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Comparative assessment of brain and circulating oxidative stress biomarkers in weaned New Zealand White rabbits supplemented with microalga *Chlorella vulgaris* biomass

Sikiru A.B.[1,2*], Arangasamy A.[2], Makinde O.J.[3], Sa'aci Z.A.[4], Opoola E.[5], Kubrah, S.A.[4], and Bhatta R.[2]

[1]*Department of Animal Production, Federal University of Technology, Minna, Nigeria.*
[2]*Reproductive Physiology Laboratory, ICAR – National Institute of Animal Nutrition and Physiology, Bangalore, India.*
[3]*Department of Animal Sciences, Federal University, Gashua, Nigeria.*
[4]*Department of Agricultural Technology, Federal Polytechnic, Bida, Nigeria.*
[5]*Department of Animal Sciences, Ahmadu Bello University, Zaria, Nigeria.*

[*]***Corresponding Author:*** *akeembaba01@gmail.com* ***Phone Number:*** *+234-816-094-2976*

Target Audience: *Animal Scientists, Biologists, Neurologists, Pharmacologists and Veterinarians,*

Abstract

The brain is central to human and animal well-being but it requires a high amount of oxygen for its normal functioning and this makes it an organ highly vulnerable to oxidative stress damage. Therefore, for the promotion of normal physiological and cellular functions of the brain, antioxidant intake is very critical. This study investigated the antioxidant enzymatic activities in the brain by measuring activities of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) in connection with the circulating oxidative stress biomarkers of the serum and liver of New Zealand White rabbits supplemented with microalga Chlorella vulgaris biomass in addition to regular basal diets. The study involved a random distribution of 40 rabbits of eight weeks old into five experimental group using completely randomized design. The rabbits were observed for a period of 120 when they are being supplemented after which their blood, brain, and liver were collected for analyses. The results show that the total antioxidant capacity was higher in the brain of the supplemented rabbits ($P<0.05$). Although, there was no significant difference in the brain malondialdehyde concentrations, there were higher activities of antioxidant enzymes in the brain of the supplemented rabbits ($P< 0.05$). There was a lower concentration of the circulating malondialdehyde (MDA) in the serum and liver of the supplemented rabbits. The study concluded that Chlorella vulgaris intake led to reduced circulating malondialdehyde and increased activities of the brain antioxidant enzymes in the rabbits. The study indicated that the microalga Chlorella vulgaris contains antioxidant compounds that can cross the blood-brain barrier, which could be a very important therapeutic agent against oxidative stress-induced brain complications in animals and humans.

Keywords: *Brain, blood-brain barrier, Chlorella vulgaris, Oxidative stress, Rabbits.*

Description of the problem

The brain is one of the most important organs in the body playing roles ranging from coordination of neurological functions to energy metabolism. Physiologically, the brain exerts central effects throughout the bodily functional systems. Primarily, the brain is responsible for neurotransmission and receptor activities, metabolism, perception, motor control, homeostasis, learning, and memory management[1]. The brain is an independent organ from the

body's peripheral circulation system by being delimited with the blood-brain barrier which is a semi-permeable border preventing direct interaction between internal activities of the brain and the body circulatory system[2]. The blood-brain barrier is also responsible for preventing the crossing of pathogens and unwanted molecules into the brain. However, under some disease conditions and functional failures such as epilepsy, schizophrenia, liver damages, brain trauma, oedema, and neurodegenerative diseases; the blood-brain barrier becomes weaken thereby allowing the passage of unwanted materials including pathogenic organisms into the brain and this situation could be reportedly aggravated by oxidative stress[3].

