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EFFECT OF *EMBLICA OFFICINALIS* (AMLA) ON APOPTOTIC CELL DEATH FOLLOWING ISCHEMIA REPERFUSION INJURY IN RATS

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ABSTRACT

Cell death following ischemia reperfusion injury leads to life threatning problems in patients suffering from cerebral stroke. The aim of this study was to investigate the neuroprotective effects of *Emblica Officinlis* (amla) fruit extract on neuronal apoptosis following cerebral ischemia reperfusion injury.1 hour ischemia followed by 24 hours reperfusion, rats were orally administered amla fruit extract. Apoptotic cells in brain were determined by performing flow cytometry. The results showed that amla extract significantly decreased the apoptotic cells by flow cytometry 24 hours after the ischemic injury compared with the MCAO group. This study suggests that amla may be used for treatment of ischemic stroke as a neuroprotective agent.

KEYWORDS: **Apoptosis, Ischemia, Flow cytometry, Rats**

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INTRODUCTION

Emblica Officinalis (Amla) is a traditional Indian medicine and widely used to treat many diseases. Cerebral stroke has emerged as one of the largest reasons of disability worldwide and cause deleterious effects on normal healthy life [1.] The Brain ischemic injury involves so many mechanisms. Some studies demonstrated that severe transient ischemia serves worse compared to permanent ischemia [2,3].The Cell death examination is an important factor in cerebral stroke in the study of morphological and biochemical changes in cell [4,5,6] Apoptosis and necrosis are two main phenomena in cell death[7-9]. Apoptosis and necrosis are extremely crucial for cell development and survival in neurological diseases and they both have distinguished mechanism[10].Necrosis causes a direct effect on injured/damaged cell and the cell begin to die. On the other hand, some cascade events / mild stimuli are responsible for apoptotic events like death receptors and some protein complexes[11].Necrosis occurs in areas that are severely affected by the injury. Cell death events depend on temperature exposure eg. 43^0C -44^0C temperature is responsible for apoptosis and higher temperatures are responsible for necrosis in murine P-815 cells[12]. Acute and chronic neurodegenerative disorders are characterized by apoptosis[9,13,14]. Delayed neuronal death occurs in transient cerebral ischemia[15].Some studies showed that, protein synthesis inhibitors play a major role in delayed cell death through the apoptosis activation[16-18]. In ischemic insult, necrosis occurs in the core of the lesion and there is apoptotic cell death in the penumbra[19-21]. The goal of the present study was demonstrated whether amla fruit extract treatment could prevent or diminish tissue damages of both the cortex and the striatum caused by cerebral ischemia/reperfusion (I/R) injury in rats.

MATERIALS AND METHODS

Chemicals and kit

Collegenaae type II powder and Apo Alert Annexin V- FITC (Fluorescein isothiocyanate) Kit were obtained from Gibco by Life Technologies, USA and Clontech,USA unless otherwise specified.

Amla fruit extract

The standardized lyophilized hydroalcoholic extract of *Emblica officinalis* (amla) fruit (HAEEO) was procured from Sanat Products Pvt. Limited, New Delhi, India (A WHOGMP and ISO 9001 Accredited Herbal Extract Company).

Animals

Adult male Sprague-Dawley (SD) rats weighing 260±20g were procured from National Animal Laboratory Centre (NALC) of Central Drug Research Institute (CDRI), Lucknow, INDIA and used throughout thestudy. All animal experiments were conducted after approval and in accordance with the strict guidelines of the Institutional Animal Ethics Committee (IAEC) vide no.46/IAEC/2013 dated 23.9.2013.Rats were allowed food and water *ad libitum* and maintained 12 hours light-dark cycle before the experiment. Rats were divided into four groups. 6 rats in each group. Amla dose was decided from the work of Mahaveer Golechha et al, 2011 [22]. Group I - Normal control group. Rats were given only saline without ischemia reperfusion. Group II – Middle cerebral artry occlusion (MCAO) group. Rats were given only ischemia reperfusion without any treatment. Group III- Rats were given amla fruit extract (dissolve in normal saline) 500 mg/kg orally 3 hours post to ischemia reperfusion. Group IV- Rats were given amla fruit extract (dissolve in normal saline) 500 mg/kg orally 1 hour prior to ischemia reperfusion.

