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CARDIOPROTECTIVE ROLE OF AMLODIPINE UNDER ACUTE HYPOBARIC HYPOXIA: ROLE OF AKT-1 IN REDOX BALANCE

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ABSTRACT: Hypobaric hypoxia induced systolic heart dysfunction and arrhythmia is prevalent in ascendants. L-type calcium channel blocker amlodipine plays important role in modulating cardiac function. We tested if amlodipine could impart cardio protection and restore oxidative balance under hypobaric hypoxia, by modulating HIF-1 α activity and calcium accumulation, thus, protects from cardio-toxicity. Experimental animals, male Sprague-Dawley rats (180-200 g), were randomized into four groups, i.e. normoxia control (N), normoxia + amlodipine (N+AML), hypoxia control (H) and hypoxia + amlodipine (H+AML) (n=8), and exposed to hypobaric hypoxia for three time points i.e. 24 h, 48 h and 72 h to assess cellular damages. Amlodipine treatment not only restored systolic blood pressure and heart rate to normal levels under hypobaric hypoxia, but also regulated HIF-1 α expression, Akt/p-Akt levels and intracellular Ca²⁺ levels ([Ca²⁺]_i). Biochemical markers of oxidative damage, i.e. glutathione reduced (GSH), glutathione oxidized (GSSG), lipid peroxidation (MDA) and free radical generation by reactive oxygen species (ROS) estimation were significantly restored by intraperitoneal administration (10 mg / kg b.w.) of amlodipine. Amlodipine was found effective in regulating systolic heart function and restoring plasma MDA levels, thus help protect from hypobaric hypoxia induced damages.

INTRODUCTION: Hypobaric hypoxia, a patho-physiological condition arising from ascent to extremely high altitude, i.e. altitude $\geq$ 5500 m, is one of the major causes of pressure and volume overload induced cardiovascular pathologies appearing in the form of cardiomyocytes metabolic-insufficiency and impaired homeostasis¹. Hypobaric hypoxia is preceded by hypoxemia and increased pathological impairments and is depicted by change in physiological levels of oxidative-stress markers GSH, GSSG, lipid peroxidation and free radical generation, leading to apoptosis².

One of the early major (patho-) physiological effect of hypobaric hypoxia is depicted in the form of cardiomyocytes death and augments irregular heart rate and systolic heart function, adding to clinical complications which may lead to arrhythmia, tachycardia, hypertension and heart failure³.

Previous studies suggest that on ascent to very high altitudes, cardiovascular impairments appear primarily in the form of increased palpitation, heart rate and blood pressure⁴. These insufficiencies, along with initiation of stiffness in cardiac muscle aggravate the ascent-induced cardiac maladies. The symptomatic effects of sudden ascent to very high altitudes appear in the form of increased oxidative stress and hyper-accumulation of cytosolic calcium ultimately leading to increased cell death. Concomitant effects of hypobaric hypoxia appear in the form of increased cell-death and impaired

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cardiac-muscle contractibility. These hypobaric hypoxia induced cardiac insufficiencies may ultimately lead to myocardial damage, infarction and heart failure.

Therapeutic interventions that regulate/restore systolic heart function, intracellular calcium loading and oxidative damage could help improve (patho-) physiological condition. Various drugs⁵ and antioxidants⁶ have been assessed for their efficacy for preventing oxidative damage but, a potential candidate with comprehensive properties to improve physiological conditions under hypoxia remains unknown. L-type calcium channel blocker amlodipine has been known for its therapeutic cardiac effects⁷ and regulation of hemodynamic function.

However, its potential role in improving hypobaric hypoxia induced cardiac oxidative damage and hemodynamic deregulation remains unknown. We intended to check if long-acting calcium channel blocker amlodipine plays a role in regulating oxidative damage, and cardiomyocytes apoptosis, thus having cardio-protective effects under acute and severe hypobaric hypoxia.

MATERIALS AND METHODS:

Experimental Animals: The protocols used for handling animals were approved by Institutional Animal Ethical Committee of DIPAS, DRDO (27/1999/CPCSEA). Experimental animals, age-matched, male Sprague-Dawley rats, weighing from 160g to 180g, were bred and kept at Experimental Animal Facility, DIPAS, DRDO (ISO certified 9001:2008). All animals had equal access to normal rat chow and water. Experimental animals were randomized into four groups, i.e. normoxia control (N), normoxia + drug (N+AML), hypoxia control (H) and hypoxia + drug (H+AML)(4 groups, each group n=8), and exposed to hypoxia for three time points i.e. 24 h, 48 h and 72 h.

Animals were exposed to normal 12 h light and dark cycles. Drug treatment was given to the rats as 10mg / kg b.w. Amlodipine-besylate (Sigma, A5605), intraperitoneally (IP). Hypobaric hypoxia exposures were given to the animals for 24, 48 and 72 hours in animal decompression chamber (Ballice Instruments, India), at 25000 feet altitude,

with air-flow rate of 1 ml oxygen / animal / minute (corresponding to partial pressure of oxygen nearly 58 mm Hg) (n=8, three groups). Animal dosing, replenishment of food, water and changing of bedding were performed everyday at a fixed time at 10:00 am, daily.

