, which protectsmtDNA against ROS-induced damage in ischemia (7).PGC-1αcontrols antioxidant genes via Nuclear factor erythroid 2-related factor 2(Nrf2) which is a key cytoprotective transcription factor via upregulation of the antioxidant proteins(8)

Amlodipine is a lipophilic, long-acting, third-generation dihydropyridine (DHP) Calcium channel blocker. Amlodipine is used in management of hypertension/angina patients with a suggested prophylactic role against complications including cerebral stroke (9).

With a high bioavailability of 60% to 80%, amlodipine usually reduces blood pressure to baseline within a week without causing any harmful rebound spikes (10)

Prior research reported amlodipine's role as an antioxidant renal ischemia reperfusion. The investigated mechanism included upregulation Nrf2 and Sestrin2, improvement of mitochondrial biogenesis by promoting the Sestrin2/PGC-1α/TFAM pathway. (11)

While these findings pave the way for further investigation of these mechanisms in cerebral ischemia, the exact mechanism of amlodipinein cerebral ischemia is not studied in vivo before which is our target study

Material and Methods

Chemicals and drugs

The amlodipine was provided by Amreya Pharmaceutical Industries (Amreya, Alexandria, Egypt). Rats were fed standard rat diet (3.8 kcal/g; 63.4% carbohydrate, 25.6% protein, and 11.0% fat).

Experimental animals

The study was conducted on 30 adult male rats of the local strain weighing 200–240 g that were kept in standard, well-ventilated animalenclosures at room temperature with food and water ad libitum. Rats were monitored daily for any illnesses or cage aggression signs. The animal handling protocolswere approved by the ethics committee of Faculty of Medicine, Tanta University (approval code number: (36264PR802/0/24).

Experimental design

After a week of acclimation, the rats were randomly allocated to one of three groups (10 rats each).Group I (Sham-operated group) with no ischemia which received normal saline. Group II (Ischemic Group).Middle Cerebral Artery Occlusion (MCAO) model. Group III (Amlodipine group) receivedAmlodipine that was administered via gavage and normal saline was used as the vehicle. The highest and most effective dose of amlodipine in previous studies of organ ischemia/reperfusion in rats that had no side effects (10 mg/kg) was administered in this study(12)10 minutes prior to developing MCAO model. Another dose was given 24 hours after MCAO.

MCAO Model

Focal cerebral ischemia was induced by unilateral middle cerebral artery occlusion (MCAO) according to previous methods by blocking the origin of the right middle cerebral artery(13). Briefly, after general anesthesia (1.5% isoflurane in 30% O_2 and 70% N_2O), exposure of the right common carotid, internal carotidand external carotid arteries, a monofilament nylon suture (external diameter 0.28–0.38 mm) with a rounded tip coated with silicon was inserted into the right internal carotid artery until facing faint resistance. After 30 minutes, thefilament was withdrawn to allow reperfusion. In the sham group, the same surgical procedure was performed without closure of the middle cerebral artery. We selected 30 min MCAO because previous studies have shown that effects of ischemic brain damage could be clearly differentiated with this short length of ischemic insult with lower mortality rates and milder neurological deficit allowing for more extensive neurobehavioral evaluation. (14).

Rotarod test:

This neurobehavioral test was carried after 72 hours of reperfusion in accordance with the methodology of (15)with some modification. Each rat was trained four times, 3 days before the MCAO. The Rat RotaRod NG (47750; Ugo Basile, Gemonio, Italy) has a rotating shaft and four compartments.Rats were placed on the rod rotation apparatus for 15 seconds, and the recording was started when their gait became stable with maintained balance. The speed of the rotating rod was increased from 5 rpm to 40 rpm within 1 min, and the standing time was recorded. The trial timed out at 300 s. The test was repeatedfivesubsequent times with a 1-min rest period in their cage between trials. The maximum and minimum values were removed, and the values of the remaining three trials were averaged.

