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PROTECTIVE EFFICACY OF ETHYL 3, 4- DIHYDROXY BENZOATE AGAINST EXERCISE INDUCED DAMAGES: PUTATIVE ROLE IN IMPROVING PHYSICAL PERFORMANCE

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Keywords:

Antioxidant, exercise, Hypoxia Inducible factor-1α, Prolyl Hydroxylase enzyme, muscle damage, Oxidative stress

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ABSTRACT: Training under hypoxic conditions is known to enhance physical performance. The possible mechanism might be the stabilization of Hypoxia Inducible Factor (HIF), which otherwise is degraded under normoxia by the action of Prolyl Hydroxylase domain enzymes (PHD). Stabilization of HIF leads to upregulation of its target genes leading to improved erythropoiesis, angiogenesis and antioxidant status as an adaptation to combat training induced stress. Present study investigates efficacy of Ethyl 3, 4 Dihydroxybenzoate (EDHB) in stabilizing HIF-1α which leads to elevated levels of Vascular Endothelial Growth Factor, Erythropoetin, Hb and Hct, indicating improved angiogenesis and blood oxygen carrying capacity. In the present study we have reported 1.5 times increase in endurance performance in rats supplemented with EDHB with or without training as compared to respective controls. Significant improvement in antioxidant status as observed by increase in GSH, SOD, GST and decrease in MDA and protein oxidation, enhanced expression of anti-oxidative proteins Heme-Oxygenase, Metallothionein, Nuclear Factor Erythroid 2-Related Factor contributed in decreasing exercise training induced oxidative stress in EDHB supplemented trained rats. Enhanced levels of anti-inflammatory and reduced levels of pro-inflammatory cytokines might be additional factors responsible in decreasing muscle damage as observed by histopathological studies. The major outcome of the study is preconditioning with PHD inhibitor EDHB results in stabilization of HIF-1α, thus boosting erythropoiesis, antioxidant status and anti-inflammatory response which, in concert result in improvement of physical performance. The study thus underscores the potential of EDHB as a therapeutic agent for improving endurance performance by facilitating hypoxia adaptation in the skeletal muscle.

INTRODUCTION: Advantages of training performed under hypoxic conditions to improve the physical performance in defence personnel, mountaineers, athletes and pilgrims is the subject of much debate and importance these days. Hypoxic stimulus elicits specific molecular responses in skeletal muscle tissue possibly inducing muscular and systemic adaptation, which either are absent or found to be in a lesser extent after training under normoxic conditions.

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As a consequence of increased levels of the regulatory subunit of HIF after training under hypoxic conditions, the levels of mRNA for myoglobin, VEGF and glycolytic enzymes, such as phosphofructokinase increased in a hypoxia dependent manner [1, 2]. These results support the involvement of HIF-1 in the regulation of adaptation processes in skeletal muscle tissue that compensate for the reduced availability of oxygen during training.

Recently a family of O_2- dependent enzymes PHDs has been identified as critical oxygen sensors and thus regulate transcriptional response of HIF-1 α at varying oxygen conditions [3]. HIF is a heterodimer which consists of two subunits one is constitutively expressed β subunit and another is α subunit. HIF-

1α gets hydroxylated immediately after synthesis by PHDs which allows the binding of von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to HIF-1α, thus leading to its degradation by proteosome [4]. However, when there is reduction in intracellular oxygen levels, activity of PHDs decreases resulting in diminished hydroxylation. This leads to stabilization and nuclear translocation of HIF-1α protein which then combines with β subunit and binds to hypoxia responsive elements in target genes along with its co-activators and elicits various hypoxia adaptive cellular responses like angiogenesis, erythropoiesis, glycolysis and protects against metabolic stress [5, 6].

In skeletal muscles, all these physiological adjustments lead to increase in oxygen delivery and metabolite utilization that result in enhanced performance[7]. Furthermore, strenuous exercise is known to cause oxidative stress and inflammatory responses in skeletal muscles which leads to muscle damage. Previous studies carried out in our laboratory have shown that preconditioning by oral cobalt supplementation, a hypoxia mimetic, enhances endurance performance and also protects muscle from exercise induced oxidative damage in rat skeletal muscle [8]. Most importantly, supplementation of hypoxia mimetics is practical and feasible way of inducing hypoxia to enhance physical performance.

One of the most important class of PHD inhibitors is analogue of 2-oxoglutarate e.g. EDHB. It is also known as protocatechuic acid and is present in plant foods such as olives, roselle, du-zhong, and white grapewine [9]. It has been reported to have antioxidant [10, 11], neuroprotective [12], anti-inflammatory, cardioprotective [13] and antiulcer activity. Earlier study by Kasiganesan have shown the effect of EDHB on hypoxia tolerance as well [14].

The aim of the present study was, therefore, to investigate modulatory role of EDHB in facilitating improvement in physical performance by altering the expression of various hypoxia responsive genes. The study highlights the protective efficacy of EDHB by stabilizing HIF-1α thus improving angiogenesis, erythropoiesis, antioxidant status and anti-inflammatory response, thereby boosting physical performance, suggesting the potential of EDHB as a therapeutic agent by facilitating hypoxia adaptation in the skeletal muscle.

Methods:

Ethical Approval:

All animal procedures were approved by the Institutional Animal Ethic Committee, Defence institute of physiology and allied sciences and were in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animal, India. Efforts were made to minimize animal suffering and number of animals used for experimental purpose. Male Sprague-Dawley rats (170 ± 10 g) were used for the study. Animals were maintained in the institute animal house facility at (24±2°C) with 12-h light/dark cycle, relative humidity was maintained at 40-50%. Animals were fed standard pelletized diet (Lipton India Ltd.) and water *ad libitum*. Body weight, food and water intake were measured daily. Animals were maintained under the surveillance of a qualified veterinarian from institute.

MATERIALS:

All chemicals were purchased from Sigma (St. Louis, USA) and SRL (Mumbai, India). Antibodies were purchased from Santa Cruz Biotech (CA, USA).

Dose response studies with EDHB:

Rats (170 ± 10 g, 6-7 weeks old) were selected on the basis of their ability to run on treadmill (Columbus Instruments, Columbus) with an adjustable belt speed (0–99.9 m/min). A low-voltage, electric stimulating bar located at the rear end of the treadmill encourages rats to run. Electric bar was set to deliver 0.2-0.3 mA, which gave a mild shock but did not injure the animal. Rats were randomly categorized into four groups (n=8/group) and were subjected to treadmill training for 10 days starting for 20 min with belt speed 6 m/min-15m/min till reached 50 min [15].

Rats were supplemented with different doses of EDHB (25-150 mg/kg bw) and control rats with saline i.p. on last three days of training. On 11th day, running time till exhaustion was measured in a blinded fashion at belt speed of 24 m/min with 5% inclination. Animals were considered exhausted

when unable to run on treadmill despite in contact with the shock bar at the rear end of the treadmill.

From the results of above experiment, minimal effective dose was found to be 50 mg/kg bw, therefore, further experiments were carried out at this concentration.

Endurance performance:

In a separate set of experiment, rats were randomly divided into 4 groups (n=8/group) according to treatment and exercise conditions: (1) control sedentary (CS) (2) EDHB treated sedentary (DS) (3) control training (CT) (4) EDHB treated training (DT). Rats in treated groups were supplemented with 50 mg/kgbw EDHB (i.p.) as described above while control groups were supplemented with same amount of vehicle. Training protocol was same as mentioned above. On 11th day, running time till exhaustion was measured on treadmill. Animal were sacrificed 30 min after exhaustion time measurement.

