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Effects of *Nigella sativa* oil on the cerebellum of early-weaned Wistar rats

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ABSTRACT

Weaning period is a vulnerable phase in which malnutrition can set in and this frequently results in loss of priceless mental abilities and occasionally, physical problems. The current study sought to determine if Nigella sativa oil (NSO) could protect Wistar rats against behavioural and biochemical alterations brought on by early weaning. Twelve rats pups were selected at random. Rats in the control group were weaned on Postnatal day (PND) 28, while the second and third groups (the early weaned (EW) groups) were weaned on PND 18. Following weaning, the second group (EW) were given standard rodent feed and the third group (EW + NSO) additionally received 25 ml/kg of Nigella sativa oil till the PND 35. On PND 33, the rats had open field tests to measure their exploratory and locomotor behavior, and their brains were excised on PND 35. For histochemical analysis, Cresyl Fast Violet, and Hematoxylene and Eosin stains were employed. Cerebellar tissue slices were also processed utilizing glutathione peroxidase (GPx), malondialdehyde (MDA), and superoxide dismutase (SOD) as oxidative stress markers. EW rats showed an increase in locomotor and exploratory behavior as well as a significant decline in cerebellum SOD, GPx, and MDA levels. EW + NSO rats showed lower locomotor and exploratory activity levels, higher levels of oxidizing enzymes, and lower MDA levels. These results show that NSO protected the cerebellum against oxidative stress-related damage that occurs following early weaning. In this investigation, oral mode of administration was used.

Keywords: Weaning, motor activity, *Nigella sativa*, oxidative stress, *Pups, Early weaning*

INTRODUCTION

Weaning is the process by which an infant or a young mammal gets accustomed to other types of food different from its mother's milk and stops breastfeeding[1]. It can also be described as the permanent deprivation from breast-milk and commencement of nourishment with other food[2]. The normal weaning period for rats is between postnatal days (PND) 21 - 28, while the early weaning period is PND 15-18 and the late weaned is at PND 35[3]. When an infant fully gets accustomed to adult feeds and stops breastfeeding, it is said to have been fully weaned. Weaning is practiced all over the world with different speculations on what time is safe to wean. Early weaning is a process by which a young infant is weaned at an age younger than the age it should be weaned. Various studies have reported the implications of improper weaning practices, which include malnutrition, growth retardation, infection, diseases and high mortality[4, 5].

Medicinal plants have been employed in the treatment of illnesses for centuries in different parts of the world. One of such plants is *Nigella sativa* which has been reported to be utilized for its numerous health benefits[6]. The greater part of the remedial properties of this plant is due to the presence of thymoquinone which is a major active chemical component of the essential oil[7], and additionally, dithymoquinone, p-cymene, carvacrol, 4-terpineol, t-anethol, sesquiterpenelongifolene, α-pinene, thymol, etc.[8].

Nigella sativa oil (NSO), also known as Black Seed oil, has been widely studied for its biological activities and restorative potential[9]. The beneficial properties of NSO includes anti-hypertensive[9,10], immune-modulatory[10] and anti-oxidant properties[10,11]. In this study, we report the possible alleviating effects of NSO in early weaning-induced cerebellar histologic and oxidative damage and the resulting implications on motor function.

MATERIALS AND METHODS

The *Nigella sativa* oil (100 % Black Seed; HUSNA Black Seed Oil, Fazhab Agency, Karachi, Pakistan) was purchased from a reputable store in Ilorin, Kwara State, Nigeria.

Experimental Animals: Eight female and four male adult Wistar rats weighing between 150-170 g were obtained at the Zoological Garden, Ilorin**.** They were housed in the Animal Facility of the Faculty of Basic Medical Sciences, University of Ilorin and fed adequately under a natural 12 hours light and dark rhythm at room temperature with proper ventilation. This research was carried out accordingly by complying with the guidelines of the University of Ilorin Ethical Committee.

Female rats were mated with male rats (2:1) and monitored from the day of onset of pregnancy to the day of parturition. When the female rats littered, the pups were divided into three groups, consisting of four rats each:

Normal Weaning (NW) Group: weaned at PND 28;

Early Weaning (EW) Group: weaned at PND 18;

Early Weaning plus *Nigella sativa* oil (NSO): weaned at PND 18 and administered with 25 ml/kg of NSO from PND 19 - 35.