Focal cerebral ischemia model

Transient focal cerebral ischemia was induced by MCAO using the filament model as previously described by Longa et al.,1989[23]. In brief, animals were anaesthetized with chloral hydrate (300mg/kg i.p.). During the period of surgery, the body temperature of the animals was maintained constantly at 36.5±0.5°C by the use of a thermo regulated dissecting heating surgical table. Briefly, the left common carotid artery (CCA) was exposed through a midline incision in the neck region. The neck muscles were carefully separated further to expose internal carotid artery (ICA) and

external carotid artery (ECA). A 3-0 monofilament nylon suture (Ethicon, Johnsons & Johnsons Ltd. Mumbai) with a blunted tip was introduced into the ECA lumen through a small nick and gently advanced from ECA to the ICA lumen (about 20-22 mm from the CCA bifurcation) to block middle cerebral artry (MCA). The ECA stump was tightened by thread around the intraluminal nylon suture to prevent bleeding. Recirculation/reperfusion of cerebral blood flow (CBF) was allowed by removing the monofilament carefully after 1 hour ischemia followed by 24 hours of reperfusion. In SHAM-operated animals, all the procedures except for the insertion of the nylon filament were carried out. Animals were allowed to recover from anaesthesia and on regaining the righting reflex were transferred to cages in a room, with temperature maintained at 26 +0.5^{o}C and were allowed food and water *ad libitum*.

Flow-Cytometric Analysis of Apoptotic/Necrotic Cell Death

The Flow cytometry analysis was done for the determination of apoptotic population by using Annexin V- FITC Apoptosis detection Kit The rat brains were quickly removed under chilled condition and brain parts viz. cortex and striatum dissected out and a single cell suspension (SCS) was prepared using collagenase enzyme treatment containing 3mM $CaCl_2$. The brain homogenate (5-10 vols) was incubated with collagenase (150 units) at 37°C for 4 hours. After incubation, supernatant (SN) was collected and further centrifuged at 500g for 5 minutes. The pellet was suspended in HEPES-buffered Hanks' (HBH) solution (pH 7.4) at a density of 10^5 - 10^6 cells/ml. Added 2 µl of annexin conjugate and 5 µl propidium iodide (PI) to 200 µl of SCS. Cells were incubated for 45 minutes at 37°C in dark. The readings were taken in a flow cytometer (BD Biosciences, Francisco, USA) at log FL1 (X-axis; FITC) excitation (488 nm) and emission (515 NM).

Statistical Analysis

Data are represented as mean ± standard error of mean (SEM). Student's t-test for unpaired comparison will be used for comparison of two means and comparisons among different groups will be made using the one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. A p value of <0.01 and <0.001 was consider as statistically significant.

RESULTS

Effect of 3 hours post treatment of amla fruit extract on Flow Cytometric Analysis

The present study estimated the number of cells that was actively undergoing apoptosis following I/R injury in the striatum and cortex at the reperfusion time points of 24 hours by flowcytometry. It was observed that besides necrotic changes, cells also appeared to be damaged by apoptotic mechanism. After 1 hour of ischemia followed by 24 hours of reperfusion, there was a significant increase (P<0.001) in Annnexin V-FITC positive cells in the striatum as compared with that of the SHAM group (Fig.1A). In the cortex, in comparison to sham there was also a significant increase (P<0.001) in Annexin V-FITC positive cells after 24 hours over SHAM (Fig.2A).Amla fruit extract can significantly decrease (P<0.01) the percentage of apoptotic cells compared with the MCAO group in striatum as well as the cortex. Amla fruit extract treatment reduced the Annexin V-FITC positive cells in the in the striatum region (Fig.1B) and in the cortex region (Fig.1C). The percentage of apoptotic cells were significantly decreased with amla treatment (43.67% ± 0.84 to 40.33% ± 0.91) in the cortical region and (55.50 % ± 1.17 to 49% ± 1.82) in the striatal region as compared to the respective MCAO group. These results suggest that amla fruit extract specifically protects cells from apoptosis.

