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Biochemical Investigation into the Benefit of Quercetin Supplementation in Hypoxic Mice

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Abstract

Context: Since the proper flow of oxygen into the human body signifies one of the core features of living organisms, a shortage in supply poses a significant threat to survival.
Objective: This study aimed to investigate the effects of intermittent hypoxia on mice and the benefit of pre-administered quercetin on such effects.
Materials and Methods: Thirty (30) mice were procured and shared into five (5) experimental groups comprising six (6) mice each (n=6). Group I was the normal control group, group II was the negative group and groups III-V were the quercetin groups. Group I and II received distilled water while III-V received quercetin in different doses (10, 20 and 40 mg/kg p.o.). Also, all groups were subjected to the hypoxia protocol except group I. All treatments were carried out for seven (7) days. Afterwards, blood obtained via cardiac puncture, brains and lungs of select mice were respectively subjected to haematological evaluation and intense biochemical analysis. Also, specific lung tissue was subjected to histology using Hematoxylin and Eosin staining procedure.
Results: Quercetin significantly ($p<0.05$) slowed the rate of possible progression of systemic inflammation by enhancing antioxidant systems in the lungs and brain by increasing glutathione, catalase and superoxide dismutase activity in addition to displaying anti-inflammation by inhibiting the activity of myeloperoxidase. Also, quercetin significantly increased red blood cell and haemoglobin content when compared with the model group.
Conclusion: Quercetin attenuated hypoxia-induced biochemical alterations in major organs of the body which can be accredited to its antioxidative, neuroprotective and anti-inflammatory properties.

Keywords: Blood, Brain, Hypoxia, Lungs, Prooxidants, Antioxidants

1. Introduction

Oxygen is the element at the very heart of aerobic metabolism in mammals. A shortage in its supply, known as hypoxia, is the absence of sufficient volume of oxygen within the cell.[1] Hypoxia mostly originates from hypoxemia, dysfunctional oxygen delivery system, and/or impaired oxygen uptake system.[1] Since oxygen is regarded fuel for living organisms, high-altitude dwellers at above 3000m may experience progressive damage to the lungs, brain, heart, and other organs due to the insufficiency of oxygen available for inspiration.[2-4]

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Over the years, many preclinical studies have employed various models and techniques in a bid to enlist the physiologic and biochemical effect of hypoxia on selected organs of the human body such as the heart, brain, liver, kidneys, blood, etc, while others investigated the possible benefit of different drugs on these affected organs. In some of those

studies, hypoxia significantly induced morphological lesions in specific brain regions concomitant with behavioural/performance deficits, a decline in antioxidant activity,[5-7] an increase in leukocyte count which signals inflammation[8] and a reduction in haemoglobin content.[9]

In a bid to contribute to the existing literature on hypoxia, the authors of the current research sought to investigate the biochemical effects of hypoxia on major organs of the body and the possible benefit of pre-administered oral preparations of quercetin using Swiss albino mice.

2. Materials and Methods

2.1 Materials

Quercetin (QCN; 3, 3′, 4′, 5, 7-pentahydroxyflvanone; Sigma-Aldrich®, St. Louis, MO, USA) was the model drug used in this study. Dimethyl sulfoxide (DMSO) was selected as its solvent.

2.2 Subjects

A total of thirty (30) Swiss albino mice (male; n = 30; wt = 26.0±2.0 g) were used in the present study. They were provided by the animal house of Basic Medical Sciences, Delta State University, Abraka. Afterwards, they were allowed a week of acclimatisation at room temperature. According to the NIH guide for laboratory animals (NIH Publications No. 8023, revised 1978), the mice were exposed to equal hours in the light as well as the dark cycle environments while receiving rodent pellet diet and water ad libitum. All procedures in this experiment were in accordance with the ARRIVE guidelines and also approved by the ethics committee of the faculty.

2.3 Experimental design and treatment groups

At the end of the weeklong acclimatisation period, the mice were randomly divided by alternation into five (5) groups made up of six (6) animals each (n=6). Thereafter, grouping was done based on the substance of administration and exposure to hypoxic stress (HS).

