



UNIVERSITI PUTRA MALAYSIA

***EFFECTS OF DIETARY EDIBLE BIRD'S NEST SUPPLEMENTATION ON
COGNITIVE FUNCTION OF TRANSGENERATIONAL MICE***

OBAIDULLAH MAHAQ

FPV 2021 1



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COGNITIVE FUNCTION OF TRANSGENERATIONAL MICE**

By

OBAIDULLAH MAHAQ

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of
Master of Veterinary Science**

November 2020

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DEDICATION

To my beloved mother and my loving father who was always wishful thinking of my higher education. To the pillar of my strength my spouse for your endless attention, love and devotion in my life and cute sons (Subhanullah, Habibullah and Hozaifa), your invaluable love and support were my main motivations for completing my master journey. I am really astonished by your patience in bearing my absence for more than two years. My heartfelt thoughts also go to my siblings, who are always honestly happy with my progress.

To my academic family in Afghanistan, let us aspire to restore our nation to its former glory, as the centre of knowledge and grandeur that it once was.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Veterinary Science

EFFECTS OF DIETARY EDIBLE BIRD'S NEST SUPPLEMENTATION ON COGNITIVE FUNCTION OF TRANSGENERATIONAL MICE

By

OBAIDULLAH MAHAQ

November 2020

Chairman : Associate Profesor Hafandi Ahmad, PhD
Faculty : Veterinary Medicine

Edible bird's nest (EBN) is well known as a natural food product rich in glycoproteins such as sialic acid, minerals, and essential amino acids. Evidence from epidemiological studies suggests that EBN dietary supplementation improved brain cognitive functions. In mammals, the highest absorption of sialic acid from EBN occurs in the brain where it participates as an integral part of ganglioside structure in synaptogenesis and neural transmission. Sialic acid in EBN is vital during rapid brain growth particularly for preterm infants. While EBN dietary supplementation has been associated to enhance brain functions in infants, the effects of multiple generations of dietary EBN on cognitive function remain unclear. Thus, this study aimed to determine the effects of dietary EBN supplementation from different locations (e.g south (S) and north (N) of Peninsular Malaysia, Sabah (B) and commercial (C) on the cognitive function of transgenerational mice. To address these issues, C57BL/6 breeder mice (F0) were fed with different sources of EBN for six weeks (10 mg/kg) using oral gavage. Then, all animals were bred to obtain first generation (F1) until the second generation (F2) animals. At six weeks of age, F1 and F2 animals were tested for brain cognitive function by Y-maze test. Histological study for neuron density and distribution were analyzed using the hematoxylin and eosin procedure. The active compounds of EBN were determined using HPLC and the brain genes expression associated with cognitive function (e.g GNE, ST8SiaIV, SLC17A5, and BDNF mRNA) were analyzed using real-time PCR. Results showed that dietary EBN supplementation improved cognitive performance of F0, F1 and F2 mice by significantly increased the number of entries (9.04 ± 0.15 ; $P < 0.05$) and the time spent (2.40 ± 0.4 min; $P < 0.05$) in the novel arm of Y-maze test compared to control. This could indicate that the breeder mice (F0) absorbed sialic acid from EBN which crossed the placenta to contribute fetal brain function and development in the third trimester. In addition, EBN supplementation improved neuron development in the brain hippocampus of F0, F1 and F2 generations by significantly increased the number of neurons (32.74 ± 4.80 ; $P < 0.05$) compared to control (21.78 ± 2.86). In PCR analysis,

mice maintained on EBN supplementation significantly increased the expression level of GNE (1.6-fold; $P < 0.05$) in both F0 and F1 of EBN-N group. Interestingly, this gene was upregulated only in F1 mice (1.64-fold) especially in the EBN-C group. Expression level of ST8SiaIV was significantly increased (2-fold; $P < 0.05$) in the F0 of EBN-N group and in the F1 of EBN-C group (1.83-fold). The BDNF levels were significantly increased in EBN-S (2-fold; $P < 0.05$), EBN-N (1.5-fold) and EBN-C (1.6-fold) F1 animals compared to control (1-fold). However, SLC17A5 expression was not significantly increased ($P > 0.05$) in all groups of F0 and F1 animals. These results indicate that increased number of neurons and the variation level of genes expression due to the presence and metabolism of sialic acid in mammals associates with increased cognitive performance. In conclusion, EBN extract supplementation for six weeks with higher sialic acid content improve the cognitive function of transgenerational mice.

Keywords: Edible bird's nest (EBN), cognitive function, neuron density, gene expression, transgenerational mice

