ANTIDEPRESSANT EFFECT OF STINGLESS BEE HONEY IN CHRONIC STRESS MICE MODEL

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ANTIDEPRESSANT EFFECT OF STINGLESS BEE HONEY IN CHRONIC STRESS MICE MODEL

by

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LIST OF SYMBOLS

α	Alpha
β	Beta
°	Degree
δ	Delta
=	Equal
<	Less than
µ	Micro
>	More than
x	Multiple
%	Percentage
±	Plus-minus

LIST OF ABBREVIATIONS

5-HT	5-hyroxytryptamine receptors or serotonin
ACTH	Adrenocorticotropic hormone
ANY-maze	Animal behaviour video tracking software
AVN	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
cm	Centimeter
CNS	Central nervous system
CA	*Cornu ammonis*
CRH	Corticotrophin releasing hormone
CRS	Chronic restrain stress
cAMP	Cyclic adenosine monophosphate
DG	*Dentate gyrus*
DePeX/DPX	Dibutylphthalate polystyrene xylene
DA	Dopamine
EPM	Elevated plus-maze
Km	Enzyme kinetics
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FST	Forced swimming test
GABA	Gamma aminobutyric acid
GIT	Gastrointestinal tract
GR	Glucocorticoid receptor
GluA1	Glutamate receptor subunit type 1
g	Gram
HPLC	High performance liquid chromatography
HED	Human equivalent dose
HMF	Hydroxymethyl-2-furfural
HPA	Hypothalamic-pituitary-adrenal axis

IHC	Immunohistochemistry
IHC standard	International honey commission standard
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IP injection	Intraperitoneal injection
Kg	Kilogram
MDD	Major depressive disorder
MS	Malaysian standard
MHz	Megahertz
µL	Microliter
mg	Milligram
mL	Millilitre
MR	Mineral receptor
MAOI	Monoamine oxidase inhibitors
NGF	Nerve growth factor
NT4	Neurotrophin- type 4 receptor
NT3	Neurotrophin-type 3 receptor
NMDA	N-methyl-D-aspartate
NE	Norepinephrine
NDRI	Norepinephrine-dopamine reuptake inhibitor
NMR	Nuclear magnetic resonance
NAc	Nucleus accumbens
OCD	Obsessive-compulsive disorder
ppm	Parts per million
PPAR-α	Peroxisome proliferator-activated receptor alpha
PI3k	Phosphatidylinositol 3-kinase
PLC-γ	Phospholipase C-gamma
PSD-95	Postsynaptic density 95
PFC	Prefrontal cortex
rpm	Revolutions per minute

RNA	Ribonucleic acid
RER	Rough endoplasmic reticulum
SSRI	Selective serotonin reuptake inhibitor
SNRI	Serotonin-norepinephrine reuptake inhibitor
SEM	Standard error of the mean
SBH	Stingless bee honey
TCA	Tricyclic antidepressant
TrkB	Tropomyosin receptor kinase B
UPLC	Ultra-performance liquid chromatography
VPN	Ventral posterior nucleus
VTA	Ventral tegmental area
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

LIST OF APPENDICES

KESAN ANTIDEPRESI MADU KELULUT DALAM

MODEL TEKANAN KRONIK MENCIT

ABSTRAK

Pendedahan kepada tekanan kronik boleh meningkatkan risiko permasalahan kesihatan termasuklah kebimbangan dan kemurungan. Kajian ini dijalankan bertujuan untuk menilai kesan antidepresi madu kelulut (SBH) dalam model tekanan kronik mencit bagi kajian tingkah laku, fisiologi serta analisis histopatologi. Empat puluh sembilan mencit betina *Swiss* albino dibahagikan kepada tujuh kumpulan dan didedahkan kepada tekanan kronik selama 28 hari. Kromatografi cecair berprestasi ultra (UPLC) digunakan untuk menilai sifat fizikokimia dan profil gula dalam sampel SBH. Penilaian tingkah laku dinilai menggunakan ujian labirin jajaran tinggi (EPM) dan ujian renang paksa (FST). Kortikosteron, serotonin, dopamin, fenilalanin dalam serum dan BDNF dalam tisu otak dinilai menggunakan ujian imunosorben berkaitan enzim (ELISA). Analisis biologi termasuk pewarnaan cresyl violet dan analisis imunohistokimia telah dijalankan. Keputusan menunjukkan sifat fizikokimia danprofil gula dalam sampel SBH memenuhi julat MS2683. Pendedahan tekanan kronik kepada mencit menunjukkan pembentukan kebimbangan dan perubahan tingkah laku seperti kemurungan dalam EPM dan FST dimana SBH memperbaiki tingkah laku sepertikemurungan dalam FST dengan mengurangkan masa pergerakan. Selain itu, kumpulan yang dirawat SBH menunjukkan penurunan kortikosteron dan peningkatan serotonin dan dopamin serta peningkatan fenilalanin dalam sistem aliran. Kumpulan yang dirawat SBH juga mengimbangi ekspresi protein BDNF

dan meningkatkan neuron hipokampal serta meningkatkan ketebalan lapisan di bahagian piramidal dan granular. Selain itu, SBH membalikkan kesan tekanan kronik pada mencit noda imunohisto IL-6 dan IL-1β. Secara keseluruhan, SBH telah menunjukkan potensi untuk bertindak sebagai makanan tambahan bagi pengurusan kemurungan. Penemuan ini menunjukkan keperluan untuk analisis lanjut dalam kajian klinikal.