I. Group I mice received vehicle of 10 ml/kg p.o. 5% DMSO and were allowed normal air within the laboratory, thus considered the normal control group;

II. Group II mice also received vehicle p.o. and were exposed to hypoxic stress, thus considered the negative control group;

III. Group III mice received 10 mg/kg quercetin in addition to exposure to hypoxic stress (i.e., QCN 10 mg/kg p.o. + HS);

IV. Group IV mice received 20 mg/kg quercetin in addition to exposure to hypoxic stress (i.e., QCN 20 mg/kg p.o. + HS);

V. Group V mice received 40 mg/kg quercetin in addition to exposure to hypoxic stress (i.e., QCN 40 mg/kg p.o. + HS).

These administrations were carried out daily for seven (7) consecutive days.

Induction of hypoxic stress was achieved by daily locking each mouse in a 250 mL container for 20 mins.[10] Note that exposure to the hypoxic stress protocol was done one hour after the drug administration for each day (Fig. 1). Afterwards, on the eighth day, bioassays of the extracted tissues and organs were carried out.

2.4 Collection of blood for haematological assays

Whole blood samples of selected mice from all groups were collected from the exposed heart into heparinized tubes.[11] An analysis including white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb) estimation and other blood volume parameters such as packed cell volume (PCV) were carried out. For more accuracy, these tests were carried out within ~10 min of blood withdrawal.[12]

2.5 Tissue Preparation for Biochemical assays

Also, selected mice from all groups were euthanized by exposure to chloroform and their respective brain and lungs were harvested. Thereafter, each brain and lung sample was weighed and homogenised in different 10% w/v phosphate buffers (0.1M, pH 7.4). The homogenates were then centrifuged at x104 rpm for 15 min at 4°C. The resulting supernatants were immediately extracted and used for the oxidant activity assays.[13]

2.5.1 Glutathione (GSH) concentration

To determine GSH concentration in brain and lung tissues, 0.4 ml of 20% TCA was added to 0.4 ml of the individual homogenates and centrifuged using a cold centrifuge at 10,000 rpm for 20min to obtain

the supernatants. Following the Beutler method,[14] 0.25ml of that supernatant was added to 2ml of 0.6mM DTNB to produce 2-nitro-5-thiobenzoic acid which triggers a change in absorbance. This change was measured using a UV/VIS spectrophotometer (Techmel and Techmel® USA) at 412 nm against a blank. The concentrations of GSH in the lung and brain tissues were expressed as µmol/g tissue.

2.5.2 Malondialdehyde (MDA) concentration

The degree of lipid peroxidation, MDA, was approximated using Okhawa's method.[15] A mixture of 0.4 ml of the individual brain and lung supernatants, 1.6 ml of Tris–KCl buffer, and 0.5 ml of 30% TCA was formed. Then, 0.5 ml of 0.75% TBA was added and placed in an 80°C water bath for 45 min. Afterwards, the reaction was cooled and centrifuged for 15 min at 3x103 rpm. The colour of each resulting supernatant was measured with a spectrophotometer at 535 nm and expressed as µmol/g tissue.

2.5.3 Superoxide dismutase (SOD) activity

This was carried out according to Sirota,[16] with minimal modifications and SOD activity was expressed as unit/min/mg protein.

2.5.4 Catalase activity

Catalase (CAT) activity was estimated using a modified method described by Luck[17] and Aebi.[18] The catalase enzyme activity was expressed as µmol of H_2O^2 decomposed per minute/mg protein.

2.5.5 Nitrite concentration

Brain and lung nitrite concentration was determined using Greiss reagent in line with Giustarini's study.[19] Greiss reagent (100 µl) was added to 100 µl of the individual supernatants and absorbance was measured at 540 nm. The nitrite concentration was therefore estimated from a standard curve obtained from sodium nitrite (0-100 uM).

2.5.6 Myeloperoxidase activity

Lung myeloperoxidase (MPO) activity was determined based on established protocols by Eduviere[20] and Gorudko.[21] Finally, MPO activity was estimated by adding 0.2 ml of the supernatant to 2.8 ml of a mixture containing 0.167 mg/ ml O-dianisidine in the potassium phosphate buffer and 0.15 mM H_2O^2. Then, the change in absorbance at 450 nm was monitored over 3 min using UV/VIS spectrophotometer.