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Veterinari Sains

KESAN SUPLEMEN SARANG BURUNG YANG BOLEH DIMAKAN PADA FUNGSI KOGNITIF TIKUS TRANSGENERASI

Oleh

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Sarang burung yang boleh dimakan (EBN) dikenali sebagai produk makanan semulajadi yang kaya dengan glikoprotein seperti asid sialik, mineral dan asid amino penting. Bukti dari kajian epidemiologi menunjukkan bahawa suplemen makanan dari EBN meningkatkan fungsi kognitif otak. Di dalam mamalia, penyerapan tertinggi EBN berlaku di otak di mana ia mengambil bahagian penting bagi struktur gangliosid di dalam sinaptogenesis dan transmisi saraf. Asid sialik dalam EBN sangat penting semasa pertumbuhan otak yang cepat terutama bagi bayi prematur. Walaupun suplemen makanan EBN dikaitkan untuk meningkatkan fungsi otak pada infan, kesan pemakanan EBN pada fungsi kognitif melalui beberapa generasi adalah tidak jelas. Oleh itu, kajian ini bertujuan untuk mengetahui kesan suplemen EBN dari lokasi yang berlainan (cth. selatan (S) dan utara (N) Semenanjung Malaysia, Sabah (B) dan komersial (C) terhadap fungsi kognitif tikus dalam beberapa generasi. Bagi menangani isu ini, tikus pembiakan CJ57BL/6 diberi makan dengan sumber EBN yang berbeza (10 mg/kg) menggunakan oral gavaj. Kemudian, semua haiwan dibiak untuk mendapatkan generasi pertama (F0) haiwan sehingga generasi kedua (F2) haiwan. Pada usia 6 minggu, haiwan F1 dan F2 diuji bagi fungsi kognitif otak menggunakan ujian Y-maze. Kajian histologi bagi ketumpatan dan keselarakan sel neuron dianalisis menggunakan prosedur haematoxylin dan eosin. Aktif kompaun bagi EBN ditentukan menggunakan HPLC dan ekspresi gen otak yang berkaitan dengan fungsi kognitif (cth. GNE, ST8SiaIV, SLC17A5 dan BDNF mRNA) dianalisis dengan menggunakan real-time PCR. Hasil kajian menunjukkan bahawa suplemen pemakanan EBN meningkatkan prestasi kognitif tikus F0, F1 and F2 dengan peningkatan jumlah kemasukan secara signifikan (9.04 ± 0.15 ; $P < 0.05$) dan masa yang dihabiskan (2.40 ± 0.4 min; $P < 0.05$) di bahagian novel bagi ujian Y-maze berbanding kumpulan kawalan. Ini dapat menunjukkan bahawa tikus F0 menyerap asid sialik dari EBN yang melintasi plasenta bagi menyumbang fungsi dan perkembangan otak janin pada trimester ke tiga. Sebagai tambahan, suplemen EBN meningkatkan perkembangan neuron di hipokampus otak pada generasi F0, F1 dan F2 dengan peningkatan bilangan

neuron yang signifikan (32.74 ± 4.80 ; $P < 0.05$) berbanding dengan kumpulan kawalan (21.78 ± 2.86). Dalam analisis PCR, tikus yang dikekalkan pada suplementasi EBN secara signifikan meningkatkan tahap ekspresi GNE (1.6-kali ganda; $P < 0.05$) pada kedua-dua F0 dan F1 dari kumpulan EBN-N. Menariknya gen ini meningkat pada tikus F1 (1.64-kali ganda) terutamanya pada kumpulan EBN-C. Tahap ekspresi ST8SialV meningkat dengan ketara (2 kali ganda; $P < 0.05$) pada kumpulan F0 EBN-N dan dalam kumpulan F1 EBN-C (1.83-kali ganda). Tahap BDNF meningkat dengan ketara pada haiwan EBN-S (2 kali ganda; $P < 0.05$), EBN-N (1.5-kali ganda) dan EBN-C (1.6-kali ganda) berbanding dengan kumpulan kawalan (1-kali ganda). Walaubagaimanapun, ekspresi SLC17A5 tidak meningkat secara signifikan ($P > 0.05$) pada semua kumpulan haiwan F0 dan F1. Hasil kajian ini menunjukkan bahawa peningkatan bilangan neuron dan tahap variasi ekspresi gen adalah kerana kehadiran dan metabolisme asid sialik pada mamalia berkaitan dengan peningkatan prestasi kognitif. Kesimpulannya, suplemen ekstrak EBN dengan kandungan sialik asid yang tinggi selama enam minggu meningkatkan fungsi kognitif tikus transgenerasi.

Kata kunci: Sarang burung yang boleh di makan (EBN), fungsi kognitif, kepadatan neuron, ekspresi gene, tikus transgenerasi

ACKNOWLEDGEMENTS

First, of all, I would like to give thanks and praise to almighty Allah, and my parents for their countless support and encouragement during my studies.

My deepest appreciation goes to my supervisor, Assoc. Prof. Dr. Hafandi Ahmad and my co-supervisors Assoc. Prof. Dr. Mohd Hezmee Mohd Noor and Assoc. Prof. Dr. Hasliza Abu Hassim for their support in my master journey. Assoc. Prof. Dr. Hafandi Ahmad taught me the skills of designing and performing scientific research work that are of the highest quality. In addition, he has provided me with encouragement and advice whenever I needed them. I am also grateful to my co-supervisors, Assoc. Prof. Dr. Mohd Hezmee Mohd Noor and Assoc. Prof. Dr. Hasliza Abu Hassim who often took time off from their busy schedules to discuss with me on my research work. Through these discussions, I was able to obtain useful scientific advice and knowledge which greatly aids in my understanding on the field of animal physiology. I would also like to express my gratitude to the Higher Education Development Program (HEDP) and the Ministry of Higher Education of Afghanistan for providing me with the research scholarship for my master's degree studies.

