



Long Term Consumption of Coconut Oil Diet Increased Anxiety Related Behaviour in CD1 Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author SAB designed the study, wrote the first drafts, final manuscript and financed the process. Author OAS performed the statistical analysis and managed the analyses of the study. Author UAO provided the animals and wrote the protocol. Author MAA managed the literature searches while authors GNU and MAA performed the experiment. All authors read and approved the final manuscript.

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ABSTRACT

The effect of prolonged consumption of virgin coconut oil (VCO) diet on anxiety was assessed in CD1 mice using the Open field (OF), Elevated plus maze (EPM) and Light/dark box (LD box) tests. Thirty male CD1 mice (28.4 - 32.5 g bw) divided into 3 groups (n=10 each) were given normal rodent chow (control), 5% (w/w) and 20% (w/w) VCO diet respectively and water *ad libitum* for 23 days. The OF test showed increased grooming ($p < 0.05$) in both 5% and 20% VCO groups versus control, and higher Freeze frequency in both VCO diet groups versus control, with the 20% diet

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group being higher than that for 5% diet group ($p < 0.05$). Stretch attend postures (SAP) were similarly higher in the 5% and 20% diet groups ($P < 0.05$) versus control, and higher ($P < 0.05$) in the 20% VCO diet versus 5% VCO diet group. In the EPM test, the open arms duration was lower ($p < 0.05$) in the 20% VCO diet group. SAPs in the EPM test were higher in both 5% and 20% VCO diet groups ($p < 0.05$), with that for the 20% VCO diet group being higher than that for the 5% VCO diet group ($p < 0.05$). In the LD box test, SAP was significantly higher in 20% VCO diet group versus control ($p < 0.05$). All behaviours scored in the three tests indicate increased anxiety in the VCO diet groups. Thus long-term consumption of virgin coconut oil diet increased anxiety-related behaviour in the mice, with the 20% diet causing a greater effect.

Keywords: Coconut oil; anxiety; CD1 mice.

1. INTRODUCTION

Coconut oil is one of the world's most commonly used oils. It has been used extensively for both edible and non-edible purposes the world over [1]. It is one of the most desirable natural oils for confections, bakery goods, deep fat frying and for non-edible purposes such as manufacture of cosmetics among others. This is due to two of its special characteristics: high degree of saturation and good stability [2]. Coconut oil is very stable to oxidative deterioration when exposed to atmospheric oxygen. Its high degree (90%) of saturated fatty acids content makes it different from other vegetable oils. Methods of processing, however, may have some influence on the stability of the oil [3]. Although the components of the coconut oil may differ slightly with the different laboratories and techniques, the main important components of virgin coconut oil, which affect its characteristics are: triacylglycerols, fatty acids, phospholipids, tocopherols, trace metals, sterols, volatiles, and mono-and-di-acyl glycerols [4,5,6]. About 50% of the fatty acids in coconut oil are the 12-carbon lauric Acid. When coconut oil is enzymatically digested, it also forms a monoglyceride called monolaurin [7].

The most significant physical properties of virgin coconut oil is that, unlike most fats, both natural and hydrogenated, it does not exhibit a gradual softening with increasing temperature, but passes rather abruptly from a brittle solid to a liquid, within a narrow temperature range [4]. Virgin coconut oil consists of lauric, myristic and palmitic acids, with lauric acid predominating (47.5%) therefore making coconut oil a medium chain triglyceride [8].

The beneficial effects attributed to the use of coconut oil are enormous. Coconut oil has been reported to have very potent anticancer properties. In laboratory animals chemically induced with cancers, the addition of coconut oil

in the diets of the animals attenuated the carcinogenic effects [9]. Virgin coconut oil has been reported to increased high-density lipoprotein (HDL) and decreased low-lipoprotein (LDL) peroxidation in laboratory rats [1]. It has also been reported to offer protection from cardiovascular disease [10]. Coconut oil has been reported to aid diabetics by balancing blood sugar levels as the medium chain triglycerides (MCTs) improve insulin secretion and insulin sensitivity [11]. It has also been reported to reverse the effect of toxic agents to the liver and other organs in the body such as the intestine, colon, kidneys, and pancreas. Coconut oil has even used in the formulation on a repellent used to prevent tungiasis-causing sand fleas from invading the body [12]. Some of coconut oil's major components, lauric acid and monolaurin have both been reported to be able to kill harmful pathogens like bacteria (e.g. *Staphylococcus aureus*), viruses and fungi (e.g. the common yeast in human, *Candida albicans*) [7,13,14]. Coconut oil has also been used topically to treat paediatric atopic dermatitis [15].

On the peripheral nervous system in animal models, coconut oil is said to be acutely anti-inflammatory, although chronically neutral. [16] In some research by Song et al. [17], 5% coconut oil diet was shown to have anti-stress, anti-anxiety, and anti-inflammatory effects. It is also reported to have the potential to aid in the protection and treatment of number of health problems due to its documented anti-mutagenic, antioxidant, anti-microbial, anti-inflammatory, analgesic, and antipyretic activities [18]. In another report, Joette Calabrese, a homeopathic consultant and educator was said to have had success using coconut oil to manage anxiety in her patients [19]. In some anonymously authored internet publication [20], some individuals reported increased anxiety following consumption of coconut oil.

