Preventing depression through multivitamins: A comparative behavioral analysis in rodent model

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LIST OF ABBREVIATIONS

CUMS   Chronic Unpredictable Mild Stress

SSRIs   Selective Serotonin Reuptake Inhibitors

SNRIs   Selective Norepinephrine Reuptake Inhibitors

TCAs   Tricyclic Antidepressants

MDD   Major Depressive Disorder

5-HT   Serotonin

NE   Norepinephrine

DA   Dopamine

BDNF   Brain-derived neurotrophic factor

CRF   Corticotropin Releasing Factor

HPA   Hypothalamic-Pituitary-Adrenal

MAO   Monoamine Oxidase

CBT   Cognitive Behavioral Therapy

NMDA   N-Methyl-D-Aspartate

SAMe   S-Adenosyl Methionine

SAH   S-Adenosyl Homocysteine

TRH   Thyrotropin Releasing Hormone

TSH   Thyroid Stimulating Hormone

FST   Forced Swim Test

OFT   Open Field Test

ABSTRACT

Chronic daily life stress is among the leading causes of depression, a predominantly prevalent psychological disorder affecting millions of people globally. To counter depression, numerous known and commercially available antidepressant medications, such as SSRIs, SNRIs, and TCAs, exist. However, all these medications present a wide range of unpleasant side effects. Apart from these conventional antidepressants as treatment option, various vitamins are associated with depression management such as folates. Folate is a vital component of brain health which plays a role in various pathways, particularly in neurotransmitter synthesis, such as serotonin and dopamine. The purpose of this investigation is to examine the prospect of folate as a prevention strategy against depression. Components such as L-Methyl folate, Vitamin B2, and Vitamin D3 were used in combination on CUMS model of *Sprague dawley* rats. Animals used in this study, both male and female, were categorized into three distinct groups; the control group was given neither stress nor vitamins, the second group was subjected to stressors and subcutaneous saline injections, and the third group was subjected to stressors and subcutaneous vitamins injections daily for 21 days. Anhedonic behavior, locomotor activity, and despair-like behavior were analyzed by sucrose preference test, open field test, and forced swimming test to assess the preventive effect of folate in combination with these vitamins on the behavior of animals exposed to stress. The data revealed that the combination of L-Methyl folate with vitamins significantly prevented anhedonia, as evidenced by a sucrose preference of 80% ($p < 0.0001$). Moreover, it effectively preserved locomotor abilities, with rats covering a total distance of 80 meters in apparatus of OFT, in comparison to the 50% noted in depressed rats ($p < 0.03$). Additionally, the combination therapy prevented despair-like behavior, as shown by a decrease in immobility percentage to 50% compared to the 70% observed in depressed group ($p < 0.01$). These findings indicate that folate in combination with vitamin B2 and D3 can serve as a potential preventative candidate for stress-related mental health disorders.

CHAPTER 1: INTRODUCTION

1.1. Stress

The research literature has proven a strong correlation between the risk of depression and stressful life events(Gómez Maquet et al., 2020; Kendler et al., 1999; Shapero et al., 2014). The idea that stress can start cognitive and potentially biological processes that raise risk for the condition is central to the majority of modern theories of depression (Brush, n.d.; LeMoult, 2020a). In prefrontal cortex and hippocampus, reduction in BDNF expression is commonly due to chronic stress(X. Hu et al., 2023; Lipska et al., n.d.); on the other hand, rise in BDNF expression in specific regions of brain i.e. the amygdala and nucleus accumbens (NAc) is also due to chronic stress in a way thought to be associated with maladaptive outcomes (Lakshminarasimhan & Chattarji, 2012).

1.2. Depression

According to World Health Organization (WHO), depression can be defined as a mood disorder having symptoms such as melancholy, loss of interest, anhedonia (which is defined as loss of pleasure), loss of appetite, guilt feelings, very low self-worth or low self-esteem, disturbed sleep pattern, fatigue, and difficulty in concentrating (WHO, 2023). A person experiencing depression may report feeling powerless and hopeless to varied degrees, having trouble concentrating, sleeping through the night, losing their appetite, losing interest in activities they used to enjoy, feeling of extreme sadness and guilt, and possibly even having recurrent thoughts of suicide (Suma P et al., 2023). One's ability to

operate cab be severely impaired or damaged by depression. Depression can also become persistent or recurrent.

In general, depression is a widespread mental illness that impacts all peoples regardless of their ages, genders, races, and ethnicities. It has adverse effects on relationships(Santini et al., 2015), physical wellbeing, and cognitive function, and it can lead to disability(Brenes et al., 2008) and an increase in the burden of disease. The word "depression" is used to refer to a broad range of disorders, from moderate, transitory low mood states that majority of peoples experience at various points in their lives to serious mental disorders. According to popular belief, depression is an illness that typically comes and goes and is probably to manifest itself or occur during particular phases of an individual's life cycle.

Major depressive disorder (MDD) and dysthymic disorder are two forms of depression(Bockting & Andersson, 2012) that are considered to be influenced by hereditary and biological factors, whereas major episodes of depression and mild form of depression might be reactions to adverse life events. More specifically, major depressive disorder which is the most researched form of depression, is defined as one or more major episodes of depression, accompanied by four (or at least three, if both melancholy and loss of interest are present) accompanying manifestation of depression for at least two weeks. Other symptoms that may manifest in different tiers of intensity include Changes in appetite or weight, disturbances in sleep patterns, variations in psychomotor activity, low energy levels, feelings of guilt or worthlessness, difficulty concentrating, and trouble thinking clearly or decisions making, or recurring thoughts of suicide or plans, or attempts(Otte et al., 2016).

1.3. The scope of depression prevalence

Over 350 million individuals around the world, from all age groups, are affected by depression (Ledford, 2014). Across 17 countries in the World Mental Health Survey, one out of every seventeen participants reported experiencing a minimum of one depressive episode in the past year. Another study assessed the worldwide point prevalence of Major Depressive Disorder to be 4.7% (ranging from 4.4% to 5.0%), with a pooled annual incidence rate of 3.0% (ranging from 2.4% to 3.8%). Depression often strikes women more commonly than males, with post-partum depression affecting 10–20% of women in childbearing age. Depression frequently begins in adolescence or early adulthood(Mundy et al., 2021).

1.4. Effects of depression

Depression has a substantial effect on an individual's physical condition and overall quality of life, contingent upon the degree of the illness. It has detrimental effects on bodily processes, making it difficult for those who are affected to carry out their social and familial responsibilities. Regrettably, 80% or more of depressed individuals experience some kind of impairment in their day-to-day functioning(*APA PsycNet Buy Page*, n.d.).

The impacts of the depression, both direct and indirect, on the economy are profound and worrisome in their whole. For example, depression greatly lowers productivity in the workplace. People who are sad miss over 6 hours of productive work each week on average, and depression is responsible for nearly 20% of all missed workdays. Additionally, the likelihood of unemployment is seven times higher for depressed

individuals, which significantly lowers the labour force and pool of human capital in any given economy(Katon, 2009). Depression's entire cost to an economy is estimated to be between $30.1 and $51.5 billion a year in missed work performance, making it evident how much depression costs an economy's overall output of labor and productivity.

1.5. Folate association with depression

Growing data suggests that depression and low folate levels are related. Patients with low folate levels who are depressed are more likely to relapse, have poorer cognitive function, and are less likely to respond to antidepressant medication. The monoamine neurotransmitter theory explains the primary mechanism of depression. Clinical data from the 1950s that reserpine, which depletes central reserves of monoamines, can cause depression in some patients may provide support to this theory. The synthesis of neurotransmitters in the central brain system, such as norepinephrine (NE), dopamine (DA), and serotonin (5-HT), is closely associated with folate. Moreover, concentrations of brain-derived neurotrophic factor (BDNF)are a helpful indicator of clinical response or the amelioration of depression symptoms [14]. In a model of premenopausal depression, lower levels of serum β-endorphin (β-EP) were noted. Clinical investigations have shown a favorable correlation between interleukin-6 (IL-6) levels and depression. All of these markers have to do with the production and application of monoamine neurotransmitters(Bender et al., 2017a).

1.6. AIM:

The aim of this investigation is to inquire the potential of folate as a prevention strategy against depression. Components such as L-Methyl folate, Vitamin B2, and Vitamin D3 were used in combination on CUMS model of *Sprague dawley* rats. Behavioural assessments, including the Sucrose Preference Test for model validation, Open Field Test to check locomotor abilities and Forced Swimming Test to assess despair like behaviour, were employed to evaluate the potential protective impact of folate on rodents exposed to stress.

1.7. Objectives:

The primary objectives of this study are as follows

1. To investigate and assess the depressive like behavior in an animal model of CUMS.
2. To investigate and assess the preventative effect of multivitamins on depressive like behaviors in an animal model of CUMS.

CHAPTER 2: LITERATURE REVIEW

2.1. Depression mechanism known so far.

2.1.1. *The hypothesis of biogenic amines (Monoamine hypothesis)*

The brain houses numerous serotonergic, dopaminergic, and noradrenergic neurons. noradrenergic neurons, which originate in the brainstem, extend their projections throughout nearly every region of the brain. Norepinephrine (NE), released by these neurons, affects behavior, attention, prefrontal cortex functions, and the processing of working memory. The formation of emotionally charged memories is another function of NE. Serotonin constitutes the most extensive integrated neurotransmitter system in the brain, reaching all areas. In contrast, dopamine (DA) affects working memory, attention, and reward and motivation functions(Karabin et al., 2023).