Tissue collection:

Animals were euthanized with overdose of ketamine chlorhydrate & Xylazine. Blood was collected by renal portal vein and blood parameters were estimated immediately. Remaining blood was centrifuged at 3000 rpm for 20 min and plasma was processed immediately or kept at -80°C for further analysis. Whole gastrocnemius muscle was collected from the calf region of hind limbs. Red gastrocnemius muscle was then separated, washed in phosphate-buffer saline (PBS) twice, snap frozen after removal of hair and fatty tissue and stored at -80°C for subsequent biochemical and protein expression studies. Red gastrocnemius muscle was chosen because it is actively recruited during exercise and is highly oxidative in nature [16].

Haematological analysis:

Haemoglobin and haematocrit were analysed in heparinised blood by blood cell counter (MS-4, Desing Laboratories, France).

Biochemical Analysis:

Muscle samples were homogenized in ice-cold 0.154 M KCl fortified with protease inhibitors cocktail to obtain a 10% homogenate (w/v). A part of the homogenate was then centrifuged at 2900 x g for 10 min at 4ºC. Pellet containing tissue/cell debris was discarded and supernatant was used to determine GSH, GSSG levels, free radical production and lipid peroxidation. Other part of the homogenate was centrifuged at 14,000 x g for 30 min at 4ºC and the supernatant was used for other estimations (CK, SOD, GST, GPx, and PO). The protein content in the homogenate was determined by Lowry's method[17].

Reactive Oxygen Species Estimation:

ROS generation in the tissue was detected by using 2',7'-dichlorfluorescein-diacetate (DCFH-DA)[18]. Detection is based on the conversion of non-fluorescent DCFH-DA to the highly fluorescent compound 2', 7'-dichlorfluorescein (DCF). ROS generation was thus accessed according to Lebel and bondy [19], as modified by Kim [20]. Briefly, 50 µl of muscle homogenate was added to 2,938 µl of 0.1M phosphate buffer (pH 7.4) followed by addition of 12µl DCF-DA (1.25 mM in methanol). Assay mixture was incubated for 15 min at 37ºC and 2'7'-dichlorofluoroscein formation was determined fluorimetrically using Fluostar OMEGA, (BMG Labtech) at an excitation of 488 nm and emission at 525 nm. The fluorescent intensity parallels to the amount of ROS formed.

Lipid peroxidation and Creatine kinase (CK) level:

Tissue malondialdehyde (MDA) level was used as an index of lipid peroxidation. Measurement of thiobarbituric acid reactive substances (TBARS), which are formed by the reaction of thiobarbituric acid (TBA) with MDA, was used for determining MDA level by the method of Ohkawa et. Al [21]. Thiobarbiturate was used as standard and OD was measured at 531 nm. Levels of lipid peroxides in muscle and plasma were expressed as nmol MDA/mg protein. CK level (EC 2.7.3.2) a well-known marker of muscle damage was estimated in plasma by using commercially available kits (Randox, UK) as per manufacturer's protocol**.**

Protein Oxidation:

Protein oxidation was measured by determining the carbonyl groups after derivatization of proteins with dinitrophenyl hydrazine (DNPH) [22]. Briefly, 500µl muscle homogenate was incubated with 500µl of 10mM DNPH in 2M HCl for 60 min at

50°C. Proteins were then precipitated using 20% TCA and the unreacted DNPH was removed by centrifugation at 14,000g for 10 min. Pellet was resuspended in 1M NaOH and absorbance measured at 450 nm.

Enzymatic and non-enzymatic antioxidants:

GSH was measured in blood and muscle by the method of Kum Tatt [23]. GSH is oxidised by 5, 5'dithiobis (2-nitrobenzoic acid) (DTNB) to give GSSG with formation of 5-Thio 2-nitrobenzoic acid (TNB). The rate of formation of TNB is followed at 412 nm. GSSG levels in muscles were measured fluorimetrically by the method of Hissin & Hilf [24]. Fluorescence was measured at an excitation wavelength of 350 nm and emission at 420 nm. Activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined using commercially available kits (Randox, UK) as per manufacturer's instructions. Glutathione-S-transferase activity (GST, EC 2.5.1.18) was determined using protocol described by Habig et al [25]. The optical density was recorded at 340 nm. Total superoxide dismutase activity (SOD, EC 1.15.1.1) was determined using protocol described by Kakkar et al [26]. A single unit of enzyme was expressed as 50% inhibition of NBT (Nitro blue tetrazolium) reduction/min/mg protein.

HIF, VEGF and EPO quantification by ELISA:

Levels of HIF-1α and its target proteins EPO and VEGF were estimated in muscle homogenate (1:5 PBS) using ELISA according to the manufacturer's protocol (Quantikine R&D systems, Minneapolis, MN) and measured on microplate reader (Fluostar OMEGA, BMG Labtech).

Protein Expression Studies by western blotting:

Muscle samples were homogenized in four volumes of ice cold buffer A (0.5M sucrose, 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 1mM EDTA, 1mM DTT, 1 mM PMSF) fortified with protease inhibitors cocktail (Sigma). Homogenate was kept on ice for 15 min and nonidet (NP 40) was added to make a final concentration of 0.6% followed by centrifugation at 2000g for 10 min at 4°C. Supernatant (cytosolic fraction) was collected and stored at – 80°C. Pellet was dissolved in one volume of ice cold buffer B (20 mM HEPES, pH 7.9, 0.3 mM NaCl, 1.5mM $MgCl_2$, 20% glycerol, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF) fortified with protease inhibitors cocktail. It was kept on ice for 30 min followed by centrifugation at 20,000g for 15 min at 4°C. The supernatant containing nuclear fraction was aliquoted and stored at -80° C. Protein concentrations were quantified by Lowry's method. Protein expression of HIF-1α was studied in nuclear fraction, Epo, VEGF, myoglobin and anti-oxidant proteins HO-1, Nrf-2 and MT-1 were studied in cytosolic extract of red gastrocnemius muscle by western blot analysis. Briefly, muscle homogenate containing 50µg proteins was subjected to SDS-PAGE and electroblotted on to a nitrocellulose membrane (Millipore, USA).

The membranes were blocked with 3% BSA for 2 hr, washed with tris buffer saline with Tween 20 (0.1%), probed with respective mouse/rabbit monoclonal/polyclonal antibodies (Santa Cruz, CA, USA) for 3 hr, followed by washing with TBST and incubation with antimouse/antirabbit –IgG-HRP conjugate (1:40,000) for 2h. Membranes were incubated with chemiluminescent substrate (Sigma) and bands were developed using X-ray films. (Kodak, USA). Density of bands was quantified using Lab Works software (UVP, Bioimaging Systems, UK).

Inflammatory markers:

Protein expression of NF-κB and Cyclooxygenase-2 (Cox2) was studied in muscle homogenate of all four groups of rats by western blotting as described above. The levels of proinflammatory cytokines viz., IFN-γ, TNF-α, MCP-1 and anti-inflammatory cytokines, IL-6, TGF-β and IL-10 were estimated in plasma by ELISA (Opti-Eia kits) as per manufacturer's instruction (BD Bioscience, CA).