Coconut oil forms a major ingredient in one of the most commonly consumed special delicacy in Nigeria, coconut rice. This is perhaps because many nutritionists regard it as a wonder food or sometime as a 'super food', or because of the many health benefits, which have been attributed to the coconut oil. Some of the health benefits of the coconut oil are that it improves memory when used to treat patients with memory disorders like Alzheimer's disease, and that it is used in relieving stress and anxiety. There has however been some conflicting information, most of which are not peer reviewed, as to the effect of coconut oil on anxiety. While some people claim it has anti-anxiety effect, others say it causes anxiety-like behaviour. The aim of this research was therefore to study the effect of long-term consumption of 5% and 20% coconut oil diets on anxiety-like behaviour in mice, using the open field test, elevated plus maze and the light/dark box test.

2. MATERIALS AND METHODS

2.1 Design of Experiment

Thirty (30) male CD1 mice (28.4 - 32.5g body weight), raised under standard laboratory conditions and 12/12 hour light/dark cycle [21] were purchased from the animal house of the Department of Physiology, University of Calabar, Nigeria. After the mice were assigned to three groups (n = 10 each), a one week acclimatization period was allowed before treatment began. Mice were treated with control diet (normal pelletized rodent chow - vital feed), 5% (w/w) virgin coconut oil diet and 20% (w/w) virgin coconut oil diet. All animals had free access to both food and water. The treatment regimen was given for 23 days, following the method of Lim-Sylianco and Guevara [22], before behavioural testing was done. Mice were tested individually for 5 minutes each and once a day in the open field apparatus, elevated plus maze and the light dark transition box respectively for anxiety related behaviour.

2.2 Virgin Coconut Oil: Preparation and Administration

Virgin Coconut oil was prepared using the modified wet mill method [23]. The major differences between regular coconut oil and virgin coconut oil is the method of extraction. Whereas virgin coconut oil is extracted from the fresh milk of the coconut and is left unrefined, the regular coconut oil is made from copra, which is

dried coconut kernel. The copra is pressed and then the extracted oil is refined, bleached and deodorized. Virgin coconut oil (*Cocos nucifera* oil) was extracted from the kernel or cotyledon of matured coconuts freshly harvested from the coconut palm, by crushing/grinding the kernel which was freshly removed from the shell of the coconut fruit. After grinding, 100 ml of water at room temperature was added to 500 g of the grinded coconut. A very fine mesh was used to extract the milk from the chaff, by gently squeezing the grinded coconut in the fine mesh cloth to bring out the coconut milk, which contains coconut oil, proteins and fibre. The extracted coconut milk was kept at room temperature of about 25°C and allowed to ferment for 24 hrs. It was frozen for 20 hrs of constant power supply, such that the milk was highly blocked before removing it out of the refrigerator. After this, the milk separated into three layers (layer 1: shaft, layer 2: coconut oil and layer 3: water). The top layer containing shaft was gently removed exposing the virgin coconut oil, which was collected carefully to avoid water entering. The 5% (w/w) coconut oil diet was prepared by mixing 95 g of standard growers mash with 5 g of coconut oil while 20% (w/w) coconut oil diet was prepared by mixing 80 g of standard growers mash feed with 20 g of coconut oil.

2.3 Behavioural Assay

The open field (OF) test, which provides simultaneous measures of locomotion, exploration and anxiety [24] was used for this study. The open field apparatus is made of plywood (72 cm x 72 cm floor; 36 cm) walls, one of which is transparent for observing the animals. Blue lines drawn on the floor divide the floor into sixteen 18 cm x 18 cm squares, and central square (18 cm x 18 cm) in the middle clearly marked. Mice were tested in the apparatus for 5 minutes each and the behaviour scored includes frequency of lines crossed, rearing, grooming, stretch attends postures and freezing [25,26].

The elevated plus maze was built according to the description of Lister [27] was also used to assess anxiety. The maze has two open arms (30 x 35 cm) and two closed arms (30 x 5 x 15 cm high walls) radiating from a central square (5 x 5 cm). The floor of the maze is made of black or gray Plexiglas and the walls of clear Plexiglas. The open arms contain a slight edge (4 mm high) to prevent the mice from slipping and falling off the edge. Background lighting was provided by a

60 – watt red lamp. The experiment was recorded using a video camera as back up for rescoring of behavior and the behaviour scored included: duration in the open arms, frequency of stretch attends postures (SAP) and rearing [28].

The light/dark transition box test as described by Bisong et al. [26] was used as a test of unconditioned anxiety and exploratory behaviour. The light-dark box is a wooden box made of plywood measuring 45 x 27 x 27 cm (LxBxH) and consists of two compartments of unequal size. The small compartment (18 x 27 cm) is painted black (2/5 of the box) and the larger compartment (27 x 27 cm) is painted white (3/5 of the box). These compartments are connected by a door (7.5 x 7.5 cm) located at floor level in the center of the wall between the two compartments. The floor is divided into 9 x 9 cm squares and is covered with Plexiglas. A 60-Watt table lamp located 40-cm above the center of the white compartment provides bright illumination of white light. Behaviours scored included: Frequency of lines crossed, rearing, transitions and SAPs [29,30].