ANTIDEPRESSANT EFFECT OF STINGLESS BEE HONEY

IN CHRONIC STRESS MICE MODEL

ABSTRACT

Chronic stress exposure can increase the risk of developing health problems including anxiety and depression. The study primarily aimed to evaluate the antidepressant effects of stingless bee honey (SBH) in chronic stress mice model on behavioural, physiological as well as histopathological parameters. Forty-nine *Swiss* albino female mice were divided into seven groups and underwent 28 days of chronic stress paradigm. Ultra-performance liquid chromatography (UPLC) was used to evaluate the physicochemical properties and sugar profiles of SBH samples. The behavioural assessment was assessed using an elevated-plus maze (EPM) and forced swimming test (FST). The corticosterone, serotonin, dopamine, phenylalanine in serum and BDNF in brain tissue were assessed using enzyme-linked immunosorbent assay (ELISA). The biological analysis including cresyl violet staining and immunohistochemistry analysis targeting IL-6 and IL-1β was carried out. The results showed that the physicochemical properties and sugar profiles of SBH samples met the range of Malaysian Standard (MS2683). Chronic stress exposure to the mice elevated anxiety level in EPM and SBH potentially to alleviate the depression-like behaviour in FST by reducing immobility time. Furthermore, groups treated with SBH exhibited a reduction in neurohormonal levels including corticosterone, along with elevated serotonin and dopamine, as well as increased phenylalanine in serum. SBH-treated groups alsoupregulated the BDNF protein expression and improved the

hippocampal neuronal as well as increased the thickness layer of pyramidal and granular regions. Besides, SBH also potentially reversed the effect of chronic stress mice as observed by the IL-6 and IL-1β protein expressions. Overall, SBH has demonstrated promising potential as a dietary supplement for managing depression. These findings highlight the need for further analysis in clinical studies.

CHAPTER 1

INTRODUCTION

This chapter provides an overall understanding of this study, encompassing the research background, rationale, research questions, objectives, and hypotheses.

1.1 Research Background

Depression is the most frequent behaviour-debilitating disease and the most common mental condition among mood disorders affecting about 10% of the adult population (Hasin et al., 2018; Malhi & Mann, 2018; Vos et al., 2016). The symptoms or characteristics of depression patients can be observed as mood swings, insomnia, misery, lack of energy and no pleasure in life as well as attempting suicide (Hawton et al., 2013; Pu et al., 2017; Ser- et al., 2019). Physical or emotional stress can trigger psychopathology in individuals which can lead to major depression (Cirulli et al., 2009). For example, a child who has abusive parents may endure early-life stress daily throughout his childhood. Likewise, a worker who has a heavy workload and unsupportive co-workers may encounter psychological stress every time he/she comes to work. Several mechanisms of depression have been detected and altered including serotonergic, noradrenergic, dopaminergic and glutamatergic systems, elevated inflammation, HPA axis abnormalities, vascular changes, decreased neurogenesis and neuroplasticity (Dean & Keshavan, 2017). Studies have shown that depression is a mood disorder associated with alterations in central nervous system (CNS) structure and function as well as dysfunction in peripheral systems. In addition, clinical evidence reported that depression is associated with the hypothalamic-pituitary-adrenal (HPA) axis, monoamine neurotransmitters and neurotrophins including brain-derived neurotrophic factor (BDNF) which can

dramatically change the behavioural and neurological activity in individuals (Cirulli et al., 2009; Cirulli & Alleva, 2009; Naoi & Maruyama, 2017; Schmidt & Duman, 2007). Hence, models of chronic stress have been designed to mimic human depression in rodents (Abelaira et al., 2013; Jiang et al., 2022; Petković & Chaudhury, 2022).