Histopathological studies:

After exhaustion time measurement, animals were anaesthetized (ketamine chlorhydrate 80 mg/kg bw & Xylazine 20 mg/kg bw) and were immediately perfused with ice cold PBS followed by fixation in 4% para-formaldehyde (dissolved in 0.1 M PBS pH 7.4). Red gastrocnemius muscle was removed carefully, fixed in same fixative for 24 hr at room temperature, dehydrated through graded alcohol and embedded in paraffin. Approximately 5µm sections were made and stained with haematoxylin

and eosin according to standard procedures for morphological analysis. The sections were analysed under Labcom (Germany), trinocular research microscope at 40x. The photomicrographs were captured by Canon (Tokyo, Japan) digital camera attached to light microscope.

Statistical analysis

All the experiments were performed two times. Data are presented as mean ± SD. Data was analysed using one–way analysis of variance with post-hoc bonferroni analysis using standard statistical software package for social science (version16.0, SPSS Inc., USA). $p < 0.05$ and $p < 0.01$ were considered as significant.

RESULTS:

EDHB boosts physical performance:

There was 2.4 times increase in running time till exhaustion in the animals given training only (CT) (81.3 ± 9.5 min) as compared to control sedentary (CS) (34 ± 5.8 min). Supplementation of different doses of EDHB (25, 50 and 100 mg/kg bw) along with training further increased exhaustion time (107± 7.4, 130.3 ± 6.4 and 140.5 ± 5.5 min respectively) significantly ($p<0.01$) as compared to control trained rats.

Maximum increase was observed at 50 and 100 mg/kg bw EDHB and effect was almost same in both these groups. Therefore, 50 mg/kg bw EDHB was used in all the further studies (**Fig. 1a**). This optimum dose was administered rats in another set of experiment as mentioned earlier and running time till exhaustion was determined in all groups. EDHB preconditioning resulted in 1.5 times increase in exhaustion time in sedentary as well as trained rats as compared to respective controls (**Fig. 1b).**

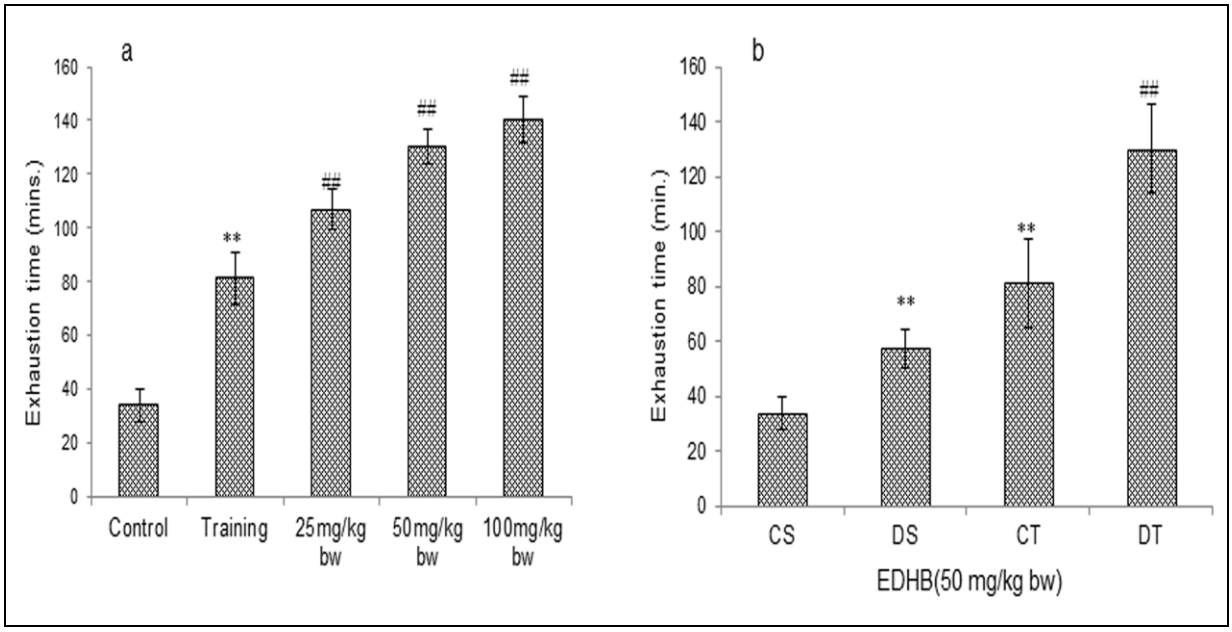

FIG. 1: ENHANCED PHYSICAL PERFORMANCE BY EDHB SUPPLEMENTATION. a) DOSE RESPONSE STUDIES OF EDHB. b) EXHAUSTION TIME MEASUREMENT AT OPTIMUM DOSE 50mg/kg bw. EXPERIMENT WAS PERFORMED TWO TIMES. VALUES ARE MEAN ± SD (n=8 per group). **p<0.01 vs. CS;; ##p<0.01 vs. CT. CS- CONTROL SEDENTARY, DS- DRUG SEDENTARY, CT- CONTROL TRAINING & DT- DRUG TRAINING.

EDHB supplementation improves blood oxygen carrying capacity:

Hct and Hb levels were assessed to study blood oxygen carrying capacity. There was 11% increase in Hb level in EDHB supplemented sedentary rats as compared to control sedentary. Training along with EDHB supplementation led to further significant increase in Hb as compared to training without EDHB (CT vs DT 24%). Significant ($p<0.01$) increase was also observed in RBC count (CT vs DT 29 %) and Hct (CT vs DT 27%) in rats supplemented with EDHB along with training ($p<0.01$) as compared to respective controls (**Table 1).**

TABLE 1: EFFECT OF EDHB ON BLOOD OXYGEN CARRYING CAPACITY. BLOOD OXYGEN CARRYING CAPACITY WAS MEASURED BY ESTIMATING LEVELS OF HB, RBC AND HCT.

Parameter	CS	DS	CT	DT
Hb (gm/dl)	15.22±0.5	16.96±0.2	14.84±0.6	18.4±0.5##
RBC (M/mm3)	50.32±1.7	54.6±0.8	47.87±2.0	61.84±1.4##
Hct (%)	7.894±0.4	8.424±0.6	7.265±0.5	9.24±0.5##

Values are mean ± SD (n=8 per group). ##p<0.01 vs. CT. CS- control Sedentary, DS- Drug sedentary, CT- control training & DT- drug training.

EDHB attenuates training induced oxidative stress:

There was increase in ROS level in animals after EDHB supplementation as compared to control but lipid and protein oxidation were lowered as observed by decrease in muscle MDA (19.75%, p<0.01) and plasma MDA (16.55%, p<0.01) along with muscle protein oxidation levels (5%, ns). Decrease in ROS levels was observed in plasma of both the training groups with/ without EDHB supplementation as compared to respective control groups (**Fig. 2a**).

Training along with EDHB resulted in further decrease in muscle and plasma MDA (38.72%, p<0.01) and protein oxidation (9.94%, p<0.05) levels as compared to control group with training (**Fig. 2b & 2c**). Creatine kinase which is a well-known marker of muscle damage was measured in plasma. It was found to be decreased in EDHB supplemented groups with or without training as compared to their respective controls (CS vs DS 17.59 %, CT vs DT 16.32 p<0.01). Training increased the CK release in plasma whereas EDHB supplementation along with training restored it to levels similar to control sedentary (**Fig. 2d**).

