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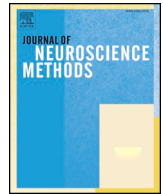
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Frequent exposure to varied home cage sizes alters pain sensitivity and some key inflammation-related biomarkers

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ABSTRACT

Background: Nature and size of rodent cages vary from one laboratory or country to another. Little is however known about the physiological implications of exposure to diverse cage sizes in animal-based experiments.

Method: Here, two groups of male Swiss mice (Control group – Cage stationed, and Test group – Cage migrated) were used for this study. The cage-migrated mice were exposed daily to various cage sizes used across laboratories in Nigeria while the cage-stationed mice exposed daily to different but the same cage size and shape. At the end of the 30 days exposure, top-rated paradigms were used to profile changes in physiological behaviours, and this was followed by evaluation of histological and biochemical metrics.

Results: The study showed a significant ($p < 0.05$) decrease in blood glucose levels (at 60 and 120 min of oral glucose tolerance test) in the cage-migrated mice compared to cage-stationed mice. Strikingly, peripheral oxidative stress (plasma malondialdehyde) and pain sensitivity (formalin test, hot-and-cold plate test, and von Frey test) decreased significantly in cage-migrated mice compared to cage-stationed animals. Also, the pro-inflammation mediators (IL-6 and NF- κ B) increased significantly in cage-migrated mice compared to cage-stationed mice. However, emotion-linked behaviours, neurotransmitters (serotonin, noradrenaline and GABA), brain and plasma electrolytes were not significantly difference in cage-migrated animals compared to cage-stationed mice.

Conclusion: Taken together, these results suggest that varied size cage-to-cage exposure of experimental mice could affect targeted behavioural and biomolecular parameters of pain and inflammation, thus diminishing research reproducibility, precipitating false negative/positive results and leading to poor translational outcomes.

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1. Background

Animal experimentation remains an indispensable component in science-driven medicine despite ethical controversies and disputed public philosophy. Most recorded advances in medicine commenced as controlled translational scientific endeavors in animals. Even where *Replacement* (from “Three Rs”) principle is adopted, efficacy of a new medical device or novel therapeutic is assessed in animals prior to clinical use in humans (Reynolds et al., 2018). However, success rate of most therapeutics during clinical trial in human remain low (Arrowsmith, 2011). Persuasive claims for these failure-littered trials bordered on inadequate study design, improper dose selection, inappropriate efficacy markers, wrong approach to data analysis and erroneous interpretation. The heterogeneity in habitat during laboratory study is mostly ignored when considering factors that could elicit flawed extrapolation or translational success in human.

Diverse habitat exposure during preclinical/laboratory studies is usually in bid to fulfill ethical guidelines on provision of adequately cleaned and conducive animal housing (Russell and Burch, 1956), as well as accomplishing other laboratory procedures. Procedures such as administration of substances, change of bedding, animal training and so on, require temporary or permanent transfer of research animals to new cages (Reeb-Whitaker et al., 2001) or environment. While acute transfer exposes the animals to various physiological stressors (Balcombe et al., 2004), effects of consistent long term cage-migration receives little or no attention from scientists. There is paucity of data on the possible effects of frequent long-term cage-to-cage exposure of experimental animals on physiological adaptations that could compromise targeted biological variables in a study.

The likelihood of cage-to-cage exposure of animals impacting on biological variables is further fortified by the fact that environment is a critical stimulus for all species and any form of environmental change may have a consequential effect on health (Tuggle et al., 2018). Thus, poorly funded laboratories (especially in developing countries) with shortage of proper equipment may adjust housing conditions from time-to-time and might be unknowingly altering vital variables in the experimental animals. In view of this, we stated our hypothesis as “frequent exposure of experimental animals to different sizes cage environments leads to behavioural changes”. Behaviour relating to innate explorative habit of rodents (emotional behaviours) and to pain stimuli were favoured. Other relevant parameters such as neurotransmitters (serotonin, noradrenaline and GABA) that involve in the physiology of these behaviour, organic molecule (glucose) necessary for energy release for the planning and execution of these behaviour, markers for oxidative stress (malondialdehyde) and inflammation (NGF, IL-10, IL-6, IL-1 β , TNF- α , and NF- κ B) that have both direct and indirect impact on the selected behaviours were all evaluated. Lastly, primary areas of the brain relating to pain processing (anterior cingulate cortex) and general motor function (motor cortex) were probed for histological changes (using hematoxylin and eosin and Cresyl fast violet stains) and antigen-linked activations (using GFAP and Ki-67 anti-bodies).

Using Swiss mice, our findings revealed that this less considered procedural act of exposing experimental animals to different sizes cage environments could mar certain animal experimentations, diminish reproducibility and precipitate poor translational outcome in animal-based research.

2. Results

Data here stated were assessed in triplicate and the averages were taken as the value for each parameter.

2.1. Weights and blood glucose rating in cage-to-cage migration of mice

Body weights were monitored for over four weeks and it showed normal weekly oscillation of body weights in both groups of animals.

However, the change in body weight was not significantly different ($p > 0.05$) in the cage-migrated animals compared to cage-stationed mice (Fig. 1A). Also, the relative weights of organs (brain, heart, kidneys, liver lungs, pancreas and testis) to body weight in the cage-migrated mice were not significantly different compared to the cage-stationed animals (Fig. 1B–H).

We then tested whether cage-to-cage exposure has effect on fasting blood glucose level. There was a significant increase in fasting blood glucose level in the cage-migrated mice compared to cage-stationed animals (Fig. 2A). Though the result falls within the normal range of fasting blood glucose in intact mice, the recorded increase piqued our interest to investigate how the cage-migrated mice with elevated fasting blood glucose would handle oral glucose load. Oral glucose treatment led to an initial increase in blood glucose levels of both groups at 60 min, but there was no significant difference in the assessed values. At 120 min, there was a significant decrease in blood glucose level in the cage-migrated mice compared to cage-stationed animals (Fig. 2B).

2.2. Behavioural alterations in cage-to-cage-migrated mice

Several conditions and environments are known to influence behavioural changes in rodents. Using various validated paradigms, the present study examined if cage-to-cage exposure of mice is capable of changing mood-/emotion- and pain- related behaviour.

The analysis of data collated from Open Field Maze (OFM) depicted that cage-to-cage migration of mice did not significantly change emotion-linked behaviour. Though, horizontal (ambulation) locomotion decreased significantly ($p < 0.05$) in cage-migrated mice compared to cage-stationed animals (Fig. 3A); vertical (rearing) locomotion was not significantly different in cage-migrated animals compared to cage-stationed mice (Fig. 3B). Also, cage-migrated animals displayed significant ($p < 0.05$) increase in grooming behaviour on the OFM compared to the cage-stationed mice (Fig. 3C). In contrast, the time spent probing the central arena of OFM was not significantly ($p < 0.05$) different in cage-migrated mice compared to cage-stationed animals (Fig. 3D). To support these evidences, animals were further exposed to Light and Dark Maze. Number of entry into the dark compartment, number of stretch into the light box and total time spent in the dark section were not statistically significant in the cage-migrated animals compared to cage-stationed mice (Fig. 3E, F and G). To confirm the trend of data presented above, mice were then exposed to tail suspension test. The frequency of turning attempt and the total motionless period in the cage-migrated mice were again not significantly different compared to cage-stationed animals (Fig. 3H and I).

Following comparison of emotion-related data that showed similarity between the two groups, it was hypothesised that pain-associated behaviour would be unperturbed since compelling evidences in the literature only linked change in emotional state to alternated pain sensitivity. Investigating this hypothesis, the results showed decrease in chemical noxious-elicited behaviour in cage-migrated mice. Precisely, there was a significant ($p < 0.05$) decrease in early and late phase paw licking behaviour in cage-migrated mice compared to cage-stationed animals (Fig. 4A and B). The observed decrease in pain sensitivity was further probed to ascertain whether this was peculiar to chemical noxious alone. Response to thermal noxious also showed similar decrease in pain sensitivities. In the cage-migrated mice, there was a significant increase in time taken to exhibit jumping and limb withdrawal behaviour on the cold- and hot-plate respectively when compared to cage-stationed animals (Fig. 4C and D). Also, threshold for mechanical noxious stimulus was significantly increased in cage-migrated mice compared to cage-stationed animals (Fig. 4E). Combined, these results demonstrated that varied sizes cage-to-cage exposure decreased pain sensitivity in mice.

