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Research Paper

The Efficacy of N-Acetyl-Cysteine (NAC) Supplementation in FST Model for Screening Antidepressants

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ABSTRACT

Introduction: The model for screening antidepressant-like activity in pre-clinical drug studies include, rat forced swimming test (FST). The reports on N-acetylcysteine (NAC) as an antioxidant supplement in stress related disorder is well documented. This study was aimed at potential antidepressant mechanism of N- Acetyl Cysteine (NAC), a glutamate precursor on FST animal model for screening antidepressant drugs using fluoxetine, a selective serotonin reuptake inhibitors (SSRIs) as standard antidepressant drug.

Methods: Thirty adult male Wistar rats used for this study were randomly divided into six groups each with five (n=5) rats. The control group (A) received 1 ml of normal saline daily, group B served as the FST model, group C received 200mg/kg/day of NAC, group D received 20mg/kg/day of fluoxetine, group E the FST model treated with 200mg/kg/day of NAC, and F is the FST model treated with 20mg/kg/day of fluoxetine. Drugs were given orally. The effects of NAC on brain weights, the FST paradigms, sucrose preference test (SPT) for anhedonia were assessed and data analyzed using ANOVA where Tukey post-hoc test for statistical significance was set at ($p < 0.05$). The brains fixed in 4% paraformaldehyde, were processed and the paraffin embedded tissue were serially sectioned at 5 µm thick to be stained using Haematoxylin and Eosin (H and E) stain, immuno-histochemistry for synaptophysin (p38) and astrocytes (GFAP) activities in the prefrontal cortex (PFC).

Results: Findings showed that NAC prevented FST-induced anxiety-like behaviors demonstrated by an increased SPT (that alleviates anhedonia), mobility time, and reduced immobility time. NAC caused an increase in brain weights and prevented FST-induced neurodegeneration, the proliferation of reactive astrocytes, and diminished synaptophysin immunoreactivity in the PFC similar to that seen in fluoxetine a standard anti-depressant drug.

Conclusion: NAC treatment significantly exhibits its neuroprotective mechanism via inhibiting the proliferation of reactive astrocytes, which protects neurons and synapses from oxidative tissue damage induced by FST, hence an increase in synaptophysin activity that culminates in increased neural activity, increased SPT, and reduced immobility time.

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Highlights

- It demonstrates NAC's potential to enhance neuron proliferation, and increase synaptic density while preventing FST-induced physiology stress loss of neuronal cells and synapses.
- NAC antidepressant effects are seen in a decline in immobility time in FST.
- NAC also increased sucrose preference in the FST model alleviating the anhedonia effects of FST
- NAC neuroprotective and neuro-anti-inflammatory effects are seen in a decline in astrocyte proliferation 9 an increase in astrocyte proliferation is a response to inflammation, and oxidative damage mediated by FST.
- Synaptophysin is a synaptic protein that increases in expression due to increased synaptic activity.
- NAC protects against oxidative damages induced loss of neuronal or synaptic activity linked with a decline in synaptophysin expression.

Plain Language Summary

This study evaluated the processing by which NAC is an antioxidant with the ability to increase intracellular production of glutathione exhibits antidepressant effects. Depression is a neurodegenerative and psychiatric disorder, ravaging a large population of the world and new therapies with fast and long-lasting antidepressant effects should be discovered. This aforementioned fact is linked to drugs that target the glutamate system. Over the years, animal models that involve the use of forced swim test (FST) to induce physiologic stress provide translationally relevant information on major depression for initial screening. N-acetylcysteine as a potential antidepressant drug (NAC) was administered and fluoxetine was used as the baseline antidepressant drug. The prefrontal cortex being area affected by depression was evaluated for changes in neurons, neuroinflammatory biomarker astrocytes, synaptophysin for synaptic activity, and neuro-behavioral changes using a sucrose preference test and changes during a forced swim test. NAC prevented loss of brain weight, neurons, and synapse formation requires for neural or synaptic activity seen in an increase in synaptic expression while ameliorating reactive astrocyte proliferation. This finding demonstrates the anxiolytic effects of NAC as a potential antidepressant drug for managing depressive disorders and it should be implored clinically as an antidepressant drug.

1. Introduction

Depression is caused by chronic psychological stress or trauma and it is the leading cause of death worldwide hence an issue of global concern for an individual and the society at large (Fitzgerald, etal., 2019; Zhang et al., 2019). It is the most common neurodegenerative (ND) or psychiatric disorder (Ossig & Storch, 2015). According to WHO reports (2017), the number of people living with depression increased by 18% from 2005 to 2015. Furthermore, depression affects 322 million people, i.e., 4% of the world's population (Furukawa et al., 2019), and only 50% of those afflicted are considered non-responsive to antidepressant treatment (Parise et al., 2013). The pathogenesis of depression is associated with neuro-inflammatory processes and oxidative stress (Frodl & Amico, 2014; Kiraly et al., 2017). Glutamatergic dysfunction is also an important pathological mechanism in depressive disorders (Costa-Campos et al., 2013; Wright et al., 2016). Currently, N-acetylcysteine (NAC) is an antioxidant supplement being evaluated for its possible antidepressant therapy (Yang et al., 2018). NAC is a glutathione precursor and glutamate modulator (Costa-Campos et al., 2013), with antioxidant and neurovascular-protective effects (Chen, et al., 2008). It could ameliorate neuropathogenesis due to its ability to cross the blood-brain barrier (Pallanti, et al., 2014). Within the brain tissue, NAC as a precursor for glutathione synthetase (GSH) prevents neuronal damage caused by reactive oxygen and nitrogen species (Magalhães et al., 2011; Miguel et al., 2012). NAC mechanism of action for its neuroprotective potential is related to its ability to regulate neuroinflammation due to neuron dysfunction and apoptosis thereby promoting neurogenesis and repairing neuronal damage, as well as normalizing glutamate dysregulation linked to N-methyl-D-aspartate hypofunction (Dean et al., 2011; Zavodnick & Ali, 2014; Deepmala et al., 2015; Jakobsen et al., 2017). Various studies reported