However, *in-vitro* studies have demonstrated the capacity of the antioxidant crossing of the blood-brain barrier and also under *in-vivo* condition; metabolites of antioxidants were found to cross-over into the brain. This is because the metabolites were isolated from the brain and they were reported to be responsible for playing a lipophilic role by preventing lipid peroxidation in the brain[4]. Furthermore, according to Faria *et al*.[5], there was an *in-vivo* transcellular transport of catechin, quercetin, and cyanidin-3-glucoside in the brain of a rat model. These compounds are antioxidants with potentially protective effects against cancer and cardiovascular diseases[6]. Evidence supporting antioxidant crossing of the blood-brain barrier can also be deduced from results of carotenoids evaluation in the human brains which showed that carotenoids including lutein, zeaxanthin, cryptoxanthin and carotenes were found in the brain of babies. Although, significantly higher levels of these carotenoids were found in termed babies compared with preterm babies[7].

Based on the above premise, exogenous supplementation of microalgae and other materials rich in carotenoids can contribute to the protection of the brain against oxidative damage for the management of brain-related diseases and promotion of physiological well-being. Lutein and zeaxanthin which are reported to be associated with enhanced brain functions are present in abundant quantities in microalgae such as *Chlorella vulgaris*[8]. Meanwhile, internal synthesizing of these carotenoids is not possible and since they have the potential of crossing over the blood-brain barrier then they could be obtained by the brain from the pools of antioxidants in the serum and liver[9–11]. Therefore, this study was carried out on the hypothesis that *Chlorella vulgaris* biomass is rich in antioxidants, and when supplemented to diets meant for rabbits; its antioxidant principles can prevent circulating malondialdehyde and then enhance the brain antioxidant enzyme activities.

Materials and Methods

Chlorella vulgaris biomass supplementation

The *Chlorella vulgaris* biomass used in this study was an organically produced commercial microalga rich in antioxidant and other bioactive beneficial compounds procured from Seagrass Tech (Pvt.) Ltd, 600087 – India. The microalga was manufactured organically without use of synthetic chemical in closed batch production system, it was delivered as a fine green powder biomass screened for its safety of use in animal experimental consumption by evaluation of its heavy metal and microbial compositions. These assessments indicated that the microalga in its biomass form used in this study is safe for animals' consumption; all the details provided about

the *Chlorella vulgaris* biomass here were reported in a previous study[8].

Study location

The was carried out at the Experimental Livestock Unit (ELU) of the National Institute of Animal Nutrition and Physiology (NIANP), Bangalore, India. The agro ecological variables of the area indicated that it is located in a Tropical Savannah zone and it is located on coordinates 12.97^{o} North (longitude) and 77.56^{o} East (latitude). The average monthly temperature is 23.90 ^{o}C while warmest temperature is 27.60 ^{o}C, average annual rainfall is 970 mm and usually spread clearly in two distinct seasons as dry and wet seasons[12].

Animal ethics and approval of animal experimentation protocol

The approval for use of the rabbits for the experiment was obtained from joint approval of the Institutional Animal Ethics Committee (IAEC) of the National Institute of Animal Nutrition and Physiology, India, and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The animals were obtained at 8 weeks old from the Animal Facility of Biogen Laboratory (Pvt.), Ltd., India, and were bred following the guidelines for laboratory animals' management for experimental purposes in India.

Experimental design, diets and animal management

Completely randomized design was implemented for this study whereby the rabbits were randomly distributed into five experimental groups designated as Control, T_1, T_2, T_3 and T_4, respectively. There were 40 weaned rabbits in all with four replicates per dietary treatment, each replicate had two rabbits giving a total of 8 rabbits per each of the experimental group. The rabbits were provided with commercial laboratory pelletized rabbit feed *ad-libitum* as basal feed, while 0, 200, 300, 400 and 500 mg/kg of the *Chlorella vulgaris* were supplemented per each rabbit in the control, T_1, T_2, T_3, and T_4, respectively for 120 days period. Clean drinking water was provided through automatic nipple drinkers while the animal room was maintained at normal room temperature on a daily 16 hours light regime.

