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Original Article

Neferine, a bisbenzylisoquinoline alkaloid, offers protection against cobalt chloride-mediated hypoxia-induced oxidative stress in muscle cells

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ABSTRACT

Background: Neferine, a bisbenzylisoquinoline alkaloid, isolated from *Nelumbo nucifera* has a wide range of biological activities. Cobalt chloride ($CoCl_2$) was known to mimic hypoxic condition. In the present study, we assessed the cytoprotective effect of neferine against $CoCl_2$-induced oxidative stress in muscle cells.

Methods: Rhabdomyosarcoma cells were exposed to different concentrations of $CoCl_2$, and the IC_{50} value was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Lactate dehydrogenase and NO assays were performed in order to determine the cytotoxic effect of $CoCl_2$. Reactive oxygen species generation and cellular antioxidant status were determined for evaluating oxidative stress. For analyzing the effect of neferine on $CoCl_2$-induced apoptosis, propidium iodide staining was performed.

Results: The results of the present study indicate that $CoCl_2$ induces cell death in a dose-dependent manner. Neferine pretreatment at 700 nM concentration offers better cytoprotection in the cells exposed to $CoCl_2$. Lactate dehydrogenase and NO release in the culture medium were restored after neferine pretreatment. $CoCl_2$ triggers time-dependent reactive oxygen species generation in muscle cells. Further, results of propidium iodide staining, mitochondrial membrane potential, and intracellular calcium accumulation confirm that neferine offers protection against $CoCl_2$-induced hypoxic injury. Depleted activities of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and

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glutathione S-transferase due to $CoCl_2$ exposure were also reinstated in the group that received neferine pretreatment.

Conclusion: Our study suggests that neferine from *N. nucifera* offers protection to muscle cells by counteracting the oxidative stress induced by $CoCl_2$.

1. Introduction

Cobalt chloride ($CoCl_2$) has widely been used to mimic hypoxia in cell culture, and it is known to activate hypoxic signaling. Hypoxia is a pathophysiological condition characterized by an increase in reactive oxygen species (ROS) and a change in intracellular redox level. Oxygen flux in the mitochondria of skeletal muscle increases up to 100-fold at high altitude and during physical exercise, which in turn results in an increased generation of free radicals. Physical exercise results in oxidative stress, which is associated with increases in lipid peroxidation (LPO) and protein oxidation.[1] High-altitude physiology may be divided into studies of short-term changes that occur with exposure to hypobaric hypoxia (the acute response to hypoxia) and studies of longer-term acclimatization and adaptation. Acute exposure to the ambient atmosphere at extreme altitude (for example, above 8000 m) is rapidly fatal.[2] Acclimatization is the set of beneficial processes whereby lowland humans respond to a reduced inspired partial pressure of oxygen. These changes tend to reduce the gradient of oxygen partial pressure from ambient air to tissues (classical oxygen cascade), and are distinct from the pathological changes that lead to altitude illness. Adaptation to high altitude describes changes that have occurred over a number of generations as a result of natural selection in a hypobaric hypoxic environment, and this can be observed in some groups of high-altitude residents.

Cellular response to hypoxia may provide important clues about impaired cellular function and neuronal cell death. ROS have been proposed to act as second messengers in redox-sensitive signal transduction pathways and can damage biomolecules.[3] Reactive oxygen intermediates, superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH), are produced mainly in mitochondria. ROS act as physiological modulators of some mitochondrial functions, but may also damage mitochondria. Oxygen-derived radicals are implicated in LPO events and are critical in injuries after ischemia.[4,5]

Physical exercise leads to temporary ischemia in muscles. During strenuous exercise, muscle oxygen consumption increases tremendously to as high as 100–200 times that in normal resting conditions.[6] The sudden increase and influx of oxygen cause a calcium overload in cells, leading to an influx of inflammatory cells into reperfused tissue. This will lead to the generation of ROS that are considered responsible for muscle fatigue during exercise.[7] Hence, oxidative stress plays a significant role in the initiation and progression of fatigue. Oxidative stress, more specifically LPO, is known to play an important role in the pathophysiology of exercise intolerance at high altitude. Exposure to high altitude appears to decrease the activity and effectiveness of the antioxidant enzyme system. Therefore, supplementation of antioxidants seems to be a necessary step to prevent or decrease the high-altitude-exposure-associated oxidative stress.[8]

Traditional knowledge reveals many medicinal uses of lotus plant. Neferine is a bisbenzylisoquinoline alkaloid extracted (isolated) from the green seed embryo of *Nelumbo nucifera* Gaertn (lotus), which has a wide range of pharmacological activities. Lotus seeds are in high demand in Ayurvedic medicinal preparations, and are widely used in folk medicines to treat tissue inflammation, cancer, diuretics,[9] and skin diseases and as an antidote to poison.[10] Embryos of lotus seeds are used in a traditional Chinese drug, called "LianZiXin," which is used in the treatment of nervous disorders, insomnia, high fevers (with restlessness), and cardiovascular diseases (e.g., hypertension and arrhythmia).[11] The major phytochemicals present in lotus seeds are alkaloids (e.g., dauricine, lotusine, nuciferine, pronuciferine, liensinine, isoliensinine, roemerine, nelumbine, and neferine).[12,13] Neferine serves as a free radical scavenger, enhancing superoxide dismutase (SOD) activity and inhibiting LPO due to the hydroxyl group present in its structure.[14] Recently, anticancer potential of neferine in HepG2 and A549 cells, and cardioprotective effect against isoproterenol-induced myocardial ischemia have been reported from our laboratory.[15–17]

2. Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium, fetal bovine serum, and all other cell culture reagents were obtained from Hi-Media Laboratories, Mumbai, India. Neferine, $CoCl_2$, 3,3′-dihexyloxacarbocyanine iodide (DiOC6), carbonylcyanide-m-chlorophenylhydrazone (CCCP), 2′,7′-dichlorofluorescein diacetate (DCF-DA), and Fura 2/AM were obtained from Sigma-Aldrich (St Louis, MO, USA). Reagents for enzyme assays were obtained from Merck Specialities Chemicals, Mumbai, India.