Blood and brain tissue collection

After rotarod test was carried out, the rats were given an intraperitoneal dose of pentobarbital (50 mg/kg)(16) and then sacrificed by cervical dislocation. Collected blood samples were centrifuged at 3,000 rpm for 10 minutes, and separated serum was kept 4 °C. Brains were dissected very quickly and carefully to avoid any mechanical trauma, washed with ice cold saline, and then divided into pieces which were either wrapped in aluminum foil and stored at –80°C till used for preparation of brain tissue homogenate, or fixed in 10% formaldehyde solution for histopathological and immnuohistochemical studies. The sacrificed animals were packed in special package according to safety precautions

and infection control measures and sent with hospital biohazard

Biochemical assays of inflammatory, apoptotic, and redox and mitochondrial markers

Serum oxidative DNA damage (8-hydroxy-2'-deoxyguanosine level) was measured in all prepared sera of all groups as described by(17)

Tissue samples were homogenized in a phosphate buffer (10% (w/v), 50 mM, pH 7.4), centrifuged at 7,700 ×g for 30 minutes andseparated supernatant was separated was kept at −80 °C until analysis. The concentration of the inflammatory marker tumor necrosis factor-alpha (TNF-α),Reactive oxygen species (ROS), TFAM and PGC-1α levels was measured using thecommercially available enzyme-linked immunosorbent assay (ELISA) kit (Catalog no. MBS355371, MBS039665,MBS942857, MBS762203; MyBiosource, San Diego, CA, USA) respectively in accordance with the manufacturer's instructions. The antioxidant marker Superoxide Dismutase (SOD) activity was estimated using a kit from Bio-Diagnostic Co., Egypt, according to(18). The concentrations of malondialdehyde (MDA), a lipid peroxidation marker, were determined using a previously described method(19). The protein content was determined using the Lowry technique with bovine serum albumin(20)

Relative gene expression of nuclear factor erythroid 2-related factor 2 (Nrf2)

The frozen tissues were processed, total RNA was extracted using the Qiagen RNeasy Total RNA isolation kit (74,104) according to the manufacturer's guidelines (Qiagen, Hiden, Germany), then cDNA was synthetized using the SuperScript III First-Strand Synthesis System for RT-PCR kit (18,091,050) (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The PCR was performed using the PCR Master Mix Power SYBR Green (Life Technologies CO., Carlsbad, California, USA), following the manufacturer's guidelines. The Nrf2 mRNA expression was measured in relation to the housekeeping gene(GAPDH) to normalize the mRNA transcripts. The primers (designed by Primer3 software): rat Nrf2 forward primer (5′-TAGCAGAGCCCAGTGGCGGT-3′), reverse primer (5′-TGCTCTGGGGATGCTCGGCT-3′) (GenBank Accession No. NM_031789.2); rat GAPDH forward primer (5′-GGTGAAGTTCGGAGTCAACGGA-3′) and reverse primer (5′-GAGGGATCTCGCTCCTGGAAGA-3′) (GenBank Accession No. NM_017008). The relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ formula (18)

Histopathological and immunohistochemical examination

Brain tissues were fixed in 10% buffered formalin and embedded in paraffin and cut into 4-µm-thick sections with a microtome for staining with hematoxylin and eosin (H&E) stain.

For immunohistochemical examination of caspase-3,dewaxing and rehydration of sections was done in a descending series of alcohols. Thenincubation in 3% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase activity. Antigen retrieval was done in a microwave oven for 10 minutes then rinsing with PBS. Blocks were prepared with 5% bovine serum at room temperature followed bytreatment with anti-caspase-3 (1:100; catalog no. ab2302; Abcam-Cambridge, UK). After rinsing, counterstaining was done with

hematoxylin. The immunohistochemical reactivity was detected at a concentration of 0.06% of 3.3′-diaminobenzidine (DAB) (Dako). Dehydration in a series of xylene and ethanol for two minutes at 70, 90, and 100% was done before examining the sections using an Olympus BX 50 Automated light microscope,

Statistical analysis

All data were statistically analyzed using the Statistical Package for the Social Sciences software Version 23.0 (SPSS IMB, NY, USA) and presented as means and standard deviations. The data were analyzed and compared among groups using a one-way analysis of variance, followed by Tukey's post hoc test for intergroup comparisons. Statistical significance was considered at p-values less than 0.05.