that NAC can increase neuron connectivity in the brain (Carmeli et al., 2012), the level of anti-inflammatory microglia (Haber et al., 2018), and modulation of glutamate pathways (Fond et al., 2014; McQueen et al., 2018) in psychiatric conditions (Mullier et al., 2019) which is one of its psychopharmacological effects. Kohler et al. (2014) reported that anti-inflammatory agents can be used as a treatment measure to ameliorate depression. The recommended oral dosage of NAC is between 2000 and 2400 mg/d in humans (Liang, Green, & Pak, 2018). Animal models for screening antidepressant-like activity in several pre-clinical models, including the rat forced swim test (FST) (Ferreira et al., 2008; Slattery and Cryan, 2012), testing for anhedonia using sucrose preference test (Ceren et al., 2018). The prefrontal cortex (PFC) is an area commonly implicated in major depression disorders (Schubert, et al., 2015; Fogaça and Duman, 2019). Synaptophysin is an integral membrane glycoprotein present in presynaptic vesicles of all neurons and is involved in synaptic transmission, synaptic biogenesis, initiating neurotransmitter release, synaptic vesicle endocytosis, and synapse formation (Gudi et al., 2017) while astrocytes are specialized glial cells which provide structural and functional support for neurons (Şovrea and Boşca, 2013). This study aimed to assess the mechanism of possible antidepressant effects of NAC by evaluating changes in PFC histology, synaptophysin, and astrocytes activities in addition to behavioral changes in the FST indexes and anhedonia status in the animal FST model.

2. Materials and Methods

Experimental animals

Thirty adult male Wistar rats with an average weight of 250 g were used for this study. They were procured and housed in the Animal House of the Anatomy Department, Bingham University, Karu, Nasarawa State Nigeria, and allowed 7 days of acclimatization before the study. Experimental animals were cared for according to guidelines for the care and use of animals in research (National Research Council, 2011). The study was approved by the Departmental Research, Animal Care and Use Ethics Committee. The rats were housed in well-aerated metallic cages. They were fed with rat pelleted feed (Vital Feeds Limited Nyanya, Nasarawa State) and water ad libitum. They were maintained in standard pathogen-free (SPF) laboratory conditions maintained at 12 h light/dark cycle, the temperature of 37±2°C and 60±5% relative humidity (lights on at 07:00 AM). The behavioral procedures were carried out between 08:00 AM and 12:00 PM in specially equipped rooms for behavioral testing within the animal facility.

Experimental drug of study and dose

Fluoxetine (prozac)

Fluoxetine (Medibios Laboratories PVT Ltd, India) was purchased from H-Medix, Abuja, Nigeria, and was used for this study. Fluoxetine affects serotoninergic neurons (Ohira et al., 2019). Its recommended oral dose in neurological studies about antidepressant and neuroprotective effects in rats is taken to be 20 mg/kg d according to some studies (Abdel-Salam et al., 2013; Klomp, et al., 2014; Torrisi et al., 2019; Furukawa et al., 2019; Costescu et al., 2019).

N-acetylcysteine

N-acetylcysteine (NAC) (Swanson, 60-mg capsule) was purchased from H-Medix Abuja, Nigeria. NAC was given orally 60 min before the FST procedure according to Erdinc et al., (2019) and Magalhães et al., (2019) methods. One hour later they were subjected to FST for 5 min and the immobility time was recorded. The study dose of NAC was taken as 200 mg/kg/ d taken below the reported toxic dose of 500-2000 mg/kg (Johnson, et al., 1983).

Experimental animals grouping with experimental design and protocol

The experimental animals were grouped as follows:

1. **Control (A):** The rats were given pelleted food and water ad libitum plus 30% sucrose solution for 3 days without FST.

2. **FST animal model (B):** The rats were given pelleted food and water ad libitum plus FST. They were also exposed to a 30% sucrose solution to test for anhedonia.

3. **NAC-treated (C):** The rats were given pelleted food and water ad libitum plus 30% sucrose solution, as well as 200 mg/kg NAC orally.

4. **Fluoxetine treatment (D):** The rats were given pelleted food and water ad libitum plus 30% sucrose solution, as well as fluoxetine 20 mg/kg orally.

5. **NAC-treated FST animal model (E):** The rats were given pelleted food and water ad libitum plus 30% sucrose solution, FST, and 200 mg/kg NAC orally.

6. Fluoxetine-treated FST animal model (F): The rats were given pelleted food and water ad libitum plus 30% sucrose solution, FST, and fluoxetine 20 mg/kg orally.

Behavioral studies

Forced swim test (FST)

The forced swim test (FST) is the commonly used animal model for screening antidepressant-like behavior in animals for antidepressant drug screening because it can induce physiological stress via forced swimming (Porsolt et al., 2001; Slattery & Cryan, 2012). FST has strong predictive validity for the short-term administration of antidepressant compounds that can reduce immobility time in the FST (Cryan, et al., 2005; Yankelevitch-Yahav et al., 2015). The rats were placed individually in a transparent cylindrical tank (50 cm diameter, 60 cm height) filled with water (35.2±1°C). The water was changed between testing sessions. Each rat received the study drugs an hour before the FST (Porsolt et al., 2001). First, a pretest of 15 min (for habituations), and then a test for 5 min following drug treatment (Slattery & Cryan, 2012; Stratinaki et al., 2013) is administered. Swim sessions were video recorded and the immobility, swimming, and climbing behaviors were scored at the end of each test session (Cryan, et al., 2005; Fischer et al., 2015; Pereira et al., 2015) and timed using a stopwatch. The 5-min test was scored by a trained blind observer (Castagné et al., 2010). All procedures were conducted between 9:00 AM and 12:00 PM.

Sucrose preference test (SPT) and water intake Test

Sucrose preference test (SPT) is a sensitive screening test as a measure of anhedonia in rodents, a decline in SPT shows a significant face validity for chronic stress and antidepressant treatment (Razmjou, et al., 2015; Liu et al., 2018). In this study, the rats were habituated to a 1% sucrose solution for 48 h before the test day. Rats were deprived of water and food for the 12hrs. Then the 1-hour preference test by exposure to 100 mL of 30% sucrose solution and water delivered in identical bottles (Razmjou et al., 2015). Sucrose and water consumption were determined by measuring the change in the volume of fluid consumed (Çorumlu et al., 2015; Watson et al., 2020). The bottles containing water and sucrose were weighed before and after this period, and sucrose preference (%) was determined (Çorumlu et al., 2015).