Collection and preparation of the blood, brain, and liver samples

Samples of blood were collected through the bleeding of the marginal ear veins from all the animals in each group while a random selection of four rabbits per group was implemented for choosing of rabbits which were sacrificed for collection of the brain and liver samples which were used for downstream oxidative stress evaluation. The blood samples were allowed to clot at room temperature after which serum was harvested from each sample[8]. Then, the entire brain of each decapitated rabbit was removed after opening the rabbits' skulls using a laboratory bone saw. The harvested brain samples were transported immediately on ice cold containers and preserved in – 80 ^{o}C refrigerators (Thermo Fischer Scientific, USA).

Determination of malondialdehyde concentrations and activities of the antioxidant enzyme activities

Oxidative stress biomarkers were determined as described in a previous study[13]. The concentration of malondialdehyde in the serum, brain, and liver samples was used as a marker of lipid peroxidation and the chemicals used for this

assay include sodium-n-dodecyl (Calibiochem, USA), acetic acid (Fischer Scientific, India), thiobarbituric acid (Sigma-Aldrich, USA), n-butanol, and pyridine (Fisher Scientific, India) which were all used to prepare lipid peroxidation assay according to procedures described by Ohkawa *et al*[14]. The assay contained 8.1 % sodium-n-dodecyl, 20 % acetic acid, 0.8 % thiobarbituric acid used in a reaction mixture containing 200 µL of sodium-n-dodecyl and samples each; and 1500 µL of thiobarbituric acid and acetic acid were boiled in the water bath for 60 minutes at 95 ^{o}C. After boiling, the mixtures were cooled on ice then the volume was made up to 5000 µL with distilled water and a mixture of n-butanol and pyridine; the new volume was centrifuged at 4000 rpm for 10 minutes at 4 ^{o}C. Organic supernatant layers collected there from were measured at 532 nm wavelength in a Multiskan microplate reader. The activities of antioxidant enzymes were determined using chemical assays for superoxide dismutase, catalase, and glutathione activities as described in a previous report[8].

Results and Discussion

There was significant (P< 0.05), effect of the *Chlorella vulgaris* intake on total antioxidant capacities of the rabbits' brain. The minimum total antioxidant capacity was found in the control diet (T_1) (4.62±0.23 mmol/mg) while maximum total antioxidant capacity (7.57±0.28 mmol/mg) was recorded in the T_2 group with 200 mg/kg *Chlorella vulgaris*. Similarly, the intake of the microalgae significantly affected the serum total antioxidant capacities (P< 0.05); but there were no significant differences in the total antioxidant capacities of the liver samples of the rabbits (Table 1). Although, rabbits in the T2 had the highest total antioxidant capacity (6.59±0.49 mmol/g) compared with the control group (5.46±0.09 mmol/g).

Table 1: Comparative effect of *Chlorella vulgaris* intake on total antioxidant capacities of New Zealand White rabbits

Parameter	Control 0 mg/kg	T1 200 mg/kg	T2 300 mg/kg	T3 400 mg/kg	T4 500 mg/kg
Brain (mmol/mg)	4.62±0.23^{b}	7.57±0.28^{a}	7.06±0.76^{a}	4.68±0.14^{b}	7.03±0.02^{a}
Serum (nmol/mL)	3.79±0.04^{b}	9.24±0.32^{a}	7.53±1.40^{a}	3.33±0.20^{b}	8.02±0.05^{a}
Liver (mmol/g)	5.46±0.09	5.90±0.38	6.59±0.49	6.02±0.44	6.04±0.06

Means with different superscripts along the same row are significantly different (P<0.05).

