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Caffeine Improves Neurobehavioural Impairments in Fructose-Fed Swiss Mice via Multiple Neurochemical Mechanisms

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ABSTRACT

Caffeine is a substance that has attracted more research attention in recent years as it forms the main ingredient in many beverages, including coffee. It has stimulant activity with low toxicity. Fructose on the other hand has been shown to impair neurocognitive functions in experimental animals. This study investigated the effect of caffeine on fructose-induced neurobehavioural impairment in mice. Twenty-seven Swiss mice (n=9) were randomly grouped as follows: Control (water and rat chow *ad libitum*); fructose treated group (100% fructose solution *ad libitum*); and fructose-caffeine treated group (100% fructose solution + 1 g/L of caffeine *ad libitum*). Treatment lasted for 6 weeks after which the mice were subjected to Morris water maze, Y-maze, elevated plus maze and beam walk tests. Thereafter, the animals were humanely sacrificed. Whole brain samples were harvested and gently homogenized in sodium phosphate buffer, and cold centrifuged for neurotransmitters assay. The results showed that fructose significantly ($p<0.05$) increased body weight, impaired memory functions and inhibited the brain levels of dopamine, serotonin and acetylcholine. Caffeine co-administered with fructose was however, found to significantly ($p<0.05$) reduce body weight, improved memory and locomotor performances and increased brain levels of the aforementioned neurotransmitters and enzyme. It was concluded that fructose impairs neurobehavioural activities via impaired brain neurotransmitter and enzyme activities, which was ameliorated by caffeine.

Keywords: *Caffeine, Fructose, Morris water maze, Y-maze, Elevated plus maze, Neurotransmitters*

INTRODUCTION

Nutritional status, diet and a range of nutrients play critical roles in our neurocognitive function and performance (Smith and Scholey 2014). Fructose is one nutrient that has received immense attention in recent times especially in neuroscience research. It has been implicated in brain impairment and in the evolution of neurodevelopmental disorders via its link to obesity and type-2 diabetes (Gomez-Pinilla et al. 2021). In contrast to glucose, fructose induces hyperphagia in the brain of experimental rodents (Luo et al. 2015). Endogenous production of fructose in the brain via the polyol pathway exacerbates its toxic effect on the brain via production of free radicals (Hwang et al. 2017). Recent findings have linked overactivation of cerebral fructose metabolism to the aetiology of Alzheimer's disease via reduced mitochondrial energy production, neuroinflammation and insulin resistance (Johnson et al. 2020; Spagnuolo et al. 2020). Caffeine is a globally consumed substance because of its brain stimulating functions. It is commonly used to sustain wakefulness, enhance memory and improve physical performance (Cappelletti et al. 2015; Lin et al. 2021). Caffeine is a non-specific adenosine receptor antagonist which has shown efficacy in retention of memory in mice trained on the Morris water maze (MWM). In human experiment, it ameliorated age-related cognitive decline and scopolamine-induced amnesia, and

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promotes explicit memory (Angelucci et al. 2002; Sherman et al. 2016). This study aimed at assessing the neurobehavioural effect of caffeine in mice fed chronically on fructose. Caffeine, which we regularly consume in coffee, could be found to possibly ameliorate the deleterious effect of fructose on human brains. Studies on the central nervous system effect of caffeine on fructose-fed mice are limited and this research seeks to explore that.

MATERIALS AND METHODS

Chemicals and Drugs

Caffeine (1,3,7-trimethylxanthime, analytical grade, Shandong Xinhua Pharmaceutical Company, China) and 100% solid fructose (Anhui Elite Industrial Co. Ltd, China) were purchased from Stevemoore Chemical Company Zaria, Kaduna State, Nigeria.

Mazes

The Morris water maze, Y-maze, elevated plus maze and the beam walk apparatus were all constructed locally according to the specific protocol in literature.

Ethical Approval

This study was done in accordance with the laid down procedures of the Basic Science Research Ethics Committee of the College of Health Sciences, Benue State University, Makurdi, Nigeria which is in line with the guideline of the Institute for laboratory Animal Research, USA (NRC 1996).

