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Ameliorative effects of bromelain on cobalt chloride-induced hypoxia in Caco-2 cell line

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***Abstract*: Hypoxia is a frequently encountered condition at high altitude. Hypoxia-inducible factor-1α modulates vascular endothelial growth factor, glucose transporter, angiogenin and fibroblast growth factor in hypoxic condition. The aim of the study was to evaluate the promising benefits of bromelain (BR; 7 µg/ml) against cobalt chloride ($CoCl_2$; 100 mM) induced hypoxia in Caco-2 cell line. The inference drawn from the study was $CoCl_2$-induced hypoxia regulates hypoxia-inducible factor-1α by normalizing oxidative insult, which further leads to changes in cell morphology, DNA damage and apoptosis. BR pre-treated cell line has shown a noteworthy protection against $CoCl_2$-induced cell morphology and apoptotic cell death. The study also confirms that the supplementation of BR could be helpful in regulating appetite at high altitude environments.**

***Keywords:* Hypoxia, Cobalt chloride, HIF-1α, Bromelain.**

1. INTRODUCTION

Bromelain (BR) is one of the active component present in the *Ananas comosus*, which belongs to sulfhydryl protease and is used medically to aid digestion.BR also comprises endopeptidases, peroxidase, acid phosphatase, glycoproteins, carbohydrates and organically bound calcium, it is also commercially available as a nutritional supplement and it has been shown to be effective in promoting digestion, wound healing, fibrinolytic, anti-thrombotic and as an anti-inflammatory agent [1]. It remains stable at pH range 2 to 9, which indicates the stability of bromelain in acidic conditions of the stomach [2].

Hypoxia is the major pathophysiological feature of various diseases, such as ischaemia, cancer and inflammatory bowel syndrome (IBD) and it happens when oxygen supply fails to fulfil regular physiological requirements [3]. Hypoxia influences the cell to adapt to low oxygen concentrations via several transcriptional factors which are important in the apoptotic pathway viz., hypoxia-inducible factor-1α (HIF-1α), tumor protein p53 (p53) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). Recent reports have demonstrated that HIF-1α has a major role in high altitude hypoxia or chemically induced hypoxia. HIF-1α mediates adaptive responses, including angiogenesis, metastasis, glucose metabolism, cell growth and apoptosis [4]. Prior studies suggested that HIF-1α plays a vital role in paracellular barrier functions which includes epithelial barrier of the intestine. The epithelium of intestine is imperative in absorption of fluids, ions and other nutrients in the lumen [5]. Besides HIF-1α has the function to trans-activate various factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiogenin (ANG) and glucose transporter 1 (GLUT 1).

Cobalt chloride ($CoCl_2$) is a mimetic agent for hypoxia, which is commonly used in cell line studies to induce hypoxia [6]. Keeping above facts into consideration, the study was designed to assess the possible effect of BR against $CoCl_2$-induced hypoxia in human epithelial colorectal adenocarcinoma cell line (Caco-2 cells). Cell morphology and viability, mitochondrial membrane potential (MMP), anti-oxidant enzymes, gut hormones, HIF-1α and other relevant genes were assessed to know the benefit effect of BR pre-treatment on $CoCl_2$-induced hypoxia.

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2. METHODOLOGY

2.1. Cell viability studies: MTT and LDH leakage

Caco-2 cell line was procured from NCCS (National Centre for Cell Sciences), Pune, India. 80 % confluent cells were seeded into 96-well plates and incubated in CO_2 incubator (Thermo fisher, India) for one day prior to treatments. MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess cell viability [7]. Lactate dehydrogenase (LDH) leakage was measured in the cell culture medium using plate reader at 450 nm according to manufacturer's instructions (Agappe-11407002, India).

2.2. Mitochondrial membrane potential (MMP) and oxidative stress estimation

Mitochondrial membrane damage induced by $CoCl_2$ and protective effect of BR was determined by means of the rhodamine 123 [7]. Oxidative stress was determined by reactive oxygen species (ROS) production and the release of total nitric oxide levels (NO). ROS was estimated by 2′-7′-dichlorodihydrofluorescein di-acetate (DCFH-DA) assay. The amount of nitrite generated was estimated using griess reagent to estimate the NO levels [8].