Result:

Effect of amlodipine on ischemic neurological deficit

The results of this work demonstrate that ischemia/reperfusion induced manifest neurological deficit as evident by the significantly decreased latency to fall when compared to sham group which was significantly improved by amlodipine treatment. (Fig. 1(A))

Effect of amlodipine on ischemia-induced oxidative DNA damage

Oxidative stress evidently induced DNA damage as indicated by the significant increase in 8-OHdG in ischemic group which significantly decreased when compared to amlodipine group (Fig. 1 (B)).

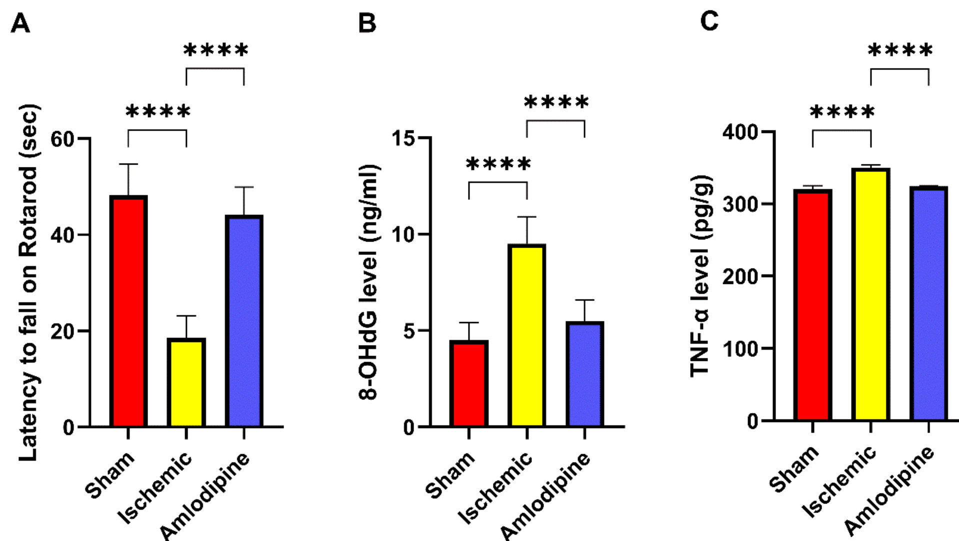

Fig. 1.(A) Effect of amlodipine on latency to fall on rotarod (sec);(B) Effect of amlodipine on 8-OHdG level (ng/ml) and C) Effect of amlodipine on TNF-α level (pg/g):(n=10), Values are displayed as mean ± SD; statistical analysis was conducted using one-way ANOVA with Tukey's post hoc, *, ***, **** denoting a statistically significant difference at ($P < 0.05$). ($P < 0.05$), * ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$).

Effect of amlodipine on ischemia-induced inflammatory stress

Fig. 1(C) demonstrates amlodipine treatment ameliorated he ischemic inflammatory stress as evident by the significantly decreased TNF-α in comparison to its significantly elevated level caused by ischemia.

Effect of amlodipine on redox state in ischemic brain

Ischemia induction by MCAOcaused significant increase of ROS production (Fig. 2(A)) which is caused by the imbalance between the significantly increased lipid peroxidation as evident by the significantly elevated MDA level(Fig. 2(B)) and significantly decreased antioxidant defense as evident by decreased SOD activity (Fig. 2(C)). These events indicating oxidative stress were significantly improved by amlodipine treatment.