Tissue collection: Rats were anaesthetized with intraperitoneal administration of ketamine (80mg / kg b.w.) and xylazine (10mg / kg b.w.). The chest cavity was cut open and the hearts were perfused with phosphate-buffered saline (pH 7.2), by ventricular puncture, using a scalp-vein set (Romsons, 24 Gauge), with a 50 ml syringe (DispoVAN), maintaining a flow rate of 50 ml / 5 minutes. The hearts were immediately removed and snap-frozen in liquid nitrogen (-180°C), for further processing.

The stored tissues were finely chopped using a sterile scalpel and mixed with 1:1 (w / v) of 0.154 M KCl for homogenization. The minced tissue was finely grinded using a sterile homogenizer (Kinematica, Switzerland) on ice centrifuged at 8000 rpm (ThermoScientific, HERAEUS, Fresco 21) for 15 minutes, at 4° C. The supernatant containing cytoplasmic extract was collected immediately for experimental analysis.

Hemodynamic studies: Hemodynamic studies were done in animals as soon as the hypobaric hypoxia exposures were completed, i.e. at the end of 24, 48 and 72 h. The animals were taken out of the hypobaric hypoxia chamber and blood pressure and heart rate of experimental animals were measured during the course of experiment to assess the effect of amlodipine on systolic heart function using tail-cuff plethysmography, and recorded digitally. Anesthesia was not given to the animals during the procedure.

Protein Estimation: The protein estimation in the cell lysate was done with the Lowry's method⁷. Absorbance was read at 660 nm (FLUOstar Omega, BMG LABTECH) and protein concentrations were calculated against an internal standard of BSA.

Estimation of $[Ca^{2+}]_i$: The $[Ca^{2+}]_i$ concentrations were estimated in cytosolic fractions of hearts using QuantichromeTM Calcium Analysis Kit

(Bioassay Systems, U.S.A.), according to manufacturer's instructions. Briefly, the phenolsulphonaphthalein dye forms a very stable blue colored complex specifically with free calcium. The intensity of the color, measured at 612 nm, is directly proportional to the calcium concentration in the sample.

Antioxidant study

Oxidative stress assessment: The extent of oxidative stress was measured as a measurement of free radicals generation for estimation of ROS was determined in cytoplasmic extract prepared from excised hearts by using 2, 7- dichlorofluorescein diacetate (DCFH-DA) as described by Cathcart *et.al.*⁸.

Accordingly, DCFH-DA, a non-fluorescent diester is cleaved by cellular esterase to form dichlorofluorohydrate (DCFH) and acetic acid. The DCFH then reacts with reactive oxygen species to produce "fluorescein", whose fluorescence (absorbance at 485 nm and fluorescence at 530 nm) is directly proportional to the free radical content in the sample.

Estimation of lipid peroxidation: Lipid peroxidation was measured in the cytosolic fraction prepared from excised hearts in the terms of formation of malondialdehyde (MDA) as described by Utley *et al.*⁹ When tetra ethoxy-propane, which correlates quantitatively to MDA during reaction, was reacted with thiobarbituric acid (TBA), their hydrolysis by acid causes the formation of pink colour compound, which was measured spectrophotometrically at 531 nm (FLUO star Omega, BMG LABTECH).

Estimation of Reduced Glutathione (GSH) and Oxidised Glutathione (GSSG): The reduced glutathione (GSH) and oxidised glutathione (GSSG) levels in whole heart cytosolic homogenates were determined by the method of Hissin and Hilf¹⁰. The thiol group of GSH reacts with o-phthaldehyde (OPT) to form a complex which gives fluorescence with an excitation at 350 nm and emission at 420 nm. The thiol group of GSSG reacts with o-phthaldehyde (OPT) to form a complex which gives fluorescence with an excitation at 350 nm and emission at 420nm (FLUO star Omega, BMG LABTECH).

Measurement of SOD activity: The measurement of superoxide dismutase (SOD) activity was done according to method previously described¹¹. Briefly, xanthine and xanthine oxidase were used to generate superoxide radicals, which react with 2, 4-iodophenyl-3,4-nitrophenol-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction.

Measurement of ATP concentration: Intracellular ATP concentration was measured using commercially available ATP determination kit (A22066, Molecular Probes), using bioluminescence generated by luciferase activity.

Measurement of AMPK α expression, Akt and p-Akt levels: The tissue p-AMPK α ([pT172] ELISA kit, INVITROGEN, USA) expression and Akt (Akt/Erk Activation InstantOne™ ELISA, 85-86014-11) and p-Akt (pS473, ELISA kit, INVITROGEN, USA) levels were measured using commercially available ELISA kits according to manufacturer's instructions. Briefly, the method involves a solid phase ELISA in which samples were incubated with antigen specific pre-coated monoclonal antibody. An HRP-conjugated secondary antibody was added for development of colour. The absorbance was then read optically at 450 nm.

Western Blot analysis: Cytoplasmic proteins were resolved in 10% gel and transferred to polyvinylidene difluoride membrane. The membranes were incubated with primary antibody HIF-1 α (SIGMA, H6536, 1:1000), Akt1/2/3 (SantaCruz, sc8312, 1:500), p-Akt1/2/3 (Invitrogen, 34-8400, 1:1000) and p-AMPK α (Santa Cruz, sc101630, 1:500) overnight at 4° C. HRP-conjugated secondary antibody (Santa Cruz, sc2005, 1:25000) was added and the membranes were developed on photographic film.