2.2. Cell culture

Rhabdomyosarcoma (RD) cells were obtained from NCCS (Pune, India) and grown to confluence in 25 cm^2 flasks with Dulbecco's modified Eagle's medium and 10% fetal bovine serum (v/v), containing 100 units/mL penicillin, 30 μg/mL streptomycin, and 20 μg/mL gentamycin in a CO_2 incubator with 5% CO_2. Cells at 80% confluence were used for all the assays. All assays were carried out within 20 passages, between Passage No. 40 and Passage No. 60, to ensure uniformity of cell population and reproducibility.

2.3. Cell proliferation assay

Cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, because reduction of tetrazolium salts is widely accepted as a reliable method to examine cell viability/proliferation.[18] The cells were seeded at a density of 1×10^4 cells per well, in 200 μL Dulbecco's modified Eagle's medium, and were allowed to attach overnight in a CO_2 incubator. Neferine dissolved in 50% (v/v) ethanol (final solvent concentration not exceeding 0.1%) was added to the cells. After different exposure times, 20 μL of MTT (5 mg/mL) was added and incubated at 37 °C for 4 hours after aspirating the medium with neferine and $CoCl_2$. The purple formazan crystals formed were dissolved in 200 μL dimethyl sulfoxide, and the absorbance was read at 570 nm in a microquant plate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.4. Lactate dehydrogenase release and NO assay

Cytotoxicity induced by $CoCl_2$ was evaluated by measuring lactate dehydrogenase (LDH) and NO release by the cells. LDH leakage is a measure of membrane integrity. Nitrite is the stable oxidation product of NO produced by iNOS. The cells were seeded in triplicate in a 24-well plate at a density of 1×10^5 cells/well and were allowed to attach overnight in aCO_2 incubator at 37 °C. Cells were treated with appropriate concentrations of neferine and $CoCl_2$ for 24 hours and 48 hours, respectively; the cells were harvested and the supernatant (spent media) was used for the assay.

LDH activity was determined from the linear region of a pyruvate standard graph using regression analysis and expressed as % leakage, as described previously.[19] The amount of LDH released was expressed as a percentage. NO assay was carried out by the measurement of nitrite using Greiss reagent in the spent medium.[20] The amount of nitrite was expressed as nmoles of nitrite released.

2.5. Estimation of ROS

Relative changes in intracellular ROS in RD cells were monitored using a fluorescent probe, 2′,7′-dichlorofluoresceindiacetate (DCF-DA). The treatment groups were maintained as described for LDH leakage, and the time course for ROS generation by $CoCl_2$ was estimated. Cells were incubated with 10 μM of DCF-DA and incubated for 30 minutes, followed by incubation with $CoCl_2$ for different time periods (15 minutes, 30 minutes, and 1 hour). One treatment group with 50 μM H_2O_2 was included to serve as a positive control. In the neferine pretreatment samples, the cells were treated with 700 nM neferine for 24 hours and then exposed to DCF-DA and $CoCl_2$ for 30 minutes each. The cells were then harvested, centrifuged, washed, and resuspended in phosphate-buffered saline (PBS), and read in a Hitachi spectrofluorimeter (excitation 480 nm, emission 520 nm). The estimations were carried out thrice in triplicate, using the same number of cells per treatment group to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.

2.6. Lipid peroxidation

Malonaldehyde, an indicator of LPO, which is formed during the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. LPO content in different treatment groups was estimated according to Ohkawa et al.[21] All experiments were carried out thrice in triplicate to ensure reproducibility, and the LPO content was expressed as nmoles of Thiobarbituric acid (TBA) reactants.

2.7. Cellular antioxidant status

After completing the treatment schedule, cell extracts were prepared by sonication in 50 mM Tris, 5 mM EDTA, 10 μg/mL phenyl methyl sulfonyl fluoride, pH 7.6. The cell debris was removed by centrifugation at 1800 g for 5 minutes at 4 °C, and the protein content of the supernatant was determined by the Lowry method and used for antioxidant assays. Both reagent blanks and enzyme blanks were measured for all assays. All assays were carried out in the linear range and expressed as specific activities of enzymes.

The intracellular concentration of reduced glutathione (GSH) was estimated by the fluorimetric assay, as described by Hissin and Hilf.[22] Cells from the different treatment groups were treated with 25% ortho-phosphoric acid followed by sonication. The values obtained were calculated based on the linear region of a GSH standard graph using regression analysis. All experiments were carried out thrice in triplicate to ensure reproducibility, and the amount of GSH was expressed as nmoles of GSH/mg protein.

SOD activity was measured following the procedure described previously by Marklund and Marklund,[23] and expressed as the amount of enzyme required for 50% inhibition of pyrogallol auto-oxidation. Catalase was determined following the method described by Aebi.[24] Catalase activity in the samples was calculated based on the extinction coefficient of hydrogen peroxide and expressed as specific activity. Glutathione S-transferase (GST) was determined by the method of Habig et al.[25] The enzyme activity was calculated based on the extinction coefficient of Glutathione-1-chloro-2,4-dinitrobenzene (GS-CDNB), and expressed as specific activity. The activity of glutathione peroxidase (GPx) was determined following the method described previously by Rotruck et al.[26] The amount of GSH consumed was calculated from a standard graph, using linear regression. The enzyme activity was expressed as units/mg protein.

