IJPSR (2015), Vol. 6, Issue 7 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 13 November, 2014; received in revised form, 30 January, 2015; accepted, 18 March, 2015; published 01 July, 2015

THE EFFECT OF AMLODIPINE IN AMELIORATING THE HYPOBARIC HYPOXIA INDUCED NEURONAL DAMAGES AND DYSFUNCTION

Varun Bhardwaj, Sarita Nehra, Anju Bansal, Shweta Saxena, Mrinalini Singh and Deepika Saraswat*

Department of Experimental Biology, Defence Institute of Physiology and Allied Science, Defence Research and Development Organization, Lucknow Road, Timarpur, New Delhi-110054, India

Keywords:

Calcium Channel Blocker(CCB), Amlodipine, Hypoxia, vascular leakage, Brain water content.

Correspondence to Author:
Deepika Saraswat

Scientist 'E', Department of Experimental Biology, Defence Institute of Physiology and Allied Science, Lucknow Road, Timarpur, New Delhi-110054, India.

E-mail: deepika_saras@yahoo.com

ABSTRACT: Calcium accumulation and calcium overload in the intracellular organelles has been known to be the main reason for cellular abnormalities related to oxidative stress. The study deals with the effects of an important calcium channel blocker (CCB)amlodipine in countering the hypoxia induced oxidative stress, *in vitro* and *in vivo*. We examined the intracellular calcium changes along withReactive Oxygen Species(ROS), lipid peroxidation by malondialdehyde(MDA) and reduced glutathione levels(GSH). Amlodipine displayed its maximum efficacy in attenuating oxidative damage during hypoxic insult of 0.5% hypoxia for 24 hours at concentration of 12.5nM. Amlodipine was also found effective in controlling apoptosis by maintaining Bax/Bcl-2 ratio, Cox-2 and VEGF levels significantly offering neuroprotective activity in N2a cells.In hypobaric hypoxia subjected (25,000 ft, 280mmHg and 28°C for 24 h) Sprague Dawley (S/D) rats, intracellular calcium levels were found to be lowered significantly in amlodipine treated group (10mg kg^{-1} body weight) when compared to control group. Vascular leakage and brain water content were also found to be significantly decreased upon treatment with amlodipine compared to control animals. Similarly, antioxidant status significantly improved in brain and was evaluated using ROS, MDA and GSH levels. These results suggest a protective role of amlodipine against oxidative damage and edema formed in response to hypobaric hypoxia.

INTRODUCTION: The survival and effective functioning of the brain and its cells is highly dependent on the balance between the brain's ability to utilize oxygen and the latter's relative availability in the vascular system in the immediate surroundings. An extreme reduction of brain oxygen supply leads to neuronal death[1]. The brain with its high lipid content and low antioxidant levels and higher per gram tissue free radical generation compared with other organs, is particularly susceptible to hypobaric hypoxia and oxidative stress [2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(7).2770-80

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(7).2770-80

The partial pressure of oxygen decreases as altitude increases, yet humans are capable of living at altitudes as high as 4000m. The ability of the brain to function at such high altitudes involves physiological adaptation and under prolonged

stress, these adaptive responses may contribute to problems associated with high altitudes, such asneuro-physiological impairment and increased occurrence of headaches. It is also reported that chronic high altitude hypoxia can lead to permanent neuronal damage in the non-acclimatized human brain[3].

Calcium signaling in brain during hypoxia is initiated by hyper-influx of calcium into cytosol via ion channels which include N-methyl D-aspartate (NMDA) receptor channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor channels, voltage gated calcium channels, Na-Ca exchanger channels, plasma membrane Ca^{2+} ATPase (PMCA) channels and other nonspecific ion channels. Hypoxia also activates intracellular calcium release from ligand gated channels, store operated calcium channels, IP-3 channels, ryanodine receptor channels and CatSper protein(cAMP mediated Calcium channel).

During sustained hypoxia, owing to long term activation of L-type Calcium Channels(LTCC) and the subsequent increase of the intracellular Ca^{2+} concentration, it is widely considered the single most important contributor to neurodegeneration and neuronal cell death[4]. As a consequence, calcium channels are important targets for the development of neuroprotective strategies, given their central role in depolarization- induced Ca^{2+} influx.

Calcium channel blockers are widely used agents in the treatment of a variety of cardiovascular diseases[5-8]. They are also under trials as effective pharmaco-therapeutic agents for some types of brain dysfunction by regulating neuronal Ca^{2+} homeostasis. In *in-vitro* systems, dihydropyridine(DHP) blockers of LTCC channels, such as amlodipine, nimodipine, israpidine and nicardipine have shown neuroprotective effects by reducing the intracellular rise in calcium. However, results from *in vivo* models have been highly variable whereas some studies found neuroprotective compounds mediate their beneficial effects, at least in part, through LTCC channels [9]. Amlodipine besylate is a third generation CCB which is used to treat hypertension and appears beneficial with minimal long term effects when compared to other CCBs[10]. Amlodipine besides being a calcium channel blocker has anti-inflammatory, antioxidant and anti-apoptotic activity[11-15]. Therefore the present study was designed to evaluate the efficacy of amlodipine besylate in ameliorating the effect of hypobaric hypoxia on brain.

MATERIALS AND METHODS:

Experimental design:

The experiments were divided into two phases. In phase I, murine N2a cells were used as *in vitro* model for optimization of amlodipine dose under hypoxia for maximum improvement in cellular viability. The same dose was then used for evaluation of efficacy of amlodipine in ameliorating hypoxia induced intracellular calcium homeostasis, apoptosis and maintenance of redox balance. In the phase II of studies, male Sprague-Dawley rats were used as animal model to evaluate efficacy of amlodipine in ameliorating hypobaric hypoxia induced oxidative damage and intracellular calcium homeostasis in brain.

Cell Culture Maintenance:

Murine neuroblastoma (N2a) cells were procured from National Centre for Cell Science (NCCS) Pune, India and maintained in the laboratory in Minimal Essential Medium (MEM) (Sigma) at 5% CO_2, 95% N_2 at 37°C in the incubator (Galaxy 170R, New Burnswick). MEM powder was dissolved in 1 liter sterile distilled water. To this medium antibiotics gentamycin sulphate (Sigma) (100mg L^{-1}), streptomycin sulphate (Sigma) (100 mg L^{-1}), and sodium salt of ampicillin (Sigma) (100 mg L^{-1}) were also added, along with sodium bicarbonate (Sigma) (2.2g L^{-1}). The pH of the medium was adjusted to 7.2 ± 0.2. Fetal bovine serum (FBS) was added to the incomplete medium to give a final concentration of 10% (v/v).