Monoaminergic systems significantly impact various behavioral symptoms of depression, including low mood, diminished alertness, lack of motivation, fatigue, and changes in psychomotor activity, either increased or decreased. The behavioral and somatic changes associated with depression, such as those related to appetite, sleep, sex, pain response, body temperature, and circadian rhythm, have been linked to changes in cerebral 5-HT levels. Post-mortem studies have consistently shown that the brains of depressed patients exhibit lower levels of 5-HT than those of non-depressed patients. Similar to the way, transmission of dopamine improve the cognitive outcomes such as motivation and decision-making, aberrant dopaminergic activity has also been connected to decreased motivation, focus, and aggression. Additionally, A diverse array of depression symptoms, encompassing

disturbances in sexual function, appetite regulation, aggression, concentration, interest, and motivation, are modulated by reduced NE levels in tandem with 5HT and DA(Elhwuegi, 2004).

Evidence exists to substantiate the contribution of distinct neurotransmitters in both the initiation and manifestation of major depression clinically. The essential functions of the brain heavily relies on the presence and functioning of various neurotransmitters at the synaptic junctions of its numerous neurons. The "monoamine hypothesis" postulates that decreased neurotransmission and worse cognitive function may result from lower levels of these three primary monoamine neurotransmitters (NE, 5HT and DA) and may cause depression. Anomalies in neurotransmitter receptor function and decreased activities of protein transporters might potentially contribute to the observed functional deficit of monoamines in depression, as suggested by evidence(Perez-Caballero et al., 2019).

2.1.1.1.   *Functional monoamine deficiency.*

As per the hypothesis of monoamines, the degradative activity of monoamine oxidases within the synaptic cleft is what causes the functional shortage of these neurotransmitters.

Experimental evidence showing elevated enzyme activity of monoamine oxidase in depressed people supports this. The ongoing activity of these enzyme systems leads to a substantial decrease in the levels of biogenic amines, consequently leading to the diminished neurotransmission evident in depression. To replenish the depleted levels of monoamine neurotransmitters, antidepressant drugs known as monoamine oxidase inhibitors are utilized in the treatment of depressive disorders. This theory serves as the foundation for this practice(Olayinka et al., 2023).

There are further explanations for neurotransmitter deficiencies. For example, the stress response brought on by depression may cause certain neurotransmitters, such as glutamate, NE, and histamine, to interact in a way that reduces the production of the substrate, 5HT. New research indicates that this effect may be reversed and that 5HT levels may be restored by employing a multimodal treatment strategy that targets multiple neurotransmitters. If this is the case, then decreased depressive symptoms ought to coincide with elevated levels of 5HT (as well as DA and NE) in the brains of depressed individuals. Monoamine deficiency could be a contributing element in the pathophysiology of depressive disorder, as indicated by the absence of improvement observed in some studies even after neurotransmitter levels were enhanced(Hersey et al., 2022). Conversely, other studies have demonstrated that elevated neurotransmitter levels, resulting from antidepressant therapy, correlate with a reduction in depressive symptoms.

2.1.2. *Diminished activity of transport proteins*

Transport proteins are essential for nerve-nerve and monoaminergic transmissions, as they are for the majority of human body system cell-to-cell communication. To ensure that neurotransmitters are available for continued neurotransmission, transport proteins that are available in the brain improve or help pre-synaptic reabsorption of neurotransmitters. Transport proteins contribute to a reduction in neurotransmitter availability in the synaptic cleft by promoting neurotransmitter re-uptake, which lessens the amount of neurotransmitter breakdown by monoamine oxidase enzymes. Therefore, lower quantities or decreased activity of transport proteins might contribute to the reduced levels of monoamine neurotransmitters seen in depression. Notably, it has been observed that

reduced protein transporter function occurs in depression, corroborating their proposed involvement in the onset of depression(Owens & Nemeroff, 1998).

2.1.3. Abnormalities of receptor functions

Changes in receptor functioning may also result in abnormal neurotransmitter functionality and depression. Such anomalies may arise from altered downstream signal transduction cascades leading to aberrant or inefficient transmission, or from poor neurotransmitter-receptor coupling (often caused by changes such as alterations in receptor affinity to neurotransmitters or reductions in receptor numbers may occur).

Several biomolecular studies have shown that depressed people exhibit these anomalies in receptor function. For example, research has shown that individuals with depression exhibit changes in both the quantity and sensitivity of receptors associated with serotonin (5-HT1 and 5-HT2) present in the brain, along with increase in sensitivity at pre-synapse of α2-adrenoceptors, that influence norepinephrine production in the brain. These findings suggest new possibilities for treatment. Additionally, studies which has used cells of periphery as models and brain tissue derived from post mortem have revealed changes in G-proteins and protein kinases—crucial elements of signal transduction pathways—throughout different stages of the cyclic adenosine monophosphate (cAMP) pathway(Wang et al., 2021).

2.1.4. Genetic factors

Strong proofs for a genetic component to depression progression have been extracted from the family, twin, and children studies of adoption as well as epidemiological data.

Heritability seems to be notably higher in women compared to men, which aligns with the increased frequency and prevalence rate of most types of depression observed in females (Kendall et al., 2021).

The emergence of depression has been connected to numerous genetic factors, although only a small number of genetic variants are significantly correlated with major depressive disorder (MDD). Genes associated with depression include the dopamine receptor gene (DRD4)(Ptáček et al., n.d.), serotonin transporter protein (SLC6A4), dopamine transporter protein (SLC6A3), apolipoprotein E (APOE2 and APOE4), methylenetetrahydrofolate reductase (MTHFR 677T), and guanine nucleotide-binding protein (GND3).

Further information regarding precise correlations between above mentioned genes and depression can be found in studies which are relevant to function of above mentioned "susceptibility genes of MDD" contributing in the onset of depression. For instance, there is a strong correlation between depression and some of the isoforms of apolipoprotein E (APOE ε2 and ε4). Of these, APOE ε4 has been linked to the onset of depression because it significantly correlates with the physical structure of the brain being diminished in depressed people and their cognitive function being reduced. Conversely, APOE A2 is thought to be a gene that protects against MDD. Nevertheless, some research has not been able to establish a meaningful relationship between depression and APOE(Feng et al., 2015).

In a similar vein, it has been demonstrated that postmortem samples of depressed persons and those with current MDD express higher levels of the dopamine receptor gene DRD4. Compared to non-carriers, SCL6A4(C. Hu et al., 2020) gene carriers have noticeably

higher amygdala activity and heightened reactivity to social stress, emotional, and unpleasant stimuli. Interestingly, pharmacological approaches targeting specific genes among these (e.g., GNB3, SCL6A3, SLC6A4) have been used to treat depression, supporting the theory that genetics contributes to the pathophysiology of depression.

2.1.5. *Environmental stress factors*

The relationship between environmental stress, negative life experiences, and the onset of depression is extensively recognized, with numerous researchers identifying a high incidence of severe life events before depression onset. Critical incidents leading to depression may result from a range of life events, including job loss or redundancy, divorce, marital separation, the death of a spouse, retirement, social isolation, unintended pregnancy, childhood abuse, sexual assault, experiences of war, and major accidents. Such stressful experiences can impede recovery and elevate the risk of depression relapse(Nabeshima & Kim, 2013).

Depression and the stress response system are interlinked through various shared mediators and neural pathways. Stress significantly influences the initiation and progression of depression. Conversely, the lack of stress might provide some defence against depression, indicating that malfunctioning of the stress response system might lead to depression. Animal studies provide evidence that exposure to stress reshapes the brain's structure and affects its function particularly in the nucleus accumbens, hippocampus, amygdala, and prefrontal cortex, contributing to depression(LeMoult, 2020b).

Research involving monozygotic twins has demonstrated the influence of environmental stressors on depression development. Disparities in life experiences and environmental

factors between monozygotic twins who exhibit differing major depression statuses have been connected to the onset in the twin impacted by depression. Stressful events such as a distressing breakup, a troubled marriage, separation, severe cognitive impairment from traumatic brain injury, and overwhelming feelings of remorse have been implicated (due to actions adversely affecting others, such as causing death), occupational stress, job loss, and career setbacks have been identified as contributing factors(Nabeshima & Kim, 2013).

Notably, the affected twin experienced a greater frequency of stressful life incidents and prolonged exposure to stressors. Moreover, the nature and duration of these stressors can affect the characteristics of the episodes of depression.

Previous studies indicates that the occurrence of both sudden and prolonged challenging life incidents have the potential to initiate the development and relapse of major depression(Burcusa & Iacono, 2007). Conversely, favorable occurrences in life like marriage, nurturing connections, the arrival of a newborn, achievements in academics and profession, social backing, receiving gifts and aid, financial self-sufficiency, and various financial aids have been demonstrated to enhance outcomes in depression and could potentially offer a shield against the onset of depression.

2.1.6. Endocrine factors

Numerous abnormalities within the system of endocrine glands have been recognized as potential factors in the genesis of depression. It encompass fluctuations in levels of growth hormone (GH), imbalances in thyroid hormones, and dysregulation of the Hypothalamus-Pituitary-Adrenal (HPA) axis(Gold, 2021). Below, we delineate the potential mechanisms

by which these disruptions in the endocrine system could contribute to the onset of depression.