The treatment used was adopted from the work of Richter et al., 2016.

Weight Assessment: The rat pups were weighed through a period of 18 days. Weights were taken at PND1, PND4, PND7, PND19, PND14 and PND17.

Neurobehavioral Evaluation: The rats were subjected to neurobehavioral studies using the open field test (OFT) paradigm on PND 33 to assess locomotion and exploratory behavior. The rats were individually placed in the middle of the apparatus and left to explore the paradigm (a well illuminated wooden box, divided into 4 × 4 squares) for a 5-minute session under video surveillance. The number of lines crossed and the rearing frequency were recorded with a video camera and analyzed [10].

Histological and Histochemical Evaluations: At PND 35, the rats were euthanized with Ketamine (25 ml/kg) via intramuscular injections and their brains were dissected out. Thereafter, the cerebellum of each rat was separated from the whole brain and fixed in 4% paraformaldehyde solution for 24 hours. The tissues were then dehydrated through ascending grades of alcohol, cleared in xylene and embedded in paraffin blocks. Cerebellar tissue sections (5 μm in thickness) were stained with Hematoxylin and Eosin (H&E) stains for light microscopy histological examination of general features and cresyl fast violet (CFV) stain for Nissl substance demonstration.

Biochemical Evaluation: Cerebellar tissues of the hemisphere not used in histological analysis were removed from the brains of the rats in each of the groups, dipped in 30% sucrose solution, homogenized and centrifuged for 10 minutes at 2500 revolutions per minute. The supernatant was then collected and assessed for oxidative status using assay kits for superoxide dismutase, glutathione peroxidase enzymes and malondialdehyde[12].

Data analysis: Data from the study were analyzed using one-way analysis of variance (ANOVA), and subjected to *post hoc* Turkey's multiple comparison test. The results were expressed as mean±SEM (standard error of mean) and value $p<0.05$was considered statistically significant. Statistical analyses were performed using Graph Pad Prism software (version 5.0, La Jolla, CA).

RESULTS

Morphological Observations: Early weaned rats had a significant reduction in body weight when compared with the rats weaned at PND 28 ($p < 0.01$), but higher body weight than those that received NSO intervention. The EW + NSO rats had the least body weight, and this was significant when compared with the NW rats ($p < 0.01$) (Fig. 1A). The rate of weight gain of the early weaned rats lagged immediately after weaning compared with the other groups (Fig. 1B) until PND 29 when the rate of weight gain, though not statistically significant, became higher than the early weaned rats that received NSO intervention.

Neurobehavioural Observations: The results of the open field tests revealed that rats weaned early (EW) recorded high

rearing frequency compared with rats weaned normally (NW) at PND 28, and this difference was significant ($p < 0.05$). However, rats that received NSO intervention following early weaning (EW+NSO) had a significantly reduced rearing frequency compared with those that did not receive NSO after early weaning.. The rats that received NSO intervention (EW+NSO) had a slight reduction in rearing frequency when compared to the control, and this difference was not statistically significant (Figure 2A).

Findings showed the frequency of lines crossed by rats in the open field apparatus (Figure 2B) were similar to that of the rearing frequency. Early weaned (EW) rats had the highest frequency of lines crossed compared with both NW and EW+NSO ($p < 0.001$). Rats administered NSO following early weaning (EW+NSO) had relatively same line crossing frequency compared with the rats that were weaned at PND 28 (NW).

***Nigella sativa* oil counterbalances early weaning-induced oxidative stress:** Superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes and malondialdehyde (MDA) were assessed as markers of oxidative stress (Fig. 3). Early weaned (EW) rats had a reduction in the activity of SOD and GPx when compared with normal weaned (NW) rats, though not significant statistically. However, the rats that received NSO intervention after early weaning recorded significant elevation in the cerebellar levels of both SOD (Fig. 3A) and GPx (Fig. 3B) when compared with the rats that were not given NSO ($p < 0.05$). The EW+NSO rats also had higher activities of SOD and GPx compared with the NW though not statistically significant.

Malondialdehyde levels, a marker of lipid peroxidation, was significantly elevated in early weaned rats (Fig. 3C) compared with both the Control (NW) and EW+NSO rats and this differences were significant ($p < 0.05$). No significant change was observed in the cerebellar level of MDA in rats weaned at PND 28 (NW) and those that received NSO after early weaning (EW+NSO).