The mixture was then filtered into another sterile medium bottle through syringe filter (Millipore, 0.22µm), using a 10ml syringe. The sterility of the medium was checked by keeping the medium at 37°C in the incubator overnight. The sterile medium was kept at 4°C for storage and further use. All the cell culture experiments were conducted under strictly sterile environment. The normoxic experiments were carried out at 21% O_2, 5% CO_2 at

37°C in a normoxia incubator(Galaxy 170R, New Brunswick) while hypoxic experiments were carried out at 0.5% O_2, 5%, CO_2 at 37°C in hypoxia incubator(Galaxy 170R, New Brunswick).

Collection of the cells was done by de-adherence from the culture flask by trypsinization (0.1% v/v, Sigma). The cells were counted using Neubauer haemocytometer and seeded in 24-well plates (Nunc, Denmark), with a cell count of 4 x 10^4 cells per well. The N2a culture plates were then incubated in the CO_2 incubator (Galaxy 170R, New Burnswick), maintaining 37°C temperature and 5% CO_2 overnight. The adhered cells were grown to 70- 80% confluence.

***In-vitro* Experiments:**

Stock (concentration) solution of amlodipine was prepared and different dilutions were made by serially diluting the stock solution. The different concentrations of the drug used for experiments were 100nM, 50nM, 25nM, 12.5nM, and 6.25nM.

N2a cells seeded in 24wellwere kept in the incubator (Galaxy 170R, New Burnswick) with 21% O_2i.e. normal condition and one set was kept in the CO_2 incubator with 0.5% O_2(5% CO_2, 95% N_2) i.e. hypoxic condition for 24 h. After incubation the plates were removed and processed for further biochemical estimations. Cellular contents were suspended in 500µl cell RIPA (Radioimmune-precipitation) buffer (10mM HEPES, 38mM NaCl, PMSF 25µM), with protease inhibitor cocktail (MP Biomedicals, USA) (1µL/ml buffer) centrifuge at 8000rpm for 30 minutes at 4ºC. The supernatant containing cytosolic fraction was collected and stored at -80ºC for further analysis. All the biochemical estimations were performed in freshly isolated cytosolic fractions. Experiments were repeated thrice to avoid errors.

Animal Experiments:

Age-matched male Sprague-Dawley (S/D) rats (180 ±20 g) were used in all the experiments. Animals were maintained in the Experimental Animal Facility of the institute at (24±2°C) with 12-h light/dark cycle and 45-50% relative humidity. Animals were fed with standard pelletized diet (Lipton India Ltd.) and water *ad libitum*. All animal experimental procedures were approved by the Institutional Animal Ethic Committee (Authorization No: 27/1999/CPCSEA) and were in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animal, India (CPCSEA).

Rats were randomly divided into 4 groups (n=6) according to the treatment: (i) Normoxia Control (N); (ii) Hypoxia control (H); (iii) Normoxia+ Drug (N+D); (iv) Hypoxia+ Drug (H+D). The animals were administered with amlodipine (5 mg kg^{-1} b.w.) in phosphate buffered saline, (PBS) intra-peritoneal prior to hypobaric hypoxia exposure, whereas PBS alone was administered in the control groups as vehicle control. The rats were exposed to hypobaric hypoxia in animal decompression chamber (Ballice Instruments, India) at simulated altitude of 25,000 feet, 280 mmHg at28°C for 24 h. The airflow in the chamber was 2 $Lmin^{-1}animal^{-1}$, with relative humidity maintained at 50-55 %. After exposure to hypobaric hypoxia, the animals were sacrificed using overdose of ketamine (80 mg kg^{-1}) and xylazine (10mg kg^{-1}). Brain tissue was excised out after perfusion with ice cold phosphate buffered saline (PBS, pH 7.2).

Samples were washed in ice-cold PBS twice to remove blood and immediately snap frozen in liquid nitrogen and stored at -80°C for further analysis.The stored tissues were finely chopped using a sterile scalpel and mixed with 1:1 (w/v) of 0.154M KCl, fortified with protease inhibitor cocktail (MP Biomedicals, USA) (5µL ml^{-1} buffer) for homogenization. The minced tissue was finely grinded using a sterile homogenizer (Polytron PT1200 E, Kinematica, Switzerland) on ice and centrifuged at 10,000 rpm (HERAUS, Fresco 21, Thermo Scientific) for 30 min at 4°C. The supernatant containing cytosolic fraction was collected for immediate biochemical analysis or stored at -80°C for further use.

Estimation of Malondialdehyde (MDA):

MDA level was used as an index of lipid peroxidation under hypobaric hypoxia. Measurement of thiobarbituric acid reactive substances (TBARS), which is formed by the reaction of thiobarbituric acid (TBA) with MDA, was used for determining MDA level by the method of Ohkawa *et. Al* [16]. Thiobarbiturate was used as standard and OD was measured at 531 nm.

The levels of lipid peroxides were expressed as nM MDAmg^{-1} protein.

Intracellular Calcium estimation:

Ca^{2+} level in samples were estimated by using commercially available Quantichrom^{TM} Calcium Assay Kit (DICA-500, Bio Assay Systems) and performed according to manufacturer's instructions.

Free Radical Estimation by DCFH-DA Method:

The production of free radicals was determined by using 2,7-dichloroflourescein diacetate(DCFH-DA) as described by Cathcart *et al* [17]. Briefly, 30 µl of homogenate was added to a tube containing 2.938µl of 0.1M phosphate buffer (pH 7.4) and 12µl of 2,7-dichlorofluoroscein diacetate (DCF-DA), (1.25 mM prepared in methanol) was added. The assay mixture was incubated for 15 min at 37ºC and 2, 7-dichlorofluoroscein formation was determined fluorimetricallyat an excitation of 488 nm and emission at 525 nm (FLUO star OMEGA, BMG Lab tech). The fluorescent intensity is directly proportional to the amount of ROS formed.

Estimation of Reduced Glutathione (GSH):

The reduced glutathione (GSH) level were determined by the method of Hissin and Hilf [18]. The thiol group of GSH reacts with o-pthaldehyde (OPT) to form a complex which gives fluorescence with an excitation at 350nm and emission at 450nm(FLUO star OMEGA, BMG Labtech).