Sucrose preference percentage (%)

Sucrose preference was calculated using this formula (Çorumlu et al., 2015; Ceren et al., 2018).

$$\text{SPT (\%)} = \frac{\text{Sucrose consumption (mL/h)} \times 100\%}{\text{Water consumption} + \text{Sucrose consumption (mL/h)}}$$

The means of three measurements were taken as follows: sucrose and water I was measured 24 h before the 15-min FST pretest; sucrose and water II was measured 24 h after the 15-min FST pretest, and sucrose and water III was taken 1 h after 5-min FST and drug administration.

Experimental animals euthanasia and prefrontal cortex excision for preservation

The final body weights were taken using a weighing balance (OHAUS Pioneer™, India). The experimental rats were then perfused transcardially with normal saline (0.9% NaCl), followed by 4% paraformaldehyde for 20 min. The animals were randomly decapitated via cervical dislocation (Magalhães et al., 2019). The whole brain was gently excised and wet weight was taken using an analytical weighing balance (OHAUS Pioneer™, India). They were rapidly fixed in 4% paraformaldehyde for tissue processing (Bancroft & Gamble, 2008).

Prefrontal cortex histological processing for staining

Coronal sections of the prefrontal cortex were dissected according to some studies (Paxinos & Watson, 2007; Abdallah et al., 2016; Amat et al., 2016) for histological tissue processing using an automated tissue processor (LEICA TP 1050) set to pass through dehydration in graded alcohols, clearing through xylene, and embedded in paraffin. The processed PFC tissue was embedded in paraffin wax and sectioned using a Lecia rotary microtome set at 5 µm ready for Hematoxylin and Eosin (H & E) staining, glial fibrillary acidic protein (GFAP) (Bancroft & Gamble, 2008; Akinrinade et al., 2015; Memudu, et al., 2020), and synaptophysin (p38) immunohistochemistry (Gudi et al., 2017).

Histological staining

The labeled prefrontal cortex sections were stained using H & E staining to demonstrate the general histological appearance of the pyramidal cells and the neuropils by following this stepwise procedure: dewaxing in two changes of xylene for 5 min each; hydration in descending grades of alcohol (100%, 90%, and 70%) for 2 min each; rinsed in running tap water for 3 min to wash off the alcohol; stained with Mayer's hematoxylin for 5 min; differentiation in 1% acid alcohol for 2 to 3 s; rinsed in running tap water for 3 min for bluing; counterstained in Eosin for 3 min; rinse in wa-

ter and dehydrated through ascending grades of alcohol: 50%, 70%, 90% and 100% for one minute each; cleared briefly in xylene and set to dry in an oven set at 80°C for 60 s. The slides were mounted with cover glass (22×50 mm) using Distrene Plasticizer Xylene (DPX) mountant (Bancroft and Gamble, 2008).

Immunohistochemical staining

Sections of the frontal cortex of the brain were immuno-stained for glial fibrillary protein (GFAP) and Synaptophysin (p38).

Immunohistochemical (IHC) staining for astrocytes

Astrocytes in the formaldehyde-fixed paraffin-embedded rat brain tissue sections were examined using glial IHC protocol (Bancroft & Gamble, 2008; Akinrinade et al., 2015; Gudi et al., 2017; Gil-Martinez et al., 2018). GFAP is commonly used as an astrocyte marker that localizes intermediate filaments and stains astrocyte cellular processes (Gudi et al., 2017; Gil-Martinez et al., 2018). The following primary antibodies were used the primary Novocastra-mouse monoclonal: GFAP-antibody Leica Microsystems-Novocastra™, United Kingdom (1:100 dilutions) and the secondary antibody (Novocastra biotinylated secondary antibodies; biotinylated donkey anti-mouse IgG, 1:200). The peroxidase-coupling was done using avidin-biotin complex (ABC Kit, Vector Laboratories, and Burlingame, CA). The immunoreaction product was visualized with 3,3′-diaminobenzidine (DAB, Dako) for chromogen development. The counterstain was done using Mayer's Hematoxylin and mounting media-DPX (Distrene Plasticizer Xylene). The procedure is as follows: the paraffin-embedded tissue sections were deparaffinized in xylene for 2 min, then they were hydrated in a descending grade of alcohol (100%, 95%, and 70%) for 2 min and brought to water (distilled water). Antigen retrieval was performed by incubation in citrate buffer (10 mM citric acid, pH 6.0) for 15 min and washed in PBS for 3 minutes. Peroxidase activity was blocked by incubating 1% bovine serum albumin (BSA; Sigma, Germany) and 0.3% Triton X-100 for 15 min in the incubating chamber at a temperature of 37°C; slides were washed in PBS for 2 min. Then, the sections were incubated in two drops of diluted primary anti-GFAP antibody (mouse GFAP-antibody Novocastra™, 1:100 dilution) for 45 min. Next, they were washed in PBS for 2 min. Afterward, two drops of the secondary antibody (biotinylated donkey anti-mouse IgG, 1:200 dilution) was added and allowed to incubate for 15 min and then washed twice in PBS for 30 s each. The immune-positive reactions were developed using a polymer, 3,3'-diaminobenzidine tetrachloride (DAB; Sigma, Germany), for chromogen development, and sections were counterstained in Mayer's hematoxylin. Negative controls were performed by omitting the primary antibody (Bancroft and Gamble, 2008). The sections were briefly dehydrated and cleared in xylene and air-dried, thereafter they were mounted with a coverslip using mounting media Distyrene Plasticizer Xylene (DPX) as described by Bancroft & Gamble (2008).