Histomlogical and Histochemical Observations: The general histological presentation of cortical cerebellar structure revealed the molecular, granular and Purkinje cell layers (Fig. 4 & 5). The outline and histomorphology of the cerebellar structures of rats weaned at PND 28 (NW) appeared to be apparently normal. However, the EW showed a widening of the white matter layer and slight increase of granular layer thickness (Fig. $4B_1$ & $5B_1$) compared with NW (Fig. $4A_1$ & $5A_1$). The Purkinje cell layer of EW (Fig. $4B_2$ & $5B_2$) and EW+NSO (Fig. $4C_2$, & $5C_2$) appeared sparse compared with NW (Fig. $4A_1$ & $5A_1$). There appeared to be an increased pyknosis in the EW group when compared with the NW and EW+NSO group. The histoarchitecture of EW at the granular layer showed loose granular cells and fragmentation, while EW+NSO appeared normal (Fig. 4 and 5).

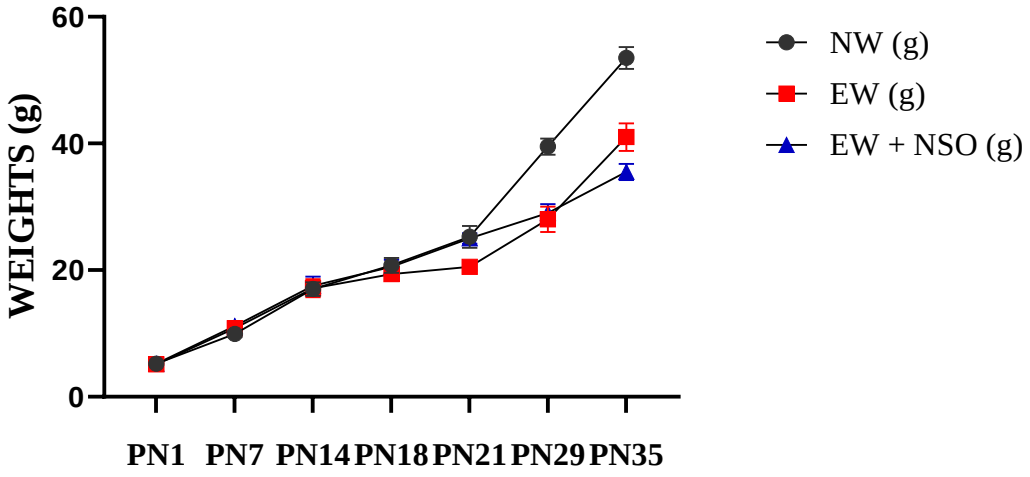

Figure 1A: Weight trend in rat pups across study period. NW= normal weaning; EW= early weaning and EW+NSO =early weaning plus *Nigella sativa* oil.

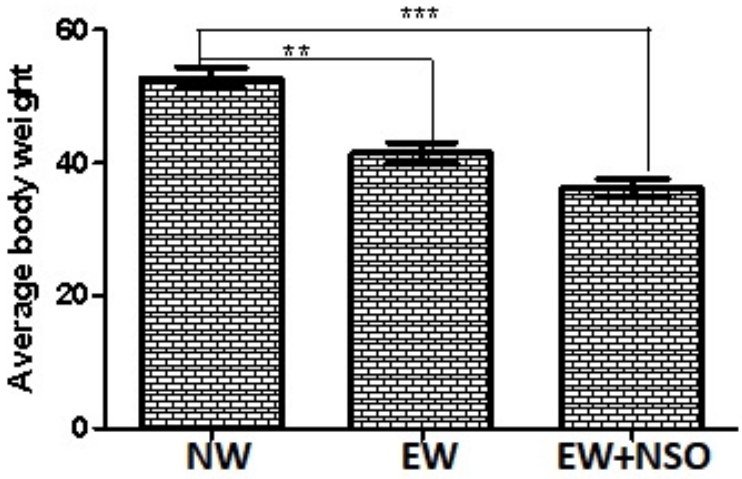

Figure 1B: Average body weight of rats at PND 35. There was significant reduction in EW (early weaning) and EW+NSO (early weaning plus *Nigella sativa* oil) groups.

