ORIGINAL ARTICLE

Changes to GFAP Immunoreactive Astrocytes in Medial Prefrontal Cortex Following Exposure to Chronic Stress and Antioxidant Supplementation in Rat Model

Mohd Asnizam Asari[1], Faizah Md Nawi[1], Mohammad Syabil Ikhwan Mohd Amin[1], Nurul Aiman Mohd Yusof[1], KNS Sirajudeen[2]

[1] Department of Anatomy, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kota Bharu, Kelantan, Malaysia
[2] Department of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

ABSTRACT

Introduction: Astrocytes are responsible for many essential functions of neurons in CNS. It has been recognised that chronic stress affects the morphology of astrocyte. Natural antioxidant such as honey has been used as one of the therapeutic strategies to lessen the damaging effect of chronic stress on our body. Therefore, the aim of the study is to explore the effect of natural antioxidant, Tualang honey (TH) on the morphology of astrocytes following chronic stress exposure. **Methods**: Thirty-two male rats were randomly divided into the 4 groups: (i) control, (ii) stress, (iii) honey, (iv) stress plus honey groups.TH was administered via oral gavage at dose of 1.0 g/kg body weight pre and post experiment. Chronic stress was exposed to animals in group (ii) and (iv) for consecutive 21 days. Anti GFAP immunohistochemistry method was employed to label astrocytes in the medial prefrontal cortex. The number of GFAP+ astrocytes and several parameters related to astrocyte processes were measured. **Results:** The present study showed that chronic stress reduced the GFAP immunoreactive astrocyte number and percentage of GFAP immunoreactive material. Chronic stress also caused a reduction in astrocyte process ramification as indicated by a reduction in astrocyte total number of processes, average length of processes and maximum number of intersections. However, antioxidant treatment using TH could not reverse these stress-induced changes to the astrocytes. **Conclusion:** These results demonstrate that chronic stress decreases the number of GFAP immunoreactive astrocyte and cause shrinking of astrocyte processes in stress-sensitive brain region, but these changes cannot be reversed by antioxidant treatment.
Malaysian Journal of Medicine and Health Sciences (2023) 19(2):35-41. doi:10.47836/mjmhs19.2.7

Keywords: Astrocyte, Glial cell, GFAP, Honey, Antioxidant

Corresponding Author:
Mohd Asnizam Asari, PhD
Email: asnizam @usm.my
Tel: +609-7676070

INTRODUCTION

Psychological stress is recognised as one of the phenomena that cause oxidative stress, in which there is an imbalance between oxidative and antioxidative status in the body (1). At cellular level, over exposure to oxidative stress can seriously injure many components of a normal cell such as cellular proteins, lipids and deoxyribonucleic acid (DNA) which if not controlled may lead to development of degenerative diseases such as Parkinson's disease, Alzheimer's disease and Huntington's disease (2). Excessive or prolong exposure to stress has been shown to be detrimental to the brain. In animal experimentation, chronic stress has been shown to alter the morphology of neurons as well as affecting their neurogenesis and synaptogenesis in certain parts of the brain (1). There is increasing evidence showing chronic stress can also affect the supporting cells of the brain, specifically the astrocytes (3).

Astrocytes, the most numerous glial cells are responsible for many essential functions of neurons in the central nervous system (CNS) such as synaptic and trophic functionality. Astrocytes also participate in mediating blood brain barrier, glial scar formation and immunomodulation (4). In addition, astrocytes have been identified as a part of internal antioxidant system of CNS which helps to protect neurons against excessive oxidative stress (5). As such, astrocytes have the potentials to be used as new biological tool to enhance neurons survival following any pathological insults to the brain. Astrocytes can be identified in brain tissue using specific immunohistochemical markers such as anti-glial fibrillary acidic protein (GFAP) antibody. Interestingly, astrocytes possess the ability to change

their morphological characteristics following exposure to some pathological insults such as to certain diseases and stress. For instance, the volume and number of processes of astrocytes become significantly reduced after exposure to chronic stress (6).

