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Research Article

Sodium-Glucose Co-Transporter-2 Inhibitors Act as AMPK Activators and Ameliorate the Depressive Symptoms Induced by Unpredictable Chronic Mild Stress in Mice

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ABSTRACT

Depression is a prevalent disorder of mental health with a highly multifaceted pathogenesis; thus, finding an effective therapy for it remains a challenge. Depression is associated with downregulation of AMPK signaling pathways and lowers AMP/ATP and ADP/ATP ratios. Brain AMPK pathway malfunction and metabolic imbalances induce depression. The unpredictable chronic mild stress (UCMS) model was created centered on the predisposition of the stress-associated assumption of depression. SGLT2 inhibitor drugs canagliflozin and remogliflozin enhance brain AMPK signaling, reduce oxidative stress, increase neurotransmitters, and improve metabolism. SGLT2 inhibitor drugs have promising antidepressant effects. Thus, in this investigation, we examined sodium-glucose co-transporter-2 inhibitors to ameliorate depression symptoms in mice induced by unpredictable chronic mild stress by improving AMPK signaling. Seven groups of male mice (Swiss albino) were divided into (n = 6). The disease groups received different stressors (unpredictable chronic mild stress model) for one week to produce depression, while normal control groups got 0.5% w/v saline (orally). The standard group received fluoxetine (10 mg/kg, orally), while the treatment groups were given canagliflozin (15 and 30 mg/kg, orally) and remogliflozin (10 and 30 mg/kg, orally). Behavioral parameters were assessed for induction of depression. Treatment with SGLT2 inhibitors showed significant (p < 0.05) antidepressant effects on behavioral, oxidative stress, neurotransmitter, brain histopathology, and gene expression evaluation of AMPK, mTOR, BDNF, and TNF- α levels. The current study found that SGLT2 inhibitor drugs have the potential to improve the AMPK signaling pathway and alleviate depression.

INTRODUCTION

Depression is a mental disorder, prevalent in nature and marked by long-term sad feelings as well as disinterest in activities that typically bring joy, coupled with an inability to perform daily tasks for a minimum of two weeks. It is a widespread ailment affecting individuals of various ages, genders, socioeconomic backgrounds, and religious affiliations both in India and globally. Approximately 322 million people were estimated to be grappling with depression on a global scale in 2015, with around 57 million affected individuals residing in India.^[1,2] Various theories

regarding the origins and triggers of depression have been proposed, including genetic susceptibility, alterations in the activity of the hypothalamic-pituitary-adrenal (HPA) axis, deficiencies in monoamines, malfunctions in specific brain regions, reduced function of gamma-aminobutyric acid (GABA), and alterations in neurotrophic and neurotoxic processes.^[3,4] Among these theories, one of the most widely accepted and empirically supported explanations revolves around the imbalance of monoamine levels in the brain.^[5] Down-regulation of any of these neurotransmitters is a hallmark feature of depression.

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Monoamines, including 5-hydroxytryptamine (5-HT), dopamine, epinephrine, and norepinephrine, have been recognized as key participants in the neurobiology of depression.^[6,7]

Depression is a psychiatric disorder that has garnered significant attention in individuals with diabetes, emerging as the most prevalent mental health condition among diabetic patients.^[8] The enzymatic part of mammalian 5' adenosine monophosphate-activated protein kinase (AMPK) was unveiled in 1994 through the successful cloning of the enzyme. The AMPK enzyme having serine or threonine kinase chiefly consists of three subunits viz. α , β and γ , which regulate metabolism centrally.^[9,10] Twelve different heterotrimeric combinations with two α -, two β -, and three γ -subunit isoforms. Adipocytes and the liver contain the α -1 isoform, whereas the brain, heart muscle, and skeletal muscle are the main locations of the α -2 isoform.^[11,12] To initiate AMPK, it is necessary to have an increase in the ratio of intracellular adenosine monophosphate to adenosine triphosphate. Additionally, one of AMPK's three upstream kinases - protein kinase activated by transforming growth factor- β (TGF- β) and liver kinase B1 (LKB1), or calcium/calmodulin-dependent protein kinase β must phosphorylate anti-phospho-AMPK- α on the α -subunit's "activation loop".^[13] These processes are crucial for the activation of AMPK. It is also possible to phosphorylate the α -1 subunit by Akt, PKA, or autophosphorylation, which can result in a variability of tissues counting the heart, adipocytes, and vascular smooth muscle cells. Activation of AMPK promotes the absorption of glucose, oxidation of fatty acids, and glycolysis.^[14] Within the liver, it enhances the absorption of glucose and the breakdown of fatty acids while inhibiting the production of new glucose, the synthesis of cholesterol, the creation of new fatty acids, and the formation of proteins.^[15] Furthermore, AMPK enhances food intake by stimulating the hypothalamus and decreases insulin production by pancreatic β -cells. Numerous triggers of 5' adenosine monophosphate-activated protein kinase (AMPK) have been identified, encompassing a range of physiological factors such as exercise, fasting, and calorie restriction, pharmacological agents including biguanides class of drugs, plant-derived flavonoids constituents stimulate the activity of AMPK pathway. In the previous studies, it is also reported that in depressive illness, AMPK is downregulated. This reduction in AMPK level in the brain is responsible for the diminution of autophagy, cell growth, and synthesis of proteins. Further, apoptotic pathways are also aroused and responsible for depression symptoms.^[13-15] The SGLT2 inhibitor class of drugs may have a potential role in the activation of the AMPK pathway, which in turn is responsible for increased autophagy, cell growth, protein synthesis and inhibition of apoptosis in brain cells. This possible mechanism of AMPK activation will be beneficial in the development of future