Western Blot for gene expression analysis:

The protein estimation in the cell lysate was done with the Lowry's method [19]. Absorbance was read at 610nm (Fluo star OMEGA, BMG Labtech) and protein concentrations were calculated. Protein expression of various proteins was studied by western blotting. 30µg protein of each sample was subjected to 10% SDS-PAGE and electro blotted using semi-dry transblot (Bio Rad, USA) on to a PVDF membrane (Millipore, USA). The membranes were blocked with 3% BSA for 2h, washed with tris-buffered saline with Tween-20 (0.01%) (TBST) and probed with respective mouse/ rabbit / goat monoclonal / polyclonal antibodies (MBL international or Santa Cruz Biotech Inc.) for 3 h at room temperature. The membranes were washed twice with TBST and incubated with appropriate anti-mouse/anti-rabbit/ anti-goat -IgG-HRP conjugate (1:30,000) for 2 h at room temperature. The membranes were then incubated with chemiluminescent substrate and luminol (Sigma) and the bands were developed using X-ray films (Kodak, USA). The changes in the protein expression levels were measured as densities of bands using Lab Works software (UVP, Bioimaging Systems, UK).

Determination of Brain water content and vascular permeability:

The permeability assays were performed by the method of Purushothaman *et al* [20] using sodium fluoresce in (Sigma) dye extravasation as an indicator of vascular leakage. After exposure to hypoxia for 24 h, all the animals were injected with sodium fluoresce in dye (15 mg kg^{-1} b.w.) in PBS through tail vein. After half an hour of injecting the dye the animals were anesthetized and perfused through left ventricle with 50ml of PBS for removing the fluoresce in from vascular bed. Brain was removed and was divided into two equal halves. Wet weight of the two halves was recorded. One half was incubated in formamide for 16 hours to extract sodium fluorescein and the flouresence was measured in supernatant at excitation wavelength of 485 nm and emission wavelength of 531nm using spectrofluorimeter (FLUO star Omega).

The values were expressed as relative fluorescence units (rfu) per gram dry weight of the tissue. The other half was dried in hot air oven at 55°C for 72hr to obtain a constant weight. The water content of the tissue was calculated as the difference between wet weight and dry weight and expressed as percentage of water per grams of dry tissue. The cerebral edema was determined by Brain Water Content. Wet-to-dry weight ratio was used as an index of tissue water content.

Edema Index = (wet weight- dry weight) x 100/ wet weight:

Statistical Analysis:

Data was expressed as Mean ± SD (n=6) for each experimental group. The results were analyzed for statistical significance using Student's t-test or one-way analysis of variance (ANOVA) with Bonferoni

post-hoc. $p<0.05$, and $p<0.01$ were considered to be significant.

RESULTS:

Cell Viability estimation:

Cell viability in 0.5% hypoxia control was reduced compared to normoxia control. In amlodipine treated hypoxia cells 12.5nM amlodipine dose increased cell viability significantly ($p<0.01$), while at higher doses the cell viability decreased significantly ($p<0.01$) when compared with hypoxia control (figure 1).This decrease in viability of cells could be attributed to certain level of drug toxicity at high levels of doses thus indicating a critical balance of Ca^{2+} is required for cell survival.

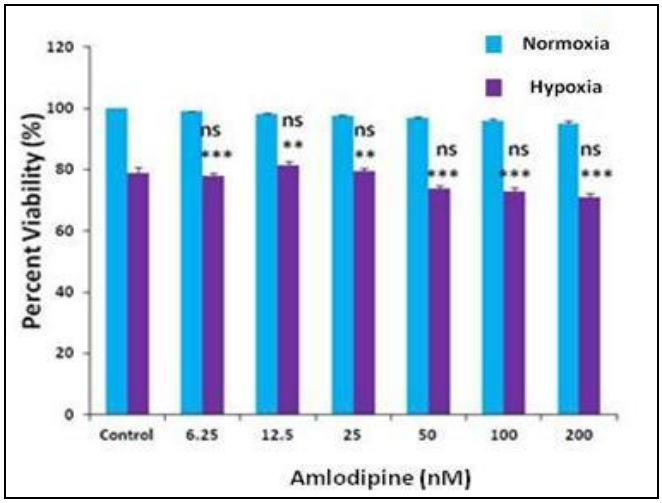

FIG. 1: PLOT OF PERCENTAGE CELL VIABILITY VERSUS DIFFERENT AMLODIPINE DOSES UNDER NORMOXIC AND HYPOXIC CONDITIONS FOR 24h. Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control whereas non-significant changes are designated as ns.

Intracellular free calcium estimation:

In N2a cells intracellular Ca^{2+} levels increased after 0.5% hypoxic exposure from normoxia control values. The intracellular calcium levels decreased progressively with increase in drug concentration in hypoxia exposed cells on treatment with amlodipine (**Fig. 2**).This decrease in Ca^{2+} levels in amlodipine treated hypoxia exposed N2a cells was statistically significant ($p<0.01$) when compared with hypoxia control at all drug concentrations suggesting as low as 6.25nM drug concentration effectively reduced Ca^{2+} levels under hypoxic conditions.

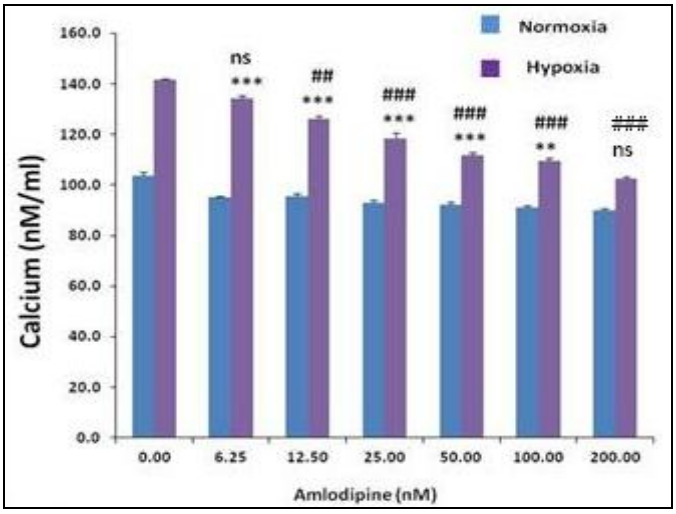

FIG. 2: PLOT OF INTRACELLULAR FREE CALCIUM (NM) VERSUS DIFFERENT AMLODIPINE DOSES UNDER NORMOXIC AND HYPOXIC CONDITIONS FOR 24h. Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns.