2.8. Protein carbonyl content

Protein carbonyl content is a cellular marker of oxidative stress. The cells were harvested and washed with PBS, and the levels of protein carbonyl content were estimated by the method previously described by Levine et al.[27]

2.9. Intracellular calcium measurement

RD cells at a density of 1×10^5 cells/well in a 24-well plate were treated with neferine and $CoCl_2$ for 24 hours and 48 hours, respectively. After treatment, the cells were washed twice

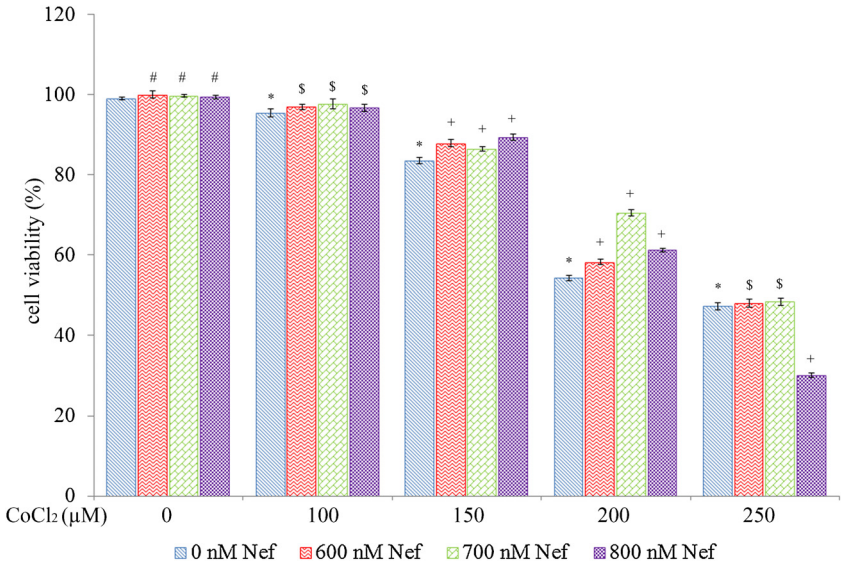

Fig. 1 – Protective effect of neferine on $CoCl_2$-induced cytotoxicity assessed by MTT assay. RD cells were treated with $CoCl_2$ (0 μM, 100 μM, 150 μM, 200 μM, and 250 μM) for 48 hours and 24 hours in the presence and absence of neferine pretreatment (600 nM, 700 nM, and 800 nM), respectively. Cell viability was assessed by MTT assay. Results shown are mean ± SEM of three separate experiments performed in triplicate.
 # $p > 0.05$, not significant compared to control.
*** $p < 0.01$, compared to control.**
+ $p > 0.05$ not significant compared to the $CoCl_2$-treated group (one-way ANOVA followed by Tukey's multiple comparison).
$ $p < 0.01$, compared to the $CoCl_2$-treated group.
ANOVA, analysis of variance; $CoCl_2$, cobalt chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nef, neferine; RD, rhabdomyosarcoma; SEM, standard error of the mean.

with buffer A (5 mM KCl, 2 mM $CaCl_2$, 0.5 mM KH_2PO_4, 137 mM NaCl, 4 mM $NaHCO_3$, and 0.2 mM Na_2HPO_4). The cells were loaded with 4 μM of Fura 2/AM in buffer A for 45 minutes. Fluorescence emission (500 nm) was monitored, with the excitation wavelength cycling between 340 nm and 380 nm, using a fluorescence spectrophotometer. The ratio of intensities at 340 nm and 380 nm excitation (F340/F380) was used as an index of $[Ca^{2+}]i$.

2.10. *Measurement of mitochondrial transmembrane potential (ΔΨM)*

Mitochondrial energization was determined as the retention of the dye DiOC6 in normal cells and its exclusion by cells with mitochondrial membrane damage. Cells were treated with neferine as described in the LDH leakage experiment. After the treatment period, the medium was flicked off and the cells were washed twice with PBS. Then the cells were treated with 50 nM DiOC6 at 37 °C for 30 minutes and washed twice with PBS. The cells were collected and resuspended in 2 mL PBS, and fluorescence intensity was measured in a fluorescent spectrophotometer using excitation/emission as 488 nm/500 nm. CCCP (50 μM) was used as a positive control for maximum Δψ M disruption. CCCP was incubated with cells for 15 minutes prior to the addition of DiOC6.

2.11. *Apoptotic cell detection by propidium iodide*

After treatment, the cells were collected, washed with PBS, fixed overnight with 70% ethanol at 4 °C, and incubated with propidium iodide at room temperature for 30 minutes; nuclear morphology was examined using a fluorescent microscope.

2.12. *Statistical analysis*

All experiments were performed three times in triplicates. Results were expressed as mean ± the standard error of the mean. Multiple comparisons were done using one-way analysis of variance followed by Tukey's multiple comparison test, using SPSS 16 software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

In RD cells, different concentrations of $CoCl_2$ and neferine were tested, and the effective dosage was fixed by calculating the cell viability using MTT assay. $CoCl_2$ induces cell death in RD cells in time- and dose-dependent manner. From this, IC_{50} of $CoCl_2$ for 48 hours was found to be 200 μM, and pretreatment with neferine for 24 hours at 700 nM provides better protection (Fig. 1). All experiments were carried out using this dosage and time point.

Light microscopic images revealed the morphological changes in the $CoCl_2$-treated cells. The control and neferine groups showed normal morphological characters of RD cells, while morphological changes such as cell shrinkage, rounding up, and detachment of the cells from the surface were