Effect of amlodipine on PGC1α/Nrf2/TFAM Pathway in brain ischemia:

72 hours after ischemia/reperfusion the brain tissue significantly disturbed mitochondrial balance evident in decreased mitochondrial biogenesis due to the decrease of PGC1α and TFAM levels with concomitant downregulated Nrf2 expression with subsequent inhibition of the antioxidant defense. Amlodipine treatment significantly improved the mitochondrial dynamics balance. (Fig. 3)

Effect of amlodipine on ischemic neurological damage:

Light microscopic examination of sections of cerebral cortex of rats of sham group stained with hematoxylin and eosin showed rounded granular cells with large nuclei and pyramidal cells with triangular shaped cell bodies, basophilic cytoplasm and apical dendrites. Blood vessels were seen (Fig. 4(A)). Examination of Sections of ischemic group revealed pyknotic granular cells surrounded by halos and red neurons. Some cells had karyolitic nuclei. Pyramidal cells appeared pyknotic with deeply stained nuclei. Congested, dilated blood vessels and vacuolated neuropil were also seen (Fig. 4(B)). Examination of sections of amlodipine group showed normal granular cells with open face nuclei and normal pyramidal cells with apical dendrites while few cells still appeared surrounded by halos with pyknotic nuclei. Normal blood vessel is noticed (Fig. 4(C))

Effect of amlodipine on ischemia-induced apoptosis:

Examination of caspase-3 immunostained sections and analysis of morphometric study of caspase-3 optical density revealed negative reaction in sham-operated group (Fig. 4(D)) while strong positive reaction was seen in ischemic group (Fig. 4(E)). Section of treated group showed mild positive reaction (Fig. 4(F)).

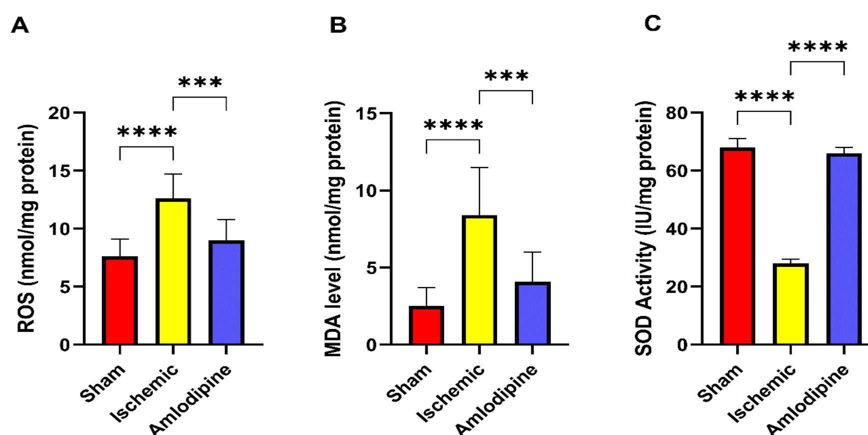

Fig. 2. A) Effect of amlodipine on ROS level (nmol/mg protein); B) Effect of amlodipine on and MDA level (nmol/mg protein) and C) Effect of amlodipine on SOD activity (IU/mg protein): (n=10), Values are displayed as mean ± SD; statistical analysis was conducted using one-way ANOVA with Tukey's post hoc, *, ***, **** denoting a statistically significant difference at ($P < 0.05$). (P < *0.05),* * ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$).