Next, I would like to thank the Centre of Excellence (CoE) Swiftlets, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Department of Veterinary Services Malaysia, and Ministry of Science and Technology (MOSTI) Malaysia (Project Number: 6371401-10301) for providing the grant for the project of edible bird's nest. Throughout the course of my master's program, it has been a great pleasure to work with the post-graduates from Anatomy laboratory, Mr. Mohd Adha P. Rameli, Mrs. Rosmawati Mohd Hanipah from physiology laboratory and Mrs. Nur Farhana Harun from nutrition laboratory. Without them, I am certain that my research work would not have proceeded so smoothly. I am grateful to all their assistance and wish them the best of luck in the future. Also, I would like to thank Mr. Mohd Qayyum and other EBN industries for their assistance in obtaining the edible bird's nest from South and North Peninsular Malaysia as well as Sabah. In addition, I wish to express my thanks to Mr. Saipuzaman Ali and Mr. Mohd Jamil Samad from the Serology laboratory for their valuable technical advice and training in the analytical instruments.

Last but not least, I am always grateful to my friends for their continuous support and understanding throughout the journey of my master's program.

Finally, my deepest gratitude goes to my dear parents my wife and all family for their understanding, financial and moral support.

Thank you to everyone who had contributed direct or indirectly in making this research successful.

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

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Date: 11 February 2021

TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	3
2.1 General overview	3
2.2 Edible bird's nest (EBN)	3
2.2.1 Swiftlets	4
2.2.2 Swiftlet ranches	6
2.2.3 Grading of EBN	7
2.2.4 Harvesting and cleaning processes	8
2.2.5 Health benefits of EBN	9
2.2.6 Nutritional composition of EBN	10
2.2.6.1 Moisture	10
2.2.6.2 Protein	11
2.2.6.3 Amino acids	11
2.2.6.4 Mineral elements	11
2.2.6.5 Fatty acids	12
2.2.6.6 Carbohydrates	12
2.2.6.7 Sialic acid	13
2.2.7 Factors affecting the nutritional composition of EBN	13
2.2.8 Biosynthesis and metabolism of sialic acid	13
2.3 Cognitive function	15
2.3.1 Effects of diets on cognitive function	16
2.3.2 Evaluation of cognitive function	17
2.4 Effects of EBN on cognitive function	17
2.5 Effects of EBN on brain neurons	18
2.6 Effects of EBN on gene expression	19
2.7 Summary	20
3 MATERIALS AND METHODS	21
3.1 Experimental design	21
3.2 EBN sample collection and preparation	23
3.3 Sialic acid determination using HPLC	24

3.4	Animals	26
3.4.1	Experimental location and animal housing	26
3.4.2	Treatment diets	26
3.4.3	EBN extract preparation for experimental animals	26
3.4.4	Animal breeding program	27
3.5	Cognitive function assessment technique by Y-maze test	27
3.6	Brain neuron density by hematoxylin and eosin (H&E) technique	28
3.6.1	Neuron cell count	29
3.7	Brain gene expression using real time qPCR	30
3.7.1	Tissues preparation	30
3.7.2	RNA extraction	31
3.7.3	cDNA synthesis	31
3.7.4	Primer design	31
3.7.5	qPCR primer validation	31
3.7.6	of standard curve and melting curve for Real-time quantitative PCR (qPCR)	32
3.7.7	Real-time Polymerase chain reaction (RT-PCR)	32
3.7.8	Gene expression analysis	33
3.8	Statistical analysis	33
4	RESULTS	34
4.1	Effects of EBN on cognitive performance	34
4.2	Effects of EBN on brain neuron histology	38
4.2.1	Neuron density and population in the brain hippocampus	38
4.2.2	Histological structures of the neurons	41
4.3	Effects of EBN on gene expression associated with cognitive function	45
5	DISCUSSION	48
5.1	Effects of EBN cognitive performance	48
5.2	Effects of EBN on brain neuron population in the hippocampus	49
5.3	Effects of EBN on brain gene expression associated with cognitive function	51
6	SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH	55
6.1	Summary and conclusion	55
6.2	Recommendations	56
	REFERENCES	57
	APPENDICES	75
	BIODATA OF STUDENT	82
	LIST OF PUBLICATIONS	83

LIST OF TABLES

Table	Page
2.1 Scientific classification and nomination of swiftlet bird	5
3.1 Retention time of HPLC for the sialic acid in different EBN samples was compared with the retention time of the sialic acid standard (N-Acetylneuraminic acid)	25
3.2 The sequences of forward and reverse primers with its amplicon sizes designed for the reference and target genes used in this experiment	32



LIST OF FIGURES

Figure	Page	
2.1	The countries in the Southeast Asia where EBN is produced. Edible bird's nest has been seen in dark green-shaded areas	5
2.2	Two species of swiftlets found in Southeast Asia including Malaysia	6
2.3	The processes of EBN production start from nesting until cleaning and selling in the market	7
2.4	Anatomical and histological structures of the hippocampus with its different areas and the CA1 region of the hippocampus in the coronal section located on the proximal direction	16
3.1	A flowchart diagram summarizing the whole research procedures designed for the study. F0 = maternal mice, F1= first generation, F2 = second generation, H&E = hematoxylin and Eosin	22
3.2	The grounded powder of EBN samples collected from four different geographical locations	23
3.3	Schematic description of the Y-maze test	28
3.4	Neuron counting at the CA1 region of the brain hippocampus	30
4.1	Total number of entries in the novel arm of Y-maze across the experimental groups from F0 generation	35
4.2	Time spent in the novel arm of Y-maze across the experimental groups from F0 generation	36
4.3	Total number of entries in the novel arm of Y-maze across the experimental groups from F1 generation	36
4.4	Time spent in the novel arm of Y-maze across the experimental groups from F1 generation	37
4.5	Total number of entries in the novel arm of Y-maze across the experimental groups from F2 generation	37
4.6	Time spent in the novel arm of Y-maze across the experimental groups from F2 generation	38
4.7	The mean of neuron's population in the CA1 region of the brain hippocampus across the experimental groups from F0 generation	39