Histopathology assessment by haematoxylin and eosin/Masson's trichrome staining: To assess muscle damage by collagen accumulation and cellular viability under hypobaric hypoxia, Masson's trichrome staining and haematoxylin and eosin staining were performed in cardiac tissue. Cardiac tissues were fixed in 4 % neutralized paraformaldehyde (PFA, in PBS) in PBS and stored

for histopathological staining. Nearly 4-5 μm thin paraffin sections were taken and stained for haematoxylin and eosin and Masson's trichrome staining to visualize cellular viability and muscular damage.

Statistical Analysis: Data was expressed as Mean $\pm$ SD (n=8) for each experimental group. The results were analysed for statistical significance using one-way ANOVA, $p < 0.05$ was considered to be significant.

TABLE 1: TABLE SHOWING CHANGES IN SYSTOLIC BLOOD PRESSURE (sBP) AND HEART RATE (h.r.) OF ANIMALS (n=8) UNDER VARIOUS EXPERIMENTAL CONDITIONS.

Experimental group	sBP/h.r.	sBP /h.r.	sBP /h.r.
	24 h	48 h	72 h
N	122.57 $\pm$ 5.33/333.37 $\pm$ 15.27	134.41 $\pm$ 3.45/336.45 $\pm$ 14.25	128.33 $\pm$ 4.11/418.23 $\pm$ 6.33
N+AML	112.44 $\pm$ 4.15/326.83 $\pm$ 9.36	127.54 $\pm$ 7.11/339.22 $\pm$ 17.54	123.69 $\pm$ 3.29/413.43 $\pm$ 16.37
H	138.79 $\pm$ 6.72/316.33 $\pm$ 12.18	141.22 $\pm$ 6.24/417.27 $\pm$ 14.05	134.27 $\pm$ 6.71/384.19 $\pm$ 11.14
H+AML	125.66 $\pm$ 4.57/321.78 $\pm$ 11.22	131.36 $\pm$ 5.22/426.34 $\pm$ 11.10	133.55 $\pm$ 8.16/403.23 $\pm$ 14.77

Systolic Blood Pressure Along With Heart Rate Increased In Hypobaric Hypoxia Control Animals Whereas Decrement To Near Normal Levels Was Observed In H+AML Groups, Showing Effectiveness Of Amlodipine In Maintaining Systolic Blood Pressure And Heart Rate Under Acute Hypobaric Hypoxia. Values are represented as mean $\pm$ SD.

Ameliorative effect of amlodipine on ROS generation: Free radical generation is considered as one of the major outcomes of hypoxia stress. The efficacy of any drug in decreasing ROS generation is considered crucial for supporting homeostasis. Amlodipine was found very effective in decreasing ROS generation in heart tissues, at all time points in H+AML groups i.e. 6983.68 RFU / minute / mg protein, 6914.8 RFU / minute / mg protein and 4949.96 RFU / minute / mg protein at 24 h, 48 h and 72 h respectively (**Fig. 1**).

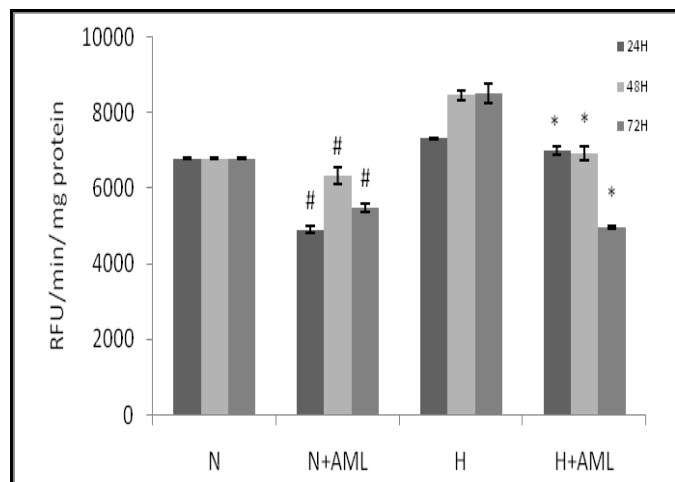


FIGURE 1: Figure showing ameliorative effect of amlodipine on the ROS generation in animals (n=8). Values are Mean $\pm$ SD (n=8). Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

RESULTS:

Hemodynamic Regulation by amlodipine: It was found that amlodipine treatment helped in maintaining systolic blood pressure of animals under hypobaric hypoxia. The blood pressure was found best regulated in 24 h group of animals and slight decrement was observed in 48 h and 72 h groups of animals. Similarly, it was observed that heart rate of animals was best restored in 24 h and slight decrement was observed in 48 and 72 h hypobaric hypoxia exposed animals (**Table 1**).

Ameliorative effect of amlodipine on lipid peroxidation: Lipid peroxidation, measured as MDA levels, increases in hypoxia, which in turn promotes ROS generation by oxidation of lipids and proteins. The accumulation of oxidized lipids and proteins is highly toxic to cells. In the present study, it was found that amlodipine was efficiently capable in reduction of lipid peroxidation at all the time points in H+AML groups, i.e. 10.944 n mole / μg protein, 15.5299 n mole / μg protein and 16.71 n mole / μg protein at 24 h, 48 h and 72 h respectively (**Fig. 2**).