One of therapeutic strategy to combat the negative effects of chronic stress on our brain is to use natural antioxidant which are widely distributed in food and plants.. For instance, it has been shown that antioxidant content of a plant, Plicaria incisa protected astrocytes from hydrogen peroxide-induces oxidative stress (25). One of the world's oldest natural products that contain high level of antioxidant is honey. As a matter of fact, honey is the only insect-derived natural product that has stood the test of time, being used for spiritual, therapeutic and nutritional purposes for thousands of years (7). Honey contains hundreds of different components which include carbohydrates (fructose, glucose), amino acids, vitamins, minerals, organic acids and antioxidants (26). Antioxidant contents of honey include flavonoids, phenolic acids, ascorbic acid and carotenoids which are responsible for many therapeutic effects of honey (7).

Tualang honey, a multifloral jungle honey produced by stingless honeybee (*Apis dorsata*) has been demonstrated to possess high amount of natural antioxidants (7). In fact, Tualang honey has been shown to contain greater amount of phenolic acids and flavonoids, giving it greater radical scavenging activity compared with other types of honey (8). Previous studies have shown that Tualang honey possesses some anti-stress effects. Tualang honey supplementation has been shown to protect animals from stress-induced elevation in adrenocorticotropic and corticosterone hormones (27). In addition, Tualang honey has been reported to reduce pro-inflammatory cytokines in the brain flowing exposure to chronic stress (9). However, whether Tualang honey has similar protective effects on the brain glial cells is large unknown at present time. As mentioned earlier, astrocytes serve as an important role in supporting, maintaining, and protecting the overall activities of the neurons (4). Therefore, any damage or reduction in astrocyte functions may impair the structural plasticity and activities of neurons. As such, the aim of the present study was to evaluate using immunohistochemistry method, changes in astrocyte number and morphology in a stress-responsive brain region in rat model of chronic stress. In the present study, medial prefrontal cortex was chosen as it is one of the prominent stress-responsive areas of the brain and easily assessable for immunohistochemical analysis.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats were obtained from Animal Research and Service Centre of Universiti Sains Malaysia (USM), Health Campus. The animals were kept in plastic cages of three rats per cage on sawdust bedding with light cycle 12:12 light: dark. All animals had free access to standard rat chow and water ad libitium and were allowed to habituate to the surrounding environment and experimenters one week prior to the start of actual experiment. This study was carried out in accordance with USM guidelines for the care and use of animals in research and approved by the Animal Ethics Committee of USM (Approval number: USM/IACUC/2020/ (123)(1066).

Experimental design

A total of 32 rats were randomly divided into the four different experimental groups (8 rats per group): (1) control group (Con), (2) stress group (S), (3) honey group (H), (4) stress plus honey group (S+H). Animals in group 2 and group 4 were exposed to chronic stress for consecutive 21 days period. To minimise possible indirect exposure to stress, rats in the group 1 and group 3 were placed in separate room throughout the experimental period. Tualang honey dose of 1 g/kg body weight twice daily (via oral gavage) used in the present study has been shown to be effective to alter the pro-inflammatory cytokines in the brain (9). Equal volume of normal saline was given to rats in the group 1 and group 2 to ensure that all animals were exposed to similar amount of stressful environment during the experiment. Tualang honey (AgroMas) was purchased from the Federal Agricultural Marketing Authority (FAMA) of Malaysia which had been treated to reduce the water content and sterilised using gamma irradiation.

Stress protocol

The method of stress used in the current study has been previously described (9). Briefly, rats were exposed to a five-hour session of restraint plus 15 minutes of swim test per day for 21 days. Swim stress test was included in stress regimen to minimise the occurrence of habituation normally associated with restraint stress protocol (10). Restrainer was constructed using fine gage plastic mesh in which both ends were secured with butterfly metal clips. The restrainer was adjusted just to fit the rats but without causing any pain to the animals. During swim test, rats were allowed to swim freely in a plastic container for 15 minutes. At the end of 15 minutes, rats were dried and returned to their home cages.