The brain regions that are usually affected by depression include the hippocampus, amygdala, midbrain and prefrontal cortex (PFC) (Gryglewski et al., 2014; Hare et al., 2017). The area of brain regions is highly sensitive to stressful stimuli and may reduce the number of neurons and the size of synaptic contacts, changes in the expression of synaptic proteins and also reductions in trophic factor expression. These changes are observed in post-mortem analysis of tissue from depressed individuals (Kang et al., 2012; Ota et al., 2014; Rajkowska et al., 2007). Additionally, pro-inflammatory cytokines including IL-6, TNF-α and IL-1β also played a role in neuroinflammation and neurodegeneration in various brain regions (Afridi & Suk, 2021; Wang et al., 2015). According to (Alkadhi, 2013; Hettema et al., 2015; Kendler et al., 1999), stress exposure will lead to depression in human life and prolonged chronic stress in animal models was used as it is a common experimental that can dysregulate hypothalamic-pituitary-adrenal (HPA) axis and produces depression-like physiological as well as behavioural changes.

It is critical to identify an effective therapeutic anti-depressive alternative medicine for controlling everyday stress hormone levels. Monoaminergic antidepressants are currently available and have been prescribed to major depressive disorder (MDD) patients since their fortuitous discovery in the 1950s. Brain monoaminergic activity including serotonin and dopamine can be reduced due to depression, however, this activity can be increased by using antidepressant drugs

(Andrade & Rao, 2010; Schildkraut, 1965). Different mechanisms involve and may increase the availability of brain monoamine including blocking the reuptake of the monoamine from the synapse, inhibiting the intra-neuronal metabolism of the monoamine, or blocking the pre-synaptic inhibitory auto- and hetero-receptors (Hensler, 2003). Therefore, persistent research efforts in findings and improved monoaminergic antidepressants as selective agents including serotonin reuptake inhibitors (SSRIs) along with quality-of-life management have been introduced. However, there are still clinical limitations existing in these compounds (Trivedi et al., 2006). Depressed patients, for instance, must wait weeks or months to benefit from the antidepressants' efficacy and one-third of those treated do not respond. Usually, after taking antidepressants, patients often suffer side effects such as nausea, insomnia, weight gain or loss, decreased libido, drowsiness, fatigue and headaches (Morgan et al., 2011). These drawbacks highlight the need for new antidepressant targets and drugs to treat depression.

Honey is widely known as a natural sweetener across the world. It is widely used among natural products for various uses, some clinically and comprises approximately 200 distinct chemical compounds (Al-Hatamleh et al., 2020; Rao et al., 2016). Previous research studies of honey have confirmed its biological properties, including antioxidant, anti-inflammatory, anti-bacterial, antiviral, anti-ulcer activities and antihyperlipidemic, antidiabetic as well as anticancer properties (Al-Hatamleh et al., 2020; Erejuwa et al., 2010; Kishore et al., 2011; Viuda- Martos et al., 2008). A few studies of honey from *Apis* sp. suggest that honey possesses neuroprotective and nootropic effects. Honey has a memory-enhancing effect and anxiolytic, antinociceptive, anticonvulsant and antidepressant effects as well as has the potential to ameliorate the oxidative status of the brain (Abd Aziz et al., 2019;

Akanmu et al., 2011; Al-Rahbi et al., 2014; Aziz et al., 2014; Chepulis et al., 2009). A recent study also showed that honey from stingless bees improved spatial learning memory in the animal brain (Mustafa et al., 2019). In the earliest study, trehalulose was identified in stingless bee honey which acts as a cariogenic and has high antioxidants and these properties can be beneficial to health in humans (Fletcher et al., 2020; Hamada, 2002).

Honey from the stingless bee is a precious product and it is different from the honey bee. The stingless bees known as *Meliponinae* is the most abundant subtypeof bees on Earth and also the most diverse group of bees found in tropical and subtropical countries (Musa et al., 2018; Thakodee et al., 2018). The composition of amino acids in stingless bee honey can be influenced by the presence of the microorganisms that work in symbiosis with the bees inside the hive (Barbosa et al., 2017). The most abundant composition found in all samples of honey is proline (12.1-762 mg kg^{-1}) and phenylalanine (5.00-1231 mg kg^{-1}) (Biluca et al., 2019). According to (Meyers, 2000), tyrosine is the precursor of norepinephrine, and L-phenylalanine is the direct precursor of tyrosine. Tyrosine and phenylalanine are also precursors of dopamine, another neurotransmitter that may play a role in depression.

Therefore, we conducted a study on chronic stress mice treated with SBH to evaluate the effects on the behavioural study and to investigate the neuronal activity in the brain region as a novel treatment approach for depression. We induced 28 days of restraint stress to female *Swiss* albino mice (*Mus musulus*) and tested its behavioural test after stress followed by post-sacrifice ELISA and histopathological analysis. A previous study showed that SBH has strong capabilities to improve spatial memory performance in mice and also phenylalanine in SBH may have triggered the increase of BDNF genes in mice (Mustafa et al., 2019). In the current

study, the SBH effects on depression in chronic stress mice were evaluated. Furthermore, to our knowledge, studies on SBH and its effects on chronic stress-induced neuro-inflammation, neurogenesis and neuroendocrine are still elusive. Therefore, further studies are needed to explore the significant use of SBH as a treatment or functional food supplement for depression control.