Animal Management

Twenty-seven Swiss mice of both sexes, 7 weeks old, weighing between 15-17 g were obtained from the Animal House, College of Health Sciences, Benue State University, Makurdi, Nigeria. They were kept in standard wooden cages in the research room of the Department of Physiology, Benue State University, at 37℃ room temperature, and were fed with standard rat chow (Chikun Feed, Osun State, Nigeria) and water *ad libitum*. The animals were allowed a period of two weeks to acclimatize before commencement of experiment. Solid fructose (100%) was dissolved in tap water (1.5 g/L). Caffeine was constituted at a concentration of 1 g/L. The animals were randomly divided into three groups of nine mice each: Control (normal rat chow *ad libitum*); Fructose (1.5 g/L *ad libitum*) and Fructose+Caffiene (1.5 g/L *ad libitum*) for six weeks. The animals were weighed before and after the treatments. They were also subjected to the various neurobehavioural paradigms for a total of five days. The MWM test lasted for four days, while the Y-maze, elevated plus maze (EPM) and beam walk (BWT) tests were performed on the last two days.

Morris Water Maze Test

Morris water maze (MWM) as described by Morris (1980) was used to assess learning and memory in the mice. An inflatable pool (1.47 m in diameter, Intex® Development Company Limited, Hong Kong) was used for the test. The pool was divided into four quadrants and filled with water at 29℃ to a depth of 14 cm to allow the animal to swim freely. It was used to assess visuospatial memory which involves using extra maze cues to find the location of a hidden escape platform (Vorhees and Williams 2006). A sealed cylindrical plastic container submerged 1 cm below the water surface in the escape quadrant served as the escape platform. The animals were subjected to a 3-day acquisition training phase with starting points changed sequentially in the various quadrants. The time (escape latency) to locate the hidden platform after 120 sec of exploration on each day was noted. In case an animal fails to locate the hidden platform it was assisted to the platform and allowed to stay there for 30 sec to build cohesive visuo-spatial memory and appropriate representation of the pool. On the fourth day a single probe trial was done; the animals were released into the pool from quadrant 4 (Q4) without a hidden platform to explore for 120 sec. The total time spent in Q4 in search of the removed platform was recorded.

Y-Maze Test

The Y-maze was also used to assess working and spatial memory. It consisted of three identical arms A, B and C at 120° to each other. This maze, tests the innate preference of an animal to explore an unexplored arm (Hughes 2004). One of the arms of the maze was blocked with a shutter, allowing for a 5-min exploration of only two arms. After a 30 min delay, trial 2 was commenced in a similar manner, this time with all three arms open for another 5 min exploration. After each trial, the maze was wiped with a cloth dipped in 70% ethyl alcohol and allowed to dry, to remove any olfactory clue. The number of maximum spontaneous alternations (total number of arms entered minus two) and actual alternations (i.e ABC, ACB, BAC, BCA, CAB, or CBA). Percentage alternation was therefore calculated thus:

$$\text{Percentage alternations} = \frac{Actual\ alternation}{Maxium\ alternation} \times 10$$

Elevated Plus Maze (EPM) Test

The EPM is used in testing for anxiety in experimental animals (Almosawi et al. 2018). It was constructed from wood and consists of two open arms (25 × 5 cm) and two enclosed arms of the same size at opposite sides. The enclosed arms were 15 cm high, and all raised 55 cm above the floor. The entire apparatus was cleaned using 70% ethanol between each trial. Each mouse was individually placed at the edge of the open arm and allowed to freely explore the apparatus.

The time spent in the open arms before entering the closed arm (transfer latency) was recorded for 10 min. An entry was recorded when all four paws entered the closed arm.