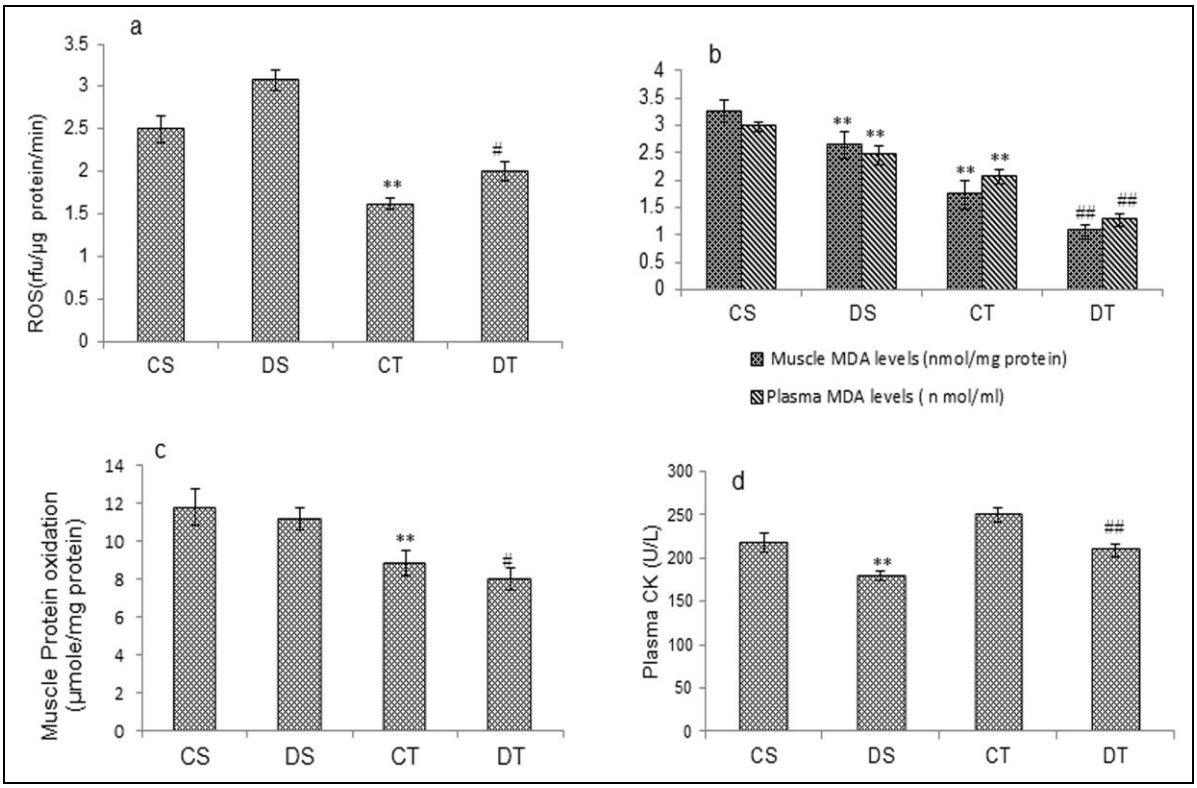

FIG. 2: EDHB SUPPLEMENTATION ALLEVIATES OXIDATIVE STRESS: a) REACTIVE OXYGEN SPECIES WAS DETERMINED IN FRESH PLASMA USING DCHFDA AS A PROBE AND VALUES ARE EXPRESSED AS RELATIVE FLUORESCENT UNIT (r.f.u.); b) LIPID PEROXIDATION WAS MEASURED COLORIMETRICALLY BY MDA ESTIMATION IN PLASMA AND MUSCLE; c) PROTEIN OXIDATION WAS ESTIMATED BY USING DNPH; d) CREATINE KINASE LEVELS WERE ESTIMATED IN PLASMA AS A MARKER OF MUSCLES DAMAGE. EXPERIMENT WAS PERFORMED TWO TIMES.

Values are Mean ± Sd (N=8 Per Group). **P<0.01 Vs. Cs; ##P<0.01 Vs. Ct; #P<0.05 Vs. Ct. CS- Control Sedentary, DS- Drug Sedentary, CT- Control Training & DT- Drug Training.

Augmentation of antioxidant status:

EDHB influences glutathione status:

During physical exercise the extent of oxidative damage is not only determined by oxidative stress but also by antioxidant defense. EDHB supplementation ensued increase in antioxidant status and antioxidative enzymes. There was increase in GSH level in the plasma (67%) and muscle (13.79%) of EDHB sedentary treated rats as compared to control sedentary (CS vs DS, $p<0.01$). Endurance training alone also induced GSH level, however EDHB supplementation along with training further increased GSH in plasma (66.9%)and muscle (33.1%) as compared to training alone group (CT vs DT, $p<0.01$).

There was reduction in GSSG levels in muscle significantly ($p<0.01$) in EDHB supplemented group (16.9%) as compared to control sedentary. Though endurance training also decreased GSSG level, EDHB with training further resulted in significant decrease in GSSG ($p<0.01$) as compared to training alone. Overall increase in GSH/GSSG ratio was observed in EDHB supplemented rats even without training as compared to control and this ratio was further enhanced with EDHB supplementation along with training (**Table 2**).

TABLE 2: EFFECT OF EDHB ON GLUTATHIONE STATUS AS AN ANTIOXIDANT MARKER.

Biochemical Parameter	CS	DS	CT	DT
Plasma GSH (mg/dl)	12.55±1.13	21.02±1.8**	15.27±1.7*	25.49±2.3##
Muscle GSH (µmol/mg protein)	49.5±7.7	56.33±7.2**	63±7.8**	83.83±8.1##
Muscle GSSG (µmol/mg protein)	45.16±1.7	37.5±1.04**	29.16±3.1**	20.66±1.2##
GSH/GSSG ratio	1.09±0.16	1.50±0.2**	2.17±0.4**	4.05±0.5##

Values are mean ± SD (n=8 per group). **p<0.01 vs. CS; *p<0.05 vs. CS; ##p<0.01 vs. CT. CS- control Sedentary, DS- Drug sedentary, CT- control training & DT- drug training.

EDHB augments antioxidant enzyme activities:

EDHB supplementation without training increased enzyme activities of GST and SOD ($p<0.01$) in blood as well as in muscle tissue as compared to control sedentary rats. However, training along with EDHB resulted in further increased activity of GST and SOD when compared with unsupplemented trained rats. No significant change was observed in GPx activity on EDHB supplementation as compared to control (**Table 3**).

TABLE 3: EFFECT OF EDHB SUPPLEMENTATION ON ANTIOXIDANT STATUS BY ENHANCED ANTIOXIDANT ENZYME ACTIVITIES (A). IN MUSCLE TISSUE. (B). IN BLOOD/PLASMA SAMPLE.