antidepressant drugs. Based on these considerations of AMPK activation, we select SGLT2 inhibitor drugs, namely canagliflozin and remogliflozin, to assess the pharmacological effect on depression induced by the UCMS model.

MATERIAL AND METHODS

Drugs and Chemicals

Canagliflozin was received as a gratis sample from Dr. Reddy's Laboratories in Hyderabad, India. Remogliflozin was received as a courtesy sample from Glenmark Pharmaceuticals Limited in Mumbai, India. Fluoxetine was received as a gratis sample from Palam Pharma Pvt. Ltd in Ahmedabad, India. Sandeep Organics Pvt. Ltd. India supplied all of the analytical grade chemicals and other reagents utilized in the experiment.

Animal Experiments and Protocol

Male Swiss albino mice, with a regular weight of 25 ± 2 g and 7 to 8 weeks old, were obtained from the Zydus Research Centre located in Ahmedabad provided the mice, which were then accommodated at the Sumandeep Vidyapeeth Deemed to be University - Department of Pharmacy. Typical conditions, such as a 12-hour light and dark cycle, $22 \pm 5^\circ\text{C}$ temperature, and regulated humidity at $55 \pm 5\%$, were maintained in the animal facility. The Institutional Animal Ethics Committee (IAEC) of Sumandeep Vidyapeeth permitted all experimental techniques defined in this work. Eligible under the standards of the Ministry of Social Justice and Empowerment, the Government of India's - Committee for Control and Supervision of Experiments on Animals (CCSEA) is the University of Vadodra's Department of Pharmacy. Protocol number SVU/DP/IAEC/2022/11/59 was adhered to during the experimentation process. Earlier initiation of the experiment, the mice were given a week to adjust to their new environment.

The animals' weights were recorded on the first day of the experiment so that the dosages could be calculated. All medication solutions were prepared by dissolving them in normal saline, and dose selection was done according to previous research. The different stressor approach was used to generate depression as per unpredictable chronic mild stress model-induced depressive symptoms in mice.^[13-15] Stressors included deprivation of food and water for a full day, cages sloping 45° , vibration exposure, swimming in 4° water, bedding deprivation for 16, 6 hours in a wet cage, 2 hours of restrained motion, 2 hours of constraint, and 2 hours of pairing. Table 1 presents the specifics of the stressors for a week. Following the conclusion of the stressor exposure, behavioral assessments were conducted to confirm the presence of depression. The mice were allocated randomly into seven groups, as outlined in Table 2. The treatment regimen

Table 1: Details of stressor procedure

Days	Early in the morning	In the afternoon	In the evening
Monday	Vibration of the cage	Wet pens	Swimming in cold water
Tuesday	Combining	sloping cage at a 45-degree angle	Constraint
Wednesday	Dry heat	Constraint	dumping of bedding
Thursday	Lack of food and water	-	-
Friday	Vibration of the cage	sloping cage at a 45-degree angle	Swimming in cold water
Saturday	Wet pens	Restrained motion	Constraint
Sunday	Pairing	Dry heat	dumping of bedding

spanned a duration of 4 weeks. After the treatment period, animals underwent evaluations encompassing behavioral analyses, biochemical and oxidative stress assessments, neurotransmitter, gene expression, and histopathological examinations. Brain samples were collected to measure neurotransmitters, oxidative stress parameters, and histopathological and gene expression features. Blood samples were obtained on the 36th day, the retro-orbital area was punctured under light anesthesia, and the samples were then stored in tubes containing EDTA. The serum was extracted by centrifuging the blood for 20 minutes at 5000 rpm after it had clotted for 15 minutes. The resultant serum was kept at -20°C for evaluation of antidepressant activity, i.e., body weight, behavioral parameters including FST, TST and locomotor activity measurement for stress-induced behavioral parameters were chosen. Biochemical parameters of glucose, total protein, cholesterol, triglyceride, and C-reactive protein (CRP). Neurotransmitter assay of dopamine and serotonin levels. Oxidative stress parameters include analysis of lipid peroxidation, reduced glutathione, superoxide dismutase, and nitrite levels. Gene expression of AMPK, TNF- α , mTOR, and BDNF, and histopathology of the brain. Detailed test procedures are described as follows:

Body Weight

During the experiment, body weight was to be dignified and noted from the first day until the 36th day.