Beam Walk Test (BWT)

The BWT was used to assess motor coordination and balance. The apparatus consists of a 1 m long beam with a flat surface 12 mm wide, placed 50 cm above floor standing on two erect poles. A black box was placed at the end of the beam as the finish point. A 60 Watt lamp bulb was used to illuminate the start point serving as an aversive stimulus. The time to traverse the beam was measured sequentially. A soft material to cushion a fall, stretched below the beam. The animals were placed at the end of the beam and allowed to traverse it at three consecutive times. The time taken to enter the escape box was measured for each animal. Video recordings were used for finer analysis of slipping and other observable motor deficits. The beams and box were wiped with 70% ethanol after each test. Performance on the beam was quantified by measuring the time it takes for the mouse to traverse the beam and the number of paw slips that occur in the process (Luong et al. 2011).

Organ Harvest and Neurotransmitter Assay

Twenty-four hours following the behavioural tests, the animals were subjected to light anaesthesia under chloroform vapour in an enclosed plastic chamber and craniotomy was done, whole brains harvested, homogenized with sodium phosphate buffer (pH 7.5) and centrifuged. The supernatants were decanted and refrigerated in plain sample bottles.

Acetylcholinesterase Activity

Acetylcholinesterase activity was measured according to the modified method of Ellman et al. (1961). The principle of the method involves the hydrolysis of acetylthiocholine iodide by acetylcholinesterase to produce thiocholine. Thiocholine was allowed to react with the -SH reagent 5,5′-dithiobis-(2-nitrobenzoic acid), which was reduced to thionitrobenzoic acid, a yellow coloured anion whose absorption was read spectrophotometrically at 412 nm. The results are expressed as µmol SH/min/g.

Neurotransmitter Analysis

Dopamine was assayed based on the hydroxyindole principle as described by Atack (1973). N-ethanolic (50%)-HCl eluate is added to a cation exchange purified sample. Fluorescence readings were taken at 360-470 nm. Serotonin estimation was done by a fluorometric method as described by Bogdanski et al. (1956). The procedure involves the use of a spectrophoto-fluorometer which can activate and measure emitted fluorescence continuously from 250 to 650 mµ. Serotonin was activated maximally at 295 mµ and emitted fluorescent light which showed a maximum at 550 mµ.

Data Analysis

Data obtained from the study were expressed as mean ± standard error of mean. The differences between the groups were analyzed by one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons using statistical package for social sciences (SPSS version 23). Values of $p < 0.05$ were considered significant.

RESULTS

Effect of Caffeine on Body Weight

The fructose-only group (Fig. 1) had a significantly higher body weight ($p<0.05$) when compared to the initial body weight and that of the control group. The fructose+caffeine treated group showed a significantly lower change in body weight when compared to the fructose group.

Effect of Caffeine on Memory in Fructose-Fed Mice

The time spent in the target quadrant during probe trial in the MWM and the percentage spontaneous alternations on Y-maze both measure memory. The caffeine-supplemented group showed a significantly higher time spent in the target quadrant (Fig. 2) and percentage spontaneous alternations (Fig. 3) compared to the fructose only group ($p<0.05$) which performed poorly.

Effect of Caffeine on Locomotion and Anxiety

The transfer latency in the EPM was significantly shorter in the caffeine treated group (Fig. 4)

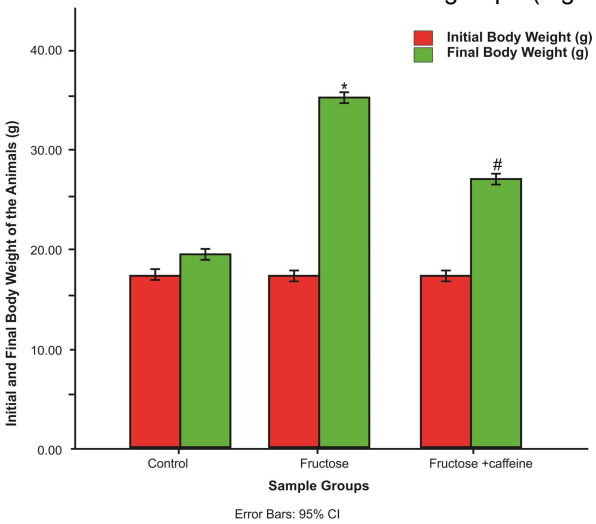

Fig 1: Change in body weights across the various groups. * indicates statistically significant difference in relation to the initial weight in the fructose group and the control. # indicates statistically significant difference in relation to the fructose group ($p<0.05$).

compared to the other groups (p<0.05). The caffeine-treated group had a significantly shorter time to cross the entire beam length when (Fig. 5) compared to the other groups (p<0.05).