NW = Normal weaning. ** and *** were significant levels of difference at $p < 0.01$ and $p < 0.005$ respectively

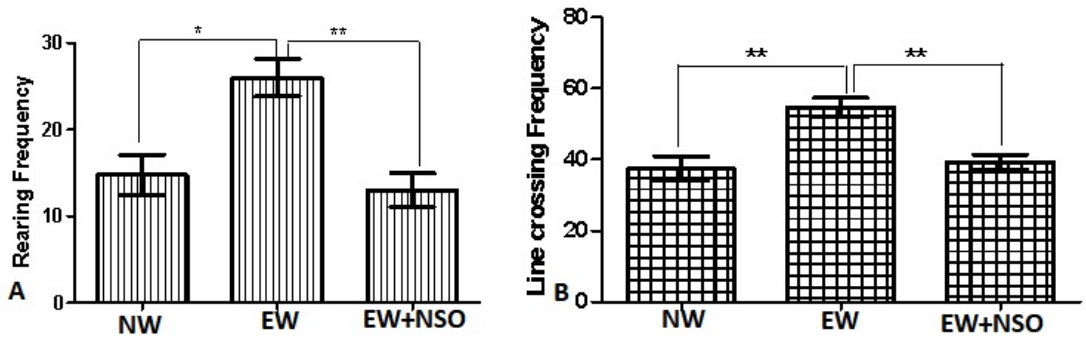

Figure 2: The rearing frequency (A) and line crossing frequency (B) of rats in the open field tests.

Early weaned rats had significantly increased rearing and line crossing frequencies. NW - normal weaning at PND 28, EW - early weaning at PND 18, EW+NSO - early weaning at PND 18 with NSO. * and ** represents significant levels of $p < 0.05$ and $p < 0.01$ respectively.

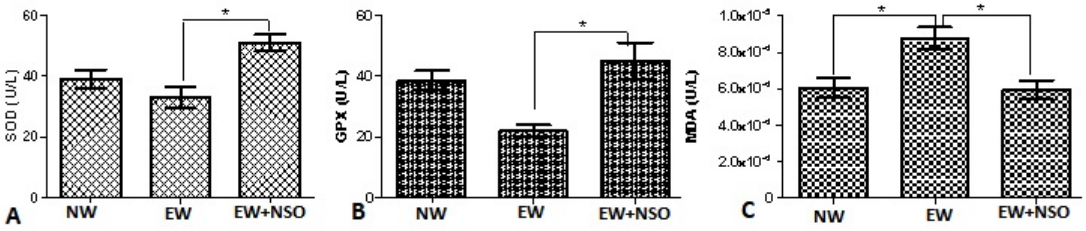

Figure 3: Cerebellar levels of superoxide dismutase (SOD) (A), glutathione peroxidase (GPx) (B) and malondialdehyde (MDA) (C) of Wistar rats.

NW- normal weaning at PND 28, EW- early weaning at PND 18, EW+NSO - early weaning at PND 18 with NSO. Early weaned rats had significant reduction in SOD and GPx enzymes and raised MDA level compared with EW+NSO (*$p < 0.05$).