Fig. 1a. The Light/Dark box



Fig. 1b. The open field apparatus



Fig. 1c. The elevated plus maze

2.4 Statistical Analysis

Data from the research were analysed using One Way Analysis of Variance (ANOVA) and post hoc LSD tests. The statistical software SPSS 17.0 and Microsoft Excel, 2010 version were employed. Results were presented as means \pm standard error of means (SEM) and the probability level $P < 0.05$ were accepted as significant.

3. RESULTS

3.1 Comparison of Behaviour Score in the Open Field Apparatus for Mice Treated with 5% and 20% Virgin Coconut Oil Diets

The frequency of line crossing in the open field apparatus was not significantly different between 5% virgin coconut oil (VCO) diet, 20% VCO diet and control groups of mice. The mean frequencies of line crossing for the control, 5% coconut oil diet and 20% coconut oil diet groups of mice were; 101.3 ± 4.20 ; 108.7 ± 9.09 and 95.9 ± 5.94 respectively (Fig. 2). The mean frequencies of grooming were 2.9 ± 0.52 , 5 ± 0.25 and $5.6 \pm 0.74/5$ min for the control, 5% coconut oil diet and 20% coconut oil diet respectively. The frequency of grooming for both 5% and 20% virgin coconut oil diet treated mice was significantly ($P < 0.01$) higher compared to the control group (Fig. 3.). The duration of grooming, $6.3 \pm 0.94s$, $29.1 \pm 6.07s$ and $35.1 \pm 5.66s$ for the control, 5% virgin coconut oil diet and 20% virgin

coconut oil diet groups of mice respectively, followed a similar trend as the frequency of grooming. These were also significantly higher in the both groups treated with virgin coconut oil ($P < 0.001$) when compared to control (Fig. 4).

The mean frequency of freeze for the groups was 1.3 ± 0.03 , 1.5 ± 0.04 and 1.8 ± 0.04 respectively for control, 5% and 20% virgin coconut oil diet. The freeze frequency for 5% VCO diet treated mice was significantly ($P < 0.05$) higher compared to the control group. The frequency of freeze for 20% VCO diet treated mice was also significantly

higher compared to both control ($P < 0.01$) and the 5% VCO diet ($P < 0.05$) (Fig. 5). The frequency of stretch attends posture (SAP) of mice in the three groups (control, 5% and 20% VCO diets) were; 8.4 ± 0.60 , 10.5 ± 0.57 and $13.1 \pm 0.79/5$ min respectively (Fig. 6). The frequency of SAP for 5% VCO treated mice was significantly higher when compared to control group ($P < 0.05$). The frequency of SAP for 20% VCO diet treated mice was significantly high compared to control group ($P < 0.01$) and also higher than the 5% VCO diet treated mice ($P < 0.05$).

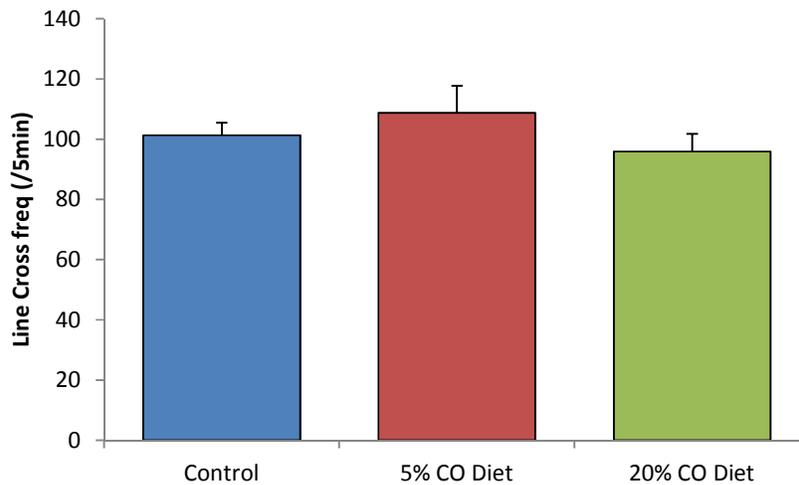


Fig. 2. Comparison of frequency of line cross in the open field apparatus between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
Mean \pm S.E.M = Mean values \pm Standard error of means

