ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 4, Issue 3, pp: (106-114), Month: July - September 2016, Available at: www.researchpublish.com

Ultrastructural Changes of CA1 Subfield of Hippocampus of Experience Stressed Rat after Acute Heat Stress

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Abstract: **The aim of the present study is to use the transmission electron microscope to characterize the cell death of pyramidal cells from the CA1 subfield of hippocampus after 15 minutes received thermal stress in stress-experienced Sprague Dawley rats. Findings - the rats exposed to acute heat at 42 ºC, the nucleus membrane was destroyed and indeed some of the intracellular went out. In conclusion, acute thermal stress really caused injuries into neurons of the CA1 subfield of hippocampus. Repeated forced swimming stress early in life had enough influence to manipulate heat transmission. Therefore, less alteration are presented by the thermal stress in the experience stressed rat.**

Keywords: **CA1, pyramidal cell, cell death, thermal stress, and experience stressed rat.**

I. INTRODUCTION

The body needs a constant temperature to gain a maximum growth. What will happen when the body systems face the stress? In addition to haze phenomenon occurs due to global warming problem that we are facing now, therefore we need to think on how to train our body to balance up inducer given to us especially the brain cells who is the king of cells of the body. Hippocampus is a structure which is located in the frontal area under the cortex and having a role in learning and memory [1]. It is also has high amounts of glucocorticoid receptors, which make it more vulnerable to long term stress than most other brain areas [2]. The hippocampus has been extensively used to measure neuronal death after stress because its simple arrangement and homogenous pattern damage within each region. For example, the dorsal hippocampus and particularly the CA1 pyramidal cells show a significant degree of vulnerability to transient ischemic damage of short duration of vessel occlusion [3,4].

The hippocampus has been implicated in many of the functions that changes by early adverse experience; first, the hippocampus mediates negative feedback of the HPA axis. Lesions of the hippocampus or the fornix, its efferent hypothalamic projections, resulted in a slower restoration of basal measures of the HPA axis after stress [5]. Second, the hippocampus is an important structure in regulating behavioral measures of anxiety. Rats with hippocampal lesions appear to be less anxious when behaviours such as exploration of a new environment, social interaction and neophagia are measured [6]. Third, the hippocampus has a well-described role in spatial navigation learning [7]. Because all of these processes in the adult are influenced by maternal separation during early life, abnormal hippocampal development may underlie some abnormality in the cognitive performance later in life.

Cell death can be divided into two types: necrosis and apoptosis, and electron microcopy (EM) is a must to distinguish these two features. The present morphological findings of early organelles (mitochondria, rough endoplasmic reticulum (RER), Golgi apparatus) swollen, disaggregation of polyribosomes and subsequent lethal findings of mitochondrial flocculent densities, and cell and nuclear membrane breaks are clearly indicative of neuronal necrosis not apoptosis.

Apoptosis is an active and physiologic process that requires the cell expend energy in causing its own demise. Initially, an individual cell become loose or detached from its neighbours and morphologically shrinks. The cell membrane become contorted and folded into blebs. Intracellular proteases activate enzymes that begin to cleave the cytoskeletal framework [8]. The chromatin become condensed near the nuclear membrane and is cut into regular repeating lengths. The blebs of the intact cell membrane, many of which now contain condensed nuclear fragments or apoptotic bodies, pinch off as separate packets. These packets may undergo phagocytosis by macrophages. The purpose of this study is to see more details on the CA1 neurons field of hippocampus after received the acute thermal stress on the control and experience stressed male rats.

II. MATERIALS AND METHODS

Animal

For this study, 24 adult male Sprague Dawley rats from Animal Room of Physiology Department USM were used. The animals were kept under a constant 12:12 hours light-dark cycle at room temperature of 25 ± 1ºC and were maintained with food and water *ad libitum*.