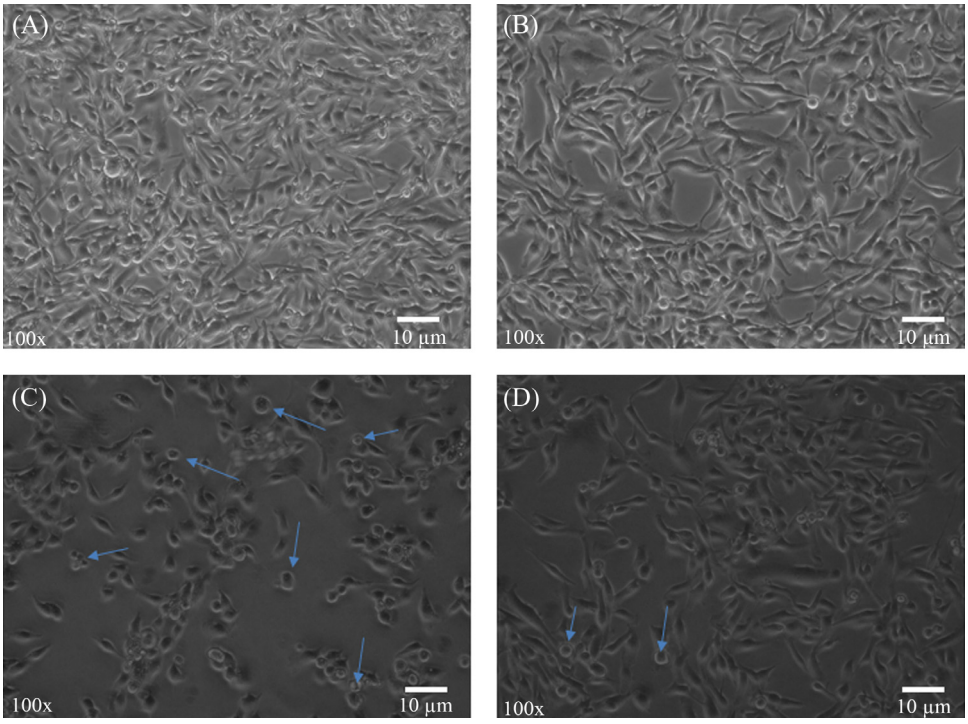

Fig. 2 – Effect of neferine on $CoCl_2$-induced morphological changes. Results shown are representative images of three separate experiments carried out in the following groups: (A) control, (B) neferine, (C) $CoCl_2$, and (D) neferine + $CoCl_2$ (100×). $CoCl_2$, cobalt chloride.

observed in the $CoCl_2$-treated group. These morphological changes were reduced in the group that received neferine pretreatment (Fig. 2).

Nitrite is the stable oxidation product of NO produced by iNOS. The effect of $CoCl_2$-induced hypoxia on nitrite production was determined by NO assay. Treatment of RD cells with $CoCl_2$ resulted in a statistically significant increase in nitrite production when compared to the control. Cells that received neferine treatment, followed by $CoCl_2$, showed lower nitrite production in a statistically significant manner compared to control (Fig. 3A). LDH leakage was a measure of membrane integrity. $CoCl_2$ treatment of cells resulted in a statistically significant increase in LDH leakage compared to the control. Whereas pretreatment with neferine resulted in a significant increase in the LDH level compared to the $CoCl_2$-treated group. Pretreatment with neferine did not show a statistically significant difference from the control (Fig. 3B). $CoCl_2$ treatment resulted in significantly elevated levels of protein carbonyl content compared to the control, which may be attributed to oxidative stress. Pretreatment of cells with neferine resulted in a significant decrease in protein carbonyl content levels compared to $CoCl_2$-treated cells. Cells treated with neferine alone did not show significant changes (Fig. 3C).

ROS are generated during mitochondrial respiration. We tested whether ROS generation contributed to the effect of $CoCl_2$-induced hypoxia on RD cell death. $CoCl_2$ induced ROS generation in a time-dependent manner. In $CoCl_2$-treated cells, a significant increase in ROS generation in muscle cells was observed at 15 minutes, which reached a maximum at 30 minutes. RD cells pretreated with neferine showed diminished ROS generation when exposed to $CoCl_2$ (Fig. 4).

The role of antioxidants in cytotoxicity induced by $CoCl_2$ was also investigated. Reduced GSH is a major antioxidant and protects cells from oxidative stress by scavenging peroxides in the cytosol and mitochondria; hence, measurement of the levels of GSH helps in finding the severity of oxidative damage induced by $CoCl_2$. There was considerable reduction in the levels of GSH in the group exposed to $CoCl_2$ compared with the control group. In neferine-pretreated group, the GSH level was significantly increased compared to $CoCl_2$-treated cells (Fig. 5A). LPO was one of the main manifestations of oxidative damage in cells. Lipid peroxide content measured as Thiobarbituric Acid Reactive Substances (TBARS) in RD cells was significantly increased in the $CoCl_2$-treated cells. Cells that received neferine pretreatment showed significantly reduced LPO content compared with the $CoCl_2$-treated cells (Fig. 5B).

In order to evaluate the effect of $CoCl_2$ and neferine on the activities of antioxidant enzymes, catalase, SOD, GPx, and GST assays were carried out in the various treatment groups. Catalase, SOD, and GPx are enzymes primarily involved in combating cellular oxidative stress; in cells subjected to $CoCl_2$ treatment, all the antioxidant enzyme activities were significantly decreased. Pretreatment with neferine significantly increased activities of catalase, SOD, GPx, and GST (Table 1).

$CoCl_2$-induced apoptosis was determined by propidium iodide staining. Propidium iodide staining revealed chromatin