1.2 Rationale of Study

Honey is a natural product and since ancient, it has commonly been used as a traditional treatment. Various studies have been reported on the biological and chemical properties of honey due to their extraordinary antibacterial, anti-inflammatory, anti-cancer, wound healing and also antidepressant. However, to the best of our knowledge, studies on the effects of stingless bee honey targeting chronic stress and depression particularly in physiological effects are still elusive.

Nowadays, depression has become one of the worldwide major diseases and it affects the lifetime of the person. Depression and anxiety are usually comorbid. This study provides brighter insights into potential prevention treatment using stingless bee honey in chronic stress mice model for the antidepressant. Findings from this study also answer the effect of SBH supplementation in regulating the stress associate neurohormonal as a new knowledge in the application of SBH as a safe food supplement in stress management.

Even though there are drugs that can reduce depression symptoms, major drawbacks including nausea, dizziness, loss of appetite and many more are observed. Hence, we conducted a study using stingless bee honey to elucidate the antidepressant effect in the chronic stress mice model as an alternative. This study helps in answering the question of whether the administration of stingless bee honey will affect the behavioural, physiological and neuronal changes in the brain region of the chronic stress mice model.

1.3 Research Question

What are the behavioural, physiological and histopathological changes exhibited by chronic stress mice, and whether the stingless bee honey administration has functional food supplement potential for depression and stress-related disorders affect these behaviours?

1.4 General Objective

To investigate the impact of stingless bee honey on the behavioural, physiological, and histopathological aspects of chronic stress in a mice model and determine its potential as functional food supplement for depression and stress-related disorders.

1.5 Specific Objective

1. To validate the composition of physicochemical and sugar profiles of stinglessbee honey using ultra-performance liquid chromatography (UPLC).
2. To evaluate the effects of stingless bee honey on anxiety and depressive behaviours in the chronic stress mice model using elevated plus-maze (EPM) and forced swimming test (FST).
3. To measure the serum of serotonin, dopamine, phenylalanine and the tissue homogenate of BDNF in chronic stress mice model using ELISA.
4. To evaluate the histopathological changes of hippocampus subregions in chronic stress mice model.
5. To evaluate the expression of pro-inflammatory cytokines; IL-1β and IL-6 in the hippocampus via immunohistochemical analysis.

1.6 Hypothesis

This study hypothesized that:

The UPLC analysis will reveal a distinctive physicochemical and sugar profile in stingless bee honey, characterized by unique compounds and sugar concentrations.

Stingless bee honey treatment will lead to a significant increase in the elevated-plus maze in open arms, indicating less anxiety.

Stingless bee honey treatment will lead to a significant decrease in immobility time in forced swimming test, indicating anti-depressant like effect.

Stingless bee honey treatment will lead to a significant decrease in the serum corticosterone, indicating stress reduction.

Stingless bee honey will lead to a significant increase in the serum serotonin and dopamine, indicating improves mood and behaviour.

Stingless bee honey will lead to a significant increase in the serum phenylalanine, indicating a precursor and potential impact to neurohormones.

Stingless bee honey will lead to a significant increase in tissue homogenate of BDNF, indicating neuronal survival and neuroprotection in the hippocampus.

Stingless bee honey treatment will lead to a significant reduction in the expression of pro-inflammatory cytokines, IL-1β and IL-6, in the hippocampus, indicating an anti-inflammatory effect.

1.7 Significance of the Current Study

Evidence on the role of SBH in treating depression is still elusive. Besides, knowledge is still lacking in terms of the efficacy of SBH in response to physiological, molecular and histopathological alterations caused by chronic stress. Hence, the current study aimed to elucidate the role of SBH as an antidepressant treatment of mice following chronic stress.

By grouping the mice into seven groups; normal, untreated, control (paroxetine 10mg/kg), pre-treatment honey with low dose (200 mg/kg), post treatment honey with low dose (200 mg/kg), pre-treatment honey with high dose (2000 mg/kg) and post treatment honey with high dose (2000 mg/kg), we investigated whether the treatment of SBH is significant on depression in chronic stress mice. We observed the behavioural of mice after stress on day 22 and day 29 using EPM and FST respectively. Besides, to further validate the observation, we performed an investigation on the physiological by using the blood serum and brain tissue homogenate to test on ELISA as well as evaluated the expression of pro-inflammatory cytokines and intact neurons in brain tissue of chronic stress mice.

In this study, *Swiss* albino mice were chosen as study subjects because they are the most commonly used outbred mouse species. A large number of scientists, particularly in biological and biomedical areas, had chosen *Swiss* albino as research subjects. The physiological, biochemical and immunological understandings of this type of mouse have been thoroughly recorded over time due to its wide range of applications (Irena et al., 2016). The data and observations in our current study are highlighted in this section.