2.3. Estimation of antioxidant levels and lipid peroxidation

Briefly, the cells were treated with BR and $CoCl_2$ for 24hr followed by washing with phosphate buffer (1X PBS, ice-cold).Followed by addition of lysis buffer, the lysate was centrifuged at 4500 rpm for 5-10 min and supernatant was collected to assess the antioxidant enzyme assays such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase according to manufacturer's protocol (Randox, Canada). The thiobarbituric acid reactive substance (TBARS) assay is the most widely used test for measuring lipid peroxidation [9]. The data were expressed in nmole/g protein.

2.4. Estimation of digestive hormones

Digestive hormones such as ghrelin (RA194063400R), leptin (SEA084Hu) and cholecystokinin (EIA-4393) were studied using ELISA kit following manufacturer's protocol to know the effect of BR on $CoCl_2$ induced hypoxia in Caco2 cell line.

2.5. Quantitative real-time polymerase chain reaction (qRT- PCR)

RNA (total) extraction was done using TRIzol method (Sigma Aldrich, India) followed by conversion to cDNA using cDNA converting kit (Applied Biosystems, India). The cDNA was subjected to qRT-PCR with the CFX96 Real-Time PCR system (Bio-Rad, India) using SsoFast Eva Green Supermix (Bio-Rad, India) and mean fold change of mRNA was quantified using the $2^{-\Delta\Delta Ct}$ method [10]. The following predesigned primer sets from KiCqStart (Sigma–Aldrich, India) were as follows: HIF-1α: F5'–AAAATCTCATCCAAGAAGCC-3', R5'–AATGTTCCAATTCCTACTGC-3'; VEGF:F5'–AATGTGAATGCAGACCAAAG-3', R5'-GACTTATACCGGGATTTCTTG-3'; GLUT 1:F5'–AAGTCCAGGAGGATATTCAG-3', R5'–CTACAGTGTGGAGATAGGAG-3'; ANG: F5' – TGTAAGATTCTTCCTCCTGG-3', R5'–CTTTCACAGTATCTGTCATCC-3'; FGF: F5' – CTGAGGACAATGTGATGAAG - 3', R5' – CCGTTGCTGGTTTTCTTATAG - 3'.

The expression levels of target genes were normalized to that of β-actin in each sample.

2.6. Western blot analysis

50 µg of the protein samples were loaded onto 10 % SDS-PAGE and subjected to electrophoresis [11]. The SDS-PAGE membranes were probed by incubating with the primary antibodies independently such as GAPDH, HIF-1α, GLUT 1 and VEGF (1:1,000; Santa Cruz Biotechnology, USA), and then membranes were incubated with HRP secondary antibodies conjugated to either goat anti-rabbit IgG or anti-mouse IgG (1:10,000; Sigma-Aldrich, USA). Developed bands were transferred on CL-XPosure film and the band density was analyzed by using NIH Image J software.

2.7. Statistical analysis

It was carried out by using Graph Pad Prism software version 5. Significance was determined by one-way analysis of variance followed by a Tukey's HSD-post hoc test and $p < 0.05$ is considered to be significant.

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3. RESULTS AND DISCUSSION

The present study provides possible effects of BR on chemical induced hypoxia and its protective effects on intestinal cell line. It is evident from recent studies that hypoxia is frequently encountered condition at high altitude. Intestine epithelium is a mucosal organ with complex vasculature and Caco2 is widely used for intestinal studies as it differentiates into monolayers of normal enterocytes under conventional cell culture experiments [12].

In our recent study, we have confirmed that pineapple aids digestion and antioxidant status [13]. In the present study, we are aiming to know the beneficiary mechanism of bromelain against $CoCl_2$-induced hypoxia. Initial experiments were conducted to determine toxicity of $CoCl_2$ or BR on Caco2 cell line. Cells were exposed to increasing concentration of $CoCl_2$ (25-200 mM) and BR (1-10 µg/ml) for 24 hr followed by MTT assay to know the cell viability (Results not shown). Consequently, the treatment of 100 mM of $CoCl_2$ (LD_{50}) and 7 µg/ml of BR were selected for further cell culture experiments. The morphological changes in cells are the primary indicator of cell death and cytotoxicity [14]. The morphological changes such as decreased cell size decreased cell volume, irregular borders and increased intra-cytoplasmic vacuoles when treated with $CoCl_2$. In contrast, pre-treatment with BR synchronized the apoptotic features of $CoCl_2$-induced cytotoxicity via regulated cell morphology and also reversed the nuclear damage (Fig.1).