Immunohistochemical (IHC) Staining for Synaptophysin a Synaptic Vesicle Protein (p38) Synaptophysin is an integral membrane glycoprotein present in presynaptic vesicles of all neurons in the brain tissue and is involved in synaptic, synaptic biogenesis, initiating neurotransmitter release, synaptic vesicle endocytosis transmission and synapse formation (Gudi et al., 2017). Synaptophysin is a biomarker to detect axonal damage under different neuropathological conditions and its immunohistochemistry in brain tissues post-fixed in 4% PFA and paraffin-embedded rat brain tissue was done according to Sarnat,et al. (2010) and Gudi et al. (2017) methods. The subsequent procedures were followed: paraffin-embedded sections were dewaxed in xylene and hydrated in alcohol. Sections antigen retrieval was done by incubation using 10 mM sodium citrate (pH 6.0) and phosphate-buffered saline (pH 7.6) in a microwave for 20 min. Then, the sections were washed in PBS for 3 min. Endogenous peroxidase activity was blocked by incubating in PBS containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and 0.5% Triton X-100 at room temperature for 1 h. The slide sections were washed in PBS for 2 min. The tissue sections were then incubated at room temperature using two drops of the diluted primary monoclonal antibody (Synaptophysin Rabbit Monoclonal Antibody [Product #MA5-14532] diluted in 3% BSA-PBS at a dilution of 1:20) diluted 1: 200 in 0.5% TX and 5% normal donkey serum for overnight at 4°C for 1 h in an incubation chamber; then washed in PBS for 2 min. Detection was done using an HRP-conjugated secondary antibody (biotinylated horse anti-mouse secondary antibody) (1:200, Vector Labs, Burlingame, CA, USA), in PBS for 1 h at room temperature (0.2% TX and 5% normal donkey serum for 1 h at room temperature) and then rinsed in PBS for 2 min. Then, the sections were incubated in an avidin-biotin complex linked to peroxidase (ABC Kit [VECTASTAIN kit], Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were rinsed and immunoreaction visualized with 3,3′-diaminobenzidine (DAB) for chromogen development for 20 min. Then, they were counterstained using Mayer Hematoxylin for 5 minutes

at room temperature; dehydrated in alcohol, cleared in xylene, and mounted in DPX. Synaptophysin staining in the negative control was incubated without the primary antibody, confirming that the antibody was synaptophysin-specific (Gudi et al., 2017).

Tissue photomicrography

Photomicrographs were taken using an Olympus (Tokyo, Japan) binocular Light microscope which was connected to a 5.0-megapixel AmScope camera (AmScope Inc., Irvine, CA, USA) with a 10x objective lens. The images were captured with a 40x objective lens and the phototube of the images was captured and stored using the joint photographic expert group (JPEG) format for analysis.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistics Version 13.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., LA Jolla, CA). The Student t test was used for all pairwise comparisons and 1-way ANOVA was used for all multiple comparisons followed by the post hoc Tukey test. For all analyses, differences were considered significant when P values were lower than 0.05 and significant effects are indicated by asterisks (P<0.05). Data were expressed as Mean±SD.

3. Results

NAC prevents FST-induced decline in brain weight

There was a significant decrease in brain weight of the FST model (B) group compared to the control group (A) at P<0.05 (a). Those treated with NAC (C) had no significant difference in brain weight compared with the control (A) group at P<0.05 but had a significant increase when compared with the fluoxetine (D) treated group at P<0.05 (b), when comparing the antidepressant drugs of study (Figure 1) effect on brain weight. Fluoxetine-treated FST (F) had an increase in mean brain weight compared with NAC treated FST (E) group at P<0.05, using Tukey post-hoc test. There was no significant difference in brain weights of NAC (C) and control group (A) at P<0.05.

NAC reversed FST mediated decline in immobility time

The immobility time for FST model (B) increased significantly compared with NAC-treated FST model (E) and fluoxetine-treated (F) FST group at P<0.05 using the Tukey post hoc test (Figure 2). There was no statistically significant difference in immobility time of NAC-treated FST model (E) and fluoxetine (F) treated FST group at P<0.05 using Tukey post hoc test. The mobility time in FST model (B) decreased compared with NAC-treated FST model (E) and fluoxetine-treated (F) FST group at P<0.05 using the Tukey post hoc test. But NAC-treated FST model (E) had a statistically significant increase (b) in mobility time at P<0.05 using the Tukey post hoc test compared with fluoxetine-treated (F) FST group.

NAC prevented anhedonia effects of FST in sucrose preference test

The SPT I showed that groups A (control), B (FST model), E (NAC-treated FST model) and F (fluoxetine-treated FST model) had a significant increase in SPT compared with C (NAC-treated) and D (fluoxetine treated) at P<0.05 Tukey test (Figure 3) SPT II in, B (FST model), C (NAC-treated), D (fluoxetine-treated), E (NAC-treated FST model) and F (Fluoxetine-treated FST model) decreased significantly compared to the control (A) group at P<0.05. The SPT II in groups B, D, and E had no significant difference in their means using the Tukey test at P<0.05, as well as C and F. But B (FST model) showed a significant decrease in SPT III compared to groups A, C, D, E, and F at P<0.05. Groups E (NAC-treated FST model) and F (fluoxetine-treated FST model) had a significant increase in SPT compared with B (FST model) at P<0.05 Tukey post hoc test.

NAC protects the pyramidal cells of the PFC from FST-induced oxidative tissue damage

The control (A) group has numerous pyramidal neurons with no necrosis, scanty neuropil, or vacuolation within the neuropils. The FST model (B) cortical neurons appeared necrotic within the scanty and vacuole-filled neuropil as shown in Figure 4. All neurons have the characteristic of neuron degeneration, pericellular spaces around the neurons, and homogenous cytoplasm with pruned apical and basal neurites. The NAC- (C) and fluoxetine-treated (D) groups showed numerous neurons with dense neuropil and the absence of necrotic cells. There is the presence of axonal and basal dendrite outgrowth compared with the FST model (B). The FST-treated NAC and fluoxetine-treated groups (E and F) showed dense neuropil presence of neurons with the central nucleolus, neurites, and few necrotic cells (the sign of the effect of FST-induced neuron injury or assault). The number of necrotic or pyknotic cells reduced compared with the FST-treated group (B).