Effect of Caffeine on Neurotransmitters and Enzyme

Brain levels of serotonin and dopamine were all significantly elevated (p<0.05) compared to the fructose only group. The brain acetylcholinesterase level was also significantly elevated by caffeine supplementation compared to control and fructose groups (Table 1).

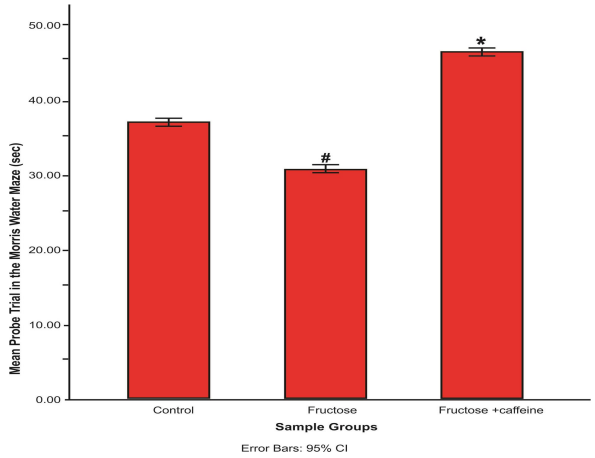

Fig. 2: Time spent in target quadrant in MWM in the various groups. * indicates a statistically significant difference in relation to the control and fructose groups (p<0.05).

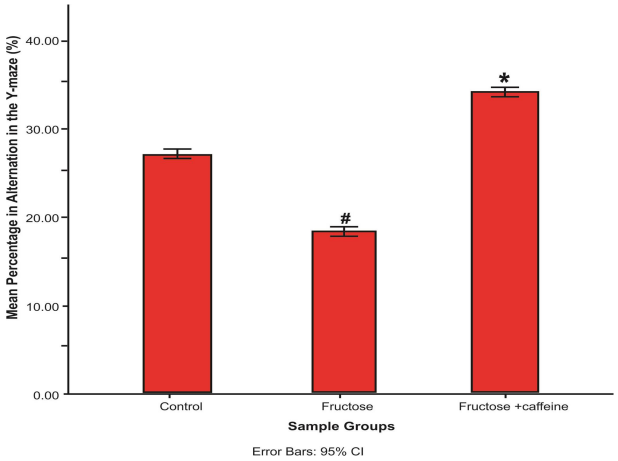

Fig. 3: Percentage spontaneous alternations in the Y-maze across the various groups. * indicates a statistically significant difference in relation to the control and fructose groups (p<0.05).

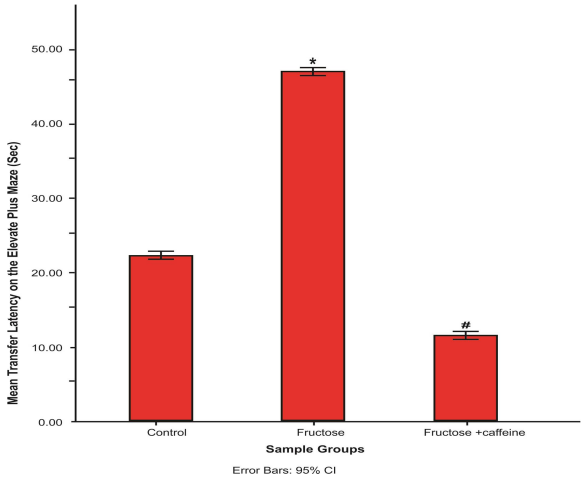

Fig. 4: Transfer latency in the EPM experiment across the various groups. * indicates statistically significant difference in relation to the control and fructose+caffeine groups. # indicates statistically significant difference in relation to the control and fructose groups