2.6 Haematological assay

Red and white blood cells were estimated by simple counting using the haemocytometer as described by Rusia and Sood.[22] Also, the packed cell volumes (PCV) and haemoglobin contents were calculated using the microhematocrit method and the cyanmethemoglobin method respectively.[23]

2.7 Tissue Preparation for Lung Histology

Following euthanization, selected mice from each group were subjected to intracardiac perfusion using phosphate-buffered saline (PBS) and 10% neutral buffered formalin (NBF). Once the lungs were pale in colour, they were excised and tissue sections obtained were further suspended in NBF until paraffin wax embedment. However, they were resuspended in fresh NBF after the first twenty-four hours (24 h). Lung tissues (5–6 µm thick) from each group were processed using the routine method for paraffin wax embedment in preparation for histology and fixed on glass slides. The Hematoxylin and Eosin stain was administered to the paraffin wax embedded sections for cell quantification[24] and the sections were subjected to microscopy and digital photography. Also, neuronal density of each section was estimated by simple neuronal counts from the photomicrographs.

2.8 Statistical analysis

All experimental data obtained were presented as Mean ± standard deviation (SD). Result analysis was done using one way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test. Graph Pad InStat® Biostatistics software was also used to determine the level of significance for all tests which was set at $p < 0.05$.

3. Results

3.1 Effect of quercetin on haematological alterations in hypoxic mice

The blood of group II mice which were exposed to only hypoxia revealed significant ($p<0.05$) changes to important blood parameters – an increase in WBC count, a decrease in RBC count, Hb content (Table 1), and also a decrease in PCV (Fig. 2) – when compared to the normal control group I ($F(4,25)=10.02$; $p<0.0001$). In all tests, the quercetin groups III-V recorded significant ($p<0.05$) reversal of these alterations.

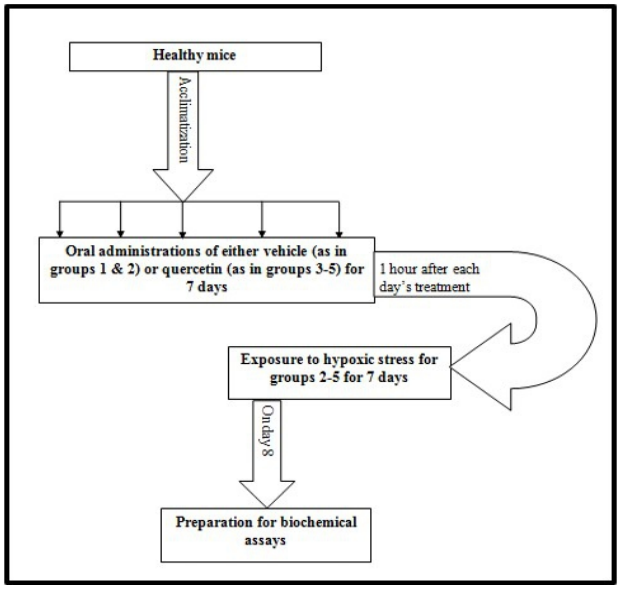

Fig. 1 Experimental schedule

Table 1: Effect of quercetin on lung oxidative profile in hypoxic mice

Groups	WBC count ($x10^3/mm^3$)	RBC count ($x10^3/mm^3$)	Hb (g/dL)
Group I	5.335 ± 1.01	11.16 ± 1.62	15.77 ±1.46
Group II	13.42 ± 1.23#	4.580 ± 1.39#	6.80 ± 1.62#
Group III	9.755 ± 0.98*	7.677 ± 1.24*	9.767 ± 1.48*
Group IV	8.333 ± 1.22*	8.843 ± 1.05*	12.45 ± 1.66*
Group V	6.472 ± 0.95*	10.76 ± 1.22*	13.29 ± 1.79*

Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II
RBC, red blood cell; WBC, white blood cell; Hb, haemoglobin

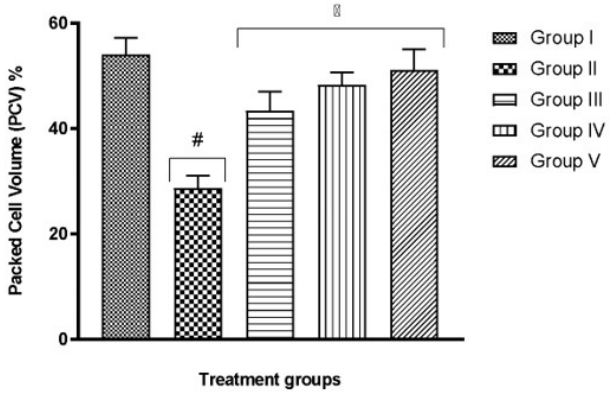

Fig. 2 Effect of quercetin on blood cell volume of hypoxic mice
Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II