NAC reversed proliferation of reactive astrocytes (astrogliosis), a neuroinflammatory response to FST induced oxidative stress

Glial fibrillary acidic protein IHC is used to display astrocytes seen to proliferate in neurodegenerative conditions or due to inflammation. The control (A) group showed well-expressed astrocytes brownish stained astrocytic processes around the neuron cell body (B) as seen in Figure 5. The FST model expressed more astrocytes when compared to the control (A) group. NAC (C) and fluoxetine-treated (D) groups expressed astrocytes mildly compared with the FST model but more compared with the control (A) group. FST-treated study groups E (NAC-treated) and fluoxetine-treated (F) expression of astrocytic activity reduced as compared with the FST model.

Immunohistochemical expression of synaptic protein synaptophysin

To identify the amount of synaptophysin protein which plays a critical role in synaptic plasticity in the prefrontal cortex, cerebellar cortex, and hippocampal CA1 neurons, the immunoactivity of synaptophysin protein was assessed immunohistochemically whereby the rate of expression of synaptophysin protein can be observed by the changes in the intensity of the brown color in micrographs stained with IHC technique. There was an accumulation of synaptophysin-positive vesicles as seen in the brownish deposition within the neuropil of the gray matter of the prefrontal cortex of the control group (A), NAC-treated (C), and fluoxetine-treated (D) as shown in Figure 6. The synaptophysin immunoreactivity was uniformly homogeneous throughout the PFC in A, C, and D, compared to the FST model (B).

4. Discussion

According to the study, there was a significant decrease in brain weight of FST-induced stressed rats (B) compared with the control, NAC-treated, and fluoxetine-treated animal groups. The decrease in brain weight of FST rats in this study correlates with Fortunato et al. (2010) report that FST-mediated physiological stress induces depression in rats that leads to a decline in brain weight. Katz, et al. (1981) reported that mild depression does not have much effect on brain weight. In this study, NAC-treated rats had no significant difference in brain weight compared with the control (A) group which supports Mohammed, et al. (2019) findings. Fluoxetine- and NAC-treated FST model rats had an increase in brain weight compared with FST model rats. The reduced brain weight observed in the FST animal model in this study is associated with a reduction in dendritic spine density (Li et al., 2011; Kang et al., 2011; Penzes et al., 2011). The increase in brain weight of NAC- and fluoxetine-treated FST rats is due to NAC and fluoxetine increasing synaptogenesis that increases cortical spine density (Berk et al., 2014).

The forced swim test is the most used antidepressant drug screening test based on an immobility response induced by inescapable exposure to stress (Bourin, 1997; Can et al., 2012) and it has a strong predictive validity for short-term study for antidepressant compounds that can reduce immobility time in FST (Cryan, et al., 2005). In this study, the immobility time for FST model (B) increased significantly compared with NAC-treated FST model (E) and fluoxetine-treated (F) FST groups. FST-induced stress causes an increase in immobility time during FST in rats (Kawaura et al., 2016; Wright et al., 2016; Adu-Nti, et al., 2019). In this study, NAC- and fluoxetine-treated FST groups had a statistically significant reduction in immobility time. Fluoxetine (20 mg/kg) treated with FST caused a significant decrease in the immobility time (David et al., 2009; Abdel-Salam et al., 2013) and this finding correlates with our report in this present study. A dose of 20 mg/kg of fluoxetine significantly increased the mobility time and this increase in the mobility time in the Porsolt swim test (FST) is considered an expression of an antidepressant effect (Costescu et al., 2019) which supports reports in this present study. Fluoxetine is a selective serotonin reuptake inhibitor, and has demonstrated the potential to alleviate behavioral depression in the forced swim test possibly due to its ability to suppress cholinergic activities in the nucleus accumbens, or by inhibiting the reuptake of serotonin at synaptic terminals or inhibition of noradrenaline and dopamine reuptake (David et al., 2009). The antidepressant-like effect of NAC is dependent on glutamate transport (Fond et al., 2014) because NAC can increase glutamate in the synaptic cleft in a glutamate transporter-dependent manner (Wright et al., 2016).

The two-bottle choice procedure for assessing sucrose preference is a significant test to investigate anhedonia (i.e., inability to feel pleasure) in stress-based in laboratory rodent models of depression (Eagle, et al., 2016). A reduced sucrose preference is used as an index of anhedonia (Çorumlu et al., 2015) in the FST animal model for depression. It has been reported that anhedonia (loss of interest in normally pleasurable and rewarding activities) is a core symptom of depression (Watson et al., 2020). In this study, the test drug for antidepressant potential (NAC) and fluoxetine (a standard antidepres-

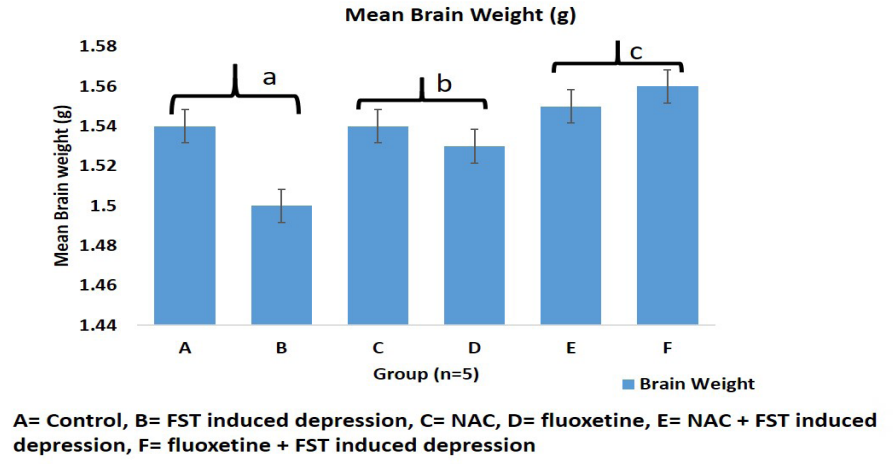

Figure 1. Graphical representation of mean brain weights of experimental Wistar rats

Data were analyzed using 1-way ANOVA and expressed as Mean±SD. Statistical Significance is taken at P<0.05 (*) using the Tukey post hoc test for multiple comparisons.