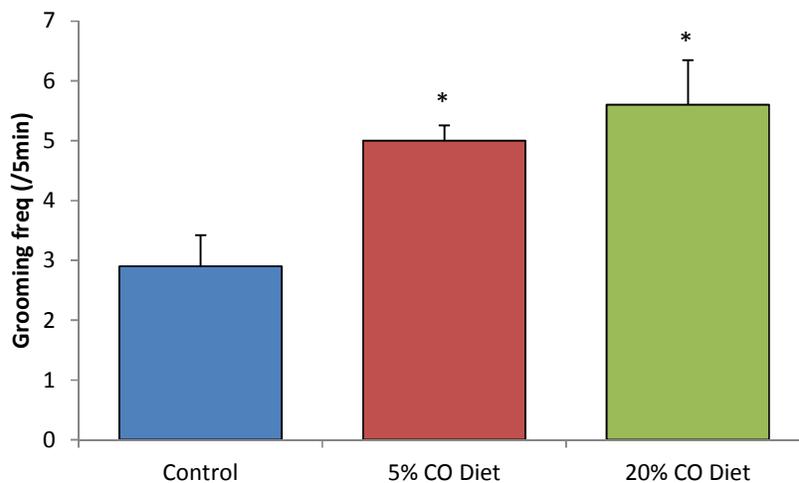


Fig. 3. Comparison of frequency of grooming in the open field apparatus between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
** $P < 0.05$ vs control*

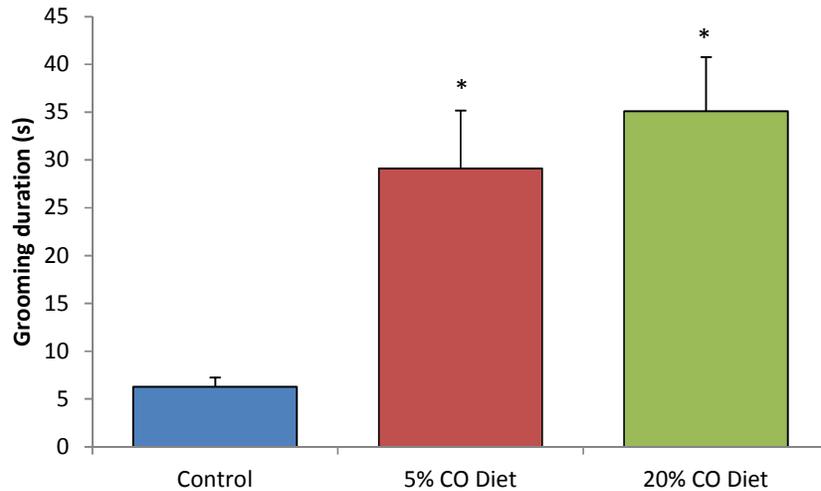


Fig. 4. Comparison of duration of grooming in the open field apparatus between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
 *** – Significant at $p < 0.001$ compared to control

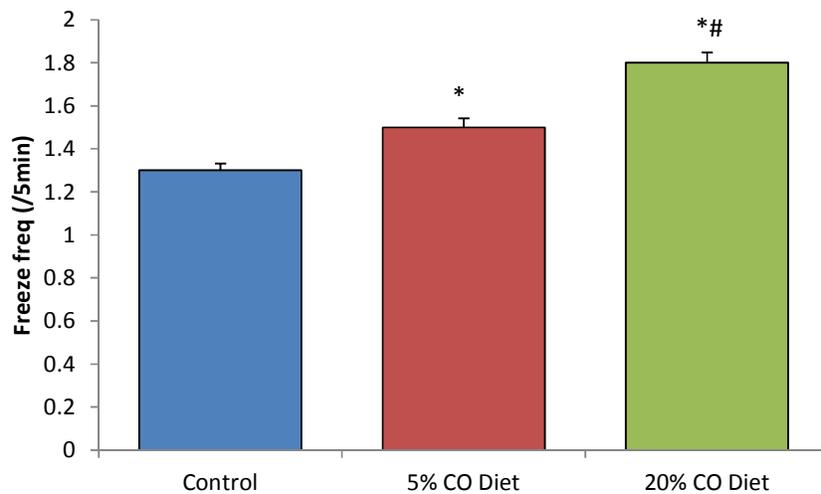


Fig. 5. Comparison of frequency of freezing in the open field apparatus between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
 * - Significant at $p < 0.05$ compared to control; # - significant at $P < 0.05$ vs 5% virgin coconut oil diet

3.2 Comparison of Behaviour Score in the Elevated Plus Maze Test for Mice Treated with 5% and 20% Virgin Coconut Oil Diets

The mean duration in the open arms of the elevated plus maze were; $100 \pm 11.67s$, $78.8 \pm 13.46s$ and $52.5 \pm 6.35s$ for the control, 5% and 20% virgin coconut oil diets respectively during the 5 minutes test period (Fig. 7). The open arms duration for the 5% virgin coconut oil diet treated

mice did not differ from control but the open arms duration for the 20% virgin coconut oil diet group was significantly lower compared to the control ($P < 0.01$). The frequency of stretch attends posture (SAP) in the elevated plus maze (EPM) were; 4.1 ± 0.45 , 6.5 ± 0.79 and 10.2 ± 1.16 for the control, 5% and 20% virgin coconut oil diets respectively (Fig. 8). The frequency of SAP for 5% coconut oil diet treated mice was significantly ($P < 0.01$) higher compared to control group. The frequency of SAP in the EPM test for 20%