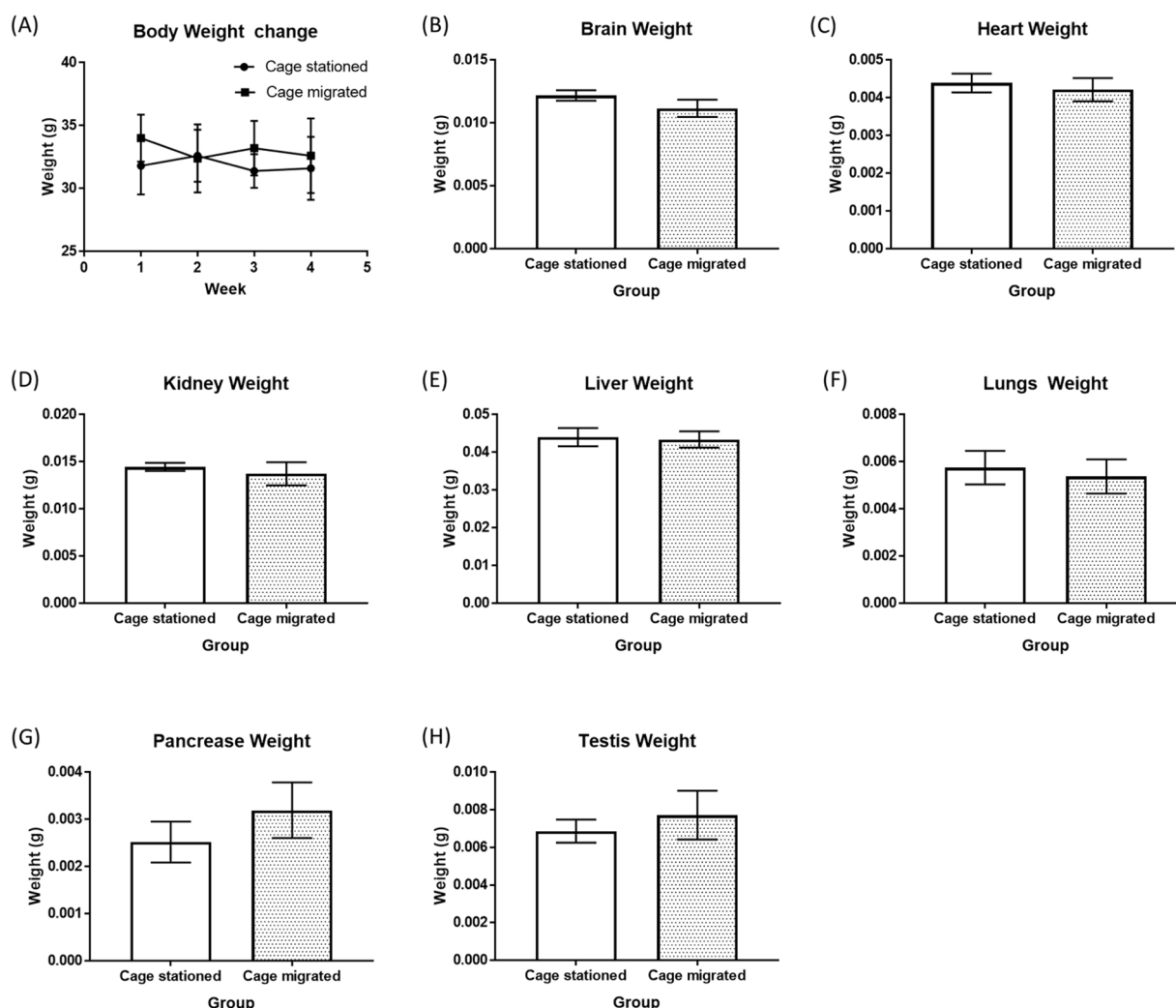


Fig. 1. Overview of changes in body weights and organ/body weight ratios. (A) Changes in Body weight over four weeks. (B) to (H) Changes in organosomatic weight ratio in the brain, heart, kidney, liver, lungs, pancreas and testis respectively; (n = 6).

2.3. Biomolecular changes in the brain and plasma of mice exposed to varied sizes cage

Notable neuroprotective and inflammatory mediators were assessed to evaluate the biomolecular implications of cage-to-cage exposure on experimental animals. The targeted biomarkers were those documented in the literature to have either beneficial or detrimental effect on mood and pain sensation.

Measured neuroprotective and anti-inflammatory mediators; nerve growth factor (NGF) and IL-10 were not significantly ($p < 0.05$)

changed in the cage-migrated mice compared to cage-stationed animals (Fig. 5A and B). In contrast, IL-6 level in the cage-migrated mice was significantly increased compared to cage-stationed animals (Fig. 5C). Other proinflammatory mediators (IL-1 β and TNF- α) measured appeared to be tractable as they revealed no significant changes in cage-migrated mice compared to cage-stationed animals (Fig. 5D and E). Then, we hypothesised possible elevation of NF- κ B since IL-6 is one of its activating signals. As predicted, NF- κ B increased significantly in cage-migrated mice compared to cage-stationed animals (Fig. 5F).

To further characterise effects of varied sizes cage exposure on

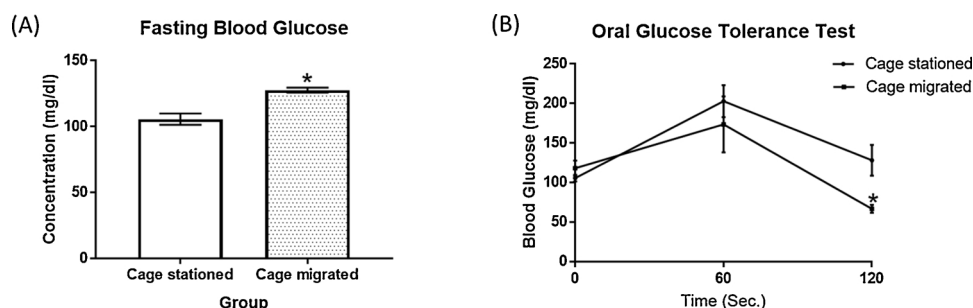
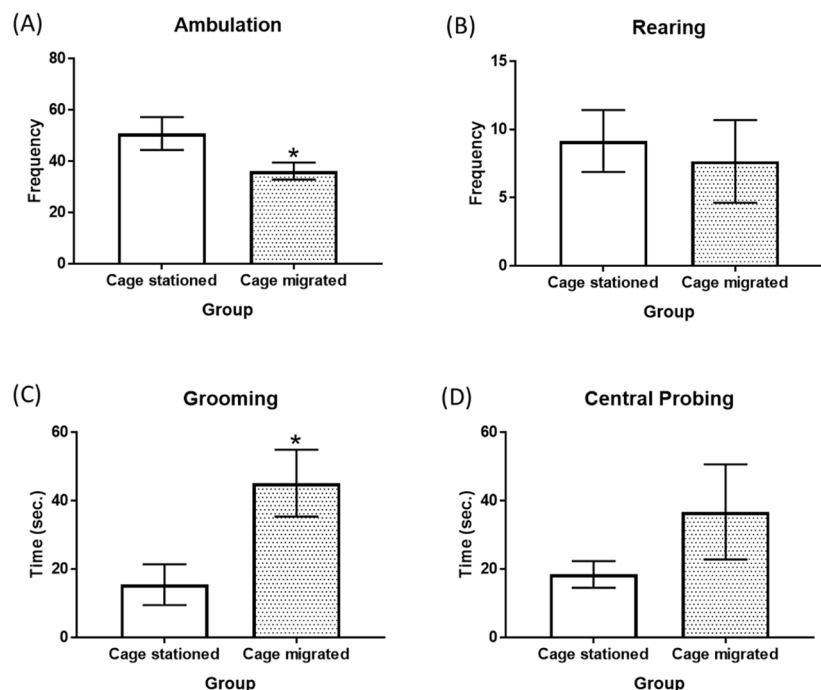
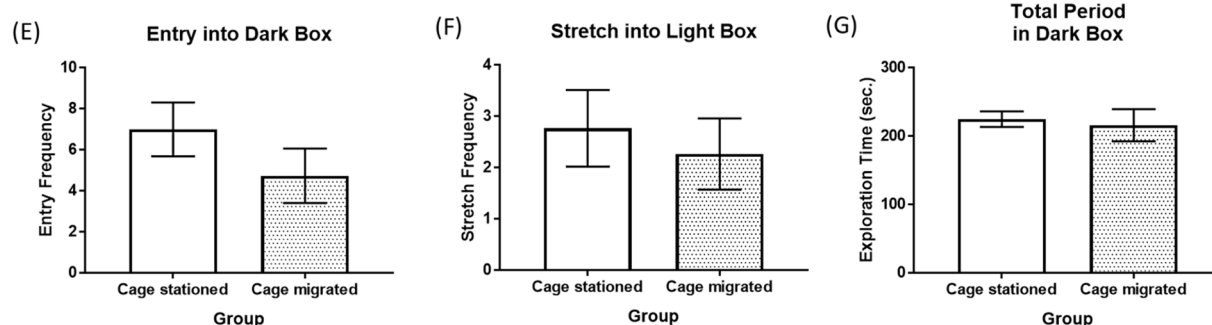