Tissue preparation and immunohistochemical procedures

About 24 hours after the final stress procedures, all rats were put under deep anaesthesia using ketamine/ xylazine mixture (90 mg/kg ketamine and 5 mg/kg xylazine, intraperitoneal) and subsequently were transcardiacly perfused with ice-cold phosphate buffer saline (pH 7.4), followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brain was extracted from the skull and then placed in the same fixative solution for 24 hours. Cerebrum were separated from the cerebellum and brain stem and only the right cerebrum

was used for immunohistochemical procedures. The right cerebrum was embedded with paraffin wax as per standard histological procedures.

Five representative coronal sections of medial prefrontal cortex of the cerebrum between +3.0 mm to +2.52 mm from Bregma were selected according to standard rat brain atlas by Paxinos and Watson (11) at a regular space interval of 100 µm with 4 µm thick section. Following hydration steps, sections were exposed to antigen retrieval treatment using citrate buffer (pH6) in microwaves for 20 minutes. To neutralize the endogenous peroxidase activity, the sections were incubated with 0.3% of hydrogen peroxide for 5 minutes. Following washing with Tris buffer saline (TBS), sections were incubated with primary antibody, rabbit polyclonal to GFAP (Abcam, dilution1:1000) for overnight incubation at 4 C. After washing, sections were incubated with a biotinylated anti-rabbit IgG secondary antibody (HRP-AEC micro-polymer IHC kits, Abcam, dilution 1:1000) for 30 minutes and then washed with TBS. Sections were visualised using 3,3'-diaminobenzidine (DAB) followed by haematoxylin counterstain. Sections were then exposed to dehydration process, mounted on poly-L-lysine glass slides and then covered slip.

Quantification GFAP immunoreactive cells and material

GFAP immunostained sections were visualized using an Olympus BX41-32PO2 microscope (Olympus, Japan) attached to a high-resolution digital camera system (Olympus XC50, Japan) and desktop computer (HP Pavilion). Using a low power objective (4x), whole area of the medial prefrontal cortex was visualised and divided by 5 x 6 rectangular grids using the CS image analysis software (Olympus, Japan). Five grid areas were randomly selected using systematic random sampling method. Each selected area was then examined using 40x objectives (N.A 0.65), with field of view of 143,550 μm^2 (width: height, 435 µm X 330 µm). Representative image from each area was captured and stored as tiff format for later analysis. The number of GFAP immunoreactive cells per view field was counted using open-source software Image J Fiji (12). The total number of immunoreactive cells were averaged across the five field of views for each animal and the data were reported as mean number of immunoreactive cells per field of view. GFAP immunoreactive cells were counted only if they have clear star-shaped appearance with uninterrupted radiating processes coming from their cell body (13).

Meanwhile, density of GFAP immunoreactive material was determined by performing thresholding operation in Image J Fiji software which distinguish between GFAP immunoreactive material from non-specific background as described by Tynan et al. (2010) (14). All images were processed under the same basic parameters to ensure the cell bodies and their associated processes were equally thresholded. Density of immunoreactive material was quantified by measuring the relative percentage of thresholded material within the selected region of interest (ROI). Data were reported as the percentage of immunoreactive material within the ROI.

Quantification of GFAP immunoreactive astrocyte processes

Using the same microscope setting as used for counting immunoreactive cells, the processes of GFAP immunoreactive astrocytes and their branching complexity were assessed within the medial prefrontal cortex. For this purpose, five clearly stained isolated GFAP-immunoreactive cells were randomly selected from the medial prefrontal cortex under 100x objective (N.A 1.4, oil immersion) and saved as tiff image for later analysis. The GFAP immunoreactive astrocytes were selected only if they displayed clear processes radiating from the cell body with no obvious artificial cut in the processes. Using Paint 3D software (Microsoft corporation), the selected cells were the cropped and the background were cleaned. Then using Image J Fiji software, the labelled immunoreactive cell were thresholded and skeleton plugging was used to generate set of data on astrocytes processes and their branching complexity. The following data were recorded in the present study: number of primary process, total number of processes (primary + secondary + tertiary processes) and mean length of processes. To further analyse astrocyte branching complicity, Sholl analysis was performed using the Sholl plugging of the Image J Fiji using start radius and end radius of 5 µm and 30 µm respectively with step size of 5 µm. Data of Sholl analysis is presented as the maximum number of intersections encountered by each astrocyte in the image. Fig.1 shows the steps taken to perform Sholl analysis. All visual quantifications were conducted by an observer, blind to the treatment groups.

