African Journal of Pharmaceutical Research & Development

Vol. 14 No.1; pp. 040-051 (2022)

QUERCETIN SUPPLEMENTATION MODULATED EXPLORATORY AND ANXIETY-RELATED BEHAVIOURS IN MICE SUBJECTED TO HYPOXIC STRESS

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ABSTRACT

A majority of central nervous system (CNS) disorders such as Alzheimer's disease, Parkinson's disease, etc. have been linked to the excess production of oxidative radicals over antioxidants; a phenomenon known as oxidative stress. More so, the level of oxygen present in the system is a crucial player in this process and thus fuels cellular functioning. This research therefore seeks to assess the effect of insufficient oxygen on specific behaviours of mice and the therapeutic potential of quercetin. Thirty (30) mice were procured for this research. They were randomly divided into five groups of six mice each (i.e., n=6) with group 1 serving as normal control receiving vehicle, group 2 as stressed control receiving vehicle and groups 3-5 receiving 10, 20 and 40 mg/kg quercetin, respectively. Only groups 2-5 were subjected to the hypoxia protocol. Afterwards, mice from all groups were tested for behavioural changes using tests for anxiety and movement patterns. Furthermore, histology of specific brain regions was carried out. Data were analyzed using one-way ANOVA and post-hoc tests at $p<0.05$ significance. The results revealed that mice that were subjected to hypoxia only exhibited behaviours that suggested increased anxiety and reduced movement patterns, and decreased number of viable neurons in specific brain regions. These effects were however significantly attenuated in the quercetin-treated groups. In conclusion, dietary quercetin is beneficial for brain health, behaviour and cellular functioning in mice exposed to hypoxia-induced stress or hypoxic environments.

KEYWORDS: **Oxygen; Hypoxia; Anxiety; Movement; Oxidative stress.**

INTRODUCTION

Generally, cells require oxygen as a precursor to drive the various cellular processes in a living organism. Maintaining a properly oxygenated internal environment is therefore crucial for the proper function of these cells and even organisms at large [1-3]. Of all known cells, the cells of the brain have been described as utilizing the most amount of available oxygen. This indicates that the brain has a significantly higher activity and energy requirement which will be adversely affected in the face of even a minute reduction in oxygen levels [4].Hypoxia, which simply refers to reduced oxygen levels, elicits a wide range of physiological changes in cells as a means of adaptation [3, 5].These responses to hypoxia depend on various factors [6-9] and have been previously linked to numerous human diseases such as inflammation, pulmonary diseases, stroke, anxiety, schizophrenia, depression etc. [3,10-11]. However, if oxygen delivery is completely shut off, neuronal death may occur within minutes as seen in most human tissues [3, 12-15]. In other studies, hypoxia has been linked to oxidative stress [9, 16-

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17]. Therefore, therapies targeting hypoxia have become viable choices for associated diseases [3]. One of such is nutritional flavonoids.

Certain flavonoids, such as quercetin (QCN), present in diet have been described as being very efficient towards improving health. Among its numerous benefits, the antioxidant and anti-inflammatory effects of quercetin are crucial in its role in neuronal survival [21, 22-24]. This study was therefore orchestrated to evaluate the effect of quercetin in mice previously exposed to hypoxia while noting specific behavioural changes.

MATERIALS AND METHODS

Animals

Thirty (30) Albino mice weighing between 22-26 g were the experimental animals used in this study. These were procured from the faculty animal house of Basic Medical Sciences, Delta State University, Abraka. Afterwards, they were housed in plastic cages at room temperature and allowed to acclimatize for about a week before commencing the experiment. In doing this, mice were exposed to equal hours of light and darkness accompanied with an adequate rodent diet. Also, proper ethical certification was acquired from the institution (REC/FBMS/DELSU/21/105).

Drug preparation

Quercetin (QCN) was obtained from Sigma-Aldrich, St. Louis, MO, USA. A stock solution was then prepared by the method earlier described [25, 26]. First, 80 mg QCN was dissolved in 40 mL distilled water to obtain the needed concentration. Quercetin was administered orally in order to truly evaluate the nutraceutical benefit of QCN. Initial pilot studies set the doses of QCN in this study at 10, 20 and 40 mg/kg.