On the other hand, the purpose of performing behavioural tests as the experimental approach is because multiple clinical types of research have found that depression and anxiety have a high degree of correlation. A previous study showed that depression and anxiety disorders are approximately 20% to 70% comorbid to each other (Dunner, 2001; Kalin, 2020). The elevated plus-maze is a simple method used to screen the anxiety responses of rodents (Pellow et al., 1985). A forced swimming test (FST) is currently the most widely used tool in depression research, especially as a screening for an antidepressant (Petit-Demouliere et al., 2005). Hence, performing these two tests allowed better interpretation, behaviourally and molecularly of chronic stress effects on anxiety and depression mechanisms of mice in our study.

This research study provides the overall picture of experimental proof of the significance of SBH treatment as an antidepressant in the chronic stress mice model. In the longer term, our findings will aid future research in the discovery of a promising pre-treatment or post treatment with SBH for stress-induced depression.

The overview of this study is explained through a conceptual framework illustrated in Figure 1.1 below:

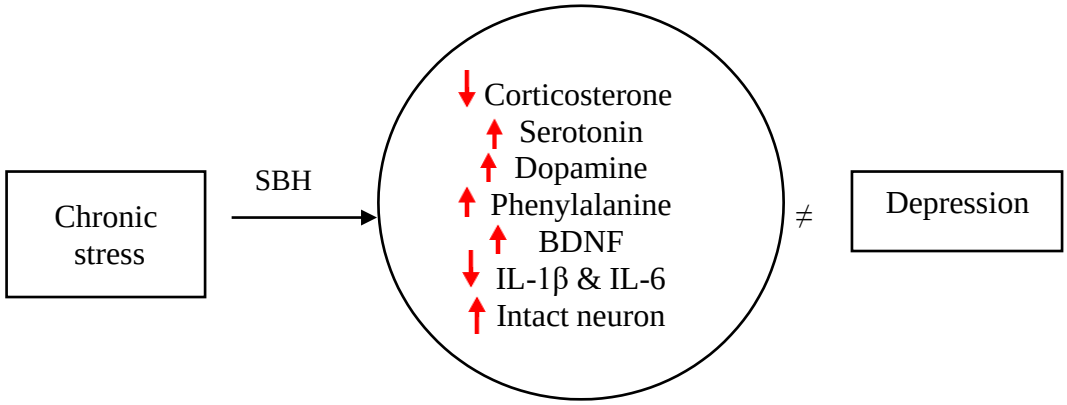

Figure 1.1 Conceptual framework of the current study. The administration of SBH to mice after exposure to chronic stress resulted in a decrease in corticosterone levels, while levels of serotonin, dopamine, phenylalanine, and BDNF increased. Additionally, there was an increase in the number of intact neurons in the hippocampus and a decrease in the levels of pro-inflammatory cytokines. These changes collectively ameliorated depression in the chronically stressed mice.

CHAPTER 2

LITERATURE REVIEW

This chapter covers topics essential for the study and describes relevant literature on anxiety and depression pathways as well as the SBH with therapeutic potentials for the prevention of stress-induced depression.

2.1 Anxiety

Anxiety disorders are characterized by a diverse array of symptoms, encompassing persistent and overwhelming worry, apprehension related to social interaction and performance, panic attacks, anticipatory anxiety and avoidance behaviours (Szuhany & Simon, 2022). There are also among the most prevalent psychiatric conditions, characterized by high rates of comorbidity, particularly generalized anxiety disorders or panic disorders, and depressive disorders. Research indicates that over 70% of individuals diagnosed with depressive disorders also exhibit symptoms of anxiety with 40% to 70% simultaneously meeting criteria for at least one type of anxiety disorder (Lamers et al., 2011; Wu et al., 2013). This comorbidity possesses challenges in devising effective treatment approaches. Behaviour tests in rodents are commonly used to study anxiety and the effectiveness of anxiolytic drugs. These tests are grouped into categories based on conditioned fear, acute fear responses or approach-avoidance conflicts. The Elevated Plus-Maze (EPM) and Open Field (OF) tests represent common animal models utilized for evaluating anxiety-like behavior and exploratory activity (Knight et al., 2021). In the elevated plus-maze (EPM) test, the animals were individually introduced into the center of a maze, positioned with their heads directed towards one of the open arms of the maze (Kurilova et al., 2023). Meanwhile, in the OF test, the animals are placed

within a brightly lit arena bounded by walls, and their behaviour in both tests are carefully observed and recorded. The test was initially developed to assess rodent emotionality by measuring urination and defecation as indicators of fearfulness. Moreover, the distance travelled during the open-field test has been further employed as an indicator of locomotor activity, facilitating the assessment on the effects of substances and the expressed of anxiety by the animals (Belovicova et al., 2017). Additionally, thigmotaxis in the open-field test, referring to the tendency of rodents to liner near to the close walls while avoiding the center or open area of the arena which is interpreted as a sign of anxiety (Zhang et al., 2023).