A. Biochemical estimations in muscles tissue				
Biochemical Parameter	Control sedentary	Drug sedentary	Control training	Drug training
GST(nmol/min/mg protein)	13.84±1.9	19.84±1.8**	20.12±1.6**	27.31±1.7##
SOD (unit/mg protein)	49.81±6.6	78.57±8.4**	85.9±8.6**	103.28±9.5##
GPx(unit/mg protein)	43.51±7.5	40.08±3.9	53.18±7.5**	43.77±1.39
B. Biochemical estimations in Blood/plasma				
GST(nmol/min/ml)	32.38±1.02	38.26±1.9*	52.32±3.2**	60.32±4.3##
SOD(U/gmHb)	91.33±3.4	98.33±2.8*	119.83±3.2**	130.16±2.2##
GPX(U/gmHb)	155.96±14.5	130.43±11.7	204.58±14.7**	140.88±13.2

Values are mean ± SD (n=8 per group). **p<0.01 vs. CS; *p<0.05 vs. CS; ##p<0.01 vs. CT. CS- control Sedentary, DS- Drug sedentary, CT- control training & DT- drug training.

EDHB enhances expression of anti-oxidative proteins:

HO-1 is a highly inducible gene expressed in response to oxidative stress, inflammation and MT is a potent free radical scavenger. Nrf2 is an important positive regulator of many antioxidant and phase II detoxifying enzymes. There was boost in the expression of HO-1 and Nrf2 in red gastrocnemius muscle of EDHB sedentary group as compared to control sedentary which was increased further with training (HO-1 $p<0.05$ and Nrf2 $p<0.01$). However, much higher expression of HO-1 ($p<0.05$), Nrf2 and MT-1 ($p<0.01$) was observed when training was given along with EDHB as compared to control training group (**Fig 3**).

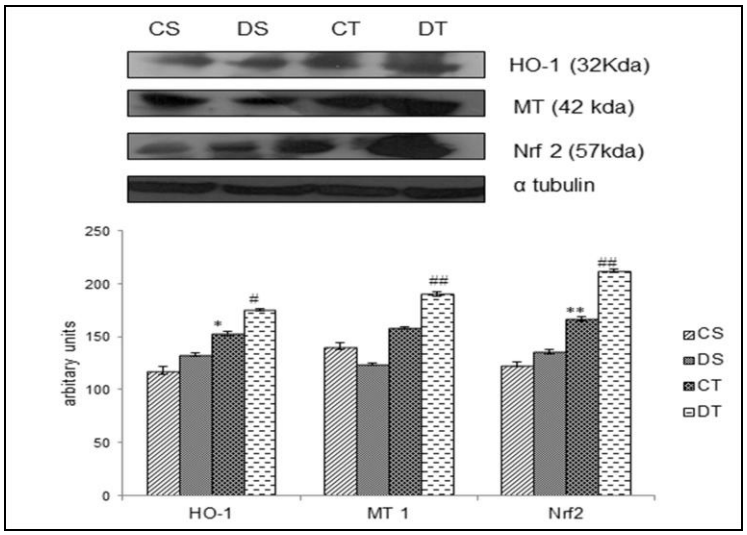

FIG. 3: EFFECT OF EDHB PRECONDITIONING ON ANTIOXIDANT PROTEINS BY WESTERN BLOTS. EXPRESSION OF ANTIOXIDANT PROTEINS HO-1, MT-1 AND NRF2 WERE STUDIED AFTER ASSESSMENT OF PHYSICAL PERFORMANCE IN RED GASTROCNEMIUS MUSCLE. DENSITOMETRIC ANALYSIS OF BLOT WAS NORMALIZED AGAINST A-TUBULIN.

Values are mean ± SD (n=8 per group). **p<0.01 vs. CS; *p<0.05 vs. CS; ##p<0.01 vs. CT; #p<0.05 vs. CT. CS- control Sedentary, DS- Drug sedentary, CT- control training & DT- drug training.

Stabilization of HIF and upregulation of HIF targeted proteins:

Expression and levels of HIF-1α in gastrocnemius muscle were found to be significantly elevated by EDHB supplementation in sedentary rats as compared to control sedentary rats (**Fig. 4**) which were further enhanced by training in EDHB supplemented/non-supplemented groups as compared to their respective controls (CS vs DS 16.30%, CT vs DT 26.66%, p<0.01, **Fig. 4b**). Thus the upregulation of HIF-1α by training alone, more so along with EDHB supplementation resulted in increased expression (**Fig. 4a**) and levels (Fig 4b) of EPO(CS vs DS 50.53%, CT vs DT 18.44%, p<0.05) and VEGF (CS vs DS 22.63%, CT vs DT 16.86%, p<0.01) as compared to respective controls.

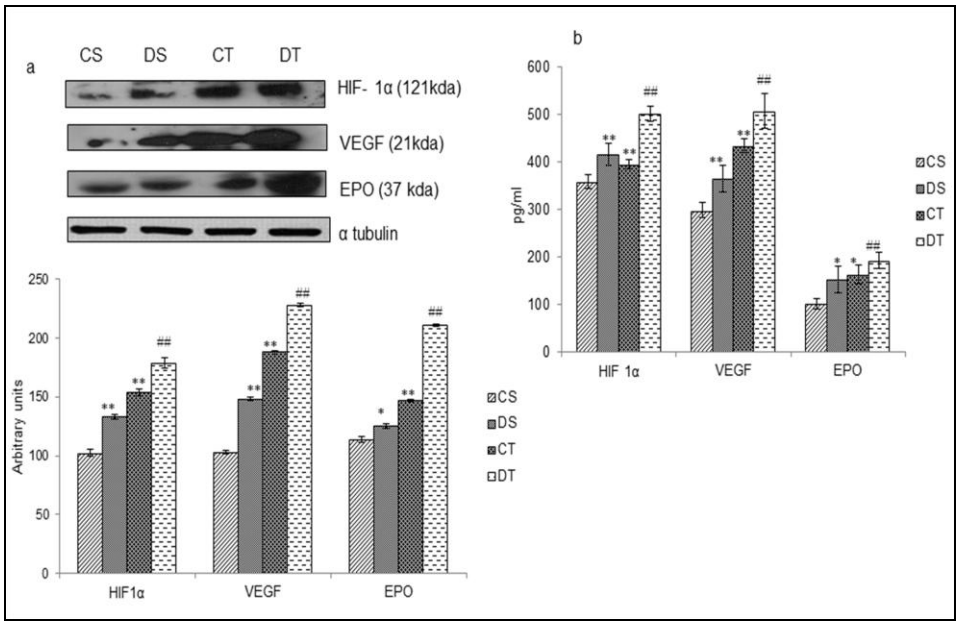

FIG. 4: EFFECT OF EDHB ON HIF STABILIZATION AND UPREGULATION OF HIF TARGET GENES. EXPRESSION OF HIF-1A AND ITS TARGET GENES WAS ANALYSED IN RED GASTROCNEMIUS MUSCLE BY A) WESTERN BLOTTING OF HIF, VEGF AND EPO. DENSITOMETRIC ANALYSIS OF BLOT WAS NORMALIZED AGAINST A- TUBULIN; B) LEVELS OF HIF-1A, VEGF AND EPO WERE ALSO ESTIMATED BY ELISA.

Values are mean ± SD (n=8 per group). **p<0.01 vs. CS; *p<0.05 vs. CS; ##p<0.01 vs. CT. CS- control Sedentary, DS- Drug sedentary, CT- control training & DT- drug training.

EDHB alleviates training induced inflammation:
There was significant decrease in the expression of inflammatory proteins viz., NF κB and Cox-2 in EDHB treated groups as compared to controls sedentary (**Fig. 5c**). Increased expression of NFκB and Cox-2 is associated with inflammation therefore; levels of circulatory pro and anti-inflammatory cytokines were estimated in plasma by ELISA to study the anti-inflammatory effect of EDHB.