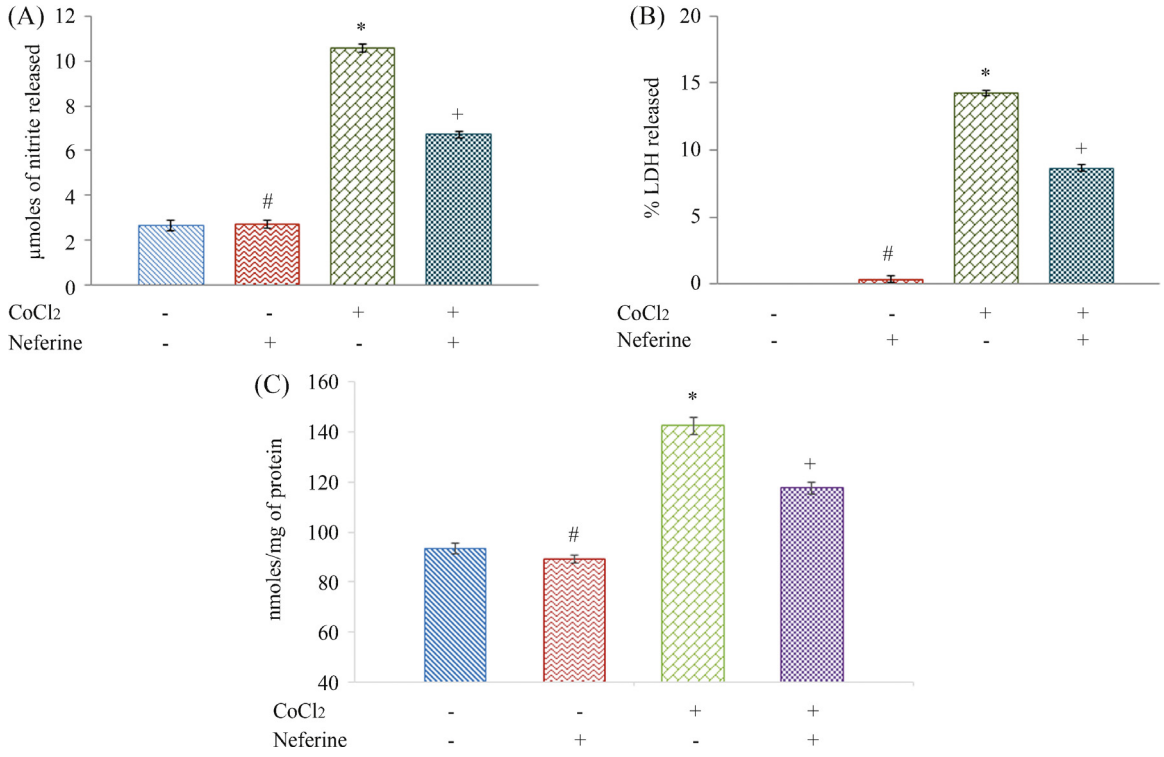

Fig. 3 – Effects of neferine on nitrite release, LDH leakage, and protein carbonyl content. (A) Nitrite release was measured using Griess reagent. RD cells were treated with neferine for 24 hours and $CoCl_2$ for 48 hours for neferine and $CoCl_2$-alone groups, respectively; for pretreatment with neferine, the cells were treated with neferine for 24 hours followed by $CoCl_2$ exposure for 48 hours. (B) LDH leakage was measured to assess membrane integrity. Results are expressed as % LDH release. (C) Protein carbonyl content was determined using dinitrophenyl hydrazine (DNPH). Results shown are mean ± SEM of three separate experiments performed in triplicate.

 # $p > 0.05$, not significant compared to control (one-way ANOVA followed by Tukey's multiple comparison).

*** $p < 0.01$, compared to control.**

+ $p < 0.01$, compared to the only $CoCl_2$-treated group.

ANOVA, analysis of variance; $CoCl_2$, cobalt chloride; LDH, lactate dehydrogenase; RD, rhabdomyosarcoma; SEM, standard error of the mean.

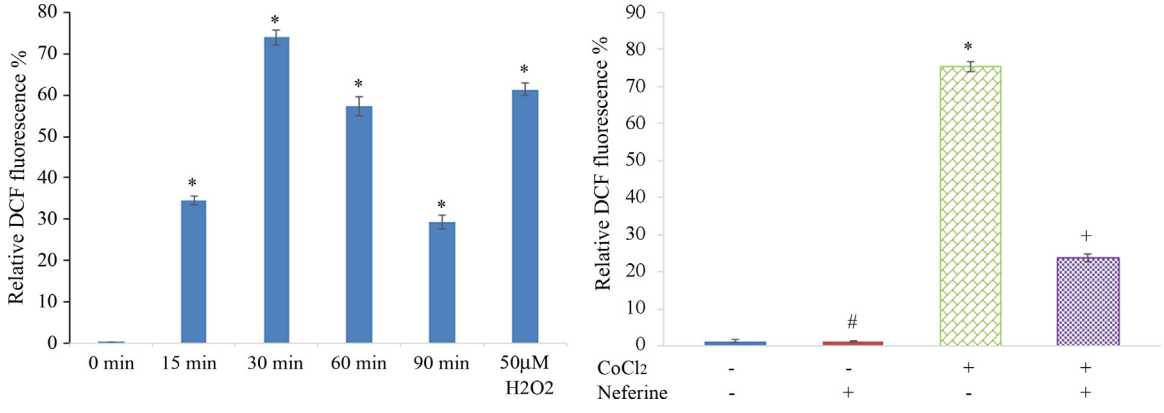

Fig. 4 – $CoCl_2$-induced ROS generation in a time-dependent manner. ROS production was assessed by the spectrofluorimetric method using 2,7-dichlorodihydrofluorescein diacetate (DCF-DA). Results shown are mean ± SEM of three separate experiments performed in triplicate.

*** $p < 0.01$, compared to control.**

 # $p > 0.05$, not significant compared to control (one-way ANOVA followed by Tukey's multiple comparison).

+ $p < 0.01$, compared to the only $CoCl_2$-treated group.

ANOVA, analysis of variance; $CoCl_2$, cobalt chloride; ROS, reactive oxygen species; SEM, standard error of the mean.