Groups

At first, the animals were divided into 2 main groups: experience (F) and non-experience stressed (C). For experience stressed group, the pups were forced to swim (FST) on three consecutive days: days 7 and 8 of age for 60 seconds, day 9 for 90 seconds in water temperature at 30 ± 1 ºC respectively. After FST, they were lived free until adult. For non-experience stressed group (C) the pups were not disturbed except during cleaning of saw dust. Both groups again were divided into another two groups: without received thermal stress (WTH) and received thermal stress (TH) at 42 ± 1 ºC for 15 minutes. The rectal temperatures were recorded using a rectal thermometer which inserted deep into the rectum, representing visceral temperature during thermal stress [9].

Thermal stress

Before the rats were exposed to thermal stress, they were sedated with thiopentobarbital at 50 mg/kg. This procedure is to ensure that both hind paws were received a similar amount of heat which set at 42 ± 1 ºC. Besides, it also made much easier and caused less injuries to animal due to manipulation of rat to stress factor.

Corticosterone level

Before they were proceeded with perfusion, blood samples were collected and centrifuged at 10,000 rpm for 10 minutes to get clear serum. The serum was stored in -20 ºC until the process for corticosterone assay. Measurement of corticosterone level was followed normal practice by commercial product (DSL-10-81100, USA).

Perfusion fixation/euthanasia

Two hours after the treatment, the rats were killed with an overdose of thiopentobarbital (100 mg/kg). Perfusion technique was applied by inserting canula into left ventricle and flushed out the blood with cold phosphate buffer saline and continue fixed with 4% paraformaldehyde. Brain was dissected out from the skull and immersed in the same fixative until ready for EM process. Mortality rate was documented if it present.

Electron Microscope (EM) Processing

Through dorsal approach, cerebral cortex of the brain was removed from the brain to see the whole hippocampus. Three small sections at 1 mm^3 of CA1 of the right hippocampus were cut and fixed in 4% glutaraldehyde for 12 hours at 4 ºC. Then, the sections were washed with 0.1 M sodium cacodylate buffer for 3 changes of 10 minutes each. Post fixed in 1% osmium tetroxide for 2 hours at 4 ºC and washed again with 0.1 M sodium cacodylate buffer. Dehydration in series of graded acetones (35 – 100%), infiltrated with 1:1 aceton resin for 1 hour, 1:3 aceton resin for 2 hours, overnight in 100% resin and followed with 2 hours 100% resin, embedded in resin and allowed to polymerize for 2 days in oven 60 ºC.

Areas of interest were selected after did semi thin section (0.5 µm) and stained with 0.5% toluidine blue. Selected ultrathin sections were put in 200 mesh grid and stained with uranyl acetate and lead citrate and observed and photographed in a blinded manner by an electron microscope technician using transmission electron microscope (TEM) (Hitachi M600) who was instructed to find six representative neurons (per experimental rat specimen) as identified by a

typical nucleus and surrounding perikaryon. Approximately 24 neurons were randomly identified, imaged at X 5000 and X 13500 magnifications and analyzed.

Two blinded investigators using an objective grading system analyzed electron micrographs of these neurons. Each investigator was asked to examine each neuron for evidence of nuclear changes (chromatin dispersion or clumping), for the presence of cytoplasmic changes including macrovacuoles or microvacuoles and for the overall shape of neuron (shrunken, swollen) and the appearance of rough endoplasmic reticulum (RER) compared with control. Similarly, each investigator was instructed to examine the perinuclear neuronal mitochondria for abnormalities in mitochondrial distribution or shape, matrix density, cristal structure and appearance of any abnormal structures compared with control group. Each finding was indicated as mild, moderate or severe depending on its frequency.

Data analysis

The differences of rectal temperature among thermal stress groups (TH C and TH F) and corticosterone level of each group were analysed by using independent T-test and ANOVA, SPPS 16.0 respectively. P values less than 0.05 were considered statistically significant and data are presented as mean ± SEM.