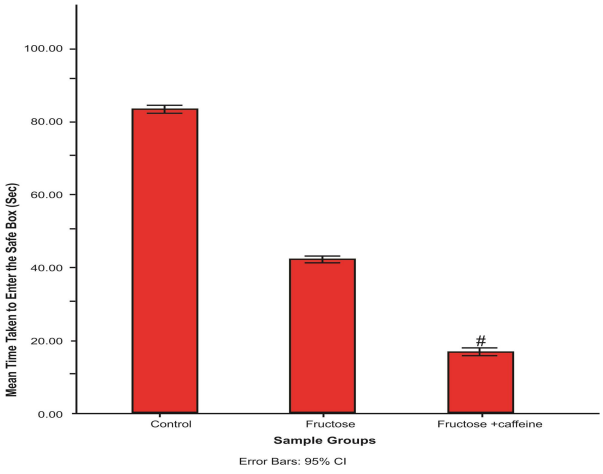

Fig. 5: Mean time to traverse the beam across the various groups. # indicates a statistically significant difference in relation to the control and fructose groups (p<0.05).

DISCUSSION

The current study assessed the effect of caffeine supplementation on neurobehavioural and neuro-chemical activities in fructose-fed Swiss mice. The present study showed significant weight gain in the fructose only group at the end of the experiment. This result corroborates Bocarsly et al. (2010), who showed that the administration of fructose to Sprague-Dawley rats for 6 and 7 months resulted in significant weight gain, and adiposity as well as dyslipidaemia. The fructose+caffeine treated group had significantly reduced weight gain in the present study. Some previous studies also agree with this: with possible mechanisms of caffeine's action being through improved energy balance, depressed appe-

tite, elevated basal metabolic rate, sympathetic drive, catecholamine-induced lipolysis and uncoupling of protein-1 in brown adipose (Kobayashi-Hattori et al. 2005; Velickovic et al. 2019).

The MWM and Y-maze tests assessed visuospatial memory. These tests are acceptable models for evaluating learning and memory in experimental animals. Locomotor activity and anxiety were assessed using BWA and EPM, respectively. The fructose treated group had a significantly reduced time spent in the target quadrant in the MWM. However, the caffeine treated group had a significantly longer time spent in the target quadrant. Similarly, in the Y-maze the caffeine treated group had a significantly higher percentage spontaneous alternations compared to the fructose treated group which showed significant reduction. The poor performance in the memory tests in the fructose only group compared with the control can also be attributed to the increased weight induction which has a strong link with metabolic disorders and cognitive decline (Stephan et al. 2010; Lakhan and Kirchgessner 2013). The positive action of caffeine on learning and memory has been established in several studies conducted in experimental animals using neurobehavioural mazes (Angelucci et al. 2002; Simons et al. 2012; Sherman et al. 2016). This present study agrees with other research that demonstrated that chronic fructose intake impairs performance in mazes. Studies have shown that fructose intake impairs learning and memory in animals, causes brain insulin resistance, neuroinflammation, and reduces neurogenesis by an oxidative stress mechanism (Lowette et al. 2015; Spagnuolo et al. 2020). Another study showed that fructose reduces cognitive function by a phosphorylation process that disrupts the mitochondria and increases free radicals (Yabe et al. 2018).

Results from the EPM showed that fructose treated group spent more time in the open arm compared to the fructose+caffeine treated group that spent lesser time. These results indicate that caffeine may have caused anxiety in our animals as they had an aversion for the open space. The result is in line with Almosawi et al. (2018), who reported that a high dose of caffeine (1g/l) causes anxiety, but low dose has anxiolytic effects. A medium to high dose of caffeine has been shown to heighten anxiety by its ability to increase the secretion of catecholamine neurotransmitters such as adrenaline (Pohanka and Dobes 2013). Our result in the fructose-only group is contrary to findings of Chakraborti et al. (2021), which showed that fructose causes anxiety and depression. More study on this will be necessary.