Table 2: Effect of quercetin on lung oxidative profile in hypoxic mice

Group	MDA (µmol/g tissue)	SOD (unit/mg protein)	CAT (unit/mg protein)	GSH (µmol/g tissue)
Group I	0.3611 ± 0.12	39.07 ± 4.76	45.34 ± 4.64	42.51 ± 5.69
Group II	0.8806 ± 0.10#	18.54 ± 4.75#	21.92 ± 4.94#	14.26 ± 4.08#
Group III	0.4825 ± 0.07*	30.25 ± 5.58*	40.44 ± 9.25*	31.43 ± 7.21*
Group IV	0.4417 ± 0.06*	32.22 ± 5.68*	43.63 ± 4.22*	36.67 ± 7.86*
Group V	0.3861 ± 0.09*	41.25 ± 6.46*	48.24 ± 8.0*	39.01 ± 6.43*

Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II
MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione

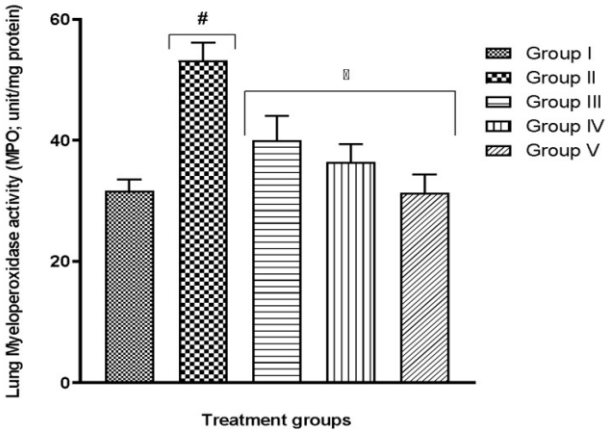

Fig. 3 Effect of quercetin on lung myeloperoxidase activity in hypoxic mice
Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II

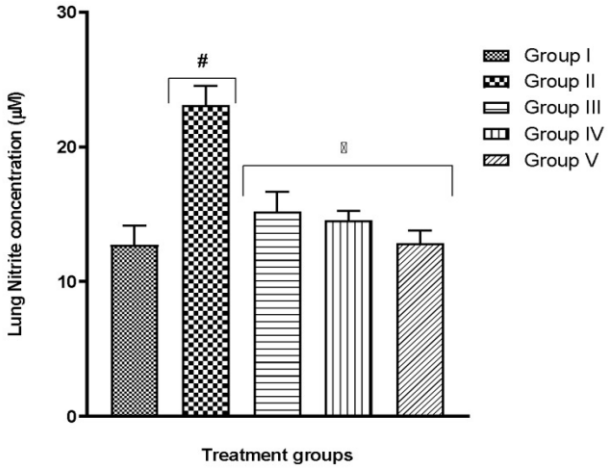

Fig. 4 Effect of quercetin on lung nitrite concentration in hypoxic mice
Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II

Table 3 Effect of quercetin on brain antioxidants in hypoxic mice

Groups	SOD (unit/mg protein	CAT (unit/mg protein	GSH (µmol/g tissue
Group I	53.40± 5.87	70.25± 6.30	40.48± 4.28
Group II	26.2± 7.33#	37.39± 7.00#	16.05± 3.71#
Group III	43.42± 5.53*	59.08± 7.89*	30.23± 3.46*
Group IV	48.99± 9.73*	64.00± 10.32*	34.61± 3.44*
Group V	52.7± 3.21*	68.53± 9.09*	38.10± 3.24*

Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II
SOD, superoxide dismutase; CAT, catalase; GSH, Glutathione

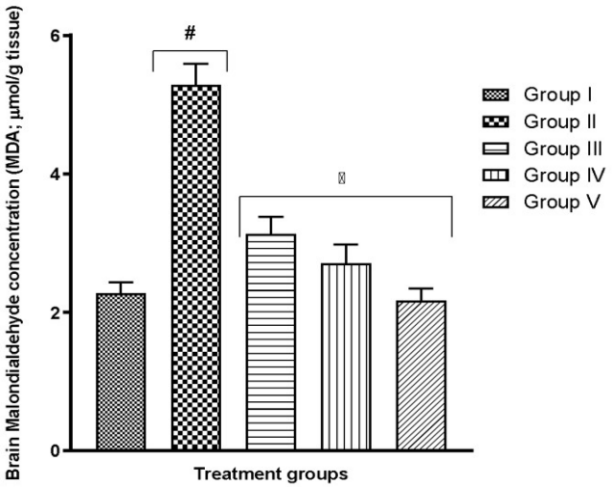

Fig. 5 Effect of quercetin on brain prooxidant level in hypoxic mice
Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II