Table 2: Animal group design protocol

Groups		Animals (Mice)
Group 1	Normal control group (0.5% w/v Saline)	6
Group 2	UCMS (Unpredictable chronic mild stress)	6
Group 3	UCMS+ Fluoxetine (10 mg/kg)	6
Group 4	UCMS+ Canagliflozin (15 mg/kg)	6
Group 5	UCMS+ Canagliflozin (30 mg/kg)	6
Group 6	UCMS+ Remogliflozin (10 mg/kg)	6
Group 7	UCMS+ Remogliflozin (30 mg/kg)	6

Behavioral Parameters

The depression symptoms of the forced swimming test

The assessment of depressive symptoms conduct often involves the use of the forced swim test. Following the conclusion of the treatment, the FST was conducted on all groups of mice. Each mouse was placed individually in 2-liter beakers filled halfway with water at 27°C. The duration of immobility for each mouse was then measured over 4 minutes.^[16]

The depression symptoms throughout the tail suspension test

The animals were adjoined by their tails from a tube approximately 10 to 12 cm above the ground for a duration of six minutes. Initially, the animals attempted to escape and reach the ground. The period during which the animals exhibited no movement or action was then recorded. Subsequently, the duration of immobility for each mouse was measured over the entire six-minute period.^[16]

Locomotor behavior

Locomotor activity serves as an indicator of animal behavior. The actophotometer was employed to monitor the locomotor behavior of mice. Each mouse was placed individually into the actophotometer, where the fundamental concept involves the animals moving from one side to another, passing through a beam, and the readings being displayed. The actophotometer recorded the baseline activity score over a period of 3 minutes.^[16,17]

Neurotransmitters assays

The levels of dopamine and serotonin were determined using the method described by Nazir S. *et al.* 2022.^[18]

Oxidative stress parameters

The methods by Bakhtiari-Dovvombaygi H *et al.* 2021 were used to estimate lipid peroxidation (MDA), estimate reduced glutathione (GSH), nitrite level, and superoxide dismutase (SOD).^[19]

Histopathology of brain

Diethyl ether overdosage was used to euthanize mice. After that, the brains were removed and stored in a formalin



solution (10%). The brain was decalcified (5% formic acid) and then paraffin-embedded. Hematoxylin and eosin were used to create and stain sections that were 5 μ m thick. The stained sections were then analyzed in a lab setting using light microscopy.^[18,20]

Gene Expression Analysis of Brain Tissue

Collection of brain

The animals were sedated with an intraperitoneal injection of 0.5 mg/kg xylazine and 80 mg/kg ketamine. The brain was placed in an RNA later solution and maintained at -20°C from the hypothalamic region (HTB) until the pure RNA could be extracted.^[20,21]

Separation of messenger RNA

With the utilization of an RNeasy kit and a slight modification to the phenol-chloroform procedure, the extraction of total RNA was accomplished. Essentially, the sample of frozen tissue was rinsed with phosphate-buffered saline to eliminate the solution, viz. RNA later. Tissue homogenization was done by using a powerful homogenizer and a specialized lysis reagent. The contents were held in a 1.5 mL tube after being filled. Once β -mercaptoethanol in 20 μ L quantity was added to the tube, it was vigorously mixed for 15 seconds and left aside at room temperature without disturbing for at least 5 minutes. Afterward, the mixture was blended with chloroform-isoamyl alcohol at the quantity of 400 μ L, for each mL of QiazolLysis reagent and strongly agitated for 15 seconds.

After waiting for 3 minutes at room temperature, the tube was centrifuged for 20 minutes at 4°C using a Sorvell SR8 centrifuge at a speed of 1200 g. The aqueous phase at the upper side was isolated in a fresh tube. In addition, 10M lithium chloride at the quantity of 200 μ L was added and thoroughly mixed. Afterward, the mixture was combined with a 0.5-fold amount of icy isopropanol and vigorously mixed for 15 seconds. The mixture was maintained at a temperature of -20°C for a period of one hour. Once removed from the fridge, the tube underwent centrifugation for 20 minutes at 4°C at 12000 g using a Sorvell ST8R centrifuge. The RNA pellet remained undisturbed during the careful removal of the supernatant. The pellet underwent centrifugation at 12000 g for 10 minutes at 4°C, following two rounds of cleaning with 70% ethanol. Using a pipette, the remaining ethanol was extracted and left to dry. Following that, the RNA pellet was dissolved in 70 μ L of RNase-free water and stored at -20°C. Using the QIAxpert System, the ratio of absorbance specifically at 260 as well as 280 nm, was utilized to assess the quantity and quality of RNA. The total RNA integrity was further confirmed by running it on a 1% agarose-formaldehyde gel. Pictures were captured using the Gel DocTM EZ Gel, and MOPS buffer- formaldehyde was used as the electrophoretic buffer.^[20, 21]