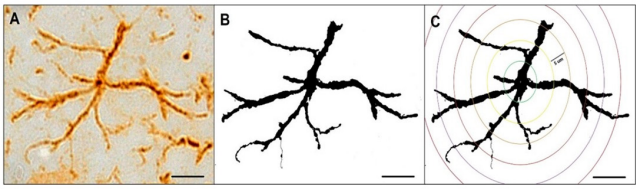

Fig. 1: Steps taken to perform Sholl analysis on GFAP immunoreactive astrocytes. (A) An unprocessed image of a GFAP immunoreactive astrocyte. (B) The thresholded image of an astrocyte with clean background. (C) The thresholded image of an astrocyte undergoes Sholl analysis in Image J Fiji software. Bar scale represents 10 µm

Statistical Analysis

All data were analysed using SPSS 26 for Windows (IBM). Homogeneity of variance was verified using Levine's test. Statistical analyses were performed using one-way analysis of variance (ANOVA) to illicit differences between the groups. Results are expressed as means ± SEM. Significance was set at a probability level of 95 % ($p < 0.05$).

RESULTS

As shown in Fig. 2, the number of GFAP immunoreactive astrocyte per field of view in the medial prefrontal cortex in the stress group was statistically less than that in the control group (Con: 15.2 ± 1.16 vs S: 9.3 ± 1.16; $p < 0.01$), whereas there was no significant difference between stress+honey group and stress group (S+H: 10.1 ± 1.33 vs S: 9.3 ± 1.16; $p > 0.05$). These results indicated that while chronic stress significantly reduced the number of GFAP immunoreactive astrocytes, honey supplementation failed to reverse this trend.

With regard to the percentage of GFAP immunoreactive material, analysis revealed that exposure to stress significantly reduced the amount of GFAP immunoreactive material in the medial prefrontal cortex in comparison to the control group (Con: 14.02 ± 0.98 vs S: 9.44 ± 0.72; $p < 0.05$) (Fig. 2). Compared with the stress group, the stress+honey group (12.59 ± 1.02) had more GFAP+ material, but the difference was insignificant (S+H: 12.59 ± 1.02 vs S: 9.44 ± 0.72; $p > 0.05$) (Fig. 2). These results indicated that stress exposure significantly reduced the amount of GFAP immunoreactive material present in the medial prefrontal cortex as predicted (Fig.3). However, honey supplementation failed to change the amount of GFAP immunoreactive materials present in the medial prefrontal cortex following exposure to chronic stress.

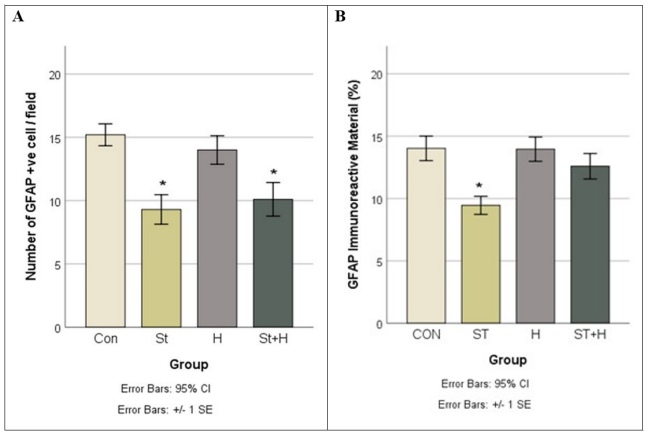

Fig. 2: Mean number of GFAP immunoreactive astrocytes per field of view in all experimental groups (A). Mean percentage of GFAP immunoreactive material in all groups (B). * denotes *p* value of < 0.05 in comparison to the control group.