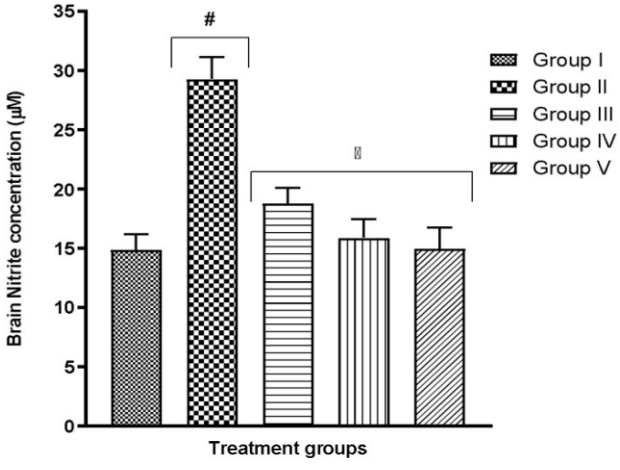

Fig. 6 Effect of quercetin on brain nitrite level in hypoxic mice
Results are expressed as mean ±SD
represents p< 0.05 compared to group I
* represents p< 0.05 compared to group II

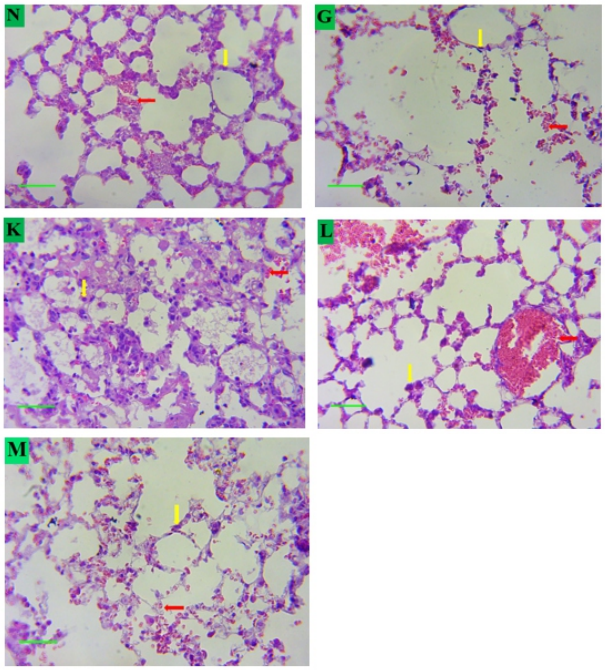

Fig. 7 Photomicrograph showing the effect of quercetin on the histology of the lung alveoli tissue in mice exposed to hypoxic stress
Key:
N – Group I.
G – Group II.
K – Group III.
L – Group IV.
M – Group V.

Red arrow: Pulmonary capillaries. Yellow arrow: Nuclei
Magnification: X400.

3.2 Effect of quercetin on lung oxidative activity in hypoxic mice

The lung tissue of group II mice which were exposed to only hypoxia revealed significant ($p<0.05$) changes to important lung oxidants – an increase in MDA content, a decline in SOD activity, CAT activity, GSH content (Table 2), and also an increase in MPO activity and nitrite level (Fig. 3 and 4, respectively) – when compared to the normal control group I ($F(4,25)=12.13$; $p<0.0001$) . In all tests, the quercetin groups III-V recorded significant ($p<0.05$) reversal of these alterations.

3.3 Effect of quercetin on brain oxidative activity in hypoxic mice

Tissues from selected brain regions of group II mice

which were exposed to only hypoxia revealed significant ($p<0.05$) changes to biologically-active brain antioxidants (Table 3) and prooxidants (Fig. 5 and 6) – a reduction in GSH content, a decline in SOD and CAT activity, as well as an increase in MDA and nitrite levels – when compared to the normal control group I ($F(4,25)=29.37$; $p<0.0001$, ($F(4,25)=14.73$; $p<0.0001$)). In all tests, the quercetin groups III-V recorded significant ($p<0.05$) reversal of these alterations.