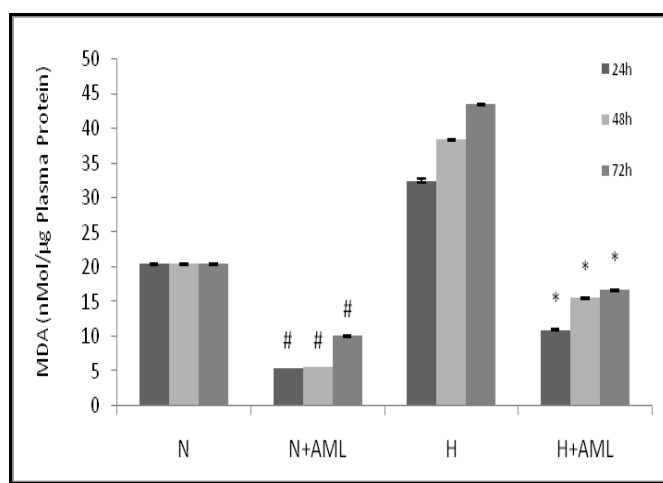


FIGURE 2: Figure showing efficacy of amlodipine treatment on plasma lipid peroxidation in animals (n=8). Values are Mean $\pm$ SD. Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

Ameliorative effect of amlodipine on GSH/GSSG ratio: The intracellular ratio of GSH and GSSG is an indicative of normal physiological homeostatic response to antioxidant stress. In the present study, it was found amlodipine was efficiently capable in regulating the GSH / GSSG ratio at all the time points in H+AML groups, i.e. 6.71, 6.41 and 6.22 at 24 h, 48 h and 72 h respectively (**Fig. 3**).

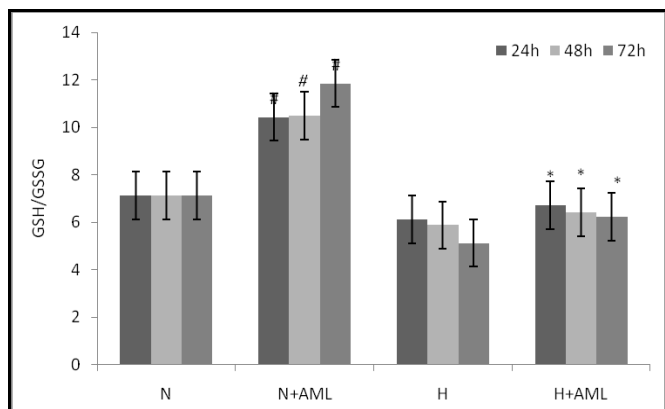


FIGURE 3: Graphical representation of restoration of GSH/GSSG levels in cardiac tissue by amlodipine treatment in animals (n=8). Values are Mean $\pm$ SD. Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

Restoration of normal intracellular Ca^{2+} levels: Acute hypobaric hypoxia causes rise in $[\text{Ca}^{2+}]_i$ levels through L-type calcium channels. These voltage gated calcium channels are crucial for regulation of normal ionic balance inside the cardiomyocytes, thus helping to maintain a normal electric potential across the cell membrane. This is crucial for maintaining rhythmicity of heart. The total $[\text{Ca}^{2+}]_i$ was well regulated by drug treatment in H+AML groups at all the time points, 0.0425 m M, 0.047 m M and 0.0689 m M at 24 h, 48 h and 72 h respectively (**Fig.4**).

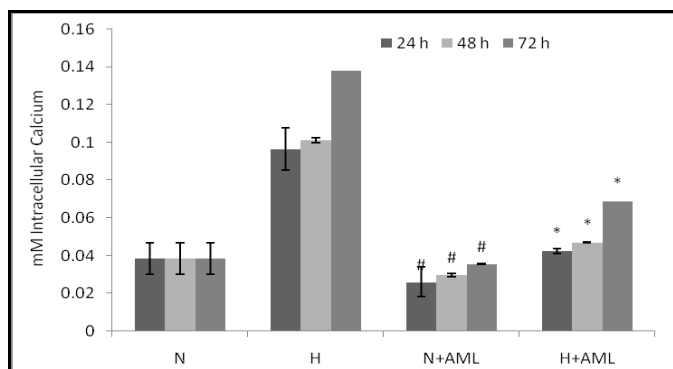


FIGURE 4: Figure showing ameliorative effect of amlodipine on maintaining total intracellular calcium levels in animals (n=8). Values are Mean $\pm$ SD. Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

Amelioration of SOD activity: Acute hypobaric hypoxia induced increase in SOD activity was observed in hypobaric hypoxia control animals (45.47 IU / min). Oxidative stress was found decreased in the form of decreased SOD activity in amlodipine treated 24 h hypobaric hypoxia exposed animals (61.56 IU / min), respectively, when compared to normoxia controls (76.46 IU / min) (**Fig.5**).

SOD activity was also found well regulated in 48 h and 72 h groups being 77.08 IU / min and 126.04 IU / min respectively.