2.1.7.   Growth hormones

Investigations into the impact of pituitary hormones on depression have concentrated on their immediate and secondary effects on the norepinephrine (NE) system. Particularly, the secretion of growth hormone (GH), which is activated by catecholaminergic processes, is diminished in individuals with depression. A notable disparity in GH responses has been detected between depressed patients and healthy controls. During GH challenge tests involving apomorphine and clonidine, individuals experiencing repeated episodes of major depression frequently demonstrate "flat" or "diminished" GH responses compared to those without depression. Nonetheless, this response can be corrected by using various α2-adrenoceptor-selective drugs, leading to a restored increase in GH secretion. It indicates that a fundamental anomalies in the growth hormone system contributes to the subdued GH response observed in individuals experiencing repeated episodes of major depression during these assessments, highlighting GH's role in depressive pathology(Karachaliou et al., 2000).

Remarkably, a diminished GH response to clonidine, a centrally acting α2-adrenergic agonist, has been recognized as a persistent trait marker for depression, remaining evident even after recovery(Algahtany et al., 2021). Additionally, suppression of the GH gene has been shown to induce depressive-like behavior in experimental animals, although the exact mechanism remains unclear.

2.1.8. *Thyroid Gland Hormones*

The thyroid gland synthesizes the most potent thyroid hormones, triiodothyronine (T3) and thyroxine (T4), in response to stimulation by Thyroid Stimulating Hormone (TSH) released by the pituitary gland. TSH release is regulated by Thyrotropin Releasing Hormone (TRH), which is controlled by the hypothalamus. T3 and T4 are crucial for managing the body's overall metabolism. Research indicates that changes in thyroid function may play a role in the development of depression. Symptoms associated with depression, such as weight loss, sleep disruptions, and psychomotor agitation, may be related to thyroid dysfunction.

Moreover, the administration of T3 has been found to be an effective supplementary treatment for many individuals with depression. Although the exact mechanisms through which thyroid hormone imbalances play a role in the development of depression are not completely understood—and some studies have found no connection between thyroid hormone precursors and depression—the increase in cortical serotonin (5HT) secretion caused by T3 and T4 suggests that thyroid hormones might have an indirect effect through the serotonergic system(Hage & Azar, 2012). Additionally, some researchers propose that hormones produced by thyroid could function as joint transmitters with norepinephrine (NE) in the system regulating adrenaline responses. Consequently, there is a hypothesis that thyroid hormone imbalances might influence depressive symptoms through indirect effects on serotonin and adrenaline systems.

2.1.9. *HPA (Hypothalamic-pituitary-adrenal) system*

The HPA (Hypothalamic-Pituitary-Adrenal) system is also thought to have a vital role in the onset of depression. Research on the HPA axis shows that individuals with depression frequently display excessive secretion of corticotropin-releasing factor (CRF) and cortisol, impaired glucocorticoid feedback mechanisms, insufficient inhibition of the HPA axis when external glucocorticoids are administered and disrupted signaling of corticosteroid receptors(O'Keane et al., 2012).

Some depressive symptoms, such as an overabundance of personal guilt, feelings of hopelessness, diminished appetite, loss of weight, decreased sexual drive, disturbed sleep patterns, changes in neuropsychomotor behavior, and heightened sensitivity to psychological stress factors, was associated with dysfunction in the HPA system. This might partially elucidate why depression is frequently encountered in patients having Cushing's disease (conditions typified by excessive cortisol secretion).

The regulation of HPA axis activity is significantly influenced by CRF, which entails the elevation of anterior pituitary secretion of adrenocorticotropic hormone, subsequently prompting the secretion of cortisol by the adrenal gland. CRF also serves as a neurotransmitter in brain regions beyond the hypothalamus, orchestrating behavioral, hormonal, autonomic, and immune reactions to stress, potentially contributing to its involvement in the onset of depression.

Animal studies have shown that administering CRF directly into the central nervous system induces symptoms consistent with depression, such as reduced appetite and loss of body weight, reduced sexual behavior, disturbed sleep, and varied psychomotor activity. Additionally, elevated concentrations of CRF in CSF have been observed in depressed

patients. Posthumous examinations unveil a considerable decrease in density of CRF receptor within the cortex of frontal lobe, a decline in the expression of CRF1 receptor mRNA, and heightened CRF concentrations in the brains of depressed subjects in relation to non-depressed counterparts(Juruena, 2014). The heightened CRF levels detected in the cerebrospinal fluid (CSF) of depressed individuals return to baseline after having effective electroconvulsive therapy (ECT) or the administration of the most commonly used SSRI fluoxetine drug. Furthermore, clinical trials have illustrated that the antagonist R121919 of CRF can alleviate symptoms associated with major depression. Sustained elevation of CRF concentrations in the CSF is also linked to increased chances of early relapse, and the CRHR1 gene has emerged as a potential factor impacting vulnerability to major depressive disorder after having exposure to adverse life events. Such outcomes collectively underscore the involvement of chronic CRF hypersecretion and HPA axis alterations in the pathogenesis of depression.

2.1.10. Neuroinflammation

The connection of neuroinflammation with major depressive disorder is supported through the high prevalence of major depression in medical conditions that induce notable brain neuroinflammation associated with diseases such as systemic lupus erythematosus, brain injury caused by trauma, and autoimmune demyelinating disorder such as multiple sclerosis(Furtado & Katzman, 2015). A connection has been established between the density of the translocator protein's distribution volume (TSPO VT) measurement, as well as depressive disorders. During a major depressive episode related to major depressive disorder, patients show significant elevations in TSPO VT in critical areas of the brain,

including the prefrontal cortex, anterior cingulate cortex, and insula. The translocator protein is found on the surfaces of cells involved in immune modulation, and its increased presence signifies activated microglia, which points to neuroinflammatory processes in the brain(Woelfer et al., 2019).

2.1.11. Neural development

Neural development also known as neurogenesis in adults refers to the formation of new neurons and the development of connections between neurons within the region of dentate gyrus located in hippocampus and the subventricular zone of the lateral part of the ventricles. In the context of depression pathophysiology, it has been suggested that a decrease or lack of neurogenesis capacity contributes to depression. However, several animal studies have shown that reducing neurogenesis does not necessarily lead to depression-like behaviors(Sahay & Hen, 2008). In line with the structural and functional alterations caused by depression in the hippocampus, neurogenesis is essential for restoring the hippocampal structure and function, which could potentially alleviate depressive symptoms. Importantly, various antidepressant treatments, such as SSRIs, MAO inhibitors, TCAs, ECTs, and mood stabilizers, have been demonstrated to stimulate neurogenesis, thereby improving the efficacy of depression treatment.

Furthermore, investigations conducted by Gulbins and associates have uncovered a correlation between antidepressants and neurogenesis(Park, 2019). Their research has demonstrated that antidepressant treatment notably diminishes levels of ceramide—a lipid molecule within the brain that impedes cellular growth—thereby fostering neurogenesis. However, it is imperative to acknowledge the divergent findings within neurogenesis

research and the pathophysiology of depression. For example, the induction of neural development deficiency in animal experiments does not consistently result in symptoms of depression, and certain antidepressant effects are unaffected by neural development. Nevertheless, the prevailing consensus among the majority of researchers is that neurogenesis represents a crucial aspect in comprehending the pathophysiology and management of depression.

Presently, a confluence of genetic, environmental, immunological, neurogenic, deficiencies in biogenic amines, and endocrine factors contribute in the pathogenesis of depression, with ample proof substantiating the involvement of each component. The Hypothalamic-Pituitary-Adrenal system is arguably the principal neurobiological conduit connecting these factors to the genesis of depression. Genetic predispositions and environmental stress factors exert their influence through immunological and endocrine reactions, instigating structural and functional alterations in diverse regions of brain. Consequently, this results in impaired neural development and neurotransmission, culminating in the symptomatic presentation typical of depression.

2.2.   Current treatment/prevention approaches for depression.

A wide variety of evidence-based treatments for depression are available, in contrast to other severe mental disorders, which have limited options. Antidepressant medications, in particular, have been shown to be effective compared to placebo. Starting with inhibitors of MOA and tricyclic antidepressants compounds in the late 1950s and early 1960s and extended by the FDA's approval of fluoxetine in 1987, a wide range of other pharmaceuticals have been made available, including SNRIs (like venlafaxine and

duloxetine), SSRIs (like paroxetine, citalopram, escitalopram, and fluvoxamine), and other substances (like bupropion, nefazodone, trazodone, mirtazapine, vortioxetine, reboxetine, and agomelatine). All these pharmaceuticals have demonstrated efficacy more than placebo in treating major depressive disorder and have received approval from either the FDA or its European equivalent(Nemeroff, 2020).

The development of tricyclic antidepressants, MAO inhibitors, SSRIs, and SNRIs, which increased serotonin, norepinephrine, and/or dopamine's synaptic availability, supported the monoamine theories of severe depression. Certain more recent antidepressants, however, don't directly affect these systems. For instance, bupropion, whose mechanism of action was first believed to be an inhibitor of dopamine and norepinephrine absorption, does not reach the concentrations necessary to produce these effects at typical clinical doses. Antidepressants like nefazodone, mirtazapine, and mianserin have relatively mild impact, and others, like agomelatine and tianeptine, which are not accessible in the United States, do not exert a direct effect on either monoamine neurons or their associated receptors.

These findings highlight significant questions regarding how antidepressants work and the underlying core mechanisms of depression. They also imply that SSRIs might not primarily operate through these pathways and suggest that new antidepressants, like esketamine, which have minimal impact on monoamine circuits, could be developed.