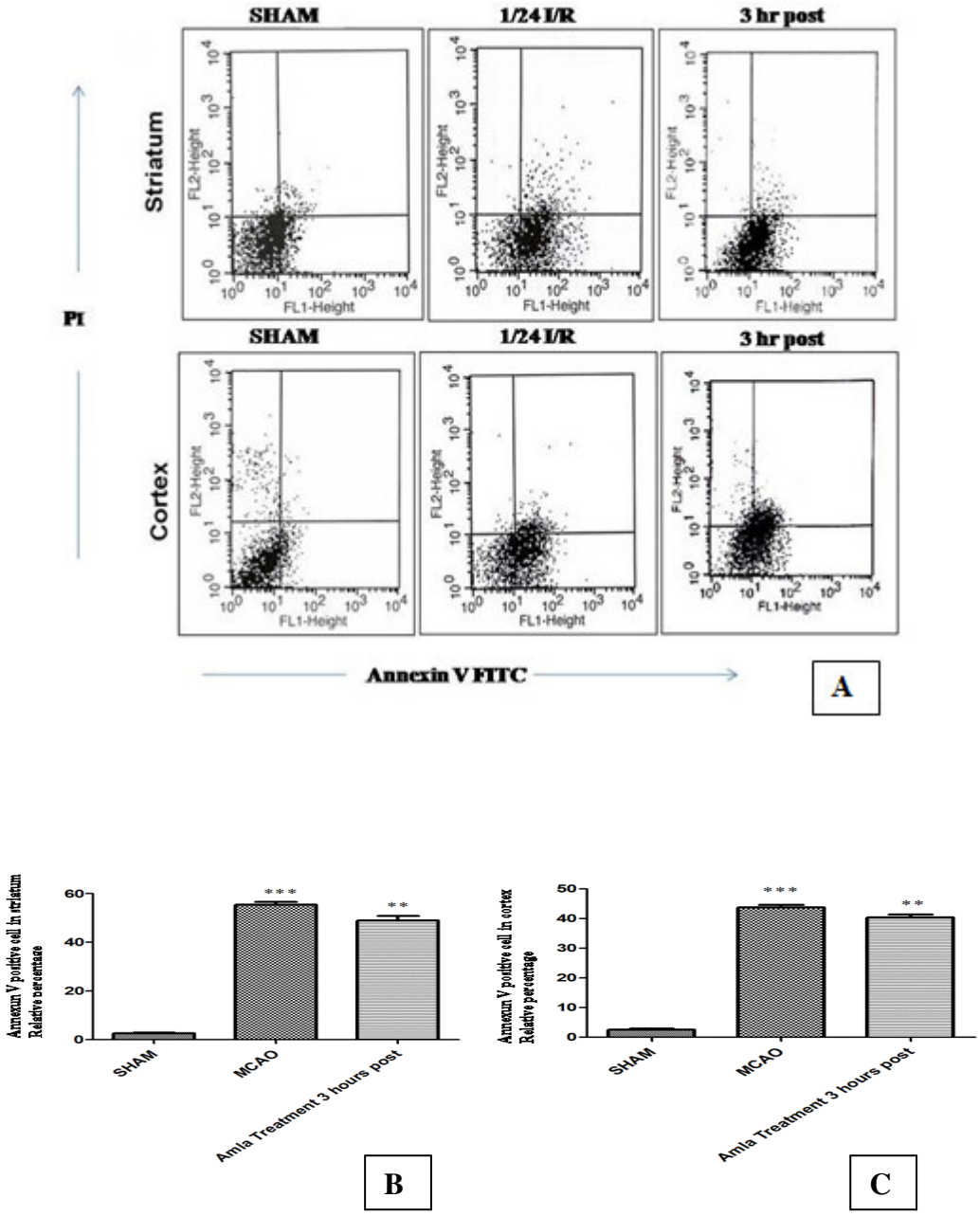

Figure 1

A) Representative flow cytogram of an Annexin V binding (abscissa, FL1) in the affected striatal and cortical regions of rat brain cells subjected to 1 hour ischemia followed by 24 hours of reperfusion. The numbers in the lower right quadrant represent the percentage of Annexin V-FITC positive cells. Relative percentage of Annexin V positive cells in striatum and cortical area. Amla treated groups were compared with MCAO group and data were expressed as means±SEM percentage change. 3hours post Amla treatment reduces the number of these cells (n=6) in striatum (B) and cortex (C) after I/R injury as compared to MCAO rats. (**p< 0.01 versus MCAO, ***p< 0.001 versus SHAM).