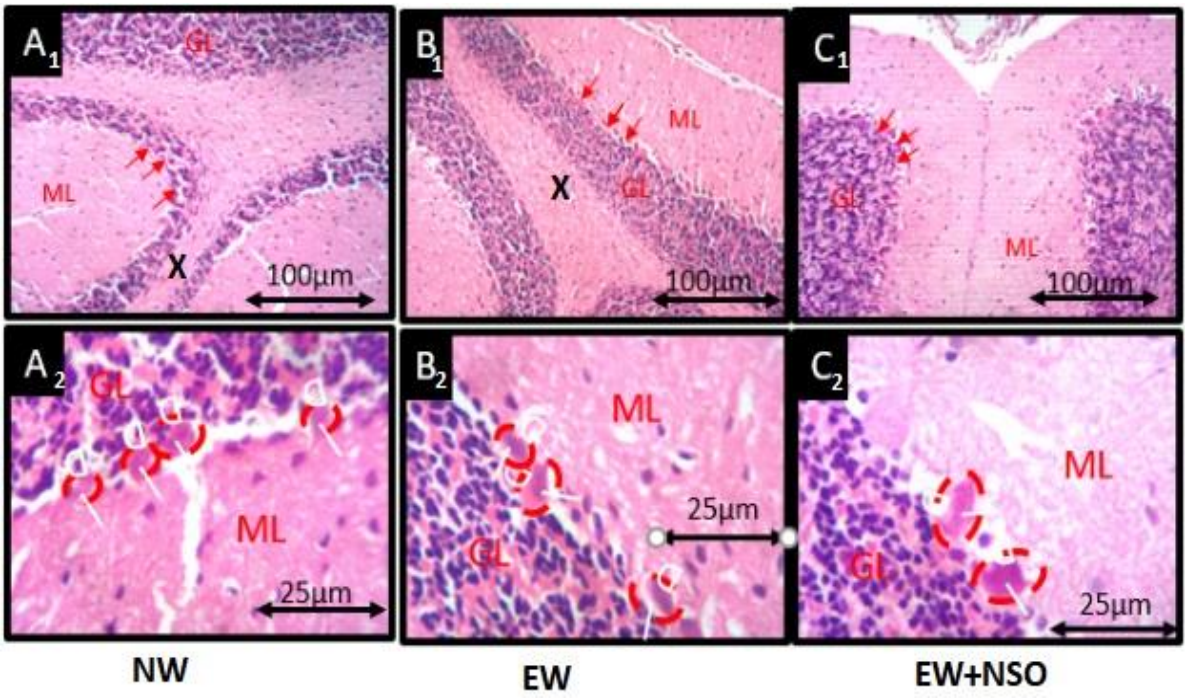

Figure 4: **Photomicrograph of sections the cerebellum of adolescent Wistar rats showing increased thickness of white matter layer (X) and slight increase in granular layer (GL) of early weaned (EW) compared with normal weaned (NW) rats.**

A_1 - NW (normal weaning), B_1 - EW (early weaning), C_1 - EW+NSO (early weaning and *Nigella sativa* oil), ML = Molecular layer, Broken Circle/Arrows = Purkinje cell layer.

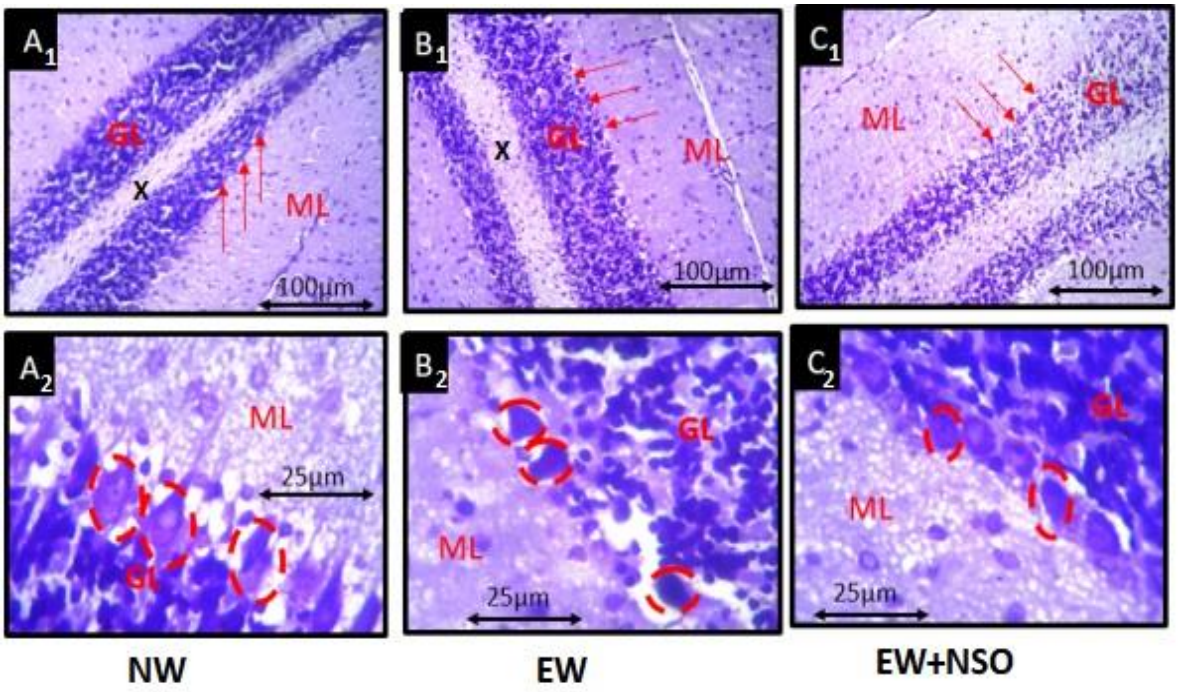

Figure 5: **Photomicrograph of the cerebellar of adolescent Wistar rats stained with cresyl fast violet.**

The early weaned (EW) rats showed fragmentation of cells of the granular layer (GL), reduced Purkinje cells (broken circles) and widening of the white matter layer (X). The Purkinje cell layer of **C_2** revealed abundant and deeply stained Purkinje cells. A - NW (normal weaning), B - EW (early weaning), C - EW+NSO (early weaning and *Nigella sativa* oil), ML = Molecular layer.