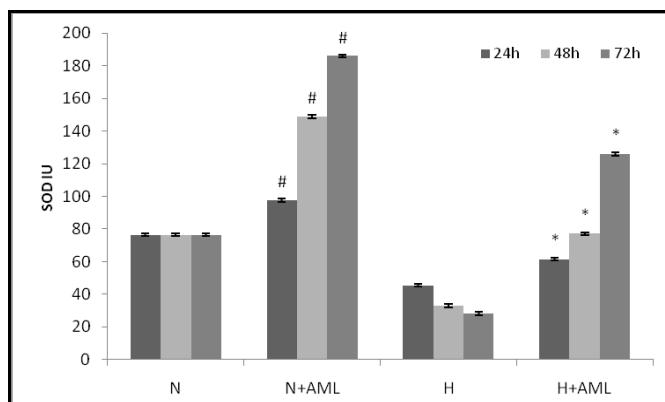


FIGURE 5: Figure showing efficacy of amlodipine on the SOD activity in cardiac tissue in animals (n=8). Values are Mean $\pm$ SD. Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

Effect of amlodipine on cellular ATP concentration: It was found that amlodipine treated groups showed restoration of cellular ATP pool, maximum efficiency depicted in 24 h group. A gradual decrease in ATP concentration was observed in 48 h and 72 h groups of animals (**Fig. 6**).

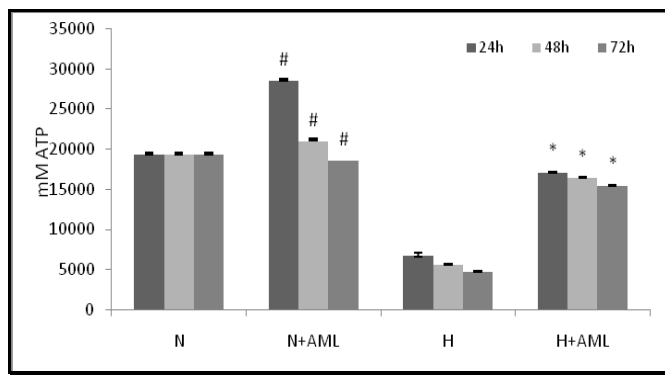


FIGURE 6: Figure showing efficacy of amlodipine on restoration of cellular ATP pool in cardiac tissue in animals (n=8) in various experimental groups. Values are Mean $\pm$ SD. Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

Regulation of p-AMPK α expression, Akt and p-Akt levels: Amlodipine was found effective in regulating p-AMPK α (Fig. 7) expression along with Akt and p-Akt levels (Fig. 8) in the cardiac tissue. in H+AML and N+AML groups, in which, a significant lowering of p-AMPK α expression was seen in all the time points, i.e. nearly 315 IU / ml, 350 IU / ml and 365 IU / ml in 24 h, 48 h and 72 h N+AML groups as compared to 389 IU / ml normoxia control. Also, the H+AML groups showed parallel regulatory effect, nearly being 428 IU / ml, 495 IU / ml and 640 IU / ml respectively as compared to hypoxia controls.

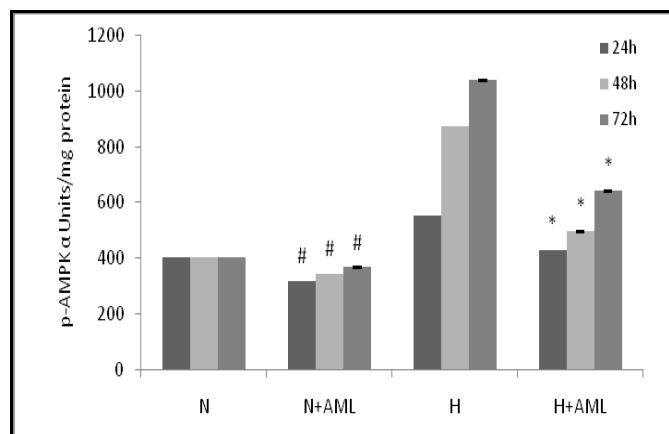


FIGURE 7: Figure showing efficacy of amlodipine on regulating the p-AMPK α expression in cardiac tissue in animals (n=8). Values are Mean $\pm$ SD. Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

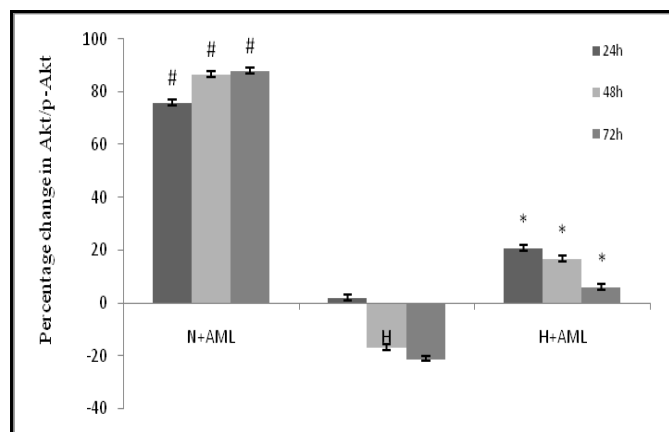


FIGURE 8: Figure showing percentage changes in Akt/p-Akt levels in experimental groups (n=8) normalized to normoxia controls. Amlodipine treatment enhances Akt/p-Akt ratio which then helps in phosphorylating downstream apoptotic targets and prevents apoptosis. A slight increase in the ratio is seen in 24 h hypobaric hypoxia exposed animals probably as adaptive physiological response to maintain homeostasis. However, the same is found decreased in 48 h and 72 h showing induction of hypobaric hypoxia induced apoptosis. 24 h H+AML animals showed maximum increment in the Akt/p-Akt levels showing maximum efficiency of amlodipine

in 24 h of hypobaric hypoxia exposure. Values are Mean $\pm$ SD. Significant differences are indicated by # at $p < 0.05$ when compared with normoxia control, and * $p < 0.05$ when compared with hypoxia control.