Effect of 1 hour pre treatment of amla fruit extract on Flow Cytometric Analysis

It was found that cells appear to lose its symmetry at 1/24 hours of I/R in the affected cortical and striatal area of MCAO rats as indicated by Annexin V-FITC positive cells (See Fig. 2A). The brain cells undergoing apoptotic changes in the cortex of MCAO increased at 1/24 hours of I/R as compared to SHAM at respective time point of I/R. Similarly, the brain cells undergoing early apoptotic changes were also detected in the striatal area of MCAO rats. The number of these cells, increase at 1/24 hours of I/R as compared to SHAM group. However, cells with apoptotic changes were greater in MCAO rats and significantly higher (P<0.001) at 1/24 hours of I/R injury as compared to SHAM

group (Fig. 2A). Amla extract reduced the Annexin V-FITC positive cells in the striatum region (Fig. 2B,P<0.001) and in cortex region (Fig. 2C,P<0.001).The percentage of apoptotic cells were significantly decreased with amla treatment in the cortical region (43.33% ± 0.84 to 36.50% ± 1.05) and in the striatal region (55.33 % ± 1.08 to 45.33% ± 1.87) as compared to the respective MCAO group.Our study demonstrated that 1 hour prior treatment was better than 3 hours post amla treatment on apoptotic cell death following 1/24 I/R injury in rats.