3.4 Effect of quercetin on lung histology in hypoxic mice

As shown in Fig. 7, alveolar epithelial cells and pulmonary capillaries responsible for tissue perfusion of the hypoxia only group (group II) were significantly damaged when compared to the normal control (group I). This could be linked to increased lung inflammation as evidenced in the increase of MPO and prooxidant activity (Fig 3 and Table 2) due to hypoxia. However, significant tissue repair and resolution of inflammatory processes was observed in groups that received quercetin as the alveolar cells were protected from inflammation-induced apoptosis and alveoli damage.

4. Discussion

Hypoxia is a common experimental model of physiological stress. As with all other stressors, there are pre-programmed sensors within the human body with instructions to induce appropriate responses to stress. The two most powerful responders are the sympatho-adrenomedullary (SAM) and the hypothalamic-pituitary-adrenal (HPA) axes.[25,26] Over the years, low oxygen supply has been considered a major threat to the normal physiologic wellbeing of organisms. As a matter of fact, a wide range of conditions have been linked to hypoxia in previous studies: cardiac arrest, seizures, arrhythmia, respiratory diseases,[27] anxiety disorders, depression and mood changes.[28] In this current study, the authors sought to investigate the simple biochemical changes that occur in the vital organ systems of mice using an intermittent respiratory hypoxia model. Therefore, the level and activity of some reactive oxygen species (ROS) and enzymes in the lungs and brain were estimated as well as the levels of relevant blood constituents.

More recent studies proceeded to link a shortage in oxygen supply to an increase in the generation of ROS.[29-39] Although these molecules are useful in particular conditions, higher levels might result in an increase in lipid peroxidation.[29,40] As a result, malondialdehyde (MDA) production may become excessive thereby frustrating the efforts of defensive enzymes superoxide dismutase (SOD) and catalase (CAT). This is evident in the present study where the highest MDA and nitrite levels were seen in the group exposed to hypoxia alone (Table 2, Fig. 4-6). It is also evident that the defence enzymes CAT, SOD and GSH in the brain and lungs were diminished in the hypoxia-only group (Table 2, Table 3). Similar to MDA, the activity of MPO was also significantly elevated in the lungs of the hypoxia-only group (Fig. 3 and 4). This increase in MPO activity in the lungs is most likely an indicator of the outset of inflammation as previously postulated that hypoxia can cause inflammation.[41]

According to Yoon and Ponka,[42] hypoxia is an important stimulus for the secretion of erythropoietin, a precursor for red blood cell (RBC) production. A previous study on hypoxia revealed that acute hypoxia increases erythropoietin levels which subsequently increase RBC production, packed cell volume (PCV), haemoglobin (Hb) concentration and total blood volume.[43-45] This does not correlate with the present study which studied the effect of a seven-consecutive day exposure to hypoxia. Here, chronic hypoxia led to a significant increase in white blood cells (WBC) which could mean inflammation just like MPO, and a decrease in RBC count, Hb concentration and PCV.

Finally, the results from this present study generally revealed that chronic hypoxia can cause damage to the central nervous system, the pulmonary and circulatory systems by encouraging oxidative stress and inflammation. However, this present study also revealed that pre-treatment with quercetin slowed the whole process of damage significantly and opposed specific system deterioration. Quercetin exhibited its anti-inflammatory activity by opposing the activity of lung MPO and excessive production of WBCs in the quercetin-treated groups.[46] These effects were further confirmed by the histological analysis of the lung alveoli tissues which described a role of quercetin in alleviating the inflammation-induced alveoli damage induced by hypoxia.[47] Also, its neuroprotective and

antioxidative activity were exhibited by potentiating the production of antioxidative defence enzymes GSH, CAT and SOD in the quercetin-treated groups.[48] Blood constitution of the quercetin-treated groups was brought towards normal through, what the authors believe is, the hepatoprotective effect of quercetin since erythropoietin secretion is done by the kidneys.[49] The clinical implication of these findings is that quercetin consumption from various sources must be encouraged in individuals who often indulge in activities or suffer from diseases that may limit their oxygen supply as a means to improve their health regardless.

In conclusion, quercetin possesses the ability to counter the adverse effects of hypoxia across the various body systems.

Declaration of interests

The authors declare that they have no conflicts of interest.

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Ethical statement

This research was approved by the institutional animal care and use committee (REF/FBMS/DELSU/21/105)

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