In the beam walk test, the fructose treated group spent a significantly longer time to cross the beam compared with the caffeine treated group that spent a lesser time to enter the safe box without evidence of slipping off the beam. A study by Rendeiro et al. (2015) supported this finding and attributed the decreased locomotion function caused by fructose to a sequence of elevated visceral fat, weight gain and decreased physical activity without any adverse hippocampal effect. The enhanced locomotor activities in the caffeine treated group agrees with the findings of Marin et al. (2011) who demonstrated that caffeine increased locomotor activities in habituated rats. The effect has been attributed to its antagonism at the adenosine receptors. The high level of dopamine from the basal ganglia has also been found to increase locomotor output (Ryczko and Dubuc 2017), which was significantly elevated in this study.

Caffeine has been known to possess neuroprotective action attributed to its antagonism of the adenosine receptors, suppression of such an enzyme as acetyl cholinesterase, and to promote the secretion of such neurotransmitters as dopamine, epinephrine, norepinephrine and acetylcholine (Angelucci et al. 2002; Persad 2011; Pohanka and Dobes 2013; Sherman et al. 2016).

This present study revealed that caffeine increases the brain level of dopamine compared to the fructose group. Caffeine antagonizes the effects of endogenous adenosine thus, enhancing the release of dopamine, as well as activating noradrenaline neurons with resultant local release of dopamine (Nehlig et al. 1992; Solinas et al. 2002). Another neurotransmitter, serotonin was elevated by caffeine in the present study. A recent human study demonstrated that caffeine reduces brain serotonin level by antagonizing the inhibition of adenosine α1 and α2 receptors (Lee and Kim 2019). This conflicting result may be due to specie difference or difference in dosage. However, our finding agrees with Yogoshi et al. (1987) and Habibzadeh (2020), who showed that caffeine increased the brain serotonin level in mice. Dopamine and serotonin are neurotransmitters necessary for the maintenance of memory consolidation when released from nerve terminals, and caffeine has the potential to cause their release in significant proportion (Lee and Kim 2019).

Acetyl cholinesterase, an enzyme was also significantly elevated in this study. This is not in line with a number of studies (Pohanka and Dobes 2013; Khadrawy et al. 2018), which showed that caffeine reduces the brain level of acetyl cholinesterase. It is

Table 1: Neurotransmitters and enzyme levels across the various groups

Neurotransmitters/Enzyme	Control	Fructose	Fructose+caffeine
Serotonin (µg/g)	0.17±0.9	0.08±0.1	0.20±0.8*
Dopamine (µg/g)	0.90±0.8	0.70±0.9	0.85±0.7*
Acetyl cholinesterase (SH/g/min)	5.00±1.2	11.00±1.3	13.00±1.5*,**

** statistically significant in relation to the control group (p<0.01);
* statistically significant in relation to the fructose group (p<0.05)

proposed that the rise in brain acetyl cholinesterase may be as a result of a positive feedback effect in response to a probable surge in acetylcholine at the brain neuronal terminals caused by caffeine. Trang and Khandar (2022) supports this proposition by demonstrating that acetyl cholinesterase controls the volume of acetylcholine at the synapse when released suddenly, and thus, plays a positive role in neural development and in morphogenesis of actively growing brains.

Conclusion

The results showed that caffeine ameliorated the deleterious effect of fructose on learning, memory, locomotion and mood by multiple neurotransmitter mechanisms. The body weight gained in the fructose treatment was also stabilized by caffeine. The possible mechanism(s) proposed is the increased release of some neurotransmitters (dopamine and serotonin) as well as the surge in acetyl cholinesterase enzyme in possible response to an initial elevation in brain acetylcholine by caffeine. Further research will be necessary to understand the molecular mechanism behind these findings.

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Conflict of Interest
None declared.

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Authors Contribution
Conceptualized, designed and supervised: IA; Data collection and analysis: VVM; Animal handling, experimentation and sacrifice: VVM, BNO and JUO

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