Evidence-based psychotherapies, such as cognitive-behavioral therapy (CBT) and interpersonal psychotherapy, have shown promise in treating major depression when used in conjunction with antidepressants. Psychodynamically oriented psychotherapies have also been shown to be beneficial in certain cases. The relative effectiveness of

psychotherapy and antidepressants is still up for question. Some argue that the two are equally helpful, while others claim that antidepressants medications are more effective for severe form of depression.

Significant achievments has been achieved in the creation and improvement of somatic nonpharmacological treatments for depression in recent years. These include deep brain stimulation (DBS), vagal nerve stimulation (VNS), repetitive transcranial magnetic stimulation (rTMS), electroconvulsive treatment (ECT), and other newer techniques including focused ultrasound and direct current stimulation. It is difficult to provide a sufficient summary of this field due to its complexity and breadth

Although ECT is widely regarded as the most effective treatment, no well-powered comparison of ECT with any antidepressant has been done for serious depression. While there is a chance that ECT will result in short-term memory loss, changes in electrode location have mostly allayed this worry. The FDA has sanctioned the use of ECT, VNS, and rTMS for treating depression.

For example, a 5-year, multisite, large-scale study on the effectiveness of VNS in severe major depression that is not responding to treatment is being carried out in association with the Centers for Medicare and Medicaid Services. With most patients with major depression not responding well to monotherapy, different augmentation and combination techniques have been investigated in an attempt to improve outcomes. Clinicians have a range of options to choose from when addressing treatment nonresponders, partial responders, or nonremitters.

Various strategies, such as lithium augmentation, which increases the effect of tricyclic antidepressants on serotonergic neurotransmission, are based on preclinical data and monoamine hypothesis(Nemeroff, 2020). Others that are less based on fundamental neuropharmacology have shown promise in turning nonresponders into remitters or responders. These include atypical antipsychotics including risperidone, aripiprazole, brexpiprazole, olanzapine, and quetiapine, some of which have been authorized by the FDA for this use.

Further agents that have shown promise in this field are thyroid hormone (T3), pramipexole (a D2/D3 agonist), pimavanserin (a serotonin inverse agonist), ketamine and esketamine, brexanolone, estrogen (in women going through menopause), and an increasing variety of psychedelic drugs such as psilocybin. It is also common to use combination therapy that combine SSRIs with additional antidepressants, such as bupropion, venlafaxine, or mirtazapine.

2.3. General Nutritional deficiencies associated with depression.

Depression is a multifaceted disorder involving numerous biological processes, like inflammation, impaired HPA axis function, disturbances in the sympathetic and parasympathetic nervous systems, and impairment in endothelial function. Research in neurobiology has revealed associations between depression and the shrinkage of cortical and limbic neurons, along with abnormalities in the connectivity and operation of neural networks(Lang et al., 2015). These alterations stem from deficits in neural structure, function, and neurochemistry, with particular emphasis on the roles of γ-aminobutyric acid (GABA) and glutamate.

One prevalent hypothesis concerning depression's pathophysiology suggests a connection between the disorder and decreased levels of monoamine neurotransmitters. Depressed individuals often exhibit decreased levels of dopamine, serotonin and norepinephrine. The production and release of these neurotransmitters are influenced by several factors, including changes in composition of plasma resulting from dietary nutrients. Furthermore, changes in dietary patterns, including shifts in dietary intake of macronutrients, vitamins, and minerals can affect mental function by modulating inflammation and impacting biochemical and cellular processes in the body (Rao et al., n.d.).

2.3.1. *Mineral Components*

To date, research has mainly concentrated on understanding how minerals contribute to the onset of depression, focusing on both dietary intake and serum deficiencies. Key minerals like magnesium, zinc, iron, copper, and selenium are known to play crucial roles in cellular function, neuromodulation, and antioxidant mechanisms. Evidence indicates that low magnesium levels can disrupt central nervous system function, particularly impacting glutamatergic signaling in the limbic system and cerebral cortex, which are critical areas involved in the development of depression. Magnesium deficiency may impact corticotropin-releasing hormone secretion, thereby elevating adrenocorticotropic hormone (ACTH) levels and potentially influencing hypothalamic–pituitary–adrenal (HPA) axis regulation(Zielińska et al., 2023). Moreover, magnesium function as an antagonist to N-methyl-D-aspartate (NMDA) receptors, and its deficiency may result in NMDA receptor overactivity, leading to neurotoxicity.

Changes in brain zinc levels have also been linked with genesis of depression, with zinc supplementation showing promise in alleviating depressive symptoms and augmenting the effects of antidepressants. Zinc's antidepressant properties may stem from its anti-inflammatory and antioxidant characteristics, along with its role as an NMDA receptor antagonist [126]. Additionally, zinc impacts hormonal control, including the regulation of cortisol, immune cell activity, neurogenesis, and synaptic communication. Recent studies have emphasized The significance of zinc transport proteins and GPR39 receptors responsive to zinc in the onset of depression and in exploring potential treatment strategies(Zielińska et al., 2023).

Selenium intake has been strongly linked to depression, with selenium playing a vital role in the proper functioning of selenoproteins that defend against oxidative damage in the brain and nervous system. Despite contradictory findings, there is a rising interest among researchers in understanding how selenium relates to depression due to its neuromodulatory function in brain health.

A study by Hongrong et al. found a vital association between elevated serum copper levels and depressive symptoms, suggesting a potential use of copper as an indicator of depressive disorders. Copper plays various roles in the nervous system, including neurogenesis, neurotransmission, and synaptic function, and it influences the function of neurotrophic factors like BDNF and NGF (Zielińska et al., 2023). However, imbalances in copper levels may contribute to depression, although copper's precise role in depression pathogenesis remains complex.

Iron deficiency, a prevalent global nutritional issue, has garnered attention for its potential involvement in mental disorders, including depression. Iron is essential for neurotransmitter synthesis and the regulation of BDNF levels, vital for emotional processes. Iron deficiency may also impact the glutamatergic system, potentially leading to mood disorders. Higher body iron levels have been associated with fewer depressive symptoms, suggesting a potential link between iron status and depression severity(Zielińska et al., 2023).

Calcium's role in depression pathophysiology involves its participation in various mechanisms, including the regulation of the HPA system and neuronal processes. Changes in extracellular calcium concentration may influence emotion regulation and neuronal plasticity, potentially affecting depressive symptoms.

2.3.2. *Vitamin D*

Vitamin D, which is a fat-soluble nutrient that the body synthesizes when exposed to sunlight, also by dietary intake, and supplementation, has garnered increasing attention for its role in various diseases, including mental health disorders. Studies suggest that vitamin D deficiency is associated with an 8–14% increased risk of depression. One proposed mechanism involves the distribution of vitamin D receptors (VDR) throughout the cortex and limbic system. and hippocampus, brain regions implicated in depression pathophysiology(Zielińska et al., 2023). Moreover, vitamin D regulates The formation of neurotrophic factors like BDNF and NGF, and modulates immunoinflammatory pathways relevant to depression.

Individuals deficient in vitamin D may experience elevated concentrations of ROS and calcium ions in nerve cells, potentially contributing to depression. Furthermore, vitamin D plays a role in maintaining optimal serotonin concentration and regulating dopamine and norepinephrine in the brain, thus impacting mood regulation. Vitamin D deficiency is a significant health concern in both depressed individuals and the general population, with research focusing on serum vitamin D-25-hydroxyvitamin D (25(OH)D) levels. Notably, vitamin D's role in prevention of postnatal depression has received attention.

Ongoing research is examining the levels of the vitamin D-binding protein gene and its link to the onset of depression, with a focus on how calcitriol (1,25[OH]2D3) affects neuronal quantity and structure through detoxification pathways and neurotrophin regulation. The effect of calcitriol on depressive symptoms could be associated with the presence of vitamin D receptors, the vitamin D-binding protein (VDBP), and the enzyme 1-alpha-hydroxylase, which converts 25(OH)D3 to 1,25(OH)2D3 within the central nervous system(Zielińska et al., 2023).

2.3.3. Vitamins of B Family

Deficiencies in various vitamins, notably those belonging to the B group, have been linked to symptoms of mental disorders. B vitamins play crucial roles in the human body, including maintaining normal nervous system function by aiding in monoamine oxidase production, DNA synthesis, methylation, and phospholipid repair. Deficiencies in B vitamins such as B1, B6, B9, and B12 have specifically been associated with depression due to their essential roles in neuronal function. Moreover, these vitamins have been found

to offer protection against hypercysteinemia, a condition associated with an increased risk of mood disorders(Zielińska et al., 2023).

Reduced levels of vitamins B9 and B12 have been associated with less effective responses to antidepressant therapy. Recent studies by Berkins et al. propose that consuming vitamins B6 and B12 might impact brain structure, suggesting that vegetarians, particularly those dealing with depression, could benefit from supplementing their diets with vitamins B6, B9, and B12 to support optimal brain health(Zielińska et al., 2023). Table 1 details the influence of these B vitamins on the nervous system and their potential implications for depression risk.

Table 1 Primary functions of specific B vitamins in the nervous system adapted from(Zielińska et al., 2023).