4.8	The average number of neurons in the CA1 region of the brain hippocampus across the experimental groups from F1 generation	40
4.9	The average number of neurons in the CA1 region of the brain hippocampus across the experimental groups form F2 generation	40
4.10	Histological structures of neurons in the area of interest from F0 generation	42
4.11	Histological structures of neurons in the brain hippocampus from F1 generation	43
4.12	Histological structures of neurons in the brain hippocampus from F2 generation	44
4.13	GNE expression level in the hippocampal region of the brain in F0 and F1 mice supplemented with different EBN extract and CRTL (control)	46
4.14	ST8SiaIV expression level in the brain hippocampus in F0 and F1 mice supplemented with different EBN extract and CRTL (control)	46
4.15	SLC17A5 expression level in the brain hippocampus in F0 and F1 mice supplemented with different EBN extract and CRTL (control)	47
4.16	Expression level of BDNF in the brain hippocampus in F0 and F1 mice supplemented with different EBN extract and CRTL (control)	47

LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AD	Alzheimer's Disease
ALDH	Aldehyde dehydrogenase
BDNF	Brain Derived Neurotrophic Factor
CA-1	Cornu Ammonis-1
CALM	Calmodulin
cDNA	Complementary Deoxyribonucleic acid
ChAT	Choline acetyltransferase
CTRL	Control
DG	Dentate gyrus
DP	Degree of polymerization
EBN	Edible Bird's Nest
EBN-B	Edible Bird's Nest collected from Borneo
EBN-C	Edible Bird's Nest Commercial
EBN-N	Edible Bird's Nest collected from North
EBN-S	Edible Bird's Nest collected from South
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-regulated protein kinase
F0	Generation 0 (Maternal mice)
F1	First Generation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GNE	UDP-N-acetylglucosamine 2-epimerase/ N-acetylmannosamine kinase
Gpx	Glutathione peroxidase

Gsr	Glutathione reductase
H&E	Hematoxylin and Eosin
HPLC	High performance liquid chromatography
HSD	Honestly Significant Difference
IL-6	Interleukin-6
JNK	c-Jun N-terminal kinases
MDA	Malondialdehyde
MFGM	Milk fat globule membrane
MMP-1	Matrix metalloproteinase-1
NCAM	Neuro Cellular Adhesion Molecule
P4R	Progesterone 4 Receptor
PAGE	Polyacrylamide Gel Electrophoresis
PCNA	Proliferating Cell Nuclear Antigen
PUFA	Poly Unsaturated Fatty Acid
qPCR	quantitative Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SIRT1	Sirtuin 1/NAD-dependent deacetylase sirtuin-1
SLC17A5	Sialin/sialic acid cotransporter
SNCA	α -Synuclein
SOD	Superoxide Dismutase
ST8SiaIV	CMP-N-acetylneuraminase-poly-alpha-2,8-sialyltransferase
TNF- α	Tumor necrosis factor-alpha
TTR	Transthyretin
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

Edible bird's nest (EBN) is a type of medicinal food secreted by the salivary glands of swiftlets (Ma & Liu, 2012). For several centuries, EBN has been used in traditional Chinese medication or as dietary supplements. Until now, it has also been consumed as a health-supportive substance to provide nutrients and reinstate body deficiency (Nabilah et al., 2018). The EBN has now been developed into different types of food products, including drinks and food additives. It is now also being used as a cosmetic ingredient (F. Ma & Liu, 2012). Although, it is one of the most expensive and popular food in the world but its compositional properties are remain debated among the researchers (Marcone, 2005). It is necessary to investigate the relationships of ingredients and biological functions of the EBN, which are yet to be determined (Ma & Liu, 2012). Mainly, EBN contains proteins, carbohydrates, amino acids, and mineral salts (Marcone, 2005). The carbohydrates are the second abundant components with a higher content of sialic acid found in EBN (Saengkrajang et al., 2013). It was suggested that sialic acid found in EBN is vitally important for brain cognitive function and neurological development (Rashed & Nazaimoon, 2010; Yew et al., 2014).

Cognitive functions are including perception, learning, memory and decision making, that contribute to foraging, walking, vocalizing, emotion and many others behaviors of daily life. In animals, the cognitive function described the exploration or spatial recognition in a novel environment (Shettleworth, 2001). The relative abundance of a particular diet or nutrients has long been recognized to influence cognitive function (Gómez-Pinilla, 2008). Dietary supplementation such as edible bird's nest considerably improve cognitive function and has a potent neuroprotection by inhibiting the mechanisms of neuroinflammation and oxidative stress in the brain and hippocampus (Careena et al., 2018). It has been reported that rats supplemented with EBN for 12 weeks improved water maze performance during four days consecutively trial and indicated that EBN dietary supplementation for 3 months could improve cognitive function and the memory of ovariectomized female rats (Hou et al., 2017). Recent studies suggest that maternal supplementation of EBN in the prenatal and early postnatal periods increases the level of sialic acid in the brain ganglioside of the offspring' which may contribute to the developing of cognitive function and learning efficiency (Xie et al., 2018). While dietary EBN supplementation has been associated to enhance brain functions in mammals, the multi-generation effects of dietary EBN supplementation on cognitive function are remain unclear. In addition, there is a paucity of information about the beneficial effects of maternal dietary EBN supplementation on brain cognitive function over several generations in mammals. Challenges in quantifying mental development in humans remained a major obstacle because of the long generation gap in humans makes it difficult to investigate the effects of maternal EBN dietary supplementation on brain development and cognitive function in their neonates. In this study, mice were used due to their features short reproductive cycle and gestation period. This enables the transgenerational effects of

EBN on mental health and cognitive function to be investigated thoroughly in this model. Therefore, the main objective of this study was to investigate the effects of multiple generations of dietary EBN supplementation on cognitive function in mice.