Fig. 2. Blood glucose homeostasis. (A) Fasting blood glucose (B) Glucose regulation in oral glucose tolerance test. *Significant at $p < 0.05$ compared to Cage-stationed group, (n = 6).

Open Field Test



Light and Dark Box Test



Tail Suspension Test

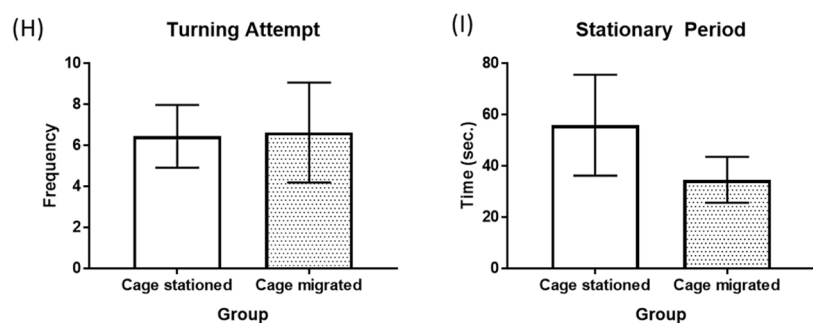
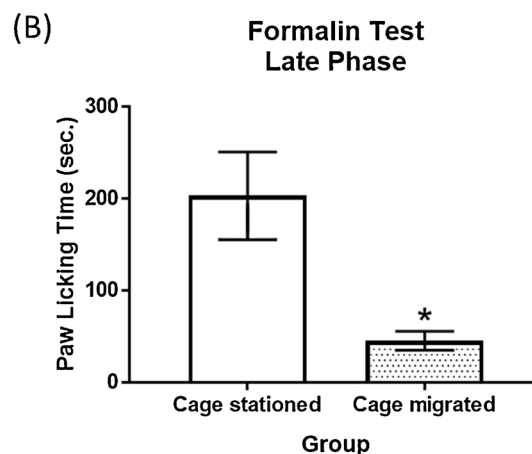
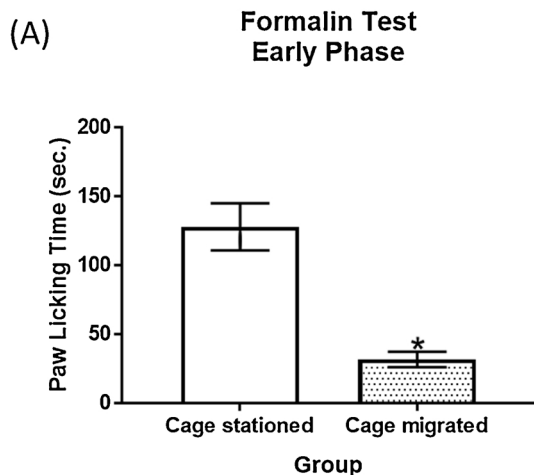


Fig. 3. Evaluation of mood-related behaviour. (A) Line crossing (horizontal locomotion) on OFM, (B) Vertical locomotion (Rearing) on OFM rearing frequency, (C) and (D) Grooming and time spent probing the central area of OFM respectively, (E) Total number of entry into dark compartment of light/dark box, (F) Number of neck stretch into the light box compartment in attempt of assessing risk, (G) Total time spent seeking safety in dark compartment of light/dark box, (H) Attempt of tail climbing on tail suspension, (I) Time spent inactive during tail suspension. *Significant at $p < 0.05$ compared to Cage-stationed group, ($n = 6$).

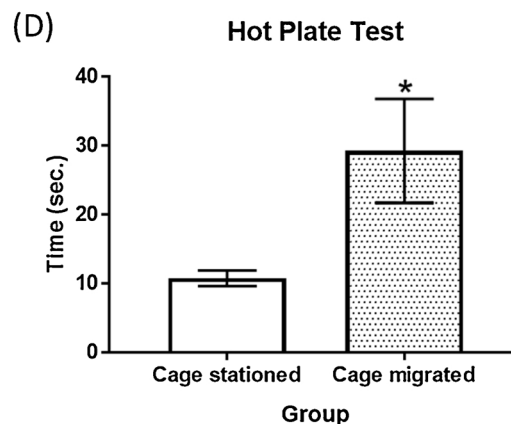
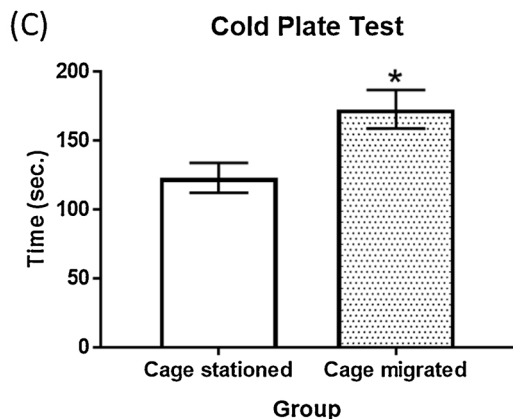
biomolecular indices in animals, critical neurotransmitters necessary for the processing of signals relating to mood and pain were evaluated. Brain levels of serotonin, noradrenaline and GABA in cage-migrated animals showed no significant difference compared to cage-stationed

mice (Fig. 6A–C). Evidence of lipid peroxidation was then checked in the animals. Brain level of MDA exhibited no significant change in the cage-migrated mice compared to cage-stationed animals (Fig. 7A). Though in the plasma, there was a significant decrease MDA in the

Chemical Noxious



Thermal Noxious



Mechanical Noxious

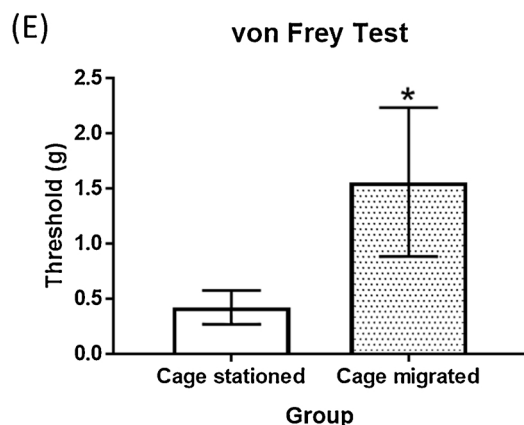


Fig. 4. Pain sensitivity in cage-to-cage-migrated mice. (A) and (B) Decreased paw licking during early and late phase in formalin pain test, (C) and (D) Total time spent on hot and cold plate, (E) Response to mechanical pain stimulus. *Significant at $p < 0.05$ compared to Cage-stationed group, ($n = 6$).

cage-migrated animals compared to cage-stationed mice (Fig. 7B).