Treatment groups

Five groups were created based on the treatment substance administered. These groups are: group 1 is the non-stress control group which received 10 mL/kg distilled water (i.e., vehicle); group 2 is the stress control group which also received 10 mL/kg distilled water (i.e., vehicle); group 3 received 10 mg/kg quercetin; group 4 received 20 mg/kg quercetin; group 5 received 40 mg/kg quercetin. Treatment lasted for seven (7) days.

Experimental protocol

Hypoxic stress was induced in mice by following the protocol earlier described by Knaupp and Aluko respectively [27, 28] with slight modifications. One hour after drug administrations, mice were individually locked in an airtight container of 250 mL capacity for 20 min daily for seven (7) consecutive days. On day 8, behavioural tests for anxiety and movement patterns were carried out between 9 am and 12pm.

Behavioural tests

Assessment of anxiety levels

- **Using the elevated plus maze (EPM) test**

The EPM apparatus is made up of two open arms (uncovered boards with dimensions) and two closed arms (covered boards with dimensions). Both arms run from a common central platform and the entire apparatus is elevated to a height of about 25 cm above the floor. To measure anxiety level, each mouse was placed at the edge of one open arm, with its head facing the centre, and allowed to explore the maze for a period of 5 min. The time spent in each arm were then recorded [29].

- **Using the light /dark transition test**

The light/dark box (LDB) consists of a rectangular box partitioned into two compartments – an illuminated one and a dark one – connected by a small opening in the wall between the compartments. Similar to the EPM, each mouse was placed in the illuminated compartment of the box and the times spent in each compartment were measured within a 5 min session [30].

Assessment of movement patterns

- **Using exploratory behaviours**

The indices of exploratory behaviour include rearing, grooming, duration of ambulation and number of lines crossed. The open field chamber was also utilised to assess exploratory behaviour in the mice. Each mouse was allowed to explore freely in the chamber for 5 min. During this time, the duration of ambulation, number of lines crossed, cumulative frequencies of rearing and grooming, respectively, were recorded [31].

- **Using the rotarod**

The Ugo Basile automated rotarod device was used in this test. Each mouse was placed on the rotating bar of the rotarod set at 5 rpm. The duration the mouse spends on the bar before falling, which is referred to as the latency of fall (LOF), was used as the index of movement patterns [32].

- **Using catalepsy**

The bar test for catalepsy was carried out by carefully placing the forelimbs of each mouse on a

horizontal plane wood surface, allowing it to walk on the bar and recording the duration (in seconds) of ataxia. Ataxia refers to the loss of motor coordination, that is, the period the mouse remained immobile [33].

Preparation of brain tissues for histology

Brain tissue slices (5–6 µm thick) of the amygdala and hippocampal cornus ammonis 1 (CA1) regions were obtained using a microtome. These slices were subsequently subjected to paraffin wax embedment used in histological studies [34]. The Hematoxylin & Eosin (H&E) staining technique was used for proper cell quantification and the sections were fixed on glass slides for microscopy (at x400 magnification) and photomicrography (using a digital camera). Viable neuronal density of each group was also extrapolated from the micrographs; neuronal cell counts were based on the number of neuronal nuclei in three (3) rectangular boxes in each slide, using pre-calibrated Image J software [35].

Statistical analysis

Statistical analysis was performed with Graph Pad InStat® Biostatistics software (version 6). One-way ANOVA and Bonferroni's Multiple Comparison tests were used for comparisons between data groups. The results are expressed as mean ± standard error of mean (S.E.M) and p values less than 0.05 ($p < 0.05$) were considered significant.

RESULTS

Effect of quercetin on anxiety-like parameters

Two major apparatuses were used to assess anxiety-like behaviour in this study – the elevated plus maze (EPM) and light/dark box (LDB) tests. In both tests, hypoxic mice of the stress control group spent significantly ($p<0.05$) more time in the closed arm of the EPM as well as the dark compartment of the LDB than mice of the non-stress control group. On the other hand, mice in the quercetin groups spent significantly ($p<0.05$) less time in the dark and more time in the light compartments of both apparatuses. Tables 1 and 2 below depict the effect of hypoxia and quercetin on the indices of anxiety-like behaviour in mice using the EPM and LDB respectively.