There was no significant (P>0.05) effect of the *Chlorella vulgaris* intakes on the malondialdehyde concentration of the rabbits' brains (Table 2). Although, the intake of the microalgae significantly (P<0.05) affected the serum and liver malondialdehyde concentrations. The serum malondialdehyde concentration of rabbits in the control group was the highest (23.90±1.65 nmol/mL), while the serum malondialdehyde concentration of the rabbits in the T_1 group was the lowest (15.36±2.24 nmol/mL). Similarly, liver malondialdehyde concentration of the control group was significantly (P<0.05) higher compared with other groups with the control having the highest (18.90±0.38 nmol/g) and T_1 being the lowest (0.53±0.15 nmol/g). Also, there was no significant difference in the brain concentration of the malondialdehyde but

there were significant differences in the activities of the endogenous antioxidant enzymes including superoxide dismutase and catalase (Fig. 1). There were higher activities of superoxide dismutase (SOD) in the treatment groups compared with the control. While mean activities of SOD were 6.68 U/mg of the brain in control animals which was the lowest; the activities of SOD in the T_4 were 13.3 U/mg of brain sample (Fig. 1).

Table 2: Comparative effect of *Chlorella vulgaris* intake on malondialdehyde concentrations of in the New Zealand White rabbit

Parameter	Control 0 mg/kg	T1 200 mg/kg	T2 300 mg/kg	T3 400 mg/kg	T4 500 mg/kg
Brain (nmol/mg)	2.37±0.55	3.15±1.10	3.90±1.30	2.40±1.36	3.25±1.10
Serum (nmol/mL)	23.90±1.65^{b}	15.36±2.24^{a}	16.87±0.94^{a}	19.08±0.91^{a}	16.77±1.33^{a}
Liver (nmol/g)	18.90±0.38^{d}	0.53±0.15^{a}	2.57±0.99^{b}	5.05±1.31^{c}	5.98±1.87^{c}

Means with different superscripts along the same row are significantly different (P< 0.05).