Histopathological assessment of cardiac tissue: Improvement in cellular morpho-histological intercellular collagen accumulation was found in amlodipine treated animals under hypobaric hypoxia as compared to hypoxia controls. It was found that amlodipine treated animals showed decreased muscle damage, collagen accumulation and damaged nuclei under hypobaric hypoxia as compared to hypoxia controls, as evident from haematoxylin and eosin staining along with Masson's trichrome staining (Fig.9).

Haematoxylin and eosin staining showed more apoptotic nuclei in cardiac muscles indicating hypobaric hypoxia induced cell death, arising due to oxidative damage and disturbed calcium homeostasis.

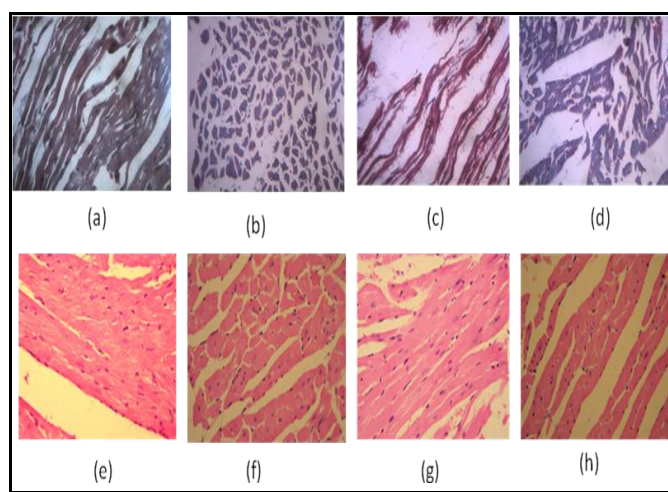


FIGURE 9: Figure showing histopathological assessment of cardiac muscles after 24 h in various experimental groups by (a to d, i.e. N, H, N+AML and H+AML respectively) Masson's trichrome staining and (e to h, i.e. N, H, N+AML and H+AML respectively) haematoxylin and eosin staining. Amlodipine treated animals showed increased cellular viability and lessened muscle damage as compared to hypobaric hypoxia control animals, indicating enhanced cellular viability and decreased muscle damage by amlodipine treatment under acute hypobaric hypoxia

Western blot: Western blot analysis of HIF-1 α indicate efficacy of amlodipine in improving cellular viability and stabilization of HIF under hypoxia. HIF-1 α expression was increased under hypobaric hypoxia controls and amlodipine treated groups showing stabilization of HIF under

hypobaric hypoxia. The expression levels of Akt/p-Akt in animals treated with and without amlodipine indicate pro-survival effect of amlodipine under hypobaric hypoxia. Also, restoration of p-AMPK α levels indicates restoration of metabolic state and maintenance of metabolic homeostasis in animals treated with amlodipine under hypobaric hypoxia (Fig. 10).

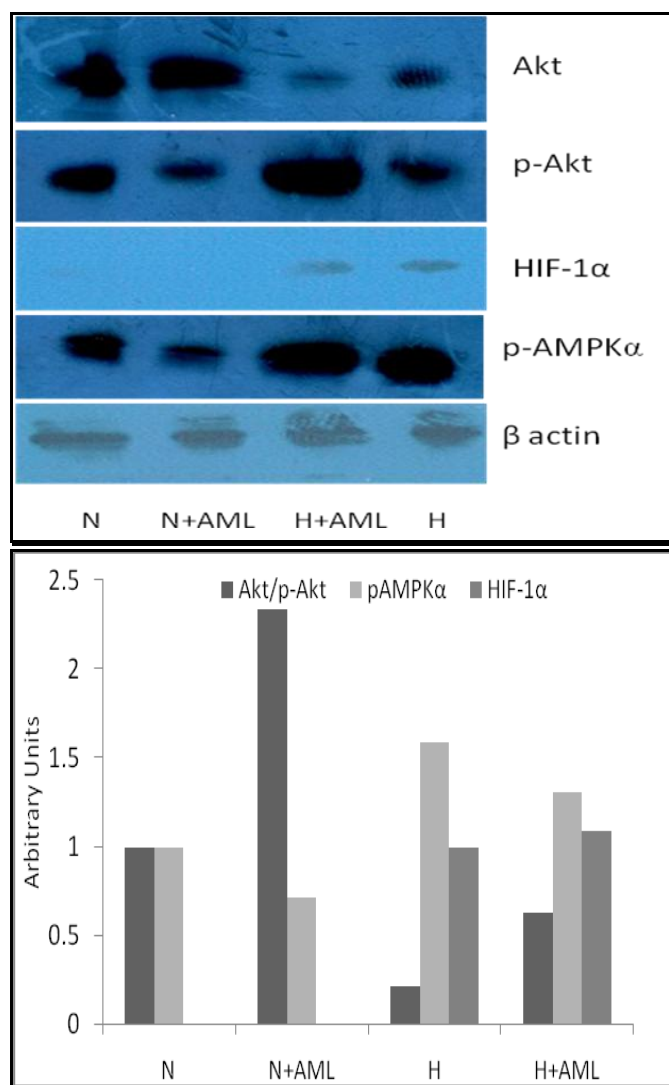


FIGURE 10: Western blot and densitometric graphs of Akt, p-Akt, AMPK α , HIF-1 α and β actin in animals exposed to 24 h hypobaric hypoxia. There was found no expression of HIF-1 α in normoxia control and N+AML groups. High expression of HIF-1 α was observed in hypoxia controls, whereas the same was found decreased in H+AML group implying stabilization of HIF-1 α post amlodipine treatment in hypobaric hypoxia.