Protein evaluation in the plasma, brain, heart, kidney, liver, lungs, pancreas and testis showed no significant difference in the cage-migrated animals compared to cage-stationed mice (Supplementary Fig. 1A–H). Also, possible perturbation in plasma and brain electrolytes was investigated. The results of estimated sodium, potassium, chloride, calcium, phosphorus and magnesium in the plasma and brains of cage-migrated mice were not significantly different compared to cage-

stationed mice (Supplementary Fig. 2A–F and Fig. 3A–F).

2.4. Histological variations at the cingulate and motor cortex in mice exposed to varied sizes cage

2.4.1. Anterior cingulate cortex

H&E-stained brain tissues showed no remarkable difference at the anterior cingulate cortex of cage-migrated mice compared to cage-

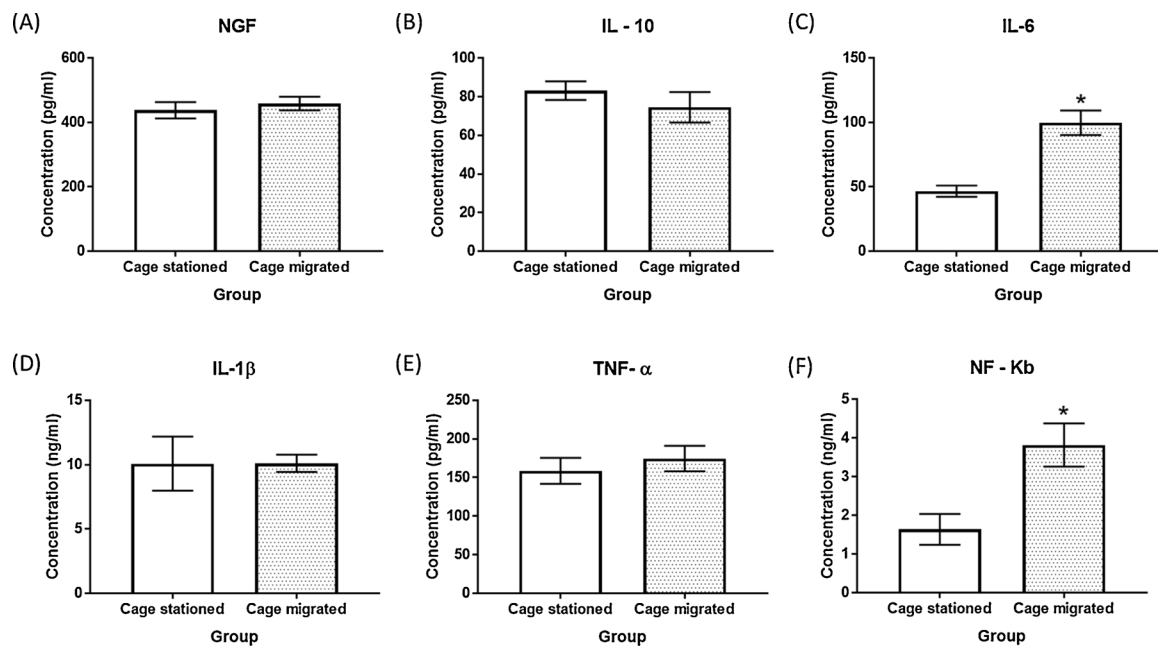


Fig. 5. Changes in anti- and pro-inflammatory markers. (A) and (B) Neuroprotective mediators, (C) to (F) Pro-inflammatory markers. *Significant at $p < 0.05$ compared to Cage-stationed group, (n = 6).

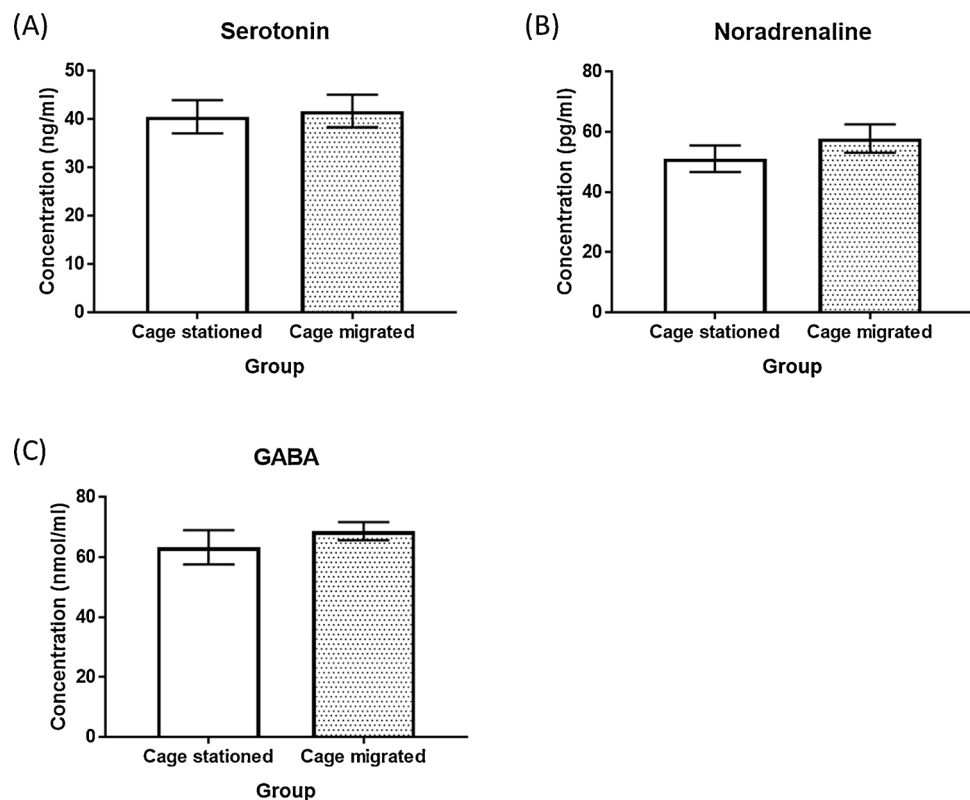


Fig. 6. Levels of relevant neurotransmitters. (A) and (B) Excitatory neurotransmitter, serotonin and noradrenaline respectively, (C) Inhibitory neurotransmitter, GABA.

stationed animals. The cytoarchitecture, distribution and morphology of the neural cells (white arrows) at the anterior cingulate cortex of cage-migrated mice appeared to be similar to that of cage-stationed animals (Fig. 8A and B). When staining for Nissl substance, cresyl fast violet-stained brain tissue of cage-migrated mice seemed to absorb more stain (hyperchromic) compared to brain tissue of cage-stationed animals. However, both groups of animals appeared to enjoy copious

population of ribosomes with probable adequate protein synthesis as the distribution and size of Nissl bodies (black arrows) looked similar in the anterior cingulate cortex of both cage-migrated and -stationed mice (Fig. 8C and D).