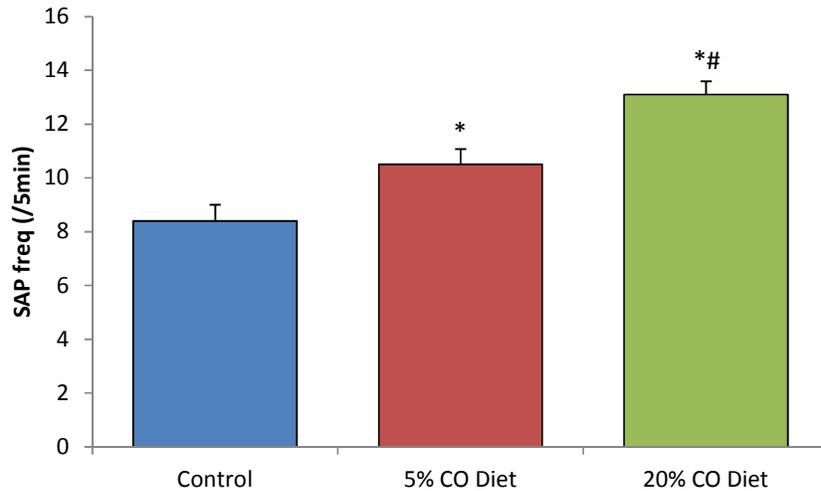


Fig. 6. Comparison of frequency of stretch attends postures in the open field apparatus between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
 * - Significant at $p < 0.05$ compared to control; # – significant at $p < 0.05$ vs 5% virgin coconut oil diet

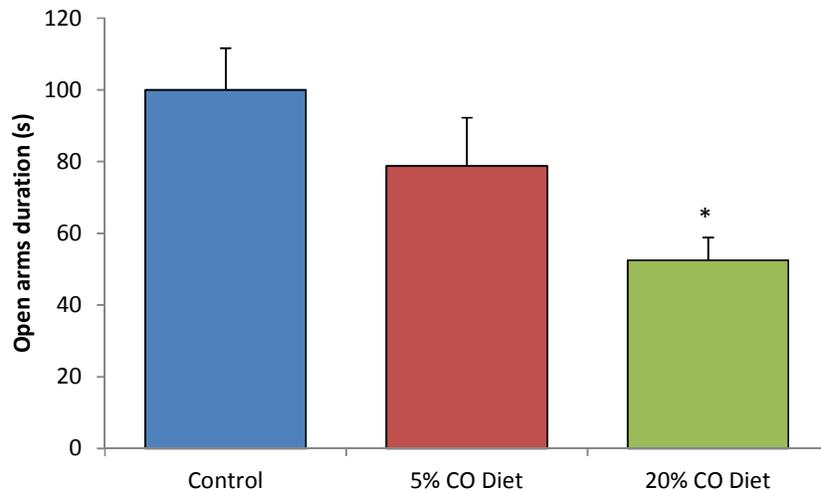


Fig. 7. Comparison of duration in the open arms of the elevated plus maze between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
 * - Significant at $p < 0.05$ compared to control

virgin coconut oil diet treated mice was significantly ($P < 0.001$) higher compared to the control group and also higher when compared to the 5% coconut oil diet ($P < 0.01$). The frequency of rearing in the EPM test were; 21.2 ± 1.70 , 16.5 ± 1.45 and $17.3 \pm 1.57/5$ min for the control, 5% coconut oil diet and 20% virgin coconut oil diet groups respectively (Fig. 9). This was significantly lower for both groups of mice treated with 5% and 20% virgin coconut oil diets ($P < 0.05$) when compared to their control.

3.3 Comparison of Behaviour Score in the Light/dark Box Test for Mice Treated with 5% (low dose) and 20% Virgin Coconut Oil Diets

The frequency of line crosses and transitions in the Light/Dark box test both did not differ between the groups, and are shown in Fig. 10 and 11 respectively. The frequency of line crosses were 83.8 ± 7.17 , 74.6 ± 5.06 and $74.6 \pm 4.57/5$ min, respectively, for control, 5% and 20%

virgin coconut oil diets; whereas the frequency of transitions were 13.6 ± 1.34 , 11.5 ± 1.66 and $12.7 \pm 1.10/5$ mins, respectively, for the control, 5% and 20% virgin coconut oil diets. The frequency of rearing for the control, 5% and 20% virgin coconut oil diet groups were 45.1 ± 2.09 , 36.7 ± 1.55 and $40.5 \pm 1.16/5$ min respectively (Fig. 12). The rearing frequency for the 5% virgin coconut oil diet treated mice was significantly ($P < 0.01$) lower compared to control group. Similarly, the frequency of rearing in the 20%

virgin coconut oil diet treated mice was ($P < 0.05$) lower compared to control. The frequency of SAP was 5.3 ± 0.80 , 6.4 ± 1.34 and $13.3 \pm 2.09/5$ min for control, 5% coconut oil diet and 20% virgin coconut oil diet respectively (Fig. 13). Although the SAP frequency in the 5% coconut oil diet group did not differ from control, the SAP frequency in the 20% virgin coconut oil diet group was significantly higher compared to control ($P < 0.01$) and the 5% virgin coconut oil diet group ($p < 0.05$).