A: Control; B: FST; C: NAC; D: fluoxetine; E: NAC+FST-induced depression; F: fluoxetine+FST-induced depression

sant drug) reversed the anhedonia effects of FST by causing an increase in sucrose preference compared with the FST model with a reduction in sucrose preference (Eagle et al., 2016). FST-induced reduction of anhedonia or sucrose preference in this study supports Dale et al. (2012), Browne and Lucki (2013), and Çorumlu et al. (2015) findings. This anhedonia status in the FST model corresponds to a reduction in sucrose consumption (mL/h) as observed in this study. NAC and fluoxetine reversed this anhedonia status of FST model rats in this study. The 2-bottle choice procedure allows for a comparison between behavioral preference for sucrose solution in drinking water compared to water only (Eagle et al., 2016). In this study, all experimental animals showed a preference for sucrose compared to water 24 hours before 15 min FST test. The FST model (B) had a significant decrease in sucrose II and water II consumption when compared with control and fluoxetine (Parise et al., 2013; Çorumlu et al., 2015).

N-acetylcysteine reverses motor dysfunction in FST animal model by attenuating neuroinflammation associated with neuron dysfunction (Deepmala et al., 2015; Jakobsen et al., 2017) and increasing neuron repair and

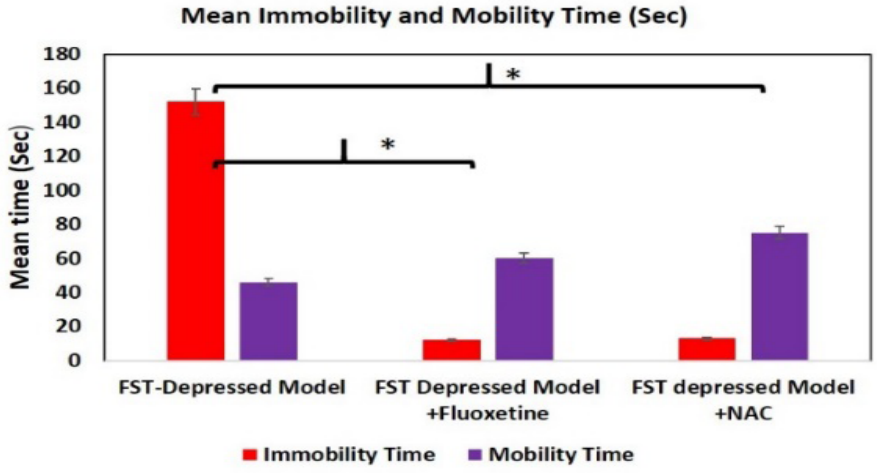

Figure 2. Graphical representation of mean immobility and mobility time of experimental Wistar rats

Data were analyzed using 1-way ANOVA and expressed as Mean±SD. Statistical Significance is taken at P<0.05 (*) using the Tukey post hoc test for multiple comparisons.

A: Control; B: FST; C: NAC; D: fluoxetine; E: NAC + FST-induced depression; F: fluoxetine + FST-induced depression.

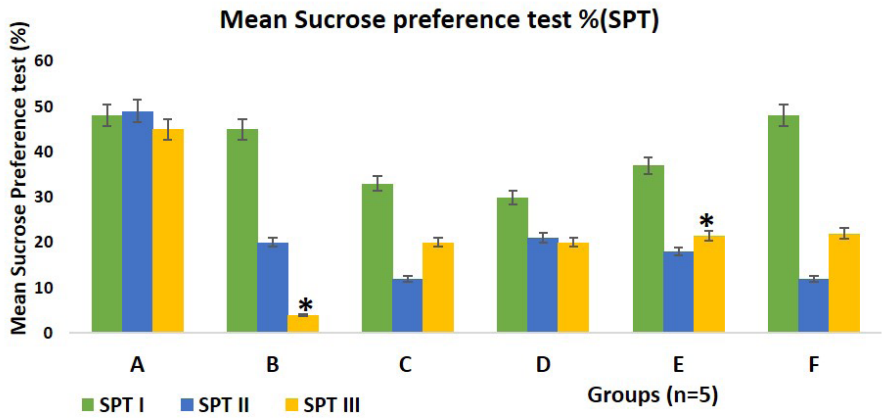

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Figure 3. Graphical representation of mean sucrose preference test (SPT) in percentage (%) to assess anhedonia status of experimental Wistar rats

Data were analyzed using 1-way ANOVA and expressed as Mean ± SD. Statistical significance is taken at P<0.05 (*) using the Tukey post hoc test for multiple comparisons.

A: Control; B: FST; C: NAC; D: fluoxetine; E: NAC + FST-induced depression; F: fluoxetine + FST-induced depression.

SPT I: measured 24 h before the FST as the pretest, SPT II: measure 24 h after 15-min FST, and SPT III: measured 1 h after 5-min FST.

connectivity (Carmeli et al., 2012; Zavodnick and Ali., 2014). The present study demonstrated the NAC neuroprotective effect on the brain damage induced by FST, the pyramidal neurons of the prefrontal cortex (PFC) area are commonly implicated in depression (Schubert et al., 2015) as post-mortem studies revealed a reduction in pyramidal cells in the PFC of depressed individuals (Fogaça and Duman, 2019). In this study, the FST PFC demonstrated numerous necrotic pyramidal neurons compared with the control with numerous py-