Effect of quercetin on exploratory behaviours and movement patterns

Hypoxic mice, that is, the stress control group demonstrated a significant ($p<0.05$) reduction in exploratory behaviours in the open field test (Table 3) when compared to the non-stress control group. Similarly, hypoxic mice exhibited significantly ($p<0.05$) reduced motor coordination and balance in the rotarod test (Figure 1) and a longer duration of ataxia in the bar test (Figure 2) when compared to the non-stress control group. On the other hand, mice that received the varying doses of quercetin exhibited significantly ($p<0.05$) enhanced motor function as observed in the various tests.

Histological evaluation of specific brain regions of mice exposed to hypoxic stress

In this current study, Figure 3 presents the photomicrographs showing the hippocampal CA1 region in selected mice from all treated groups. Slide G, which represents the stress control group shows significant ($p<0.05$) depletion in the number of normal neuronal cells when compared with slide N, the non-stress control group. Slides K, L, and M show the three respective quercetin groups, from which it can be observed that neuronal cell degeneration was significantly ($p<0.05$) reduced. This is also buttressed in Figure 4 where the neuronal density of the hippocampal CA1 region across all groups is presented.

Also, Figure 5 presents the photomicrographs showing the amygdala region in selected mice from all treated groups. Slide G, which represents the stress control group shows significant ($p<0.05$) depletion in the number of normal neuronal cells when compared with slide N, the non-stress control group. Slides K, L, and M show the three respective quercetin groups, from which it can be observed that neuronal cell degeneration was significantly ($p<0.05$) reduced. This is also buttressed in Figure 6 where the neuronal density of the amygdala region across all groups is presented.

DISCUSSION

Our findings reveal that depletion in the oxygen content of cells is involved in the pathophysiology of certain neuro-related disorders such as anxiety. Furthermore, we observed that the anxiolytic effect of quercetin during hypoxic conditions is linked to its antioxidant property.

Our results demonstrated that hypoxia resulted in increased anxiety-like behaviour in mice subjected to the elevated plus maze (EPM) and light/dark box (LDB) tests. The EPM is a known pharmacological tool based on two normal rodent behaviours: the tendency to explore the surroundings (the open arm, indicating anxiolysis) and the tendency to stay in a safe surroundings (the closed arm, indicating anxiogenesis/anxiety) [29]. In both tests, mice of

Table 1: Effect of quercetin on anxiety-like behaviour of hypoxic mice in the elevated plus maze test

Treatment	Exploration time (s)	
	Open arm	**Closed arm**
VEH 10 mL/kg	141.8±5.17	156.8±3.78
VEH 10 mL/kg + HS	98.50±5.55#	199.7±3.77#
QCN 10 mg/kg + HS	131.0±4.74*	171.7±4.09*
QCN 20 mg/kg + HS	133.5±5.23*	163.8±4.70*
QCN 40 mg/kg + HS	140.2±3.75*	157.5±5.06*

depicts significance ($p < 0.05$) compared to the non-stressed control group.
* depicts significance ($p < 0.05$) compared to the stress control group.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

Table 2: Effect of quercetin on anxiety-like behaviour of hypoxic mice in the light/dark transition test

Treatment	Exploration time (s)	
	Light compartment	**Dark compartment**
VEH 10 mL/kg	146.2±4.69	151.2±6.65
VEH 10 mL/kg + HS	88.17±5.87#	206.8±3.89#
QCN 10 mg/kg + HS	125.2±5.50*	172.0±6.86*
QCN 20 mg/kg + HS	136.7±4.48*	160.8±5.03*
QCN 40 mg/kg + HS	145.3±4.15*	150.2±6.44*

depicts significance ($p < 0.05$) compared to the non-stressed control group.
* depicts significance ($p < 0.05$) compared to the stress control group.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