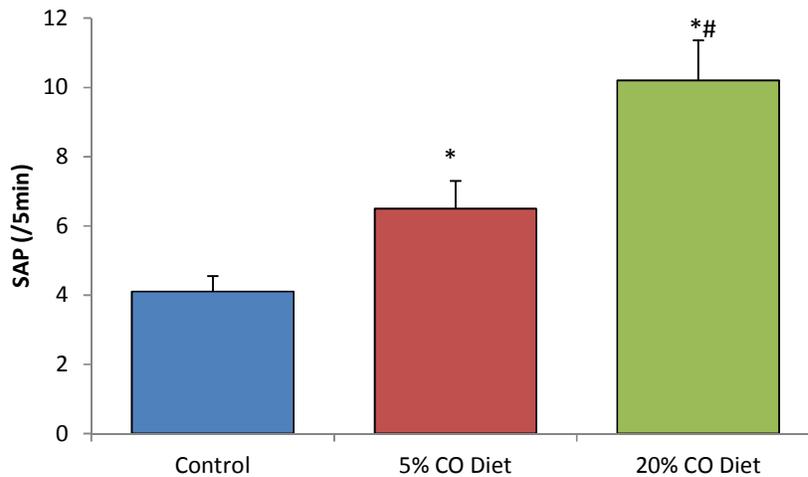


Fig. 8. Comparison of frequency of stretch attend postures in the elevated plus maze test between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
 * - Significant at $p < 0.05$ compared to control; # - Significant at $p < 0.01$ vs 5% virgin coconut oil diet

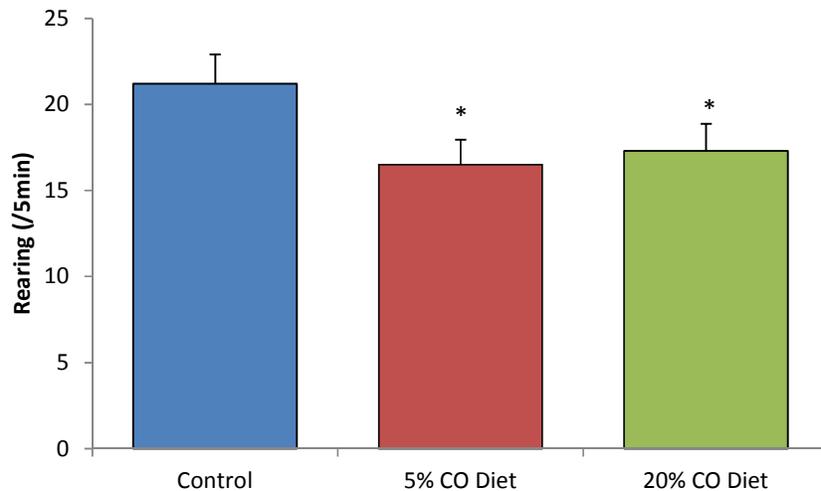


Fig. 9. Comparison of rearing in the elevated plus maze test between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet
 * - Significant at $p < 0.05$ compared to control

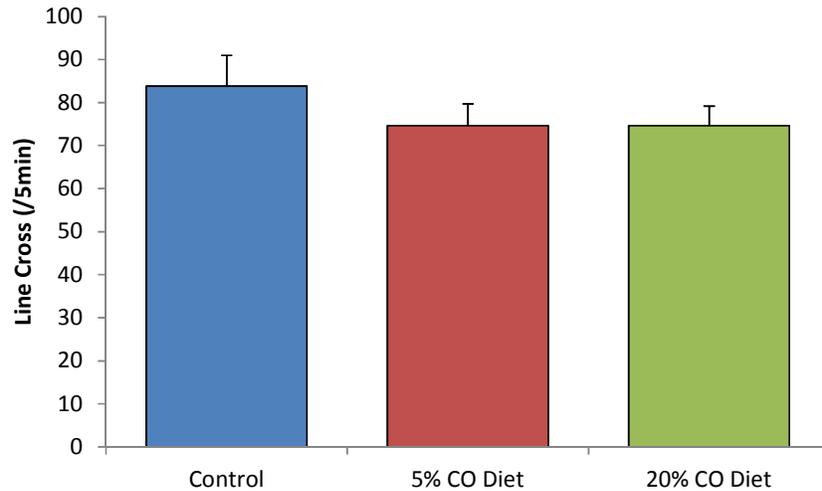


Fig. 10. Comparison of frequency of line cross in the light/dark box test between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet

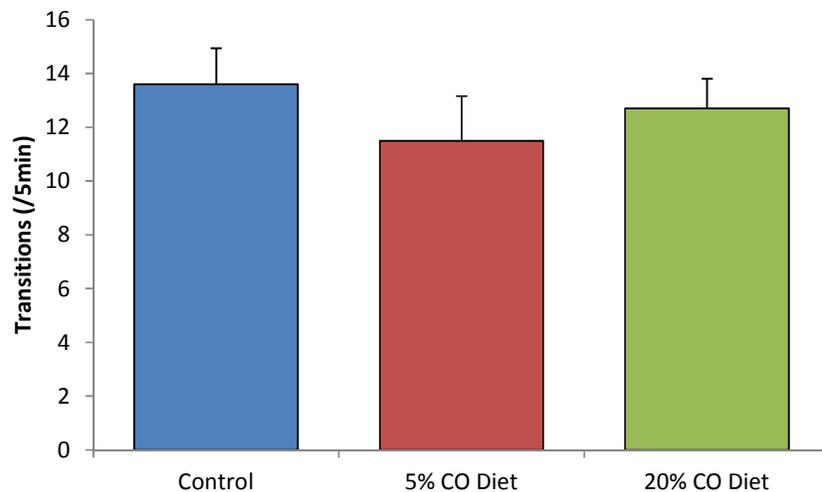


Fig. 11. Comparison of frequency of transitions in the light/dark box test between mice treated with 5% virgin coconut oil diet and 20% virgin coconut oil diet

4. DISCUSSION

The open field apparatus provides simultaneous measure of locomotion, exploration as well as anxiety and fear [24]. Among the behaviour scored, the frequency of line crosses was used to assess locomotor behaviour while frequency and duration of grooming, frequency of stretch attend posture (SAP), and frequency of freeze measured anxiety. The frequency of line crosses correlates strongly with distance travelled and so an increase in this behaviour indicates increased horizontal locomotor activity and vice versa [25]. The result of this study did not show

any difference in the frequency of line crosses among the groups, indicating that horizontal locomotor behaviour was not affected by both 5% and 20% virgin coconut oil diets.

Grooming behaviour is a displacement response and is often seen in a novel environment because of fear of novelty [31]. Increased grooming behaviour, therefore, is an indication of increased anxiety. The study showed an increase in both frequency and duration for both 5% and 20% coconut oil diets implying increased anxiety. Freezing is another behaviour that assessed anxiety [27]. An increase in this

behaviour indicates increased anxiety. The frequency of freezing was higher in both 5% and 20% virgin coconut oil diets compared to control. However, the frequency of freeze was higher in the 20% virgin coconut diet group when compared to their 5% virgin coconut oil diet counterpart. This indicates that although there was increased anxiety following treatment with 5% and 20% virgin coconut oil diets, the 20% virgin coconut oil diet caused more anxiety than the other did.

hesitant to move from its present location to a new position [32] and thus a high frequency of this behaviour indicates a high level of anxiety. The frequency of stretch attend postures in our study was higher in both 5% and 20% virgin coconut diets compared to control. However, it was higher in the 20% virgin coconut diet group when compared to their 5% virgin coconut diet counterpart. Again, this indicates that although there was increased anxiety following treatment with 5% and 20% virgin coconut oil diets, the 20% diet caused more anxiety than the 5% virgin coconut oil diet did.

Stretch attend postures are “risk-assessment” behaviours which indicate that the animal is

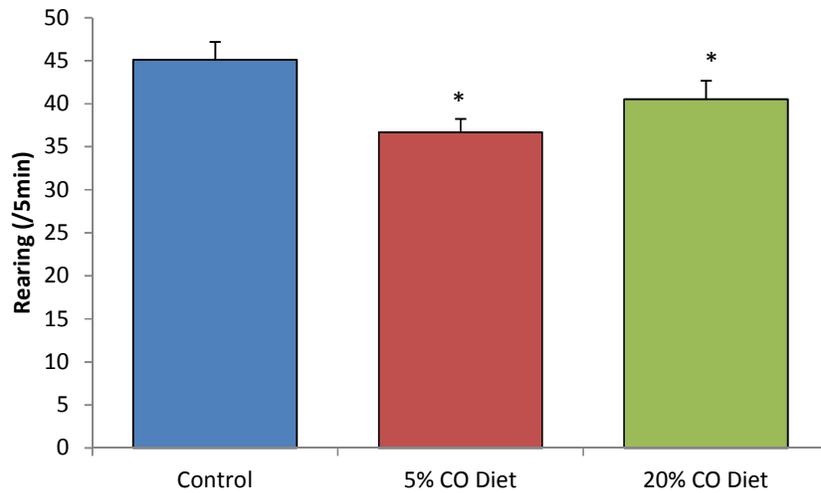


Fig. 12. Comparison of frequency of rearing the light/dark box test between mice treated with 5% coconut oil diet and 20% coconut oil diet
 * - Significant at $p < 0.05$ compared to control