There was significant decrease in plasma levels of proinflammatory cytokines IFN-γ and TNFα in EDHB treated groups as compared to their respective controls (**Fig. 5b**). MCP1 level was found to be decreased in drug sedentary group as compared to control sedentary while training with or without EDHB did not result in further change in MCP1 level. Increase in anti-inflammatory cytokines, IL-6, TGF-β, IL-10 (**Fig. 5a**) in the EDHB treated trained rats as compared to control trained rats shows anti-inflammatory property of EDHB.

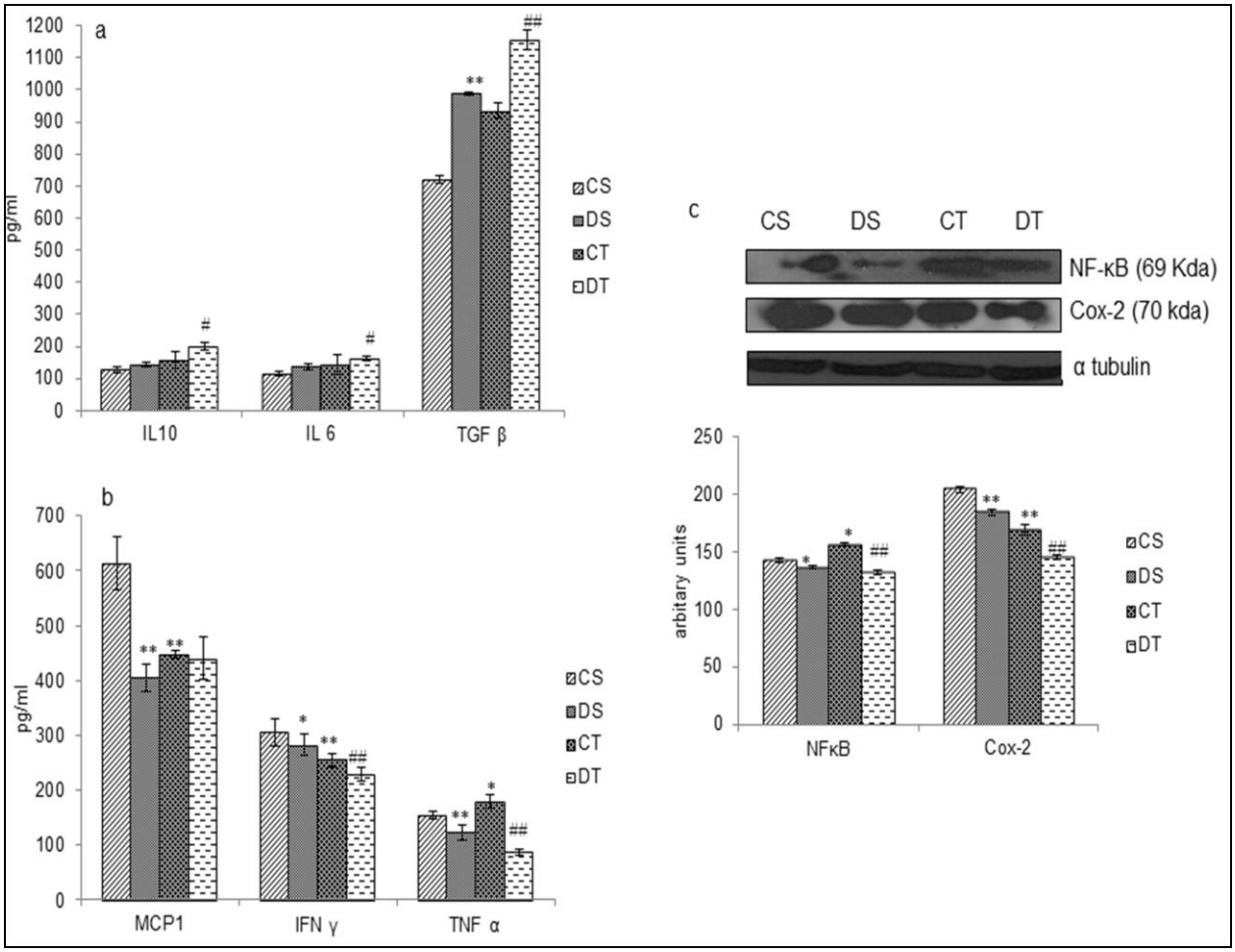

FIG. 5: EFFECT OF EDHB ON INFLAMMATORY MARKERS. AFTER MEASUREMENT OF RUNNING TIME TILL EXHAUSTION ANIMALS WERE SACRIFICED AND LEVELS OF CIRCULATORY CYTOKINES WERE ESTIMATED IN FRESH PLASMA. a) LEVELS OF ANTI-INFLAMMATORY CYTOKINES IL10, IL6 AND TGFβ; b) LEVELS OF PROINFLAMMATORY MARKERS MCP-1, IFNγ and TNFα, were QUANTIFIED BY ELISA; C) PROTEIN EXPRESSION OF INFLAMMATORY MARKERS NF-κB and Cox2 WAS STUDIED IN GASTROCNEMIUS MUSCLE. DENSITOMETRIC ANALYSIS OF BLOT WAS NORMALIZED AGAINST α-TUBULIN. EXPERIMENT WAS PERFORMED TWO TIMES.

Values are Mean ± Sd (N=8 Per Group). **P<0.01 Vs. Cs; *P<0.05 Vs. Cs; ##P<0.01 Vs. Ct; #P<0.05 Vs. Ct. CS- Control Sedentary, DS- Drug Sedentary, CT- Control Training & DT- Drug Training.

Histopathological analysis:
Histopathology studies reveal myoprotective effect of EDHB in supplemented rats as control sedentary group showed greatest degree of gastrocnemius muscle damage with rounding and reduction in fibre diameter whereas EDHB supplemented group showed improvement with no rounding or reduction in fibre diameter with only slight increase in interfascicular spaces (**Fig.6**).