General objective

To study the influences of dietary EBN supplementation on brain cognitive function of transgenerational mice

Specific objectives

1. To determine the effects of different EBN extract supplementation on brain cognitive function of F0, F1 and F2 mice using the Y-maze task.
2. To determine the influence of maternal EBN dietary supplementation on brain neuron histological changes and distribution of F0, F1 and F2 mice.
3. To assess the influence of maternal EBN dietary supplementation on brain genes expression (e.g ST8SiaIV, GNE, SLC17A5 and BDNF) associated with the cognitive function of F0 and F1 mice.

Hypotheses

1. Dietary EBN supplementation improves cognitive function of transgenerational mice by transmitting the sialic acid component from EBN in the first and second generations.
2. Dietary EBN supplementation increases brain neuron density in the hippocampus of transgenerational mice.
3. Dietary EBN supplementation increases the levels of mRNA for GNE, SLC17A5, ST8SiaIV and BDNF in the brain hippocampus in F0 and F1 mice, as the likely basis for increased cognitive function and learning memory.

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APPENDICES

Appendix - 1

A: Nutritional composition of normal pellet given to the experimental animals

Composition	EBN-S	EBN-N	EBN-B	EBN-C	CTRL
Crude Protein	23.8	23.8	23.8	23.8	23.8
Crude Fibre	5.5	5.5	5.5	5.5	5.5
Crude Fat	5.0	5.0	5.0	5.0	5.0
Ash	9.8	9.8	9.8	9.8	9.8
Calcium	1.2	1.2	1.2	1.2	1.2
Phosphorus	0.9	0.9	0.9	0.9	0.9
Non-Protein Nitrogen	53.8	53.8	53.8	53.8	53.8
Total (DM)	100%	100%	100%	100%	100%
(As % Of Total Wet Weight)					
Moisture	13.0	13.0	13.0	13.0	13.0

B: Edible bird's nest extracts, diets and water provided for experimental animals

Composition	EBN-S	EBN-N	EBN-B	EBN-C	CTRL
EBN	10mg/kg (0.2 ml)	10mg/kg (0.2 ml)	10mg/kg (0.2 ml)	10mg/kg (0.2 ml)	-
Normal saline	-	-	-	-	0.2 ml
Normal pellet	ad libitum	ad libitum	ad libitum	ad libitum	ad libitum
Water	ad libitum	ad libitum	ad libitum	ad libitum	ad libitum

Appendix – 2

The sequential procedure of Haematoxylin and Eosin staining used for brain neuron morphological examination.

Haematoxylin and Eosin procedure		
Process	Solution	Time (min)
Deparaffinization	Xylene I	2 min
	Xylene II	2 min
Dehydration	100% alcohol	2 min
	90% alcohol	2 min
	70% alcohol	2 min
Wash	Running water	2 - 3 min
Staining	Haematoxylin	5 min
Wash	Running water	2 - 3 min
	Acid alcohol	3 dips
Wash	Running water	2-3 min
Staining	Eosin	5 min
Wash	Running water	2-3 min
Dehydration	70% alcohol	2-3 dips
	90% alcohol	2-3 dips
	100% alcohol	2-3 dips
Mounting	Xylene I	2-3 dips
	Xylene II	2-3 dips
	DPX	

Serology laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Appendix – 3

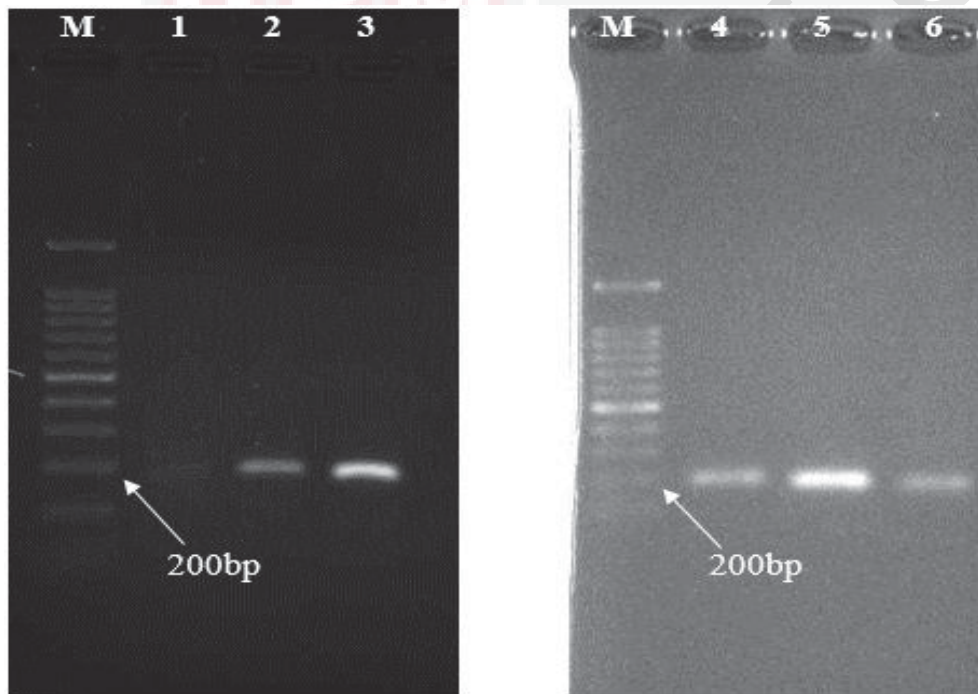
Brain hippocampal tissue samples of extracted mRNA from F0 and F1 generation animals used for reverse transcription to produce cDNA used in the qPCR.