There was a substantial increase in loss of cell viability in MTT and LDH leakage induced by $CoCl_2$ treatment when compared to control cells ($p < 0.05$). In contrast, the pre-treatment with BR significantly reduced the $CoCl_2$-induced cytotoxicity by increasing the viable cells in both the assays i.e. MTT and LDH leakage (Fig.2a and b). Furthermore, BR pre-treatment regulated MMP (Fig.2c), which is considered as an indicator of mitochondrial damage and one of the early signs of apoptosis .Previous studies have confirmed that BR has a pro-active role in regulating inflammatory markers, such as interleukins (IL-1β, IL-6), tumor necrosis factor and interferon, and resultant reduced cell death [15].

Exposure to high altitude may lead to hypobaric hypoxia resultant oxidative stress. Further it leads to increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) thereby altering redox status of the cell [16]. ROS and RNS generation in excess can lead to changes in molecular level including DNA damage and lipid peroxidation. In the present study, $CoCl_2$-induced hypoxic condition significantly generated free radicals such as ROS and RNS ($p < 0.05$), and led to apoptotic cell death. In contrast, pre-treatment of BR significantly controlled the generation of ROS, RNS ($p < 0.05$; Fig.2 d, e) and lipid peroxidation (TBARS) (Table 1). The resultant decrease in oxidative stress by BR pre-treatment is due to increased levels of antioxidant enzymes such as SOD (1.81 ± 0.02), CAT (1.54 ± 0.14), GPx (0.57 ± 0.02) and GR (13.33 ± 0.05) (Table. 1). Therefore, the study further confirms the shielding effect of BR in $CoCl_2$ induced hypoxia is all the way through reduced oxidative stress.

Expression of various genes has been found to be regulated by hypobaric hypoxic condition, which also plays a crucial role in micro environment of the cell. Angiogenesis is regulated by various factors, molecules and genes which include HIF-1, VEGF, FGF, GLUT-1, platelet-derived growth factors, basic fibroblast growth factor, epidermal growth factor, angiopoetin-1, angiopoetin-2 and matrix metalloproteinase. However, in hypoxic environments, HIF-1 plays a crucial role in the cellular and systemic oxygen homeostasis [17]. Furthermore, HIF-1 induces the transcription of various proteins, such as ANG, FGF, VEGF, GLUT-1 and erythropoietin. Moreover, these proteins/ protein products increases oxygen availability by promoting erythropoiesis and angiogenesis; further these proteins regulate the glycolytic pathway enzymes by blocking the entry of pyruvate ultimately inhibiting mitochondrial oxidative phosphorylation [17]. In the current study, it was observed that the expression of HIF-1α, ANG, FGF, VEGF, GLUT-1 were positively correlated with $CoCl_2$, and negatively correlated by pre-treatment with BR, which demonstrates that positive effect of BR on $CoCl_2$-induced hypoxia (Fig.3).

Appetite regulating hormones such as ghrelin, cholecystokinin and leptin influences the gastro-intestinal tract. These hormones play an important role in controlling hunger and satiation [18]. Other studies are confirming that ghrelin, cholecystokinin and leptin are able to induce the expression of HIF-1α by modulating several factors such as VEGF, ANG and FGF [19][20]. In the present study, cholecystokinin and leptin were significantly increased in $CoCl_2$ induced hypoxic condition when compared to the control cells ($p < 0.05$; Table. 1). In contrast, BR pre-treatment significantly reduced the levels of cholecystokinin and leptin ($p < 0.05$). Therefore, it may be hypothesized that BR pre-treatment will be helpful in regulating digestion.