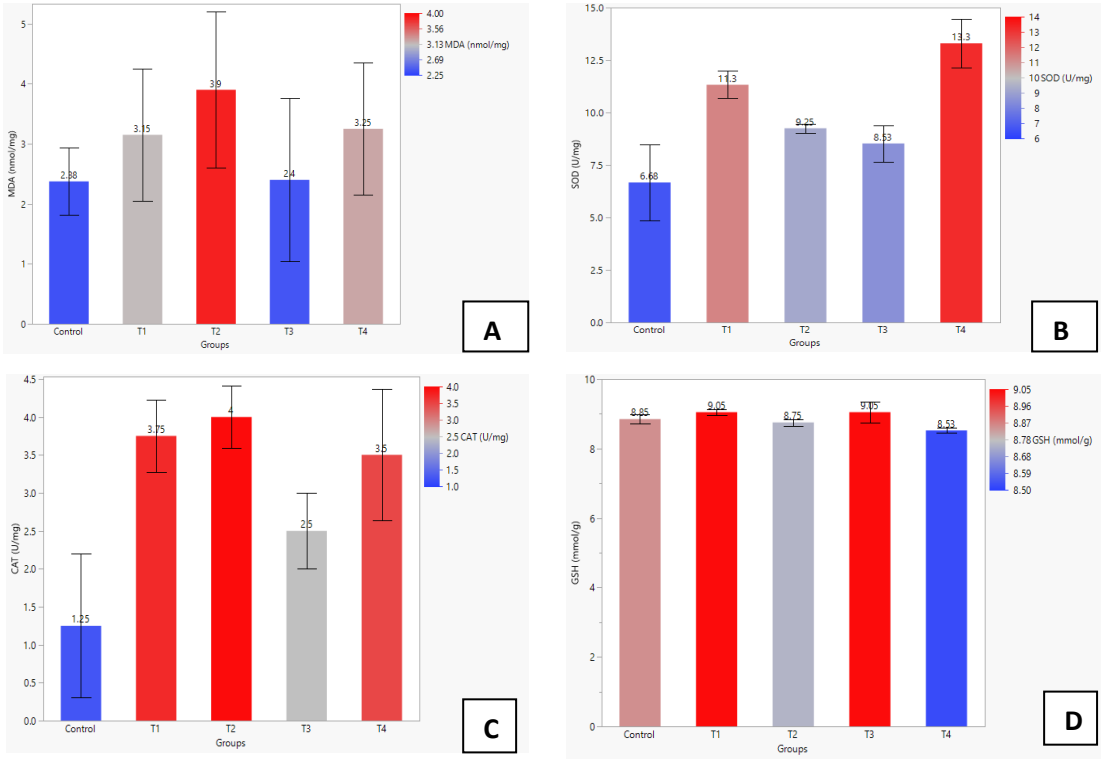

Fig. 1: Effect of *Chlorella vulgaris* supplementation on oxidative stress biomarkers in the brain of the rabbit models (a) effect on the malondialdehyde concentrations. (b) effect on the activities of superoxide dismutase enzyme (SOD) (c) effect on activities of catalase enzyme (d) effect on the concentration of reduced glutathione.

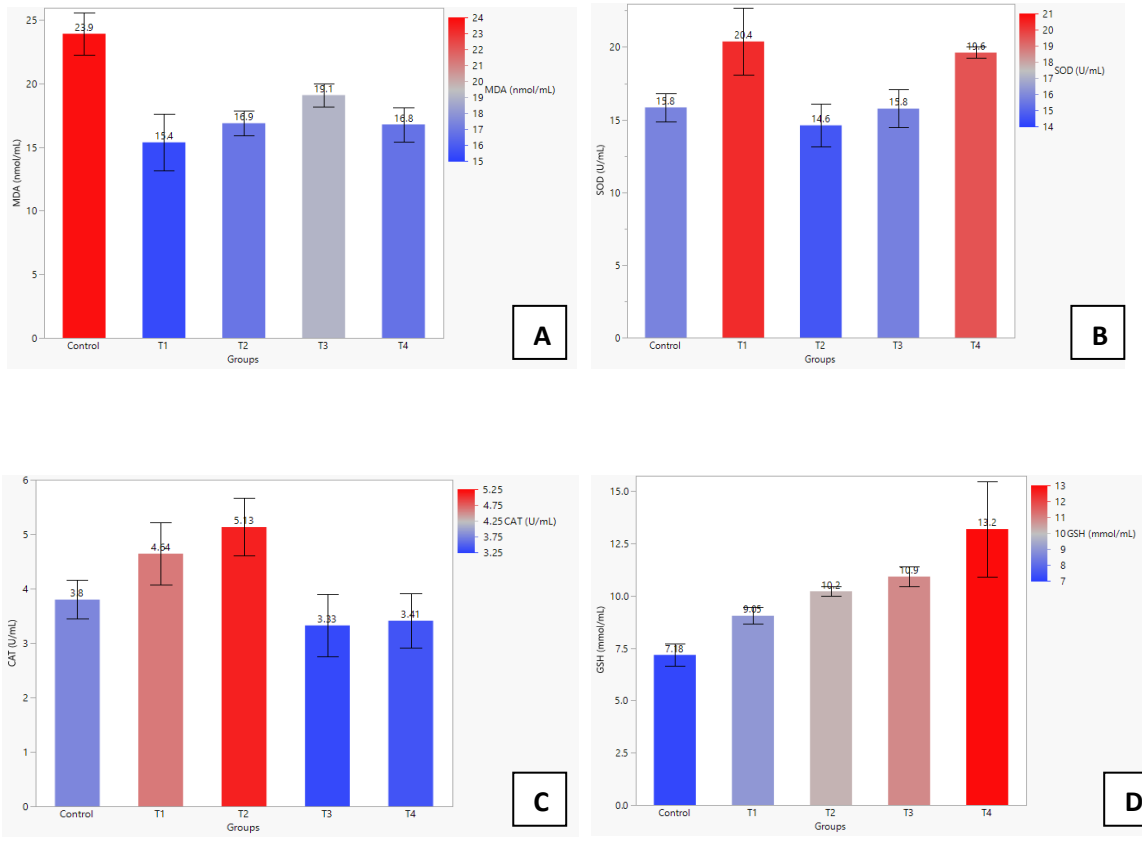

Fig. 2: Effect of *Chlorella vulgaris* supplementation oxidative stress biomarkers in the serum of the rabbit models. (a) effect on the malondialdehyde concentrations. (b) effect on the activities of superoxide dismutase enzyme (SOD) (c) effect on activities of catalase enzyme (d) effect on the concentration of reduced glutathione.