Vitamin B	Effects on the Nervous System and Risk of Depression
Vitamin B1 (thiamine)	Thiamine is an important coenzyme during the synthesis of neurotransmitters, such as acetylcholine and serotonin, for example. The most important function of thiamine is considered to be that it makes a major contribution to cellular energy metabolism and, as an essential cofactor in carbohydrate metabolism, it helps to supply energy to nerve cells [84]. An inverse relationship has been shown between thiamine levels and depressive symptoms in adults [85].
Vitamin B6 (pyridoxine)	Pyridoxine functions as a cofactor in the pathways involved in myelin synthesis and enzymatic reactions, including the synthesis of neurotransmitters such as gamma-aminobutyric acid (GABA), serotonin, and dopamine [86]. Furthermore, it controls glutamate excitability and neuronal metabolism. Vitamin B6 and magnesium both modulate neurobiological mechanisms, leading to speculation that they may exert synergistic effects [87]. Pyridoxal-5-phosphate concentrations, the active form of vitamin B6, were measured in Hispanic adults in years 2 and 5 of the study, and it was observed that depressive symptoms were higher in those with low values [88].
Vitamin B9 (folic acid)	Folic acid is involved in the synthesis and metabolism of neurotransmitters associated with depression (serotonin, dopamine, norepinephrine). In addition, it plays a vital role in the regeneration of tetrahydrobiopterin (BH4), a cofactor essential for the formation of neurotransmitters [89]. An association has been shown between lower serum folic acid levels during pregnancy and prenatal depression [90].
Vitamin B12 (cobalamin)	A specific function of vitamin B12 is to participate in the DNA synthesis of myelin-producing oligodendrocytes and in the synthesis of myelin [83,85]. Cobalamin is a cofactor of the methionine synthase enzyme, which catalyses the reaction to transfer a methyl group to a homocysteine molecule. Methionine is formed, which is the precursor of S-adenosylmethionine (SAM). SAM plays an important role in the methylation processes necessary for the normal synthesis and/or metabolism of membrane phospholipids, DNA, RNA, neurotransmitters and for the normal function of the myelin sheaths of nerve fibres [91]. Vitamin B12 deficiency may be associated with impaired glutathione peroxidase activity elevated levels of free radicals. Furthermore, the prevalence of depression tends to be higher among vegetarians due to insufficient intake of vitamin B12 [92].

2.4. Folate deficiency associated with depression.

Several investigations have indicated a possible association of low folate levels and depression. These observations imply that inadequate folate may be associated with a higher chance of developing depression, more intense depressive symptoms, longer-lasting episodes, and a greater chance of symptom recurrence(Joshua Falade*, 2020). The

fundamental neurobiological understanding of Major Depressive Disorder (MDD) involves key monoamine neurotransmitters such as serotonin (5HT), norepinephrine (NE), and dopamine (DA). These neurotransmitters contribute to the expression of symptoms of depression by acting within particular neural circuits in the brain. Another crucial element in the production of neurotransmitters is tetrahydrobiopterin (BH4), which is required to produce a number of neurotransmitters, such as nitric oxide, DA, and 5HT. Folate helps restore BH4 from its oxidized form, which is an essential step in the production of BH4. A neurochemical sensitivity to depression may arise from low folate levels as they may lead to lower levels of DA, NE, and 5HT.

A shortage in folate, in conjunction with genetic susceptibility and medicine, can result in hyperhomocysteinemia, which is defined as increased homocysteine levels. Reduced amounts of S-adenosylmethionine (SAMe) and higher levels of S-adenosylhomocysteine (SAH) can be the outcome of this metabolic imbalance, which can also upset the methylation cycle. Elevated levels of homocysteine and decreased SAMe lead to the activation of N-methyl-D-aspartate (NMDA) receptors, which play a role in excitatory neurotransmission(Joshua Falade*, 2020). The delicate balance of neurotransmitter systems can be upset by excessive NMDA receptor activation, which also increases oxidative stress and neuronal excitotoxicity. Because hyperhomocysteinemia reduces endothelial function and increases reactive oxygen species (ROS) generation, it can compromise vascular health and integrity. Endothelial dysfunction can cause mood swings and neuronal damage by reducing blood flow and nutrition supply to the brain. Control of

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mood and neurophysiology can be significantly impacted by the dysregulation of several metabolic pathways.
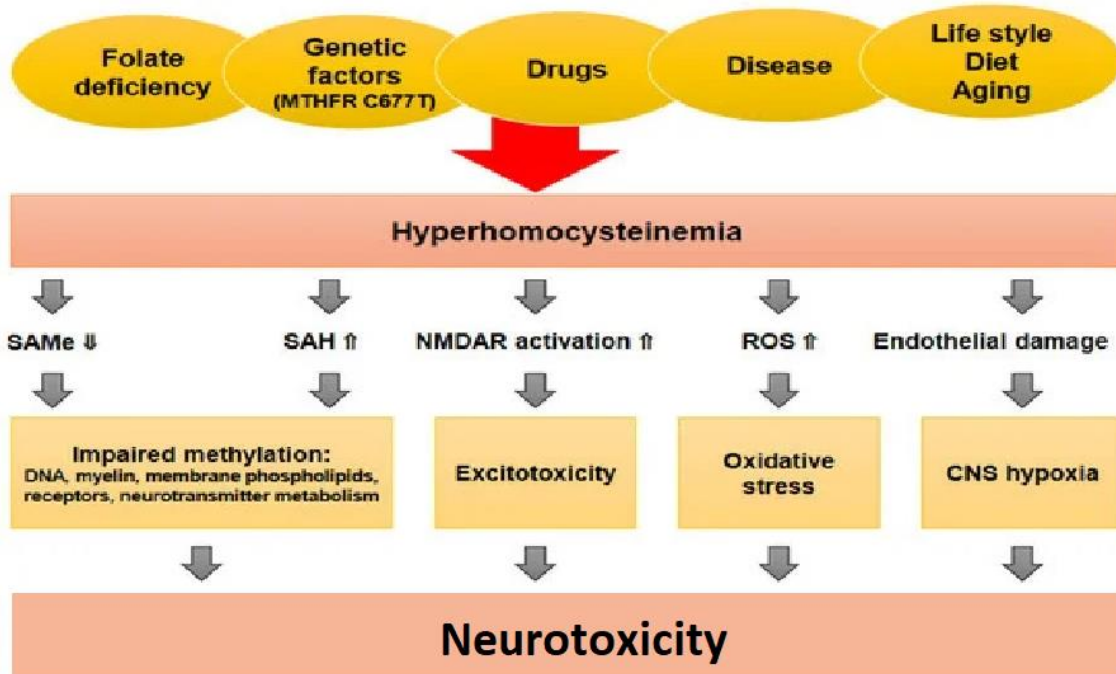
Figure 2. 1 The biochemical pathways from folate deficiency to hyperhomocysteinemia to neurotoxicity adapted from

[49].

2.5.   Potential of folate to treat depression.

Emerging investigations propose that the operation of the one-carbon cycle might impact the reaction to antidepressants. Mental health issues have been independently associated with deficiencies in any component of this cycle. However, because of its widespread implications in depression, folate levels have drawn special attention among these constituents(Coppen & Bolander-Gouaille, 2005). There is only one form of vitamin B9 that can overcome the blood-brain barrier, and that is the active form known as L-methylfolate. Studies suggest that patients with folate deficiency may not respond well to

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antidepressant drugs, which could lead to the classification of their illness as "treatment-resistant" depression. Both folic acid and L-methylfolate have shown advantages in treating depression, either as primary treatments or when used alongside other therapies. A randomized controlled trial (RCT) done by Reynolds et al. found that L-methylfolate alone was just as effective in treating mild-to-moderate depression as a traditional antidepressant. For six weeks, 20 participants received amitriptyline (150 mg/day) and 19 people received L-methylfolate (25 mg of active L-methylfolate daily)(Bender et al., 2017b). L-methylfolate had a response rate of 42%, and amitriptyline had a response rate of 35%. Remarkably, there were no reported side effects with L-methylfolate, however three trial participants had to drop out because of unbearable amitriptyline side effects. One important finding was the association between the increase in red blood cell (RBC) folate levels and the antidepressant response to L-methylfolate.

2.6. Significance of animal model for depression studies

Despite the frequency and devastating consequences of depression, studies on its aetiology are still in their early stages when compared to those on the pathogenesis of other prevalent chronic and potentially deadly multi-factorial illnesses, such as diabetes and Parkinson's disease. The primary impediment is the scarcity of approved animal models. To begin with, an appropriate animal model allows researchers to better understand the biochemical, genetic, and epigenetic elements that contribute to depression. Animal models can be used to investigate the underlying molecular changes as well as the causal link between genetic or environmental changes and depression, providing a deeper understanding of depression pathology(Czéh et al., 2016). Since no "depression gene" has been found to generate signs

of depression in mice, stress continues to be a risk factor for sadness. Second, enhanced animal models of depression are crucial for determining potential therapeutics for depression.

Over recent decades, notable advancements have transpired in comprehending the psychological constructs underpinning Major Depressive Disorder (MDD), delineating aberrant brain circuits associated with MDD, and delineating cellular and molecular modifications in this condition. This development has been facilitated, at least in part, by the utilization of rodent models, which afford researchers the opportunity to scrutinize potential brain circuits, neurophysiological systems, and molecular targets, such as anomalies in the stress axis or heightened neuroinflammation.

The manifestations of MDD encompass fundamental features such as anhedonia and a despondent mood, alongside supplementary symptoms like disturbances in sleep, alterations in weight or appetite, and changes in psychomotor activity(Becker et al., 2021). Additionally, MDD frequently coincides with related disorders such as anxiety and withdrawal from social interaction. These symptoms and associated illnesses can be readily assessed in animal models, leading to the development of various tests designed to measure these diverse features.

Animal models are essential for exploring basic biological processes and systems. They are particularly important in neuroscience, where the extensive use of invasive technologies to study the human brain remains challenging.