Table 3: Effect of quercetin on exploratory behaviour of mice exposed to hypoxia in the open field test

Treatment	Grooming Frequency	Rearing Frequency	Number of lines crossed	Ambulation (s)
VEH 10 mL/kg	54.67±3.57	59.67±4.83	118.20±7.98	202.70±10.49
VEH 10 mL/kg + HS	14.50±3.19#	17.83±3.74#	47.83±7.91#	109.00±9.91#
QCN 10 mg/kg + HS	38.33±4.18*	42.00±3.37*	90.17±7.53*	157.70±12.19*
QCN 20 mg/kg + HS	42.83±3.54*	48.17±3.05*	103.00±6.01*	176.50±9.66*
QCN 40 mg/kg+ HS	50.17±4.56*	55.50±4.60*	112.00±8.21*	196.80±12.65*

depicts significance ($p < 0.05$) compared to the non-stressed control group.
* depicts significance ($p < 0.05$) compared to the stress control group.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

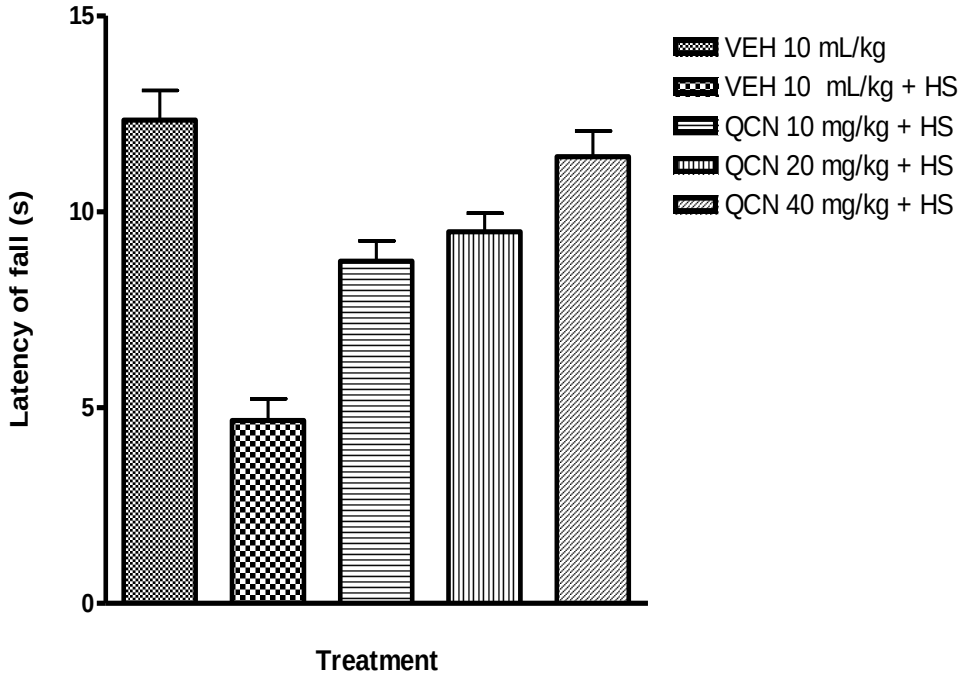

Figure 1: Effect of quercetin on motor coordination of mice exposed to hypoxia in the rotarod test.
depicts significance ($p < 0.05$) compared to the non-stressed control group.
* depicts significance ($p < 0.05$) compared to the stress control group.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