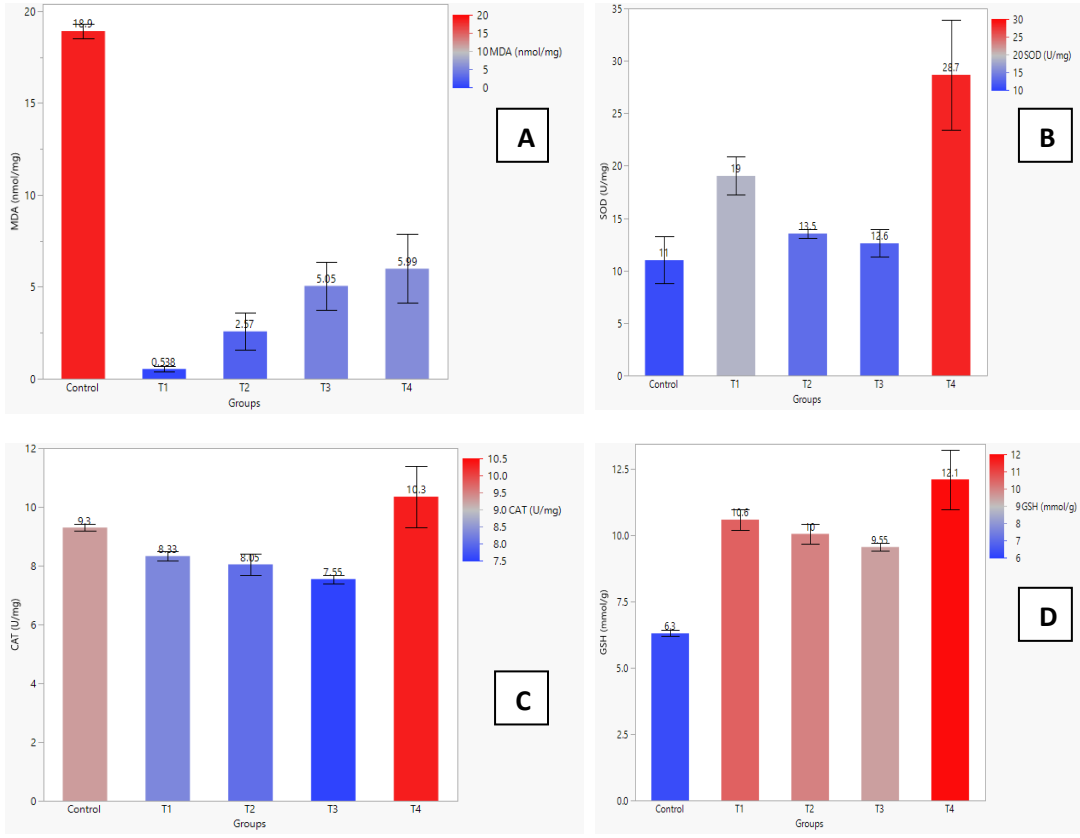

Fig. 3: Effect of *Chlorella vulgaris* supplementation oxidative stress biomarkers in the liver of the rabbit models. (a) effect on the malondialdehyde concentrations. (b) effect on the activities of superoxide dismutase enzyme (SOD) (c) effect on activities of catalase enzyme (d) effect on the concentration of reduced glutathione.