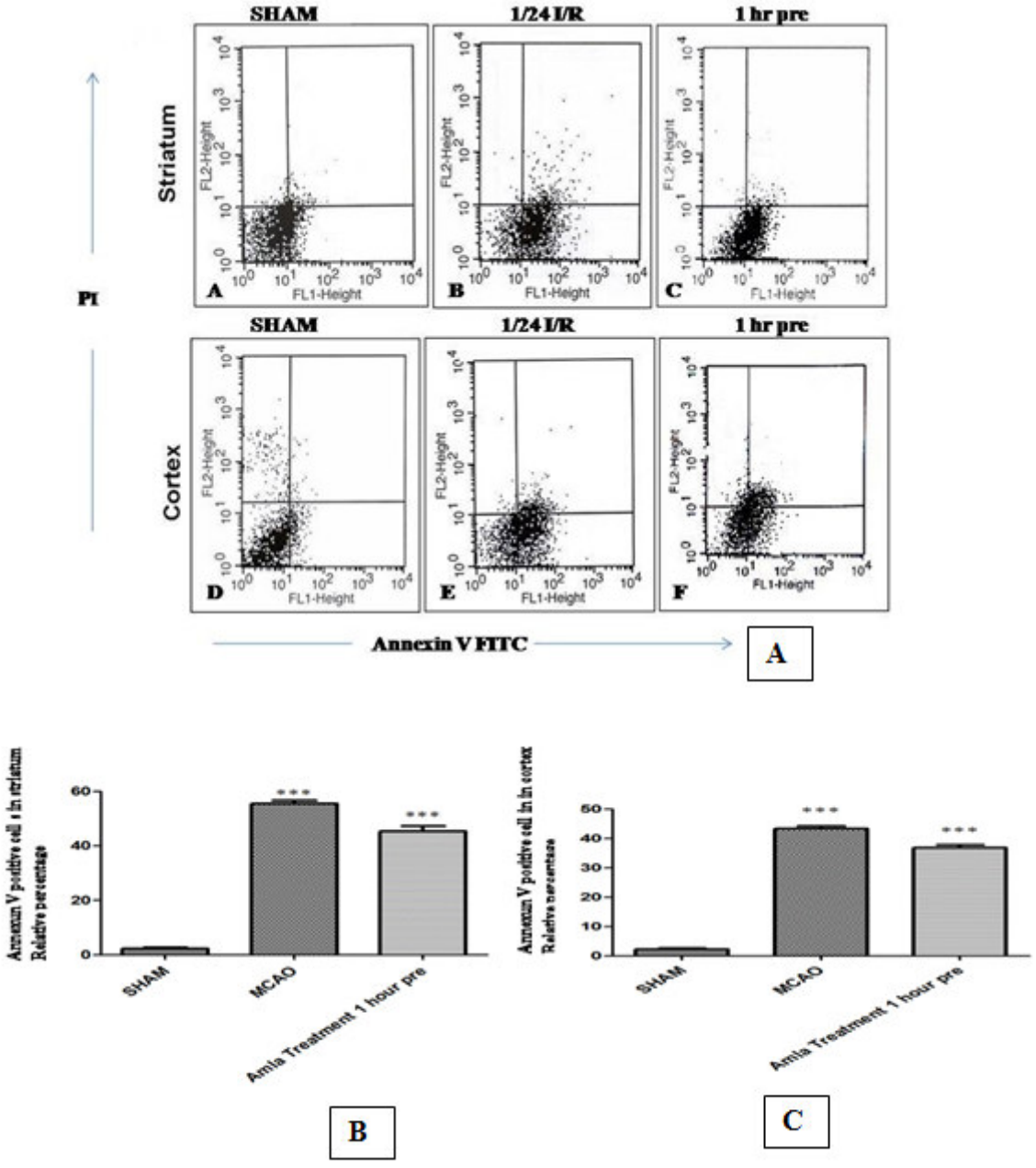

Figure 2

A) Representative flow cytogram of an Annexin V binding (abscissa, FL1) in the affected striatal and cortical regions of rat brain cells subjected to 1 hour ischemia followed by 24 hours of reperfusion. The numbers in the lower right quadrant represent the percentage of Annexin V-FITC positive cells. Relative percentage of Annexin V positive cells in striatum and cortical area. Amla treated groups were compared with MCAO group and data were expressed as means±SEM percentage change. 1 hour pre Amla treatment reduce the number of these cells (n=6) in striatum (B) and cortex (C) after I/R injury as compared to MCAO rats. (p< 0.001 versus MCAO, *** p< 0.001 versus SHAM).***

DISCUSSION

Glucose and constant oxygen flow is responsible for managing ATP (Adenosine tri phosphate) production for properly brain function[24].Neuronal damage is associated with acidosis[25]. Ischemia reperfusion injury induced neuroinflammation causes early damage in brain tissue. Translocation of PS (Phosphatidyl serine) to outer leaflet is the characteristic feature of early apoptotic cell death[26]. Apoptosis waste elimination is one of the main features of early apoptosis events. Therefore, these early apoptotic events were further confirmed by using Annexin V in order to estimate the cells that are actively undergoing apoptosis after I/R injury. A significant increase in the Annexin V binding cells was observed both in the striatum and cortex at 24 hours of reperfusion after ischemia. However, the number of both necrotic and apoptotic cells increased at 24 hours and indicating that apoptosis may also be involved in damage progression in striatum region. The number of Annexin V positive cells appeared high at 24 hours of reperfusion both in the striatum and cortex. Whereas, striatum exhibited the higher number of Annexin V positive cells as compared to the cortex. The increase in the number of apoptotic cells in the cortical region indicating that delayed infarction in this region is mainly due to apoptosis and constitutes the boundary zone of the ischemic core. This may also reflect the reduction CBF and arterial distribution. Therefore, in such regions ATP levels are conserved to carry out intrinsic neuroprotective mechanisms for survival of the cells as observed by significant increase in the number of apoptotic neurons in the cortex. Striatum is the most affected brain region following ischemic stroke. Cerebral infarction, neurons which undergoes necrosis and complex signalling pathways are the main reason for striatum tissue damage. It may be concluded from these experiments that neuronal damage induced by MCAO are of two types: acute damage in which cell volume and stain ability are altered in neurons (swelling and shrinkage) and chronic damage affecting a large number of neurons as evident by large cavitations in striatal brain regions and cellular changes. Additionally, neuronal injury in the striatal and cortical region may also depend upon the vulnerability of neurons to ischemic stress.

CONCLUSION

In conclusion, the results of the present study indicated that the increased number of apoptotic cell has been found in MCAO rats. Amla extract has a strong protective action against cerebral ischemia reperfusion injuries in rats. Amla extract could reduce apoptosis following cerebral ischemia reperfusion injury in rats. This neuroprotection may be due to amla fruit extract suppression of neuronal apoptosis by inhibiting free radicals. Amla treatment has been found to decrease apoptosis and indicates the anti-apoptotic property of amla that could be due to its strong antioxidative potential. These results suggest that anti-apoptotic treatments may have useful therapies for stroke treatment, and such treatments deserve detailed exploration.

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Conflict of Interests

The authors declare that they have no conflictofinterests.

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