III. RESULTS

Rectal temperatures were recorded only for the thermal stress groups (Table 1) and it showed that TH F was significantly higher temperature than TH C. The temperatures were increased by time for both groups (Fig. 1). However, the level of corticosterone was not significantly different ($P<0.05$) among the groups (Table 2). There was no significant changes occur in the control group (WTH C) (Fig. 2 – Fig. 4) where all the pyramidal cells almost rounded in shape. Zoom in Fig. 2 showed normal lysosome with enzyme inside (in the form of black dot particles) and endoplasmic reticulums (ER) were closed to each other and localised near to outside layer of nucleus membrane. It also has a normal mitochondrial which was rounded in shape and had a nice organization of cristae inside inner layer of it and almost of the mitochondrial lied near to outside layer of nucleus membrane. The pyramidal cell of experience stressed with no heat (WTH F) was almost similar with control group (WTH C). Only a slight alteration occurred where ERs were swollen. However its mitochondrial and lysosome were remained as normal appearances (Fig. 5 – Fig 7).

Ultrastructure examinations of the brain in non-experience heat stressed rats (TH C) were shown in Fig. 8 – Fig. 10. After 15 minutes the rats were exposed to heat at 42 ºC, the characteristic condensation of nuclear chromatin could be found in the neuron. It was worth noting that alterations of the mitochondria, including reduction of the number of cristae membranes, disruption of the surrounding membranes and swelling of the organelles, presented in these neurons. Nucleus membrane was broken and noted that some of the content move out of the cell. Fig 10 showed content of the nucleus was whitish and looked empty compare to normal pyramidal cell while most of the mitochondria appeared black and increased density. Vacuolations (empty spaces) were presented external to the nucleus membrane.

Swim experienced and received heat (TH F) group showed neuron with nucleus indented with peripheral chromatin condensation. The organelles surrounding nucleus were homogenous distributed in well preserved plasma membrane (Fig. 11). Golgi complex, lysosomes and mitochondrial were almost normal morphologies, but ER slightly swollen and dispersed each other (Fig. 12 and Fig. 13). Table 3 summarized the changes of features of the mitochondrial in each of the group and there was no rats died immediately after thermal stress.

IV. DISCUSSION

Thermal stress at 42 ºC for 15 minutes was significantly causing changes to pyramidal cells of CA1 subfield of hippocampus. Stressful experience helped the animal to compensate the brain cells even though it was not fully completed where there were minor changes occurred in the ER. Why only the ER and not other organelles are still remained inconclusive. It is probably has involved with protein damage leading to the aggregation of unfolded protein.

There were several histopathological changes, oedema, focal hemorrhages and spotty infarction, after heating the hemispheres of dog brain above 42–43 ºC for 30 minutes [10]. However, Silberman et al. [11] showed that normal rabbit brain could endure 42.4 ºC for 60 minutes without apparent histopathological or clinical damaged. Also, acute histological effects of ultrasound hyperthermia applied to the cortex of one of the brain hemispheres in cats and dogs were studied by Britt et al. [12] and Lyons et al. [13] respectively. Thermal damage was observed immediately after treatment at 43 ºC for