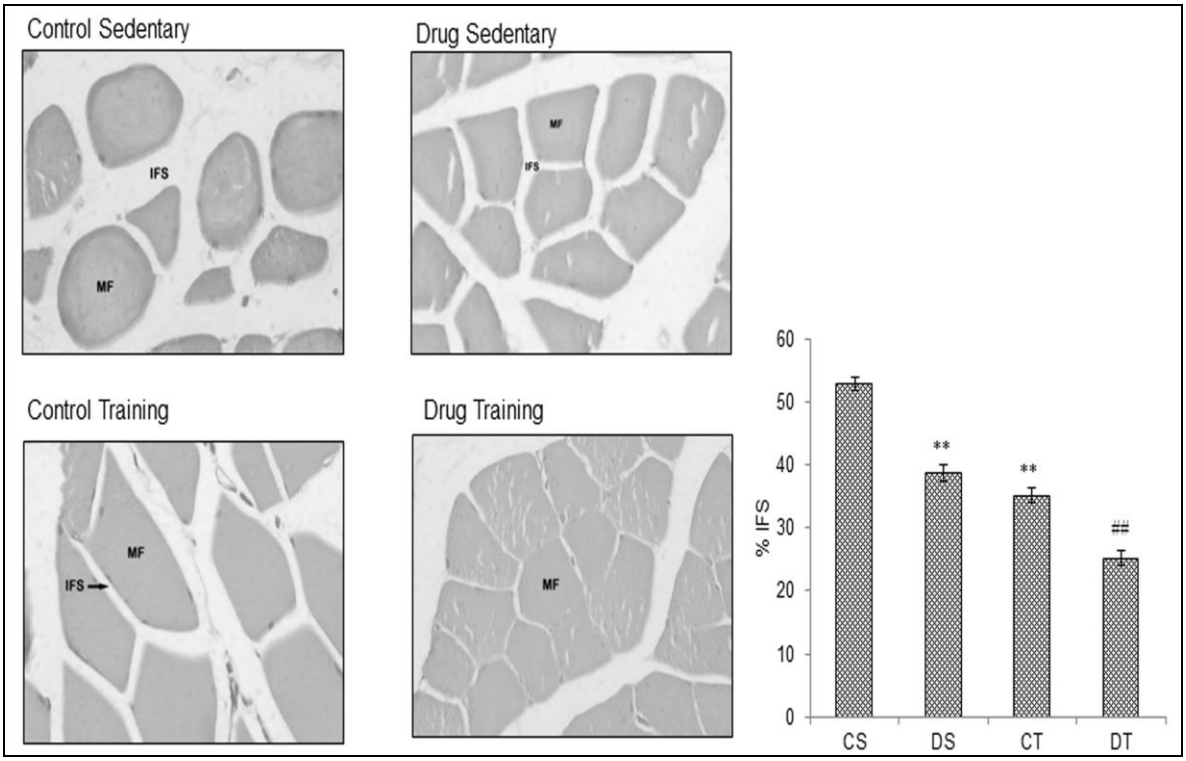

FIG.6: HISTOPATHOLOGICAL ANALYSIS ON EXERCISE INDUCED DAMAGE IN GASTROCNEMIUS MUSCLE. HISTOPATHOLOGICAL ANALYSIS OF RED GASTROCNEMIUS MUSCLE WAS DONE AFTER MEASURING RUNNING TIME TILL EXHAUSTION ON TREADMILL BY H&E STAINING. TRANSVERSE SECTION OF MUSCLES WERE ANALYSED AT 40X BY USING LABCOM (GERMANY), TRINOCULAR RESEARCH MICROSCOPE. THERE WAS DECREASE IN IFS IN EDHB SUPPLEMENTED GROUPS AS SHOWN IN GRAPH REPRESENTING % IFS. THE EDHB TRAINING GROUP SHOWED IMPROVEMENT WITH NO ROUNDING OR REDUCTION IN FIBRE DIAMETER SEEN.

IFS- Interfascicular Spaces, MF- Muscle Fibre. CS- control Sedentary, DS- Drug sedentary, CT- control training & DT- drug training. **p<0.01 vs. CS; ##p<0.01 vs. CT.

DISCUSSION: It is well-known that training under hypoxic conditions can initiate the oxygen sensing mechanism by the stabilization of HIF, that leads to induction of HIF target genes and various cellular adaptations like erythropoiesis, angiogenesis, enhanced protein synthesis which help to neutralize the effect of reduced oxygen supply [27, 28].

In present study we hypothesized that EDHB supplementation can facilitate improvement in physical performance in rats by stabilizing HIF under normoxic conditions thus inducing hypoxia responsive genes. It was observed that when training was given to rats, it boosted their physical performance as compared to sedentary control rats which was further enhanced when EDHB (50 mg/kg bw) was given along with training. Rats supplemented with EDHB performed better in both sedentary as well as in trained groups as compared to their respective controls (**Fig.1**). Increase in Hb and Hct is a key factor in enhancing aerobic performance. Increase in Hb, Hct and RBC as observed in this study strongly suggests up regulation of systemic oxygen conductance after preconditioning with EDHB. Erythropoiesis regulated by Epo boost RBC because of which Epo is widely accepted as performance enhancing hormone. In our study also it was found that there was significant elevation in the Epo levels in EDHB supplemented rats as compared to controls. Epo is known to exert both direct and indirect protective effects on muscle during exercise [29]. Increased angiogenesis is a well-known response of regular endurance training in the skeletal muscle. Epo has been reported to increase angiogenic process and one of the possible mechanisms is by enhancing the level of VEGF in tissue [30].

In conformity with these reports marked increase ($p<0.01$) in level of VEGF was observed as detected by immunoblotting and ELISA in EDHB supplemented groups as compared to respective controls. Therefore, increase in the level of VEGF, Epo along with Hb, Hct and RBC in EDHB supplemented groups implies the activation of

oxygen sensing system and increased blood oxygen carrying capacity enabling the muscle to extract more O_2 when it was made available by the cardiovascular system (**Fig.4**).

Flow of oxygen through muscles, during intense physical activity reduces to a large extent which thus leads to structural damage or inflammatory reactions due to generation of ROS and excessive free radicals. This leads to increase in oxidative damage markers such as protein carbonyls and thiobarbituric acid reactive substances and also changes in pro-oxidant- antioxidant balance [31] and thus acts as a reason for muscle fatigue, soreness and myofibril damage and finally results in decreased physical performance.

In the present study, ROS level was observed to be higher in EDHB supplemented groups as compared to their respective controls. But the levels of MDA, protein oxidation and CK were found to be decreased in plasma of EDHB supplemented animals with/without training showing the protective effect of EDHB against oxidative damage (**Fig. 2**). The increased ROS might have significance as signalling molecules as reported by other researchers also [32].

CK is released into the blood from muscles, when the cell membrane is damaged or when there is an alteration in cell membrane permeability, hence it is one of the most commonly used indicators of muscle damage[33] and decrement in the plasma CK level in EDHB supplemented rats compared to respective controls indicate muscular protection by EDHB under exercise induced oxidative stress.

To investigate basis of protective efficacy of EDHB, anti-oxidant status in animals was studied with or without EDHB administration. GSH, a tri peptide thiol is one of the most important endogenous antioxidant. It acts as an intracellular antioxidant by supplying its sulfhydryl group to reduce peroxides. Tissue GSH/GSSG ratio is known to increase with endurance training thus increase in GSH/GSSG ratio is an indication of enhanced antioxidant status that helps in protection from oxidative damage [34]. In accordance to this, we found significant increase in GSH level after training as compared to untrained controls which was further increased by EDHB supplementation. Antioxidative enzymes GST, SOD and GPx provide the primary defense against exercise generated oxidative stress and activities of these enzymes are known to increase in exercise [31].

The role of SOD is to accelerate the process of conversion of toxic superoxide radicals to hydrogen peroxide and oxygen while GPx catalyses the reduction of hydrogen peroxide and hydroperoxides formed from fatty acids, thereby effectively removing toxic peroxides from living cells. It plays an important role in protecting cells from potential damage by free radicals. In this study also, training increased enzyme activities of SOD, GST and GPx in muscle and plasma. EDHB along with training further increased the activity of SOD and GST. These results show that EDHB promotes defence against oxidative stress (**Table 2** and **3**).

Further role of HO-1, MT and Nrf2 antioxidant proteins was explored in eliciting protection against oxidative damage. HO-1 is induced by diverse stressors including heme oxidants, ischemia, hyperoxia and cytokines and has potent antioxidant and anti-inflammatory properties. HO-1 is highly upregulated by HIF-1 transcription factor [35]. Our study also shows increased expression of HO-1 in EDHB treated trained group as compared to control trained group, thus correlating with increased HIF 1α expression and improved antioxidative status on EDHB supplementation. HIF regulation by O_2 dependent hydroxylation of proline residues by PHDs provides the molecular basis for the oxygen-sensing function of these enzymes. These PHDs catalyse the reaction with the help of cofactors ascorbic acid, iron and 2-oxoglutarate.