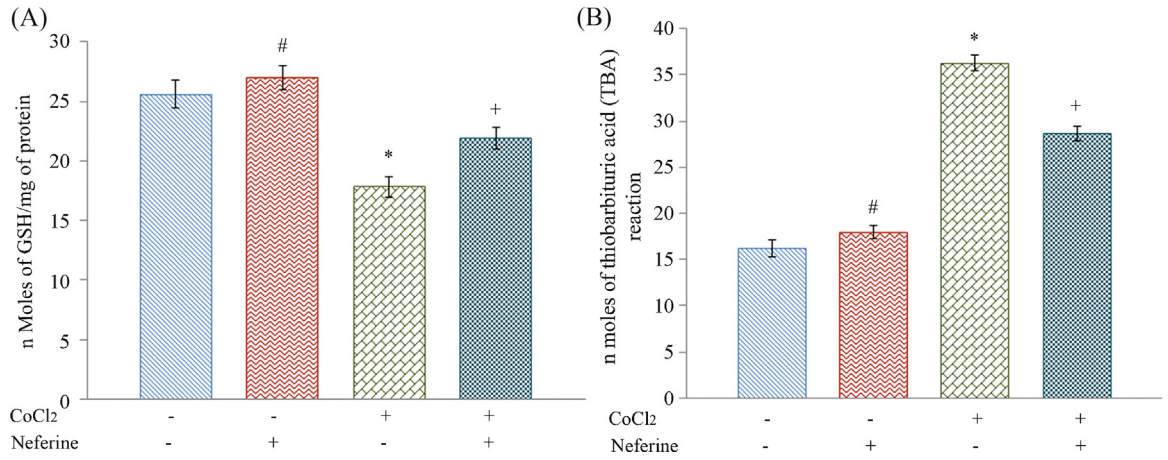

Fig. 5 – Effects of neferine on Glutathione and lipid peroxidation. (A) GSH levels were estimated using ortho-phthalaldehyde (OPT) spectrofluorimetrically. (B) A significant increase in lipid peroxidation, measured as thiobarbituric acid reactive substance (TBARS) formation, was observed in the $CoCl_2$-treated group, which was reduced by neferine pretreatment. Results shown are mean ± SEM of three separate experiments performed in triplicate.
 # $p > 0.05$, not significant compared to control (one-way ANOVA followed by Tukey's multiple comparison).
*** $p < 0.01$, compared to control.**
+ $p < 0.01$, compared to the only $CoCl_2$-treated group.
ANOVA, analysis of variance; $CoCl_2$, cobalt chloride; GSH, reduced glutathione; SEM, standard error of the mean.

condensation and formation of apoptotic bodies in $CoCl_2$-treated cells, confirming the apoptosis event. Whereas cells pretreated with neferine showed apparently normal architecture with no sign of apoptosis (Fig. 6).

Intracellular calcium was measured using Fura 2/AM and expressed as relative percentage. Intracellular calcium accumulation was higher in those cells that received $CoCl_2$ treatment. By contrast, neferine-pretreated cells showed significantly decreased calcium accumulation compared to $CoCl_2$-treated cells (Fig. 7A). When RD cells were exposed to $CoCl_2$, the $\Delta\Psi M$ was dissipated rapidly, as revealed by decreases in the fluorescence measured using a fluorescent spectrophotometer. Whereas in the neferine-pretreated cells, the $\Delta\Psi M$ was significantly higher compared to that in the $CoCl_2$-treated cells (Fig. 7B).

4. Discussion

Cobalt chloride mimics hypoxic/ischemic conditions, including ROS production, in muscle cells at high altitude. $CoCl_2$-induced cell death in RD cells may serve as a simple and convenient *in vitro* model of hypoxia-induced cytotoxicity in muscle cells. It is a well-known fact that hypoxia leads to the generation of reactive oxygen and nitrogen species, which leads to oxidative stress resulting in the oxidation of biological molecules.[28] Substantial weight loss, in particular, a considerable reduction in muscle mass, was one of the remarkable features of chronic exposure to high altitude.[29] Radák et al[30] revealed that physical training at high altitude increases the reactive carbonyl derivatives in amino acids of skeletal muscle.

Table 1 – Effect of neferine on $CoCl_2$-induced alterations in antioxidant system

Parameters	Group I	Group II	Group III	Group IV
SOD (U/mg of protein)	6937.3 ± 14.32	6893.6 ± 31.35*	5548.832 ± 23.17†	6137.3 ± 16.95‡
CAT (U/mg of protein)	298.23 ± 5.36	293.72 ± 2.31*	159.31 ± 8.81†	256.65 ± 6.41‡
GST (U/mg of protein)	42.291 ± 2.35	41.872 ± 4.81*	24.791 ± 3.76†	30.382 ± 3.29‡
GPx (U/mg of protein)	397.058 ± 8.82	391.176 ± 10.58*	296.361 ± 6.97†	358.207 ± 8.46‡

Results shown are mean ± SEM of three separate experiments performed in triplicate.
* $p > 0.05$ compared to control (one-way ANOVA followed by Tukey's multiple comparison).
† $p < 0.01$, compared to control.
‡ $p < 0.01$, compared to the only $CoCl_2$-treated group.
ANOVA, analysis of variance; CAT 1 U, the amount of catalase enzyme that consumes 1 nmole H_2O_2/min; $CoCl_2$, cobalt chloride; GPx 1 U, the amount of glutathione peroxidase enzyme that converts 1 μmole glutathione to GSSG in the presence of H_2O_2/min; Group I, control group; Group II, neferine group; Group III, $CoCl_2$ group; Group IV, neferine + $CoCl_2$ group; GST 1 U, the amount of glutathione S-transferase enzyme that conjugates 1 μmole 1-chloro-2,4-dinitrobenzene (CDNB)/min; NS, not significant; SEM, standard error of the mean; SOD 1 U, the amount of superoxide dismutase enzyme required for 50% inhibition of pyrogallol auto-oxidation.