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3.1 Figures, Graphs and Tables

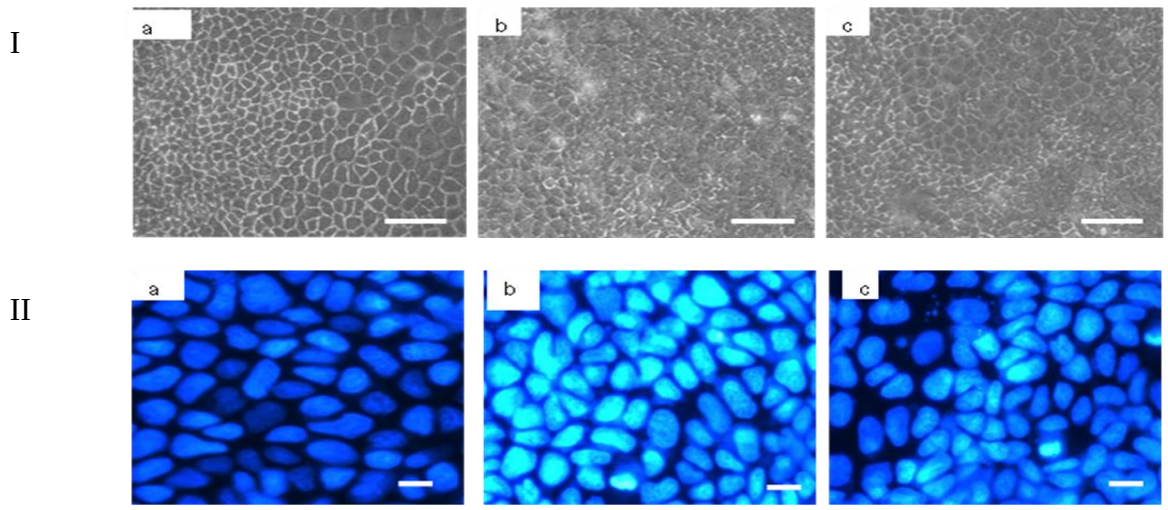

Fig.1 Protective effect of bromelain on $CoCl_2$-induced hypoxia and nuclear damage

I) Phase-contrast microscopy II) Flourescent microscopy after DAPI staining : (**a**) Control Caco-2 cells, (**b**) $CoCl_2$ (100 mM) exposed cells for 24 h, (**c**) Pre-treatment with bromelain (7 µg/ml) followed by Cobalt chloride (100 mM) exposure for 24 h. Scale bar 100 µm. Arrow marks in the image represent cells undergoing apoptosis. Scale bar 100 µm.