We also assessed the effects of stress and honey on the astrocyte branching pattern in the medial prefrontal cortex using skeleton pluggin and Sholl analysis pluggin in the Image J Fiji. Analysis of the mean number of primary branch, which is the branch that arises directly from neuron cell body, did not find significant differences between the groups (Table I). With regards to the total number of branches, analysis showed that the stress group had significantly lesser number of branches compared to the control group (C: 27.4 ± 1.76 vs S: 20.0 ± 1.10), but no significant difference was detected between stress group and stress+honey group (S+H 20. 5 ± 1.38 vs S: 20.0 ± 1.10). This data indicated a reduction the number of branches of astrocytes following exposure to chronic stress and honey supplementation could not reverse this trend.

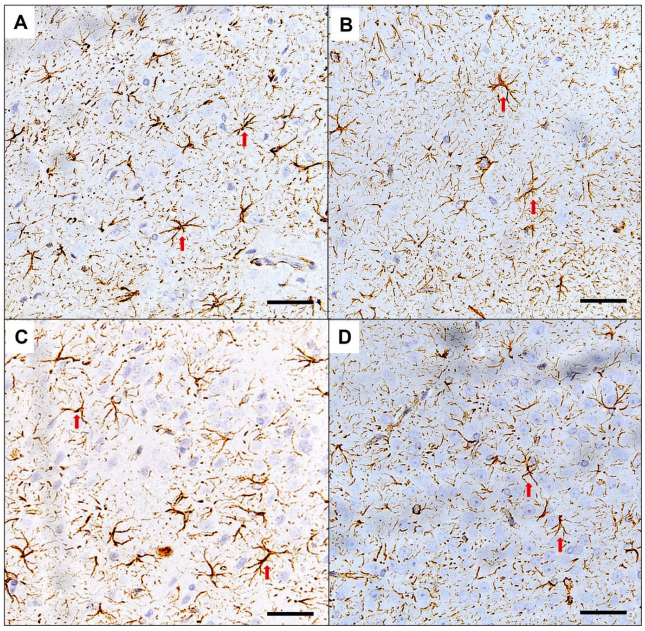

Fig. 3: GFAP immunoreactive astrocytes in the medial prefrontal cortex. (A) Control group. (B) Stress group. (C) Honey group. (D) Honey plus stress group. Red arrows pointed toward GFAP labelled astrocytes. Scale bar = 50 um

Analysis of mean branch length showed stress group had shorter average branch length compared to the control group (C: 16.8 ± 1.5 µm vs S: 11.4 ± 1.2 µm, $p < 0.05$) (Table I). However, there were no significant differences between stress+honey and stress groups (S+H: 11.5 ± 1.1 µm vs S: 11.4 ± 1.2 µm), $p > 0.05$). The results suggested that while chronic stress exposure reduced the astrocyte average branch length, honey supplementation failed to influence the mean branch length. Further analysis of astrocyte branching complexity using Sholl analysis showed that stress group had lower mean number of Sholl intersections compared to the control group (C: 31.40 ± 1.48 vs S: 23.50 ± 1.62, $p < 0.05$) whereas there is no significant different between stress+honey group and stress group (S+H: 24.50 ± 1.75 vs S: 23.50 ± 1.62, $p > 0.05$) (Table I).

DISCUSSION

In immunohistochemical studies, GFAP has long been used as a marker to identify astrocyte tissue section as it is expressed almost exclusively in the CNS. GFAP is an intermediate filament protein (50kDa) found in astrocyte cytoskeleton that is important for normal functioning of astrocytes such as maintaining blood-brain barrier and CNS white matter integrity (15). In the present study, anti-GFAP antibody has been employed to localised astrocytes present in the medial prefrontal

Table I: Morphometry of GFAP immunoreactive astrocytes in medial prefrontal cortex