DISCUSSION

Mammalian infants heavily depend on their mothers, and mother-infant interactions greatly influence neurobehavioral development [13]. In animal studies, early weaning causes long-term enhanced neuroendocrine stress responses, anxiety, and also affects neurobehavioural development[14].

In this study, early weaned pups had sudden reduction in body weight at postnatal day 18 compared to rats not weaned at same age. This was not unexpected, but a consequence of change in diet and the need for the pups to adapt to the new form of diet for their nutritional survival. Although early weaning has been associated with rapid weight gain in infancy[16], the growth pattern of early weaned pups in the current study was slower than in pups weaned at PND 28. However, around PND 35, there was a remarkable increase in growth rate beyond that of the control, although the final weight was still lower than the control. Furthermore, early weaned rats administered with NSO also showed reduced body weight. This could be as a result of increased body metabolism caused by *Nigella sativa* oil and its ability to mitigate obesity[8].

Locomotor activities are central to social behaviours; their evaluation in this study revealed a significant increase in the exploratory activities of rats that were weaned early when compared to rats that received NSO after early weaning and those weaned at PND 28. Early weaning was associated with significant increase in the number of lines crossed and the rearing frequency, which serve as indicators for assessing locomotion and exploratory behaviours. Early weaning has the tendency of causing an increase in awareness, as the rats in this category were conscious of a change in their environment when placed in the open field arena.

Early separation of pups from their dam predisposes them to serious energy deficits especially due to the need to adapt to a new diet other than breast milk[18, 19]. The period of weaning is associated with impaired immune responses and disruption of redox balance[20]. Previous studies have reported mitochondrial dysfunction and oxidative stress in early weaned experimental animals[21, 22]. Assessing the oxidative status of rats in the current study, superoxide dismutase and glutathione peroxidase enzyme activities were employed as markers, while malondialdehyde level was evaluated for lipid peroxidation. As expected, early weaned rats presented with severe lipid peroxidation and depletion in endogenous antioxidant status of the rats when compared with the control. *Nigella sativa* has anti-oxidative properties and prevents lipid peroxidation[23, 24]. This study showed that the use of NSO in early weaned rats significantly reduced lipid peroxidation in the cerebellar tissue and at the same time boosted anti-oxidative enzyme activities. Reactive oxygen species generated during the period of early weaning were gotten rid of by NSO due to its free radical scavenging properties thereby enhancing the primary endogenous antioxidant defense systems in the brain[25, 26].

Early weaning led to alterations in the cerebellar cytoarchitecture, including chromatolytic changes and apparent reduction in cell density. These alterations were more pronounced in the Purkinje cell layers which marked the transition between the granular and molecular layers of the cerebellar cortex. The granular layer consisted of granule cells that were loosely and cryptically arranged. Alteration in cellular morphology may lead to loss of signal processing, neuronal timing and synaptic efficacy in the cerebellum, and are often observed within cerebral cortex of demented patients[27]. Administration of *Nigella sativa* oil to early weaned rats resulted in less alterations and uniform cytoarchitecture similar to the control with Purkinje cells having axons penetrating the molecular layer. The well-arranged cerebellar layers and neuronal morphology in these rats suggested an appropriate interconnectivity within the cerebellar cortex. The ability of NSO to restore the chromatogenic nature of the Nissl substance following EW-induced damage might be due to its ability to prevent endoplasmic reticulum stress caused by early weaning owing to its anti-oxidative and ROS scavenging properties.

CONCLUSION

Data from this study revealed that oral administration of NSO attenuated cerebellar early weaning-induced oxidative stress by mechanisms related to its ability to decrease the levels of oxidative stress, and cerebellar tissue damage in rat pups. NSO could serve

as a true functional food and may promote well-being by reducing early weaning-induced oxidative stress and related pathologies.

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