Therefore, anxiolytic drugs were introduced and are still being used nowadays. However, the impacts of using anxiolytic drugs as a treatment for depression need to be strongly reduced because of the power of side effects on fragile patients (Curran et al., 2015; Farach et al., 2012). In consequence, the science community believes that a proper diet interpretation and if obligatory, targeted food supplementation can enhance the efficacy of therapies (Pandey et al., 2010).

2.2 Depression

Depression is a mood disorder that consists of a various collection of phenomenological features including lowered mood, loss of interest and lack of sleep, sadness, cognitive functions, anhedonia, attentional deficits, decreased energy, eating disturbances and suicide attempts (Frank, 1991; Layer, & Khan, 2012; Ser- et al., 2019). The World Health Organization predicts that major depression affects 10% of the general population and will be the second leading cause of global disability burden by 2030 (Sandmire et al., 1976). Depression usually occurs in both gender, male and female, however, female is twice higher and more likely to suffer from depression than male (Van de Velde et al., 2010). It can affect both adolescents and adults, but it is more common in the elderly (Malhi & Mann, 2018; Sjöberg et al., 2017).

The human body and brain manifested via stress, traumatic events, chronic pain and childhood difficulties are examples of the environmental causes of depression that can happen to anyone during their daily lives (Park et al., 2019). Researchers have recognised that these experiences encountered in daily life affect mental health (Layer, & Khan, 2012). In addition, depression and anxiety are highly comorbid, occurring either concurrently or sequentially (Spijker et al., 2020). These examples are thought of as sociological and psychosocial factors which are a combination of events that happen in a community and function as the workings of the human mind. The events are followed by thoughts, emotions, behaviours and prior experiences in lives (Layer, & Khan, 2012).

The use of animal models commonly in rodents can help researchers to have a better understanding of the etiology and pathogenesis of depression because

of their parallels to humans in terms of anatomy and physiology (Rydell-Törmänen & Johnson, 2019). Similarly, rats, mice and humans each contain about 30,000 genes, with 95% shared by all three species (Genome & Project, 2004; Human & Sequencing, 2001). Moreover, choosing the appropriate animal models easily can help in the search for effective treatment. In this study, we used *Swiss* Albino mice *Mus Musculus* as it is widely used in many research due to its small size, short life span, and ease of handling and maintenance (Zafar et al., 2018). It is also chosen as chronic depression mouse models have been established using various induction methods.

There are a few ways to induce depression in animals, the most widely used are environmentally induced, pharmacological models and genetically based models. The environmentally induced models of depression include early-life stress such as prenatal stress, maternal separation, pre-weaning social isolation stress and adulthood stress such as social isolation, learned helplessness, chronic mild stress (CMS), repeated restraint stress (CRS) and chronic social defeat stress. Meanwhile, for pharmacological models of depression including reserpine and corticosterone.

Genetic models usually use different strains and species of rodents such as the *Wistar Kyoto* (WKY) model, genetically-selected Flinders Sensitive Line (FSL) rat model, model of selective breeding for depressive and manic-like behaviour in mice and the Fawn-Hooded (FH/Wjd) rat (M. Becker et al., 2021).

These models appear to affect a variety of brain neurotransmitters and several molecular alterations in depressive models including dysregulation of some neurotransmitters such as monoamines, gamma amino butyric acid (GABA), glutamate, corticotrophin-releasing hormone (CRH) and arginine vasopressin (VPN)

and brain-derived neurotrophic factor (BDNF), thus, proving that depression is more than a monoamine neurotransmitter imbalance (Belmaker & Agam, 2008; Elizalde et al., 2010; Grønli et al., 2007; Hashimoto et al., 2004; Holsboer & Ising, 2010).

2.3 Modelling of Depression

Stress is widely acknowledged as the primary environmental cause generating psychopathologies, towards anxiety and depression (Hare et al., 2017; Lupien et al., 2018). The underlying molecular changes and the causal association between genetic or environmental alterations and depression can be investigated using animal models, providing a deeper overview of the pathophysiology of depression (Yan et al., 2010).

The first model for evaluating antidepressant effects was in rats in the 1960s-1970s and a few years later was introduced in mice. According to (Belzung, 1999; Borsini et al., 2002), mice and humans share more than 90% of their genes and have been extensively studied at the behavioural, molecular or cellular levels. Hence, animal models seem to be a useful tool in biomedical sciences and this is recognized as the evidenced increase in the number of active laboratories working in the field (Grønli et al., 2007; Strekalova et al., 2006; Warner-Schmidt & Duman, 2006). Nevertheless, it has been challenging to evaluate and analyse as depression is a complex disorder with many different symptoms and uncertain biological background in rodent models. Furthermore, a few factors should be taken into consideration such as the gender of animals, responses to stress, rhythmicity changes in drug effects, pharmacokinetics and possible biomarkers related to depression (Borsini, 2012; Nestler et al., 1968).