Generation	No	Sample	Concentration (ng/ μ L)	A260/A280	A260/A230	Label for analysis
F0	1	EBN-S	488.6	2.07	2.19	F0S
	3	EBN-N	132.7	2.12	2.00	F0N
	2	EBN-B	786.9	2.09	2.18	F0B
	4	EBN-C	362.5	2.09	2.10	F0C
	5	CTRL	289.1	2.10	2.10	F0CTRL
F1	6	EBN-S	418.0	2.08	2.10	F1S
	8	EBN-N	576.3	2.05	2.02	F1N
	7	EBN-B	614.8	2.08	2.17	F1B
	9	EBN-C	332.3	2.10	2.18	F1C
	10	CTRL	662.1	2.07	1.96	F1CTRL

Appendix – 4

A: Size confirmation of the primers for the target and reference genes compared with the leader for RT-PCR.

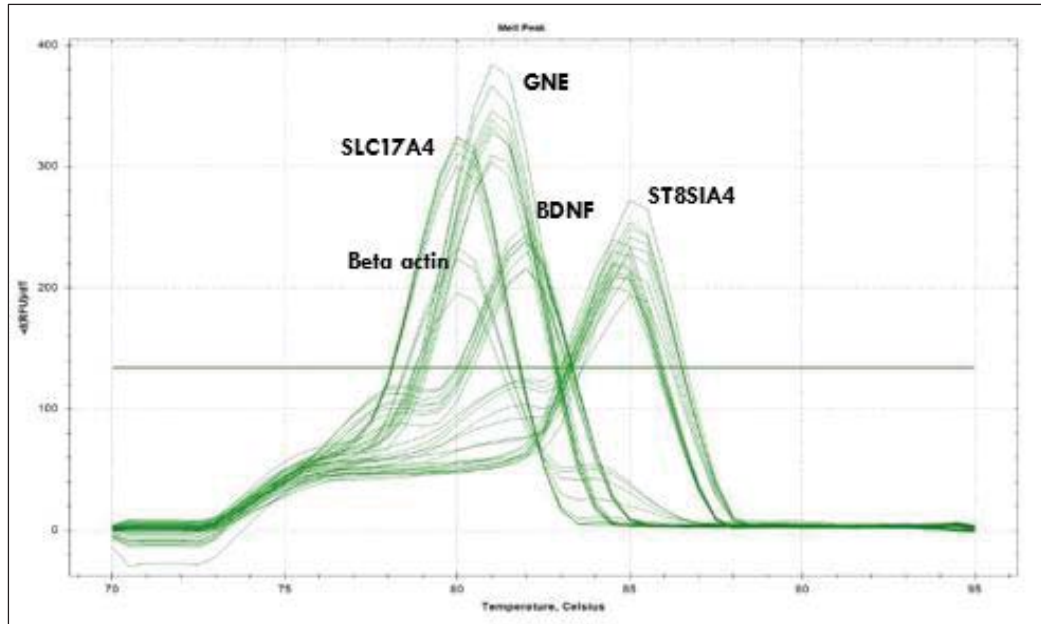
Number	Legend	Base pair
M	DNA Ladder	100bp
1	GAPDH	(176bp)
2	GNE	(188bp)
3	SLC17A5	(180bp)
4	Beta Actin	(186bp)
5	ST8SIAIV	(176bp)
6	BDNF	(183bp)



B: Size confirmation of the primers for the reference genes (1 and 4) and target genes (2, 3, 5 and 6) was identified comparing with the ladder (M).

Appendix – 5

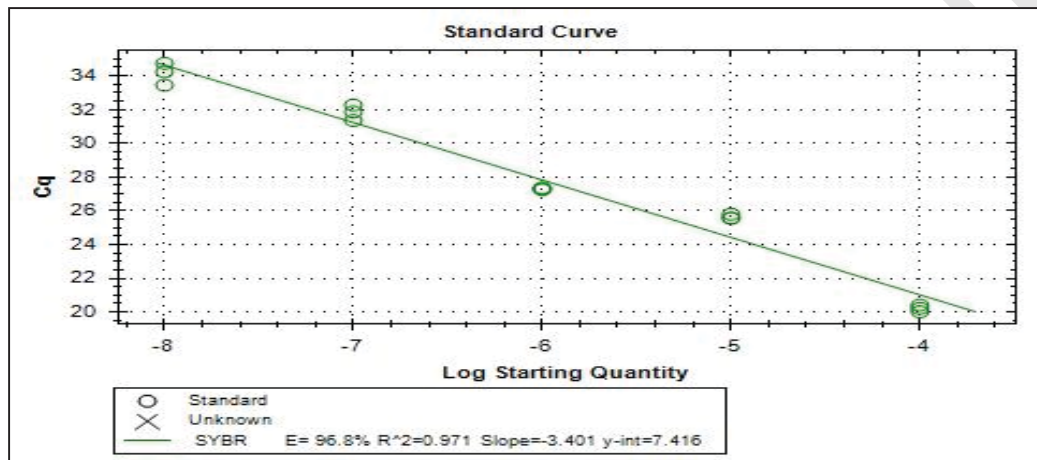
Annealing temperature optimization and the gradient annealing temperature for qPCR.