CHAPTER 3: METHODOLOGY

3.1. Animal

Total of 80 *Sprague dawley* (SD) rats of 8-12 weeks age were taken from Laboratory Animal House ASAB, NUST. The protocol used was according to the standard guidelines of Laboratory Animal House ASAB, NUST and was granted approval by Institutional Review Board NUST. All rats were kept with unrestricted access to food and water subject to 12-hour light/12-hour dark cycle maintained at a temperature of $21°C \pm 2°$. After housing, rats were given time of 7 days to acclimate to their environment before starting the protocol procedure. Rats were categorized into three distinct groups for the study as shown in figure 2. The control group, which neither received any stress nor any treatment, consisted of 11 animals (6 males and 5 females). The second group, which was exposed to stress and injected with saline, comprised 31 animals (15 males and 16 females). The third group, which was also exposed to stress but injected with folate and vitamins subcutaneously, included 38 animals (19 males and 19 females).

Control	Experimental group 1	Experimental group 2
Stress: None	**Stress:** CUMS	**Stress:** CUMS
Treatment: None	**Treatment:** Saline	**Treatment:** Natural Supplements

Figure 3. 1 Division of rats in three groups to examine the effect of natural supplements on CUMS model of rats.

3.2. Development of a Chronic Unpredictable Mild Stress Model:

Rats of experimental groups were subjected to stress for 21 days. Stressors were introduced randomly every day to maintain the element of unpredictability in protocol. The stress-inducing factors employed included deprivation of food (24h), deprivation of water (24h), Dark phase (24h), Cage Tilting (24h), No bedding (24h) and predator voice (2h). Table 2 demonstrates the overview of CUMS procedure used in this study. The rats were weighed on a weekly basis.
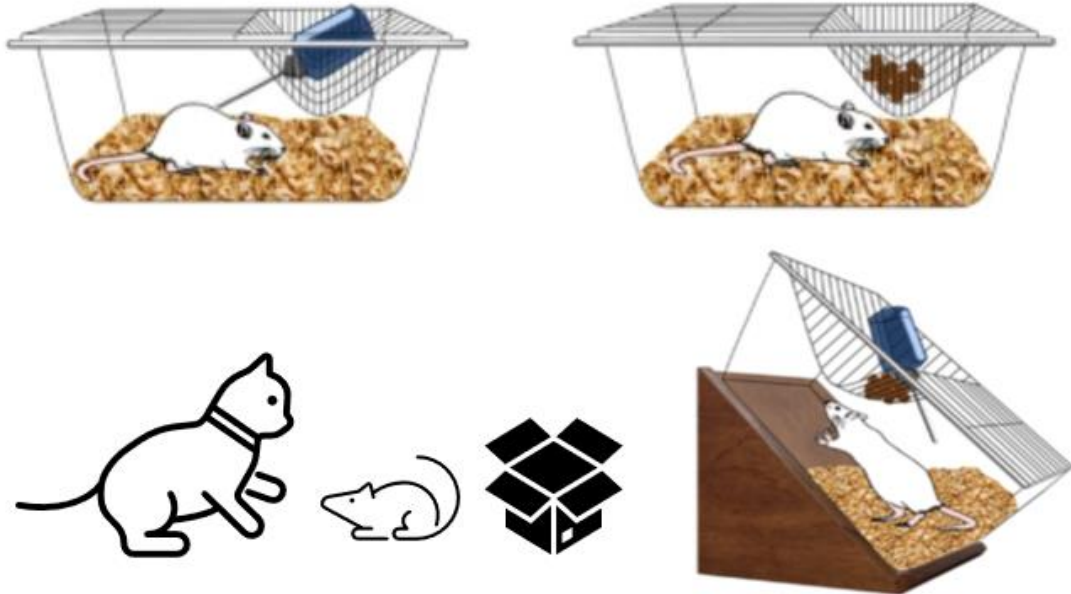
Figure 3. 2 Visual demonstration of different stressors given to rats to generate CUMS model.

Table 2 Day wise stress planner

Day	Stressors	Duration
1	Water Deprivation	24 hours
2	Food Deprivation	24 hours
3	No bedding	24 hours
4	Tilt Cage	24 hours
5	Food Deprivation	24 hours
6	Water Deprivation	24 hours
7	Predator Voice	2 hours
8	Tilt Cage	24 hours
9	24-hour dark	24 hours
10	No bedding	24 hours
11	Food Deprivation	24 hours
12	24-hour dark	24 hours
13	Water Deprivation	24 hours
14	No bedding	24 hours
15	Tilt Cage	24 hours
16	Predator Voice	2 hours
17	Tilt cage 45°	24 hours
18	No bedding	24 hours
19	Predator Voice	2 hours
20	24-hour dark	24 hours
21	Water Deprivation	24 hours

3.3. Injection Preparations:

To prepare the injections three vitamins such as L-Methylfolate with Vitamin B12 (BIO

LIFE), IN-D3 VITAMIN D3 (BRAINEX HEALTH CARE) and VITAMIN-B2

(Herbiotics) in the form of tablets were purchased from a local pharmacy in Saddar Rawalpindi. L-Methylfolate was used 2.25 mg/kg rats' weight, Vitamin D3 dosage was 2.6 mg/kg rats' weight and Vitamin B2 was 0.14 mg/kg weight of rats. All these tablets were grinned into the powder form and dissolved in phosphate buffer saline. The final volume of injection was raised to 300 microliters. Rats were given these injections subcutaneously for 21 days on a daily basis. For injections insulin syringes were used.

3.4. Sucrose Preference Test:

To validate the model generation of depression, a sucrose preference test was performed. All rats were given two bottles, one carrying 300 ml solution of 1% sucrose, and second carrying the same amount of tap water. It was done for 48 hours so that rats can adapt to the sucrose solution. After 24 hours of adaptation, side of the bottles were changed for next 24 hours to avoid the side biasness. After adaptation phase, rats were subjected to sucrose preference test for 24 hours of duration. Rats were allowed to consume pre-measured sugar solution and tap water for 24h. Then, all the bottles were withdrawn, measured, and noted. Overall theme of the sucrose preference test is demonstrated below:
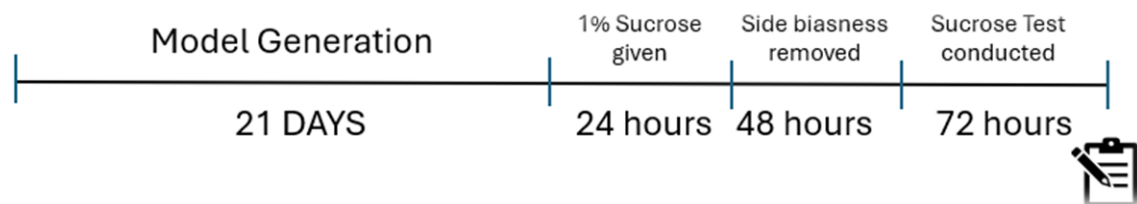

Figure 3. 3 Timeline of the Sucrose Preference Test

To calculate the sucrose preference percentage, following formula was used:

$$\text{Sucrose Preference Percentage (\%)} = \frac{\text{Sucrose Volume Consumed}}{\text{Total Volume Consumed}} \times 100$$

3.5. Behavioral Tests:

3.5.1. Open field test:

The OFT was carried out in the same way as it is often used to assess spontaneous behavior in rodents. The equipment was a black square measuring 70 cm x 70 cm $\times$ 40 cm. The arena was partitioned into central and outer zones that had been painted on its floor. A single rat was placed in the centre of the floor, and after 30 seconds of adaption, its conduct was monitored for 5 minutes using a video camera located 40 cm above the arena. After each test, the arena was sanitized using a 70% ethanol solution. Later, ANY-maze software (Stoelting Company, Wood Dale, IL, USA). To assess rats' locomotor activity, the total distance travelled was recorded. Rats' total time in the central field and freezing time were measured as an index of anxiety. (Stoelting, Wood Dale, USA). A conceptual illustration of the entire OFT is given here:
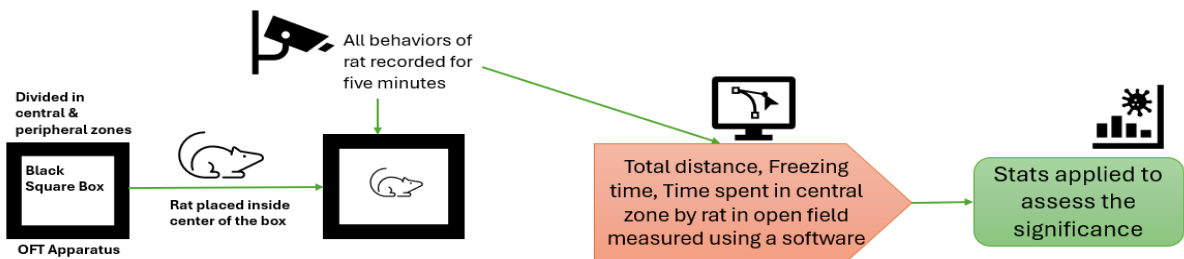