Also, glial fibrillary acidic protein revealing antibody (GFAP) showed little or no neural insult because activated astrocytes were poorly expressed in both groups of mice. However, GFAP-

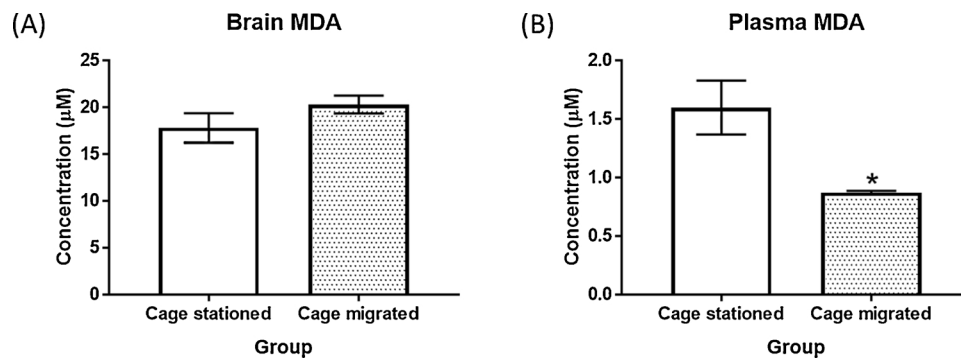


Fig. 7. Oxidative stress markers. (A) and (B) Concentration of malondialdehyde in the brain and plasma respectively. *Significant at $p < 0.05$ compared to Cage-stationed group, ($n = 6$).

immunopositive astrocytes along the either side of central sulcus that laced the anterior cingulate cortex in the cage-migrated mice appeared to be fewer compared to cage-stationed animals. Altogether, the population of reactive astrocytes in both groups was not remarkable to support possible perturbed in homeostasis or stress (Fig. 8E and F). Attempt to establish presence of varied habitats exposure-mediated neurogenesis yielded no fruitful result. There was no Ki-67-immunoreactive cells at the anterior cingulate cortex in the mice of both groups (Fig. 8G and H).

2.4.2. Motor cortex

Traditional H&E stain showed abundant pyramidal cells of Betz at the primary motor area in the brains of mice in both groups. Cells in brain of cage-migrated mice appeared to be more pronounced and packed compared to cage-stationed animals. Other than these, the cytoarchitecture and morphology of the motor cells (yellow arrows) were intact in both cage-migrated and stationed mice (Fig. 9A and B). CFV-stained brain tissue showed better profile of Nissl granules at primary motor area of cage-migrated mice compared to cage-stationed. Accurately, the Nissl substance (orange arrows) were predominantly large and mixed with small granules in both groups. Contrast to the brain tissues from the cage-migrated mice, some of the large granules in the primary motor area of cage-stationed mice have vacuole-like space (white arrows) with central “eye” (Fig. 9C and D).

GFAP-immunoreactive astrocyte cell was not evidently presence in the primary motor areas of mice in both cage-migrated and -stationed (Fig. 9E and F). Ki-67-immunopositive neural cells appeared darkly stained differentiating it from other cells. Ki-67-processed tissue showed visible darkly stained pyramidal cells in both groups though it appeared these darkly stained neurons were more in cage-migrated mice compared to the cage-stationed animals (Fig. 9G and H).

3. Methods

3.1. Mice

Male Swiss mice (11-week old, 29–34 g and at least five times backcrossed) obtained from Central Animal Facility, University of Ilorin, Nigeria were used for this study. The mice were housed in the animals' facility of Bioresearch Hub Laboratory where the experiment was carried out. Prior to any procedure, the mice were allowed to acclimatise for 1 week in a standard housing condition at a relatively constant temperature with regular light-dark cycle. Mouse pellet and water were accessible *ad libitum* before and during the experiment. All procedures here stated were as approved by the University Ethical Review Committee, University of Ilorin, Nigeria; and the proposal was issued with approval number – UERC/ASN/2019/1923.

3.2. Exposure to varied home-cage sizes

Mice were randomly divided into two, cage-stationed (Control Group) and cage-migrated (Test Group). For all the experiments performed, animals were housed in six ($n = 6$) per group. Mice assigned to cage-stationed group were kept in the same size cage of $45 \times 25 \times 15$ cm (length $\times$ width $\times$ height) for 30-day duration of the experiment. These mice were transferred to clean same size cage within a 60-second duration at 8 a.m. daily. The same toys and water bottle were returned into the cage after each transfer. Mice allotted to cage-migrated group were exposed to cages of diverse sizes. Exposure to a new cage size was done daily (every 24 h) at 8 a.m. and all mice were moved into the new sized cage within an average time of 60 s. The same toys and water bottle were returned to the new cage. From Days 1–15, mice were exposed to the following cages respectively - $45 \times 25 \times 15$ cm, $35 \times 20 \times 15$ cm, $60 \times 30 \times 20$ cm, $45 \times 15 \times 15$ cm, $50 \times 25 \times 20$ cm, $30 \times 20 \times 14$ cm, $45 \times 20 \times 20$ cm, $40 \times 25 \times 14$ cm, $60 \times 15 \times 30$ cm, $35 \times 30 \times 15$ cm, $30 \times 30 \times 14$ cm, $40 \times 24 \times 20$ cm, $50 \times 30 \times 25$ cm, $35 \times 15 \times 8$ cm, and $30 \times 15 \times 15$ cm. On Days 16–30, mice were re-exposed to the above cages respectively starting from the earliest. All cages were transparent plastics with thick gauze-like galvanized metal lids.

3.3. Weight

The body weights were measured weekly with Kerro weighing scale (Kerro Scale BL3002, Taiwan) starting from the first day of the experiment. Body weights were monitored for four (4) weeks. At the end of the experiment (Day 30), mice were sacrificed under anesthesia and weights of vital organs were taken in relative to the body weight of each mouse (organosomatic ratio).

3.4. Fasting and oral glucose tolerance test

Fasting blood glucose levels were determined in 12-h fasted mice using blood from their tail veins. The glucose levels were quantified using ACCU-CHEK glucometer. Oral glucose tolerance test was carried out following a preceding oral glucose load of 50 mg in 12-h fasted mice and subsequent measurement of blood glucose levels at 0, 60 and 120 min using ACCU-CHEK glucometer machine. Values were presented in mg/dl.

3.5. Emotion-associated behavioural studies

Emotion-related behaviours were evaluated using paradigms such as open field maze, light and dark box, and tail suspension.

Open field maze was first described by Hall in 1934 and it provides means for simple assessment of activities, and general behaviour in rodents (Hall, 1941). The open field maze used was $40 \times 40 \times 30$ cm in dimensions with a dirty white tile floor. Using black paint, the floor was