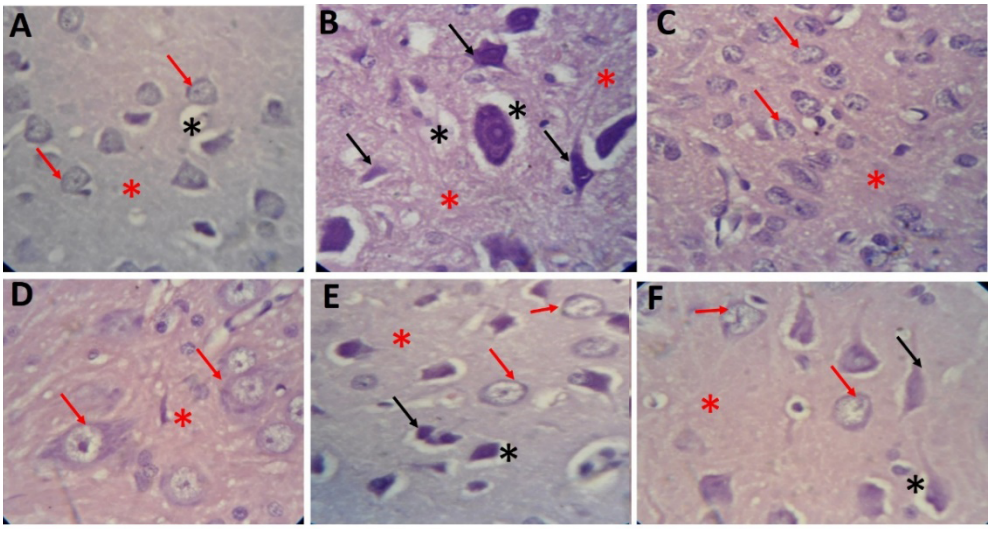

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Figure 4. Photomicrograph of a section of the formalin paraffin embedded prefrontal cortex (PFC) of adult male Wistar rats stained with hematoxylin and eosin (H & E)

Using a 100x objective lens (oil immersion) Scale bar=50µm.

A: Control; B: FST; C: NAC; D: fluoxetine; E: NAC + FST-induced depression; F: fluoxetine + FST-induced depression

Red arrows=Normal neurons, yellow arrows=axons/glia cells, Black arrows=pyknotic or necrotic neurons, Red asterisks=neuropil, and black asterisks=pericellular spaces

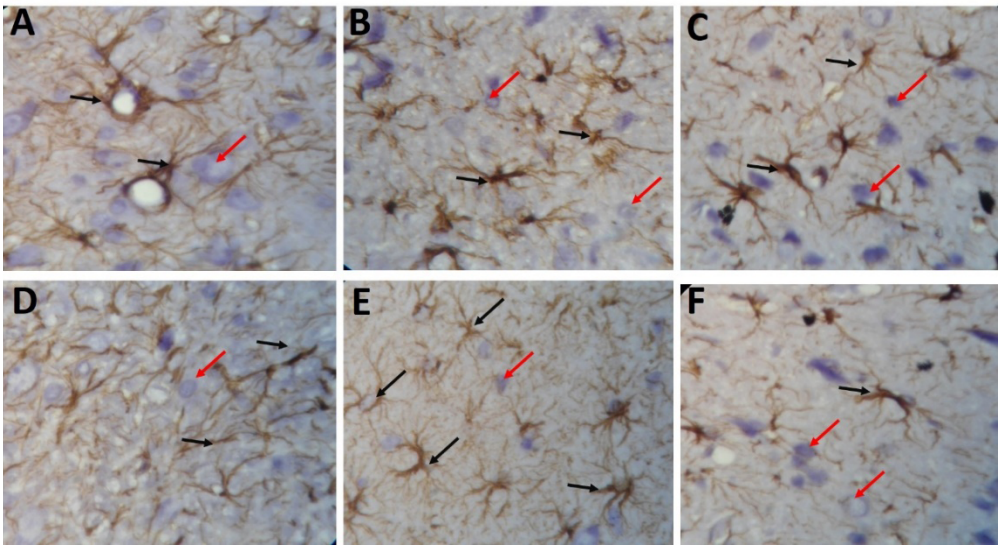

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Figure 5. Photomicrograph of a section of the formalin paraffin embedded prefrontal cortex (PFC) of adult male Wistar rats stained for astrocytic expression using glial fibrillary acidic protein (GFAP) antibody immunohistochemical staining

Using a 100x objective lens (oil immersion). Scale bar =50µm

A: Control; B: FST; C: NAC; D: fluoxetine; E: NAC+FST-induced depression; F: fluoxetine+FST-induced depression

Red arrows=Normal pyramidal neuron cell body and the black arrows=brownish coloration of the astrocytic cytoplasmic processes (intermediate filaments)

ramidal neurons having a central nucleus with axonal and dendritic outgrowths. This PFC characterization for neurodegeneration correlates with Réus et al. (2011) and Monteggia and Zarate (2015) findings. Microscopically, the PF cortical neurons have basophilic cytoplasm and peripheral processes in NAC- and fluoxetine-treated groups while the FST-induced group showed severe histopathological alteration of the neurons characterized by a shrunken darkly stained pyknotic nuclei and surrounded by wide pericellular space (degenerated neurons). NAC- and fluoxetine-treated (D) FST groups showed similar neuroprotective and neuron repair features characterized by the presence of numerous neurons with dense neuropil and the presence of axonal and basal dendrite outgrowth. NAC-treated rats reversed FST-induced oxidative stress neuronal damage (Wright et al., 2016). Antioxidant supplements such as NAC modulates these inflammatory markers thereby ameliorating FST-induced oxidative stress (Costa-Campos et al., 2013; Berk et al., 2014; Chen et al., 2016). In this study, NAC reversed neuron degeneration and this finding correlates with Wright et al. (2016) and Mohammed, et al. (2019) findings. In this study, the NAC antioxidant mechanism of action is linked with its cysteine component which is a rate-limiting component in the production of glutathione in the brain (Pallanti et al., 2014; Yin et al., 2016). Fluoxetine neuroprotective action in this study is linked with neuron proliferation, differentiation, and survival of neuron cells (Surget et al., 2008; David et al., 2009).