50 minutes to neurons in the gray matter and to myelin tracts in the white matter of the brains. In addition, Sneed et al. [14] observed that a single heat treatment at 43–44 ºC for 30 minutes produced a large cerebral lesion that consisted of central coagulation necrosis surrounded by a sharply demarcated hypervascular zone. Moreover, Lyons et al. [15] recorded definite destructive degenerative changes after local ultrasound hyperthermia on the normal dog brain. Actually, Lundgren et al. [16] summarized that the hyperthermia aggravated the epileptic brain damage of rat. Furthermore, Sharma and Cervos-Navarro [17] noticed that, in the brain of young rats, as a result of exposure to heat stress at 38 °C for 4 hours, the perivascular oedema, vacuolation and collapsed microvessels were observed. Indeed, in the brain of rats heated to 41, 42 and 43 ºC for 30 minutes, a cellular shrinkage and a vacuolation with nuclear pyknosis were noted at 4 hours after this exposure [18]. Clearly, Sharma and Horpes [19] reported that, as a result of exposure of rats to heat stress at 38 ºC for 4 hours, a disturbance and breakdown in the blood–brain barrier (BBB) and the brain oedema formation were noticed. The heatstroke induced cerebral ischemia insults and brain hypoxia in rat [20, 21] and the hippocampus showed less responsiveness to hyperthermia than the cerebellum [22]. Also, Yaqub et al. [23] depicted that a compatible degree of temperature to brain death is 43.5 ºC. Hence, together with the previous studies, the current study suggests that the exposure to high temperature caused some malformation in the brain. However, the changes were not enough to cause immediate death. When the cell received stress factor, it tried to counteract this, cells increase the expression of chaperone proteins that helped in refolding of misfolded protein and alleviate protein aggregation. Failure to do so may lead to rupture of plasma cell membrane, which resulted in the loss of intracellular contents (and show empty spaces) and later lead to necrosis. The results of many studies show that alteration in temperature of a few degrees can significantly influence performance in several tasks including signal recognition, time to respond to signals, learning performance, and word memory[24].

V. CONCLUSION

Acute thermal stress really caused injuries into neurons of the CA1 subfield of hippocampus. Repeated forced swimming stress early in life was enough to generate heat transmission which can help the neurons to manipulate the changes did by thermal stress later in life.

ACKNOWLEDGEMENT

Authors would like to acknowledge Universiti Sains Malaysia for the financial support (Res. Uni Grant 1001/PPSK/812921) that was provided for completion of this study.

Statement of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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APPENDIX - A

List of Tables:

Table 1: Rectal temperature of experience stressed rat during thermal shock.

Group	Temperature (Mean ± SEM)
TH C	37.15 ± 0.081
TH F	37.39 ±0.053*

TH F has significantly (P<0.006) higher mean rectal temperature than TH C.

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
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Table 2: Mean ± SEM corticosterone levels 2 hours after thermal stress.

Group	Corticosterone (Mean ± SEM)
WTH C	226.835 ± 7.059
WTH F	212.840 ± 4.991
TH C	218.714 ± 6.113
TH F	224.097 ± 6.113

Note that all groups had similar level of corticosterone.

Table 3: Ultrastructure features of neuronal mitochondrial after acute thermal stress of experience and non-experience stressed rats.

Group	Cristal Integrity	Intracristal Spaces	Matrix Density	Vacuoles
WTH C	Intact	Normal	Normal	None
WTH F	+	+	+	-
TH C	+	+	+++	None
TH F	+	+	+	++

-, no changes
+, indicates mild changes
++, moderate changes

List of Figures:

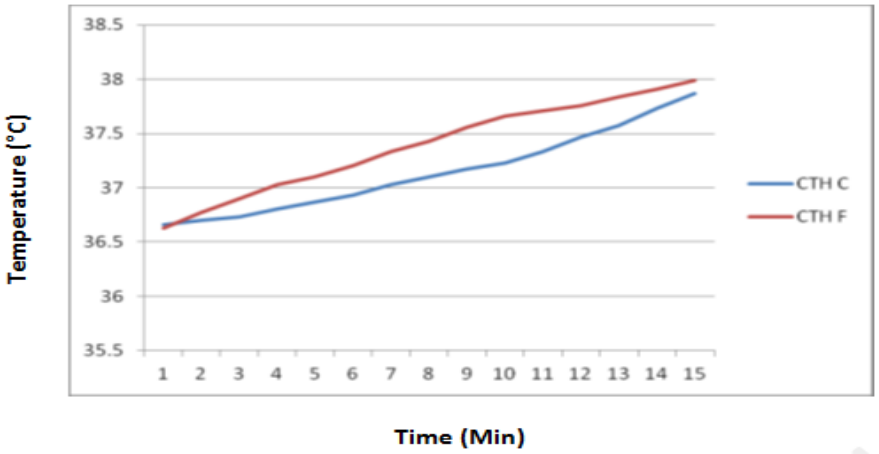

Fig 1: The increment of rectal temperature during 15 minutes of thermal stress for both groups.