There are a few models of depression were introduced which show the effect on mouse behaviours, and the mouse models have been designed to brush up the understanding of the pathophysiology and treatment of depressive symptoms. Table 2.1 represented the types of depression models in rodents that are commonly used.

Table 2.1 Models of depression as reported by (Becker et al., 2021).

	Depression models	
Environmentally induced models of depression	1. Early life stress	• Prenatal stress • Maternal separation • Post-weaning social isolation separation
	2. Adulthood stress	• Social isolation • Learned helplessness • Chronic mild stress • Repeated restraint stress • Chronic social defeat stress • Social instability stress
Lesions of the brain that may result in depression		• Olfactory bulbectomy (OBX)
Pharmacological models		• Reserpine-induced depression model • Corticosterone model of depression
Genetic models		• Wistar Kyoto (WKY) model • Genetically-selected Flinders Sensitive Line (FSL) rat model • Model of selective breeding for depressive-like (submissive) and manic-like (dominant) behaviour in mice • The Fawn-Hooded (FH/Wjd) rat • Genetically manipulated models of depression

In addition, some methods can be used for representing depression in mice which resembles human depression such as, physical and psychosocial stress that are exposed to a variety of chronic unpredictable stressors including restraint stress, social defeat stress, tail pinch in the restrainer, cold water swim, social isolation, mild foot shock, food restriction and others (Haraux, 1984; Katz et al., 1981; Paul Willner, 1997). Furthermore, it has been demonstrated that exposure to stressful episodes may affect the response to subsequent stressors, probably because not all the systems affected by stress are recovered during remission, leaving 'scars' of vulnerability that may assist the pathology's recurrence (Alves et al., 2017).

Acute stress is an important defence mechanism that prepares an individual, both physically and mentally to deal with the broad range of stressful situations that are encountered on a regular basis. Chronic stress, on the other hand, triggers several deleterious processes that jeopardise healthy living and cause a slew of complications (E. Ron De Kloet et al., 2005; McEwen, 2000). Therefore, chronic stress is considered a more precise model of depression as it involves more natural stressors and has higher face validity than other animal models of depression (Paul Willner, 2005).

In this present study, we used environmentally induced models; chronic restraint stress and social isolation. Restraint of rodents is an experimental procedure designed for studying psychogenic stress (Glavin et al., 1994; Paré & Glavin, 1986). In this model of chronic predictable stressor, animals are restrained in a narrow-restrained tube with a nose-hole for ventilation, and unable to move for 2 to 8 hours daily for 14 to 21 days followed by behavioural assessment (Becker et al., 2021; Wang et al., 2017). The previous study, showed no significant changes in behaviour compared to controls following 1 to 7 days of restraint, however when reached day

14 or 21 the changes can be observed (Wang et al., 2017). However, a previous study suggested that 28 days of chronic restraint stress was ample to induce anxiety and depressive-like behaviours as well as altered neurochemical and histopathological changes in the rodents (Kwatra et al., 2020; Zhu et al., 2014). In human daily life, this kind of stress can be continuous and predictable every day which may include daily repetition of a stressful job, social, and financial problems, familial stresses as well as constantly the previous day's workload (Wang et al., 2017). According to (Golub et al., 2004; Hudson & Chilcot, 2015), psychological distress does not necessarily involve invasive physical treatment or tissue damage to the body. Meanwhile, other studies showed the results are varied including the damage or atrophy of the area of the hippocampus (CA3), elevated corticosteroid levels along with rising aggressive behaviours, depressed behaviours as well as apoptotic cell death (Conrad et al., 1999; Wood et al., 2003; Zhang et al., 2014). This chronic stressor is the most suggested model as the animals show genetic and protein changes that are established in depression. In addition to that, chronic restraint stress was developed to mimic the stress in this modern life with good validity to explore the effect of chronic psycho-emotional stress (Woo et al., 2018; Xu et al., 2017). Its relative simplicity and straightforward workflow, make it a more practical, affordable and reliable rodent model of chronic stress, thus the model frequently employed to simulate rodent models of depression (Antoniuk etal., 2018).