Astrocytes play a fundamental role in several cerebral functions such as the uptake of glutamate and GABA by specific transporters (Goubard, Fino, & Venance, 2011) and the production of antioxidants. Astrocytes play an important role in neuropathology hence they should be targeted as an approach toward pharmacological therapies for neuro-pathological conditions (Merienne et al., 2015; Liu & Chopp, 2016). FST-induced oxidative stress is associated with astrocytic dysfunction or damage, this condition affects astrocytes' ability to detect or respond to stress-mediated elevation in glutamate levels which disturbs neuronal homeostasis leading to overstimulation of the NMDA receptors, which are responsible for modulating the cognitive functions in the prefrontal cortex (Finsterwald, et al., 2015). The FST-induced PFC has more astrocytic expression. NAC- and fluoxetine-treated PFC attenuates astrocytic proliferation compared with the FST-induced group. NAC can reverse neuroinflammation mediated by FST-induced oxidative stress (Berk et al., 2014). NAC attenuates neuroinflammatory reaction-induced astrocytic proliferation by regulating glutamate neuronal activity thereby ameliorating the neuroinflammatory reaction in the pathogenesis and pathophysiology of depression (Finsterwald, et al.,

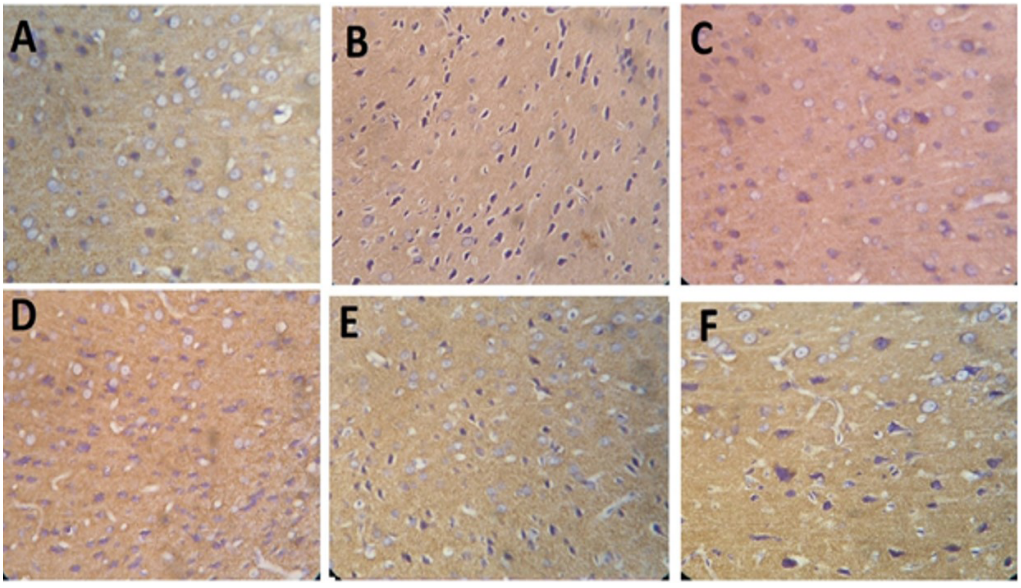

Figure 6. Photomicrograph of a section of the formalin paraffin Embedded prefrontal cortex (A), the hippocampal CA1 neurons (B), and cerebellar cortex (C) of adult male Wistar rats stained for synaptophysin (Protein p38) immunohistochemistry immunohistochemical staining

Using a 40 x objective lens (Scale bar =100 µm)

A: Control; B: FST; C: NAC; D: fluoxetine; E: NAC+FST-induced depression; F: fluoxetine+FST-induced depression.

2015; Wright et al., 2016). Fluoxetine also as an anti-inflammatory agent in posttraumatic stress disorder (Farhan and Haleem, 2016) by increasing serotonin signaling rapidly in vivo which explains its antidepressant activity in attenuating astrocytes proliferation. In this study, NAC's ability to attenuate FST-mediated astrocyte proliferation shows a NAC is a drug that exhibits its antidepressant effects by targeting astrocytic glutamate transporters to avert neurodegenerative disorders associated with excitotoxicity.

The activity of the synaptophysin protein (p38) which plays a critical role in synaptic plasticity or synaptogenesis (Sarnat et al., 2010) in the prefrontal cortex has already been studied. Loss of synaptic activity is detrimental in the pathogenesis of depression and in vivo studies to determine the role of synaptic activity and behavior as requirements to define the relation between synaptic activity and the pathophysiology of depression and therapeutics of antidepressant drugs (Tizabi et al., 2012; Sanacora,2012). In the present study, the control has an accumulation of synaptophysin-positive vesicles as seen in the brownish deposition within the neuropil of the gray matter of the prefrontal cortex. The prefrontal cortex of NAC as well as the fluoxetine-treated FST model has a uniformly intense and homogeneous synaptophysin immunoreactivity in the gray matter compared with the FST animal model. This decline in synaptophysin expression in degenerating PFC neuronal tissue correlates with Sarnat, et al. (2010) findings which indicate a loss of synaptic vesicles or no synapses (Gudi et al., 2017). This study supports reports by Karalija et al. (2012) that NAC restores the loss of synaptophysin immunoreactivity in a neurodegenerating tissue. However, Pawluski et al. (2014) reported that fluoxetine treatment significantly decreased synaptophysin expression. Here, fluoxetine increased synaptophysin expression which supports Larsen et al., (2008) report that fluoxetine affects synaptic changes and increases cell proliferation (Huang and Herbert 2006). NAC and fluoxetine have significantly increased pyramidal spine formation (Hajszan, MacLusky, & Leranth, 2005).

The discovery of antidepressants requires simple rodent behavioral tests for initial screening before undertaking more complex preclinical tests and clinical evaluation (Caoimhe, et al., 2015; Monteggia and Zarate, 2015).

5. Conclusion

In conclusion, 200 mg/kg of NAC ameliorates FST-induced anxiety-like behavior in rats by attenuating reactive astrocytes proliferation which protects against the loss of neurons and increases synaptophysin activity translating to the alleviation of anhedonia and a reduced immobility time an indicator for antidepressant drugs.

Ethical Considerations

Compliance with ethical guidelines

All experiments were approved by the Departmental Research, Animal Care and Use Ethics Committee in the Anatomy Department, Bingham University Karu, Nasarawa State Nigeria. The animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals as stipulated in by the National Research Council (2011).

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Conflict of interest

The author declared no conflict of interest.

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