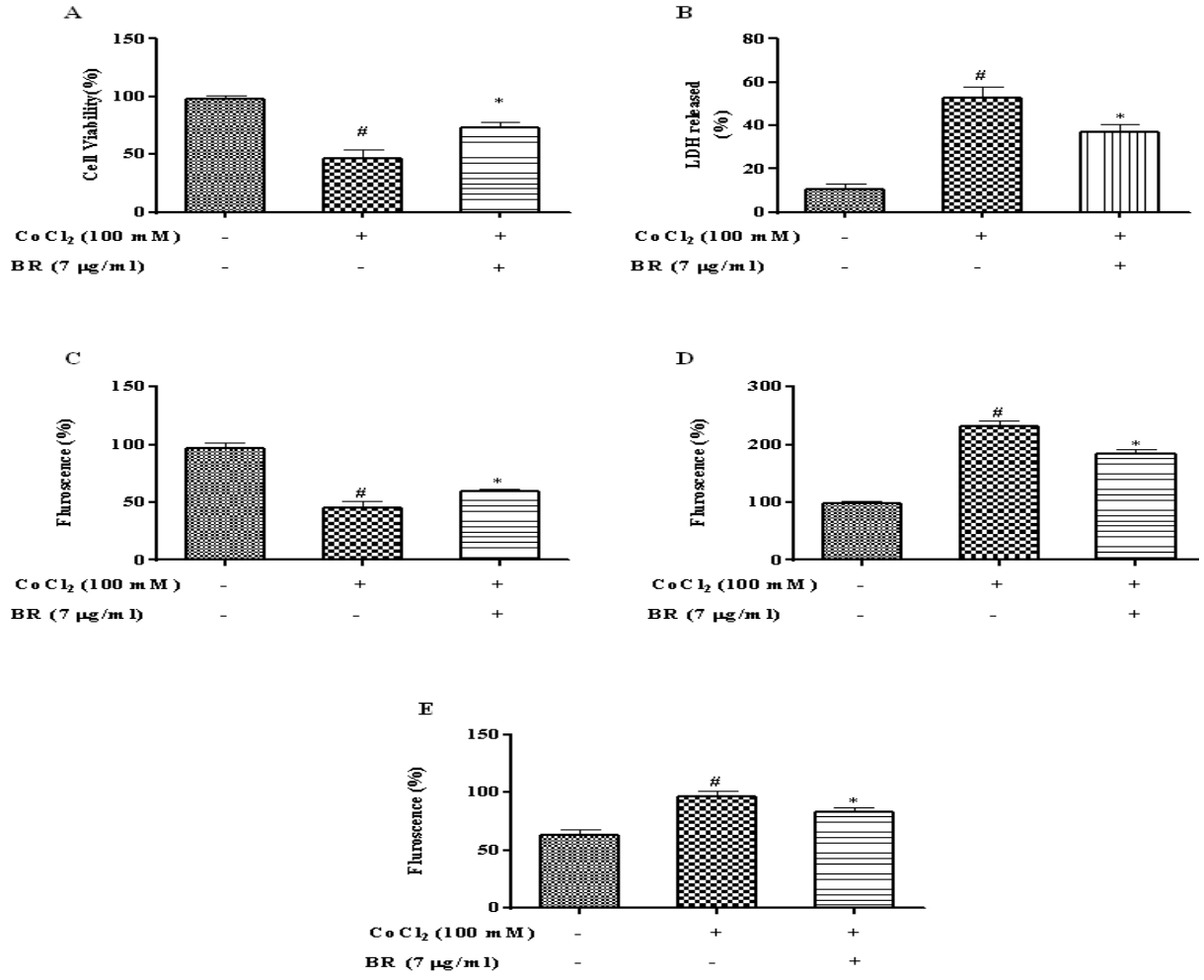

Fig.2 Effect of bromelain on $CoCl_2$-induced hypoxia: A) MTT, B) Lactate dehydrogenase C) Mitochondrial membrane potential, D) Reactive oxygen species and E) Nitrite production.

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Pre-treatment of bromelain (7 µg/ml) followed by $CoCl_2$ (100 mM) exposure for 24 h. The values of MTT were expressed as percentage cell viability while ROS, NO and MMP were expressed as relative fluorescence intensity percentage and LDH was expressed in percentage compared to $CoCl_2$ treatment. # p <0.05 versus control cells, * p < 0.05 versus $CoCl_2$ treated cells.