This study was carried out to determine antioxidant effects of the microalga *Chlorella vulgaris* intake on oxidative stress status of the rabbit brain and the circulating oxidative stress biomarker in the serum and liver of the rabbits. It was hypothesized that the intake of the antioxidant-rich microalga could lead to increased build-up of antioxidants in the blood and liver of the rabbits which could in turn aid the movement of the antioxidant compounds across the blood-brain barrier. Our data suggest that the antioxidant principles in the microalga can cross the blood-brain barrier to increase the activities of the antioxidant enzymes within the brain of the rabbits.

The present study supports the view that antioxidants can cross the blood-brain barrier as reported by previous researchers whereby supplemented resveratrol was reported to increase activities of antioxidant enzymes in the brain[15]. It was also observed that the increased activities of the antioxidant enzymes in the brain of the supplemented rabbits (Fig. 1) which corresponded with reduced circulating lipid peroxidation marker malondialdehyde. This is an observation which is also in agreement with the report from a similar view on the supplementation of *Chlorella vulgaris* which led to

upregulation of antioxidant enzymes in the brains of rats after exposure to sub-chronic low-level of lead[16]. Furthermore, data obtained in this study (Fig. 2), is a confirmation of the suggestion that the supply of certain antioxidant-rich sources could enhance circulating antioxidant enzyme activities and reduced accumulation of lipid peroxidation marker malondialdehyde[17].

It is an established principle that oxidative stress causes biochemical and performance failures such as infertility and neurodegenerative conditions, Alzheimer's and Parkinson's diseases in humans and animals at different stages of existence[18]. Meanwhile, circulating malondialdehyde in the blood and liver is a mechanism of the dysfunctions and this can negatively affect the brain, since the brain cells require a substantially higher amount of oxygen for normal functioning[19]. The result presented in (Fig. 2) of this study suggest that it is possible to reduce the circulating malondialdehyde for enhancement of the activities of antioxidant enzymes in rabbits' brains using the consumption of the microalga *Chlorella vulgaris* and this could serve as a strategy for improving all brain coordinated production performances in livestock and management of critical brain-associated diseases of humans and animals.

The intake of the microalga could be a critical nutritional intervention for the improvement of reproduction performances in both animals and humans because some of the most important hormones for reproduction are produced by the brain which include the hormones responsible for the enhancement of pubertal attainment, production of ova and sperm cells and it has been suggested that central to the infertility problems are due to oxidative stress imbalance linked with the brain which has been identified as unexplained infertilities which is a set of abnormalities associated with the brain regulation of the ovary and testicular functions[20]. Also, for the promotion of health and general well-being, it has been established that reducing oxidative stress progression could serve as a critical approach to eliminating some infectious and metabolic diseases; hence, attenuation of oxidative stress could also contribute to the management of critical human diseases including cancers[21]. The data obtained in this study support the results of other researchers who have suggested attenuation of oxidative stress via the intake of *Chlorella vulgaris* for reduction of circulating malondialdehyde to increase the activities of antioxidant enzymes. Similarly, this present study further suggested that the microalga at the rate supplemented in this study could have the potential of enhancing antioxidant enzyme activities in the brain of rabbits. This study showed that the intake of *Chlorella vulgaris* as an antioxidant source is suitable for reducing circulating malondialdehyde and protection of the brain against oxidative stress damage, and since the antioxidant compounds in the microalga enhanced brain antioxidant enzymes activities. The study further suggested that the identification of possible receptor genes could be responsible for this action and also determine the expression of the blood-brain biomarker for the development of mechanisms to support the use of the alga as a therapy for brain oxidative stress.

Conclusion and Applications

1. *Chlorella vulgaris* biomass can be used as a suitable antioxidant supplement for protection of brain against oxidative stress damages in rabbits.

2. *Chlorella vulgaris* improved both circulating and brain antioxidant defence status via reduction of malondialdehyde; hence, it is a potent antioxidant that can be incorporated into feeding of rabbits.
3. *Chlorella vulgaris* can cross the blood-brain barrier as result, it can be exploited as a potential functional feed resources for enhancement of brain function in reproduction and other related performance activities in rabbits.

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