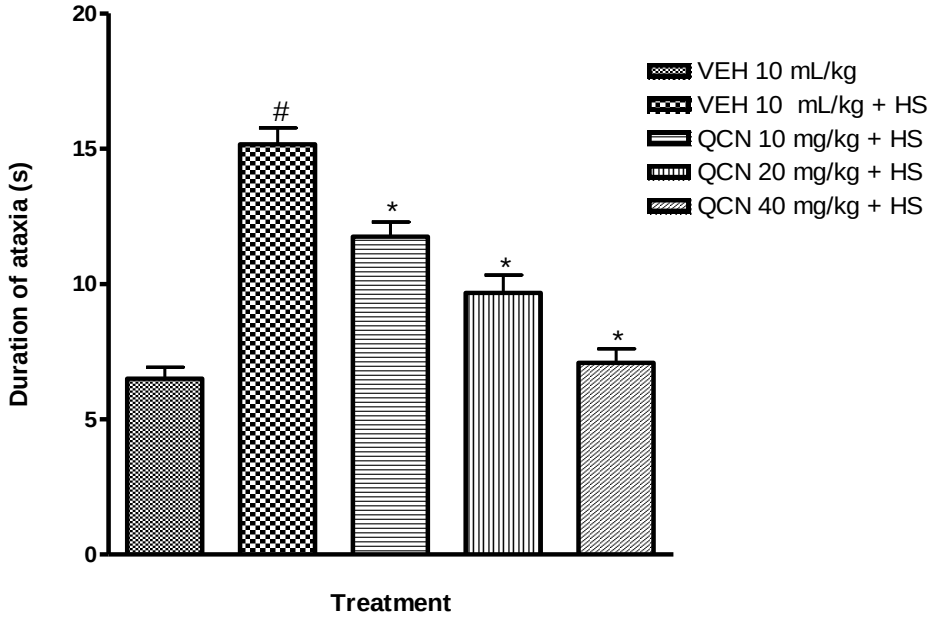

Figure 2: Effect of quercetin on cataleptic behaviour of mice exposed to hypoxia in the bar test.
depicts significance ($p < 0.05$) compared to the non-stressed control group.
* depicts significance ($p < 0.05$) compared to the stress control group.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