Intracellular Ca^{2+} levels increased in animals exposed to hypoxia as compared to normoxia controls. Whereas, in amlodipine treated animals, the intracellular Ca^{2+} levels decreased significantly ($p<0.01$) compared to hypoxia control in accordance with Ca^{2+} channel blockage induced by amlodipine (**Fig. 3**).

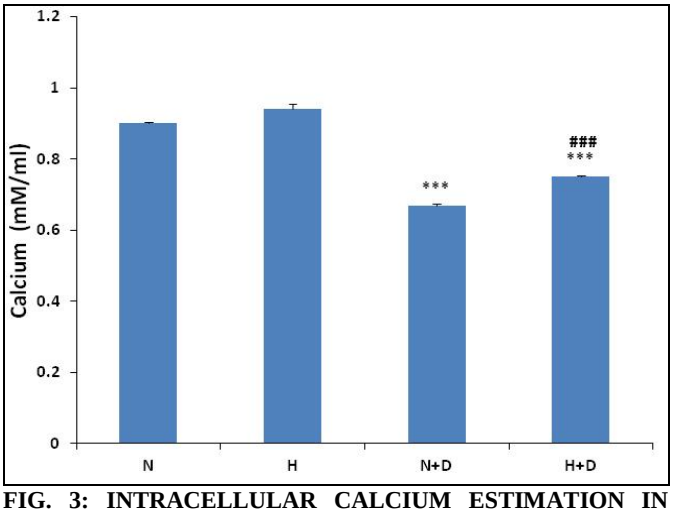

FIG. 3: INTRACELLULAR CALCIUM ESTIMATION IN RAT BRAIN TISSUE AFTER TREATMENT WITH AMLODIPINE (10mg/kg). Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas, non-significant changes are designated as ns. N-normoxia; H-hypoxia; N+D- normoxia+amlodipine; H+D-hypoxia+amlodipine.

Reactive oxygen species estimation

The control hypoxia ROS level showed a rise against normoxia ROS levels. In amlodipine treated hypoxia exposed cells, ROS value showed a U-shape curve with least at 25nM (i.e., 187.6 rfu/ mg protein/min) (**Fig.4**). These values were significantly lowered from hypoxia control except at 200nM drug concentration, at which ROS values (i.e.,229.54rfu/mg protein/min) increased compared to hypoxia control values (i.e., 223.55 rfu/mg protein/ min)

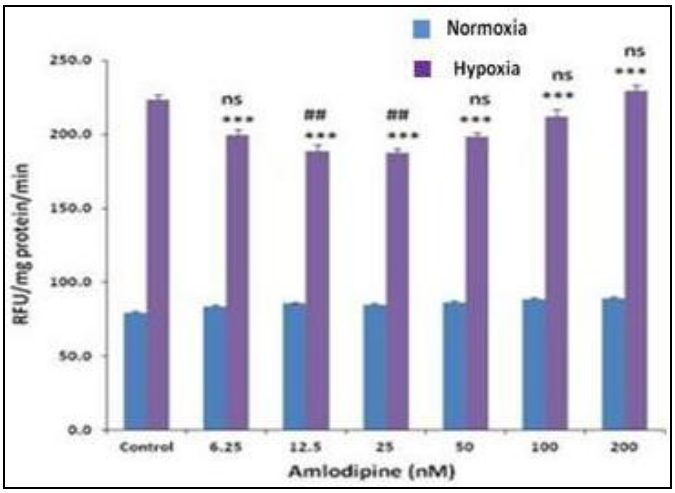

FIG. 4: PLOT OF ROS VERSUS DIFFERENT AMLODIPINE DOSES UNDER NORMOXIC AND HYPOXIC CONDITIONS FOR 24h. Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns.

Similarly, ROS generation increased in animals in hypoxia only group, whereas the treatment with amlodipine suppressed the ROS generation in hypoxia exposed drug treated animals significantly (p<0.01) when compared with both normoxia control as well as hypoxia only groups (**Fig. 5**). These findings suggest amlodipine was effective in reducing the ROS generation under critical hypoxic conditions hence having a positive impact over redox status of brain.

Malondialdehyde estimation:

The control hypoxia MDA level showed profound increase against normoxia control in N2a cells. In amlodipine treated hypoxia samples significant decrease (p<0.01) in MDA values was observed when compared with hypoxia control value. The minimum value was observed at 12.5nM of drug concentration (1nM/mg protein) (**Fig.6).**

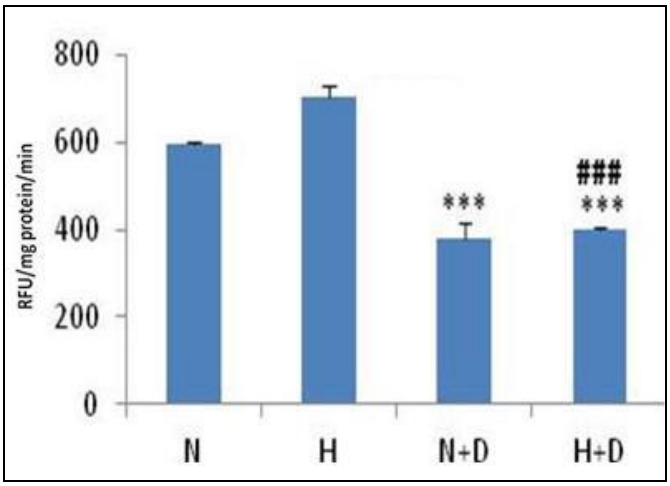

FIG.5: ESTIMATION OF ROS WITH AMLODIPINE (10mg/kg) DOSAGE IN RATS. Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns. N-normoxia; H-hypoxia; N+D-normoxia+amlodipine; H+D-hypoxia+amlodipine.