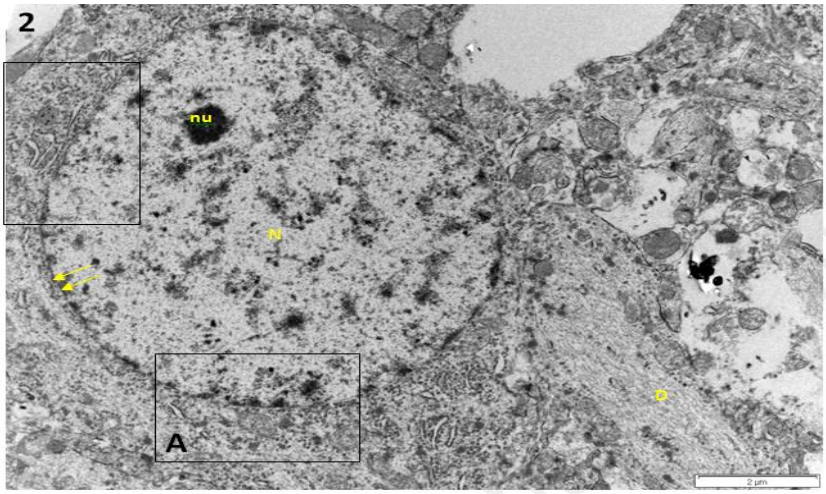

Fig 2: Normal pyramidal cell from CA1 subfield of hippocampus of non-experience no heat (WTH C) group, nucleolus (Nu), nucleus (N), dendrite (D) and nuclear membrane (yellow double arrows).

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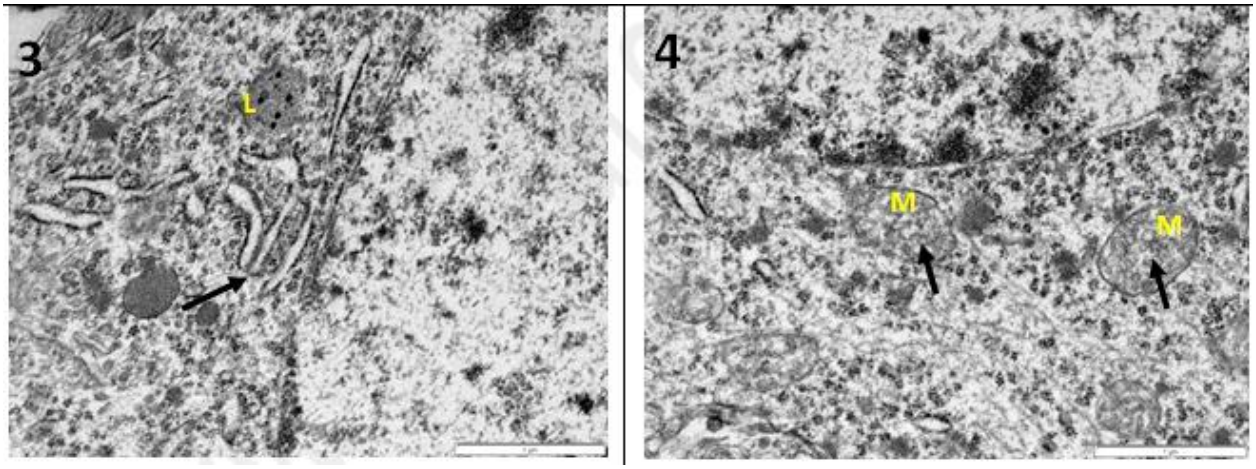

Fig 3 and 4: Zoom in Fig 2 showed normal lysosome (L) with enzyme inside and ERs (arrow) were closed each other and located near to outside layer of nucleus membrane and also normal mitochondria (M) which rounded in shape and having a proper organization of crista (thick arrow) and it present near to outside layer of nucleus membrane.