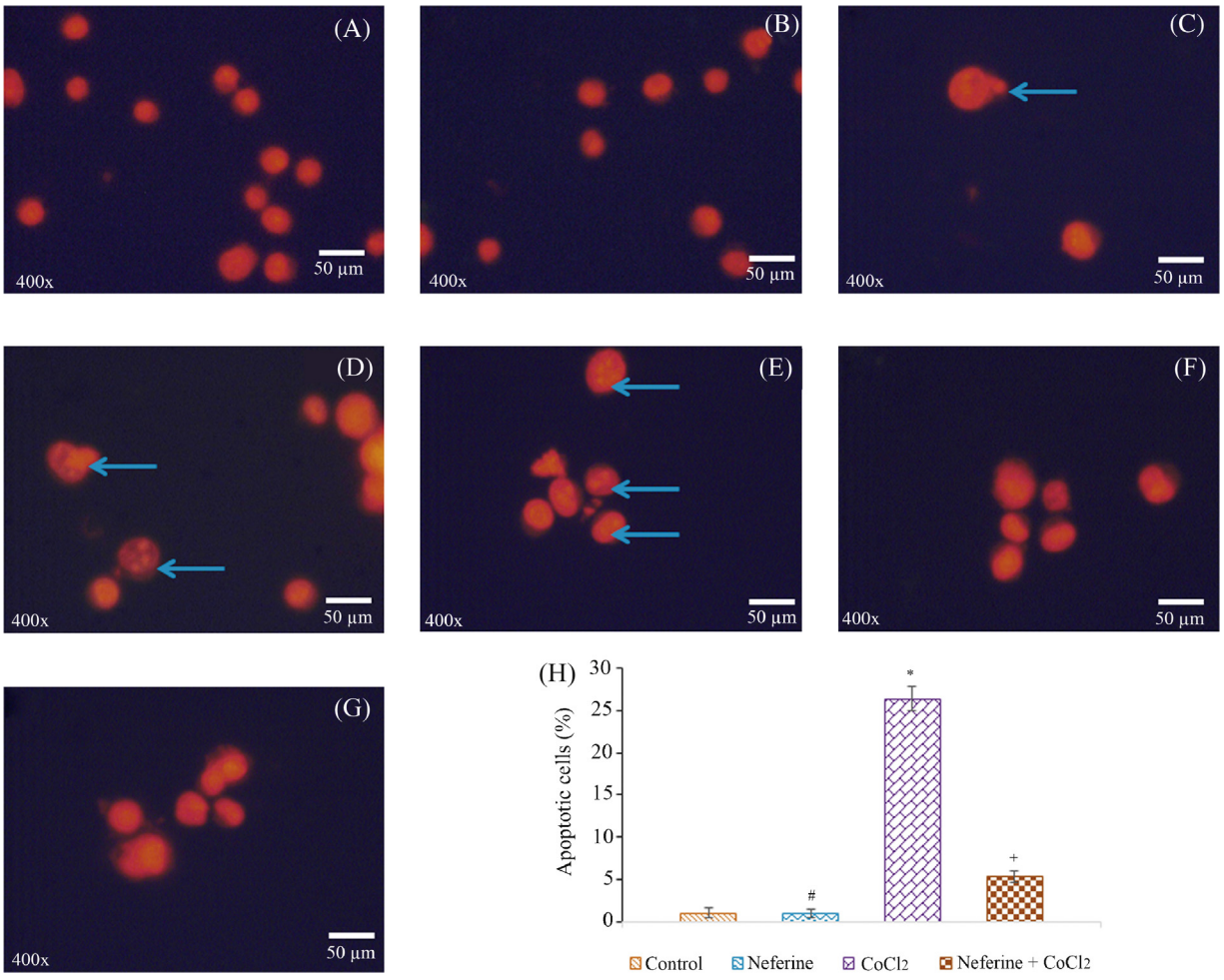

Fig. 6 – Protective effect of neferine on the formation of hypoxia-induced apoptotic bodies in muscle cells. PI fluorescent staining was used. Results shown are representative images of three separate experiments for (A) control, (B) neferine, (C–E) $CoCl_2$, (F,G) neferine + $CoCl_2$ (400×), and (H) percentage of apoptotic cells. A total of 200 cells per treatment group were screened and analyzed for the PI staining.

 # $p > 0.05$, not significant compared to control (one-way ANOVA followed by Tukey's multiple comparison).

*** $p < 0.01$, compared to control.**

+ $p < 0.01$, compared to the $CoCl_2$-treated group.

ANOVA, analysis of variance; $CoCl_2$, cobalt chloride; PI, propidium iodide.

From the MTT result it was evident that $CoCl_2$ induces cell death in a dose- and time-dependent manner. The concentration at which neferine (700 nM) offers better protection was found to be very low. To evaluate the $CoCl_2$-induced hypoxic cell death, we performed cytotoxicity assays such as LDH and NO assays. Measurement of LDH enzyme activity serves as a cellular injury marker in the biological system; hence, measurement of LDH enzyme activity may serve as an indicator of the cell injury during hypoxia. Significantly increased LDH release in hypoxia-exposed hepatocytes was previously observed by Frank et al.[31] Neferine with a strong antioxidative property protects cells against the cellular injury triggered by $CoCl_2$, which is evident from a significant decrease in the LDH in the medium of neferine-pretreated cells. Biomolecules such as DNA and protein are damaged by the NO formed by iNOS; hence, measurement of NO content in the culture medium is an indirect method of measuring the expression of iNOS, which serves as an indicator of the pathological and stress conditions. $CoCl_2$ induced a significant increase in NO production in the muscle cells (RD). NO levels in the neferine-pretreated cells were found to be significantly decreased compared to that in cells treated with $CoCl_2$ alone. Recently, it has been reported by Lalitha et al[15] that neferine possesses strong antioxidant property and maintains the membrane integrity of cardiac cells in isoproterenol-treated rats. Wang et al[13] have reported that neferine restores serum marker enzyme levels to normal in ischemic liver. Our results are in line with the above reports.

Increased intracellular ROS have been reported to contribute to $CoCl_2$-induced cell death.[32] ROS also induce apoptotic cell death associated with the loss of mitochondrial membrane potential.[33] Oxygen free radicals are highly reactive species that promote damage to lipids, DNA, carbohydrates, and proteins, and induce production of several