Absence of either of these, lead to the inhibition of these enzymes and thus stabilization of HIF-1 resulting in upregulation of its target genes VEGF and Epo[2, 36]. EDHB, an analogue of 2-oxoglutarate, is the competitive inhibitor capable of inhibiting the activity of PHDs and thus stabilizing HIF-1 under normoxic conditions [37]. Elevation of HIF-1α in EDHB treated animals resulted in upregulation of VEGF and Epo responsible for increased angiogenesis and oxygen carrying capacity of blood as observed in the present study.

MTs are small cysteine rich proteins which play cytoprotective role in stress situations and are potent hydroxyl radical scavenger [38]. Nrf2 is a member of CNC (cap 'n' collar) family of b-Zip transcription factors and an indispensable positive regulator of many antioxidant and phase II detoxifying enzymes[39].

In oxidative or neutrophilic stress Nrf2 protein stabilizes and then translocates to nucleus, where it heterodimerizes with small Maf proteins and binds to the antioxidant response element (ARE), which is a regulatory element found in the 5'-flanking regions of antioxidant and detoxification enzymes. In addition HO-1 has also been shown to be positively regulated by Nrf2 [40]. Thus augmentation in Nrf2, MT-1 and HO-1 expression by EDHB as observed in the present study might also be contributing in attenuation of oxidative stress and enhancing antioxidative status (**Fig.3**).

Increasing evidences suggest the role of inflammatory mediators in skeletal muscle damage and fatigue. Oxidative stress is thought to be one possible mechanism to activate NF-κB, which is an important transcription factor for expression of pro-inflammatory proteins. In the present study the elevated expression of muscle NF-κB by training alone was reduced ($p < 0.05$) when EDHB was supplemented along with training. Increased NF-κB resulted in elevation of TNFα and IFN-γ in plasma of training group as compared to drug training group. NF-κB induced activation of TNFα is thought to be the one possible mechanism for damage of myofibril proteins by ubiquitin-proteasome degradation pathway [41].

Also, in cultured muscle cells TNFα with IFN-γ has been known to cause a down regulation of MyoD which plays an important role in myogenesis[42]. Reduced expression of NF-κB by EDHB resulted in decrease in the plasma levels of proinflammatory cytokines IFN-γ and TNF-α. In past few years extensive research has validated the role of IL 6 in contracting muscles as an anti- inflammatory cytokine [43]. In accordance, we have seen increase in the levels of IL6, IL10 and TGFβ in trained EDHB treated rats as compared to trained controls. Thus the boost in anti-inflammatory status on EDHB supplementation might be contributing towards protection against exercise induced muscle damage. Upregulation of Cox-2 is indicative of many inflammation-associated chronic disorders and a single bout of exercise can induce Cox-2 expression which is actually regulated by NF-κB [44].

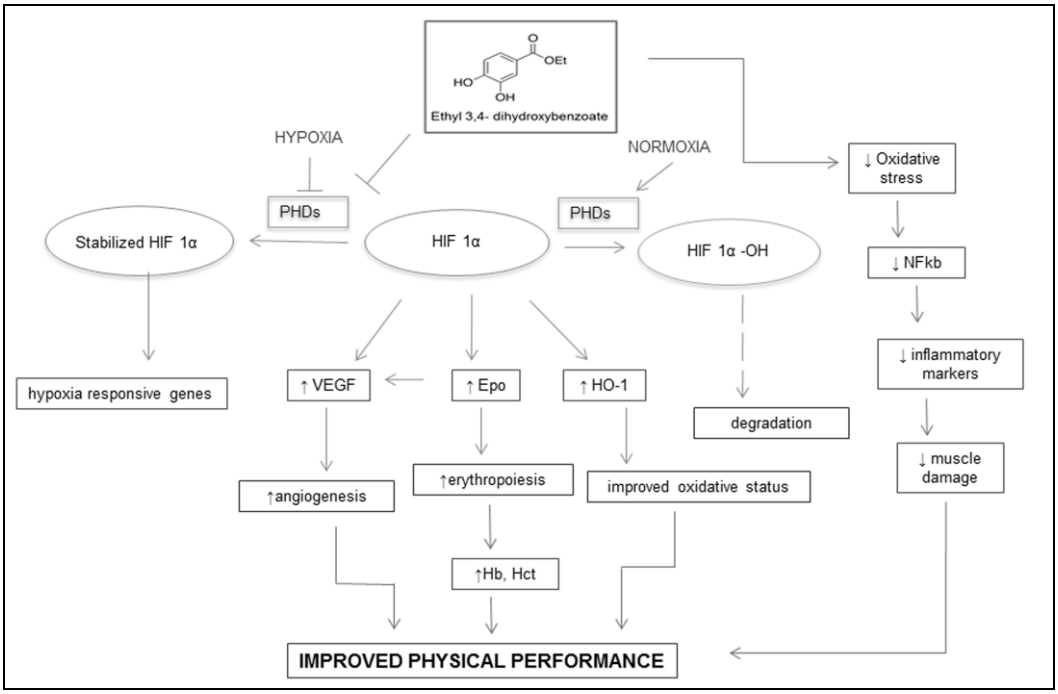

FIG. 7: PUTATIVE MECHANISM OF EDHB IN IMPROVING PHYSICAL PERFORMANCE. ↑ UPREGULATION BY EDHB SUPPLEMENTATION, ↓ DOWNREGULATION BY EDHB. HIF-1A, HYPOXIA INDUCIBLE FACTOR; EDHB, ETHYL 3,4-DIHYDROXY BENZOATE; PHDS, PROLYL HYDROXYLASE ENZYMES; VEGF, VASCULAR ENDOTHELIAL GROWTH FACTOR; EPO, ERYTHROPOIETIN; HO-1, HEMEOXYGENASE-1; HB, HAEMOGLOBIN; HCT, HAEMATOCRIT.

In present study there was decrease in the expression Cox-2 in EDHB treated sedentary as well as trained rats as compared to respective controls ($p<0.01$, **Fig.5).** Further, our histopathological studies have shown a decrease in muscle fibre damage in EDHB treated groups in comparison to controls (**Fig.6**). All these results thus suggest the protective role of EDHB in exercise induced oxidative and inflammatory damage.

CONCLUSION: Our study suggests that EDHB supplementation in rats enhances physical performance by switching on several intricate molecular pathways and protects muscle from exercise induced damage. HIF mediated augmentation in VEGF, Epo, HO-1 boosted blood oxygen carrying capacity by enhancing Hb, Hct, RBC. EDHB also protected muscle from free radical induced damage and inflammation by regulating antioxidant status and inflammatory markers. These findings provide the basis for the possible use of EDHB as a potent enhancer of physical performance and muscle protection by activation of cellular oxygen sensing mechanism helps to combats against exercise induced stresses.

AUTHOR'S CONTRIBUTION: CN and AB made substantial contributions to the conception and design, analysis and interpretation of data. CN contributed to the drafting of the manuscript, and performed experiments. DS and KB performed the western blotting studies. CN and GK carried out the biochemical assays and histological procedures. SS, DS and MS were involved in analyses of resulting data and revised the manuscript. All authors have given final approval of the manuscript.

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