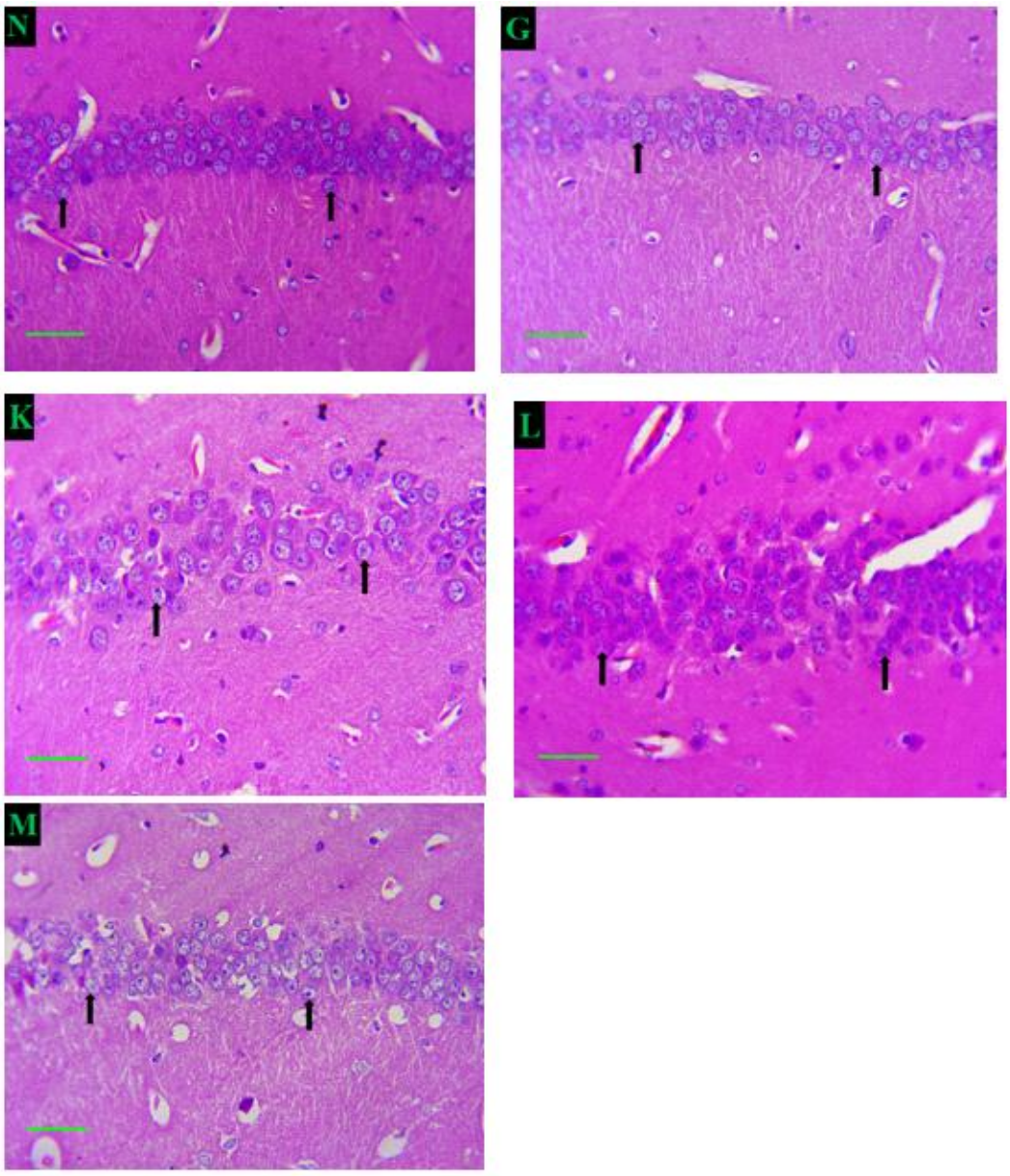

Figure 3: Photomicrograph showing the effect of quercetin on the neuronal cells of the hippocampal CA1 region in mice exposed to hypoxia.
Key:
N –VEH 10 mL/kg.
G – VEH 10 mL/kg+HS.
K – QCN 10 mg/kg+HS.
L– QCN 20 mg/kg+HS.
M – QCN 40 mg/kg+HS.
Black arrow: Normal neuronal cells.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

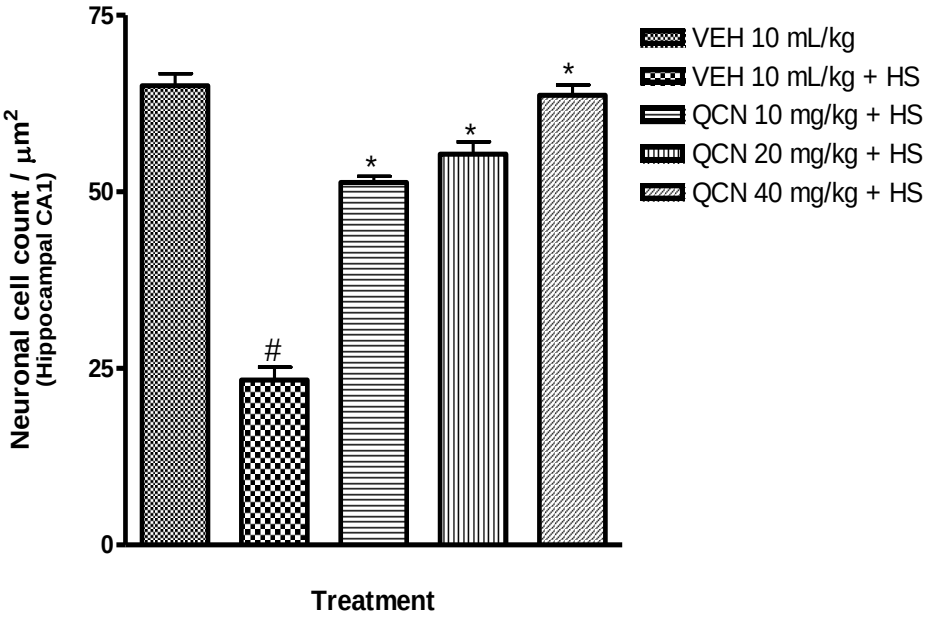

Figure 4: Effect of quercetin on viable hippocampal CA1 neurons in mice exposed to hypoxia.
depicts significance ($p < 0.05$) compared to the non-stressed control group.
* depicts significance ($p < 0.05$) compared to the stress control group.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