Group	Control (n = 8)	Stress (n = 8)	Honey (n = 8)	Stress + Honey (n=8)
Number of primary branch	5.8 ± 0.63	5.9 ± 0.72	6.0 ± 0.63	5.7 ± 0.67
Total number of branches	27.4 ± 1.76	20.0 ± 1.10*	25.5 ± 2.02	20. ± 1.38*
Mean branch length (µm)	16.8 ± 1.5	11.4 ± 1.2*	17.4 ± 1.5	11.5 ± 1.1*
Number of intersection (Sholl analysis)	31.40 ± 1.48	23.50 ± 1.62*	33.20 ± 1.58	24.50 ± 1.75*

The results were expressed as mean ± SEM. The significant difference was determined by parametric test; one way ANOVA followed by Tukey post-hoc test with $p < 0.05$ indicates statistically difference. * $p < 0.05$ versus control group

cortex following exposure to chronic stress. The medial prefrontal cortex is one of the stress sensitive areas in the brain of rats that has been studied extensively in the past (6).

The current study showed that exposure to chronic stress reduced the number of GFAP immunoreactive astrocytes as demonstrated by other researchers previously. A study demonstrated that exposure to 21 days of restraint stress caused a 38% reduction in the number of GFAP immunoreactive astrocytes in the medial prefrontal cortex of rats (6). Similarly, it has been shown that exposure to chronic unpredictable mild stress for 21 days duration caused a significant reduction in GFAP immunoreactive astrocytes as well GFAP mRNA in mouse hippocampus (16). The present study also investigated the distribution of GFAP immunoreactive material in the brain tissue section. In this variable, all immunoreactive areas labelled by GFAP (including cell body and their processes) were quantified and expressed as the percentage of immunoreactive area in comparison to the total field area. The present study demonstrated that chronic stress exposure reduced the percentage of GFAP immunoreactive material compared to the control. When considering the honey, the present study indicated that honey supplementation did not influence the number of GFAP immunoreactive astrocytes as well the percentage of GFAP immunoreactive area following chronic stress exposure.

Regarding the assessment of astrocyte branches, we found that chronic stress exposure led to reduction in the total number of branches and mean branch length in comparison to the control. In addition, in Sholl analysis, stress exposure caused a significant reduction in the number of intersections crossed by astrocyte branches. All these results suggested that chronic stress exposure resulted in morphological changes to astrocytes, that is astrocytes processes undergo structural atrophy. This finding is in parallel with the finding by Walker et al. (2013) which found that chronic stress caused produced profound atrophy of astrocyte process length, branching and volume in the medial prefrontal cortex of rats (17). Similar finding was shown by Bender et al. (2020) in acute stress condition (18). However, supplementation with honey failed to reverse the stress-induced retraction of astrocytes branches and processes.

Taken together, the present study showed that chronic stress induced astrocyte remodelling in the medial prefrontal cortex as indicated by other researchers (6, 18) . These remodelling of astrocytes may influence and produce significant changes to behaviour and physiology of our body. For instance, it has been demonstrated that astrocyte ablation in the medial prefrontal cortex caused depressive-like behaviour (19, 20). Furthermore, atrophy of astrocyte processes has been shown to lead to motor deficits (21) . The biological effect of astrocyte atrophy is not surprising considering the important involvement of astrocytes in synaptic transmission, in which fine processes of astrocytes have been documented to make contact with presynaptic and postsynaptic sites of neurons. The remodelling process of astrocytes following stress involved the synthesis of GluA1 protein via CPEB3 pathway. During stress, the production of GluA1 protein, a critical subunit of glutamate receptor become significantly suppressed and without functional glutamate receptors, astrocytes lose to communicate with each other and with neurons, causing astrocytes to retract their processes (18).

The lack of effect of honey on astrocytes following chronic stress exposure indicated that antioxidant supplementation may not have the ability to reverse stress-induced astrocyte remodelling process. Nevertheless, astrocytes are known for their role in providing antioxidant self defence against reactive oxygen species (ROS) present in the brain by influencing the NF-E2-related factor 2 (Nrf2), a master regulator of oxidative stress (22). Suppression of Nrf2 activity led to increased susceptibility of the brain to negative effects of oxidative stress while activation of Nrf2 lead to neuroprotective effects (23). A study using Manuka honey showed that Manuka honey activated Nrf2 signalling pathway in in vitro study of human dermal fibroblast (24). No similar study has been conducted using Tualang honey however it is postulated that Tualang honey would similarly activate Nrf2 pathway considering Tualang honey contains higher level of antioxidants compared to other types of honey (8). The neuroprotective effects of Tualang honey are real and confirmed by other researchers (7).