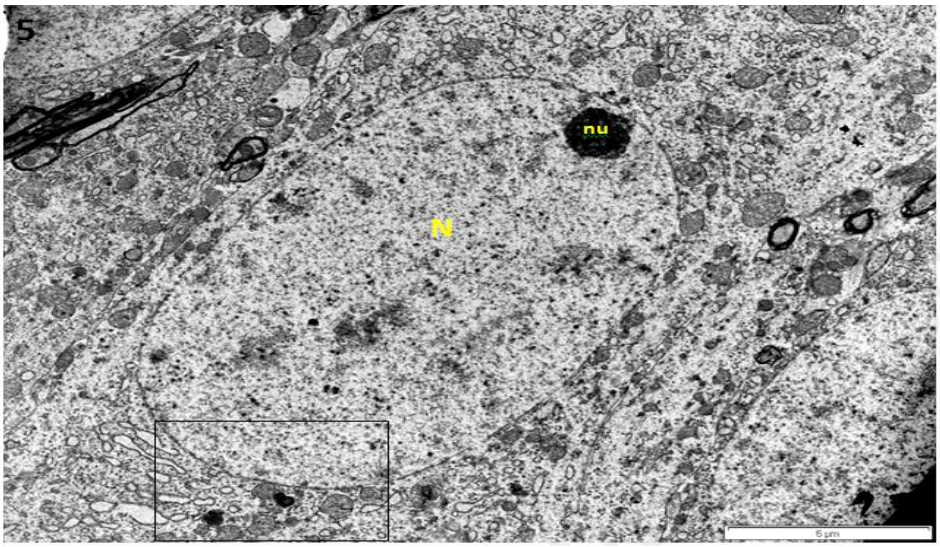

Fig 5: Pyramidal cell of swim experienced no heat (WTH F) rat was almost similar with control group (WTH C).

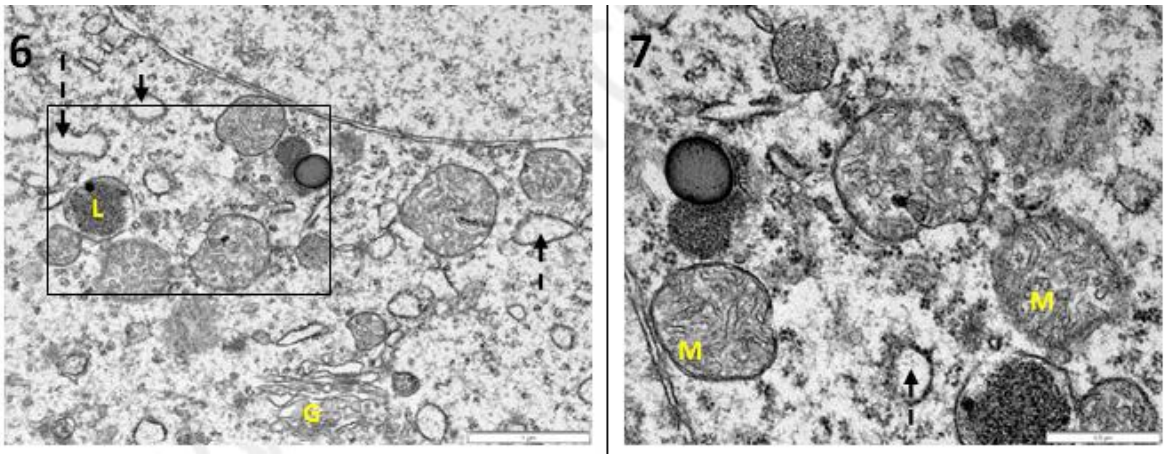

Fig 6 and Fig 7: Zoom in Fig 5 showed a slight alteration occurred in experience stressed group where ERs become swollen (dash arrow) but the mitochondrial (M), lysosome (L) and golgi apparatus (G) remained normal (arrow).