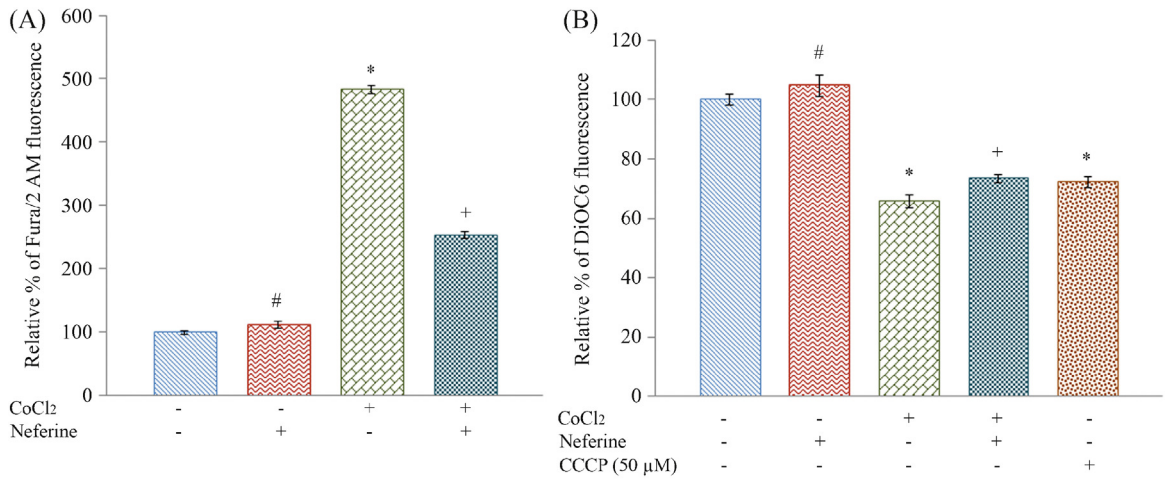

Fig. 7 – Effect of neferine on intracellular calcium accumulation and mitochondrial membrane potential on $CoCl_2$-treated cells. Results shown are mean ± SEM of three separate experiments performed in triplicate. (A) CoCl2 induced intracellular calcium accumulation in RD cells was estimated using Fura-2/AM. Results were expressed as relative % of Fura-2AM fluorescence. (B) Cells were treated with neferine (500 nM) followed by CoCl2. 50 μM CCCP was used as positive control. Results were expressed as relative % of DiOC6 fluorescence.

 # $p > 0.05$, not significant compared to control (one-way ANOVA followed by Tukey's multiple comparison).

*** $p < 0.01$, compared to control.**

+ $p < 0.01$, compared to the only $CoCl_2$-treated group.

ANOVA, analysis of variance; CCCP, carbonylcyanide-m-chlorophenylhydrazone; $CoCl_2$, cobalt chloride; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; SEM, standard error of the mean.

immune/inflammatory proteins, leading to cell death.[34] It has also been shown that antioxidants can scavenge ROS and protects cells from injury.[35] In our study, neferine with its antioxidant activity could effectively scavenge the ROS generated during $CoCl_2$ treatment.

Pathogenesis of hypoxic damage was reported to be initiated by LPO, protein oxidation, and irreversible protein modification, ultimately leading to cell membrane damage.[36] Reports of Sevanian and Hochstein[37] demonstrated that LPO was initiated by free radicals, causing alteration of membrane integrity, fluidity, and permeability. Oxidative damage to DNA and lipids in muscle tissue was reported in many patients with chronic fatigue syndrome.[38,39] Results of the present study showed significantly increased levels of TBA reactive substances in the $CoCl_2$-treated group compared to that in the control group. Generation of free radicals and activation of LPO could result in irreversible membrane damage in muscle cells. GSH is the main nonenzymatic antioxidant that protects cells from oxidative injury, by preventing the accumulation of oxygen radicals in the cells.[40] Neferine, due to its strong radical scavenging activity, restored the cellular GSH content in the $CoCl_2$-treated RD cells. A previous study by Joanny et al[41] showed that ascorbic acid restored the increased TBARS content and decreased GSH levels in the blood of the individuals subjected to the hypoxic condition, which proves that antioxidant intake will reduce hypoxia-mediated oxidative damage.

The cellular antioxidant defense system plays a role in protecting cells from ROS generated during metabolic process. Aerobic cells develop enzymatic and nonenzymatic antioxidant systems to regulate the effects of reactive oxygen and nitrogen species. The enzymatic system contains mitochondrial, cytosolic, and extracellular SOD to convert reactive superoxide to less powerful hydrogen peroxide. GPx and catalase decompose hydrogen peroxide to water.[8] The $CoCl_2$-treated cells were subjected to a continuous flux of superoxide anions and hydrogen peroxide, resulting in oxidative stress in the cells. Exposure to extreme conditions such as hypoxia results in a decline in the activities of cellular antioxidant enzymes such as SOD, catalase, GPx, and GST, and antioxidant supplementation will help in improving the cellular antioxidant status.[42] Lalitha et al[15] reported that neferine offered cardioprotection against isoproterenol-induced oxidative stress due to the strong antioxidant nature of neferine. In the present study, pretreatment with neferine improved the cellular antioxidant status in $CoCl_2$-treated cells, which matches with the earlier findings.

Membrane damage and subsequent mitochondrial dysfunction by ROS can lead to modifications in mitochondrial DNA.[38] Maintenance of the $\Delta\psi M$ is fundamental to the normal performance and survival of cells that have a high energy requirement. Pathological conditions, such as oxidative stress, adenosine triphosphate depletion, Ca^{2+} overload of mitochondrial matrix, and some physiological processes, can trigger the opening of a nonspecific permeability transition pore in the mitochondrial membrane.[43] Intracellular accumulation of calcium during hypoxia has been reported by Vecchiet et al[39] earlier. In the present study, an increase in intracellular calcium accumulation and a decrease in the $\Delta\psi M$ were observed in $CoCl_2$-treated cells, which were prevented in the neferine-pretreated group. From this result it was clear that neferine treatment protects muscle cells from $CoCl_2$-induced mitochondrial membrane damage and calcium accumulation.

The present study reveals that neferine pretreatment ameliorated $CoCl_2$-induced ROS generation, restored cellular antioxidant pool, attenuated mitochondrial transmembrane potential loss, and maintained intracellular calcium levels in muscle cells. These findings support the hypothesis that cytoprotection mediated by neferine is due, in part, to the inhibition of the oxidative stress-mediated cell death.

Conflicts of interest

All authors have no conflicts of interest to declare.

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