DISCUSSIONS: The key finding of the present work is that amlodipine treatment protects from acute hypobaric hypoxia induced oxidative imbalance, systolic dysfunction and arrhythmia. It was evident from our data that systolic blood pressure and heart rate of animals were well regulated after amlodipine treatment under severe

hypobaric hypoxia. However, since only 24 h group of animals under hypoxia showed improvement in systolic blood pressure and heart rate, it infers that effectiveness of amlodipine remained for 24 h.

A rise in blood pressure and heart rate were observed in hypoxia control animals, suggesting that extent of hypobaric hypoxia (25000 feet) was enough to induce tachycardia response, as supported by previous studies. Also, intracellular calcium levels were found restored to normal levels in 24 h group of animals, which synchronized with the maintenance of systolic heart function in 24 h hypobaric hypoxia exposed animals. It is also evident from the present study that amlodipine treatment enhanced cellular viability and decreased muscle damage under hypobaric hypoxia at 24 h of stress.

Amlodipine prevents cardiomyocytes death by affecting Akt-HIF axis

It was found that Akt/p-Akt expression level was decreased in amlodipine treated groups under hypobaric hypoxia. Amlodipine prevented phosphorylation of Akt at Ser-473 residue, thus rendering its activation and regulated apoptotic activation under hypobaric hypoxia. Also, HIF-1 α expression levels were decreased which shows the effectiveness of amlodipine in stabilizing HIF-1 α , which is known to maintain cellular viability.

The simultaneous decrement in Akt/p-Akt and stabilization of HIF-1 α stimulated the cardiomyocyte machinery towards increment in cellular viability which was further evident by Haematoxylin and Eosin staining in cardiac tissue. The similar results were found in Masson's trichrome staining of cardiac tissues which showed decreased muscle damage and increased viability after amlodipine treatment under hypobaric hypoxia.

Cardio-protection under hypobaric hypoxia

Increment in cellular viability and muscle flexibility are vital parameters under acute hypobaric hypoxia stress. It is also inferred from the present study that amlodipine imparts cardio protection under acute hypobaric hypoxia by decreasing interstitial collagen accumulation and improving cellular viability, as evident from

Masson's trichrome and haematoxylin and eosin staining. Decline in collagen accumulation prevents stiffness in cardiac muscles thus maintaining flexibility and contractibility of the muscles. It implies that amlodipine may be used as an effective therapeutic agent for controlling hypobaric hypoxia induced myocardial infarction and cardiomyopathy.

Oxidative damage under hypobaric hypoxia

The ratio of intracellular reduced and oxidized glutathione, i.e. GSH / GSSG, is a key indicative of cellular oxidative stress, higher ratio being an indicative of balanced cellular redox-state. Leakage of excessive ROS from mitochondrial complex I and III of the electron transport chain and lipid peroxidation mediated peroxide ion generation are source of free radical generation and impart lethal oxidative damage to cells¹². The tightly regulated cellular GSH / GSSG ratio, MDA content and ROS generation are parts of interlinked cascade of hypoxia induced oxidative damages¹³.

The GSH / GSSG ratio is decreased, along with increase in ROS leakage and lipid peroxidation, as a result of which, the hyperactive voltage operated calcium channels open up promoting hyper-influx of Ca^{2+} ions under hypobaric hypoxia¹⁴. As a consequence of this cascade of events, there occurs shifting of cellular respiration towards anaerobic glycolysis since glucose becomes the favoured substrate for energy production over lipids¹⁵.

The cytosolic GSH / GSSG is detrimental in providing a cellular defence to oxidative damage under hypoxia, decrement in ratio indicating increased oxidative damage. Free-radical generation, measured in the form of ROS generation, is a direct result of oxidative stress. Excess concentration of ROS in cytosol is toxic to the cells and contributes in an orchestra of mechanisms that interlink lipid peroxidation and molecular damage to cells. Lipid peroxidation, an indicative of oxidative stress is elevated in hypoxia and can also lead to accumulation of toxic compounds.

It was found in the present study that tissue GSH / GSSG ratio, levels of ROS generation and lipid peroxidation were restored to near normal levels in amlodipine treated hypobaric hypoxia exposed groups. The restoration of the oxidative stress-

markers is an indication towards efficiency of amlodipine in regulating cellular enzymatic and non-enzymatic anti-oxidant. Restoration of these crucial anti-oxidants helps in maintaining homeostasis under acute hypobaric hypoxia.

Effect of hypobaric hypoxia on cellular ATP pool under oxidative damage

A number of evidences have been previously presented that mitochondrial ROS generation is associated with imbalance of AMP/ATP ratio on exposure of acute and severe hypoxia¹⁶. One of the consequences of hypoxia induced oxidative stress and ROS generation is observed as depletion of ATP pool of the cell, which channelizes the mitochondrial machinery for more ATP generation and reserves the ATP pool for sustenance of vital functions.