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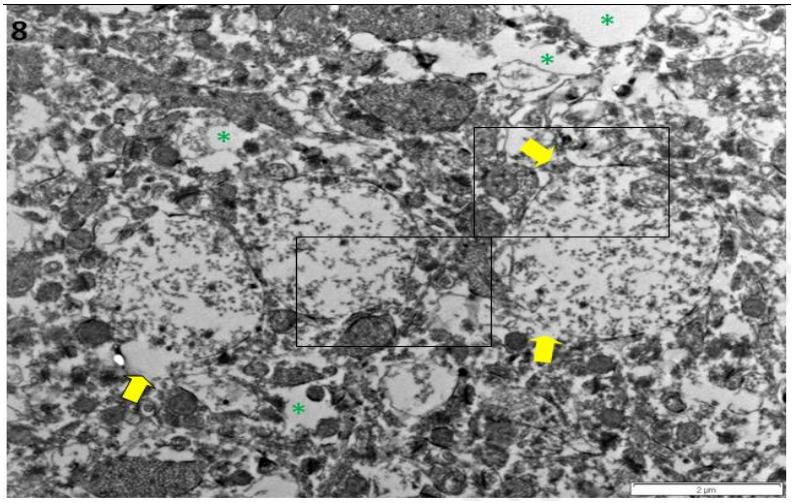

Fig 8: Non experience stressed group received heat (TH C) showed break down the nucleus membrane (yellow arrow head) of the pyramidal cell. Numerous empty spaces (*) also presented in the group.

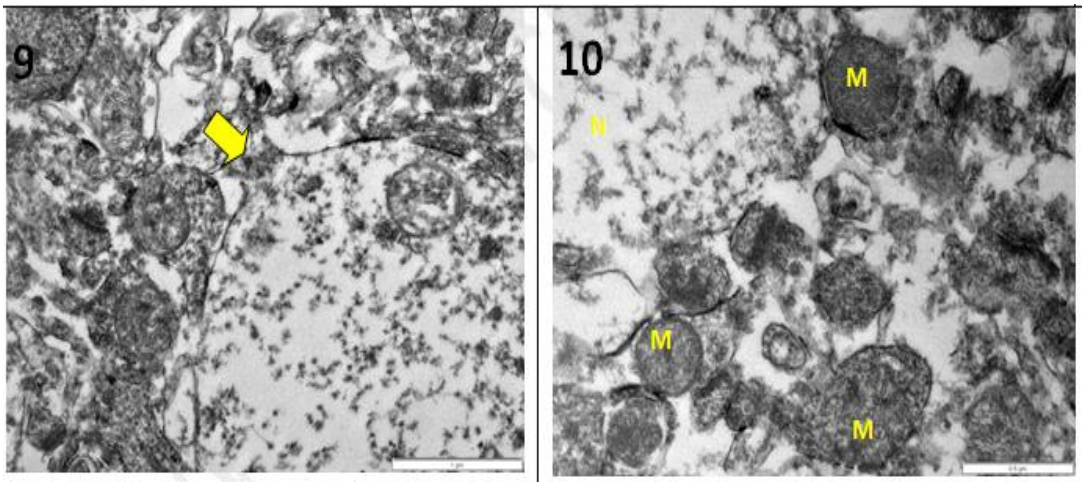

Fig 9: Zoom in Fig 8 showed nucleus membrane (yellow arrow head) was broken and some of the cellular contents came out.

Fig 10: Zoom in Fig 8 showed content of the nucleus (N) was whitish and looked empty while most of the mitochondria appeared almost black and increased density (M).