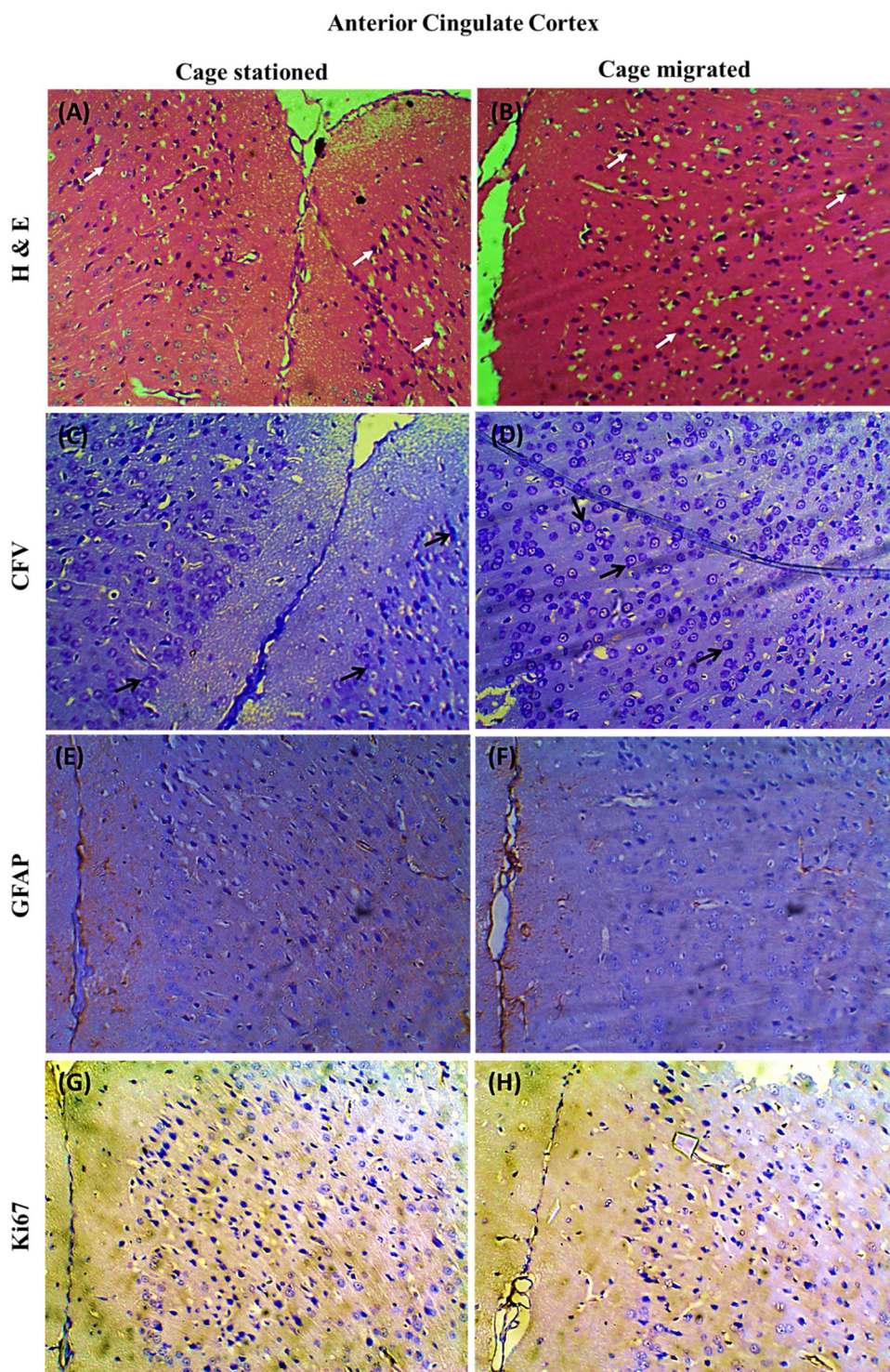


Fig. 8. Histological investigation within the anterior cingulate cortices. (A) and (B) H&E-stained sections of cingulate cortex in cage-migrated and cage-stationed mice respectively. (C) and (D) CFV-Stained sections of cingulate cortex area in cage-migrated and cage-stationed mice respectively. (E) and (F) Immunostaining of the cingulate cortex with GFAP antibody in cage-migrated and cage-stationed mice respectively. (G) and (H) Immunostaining of the cingulate cortex with Ki-67 antibody in cage-migrated and cage-stationed mice respectively. (Final magnifications (A), (B), (C), (D), (E), (F), (G) and (H) = 100x).

divided into a 16-cell of 10 cm^2 each. Each mouse was placed at the centre of the maze with the animal facing forward away from its handler. Five (5) minutes of exploration was allowed and the entire maze was cleaned with 70 % ethanol before another mouse was introduced. Total line crossing (ambulation), frequency of rearing, grooming time and time spent probing the central part of the maze were used in assessing emotional state of the mice.

Light/dark box test explores the inherent aversion to illuminated arena in rodents. The paradigm used was a two-compartment (a dark and a light) box with a total size of $83 \times 42 \times 30\text{ cm}$. Each compartment was $40 \times 40 \times 30\text{ cm}$ in size and the two compartments were linked with a tunnel of $2 \times 2\text{ cm}$. Mice were individually placed in the brightly illuminated light compartment and allowed to explore the maze for 5 min. The total number of visits and time expended in the

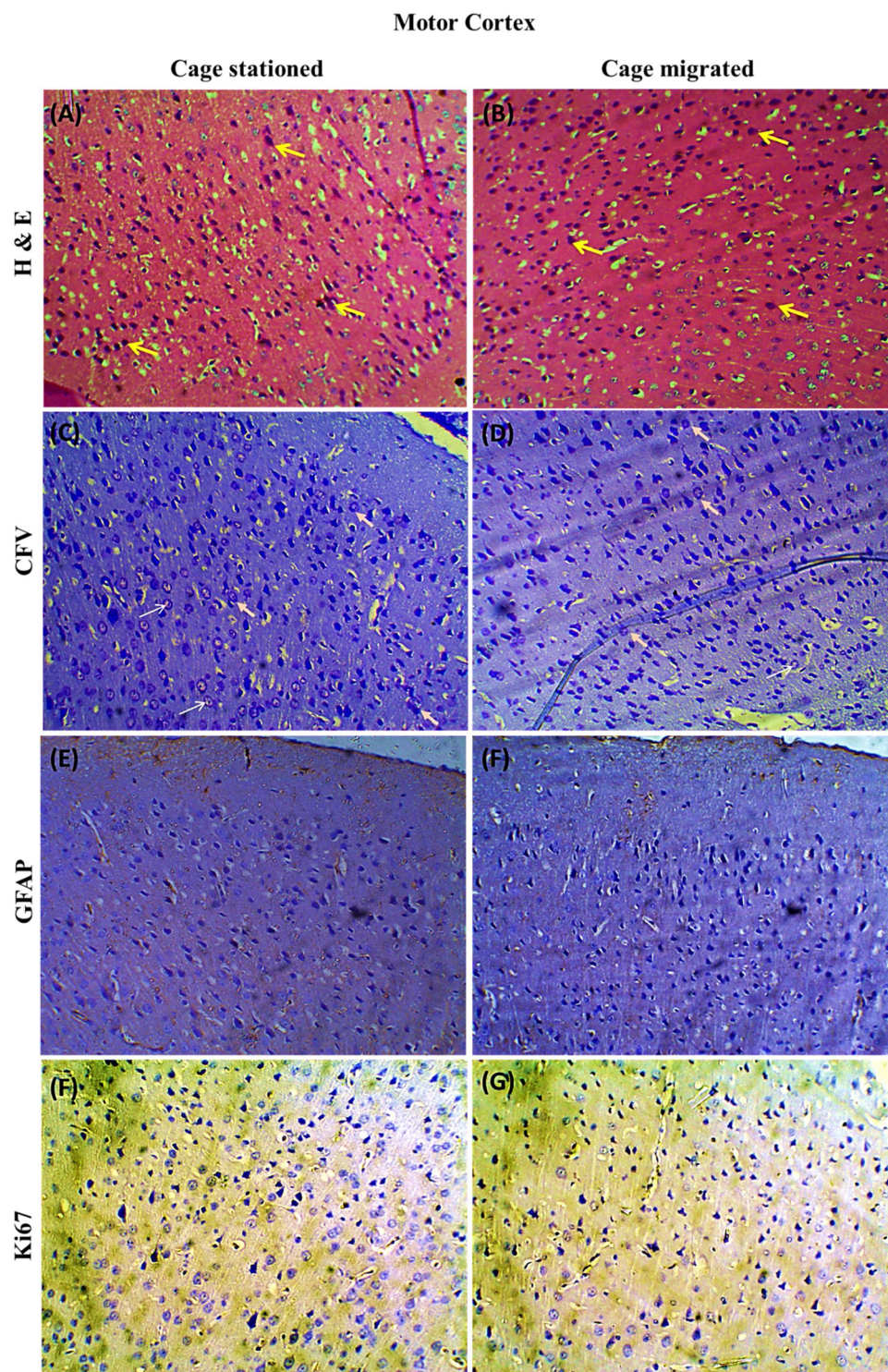


Fig. 9. Structural outcomes of motor areas. (A) and (B) H&E-stained sections of motor cortex in cage-migrated and cage-stationed mice respectively. (C) and (D) CFV-stained sections of motor cortex area in cage-migrated and cage-stationed mice respectively. (E) and (F) Immunostaining of the motor cortex with GFAP antibody in cage-migrated and cage-stationed mice respectively. (G) and (H) Immunostaining of the motor cortex with Ki-67 antibody in cage-migrated and cage-stationed mice respectively. (Final magnifications (A), (B), (C), (D), (E), (F), (G) and (H) = 100x).

light or dark compartment were used to determine the emotional state of the animals as earlier described in the literature (Crawley and Goodwin, 1980; Bourin and Hascoët, 2003).