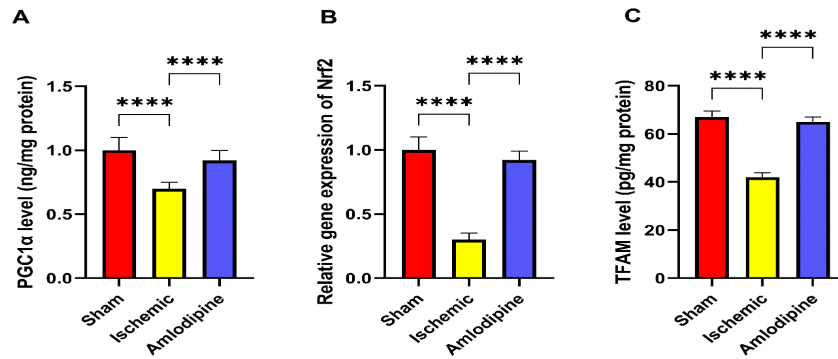

Fig. 3. A) Effect of amlodipine on PGC1α level (ng/mg protein); B) Effect of amlodipine on relative gene expression of Nrf2 and c) Effect of amlodipine on TFAM level (pg/mg protein). (n=10), Values are displayed as mean ± SD; statistical analysis was conducted using one-way ANOVA with Tukey's post hoc, *, ***, **** denoting a statistically significant difference at ($P < 0.05$). ($P < 0.05$), * ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$).

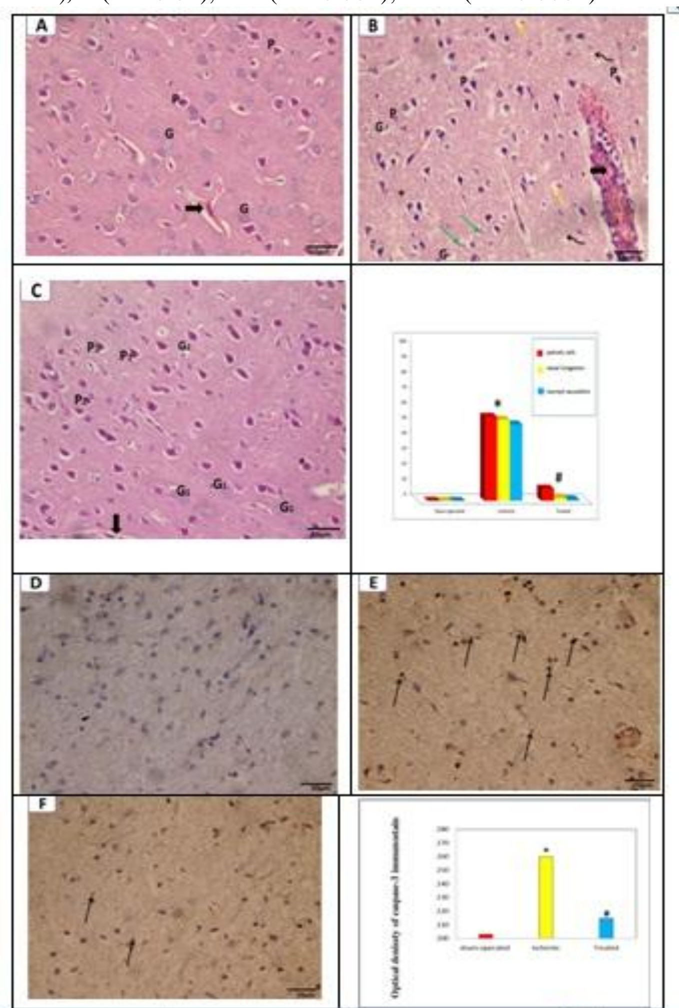

Fig. 4: Sections of cerebral cortex of sham-operated group showed rounded granular cells with large open face nuclei (G) and pyramidal cells with triangular shaped cell bodies, basophilic cytoplasm and apical dendrites(P) . Blood vessels were seen (black arrow) (Fig.4 A). Section of Ischemic group showed granular cells with pyknotic nuclei surrounded by halos (G)and granular cells with karyolitic nuclei (green arrows) , Irregular pyramidal cells with deeply stained nuclei surrounded by halos (P) were seen. Congested dilated blood vessel (black arrow), red neurons (yellow arrow) and prominent vacuolation of neuropil (curved arrow) were also noticed. (Fig.4 B). Section of cerebral cortex of treated group showed normal granular cells with open face nuclei (G1) and normal pyramidal cells with apical dendrites (P1). Normal blood vessel was seen (black arrow). Some granular cells surrounded by halos (G2) and pyknotic pyramidal cells (P2) were still seen (G2) (Fig.4 C). Examination and analysis of morphometric study of optical density of caspase-3 immunostained sections of sham-operated group showed negative reaction (Fig.4 D). Section of ischemic group showed strong positive reaction (black arrows) significant to control group (Fig.4 E). Sections of treated group showed mild positive reaction (black arrows) significant to ischemic group (Fig.4 F). (A-F ×400).Histological score (pyknotic nuclei, blood vessel congestion & neuropil vacuolation). *: significant to control group. #: significant to ischemic group.