Apart from that, social isolation is one of the most widely studied animal models for anxiety, depression and substance use disorders (SUDs) in adolescence and adulthood (Mccormick & Green, 2012; Walker et al., 2019). Previous studies, represented these stressors exhibit depressive-like behaviours, high locomotion activity, decreased time spent in the center in the open field test, increased anxiety-

like behaviours tested by the elevated plus-maze and reduced social behaviours which have been previously documented from the past studies (Amiri et al., 2015; Castillo- Gómez et al., 2017; Koike et al., 2009; Preez et al., 2020; Weiss et al., 2004). Both female and male single-housed animals appear the social behaviour, however, female animals demonstrated an unpredicted avoidance behaviour. From past studies, male and female animals have shown that they interact socially in different ways, nevertheless, previous studies work on male animals when testing social behaviours instead of female animals (Clipperton Allen et al., 2010; Koike et al., 2009; Liu et al., 2016; Van Den Berg et al., 2015; Walf & Frye, 2008). According to (Medendorp et al., 2018), the female mice were housed in the same room and the estrous cycle was synchronised, this may affect their social behaviour. The findings also showed that the area related to social behaviour showed a reduction of spine density in the medial prefrontal cortex in female mice compared to male mice (Hajszan et al., 2007). Moreover, this paradigm also expressed lower corticosteroid plasma levels and altered serotonergic and adrenergic systems along a neuroaxis (Brenes et al., 2008; Harvey et al., 2019; Lopez & Laber, 2014; Walker et al., 2019). Additionally, social isolation has a long-term effect on the animals that represented in the previous study which compared the isolated and non-isolated rodents, hence the result showedthe isolated rodents were in higher states of anxiety and became more aggressive to environmental changes (Domeney & Feldon, 1998; Einon & Morgan, 1977; Gentsch & Lichtsteiner, 1983).

Moreover, depression does not come alone and it is often comorbid with anxiety, as the previous study represented in clinical patients that both disorders were treated with antidepressants (Borsini et al., 2002). So, it is relevant to evaluate the anxiety-like behaviour before the depression test is conducted in the mouse model.

Table 2.2 below shows several experimental stressors that have been conducted on rodents to induce anxiety and depression.

Table 2.2 Experimental stressors described by (Nollet et al., 2013).

Stressor	**Description**
Social stress	Each mouse is placed individually in an empty cage
Cage change	Each mouse is placed in the empty cage of another individual and then returned to its original cage
Sawdust change	The replacement of sawdust with soiled sawdust
Without sawdust	The sawdust is removed
Damp sawdust	The water and sawdust are placed in the cage
Bath	The sawdust is removed and replaced by water
Cage tilting	Tilt the cage backward (45 degrees)
Faeces	Rodent's faeces are deposited in each cage
Restraint stress	The mice are kept in closed and ventilated tubes
Predator sounds	Broadcast a recording of cats of prey
Cycle disturbances	Change the light/dark cycle

2.4 Behavioural test

Behaviour can be observed from the result of the integration of all processes occurring in underlying organ systems in interaction with the external social and physical environment. Thus, animal models can aid in the development and prediction of therapeutic responses to pharmacological agents as well as the study mechanisms underlying specific behaviours and their pathophysiology (Bourin, 2015).

However, when experimenting with animal models, it is essential to take note that animals are not human beings so the physiological and biochemical processesare different. In terms of behavioural presentation, it is commonly agreed that an ideal animal model must meet three criteria: face validity, construct validity and predictive validity (Willner, 1984). Face validity is considered when animals mimic the disease phenotype in the same way that depressed humans do, meanwhile for construct validity, is when the pathophysiological pathways that induce disease in humans are similar in animals. The predictive validity is where animals react to an antidepressant that is also effective in humans (McKinney, 1984; Nestler & Hyman, 2010). Nevertheless, many animal models of depression fail to meet all these criteria.

In addition, when designing and reporting behavioural research in rodents, itis also critical to examine many factors that can influence behaviour. These factors can be divided into three categories: animals, housing conditions and experiment conditions. For example, in animal categories, the strain, species, sex and estrous cycle were important to consider. On the contrary, for housing conditions, the identification of animals, environmental enrichment, bedding, lighting and noise should be noted to avoid future issues. Meanwhile, for experiment conditions, the

place of testing, lighting, time of treatment and drug delivery, as well as handling, should be considered and analysed (Michelle et al., 2021). We may be able to reduce variability and improve reproducibility by carefully regulating and publicly reporting methodological factors, thereby enhancing preclinical investigations.

Tests for anxiety include open-field test, novelty suppressed feeding, elevated plus-maze, light/dark box and stress-induced hyperthermia whereas, for depression-like behaviours, the test includes such as forced swim test, tail suspension test and a sucrose preference test. These tests are commonly used and they are an important methodological tool in pre-clinical and behavioural toxicology studies (Belovicova etal., 2017).

In the current study, we used an elevated plus-maze and forced swim test for behavioural study. These tests serve as valuable tools for investigating the neurobiological basis of anxiety and depression, as well as for evaluating the efficacy of potential anxiolytic and antidepressant compounds.