Tail suspension test was used to evaluate the behavioural and physiological aspect of emotional state in experimental mice (Cryan et al., 2005). Briefly, each mouse was suspended by its tail with paper tape on a fixed retort stand. Each mouse spent 5 min on tail suspension, total

number of attempts to turn against the gravity (tail climbing) and time spent inactive were used to quantify mood of the animals.

3.6. Pain-related behavioural studies

Pain-linked behavioural responses were used to assess pain sensitivity in experimental mice subjected to noxious chemical, thermal and

mechanical stimuli.

Formalin pain test was used to assess animals' responses to noxious chemical. After 15 min habituation in a transparent glass arena, 20 μ L of 2.5 % PBS-formalin solution was injected subcutaneously in the plantar surface of right hind paw of each mouse. The total time spent on paw licking was monitored for 50 min and was used to evaluate pain perception as described in the literature (Hunskar et al., 1985). The time spent were eventually grouped into 0–5 min for nociceptive (early phase) pain and 20–30 for inflammatory (late phase) pain.

Hot- and cold- plate test were used to quantify pain sensitivity of mice to noxious thermal stimuli of 55 °C and 0 °C respectively. Each mouse was placed within the plexiglass walls positioned on a preheated iron plate surface maintained at 55 °C and the time taken for the mouse to jump was detailed as nociception threshold point. The cold plate used was a thin glass surface stand with attached perforated restriction glass chambers on it. The restriction boxes were blinded to prevent mice from seeing one another. Following 15 min acclimatisation, ice was applied to the plantar surface of the right hind paw till animal demonstrated nocifensive behaviour such as lifting the affected limb or moving away from the stimulus. Time taken to exhibit any of these behaviour was accepted as nociceptive end-point and the average of three trials within 3 min apart was recorded for each mouse.

Von Frey Test. Pain sensitivity to mechanical noxious stimulus was assessed via quantifying withdrawal thresholds of the right hind paw to calibrated filament (Lambert et al., 2009). The filament was applied with enough pressure to cause bucking against the ventral surface of hind paw and was allowed to remain for 5 s before its removal for an unresponsive mouse. From the least filaments of 0.008 g–6 g, the twelve von Frey filaments were used to probe for pain sensitivity in a continuous ascending order starting from the smallest calibration. The withdrawal threshold was accepted for a calibrated von Frey filaments if animal withdrew its hind paw at least five times from seven applied stimuli.

Note – Every maze was cleaned with 70 % ethanol solution before introducing another mouse. All behavioural studies were video-recorded and extraction of data was done by three trained persons blinded to the experiment.

3.7. Biochemical assays

Mice were sacrificed at experimental end point (Day 30) under continuous inhalation of anesthetic drug, isoflurane. Blood was collected with 1 mL syringe via cardiac puncture and was emptied into lithium-heparinized Eppendorf bottle. This was then centrifuged at 3000 rpm for 10 min under 4 °C. The plasma extracted from all samples were used for chemical analysis. Immediately after blood collection, mouse was transcardially perfused with 20 mL of cold PBS, the brain was carefully removed, homogenised, centrifuged (3000 rpm, 10 min and at 4 °C) and the supernatant was collected into plane bottle. Both the plasma and brain homogenates were used for biochemical assays of total protein, inflammatory mediators, neurotransmitters and MDA.

3.7.1. Protein assay

Bradford protein assay was used to evaluate the total protein content of the plasma, harvested organs and brain homogenates. The principle of this assay borders on capacity of protein molecule to bind on Coomassie dye. The reaction gives best result under acidic conditions and it is characterised with a colour change from brown to blue. In this study, the homogenate was diluted to obtain 50 μ g protein/30 μ L. 30 μ L of the sample or 30 μ L of the standard solution was added to separate test tubes. 30 μ L of water was added to two other test tubes for the standard curve. 30 μ L of buffer was added to the test tube containing the sample, followed by the addition 1.5 mL of Bradford reagent to each test tube. The test tubes were incubated at room temperature for 5 min after which the absorbance was read at 595 nm.

3.7.2. Inflammatory mediators and neurotransmitters assays

NGF, 1L-10, 1L-6, 1L-1 β , TNF- α , NF-K β , serotonin, noradrenaline and GABA were assessed in the brain in duplicate using ELISA kits manufactured by Elabsience, China. The samples were appropriately added to the varied kit plates and the assays were performed in accordance to the manufacturer instructions.

3.7.3. Malondialdehyde

This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid with MDA at 25 °C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm. Standard and sample (plasma or brain homogenates) solutions were prepared and 300 μ L of each was added into microwell, followed by the addition of 300 μ L of indicator to the sample and standard wells which were mixed. The mixtures were incubated for 45 min at room temperature. The absorbance of the resulting solution was taken at 532 nm using microplate reader.

3.7.4. Histological studies

On the last day of the experiment, blood was collected from anaesthetised mice, perfused with cold phosphate-buffered saline and then followed by perfusion with 4% phosphate-buffered paraformaldehyde via cardiac puncture. Sections of the brain showing motor and anterior cingulate cortices were exercised, fixed in 4% phosphate-buffered paraformaldehyde, stored under 4 °C for 24 h, processed in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Sections (5 μ m; MK 1110 rotary microtome) of the cortices were stained with hematoxylin and eosin (H&E), and Cresyl fast violet (CFV).

Immunohistochemistry was also performed on similar coronal sections of paraffin-embedded cortices tissues using anti-mouse-Ki-67 (1:100, BIO-RAD, UK) and GFAP (1:100, BIO-RAD, UK). Briefly, sections were deparaffinised with xylene and rehydrated through descending grades of ethanol (100, 95 and 70 %) and then taken to water. Sections were then subjected to heat-mediated antigen retrieval using citrate-based antigen unmasking solution (Vector®, Burlingame, CA, USA) at pH 6.0 in a steamer for 30 min. Next, sectioned tissues were washed in PBS for 2 min and were then exposed for 10 min to endogenous peroxidase blocking in PBS-0.3 % hydrogen peroxide solution. These were again washed in PBS for 2 min and were then incubated for 20 min in 2.5 % normal horse serum to enable protein blocking. Sections were finally incubated for 2 h in primary antibodies (Ki-67 - 1:100; BIO-RAD, UK and GFAP - 1:100; BIO-RAD, UK). At the expiration of incubation with primary antibodies, sections were washed in PBS for 5 min, incubated in ImmPRESS™ HRP anti-rabbit IgG polymer reagent (Vector®, Labs, USA) for 30 min and re-washed for 5 min twice. Sections were then rinsed properly under running tap water, counter-stained in hematoxylin, re-subjected to dehydration via ascending grades of ethanol (70, 95 and 100 %) and mount with Permount (Fischer Scientific, USA). Photomicrographs of the processed tissues were captured under 100X objective lens using the Zeiss Axiostar plus light microscope.

3.7.5. Statistical analysis

The present data were analyzed with 7.0 version of Graph Pad Prism. The specific tool used include unpaired Student's *t*-test and EC50 shift. Data were reported in mean $\pm$ SEM and statistical significance was accepted at $p < 0.05$.

4. Discussion

Movement of animals from one environment to another is arguably an unavoidable practice in animal-based experiments. Insight into procedural act of consistent varied size cage-to-cage exposure of Swiss mice during experiment showed consequential changes in functional and biochemical annotations. First, varied cage size-migrated animals

presented with elevated fasting blood glucose and closely-reversed oral glucose load (in tolerance test). Second, consistent exposure to different cage sizes in experimental animals precipitated decrease plasma MDA (signifying declined peripheral oxidative stress), but it increased the levels of IL-6 and NF- κ B in the brain. Third, varied size cage-to-cage exposure of Swiss mice resulted in a remarkable decrease in pain sensitivity to noxious chemical, thermal and mechanical stimuli. Fourth, neural cells distribution and morphology, mood related behaviour, central and peripheral nonspecific protein or electrolytes level, and certain transmitters (serotonin, noradrenaline and GABA) were not perturbed by diverse cage size exposure in male Swiss mice.