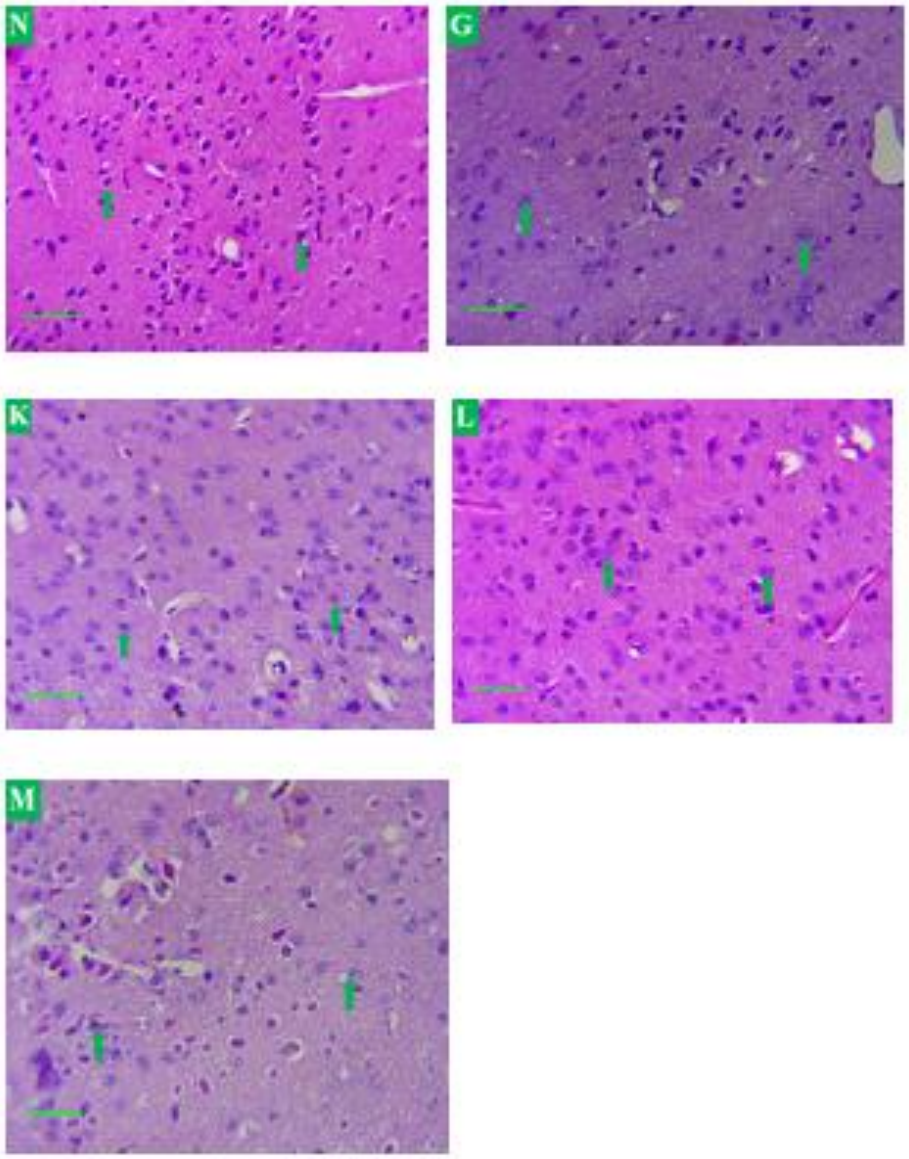

Figure 5: Photomicrograph showing the effect of quercetin on the neuronal cell counts of the amygdala in mice exposed to hypoxia.
Key:
N –VEH 10 mL/kg.
G – VEH 10 mL/kg+HS.
K – QCN 10 mg/kg+HS.
L– QCN 20 mg/kg+HS.
M – QCN 40 mg/kg+HS.
Green arrow: Normal neuronal cells.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