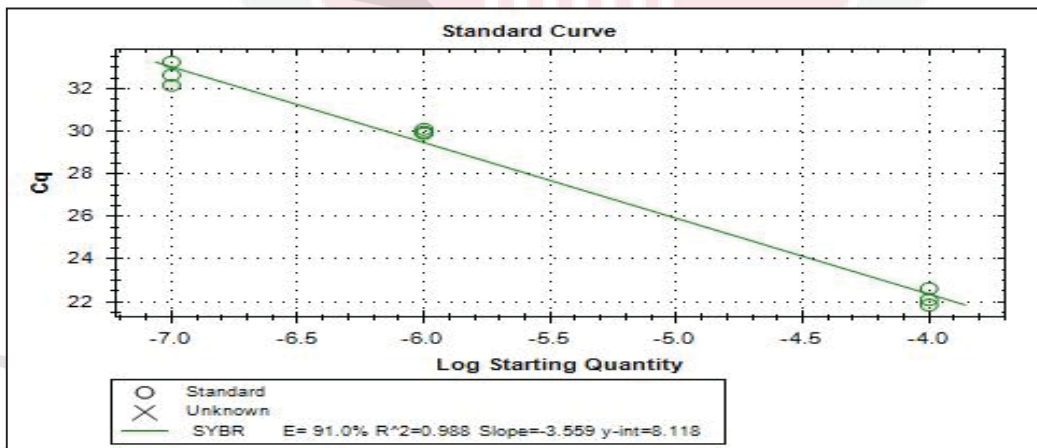
Appendix – 6

Efficiency of each target and reference gene using the serial dilution of cDNA prepared at 5-points tenfold dilution series starting from 100ng/μl to 0.01ng/μl.

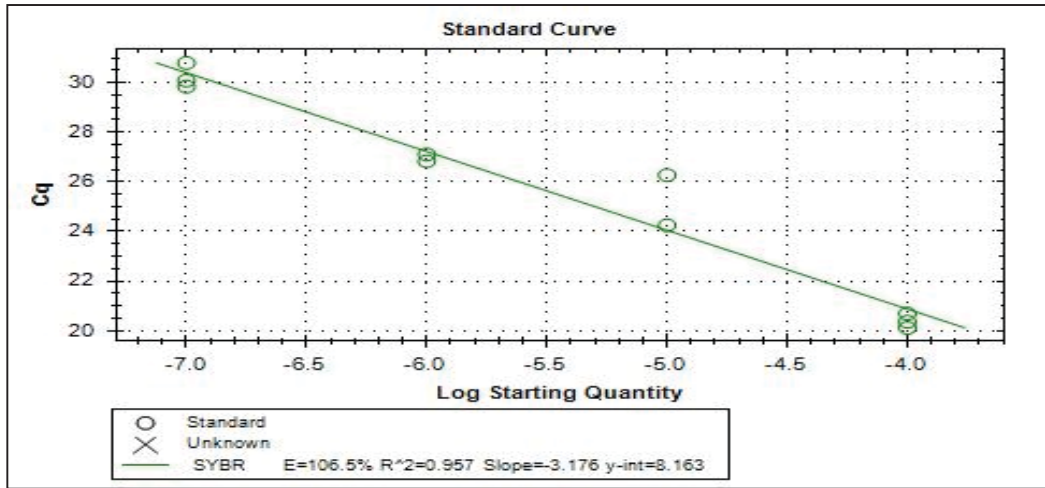
Primer	Efficiency (%) (90% - 110%)	Slope (-3.00 to -3.60)	R2 (>0.98)
Beta Actin	99.8	-3.328	0.996
ST8SIAIV	106.5	-3.176	0.957
GNE	91.0	-3.559	0.988
SLC17A5	100.00	-3.322	0.939
BDNF	96.8	-3.401	0.971