The ideal cage size and density for experimental animals has always been a welfare concern to scientists, though consensus seems to have been reached in this schema (<https://www.ncbi.nlm.nih.gov/pmc/issues/122528/>; <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1236115/?page=1>). On the other hand, there is paucity of data on the effects of exposing experimental animals to different cage sizes, thus profiling the physiology of such exposed animals will benefit biological research. The present experiment showed no mortality among the mice exposed to diverse cage sizes, body weight showed improving trend and body/organs weights did not differ significantly. Claims of lower body and organ weights upon exposure to increasing cage size (Yildiz et al., 2007) was not observed, though this was reported in rats where targeted interest was the links between cage size and density of experimental animals. The relatively constant weight recorded might be pertinent to appreciable maintenance of good ventilation and internal cage environment. Poor housing conditions had been documented to depress growth and development (Dawkins et al., 2004).

Examining the metabolic profile of mice exposed to various cage sizes showed elevated fasting blood glucose compared to its stationed counterpart. Despite this, migrated mice responded appropriately by reversing oral glucose load at the end of 120 min showing intact gut hormone releasing cells and beta cell. Supporting evidences in the literature revealed that fasted mice usually presented with significant lower blood glucose compared to least fasted or unfasted mice (Heijboer et al., 2005; Oosterveer et al., 2008). Thus, the striking increase in the recorded blood glucose level in spite of equal period of fasting for both groups was assumed to be varied cage sizes-induced or a response to stress. Since varying degrees of stress is known to increase fasting blood glucose (Ghosal et al., 2015), experimental animals were screened for one of the cellular responses to stress, oxidative stress. In evaluating stress, we reckoned that since mice have 18- to 22-day gestation period (Murray et al., 2010), then if the 30 days exposure to varied cage sizes could induce stress, such stress should have exceeded from the first two stages of responses to stress [that is, the alarm reaction and the resistance stage] to the exhaustion stage which is associated with oxidative stress (Schiavone et al., 2013). Quantifiable markers of oxidative stress, malondialdehyde was evaluated in cage-migrated mice and data showed noticeable reduction in peripheral oxidation with no clear difference centrally when compared to cage-stationed mice. Also, exposure to stress conditioning was shown to modify neuronal changes in anterior cingulate cortex, induced anxiety or depression and enhance anterior cingulate cortex excitatory nociceptive responses (Yamashita et al., 2013; Davidson et al., 2002). Our histological studies showed no striking neuronal modification, behavioural results did not support presence of anxiety or depression condition and pain perception was decreased in the cage-migrated mice compared to cage-stationed mice. Together, these suggested that elevated blood glucose level was not in response to stress. Then, protein content of the plasma and other selected organs was determined in cage-migrated mice and results did not appreciably differ compared to cage-stationed mice. Measured brain and plasma electrolytes in varied size cage-to-cage exposed mice were also not different compared to cage-stationed animals. Summarily, protein and electrolytes profiles were not susceptible to diverse cage size exposure in Swiss mice.

Results from past studies showed cage crowding could result in high

plasma IgG (Tsukamoto and Machida, 2012) and brain dopamine level (Holladay and Edens, 1987). The possibility of diverse sizes cage-to-cage exposure causing alteration in concentrations of neurotransmitters and inflammatory mediators was then investigated. Concentrations of noradrenaline, serotonin and GABA were not significantly different in migrated mice compared to its stationed counterpart. Also, with the exception of IL-6 and NF- κ B, all other quantitative data on anti-and pro-inflammatory mediators (NGF, IL-10, IL-1 β and TNF- α) were not significantly different in cage-migrated mice compared to cage-stationed mice. High IL-6 concentration showed that neural defense was not suppressed, though the reason for preferential increase of IL-6 was not known. This is more surprising as measured TNF- α was not significantly different. TNF- α prompts production of proinflammatory mediators like IL-6 and IL-1 (Le Bitoux and Stamenkovic, 2008; Soria et al., 2011) during inflammation. IL-6 with other activators in turn induce NF- κ B, a protein that plays vital roles in inflammatory responses (Karin, 2006). However, there are compelling evidences of exercise-induced increase in IL-6 concentration (Ellingsgaard et al., 2019). Thus, there is a possibility that daily exposure of cage-migrated mice to new size cage increased the animals' innate desires to explore the novel cage environments thus increased the animals' cage activities and muscular exertion similar to mild exercise.

Given the strong link between biochemical and functional metrics, phenotypic behaviour relating to mood and pain were profiled. Despite decreased ambulation and increased grooming on OFM, there was insufficient data to support claim for mood (anxiety- or depression-related) change in cage-migrated mice compared to its cage-stationed counterpart. Data from other tests (light and dark test, and tail suspension test) also reinforced this assertion. Emotional deterioration had been convincingly linked with adverse pain sensitivity in the literature (Lumley et al., 2011; Cai et al., 2018). In view of this, pain sensitivity in cage-migrated mice was postulated to remain unaltered since evaluated emotional behaviour did not vary in cage-to-cage exposed mice compared to cage-stationed. In contrast to this assumption, there was a remarkable decrease in pain sensitivity of cage-migrated mice compared to cage-stationed animals. The increased pain threshold was similar when exposed to either noxious chemical, thermal or mechanical stimuli. Structural evidences could not explain why pain sensitivity decreased. All routine-, special stain- and immuno-histological studies showed no prominent morphological, cytoarchitectural or expressional difference in the cells at the anterior cingulate cortex of the cage-migrated mice compared to cage-stationed animals. We then investigated the motor area, as this is where motor commands needed for coordinated movements are being generated. Some of these movements are main activities of evaluation in most behavioural studies. Using routine H&E stain, CFV stain, GFAP- and Ki-67-processed immuno-histochemistry on sections of primary motor cortices, we could not establish noticeable difference in morphology, cytoarchitecture or protein (GFAP and Ki-67 antigens) expression in the motor neurons of cage-migrated mice compared to cage station animals. Though, the structural results as presented was limited by inability to carry out morphometric analysis because the two brains (per group) planned for the study is not sufficient for statistical quantitative evaluation of histological variations. Summarily, there was no structural modification that could be associated with the recorded decreased pain sensitivity in diverse sized cages-exposed mice compared to cage-stationed animal. This observation is contrary to the obtainable in the literature where animals with elevated pro-inflammatory mediators were prone to increase pain sensitivity (Zhang and An, 2009; Ronchetti et al., 2017). With elevated IL-6 and NF- κ B recorded in this group, increase pain sensitivity is theoretically favoured cage-migrated mice.

In conclusion, consistent exposure of mice to varied cage sizes alters biological parameters associated with blood glucose, pain sensation, selected inflammatory markers and oxidative capacity. Owing to the observed decrease in blood glucose after oral glucose load, decreased malondialdehyde, increased IL-6 and NF- κ B, and decrease pain

sensitivity to noxious chemical, thermal and mechanical stimuli, exposure to various cage size habitats could precipitate false positive in studies where these parameters are targeted and may thus affect data accuracy or reproducibility in animal-based experiments.

Ethical approval

The protocol for the present experiment was approved and issued with approval number – UERC/ASN/2019/1923 by University Ethical Review Committee, University of Ilorin, Nigeria.

Consent for publication

Not applicable.

Authors' contribution

ALO designed the study, performed and supervised the experiments, and wrote the manuscript. KOO, KSB, JOO, MFA, OA-R, AA, DLL, AOI, AOY and SSI carried out the experiments. GLO, FAS and OJO supervised the histological studies, and reviewed the manuscript. AOA, ABN, OA, AA, WIA, OSM and OAA acquired and analyzed data, and reviewed the manuscript.

Declaration of Competing Interest

All the authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jneumeth.2020.108890>.

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