Figure 3. 4 Workflow of the Open Field Test

3.5.2. Forced swimming test:

It is a process in which rats swim in conditions that make escape impossible. The rats were introduced into a transparent plastic container (40 cm high and 25 cm in diameter) filled with tap water. up to 25 cm ± 1.5 mark The water's temperature was 24 ± 0.5 degrees Celsius. After 5 minutes, The rats were taken out of the water, towel-dried, and placed in a heated environment. Each rat had a 5-minute test session that was videotaped from the container's side perspective. The behavior measure was evaluated and defined as follows: Immobility is the lack of motion of the entire body, except for minor movements required to keep the animal's head above water. The forced swimming test protocol is illustrated in the following figure:
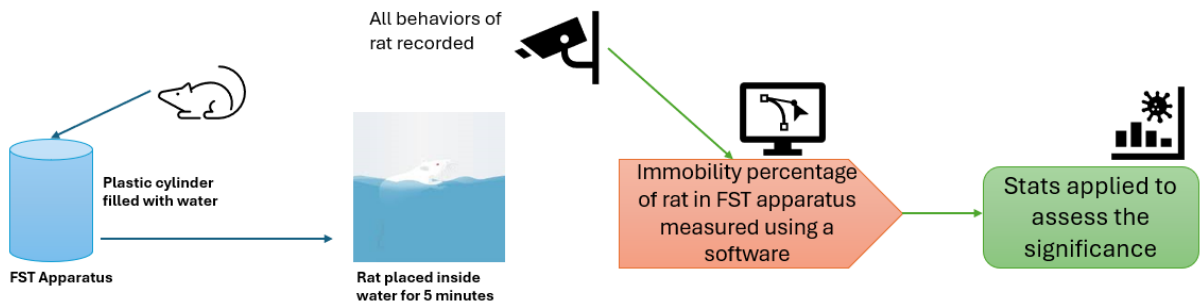

Figure 3. 5 Workflow of Forced Swim Test

3.6.   Statistical Evaluation

Statistical assessments were conducted using GraphPad Prism software (version 9.5). Data are presented as mean ± SEM and evaluated using two-way ANOVA with subsequent Tukey's test for multiple comparisons. A threshold for significance was set at $P < 0.05$.

CHAPTER 4: RESULTS

4.1. Sucrose Preference Test Results for model validation:

Sucrose preference analysis was utilized to evaluate the hedonic state in rats. After 21 days of CUMS protocol, the rats treated with saline demonstrated a huge decline in preference of sucrose solution compared to the control ($p < 0.0001$). This validates the CUMS protocol has successfully generated a model of depression. While rats exposed to CUMS stress protocol and treated with supplements mixture preferred sucrose solution over water and showed that mixture prevented the anhedonia in males completely and partially in females. **(Figure 4.1)**

Figure 4. 1 Graph demonstrates sucrose preference percentage among control and both experimental groups in male and female separately, Each value depicts the mean ± SEM. * illustrates $p < 0.01$. ** illustrates $p < 0.001$ *** illustrates $p = 0.0001$

4.2. Body Weight Gain

The total body weight gain of healthy rats and rats subjected to CUMS treated with saline and supplement mixture are indicated in **Figure 4.2**. All the changes in body weight gain of study groups in both sexes shows significant results. Both male and female Rats subjected to CUMS and given saline shows very less body weight gain. While rats subjected to CUMS and treated by mixture of supplements shows comparatively better body weight gain then the CUMS with saline group but less body weight gain than control group. While female rats shows less body weight gain as compared to male rats.
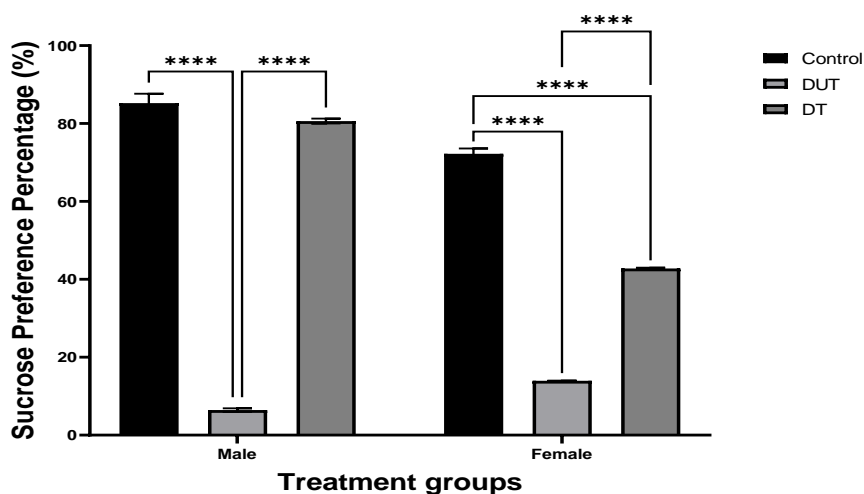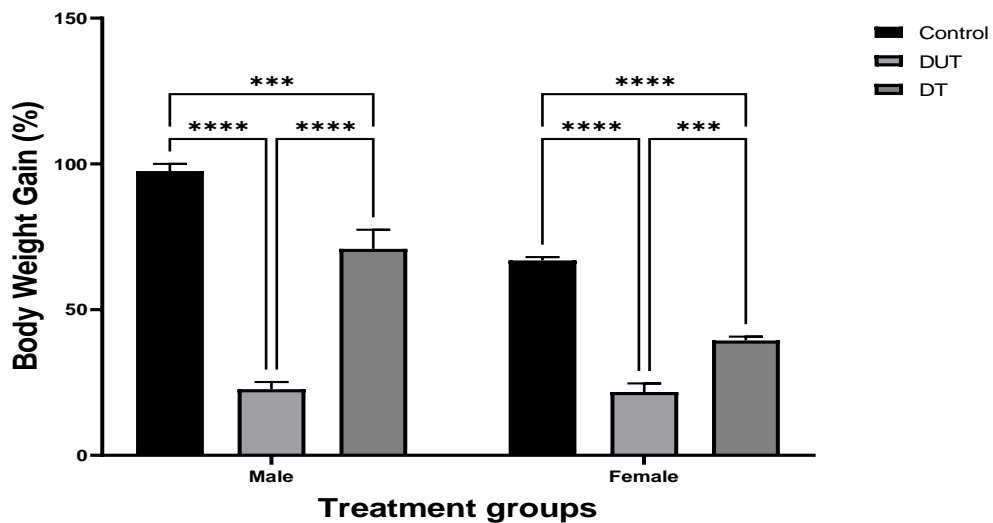

Figure 4. 2 Graph demonstrates body weight gain among control and both experimental groups in male and female separately Each value depicts the mean ± SEM. * illustrates p < 0.01. ** illustrates p < 0.001 *** illustrates p = 0.0001 and **** illustrates p < 0.0001

4.3. Behavioral results

4.3.1. Open field test:

Locomotory and exploratory behavior in open field of healthy rats and rats subjected to CUMS treated with saline and supplement mixture are indicated in **Figure 4.3**. All the changes in locomotory and exploratory behavior of study groups in both male and female shows significant results. Both male and female rats subjected to CUMS and given saline shows decrease in total distance travelled by rats and time spent in central zone. While rats subjected to CUMS and treated by mixture of supplements shows the increase in total distance travelled and time spent in central zone. While both male and female rats subjected to CUMS and given saline shows increase in time freezing. While rats subjected to CUMS and treated by mixture of supplements shows the decrease in time freezing.
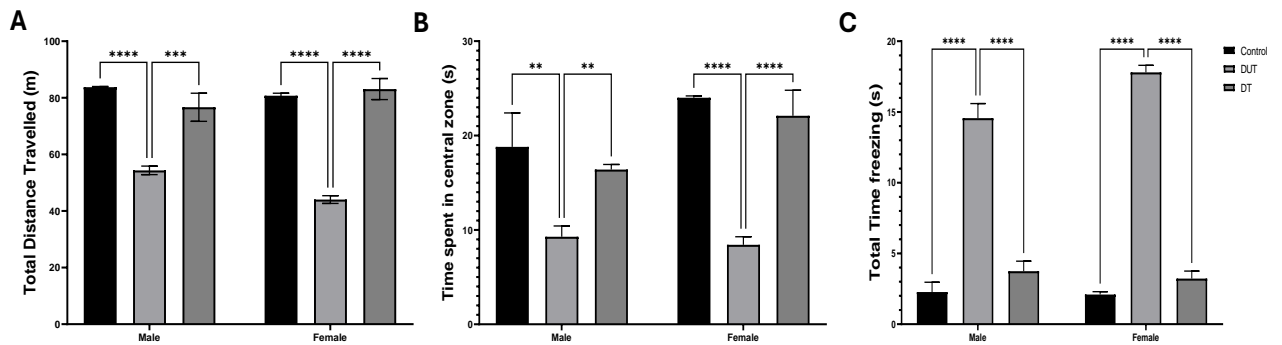
Figure 4. 3 Graph demonstrates (A) Total distance travelled by rat in the arena, (B) Time spent in central zone of the arena and (C) Total time freezing in open field test among control and both experimental groups in male and female separately.