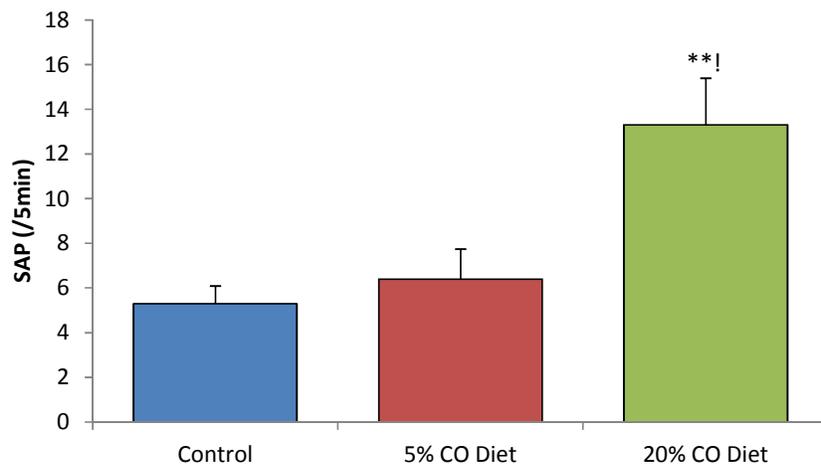


Fig. 13. Comparison of frequency of stretch attend postures in the light/dark box test between mice treated with 5% coconut oil diet and 20% coconut oil diet
 * - Significant at $p < 0.05$ compared to control

The elevated plus-maze (EPM) was also used to test for anxiety and exploration in the mice. This test exploits the conflict between the natural tendency of mice to explore novel areas and fear of open spaces. The open arms are aversive to mice because they are open and the maze is elevated [27]. The open arm avoidance (in this case decreased time spent in the open arms) also gives a measure of anxiety [28]. The closed arms provided a sense of safety because they were enclosed. Therefore, increased open arms activity indicate decreased anxiety and vice versa. Rearing behaviour is considered as exploratory, and a greater frequency of this measure shows a greater level of exploration. [25] Generally, fear related behaviours include closed arm activity, stretch attends, grooming, freezing, defecation and urination; a greater number of these measures implies a greater level of emotionality, anxiety or fear [27].

Although the open arms duration did not differ between the 5% coconut oil diet fed mice and control, it was lower in the 20% virgin coconut oil diet fed group of mice. This means an increased avoidance of the open arms by mice fed 20% coconut oil diet, indicating increased anxiety in the group. The frequency of stretch attend postures showed an increase in both 5% and 20% diets of the oil compared to their control with the 20% virgin coconut oil diet group having more stretch attend posture when compared to its 5% counterpart. This implies that although both diets increased anxiety, the 20% virgin coconut oil diet had a greater effect. The frequency of rearing was lower in both test groups of mice compared to control, indicating that at both doses 5% and 20% virgin coconut oil diets, exploration was decreased in the elevated plus maze test.

The light-dark transition box (LD Box) tests for unconditioned anxiety and exploratory behaviour. It is also based on the conflict between exploring in a novel environment and avoidance of bright light [33]. The dark compartment which is the smaller and coloured black provides a sense of safety to the mice. Ordinarily, rodents will explore novel environments; but large open areas like the light compartment is aversive to the mice [33]. Increased activity (line crosses, rearing) and transitions between the light and dark chambers is associated with non-anxious behaviour [33,34,35]. However, increased behaviour like stretch attend postures would indicate increased anxiety.

The frequency of rearing followed a similar trend as was seen in the elevated plus maze with a decrease in both 5% and 20% virgin coconut oil diet groups. The frequencies of line crosses and transitions did not differ. The frequency of stretch attend postures did not differ in the 5% diet group but was higher in the 20% diet group compared to both control and 5% diet group. This indicates an increase in anxiety in the 20% diet group over the 5% diet group.

The results of the anxiety-related behaviour scored in this study in the elevated plus-maze test (Shorter open arms activities and increased stretch attend postures (SAPs)), the open-field test (increased grooming and SAPs) and the light/dark box (increased stretch attend postures and decreased rearing) were consistent with the anxiety-related behaviour scored by Kanari et al. [36]. These behaviour are simple, straight forward and easy to deduce that there was increased anxiety in the three behavioural tests used. However, interpreting data for anxiety-related behaviour in some cases could be more complicated. In a study to compare different behavioural test situations used for measurement of anxiety, Belzung and Le Pape [37] pointed out the difficulty with which consistency could be obtained from variables of different behavioural tests.

In summary, all the anxiety related behaviour were increased in both groups of mice treated with 5% and 20% virgin coconut oil diets. The trend was the same for all the behavioural tests carried out: the open field test, elevated plus maze test and the light/dark box test. The results showed that although long-term consumption of both 5% and 20% virgin coconut oil diets increased anxiety related behaviour, the 20% coconut oil diet had a stronger anxiogenic effect. These results are however contrary to the claims that virgin coconut oil may be useful in resolving anxiety related disorders or depressive symptoms. It is not clear by what mechanism this happens. Therefore, as a continuation of this study, it would be necessary to unravel the mechanism by which prolonged consumption of coconut oil diets would cause increased anxiety.

5. CONCLUSION

Long-term consumption of coconut oil did not affect horizontal locomotor behaviour but it decreased exploratory behaviour in the mice. Long-term consumption of coconut oil increased anxiety related behaviour in CD1 mice in the

open field, elevated plus maze and light/dark transition box tests. Although coconut oil consumption is very important for other physiological functions, its long-term consumption could increase anxiety. If these results are applicable to humans, it may not be very advisable to consume coconut oil excessively over long periods.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All authors also hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Ethical approval was granted for the study by the Faculty of Basic Medical Sciences Research Ethics Committee of the University of Calabar, Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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