CONCLUSION

The results presented here are consistent with the existing literature that shows chronic stress causes retraction of astrocyte processes. However, despite the lack of positive results on antioxidant in the present study, the

real role of antioxidants particularly honey on astrocytes remodelling process remain unclear and need further investigations. One of the limitations of this study is the use of flat plane image (2D) that lacks depth perception. Future studies using other astrocyte markers and the use of more advanced three-dimensional morphology analysis will shed further understanding on this issue.

ACKNOWLEDGEMENTS

This study was supported by Universiti Sains Malaysia (USM Research University Grant No: 1001/PPSP/8012223). Special thanks to all the staffs at the Central Laboratory Unit (CRL), School of Medical Sciences, USM Health Campus for their technical support.

REFERENCES

1. Lucassen PJ, Pruessner J, Sousa N, Almeida OF, Van Dam AM, Rajkowska G, et al. Neuropathology of stress. Acta Neuropathol. 2014;127(1):109-35. doi: 10.1007/s00401-013-1223-5.
2. Sharifi-Rad M, Anil Kumar NV, Zucca P, Varoni EM, Dini L, Panzarini E, et al. Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases. Front Physiol. 2020;11:694. doi: 10.3389/fphys.2020.00694.
3. Lupien SJ, Juster RP, Raymond C, Marin MF. The effects of chronic stress on the human brain: From neurotoxicity, to vulnerability, to opportunity. Front Neuroendocrinol. 2018;49:91-105. doi: 10.1016/j.yfrne.2018.02.001.
4. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. Acta Neuropathol. 2010;119(1):7-35. doi: 10.1007/s00401-009-0619-8.
5. McBean GJ. Astrocyte Antioxidant Systems. Antioxidants (Basel). 2018;7(9):112. doi: 10.3390/antiox7090112
6. Tynan RJ, Beynon SB, Hinwood M, Johnson SJ, Nilsson M, Woods JJ, Walker FR. Chronic stress-induced disruption of the astrocyte network is driven by structural atrophy and not loss of astrocytes. Acta Neuropathol. 2013;126(1):75-91. doi: 10.1007/s00401-013-1102-0
7. Azman KF, Aziz CBA, Zakaria R, Ahmad AH, Shafin N, Ismail CAN. Tualang Honey: A Decade of Neurological Research. Molecules. 2021; 26(17):5424. doi: 10.3390/molecules26175424.
8. Kishore RK, Halim AS, Syazana MS, Sirajudeen KN. Tualang honey has higher phenolic content and greater radical scavenging activity compared with other honey sources. Nutr Res. 2011;31(4):322-5. doi: 10.1016/j.nutres.2011.03.001.
9. Asari MA, Zulkaflee MH, Sirajudeen KNS, Mohd Yusof NA, Mohd Sairazi NS. Tualang honey and DHA-rich fish oil reduce the production of pro-inflammatory cytokines in the rat brain following exposure to chronic stress. J Taibah Univ Med Sci. 2019;14(4):317-323. doi: 10.1016/j.jtumed.2019.06.004
10. Zamora-González EO, Santerre A, Palomera-Avalos V, Morales-Villagr6n A. A chronic combinatory stress model that activates the HPA axis and avoids habituation in BALB/C mice. J Neurosci Methods. 2013;213(1):70-5. doi:10.1016/j.jneumeth.2012.10.015
11. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. Academic Press; 2007
12. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nature Methods.2012; 9(7), 676–682. doi: 10.1038/nmeth.2019.
13. Eldomiaty MA, Makarenko O, Hassan ZA, Almasry SM, Petrov P, Elnaggar AM. Contribution of glia cells specifically astrocytes in the pathology of depression: immunohistochemical study in different brain areas. Folia Morphol (Warsz). 2020;79(3):419-428. doi: 10.5603/FM.a2020.0007
14. Tynan RJ, Naicker S, Hinwood M, Nalivaiko E, Buller KM, Pow DV, et al. Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions. Brain Behav Immun. 2010 ;24(7):1058-68. doi: 10.1016/j.bbi.2010.02.001.
15. Yang Z, Wang KK. Glial fibrillary acidic protein: from intermediate filament assembly and gliosis to neurobiomarker. Trends Neurosci. 2015;38(6):364-74. doi: 10.1016/j.tins.2015.04.003.
16. Ding XF, Liu Y, Yan ZY, Li XJ, Ma QY, Jin ZY, et al. Involvement of Normalized Glial Fibrillary Acidic Protein Expression in the Hippocampi in Antidepressant-Like Effects of Xiaoyaosan on Chronically Stressed Mice. Evid Based Complement Alternat Med. 2017;2017:1960584. doi: 10.1155/2017/1960584.
17. Walker R, Tynan R, Beynon S, Nilsson M. Chronic stress induces profound structural atrophy of astrocytes within the prefrontal cortex:An emerging story in glial remodeling in response to stress. Brain, Behavior, and Immunity. 2013. 32: e20-e21. doi: 10.1016/j.bbi.2013.07.082
18. Bender CL, Sun X, Farooq M, Yang Q, Davison C, Maroteaux M, et al. Emotional Stress Induces Structural Plasticity in Bergmann Glial Cells via an AC5–CPEB3–GluA1 Pathway. Journal of Neuroscience. 2020; 40(17):3374-3384. doi: 10.1523/JNEUROSCI.0013-19.2020.
19. Banasr M, Duman RS. Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors. Biol Psychiatry. 2008 ;64(10):863-70.
20. Banasr M, Duman RS. Regulation of neurogenesis and gliogenesis by stress and antidepressant treatment. CNS Neurol Disord Drug Targets. 2007; 6:311–320. doi: 10.2174/187152707783220929.
21. Saab AS, Neumeyer A, Jahn HM, Cupido A, imek AAM, Boele H-J, et al. Bergmann glial AMPA