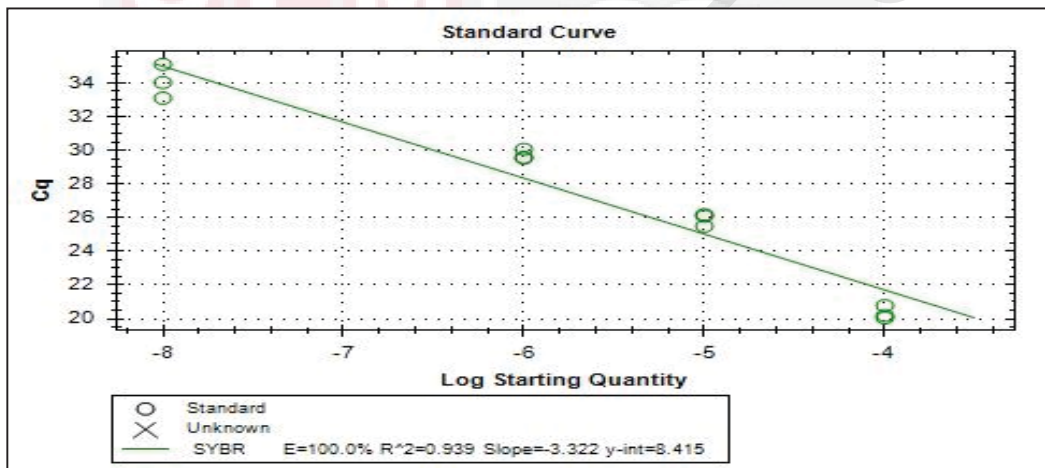
A: Beta Actin



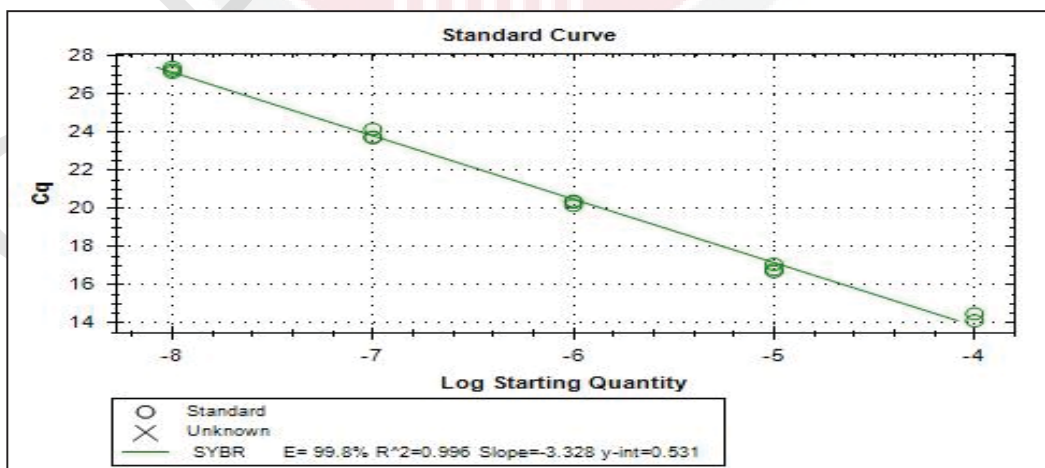
B: UDP-N-acetylglucosamine-2-epimerase/N- acetylmannosamine kinase (GNE)



C: α -2,8-sialyltransferase IV (ST8SiaIV)



D: Sialin (SLC17A5)



E: Brain derived neurotrophic factor (BDNF)

BIODATA OF STUDENT

Obaidullah Mahaq was born in Wardak Province of Afghanistan on 15th April 1989. He lives in Afghanistan with his parents since then. He started his primary studies in Osmania Tangi primary School in 1995 and completed in 2001, while he continued his lower secondary and upper secondary studies in Emam Abo Hanifa High School until year 2007. He was enrolled for his Bachelor degree in Nangahar University of Afghanistan. He was graduated with a Bachelor degree in Doctor of Veterinary Medicine (DVM) in the year 2014. At the same year, he was appointed as an assistant lecturer in Faculty of Veterinary Medicine of Shaikh Zayed University Khost Afghanistan. After four years as assistant lecturer, he was offered a scholarship from Higher Education Development Program (HEDP) to continue his study in Animal Physiology at University Putra Malaysia (UPM), Malaysia. In UPM, he continued his Master of Veterinary Science with research interests in animal physiology specific on the brain cognitive function, neuro-histology and gene expression. During his Master study in UPM, he participated in various seminars, conferences and symposiums. He was completed several postgraduate subjects such as Molecular Physiology, Applied Animal Molecular Biology, Research Methodology and Advanced Research Methods with current cgpa 4.0

LIST OF PUBLICATIONS

Obaidullah Mahaq, Mohd Adha P. Rameli, Marilyn Jaoi Edward, Nursyuhaida Mohd Hanafi, Saleha Abdul Aziz, Hasliza Abu Hassim, Mohd Hezmee Mohd Noor and Hafandi Ahmad. (2020). The effects of dietary edible bird nest supplementation on learning and memory functions of multigenerational mice. *Brain and Behaviors*. 10(11), 1–2 (published).

Obaidullah Mahaq, Mohd Adha P Rameli, Hasliza Abu Hassim, Mohd Hezmee Mohd Noor, Jalila Abu and Hafandi Ahmad (2019). Brain cellular changes of edible bird's nest supplementation on transgenerational mice. *Proceeding of Diversity in Veterinary Disease Investigation and Diagnosis*.

Obaidullah Mahaq, Mohd Adha P Rameli, Hasliza Abu Hassim, Mohd Hezmee Mohd Noor, Jalila Abu and Hafandi Ahmad (2019). Edible bird's nest supplementation improves neurons density and cognitive function of transgenerational mice. *Proceeding of Veterinary Association Malaysia*.

List of Seminars and Conferences

1. 11th Scientific Conference of Malaysian Association of Veterinary Pathology. Perdana Hotel, Kota Bharu Malaysia. 26-28 July 2019. Title of presentation: "Brain cellular changes of edible bird's nest supplementation on transgenerational mice".
2. 31st Veterinary Association Malaysia Congress 2019. Bangi Resort Hotel Selangor Malaysia. 19-20 October 2019. Title of presentation: "Edible bird's nest supplementation improves neurons density and cognitive function of transgenerational mice".