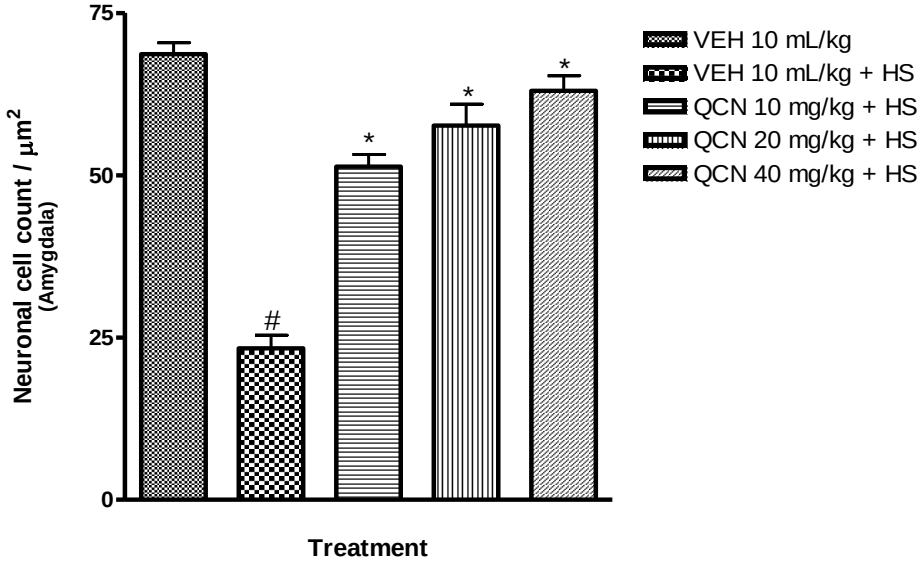

Figure 6: Effect of quercetin on viable amygdala neurons in mice exposed to hypoxia.
depicts significance ($p < 0.05$) compared to the non-stressed control group.
* depicts significance ($p < 0.05$) compared to the stress control group.
VEH – Vehicle. **QCN –** Quercetin. **HS –**Hypoxic Stress.

stressed group displayed significant preference for the closed arm/dark compartment, indicating anxiety. The results from this study were however contrary to previous studies which demonstrated that hypoxia caused a decrease [36] or did not affect anxiety levels [9]. Although the hypoxia protocol of this current study differs from the cited, the observed anxiogenesis of our results agree with another study [37]. On the other hand, mice in the quercetin groups displayed significant preference for the open arm/light compartment of both apparatuses thereby indicating anti-anxiety. This is similar to an earlier study which showed the anxiolytic property of quercetin [38].

Also in this study, the indices of motor function were evaluated. Exploratory behaviours (rearing, grooming, and ambulation) require a significant degree of motor function and are therefore sufficient indices of motor control. Hypoxic mice, that is, the stress control group moved less distance, explored itself/its surroundings less often and displayed less motor coordination in the tests for motor function. This result aligns with a previous study which showed that rats (at postnatal day 7) subjected to hypoxia exhibited a steady decline in movement patterns, duration of rearing and total distance moved [36]. On the other hand, quercetin supplementation significantly attenuated these effects by stimulating the motor abilities of the stressed mice.

Finally, histological evaluation of two mice brain regions revealed the effect of hypoxia on the brain's neuronal network. The hippocampal CA1 and amygdala of mice of the stressed group experienced significant neuronal damage with signs of neuronal cell degeneration and reduced neuronal density. This is similar to a previous study which showed that rats which were exposed to severe hypobaric hypoxia for 3 and 7 days developed oxidative stress in the cortex and hippocampal areas [9, 38].

CONCLUSION

The brain requires a steady influx of oxygen to survive. Even for a short while, hypoxia is a threat to brain neurons and their survival. However, this study has demonstrated that nutritional flavonoids such as quercetin possess beneficial properties in such hypoxic conditions. Dietary supplementation is therefore encouraged especially among those who are prone to shortage in oxygen supply regularly such as factory workers and mountain climbers.

ACKNOWLEDGMENT

Authors acknowledge Mr. Godwin Ahatty and Mr. Patrick Ayeke, of the department of Pharmacology, DELSU, Abraka for assisting with the animal handling.

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