The hypobaric hypoxia induced rise in AMP/ATP ratio is detrimental in induction of stress-sensing kinase AMPK α , which is known to be the key regulator of the ATP-generation cascade in the mitochondria¹⁷. The Ca^{2+} _i concentration and AMP/ATP pool is tightly regulated in the cells. Rise in $[\text{Ca}^{2+}]_i$ promotes ATP depletion, and hyper-influx of $[\text{Ca}^{2+}]_i$, as a result of (patho-)physiological stimulus like hypobaric hypoxia, and is known to have deleterious effect on mitochondrial machinery, resulting in excess deleterious ROS generation. Oxidative stress-induced ROS generation also activates antioxidant enzyme SOD, increase in activity of this enzyme is inferred as a direct consequence of oxidative damage under hypobaric hypoxia¹⁶.

Voltage-gated calcium channels and oxidative damage

Voltage-gated calcium channels are a route to maintain the ionic gradients across the cell membrane, thus maintaining a normal membrane potential. This helps in maintaining the excitability and rhythmicity of heart. Hypobaric hypoxia increases cellular permeability by promoting opening of membrane bound calcium channels, resulting in influx of calcium ions in the cytosol¹⁴.

One of the major reasons to this rise in total cytosolic Ca^{2+} concentration is due to opening of L-type voltage gated amlodipine sensitive Ca^{2+} gates¹⁸. Another major reason for this abrupt rise in

total $[Ca^{2+}]_i$ is loss of electrochemical gradient across the inner mitochondrial membrane, due to generation of free radicals in the form of ROS. Increased ROS generation elicits AMP levels in cells, in response to hypoxia induced oxidative stress. This rise in ROS generation causes burst of sarcoplasmic reticulum and results in cytosolic overload of calcium¹⁹.

The $[Ca^{2+}]_i$ is an important regulator in the orchestra of pathophysiological events inside the cells, ranging from hormonal balance, ion-assisted signalling, and intracellular ionic concentrations of other ions such as K^+ and Cl^- among others, well associated with hypoxia induced damages⁵.

It has been well known that hypoxia is a causative agent for accumulation of intracellular Ca^{2+} partially due to increased permeability of membrane bound L-type calcium channels and ionic leakage from sarcoplasmic reticulum in the cytosol²⁰.

This total increment in $[Ca^{2+}]_i$ leads to a plethora of events that includes activation of nuclear and cytosolic transcription factors, that direct the cells towards replenishing the ATP pools and activation of stress-induced kinase AMPK α . All these events lead to a disturbed oxidative state in the cell and finally become the causative agent for ATP depletion and accumulation of creatine, a marker of cellular injury to anaerobic respiration. These physiological parameters are also well established under hypoxia induced damages.

Various drugs have shown clinical efficiency in regulating L-type calcium channels. Amlodipine is one of the illustrious drugs used commercially as an anti-hypertensive and tranquilizer²¹. Some studies have found amlodipine as a potent cholesterol inhibitor, thus, acting as a potent anti-atherogenic²².

Also, amlodipine has been found highly potent in controlling serum concentrations of Platelet Derived Growth Factor (PDGF) and basic Fibroblast Derived Growth Factor (bFGF) in Vascular Smooth Muscle Cells (VSMC)²³. Thus, there is a huge body of evidence that amlodipine is a potential candidate to cure several cardiac

ailments, including ischemia-reperfusion and pathophysiological damages⁶ but its potential role in hypobaric hypoxia remains unexplored. Since amlodipine is known to directly control the activity of L-type calcium channels, thus, it emerged out as a potential candidate to be used in treating hypobaric hypoxia-induced oxidative damage to cells, by controlling the permeability of L-type calcium channels hence, total $[Ca^{2+}]_i$, hence imparting cardio protection.

It is evident from our data that amlodipine treatment enhanced cardio protection by enhancing cellular viability, reducing muscle damage, restoring enzymatic and non-enzymatic anti-oxidant and stress-induced sensory kinase. A strong negative correlation between ATP concentration and AMPK α expression provides an insight that increasing ATP concentrations trigger down regulation of AMPK α levels in cells under acute hypobaric hypoxia stress.

Also, the systolic heart activity was also rectified and co-regulation of systolic blood pressure, heart rate, AMPK α expression and intracellular calcium load in cardiac muscles proves that under hypobaric hypoxia, amlodipine indeed is effective in regulation of systolic heart function by regulating calcium concentration and Akt/p-Akt mediated HIF-1 α and AMPK α plays an indispensable role in the same.

Also, co-regulation of HIF-1 α with AMPK α suggests that amlodipine treatment modulated HIF-1 α activity and affected sensory expression of AMPK α under hypobaric hypoxia since as ATP concentrations lowered down, AMPK α and HIF-1 α levels increased and free intracellular calcium concentrations increased. The same were restored to near normal levels by amlodipine treatment suggesting it highly cardio protective effect under acute hypobaric hypoxia.

CONCLUSIONS: The study also confers amlodipine-mediated regulation of antioxidant status of the cardiac muscles under hypobaric hypoxia, as evident by maintenance of cellular GSH, GSSG, ROS and MDA levels. Also, the direct evidence of restoration of ROS generation that amlodipine promotes cellular aerobic

respiration and suppresses superoxide generation, along with stress-induced kinase AMPK α . Since ROS generation is well regulated in drug treated groups, and corresponding cytosolic calcium load has also been shown to well regulated, we propose that amlodipine might have an important role in regulation of mitochondrial trans-membrane permeability under hypobaric hypoxia.

STATEMENT OF CONFLICTS: The authors have no conflicts of interest.

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