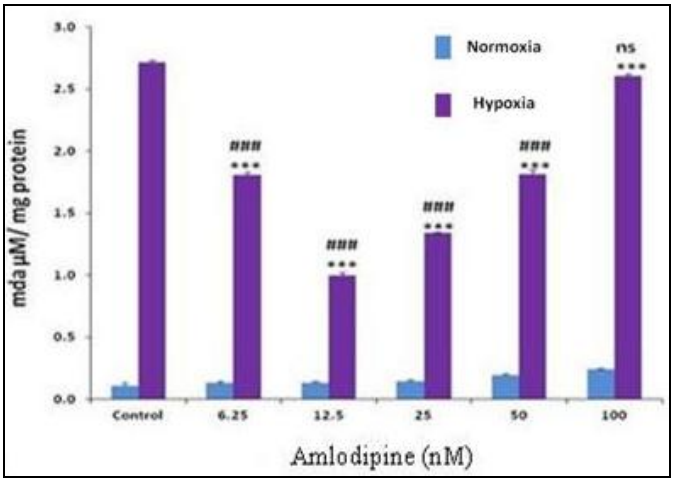

FIG.6: PLOT OF MDA VERSUS DIFFERENT AMLODIPINE DOSES UNDER NORMOXIC AND HYPOXIC CONDITIONS FOR 24H. Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns.

MDA levels increased in the hypoxia exposed animals when compared to the normoxia control (**Fig. 7**). Upon administration of amlodipine MDA levels decreased significantly (p<0.01) in both hypoxia exposed as well as normoxia group animals.

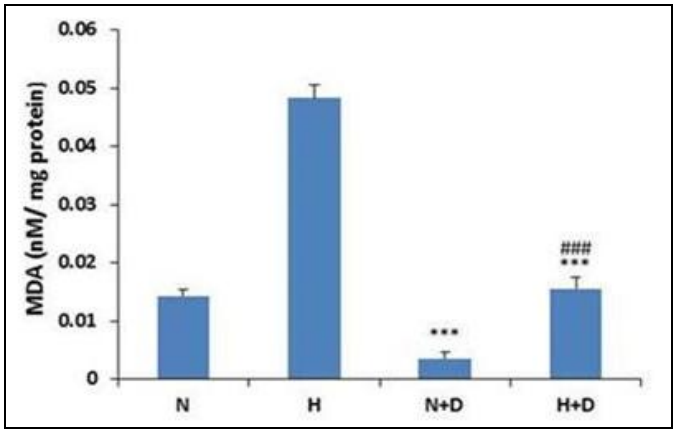

FIG.7: MALONDIALDEHYDE ESTIMATION IN RAT BRAIN TISSUE AFTER TREATMENT WITH AMLODIPINE (10mg/kg). Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns. N-normoxia; H-hypoxia; N+D-normoxia+amlodipine; H+D-hypoxia+amlodipine.

This shows that amlodipine was effective in reducing the overall effect of free radicals generated by the cells under hypoxia.

Reduced glutathione estimation:

The control hypoxia GSH level showed an approx 68% fall against normoxia GSH levels. Cells treated with amlodipine were found to have increased levels of GSH when compared with hypoxia control at drug concentrations of 6.25nM and 12.5nM (**Fig.8**). This increase in value was statistically significant (p<0.01).

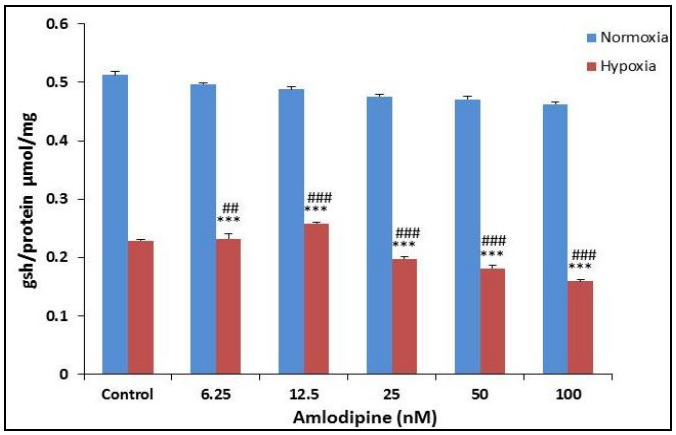

FIG.8: PLOT OF GSH VERSUS DIFFERENT AMLODIPINE DOSES UNDER NORMOXIC AND HYPOXIC CONDITIONS FOR 24H. Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns.

In *in-vivo* studies the reduced glutathione levels decreased under hypoxic condition, indicating increased oxidative stress. Whereas, upon administration of amlodipine to hypoxic group, the GSH levels increased significantly from hypoxic as well as normoxic control values (**Fig.9**).

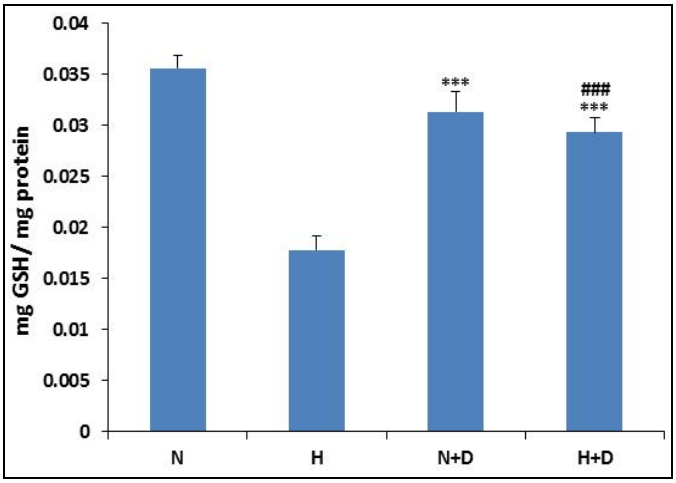

FIG. 9: ESTIMATION OF GLUTATHIONE LEVELS IN RATS AFTER TREATMENT WITH AMLODIPINE (10mg/kg). Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns. N-normoxia; H-hypoxia; N+D- normoxia+amlodipine; H+D-hypoxia+amlodipine.

Protein Expression Estimation:

The protein expression studies were carried out by western blotting (**Fig.10**).