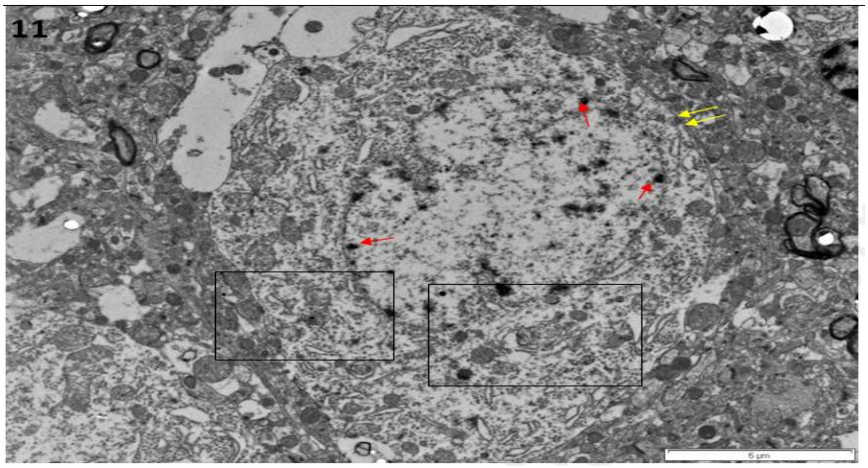

Fig 11: Swim experienced and received heat (TH F) showing nucleus indented. Some of the chromatins were clumping at the periphery (red arrow). Organelles surrounding nucleus were homogenous distributed in well preserved plasma membrane (yellow double arrows).

ISSN 2348-313X (Print)
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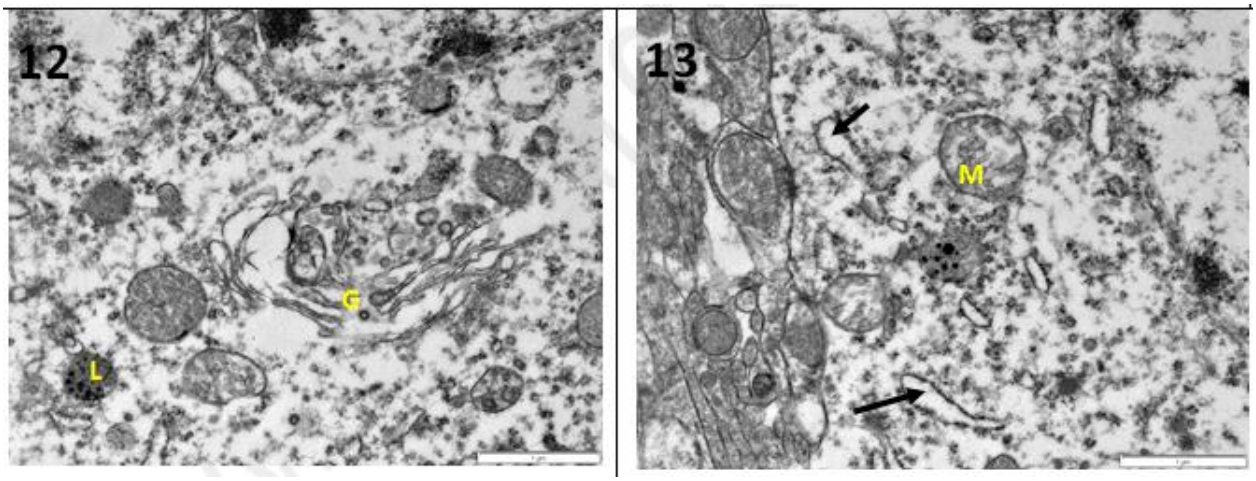

Fig 12 and 13: Zoom in Fig 11 showed Golgi complex (G), lysosome (L) and mitochondrial (M) were almost normal features but ERs were slightly swollen (black arrow) and dispersed each other.