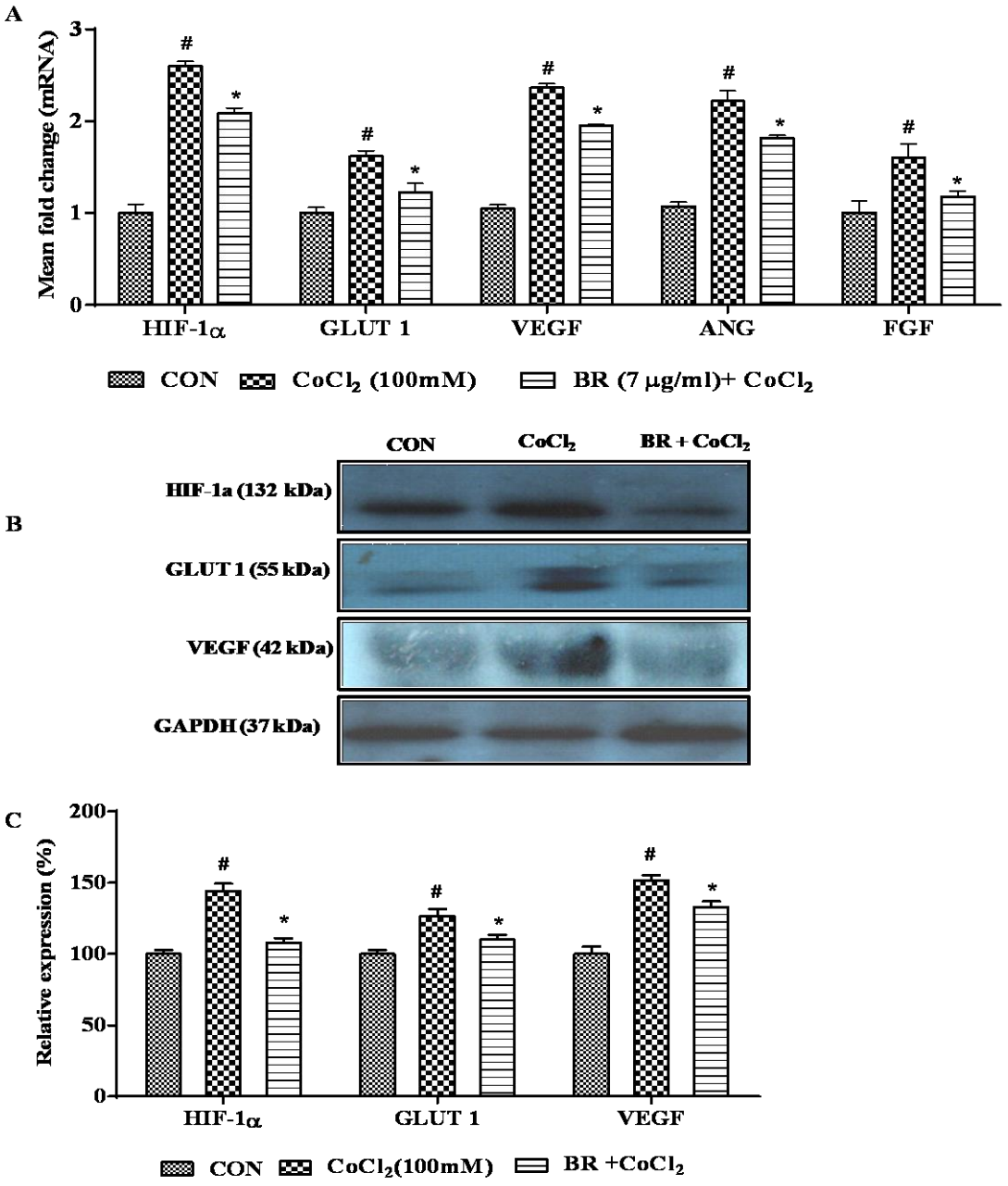

Fig .3 Effect of bromelain on $CoCl_2$-induced hypoxic markers

A) RT-PCR assay; B) Western blot and C) Densitometric analysis of western blot analysis; HIF-1α- Hypoxia inducible factor 1 alpha, GLUT 1 –Glucose transporter 1, VEGF-Vascular endothelial growth factor, ANG- Angiogenin, FGF-Fibroblast growth factor, GAPDH – Glyceraldehyde 3-phosphate dehydrogenase. Data represent the means ± SD (n = 6). Data are expressed as the mean ± standard deviation from three independent experiments. # $p < 0.05$ vs control cells, * $p < 0.05$ vs $CoCl_2$ treated cell.

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Groups	ANTIOXIDANT ASSAYS				
	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GR (U/mg protein)	TBARS (nmole/g protein)
CON	2.1 ±0.02	2.2±0.05	0.99 ± 0.4	18.21 ± 0.20	2.11 ± 0.22
$CoCl_2$ (100mM)	1.2 0.05*	1.3±0.07*	0.39 ±0.05*	11.02± 0.06*	6.55 ± 0.07*
BR (7µg/mL) + $CoCl_2$	1.8 ±0.02#	1.5±0.14#	0.57 ±0.02#	13.33± 0.05#	5.09 ± 0.04#

Groups	DIGESTIVE HORMONES		
	Ghrelin (pg/mL)	Leptin (pg/mL)	CCK (ng/mL)
CON	21.07 ± 0.07	65.16 ± 0.04	6.52 ± 0.05
$CoCl_2$ (100mM)	21.43 ± 0.12	70.61 ± 0.09*	8.12 ± 0.03*
BR (7µg/mL) + $CoCl_2$	21.53 ± 0.08	68.17 ± 0.13#	7.10 ± 0.09#

Table 1: Effect of bromelain on $CoCl_2$-induced oxidative stress and digestive hormones

Cells were pre-incubated with BR (7 µg/mL) for 2 h, followed by $CoCl_2$treatment (100 mM) for 24 h. Data are expressed as the mean ± standard deviation from three independent experiments, each performed in triplicate * $p<0.05$ versus control cells, # $p<0.05$versus $CoCl_2$ treated cell.

3.2 Acknowledgement: We would like to thank Director, DFRL (DRDO) for his valuable suggestions.

3.3 Conflict of Interest: The authors declare that there are no conflicts of interests.

4. CONCLUSION

The study concludes that the supplementation of BR could help in modulation of HIF-1α there by altering several factors of hypoxia; reducing oxidative stress and further regulating gut hormones at high altitude environments.

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