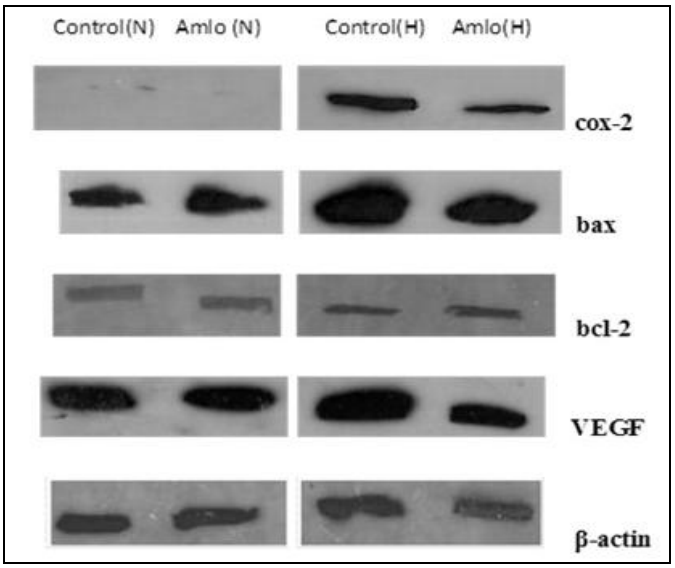

FIG. 10: WESTERN BLOT OF COx-2, BAX, BCL-2, VEGF, AND BETA ACTIN; (FROM LEFT TO RIGHT: CONTROL NORMOXIA, 12.5 nM AMLODIPINE NORMOXIA, CONTROL HYPOXIA, 12.5 nM AMLODIPINE HYPOXIA).

TABLE 1:

Sample	beta-actin	Cox-2	Bax	Bcl-2	VEGF
Normoxia control	42.34±0.01	2.68±0.06	44.02±0.01	15.06±0.01	87.50±0.01
normoxia+ Amlodipine(12.5nM)	42.31±0.03	3.02±0.01	61.55±0.02	19.02±0.01	90.53±0.02
Hypoxia control	41.72±0.01	74.65±0.009	113.69	13.44±0.1	116.15±0.06
hypoxia+ Amlodipine(12.5nM)	41.46±0.02	43.68±0.01[a;b]	77.12±0.03[a;b]	15.64±0.08[b]	58.36±0.02[a;b]

The cyclo-oxygenase (cox-2) expression in normoxia samples were negligible establishing that cox-2 expression being the inducible type has a very low basal synthesis in normal conditions. However, its expression rose in hypoxia. Whereas administration of amlodipine decreased the cox-2 expression markedly in the hypoxia exposed N2a cells (**Table 1**). The bax/bcl-2 ratio in case of hypoxic control sample was more than that of normoxic control, while administration of amlodipine to hypoxia exposed N2a cells lowered down the bax/bcl-2 ratio significantly when compared with hypoxia control (**Table 1**). Basal level expression of VEGF was evident in normoxia indicating that neuroblastoma cells display constitutive VEGF synthesis. The expression in hypoxia control was higher than normoxia control. While in amlodipine treated samples there was a decline in comparison with hypoxia control expression of VEGF (**Table 1**).

Estimation of edema index and vascular leakage

Estimation of Edema Index and Vascular Leakage reveal the changes in brain water content and Blood Brain Barrier (BBB) permeability.

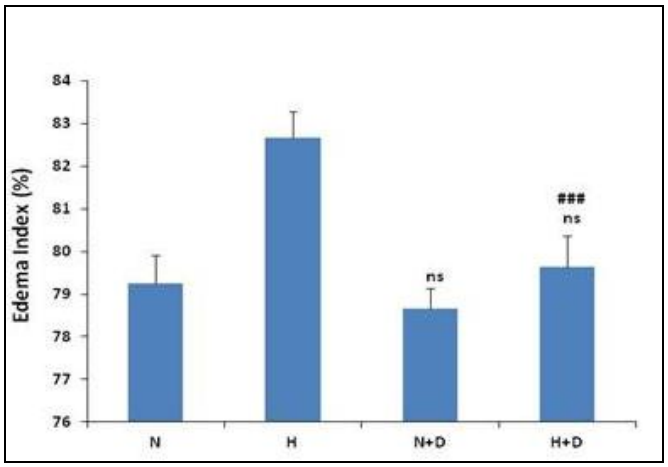

FIG. 11: ESTIMATION OF BRAIN WATER CONTENT Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns. N-normoxia; H-hypoxia; N+D- normoxia+amlodipine; H+D-hypoxia+amlodipine.

The increase in edema index denotes the increase in brain water content during hypoxia and treatment with amlodipine resulted in the reduction of brain water content back to normoxia control levels (**Fig.11**).

Vascular Leakage being an indicator for BBB permeability was evaluated with Sodium fluoresce in dye. BBB permeability was found to increase in hypoxia group of rats and significantly decreased in the groups treated with amlodipine (**Fig.12**).

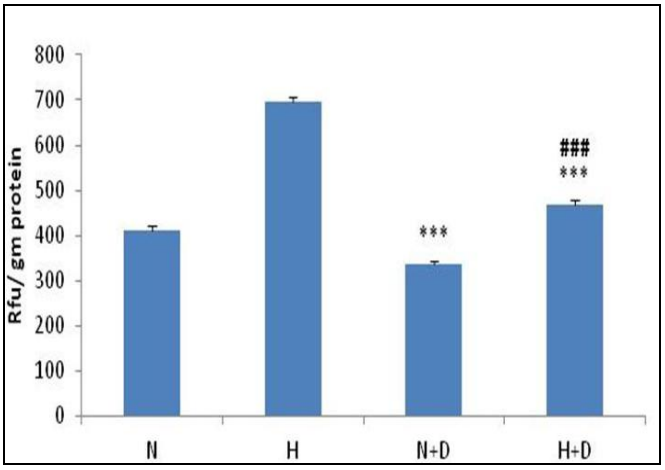

FIG. 12: ESTIMATION OF VASCULAR LEAKAGE IN BRAIN TISSUE Values are expressed as Mean±SD (n=6). Significant differences are indicated by **p<0.05 and ***p<0.01 when compared with normoxia control and ##p<0.05 and ###p<0.01 when compared with hypoxia control whereas; non-significant changes are designated as ns. N-normoxia; H-hypoxia; N+D- normoxia+amlodipine; H+D-hypoxia+amlodipine.

DISCUSSION: Amlodipine attenuated, in a dose-dependent manner, the intracellular Ca^{2+} increase elicited by VGCCs expression due to hypoxic exposure. The neuroprotective activity of amlodipine in this system was biphasic; increasing concentrations resulted in decrement of neuroprotection. This suggests that there is a favorable balance in the amount of calcium that is required for the proper functioning of calcium gated channels (CGCs) and that deficiency in intracellular calcium may compromise normal

function and ultimately promote cytotoxicity. These findings are in accordance with the findings of Mason *et al* [21]. In addition to its effect on cellular calcium levels, it was observed that amlodipine had dose-dependent anti-oxidant activity in liposomes enriched with polyunsaturated fatty acids, independent of ion channel modulation. The anti-oxidant activity of amlodipine contributes to its neuroprotective activity by reducing oxidative stress.