Discussion:

In targeting ischemic stroke, developingnew strategies is necessitated by the limitations of common clinical treatments. For instance,intravenous thrombolysis using tPA is limited by the hemorrhagic complications while mechanical thrombectomy is limited to macrovascular thrombosis while (21),(22).

To our knowledge, the current work is the first to report amlodipine's effect on PGC1α/Nrf2/TFAM pathway in cerebral ischemia in vivo. In addition toitspreviously reported role in delaying the onset of stroke in hypertensive patients, amlodipine prophylaxis mitigated the neurological deficit caused by cerebral ischemia with a possible involvement of PGC1α/Nrf2/TFAMpathway together with its direct alleviation of oxidative stress, inflammatory stress and apoptosis.

Our results demonstrated the cerebral ischemiainduced neurological deficit as evident by the significantly shortened latency to fall on rotarod.This neurological deficit is a result of the consequences of the hypoxia-induced inflammatory and oxidative stress which were aggravated by restoringthe blood flow allowing the mitochondria to produce large amount of ROS which was also demonstrated by the work of (23)

The significantly increased MDA & decreased SOD in ischemic group indicate imbalance between the ROS production & clearance. This in turn caused oxidative stress-induced membrane lipid peroxidation, DNA damage,as evident by the elevated level of 8-hydroxy-2'-deoxyguanosine level, and mitochondrial dysfunction as evident by the disruption of the PGC1α/Nrf2/TFAM pathway, ultimately leading to mitochondrial dependent neuronal apoptosis, demonstrated by the high level of caspase-3 detected immunohistochemically, resulting in the irreversible damage to brain tissue evident in histopathological examination.

(24, 25) also explained a similar mechanism to the ischemic neuronal damage eventsas those evident in our study.

It was also reported that mitochondrial dysfunction alters intracellular $Ca2+$ homeostasis leading to elevated cytoplasmic $Ca2+$through inhibition of $Na+/Ca2+$ anti-porter secondary to $Na+/K+$ ATPase failure caused by ATP depletion(26). $Ca2+$ influx also stimulates ROS production in inflammation (27)

In addition, $Ca2+$ overload and excessive ROS release were involved in altering mitochondrial membrane permeability, decreasing mitochondrial potential, inducing mitochondrial depolarization, and swelling. This results in transfer of mitochondrial material to the cytoplasm including cytochrome-C with subsequent stimulation of mitochondrial-dependent apoptosis(28,29).

Also, the high ROS levels downregulate TFAM expression, enhances its degradation jeopardizing the integrity of mtDNA(7).

However, increase in $Ca2+$ increases calcium binding protein with IQGAP1 with subsequent activation of the Nrf2 bound to IQGAP1 (28)ctivation of the Nrf2 bound to IQGAP1 (28). In addition, ttransient ischemia was reported to stimulates mitochondrial biogenesis via PGC1α/Nrf2/TFAM pathway (6) and mtDNA was reported to increase to nearly preischemic levels 24 hours after transient ischemia (31). Also, histological evidence of mitochondrial biogenesis was also found after transient global ischemia in adult rats (32)

This is also supported by the results of (33)who reported an increase ofPGC1α within 24 hours post stroke with a markedly declined level by the third day. This increasemitigates ischemic inflammation and neural damage via autophagy and mitophagy induction by regulation of ULK1 in an ERRα-dependent manner.