4.3.2. Forced swimming test:

Despair like behavior (i.e. immobility percentage) in forced swimming test of healthy rats and rats subjected to CUMS treated with saline and supplement mixture are indicated in Figure 6. All the changes in despair like behavior of study groups in both male and female shows significant results. Both male and female rats subjected to CUMS and given saline

shows increase in immobility percentage. While rats subjected to CUMS and treated by mixture of supplements shows the decrease in immobility percentage. **(Figure 4.4)**
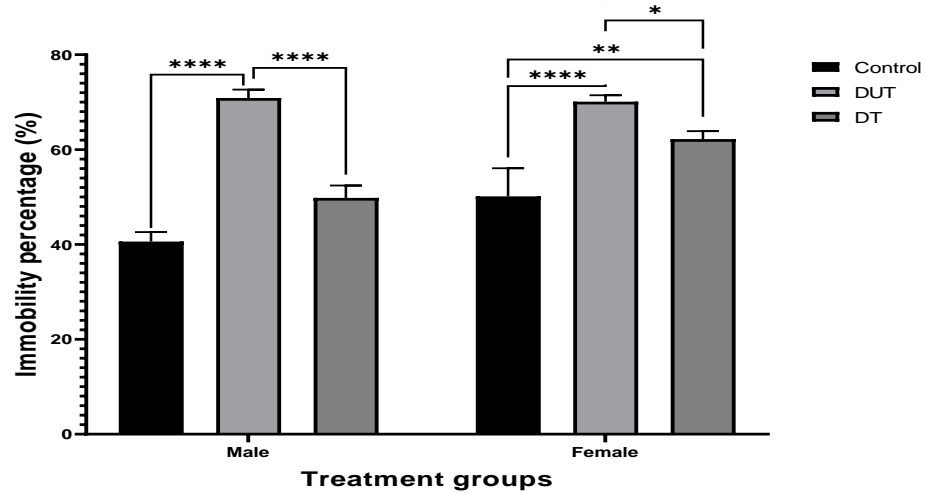

Figure 4. 4 Graph demonstrates immobility percentage among control and both experimental groups in male and female separately, Each value depicts the mean ± SEM. * illustrates p < 0.01. ** illustrates p < 0.001 *** illustrates p = 0.0001

CHAPTER 5: DISCUSSION

The present study investigated the sex-based behavioral differences in rats subjected to chronic unpredictable mild stress (CUMS), a well-established animal model for inducing depression-like states. The findings provided evidence of both anxiety and depressive-like behaviors induced by CUMS in male and female rats, highlighting a significant sex-specific variation in stress sensitivity and behavioral outcomes.

Under standard, non-stress conditions, female rats exhibited a slower rate of body weight gain compared to male rats, which is consistent with the natural physiological differences between sexes. This variation in growth rate could reflect inherent metabolic and hormonal distinctions, where males typically show a more robust anabolic profile. However, when exposed to CUMS, this natural divergence became more pronounced, with females exhibiting sharper declines in sucrose preference and longer immobility periods in the Forced Swim Test (FST) than males. These two behaviors are widely recognized as indicators of anhedonia (a core symptom of depression) and despair, respectively, pointing toward a greater vulnerability of female rats to the deleterious effects of chronic stress. This aligns with clinical observations in humans, where women are statistically more prone to major depressive disorders than men, suggesting that hormonal, genetic, and neurochemical factors contribute to this heightened susceptibility in females.

Interestingly, while female rats showed stronger depression-like behaviors, male rats displayed more prominent anxiety-like behaviors under CUMS, particularly in the Open Field Test (OFT). The greater reduction in exploratory behavior and increased anxiety-

related parameters in males suggest that CUMS might differentially influence anxiety and depressive pathways between sexes. These behavioral differences could be underpinned by sex-specific activation of neuroendocrine systems and differential regulation of stress-response pathways, though this was not directly measured in the current study.

At the biochemical level, although the present study did not assess neuroendocrine changes, it is possible that the CUMS-induced elevation in serum corticosterone concentrations reported in other studies may have played a role in the behavioral differences observed here. Female rats may experience heightened hypothalamus-pituitary-adrenal (HPA) axis reactivity in response to stress, leading to more severe depressive-like behaviors. This sex-specific hyperactivity of the HPA axis has been documented in various studies (Xia et al., 2022), and while not directly measured in this study, it could possibly explain the more significant depressive symptoms seen in females. Prolonged stress-related elevation of glucocorticoids, such as corticosterone, has been linked to exacerbation of mood disorders, impairments in neurogenesis, and dysregulation of key neurotransmitter systems, which may further explain the sex-specific behavioral outcomes.

In terms of immune response, although no direct measurements were made in this study, previous research suggests that the macrophage/T-lymphocyte hypothesis of depression may be relevant in explaining the sex-specific behaviors observed. According to this hypothesis, excessive production of proinflammatory cytokines worsens neuroinflammation and plays a crucial role in depression (Dey & Giblin, 2018). Female rats in the current study displayed more pronounced depression-like behaviors, which could be potentially due to an imbalance between Th1 and Th2 immune responses, as

reported in other studies. It is possible that females exhibit higher Th1 responses, leading to greater proinflammatory cytokine production under stress. Reduced levels of anti-inflammatory cytokines, such as IL-10 and TGFβ, may further exacerbate depressive symptoms in females (Xia et al., 2022), though this was not examined in the present research.

Additionally, while this study focused exclusively on behavioral outcomes, disruptions in monoamine neurotransmitter systems, particularly norepinephrine (NE), serotonin (5-HT), and dopamine (DA), have been strongly linked to depression. It is plausible that sex-specific differences in the regulation of these neurotransmitters could account for the behavioral disparities observed here. For example, previous studies have shown that females may have lower baseline levels of NE and 5-HT, which could result in greater vulnerability to stress-induced depression (Sun et al., 2022). The more pronounced reductions in sucrose preference in female rats may reflect impairments in the dopaminergic reward system, though these biochemical changes were not directly assessed in the current study. Future research could explore these potential mechanisms further to better understand the neurobiological underpinnings of the observed behavioral differences.

Taken together, the findings suggest that while both male and female rats are adversely affected by CUMS, the underlying mechanisms driving their behavioral responses may differ. Female rats appear to be more vulnerable to depression-like behaviors, possibly due to heightened HPA axis sensitivity, immune dysregulation, and disruptions in neurotransmitter systems, although these were not directly assessed. In contrast, male rats

showed more anxiety-like behaviors, suggesting that chronic stress differentially impacts anxiety-related pathways in males.

These findings have important implications for understanding the sex differences in stress-related psychiatric disorders in humans. Women are consistently reported to have higher rates of depression than men, and this study provides evidence of potential biological mechanisms that may underlie these differences. The sex-specific changes in behavior observed here are consistent with patterns seen in human studies, reinforcing the validity of using animal models to study the neurobiological basis of depression.

Future research should aim to elucidate the molecular and hormonal pathways that may drive these sex differences, particularly focusing on how the HPA axis, immune system, and monoamine neurotransmitters interact to influence stress responses. While this study was limited to behavioral analysis, a more comprehensive approach that includes biochemical and molecular assessments would provide a clearer understanding of the sex-specific mechanisms at play. Additionally, exploring therapeutic interventions that target these differences could lead to more effective treatments for depression, especially in women, who appear to be at greater risk.

CHAPTER 6: CONCLUSION

The findings from this investigation underscore the significant potential of folate combined with vitamins B2 and D3 in preventing depression induced by chronic daily life stress. The combination therapy effectively mitigated anhedonic behavior, preserved locomotor function, and reduced despair-like behavior in the stressed animal model. Specifically, the combination resulted in a notable sucrose preference, sustained locomotor abilities, and decreased immobility percentage in the forced swimming test, indicating a robust preventative effect against stress-related mental health disorders. Folate supplementation seems to have different effects on depression-related behaviors in female and male rats subjected to stress. Female rats exhibit reduced sucrose preference compared to males after L-methyl folate intervention, indicating a gender-specific response to stress and treatment. Folate, including L-methyl folate, can impact brain function differently in males and females, contributing to variations in behavior and responses to stressors. Both Male and female rats exhibited different behavioral responses when subjected to chronic unpredictable mild stress. HPA axis imbalance was more common in female rats. These results highlight the viability of folate and associated vitamins as a preventative strategy for depression, offering a promising alternative to conventional antidepressants, which are often accompanied by undesirable side effects. Additional research is needed to explore the pathways behind these effects and assess their potential for clinical treatment.

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ABSTRACT

Chronic daily life stress is among the leading causes of depression, a predominantly prevalent psychological disorder affecting millions of people globally. To counter depression, numerous known and commercially available antidepressant medications, such as SSRIs, SNRIs, and TCAs, exist. However, all these medicines present a wide range of unpleasant side effects. Apart from these conventional antidepressants as treatment option, various vitamins are associated with depression management such as folates. Folate is a vital component of brain health which plays a role in various pathways, particularly in neurotransmitter synthesis, such as serotonin and dopamine. The purpose of this investigation is to examine the prospect of folate as a prevention strategy against depression. Components such as L-Methyl folate, Vitamin B2, and Vitamin D3 were used in combination on CUMS model of *Sprague dawley* rats. Animals used in this study, both male and female, were categorized into three distinct groups: the control group was given neither stress nor vitamins, the second group was subjected to stressors and subcutaneous saline injections, and the third group was subjected to stressors and subcutaneous vitamins injections daily for 21 days. Anhedonic behavior, locomotor activity, and despair-like behavior were analyzed by sucrose preference test, open field test, and forced swimming test to assess the preventive effect of folate in combination with these vitamins on the behavior of animals exposed to stress. The data revealed that the combination of L-Methyl folate with vitamins significantly prevented anhedonia, as evidenced by a sucrose preference of 80% (p < 0.0001). Moreover, it effectively preserved locomotor abilities, with rats covering a total distance of 80 meters in apparatus of OFT, in comparison to the 50% noted in depressed rats (p < 0.03). Additionally, the combination therapy prevented despair-like behavior, as shown by a decrease in immobility percentage to 50% compared to the 70% observed in depressed group (p < 0.01). These findings indicate that folate in combination with vitamin B2 and D3 can serve as a potential preventative candidate for stress-related mental health disorders.

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