receptors are required for fine motor coordination. Science. 2012; 337:749–753. doi: 10.1126/science.1221140.
22. Serapide MF, L'Episcopo F, Tirolo C, Testa N, Caniglia S, Giachino C, et al. Boosting Antioxidant Self-defenses by Grafting Astrocytes Rejuvenates the Aged Microenvironment and Mitigates Nigrostriatal Toxicity in Parkinsonian Brain via an Nrf2-Driven Wnt/β-Catenin Prosurvival Axis. Front Aging Neurosci. 2020;12:24. doi: 10.3389/fnagi.2020.00024
23. Lee KH, Cha M, Lee BH. Neuroprotective Effect of Antioxidants in the Brain. Int J Mol Sci. 2020; 21(19):7152. doi: 10.3390/ijms21197152.
24. Alvarez-Suarez JM, Giampieri F, Cordero M, Gasparrini M, Forbes-Hernández TY, Mazzoni L, et al. Activation of AMPK/Nrf2 signalling by Manuka honey protects human dermal fibroblasts against oxidative damage by improving antioxidant response and mitochondrial function promoting wound healing. Journal of Functional Foods. 2016;25: 38–49. doi: 10.1016/j.jff.2016.05.008
25. Elmann A, Telerman A, Mordechay S, Erlank H, Ofir R. Antioxidant and astroprotective effects of a Pulicaria incisa infusion. Oxid Med Cell Longev. 2012;2012:157598. doi: 10.1155/2012/157598
26. Tafere DA. Chemical Composition and uses of Honey: A Review. Journal of Food Science and Nutrition Research. 2021;4(3):194–201. doi: 10.26502/jfsnr.2642-11000072
27. Al-Rahbi B, Zakaria R, Othman Z, Hassan A, Ahmad AH. Protective Effects of Tualang Honey against Oxidative Stress and Anxiety-Like Behaviour in Stressed Ovariectomized Rats. International Scholarly Research Notices. 2014; 2014: 1–10. doi: 10.1155/2014/521065