On the other hand, the current study demonstrated that amlodipine maintained the upregulation of PGC1α/Nrf2/TFAM pathway 72 hours post ischemia/reperfusion with subsequent mitochondrial biogenesis and enhanced mitochondrial function. The upregulation of this pathway was also reported in amlodipine-treated rats with renal ischemia and oxygen-glucose deprivation neuronal stem cell model via activating the PI3K/Akt pathway and decreasing Ca2+ Influx (11,34)

Amlodipine upregulated Nrf2 expression also due to decreased mRNA expression of the inhibitory Keap-1 (35). AlsoPI3K/Akt pathway activation by amlodipine was reported to upregulate Nrf2(36). However, it was also reported that amlodipine could not induce significant change in Nrf2 in neuronal cells. This may be attributed to the low dose or decreased cytosolic Ca2+ which interferes with the IQGAP1/Nrf2 interaction(30,37)

Furthermore, amlodipine was reported to mitigate oxidative stress (38) . In addition,(39) reported that targeting mitochondriainhibited ROS production and apoptosis. For instance, upregulation of TFAM was reported to decrease inflammation and ROS production in ischemia reperfusion injury(40) This is also evident in the current work by the restoration of normal levels of SOD & MDA, histopathological examination and low caspase-3 levels which can be explained by amlodipine-stimulated mitochondrial biogenesis and Nrf2-induced upregulation of antioxidant proteins.

Also, reperfusion of cells with impaired mitochondrial function and elevated cytosolic Ca2+ level aggravates the ROS production(41,42) . Thus, amlodipine's function as a Ca2+ channel blocker is another added mechanism to oxidative stress inhibition.

Moreover, the current work showed that amlodipine alleviated the inflammatory stress as evident by decreased TNF-α. This can be through its reported inhibition of inflammation as demonstrated by (43). It also can be attributed to upregulating PGC-1α as reported by (44) who demonstrated that overexpression of PGC-1α downregulate the pro-inflammatory cytokines suggesting that PGC-1α-induced mitochondrial biosynthesis alleviates mitochondrial dysfunction in cerebral ischemia byameliorating the inflammatory stress.

Altogether, amlodipine's modulation of mitochondrial function and lowering cytosolic Ca2+ withthe subsequent mitigation of oxidative and inflammatory stress reduced the ischemia reperfusion neuronal damage as evident in neurological testing and histopathological examination suggesting a strong prophylactic role for amlodipine in cerebral ischemia.

Conclusion:

It is established that amlodipine helps reducing the incidences of stroke in hypertensive patients. But it is also of great benefit in mitigating the post-ischemic hazardous effects on the brain via PGC1α/Nrf2/TFAM Pathway Activation. Thus, an amlodipine-based anti-hypertensive regimen can be of more benefit in ischemic stroke than other

drug regimens. Furthermore, the effect of amlodipine on this pathway can by targeted by future research expanding its anti-ischemic effects beyond myocardial and cerebral scopes. Also, more research is needed to compare if the effect of amlodipine is only prophylactic or if it can be combined with treatment after cerebral stroke to alleviate the ischemic damage. Monitoring the effect of ischemia on PGC1α/Nrf2/TFAM pathway immediately after ischemia, and over the time would help a better understanding of this pathway's effect and the potential role of amlodipine in its modulation.

Statements and declarations:

Funding

This study was self-funded.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This work does not include any human blood sample but include animal sample.

Data sharing statement Data sharing is applicable to this article as new data were analyzed in this study.

Informed consent Not applicable as no human blood samples were taken while compiling this original article.

Authors Contribution: The authors declare that all data were generated in-house and that no paper mill was used.

Data Availability Statement:

All data that support the finding of the current study are available upon reasonable request

Research involving human participants and/or animals.

Animals used in these experiments were treated in accordance with the procedures approved by the ethical committee of Tanta University (Approval Code Number: 36264PR802/0/24). NO human blood sample was included.

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