Furthermore, chronic administration of amlodipine in spontaneously hypertensive rats has been shown to reduce oxidative stress in brain[22]. Recent studies have also revealed that treatment with amlodipine has been effective both in reducing the size of ischemic lesion and in improving the neurological score in apolipoprotein E-defecient mice [23].

The free intracellular calcium, percentage cell viability, reactive oxygen species, malondialdehyde and glutathione levels were screened in amlodipine treated neuroblastoma N2a cells in both 21% O_2 normoxia and 0.5% O_2 hypoxia for a duration of 24 hours. In this dose dependent study, concentration range of 5 – 200 nM for amlodipine was selected. Analysis of all the above biochemical parameters in drug treated samples showed only slight variations with respect to normoxia control but when compared with hypoxia control, there was significant improvement in these parameters.

The intracellular calcium levels showed substantial rise in hypoxia control with respect to normoxia control. Suggesting that hypoxia increased intracellular Ca^{2+} concentration, which is in accordance with the findings of Luo *et al* [24] and Peterson*et al.*[25]. The hypoxic calcium levels decreased with increasing drug concentrations which confirms that voltage gated calcium channels (VGCC) play a pivotal role in modulating the intracellular calcium rise during hypoxia (**Fig.2**). Under normoxic conditions the calcium levels exhibited marginal decrease with increasing drug concentrations which suggests that few VGCCs may be in active state in N2a cells. This may be probably due to more positive resting membrane potential reported in cancerous cell lines as compared to neuronal cell lines[26].

Analysis of calcium levels in hypoxia with viability, ROS, MDA and GSH levels indicated that an optimum calcium concentration is linked with higher cell viability and GSH content (**Fig.8 & 9**) and lower oxidant levels (ROS & MDA generation; **Fig. 4, 5, 6, & 7**). In cells that had calcium concentrations either higher or lower than the optimum values were less conducive to survival and more prone to oxidative damage.

Thus there might be an optimal range of calcium concentration operating during hypoxia which might be beneficial to cell survival and adaptation. Any further rise or fall beyond this optimal range might not be conducive for cell survival in hypoxic conditions.

The percentage cell viability and GSH values under hypoxia peaked at 12.5nM. Similarly the oxidant levels measured in terms of ROS and MDA were found to be lowest at the above mentioned concentration. This clearly suggests that amlodipine offer maximum neuroprotection in N2a cells at concentrations of 12.5nM. To further investigate the efficacy of this drug concentration, western blot analysis of cox-2, Bax/Bcl-2, and VEGF expression was being carried out in hypoxia exposed N2a cells with 12.5nM amlodipine. *In-vivo* experiments were also done with conversion value of this drug dose only (i.e., 5mg kg^{-1}bw amlodipine, i.p), to study vascular leakage and brain water content.

Cyclooxygenase-2 activity has been implicated in ischemia and other neurodegenerative diseases[27, 28]. Its over expression is an indicator of lipid peroxidation as it acts on the fatty acid substrate arachidonic acid generated after phospholipase action on lipid membranes. ROS are produced by the peroxidase step of the cox reaction in which prostaglandin G2 is converted to prostaglandin H2[29]. The prostaglandins, products of COX-2 action, have clear role in blood brain barrier disruption by promoting angiogenesis which is primarily responsible for the development of cerebral edema in high altitude. The densitometric analysis after western blotting of hypoxia inducible COX-2 protein showed that 12.5 nM of amlodipine decreased the COX-2expression significantly (**Fig. 10**).

The pro-apoptotic proteinBax modulates Ca^{2+} homeostasis [30] in rat cortical astrocytes by facilitating calcium release from endoplasmic reticulum. In turn cytoplasmic calcium rise promotes Bax translocation from cytoplasm to the mitochondrial membrane and lead to apoptosis. The anti-apoptotic Bcl-2 protein present on mitochondrial membranes can bind bax and prevent apoptotic activity of bax. Thus Bax/Bcl-2 ratio is an indicator of the apoptotic state. Ratio values higher than the normal levels indicate progression of cell towards apoptosis (as inferred from **Table 1)**. Amlodipine was successful in bringing down the hypoxic Bax/Bcl-2 ratio significantly which were closer to normoxic values.

In rats exposed to hypobaric hypoxia there was a significant increase in intracellular Ca^{2+} levels along with a substantial rise in oxidative stress when compared with normoxia control levels. Upon treatment with amlodipine (12.5nM), there was a significant improvement in intracellular Ca^{2+} level shypobaric hypoxia exposed animals (**Fig. 3**). Also the oxidative stress reduced significantly in animals treated with amlodipine after exposure to hypobaric hypoxia, when compared with control animals. These results are similar to the findings of *in-vitro* studies. From the above results it is also clear that amlodipine effectively reduces edema formation and plays an active role in protection of BBB by reducing vascular leakage in brain caused due to hypobaric hypoxia (**Fig.11 & 12).**

CONCLUSION: Calcium accumulation and calcium overloading in the intracellular organelles has been known to be the main reason for cellular abnormalities related to oxidative stress. Intracellular calcium levels have been found to be lowered significantly in the drug treated groups both under normoxic and hypoxic conditions. Based on this study it can be stated that amlodipine apart from being a well-known calcium channel blocker has several antioxidant activities that could play a neuroprotective role in animals exposed to hypobaric hypoxia.

ACKNOWLEDGEMENT: The research is supported by financial assistance from Defense Research and Development Organization (DRDO), Government of India. Varun Bhardwaj is recipient of Junior Research fellowship from Council for Scientific and Industrial Research (CSIR), Govt. of India. Sarita Nehra is recipient of Junior Research fellowship from University Grant Commission (UGC), Govt. of India. We are also thankful to Mr Bhagwat Singh for providing assistance in animal procurement and maintenance for research work.

CONFLICT OF INTEREST: The authors declare no conflict of interests.

REFERENCES:

1. Xu, K. and J.C. LaManna: Chronic hypoxia and the cerebral circulation. Journal of Applied Physiology, 2006. 100(2): p. 725-730.
2. Reiter, R.J.: Oxidative processes and antioxidative defense mechanisms in the aging brain. The FASEB journal, 1995. 9(7): p. 526-533.
3. Raman, L., et al.: In vivo effect of chronic hypoxia on the neurochemical profile of the developing rat hippocampus. Developmental brain research, 2005. 156(2): p. 202-209.
4. Weir, E.K. and A. Olschewski: Role of ion channels in acute and chronic responses of the pulmonary vasculature to hypoxia. Cardiovascular research, 2006. 71(4): p. 630-641.
5. Law, M., J. Morris, and N. Wald: Use of blood pressure lowering drugs in the prevention of cardiovascular disease: meta-analysis of 147 randomised trials in the context of expectations from prospective epidemiological studies. BMJ: British Medical Journal, 2009. 338.
6. Tobe, S.W., et al.: Harmonization of guidelines for the prevention and treatment of cardiovascular disease: the C-CHANGE Initiative. CMAJ: Canadian Medical Association Journal, 2011. 183(15): p. e1135-e1150.
7. Matsui, Y., et al.: Combined Effect of Angiotensin II Receptor Blocker and Either a Calcium Channel Blocker or Diuretic on Day-by-Day Variability of Home Blood Pressure The Japan Combined Treatment With Olmesartan and a Calcium-Channel Blocker Versus Olmesartan and Diuretics Randomized Efficacy Study. Hypertension, 2012. 59(6): p. 1132-1138.
8. Chalmers, J., et al.: Effects of Combination of Perindopril, Indapamide, and Calcium Channel Blockers in Patients With Type 2 Diabetes Mellitus Results From the Action in Diabetes and Vascular Disease: Preterax and Diamicron Controlled Evaluation (ADVANCE) Trial. Hypertension, 2014. 63(2): p. 259-264.
9. Trippier, P.C., et al.: Target- and Mechanism-Based Therapeutics for Neurodegenerative Diseases: Strength in Numbers. Journal of medicinal chemistry, 2013. 56(8): p. 3121-3147.
10. Wang, J.-G., P. Yan, and B.W. Jeffers: Effects of amlodipine and other classes of antihypertensive drugs on long-term blood pressure variability: evidence from randomized controlled trials. Journal of the American Society of Hypertension, 2014. 8(5): p. 340-349.
11. Warnock, A., et al.: Amlodipine prevents apoptotic cell death by correction of elevated intracellular calcium in a primary neuronal model of Batten disease (CLN3 disease). Biochemical and biophysical research communications, 2013. 436(4): p. 645-649.
12. Choi, N.-Y., et al.: Neuroprotective effects of amlodipine besylate and benidipine hydrochloride on oxidative stress-

injured neural stem cells. Brain research, 2014. 1551: p. 1-12.
13. Salehi, I., et al.: Amlodipine attenuates oxidative stress in the heart and blood of high-cholesterol diet rabbits: cardiovascular topics. Cardiovascular journal of Africa, 2012. 23(1): p. 18-22.
14. Muraga, K., et al.: The Impact of Combined Treatment with Atorvastatin and Amlodipine in Stroke-prone Spontaneously Hypertensive Rats. Cerebral Blood Flow and Metabolism, 2014. 25(2): p. 23-30.
15. Zhang, X., et al.: Amlodipine and atorvastatin exert protective and additive effects via antiapoptotic and antiautophagic mechanisms after transient middle cerebral artery occlusion in Zucker metabolic syndrome rats. Journal of neuroscience research, 2011. 89(8): p. 1228-1234.
16. Ohkawa, H., N. Ohishi, and K. Yagi: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry, 1979. 95(2): p. 351-358.
17. Cathcart, R., E. Schwiers, and B.N. Ames: Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Analytical biochemistry, 1983. 134(1): p. 111-116.
18. Hissin, P.J. and R. Hilf: A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical biochemistry, 1976. 74(1): p. 214-226.
19. Classics Lowry, O., et al.: Protein measurement with the Folin phenol reagent. J. biol. Chem, 1951. 193: p. 265.
20. Purushothaman, J., et al.: Modulatory effects of seabuckthorn (< i> Hippophae rhamnoides</i> L.) in hypobaric hypoxia induced cerebral vascular injury. Brain research bulletin, 2008. 77(5): p. 246-252.
21. Mason, R.P., et al.: Inhibition of excessive neuronal apoptosis by the calcium antagonist amlodipine and antioxidants in cerebellar granule cells. Journal of neurochemistry, 1999. 72(4): p. 1448-1456.
22. Toklu, H., et al.: original research the protective effect of melatonin and amlodipine against cerebral ischemia/reperfusion-induced oxidative brain injury in rats. Marmara Medical Journal, 2009. 22(1): p. 034-044.
23. Mogi, M., et al.: Amlodipine Treatment Reduces Stroke Size in Apolipoprotein E–Deficient Mice. American journal of hypertension, 2006. 19(11): p. 1144-1149.
24. Luo, Y., et al.: Hydrogen sulfide prevents hypoxia-induced apoptosis via inhibition of an H< sub> 2</sub> O< sub> 2</sub>-activated calcium signaling pathway in mouse hippocampal neurons. Biochemical and biophysical research communications, 2012. 425(2): p. 473-477.
25. Peterson, B.L., et al.: Blunted neuronal calcium response to hypoxia in naked mole-rat hippocampus. PloS one, 2012. 7(2): p. e31568.
26. Kisaalita, W.S. and J.M. Bowen: Development of resting membrane potentials in differentiating murine neuroblastoma cells (N1E-115) evaluated by flow cytometry. Cytotechnology, 1997. 24(3): p. 201-212.
27. Teismann, P.: COX-2 in the neurodegenerative process of Parkinson's disease. Biofactors (Oxford, England), 2012. 38(6): p. 395-397.
28. Dudek, F.E.: Mechanisms of Seizure-Induced Inflammation of the Brain: Many Possible Roles for Neuronal COX-2. Epilepsy Currents, 2012. 12(3): p. 115-117.
29. Font-Nieves, M., et al.: Induction of COX-2 enzyme and down-regulation of COX-1 expression by lipopolysaccharide (LPS) control prostaglandin E2 production in astrocytes. Journal of Biological Chemistry, 2012. 287(9): p. 6454-6468.
30. Smaili, S., et al.: Calcium and cell death signaling in neurodegeneration and aging. Anais da Academia Brasileira de Ciências, 2009. 81(3): p. 467-475.

How to cite this article:
Bhardwaj V, Nehra S, Bansal A, Saxena S, Singh M and Saraswat D: The Effect of Amlodipine in Ameliorating the Hypobaric Hypoxia Induced Neuronal Damages and Dysfunction. Int J Pharm Sci Res 2015; 6(7): 2770-80.doi: 10.13040/IJPSR.0975-8232.6(7).2770-80.