Real-time quantitative reverse transcription PCR (Real-Time qRT-PCR)

Exploited the QuantiTect® Reverse Transcription kit and the Veriti® 96-Well Thermal Cycler were used to reverse transcribe total RNA (1- μ g), as per instructions given by the manufacturer for the synthesis of first-strand cDNA. The qPCR analysis was conducted in the CFX 96 touch system, following the instructions provided by the manufacturer. A Quantifast Probe PCR kit was used, with an equivalent quantity of cDNA from individual samples. Exon-exon spanning primers for AMPK, TNF- α , mTOR, BDNF, as well as a control gene (ACTB: β -actin) were created utilizing the blast tool named NCBI primer to ensure the removal of any gDNA contamination. The primers were synthesized by Eurofins, as indicated in (Table 3). With the 7500 software v2.3, the 7500 Fast RTPCR was configured for 40 cycles of PCR reaction conditions. These conditions involved denaturation at 95°C for 3 seconds, followed by combined annealing and extension at 60°C for 30 seconds. Utilization of QuantiFast SYBR Green PCR Kit to assess the expression of genes of AMPK, tumor necrosis factor-alpha, mTOR and BDNF, following the procedure provided by the manufacturer. Each PCR reaction sample was carefully analyzed twice, using the $\Delta\Delta$ Ct method to calculate the quantification relatively centered on the average value of Ct.^[21]

Statistical Analysis

The mean \pm SEM was the intended method of presenting the data. Following a one-way analysis of variance (ANOVA), post-tests were conducted using a computer-based fitting program (Graph Pad Prism 8.0) to identify any significant differences through multiple comparisons. To determine the statistical significance, we utilized a significance level of $p < 0.05$.

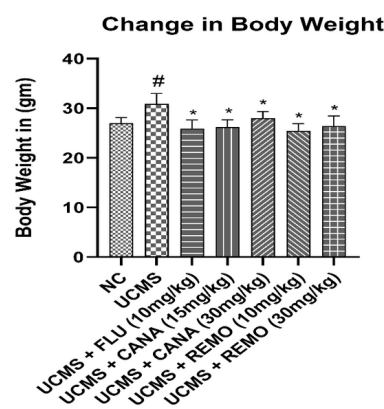


Fig. 1: Effect of SGLT2 inhibitor drugs on body weight in UCMS-induced mice model signifies a significant difference compared to the normal control group (# $p < 0.05$) and the disease control group (* $p < 0.05$). There are six animals in each group. Mean \pm SEM is used to express the values, (NC = Normal control group, UCMS = Unpredictable chronic mild stress, UCMS + Fluoxetine (FLU) treated 10 mg/kg, UCMS + Canagliflozin (CANA) treated with 15, 30 mg/kg, UCMS + Remogliflozin (REMO) treated with 10, 30 mg/kg.

Table 3: Brain tissue primer and labeled probe sequences were examined using qPCR

Name of gene	Moving order (5' >3') sequence	Reverse order (5' >3')	Size of fragments (bp)
AMPK	AGGGAGTTTAGGTTTCAGGCC	TACTCCAGGTTCCCTCTCGA	199
TNF- α	ATGGGCTCCCTCTCATCAGT	GCTTGGTGGTTTGCTACGAC	106
mTOR	CTTGCTGATCCTCAACGAGC	CCCTGACTTCATCCACCT	192
BDNF	TCCCAAGCCTGATGACCATT	TGGGCAATGAAGGAGATGGT	215
GAPDH	GCGGCATTAACCTCTGTCCTG	GTTCCTTACCTCCAGCTT	158

Table 4: Effect of SGLT2 inhibitor drugs on Behavioral parameters in UCMS Model

Groups	Forced swim test (Time in Sec)	Tail suspension test (Time in Sec)	Locomotor activity (Count)
NC	30.67 \pm 1.542	94.17 \pm 6.183	502.5 \pm 11.01
UCMS	65.50 \pm 1.962 [#]	164.7 \pm 4.208 [#]	303.3 \pm 28.58 [#]
UCMS+FLU (10 mg/kg)	34.50 \pm 3.274*	98.83 \pm 7.722*	440.8 \pm 11.65*
UCMS+CANA (15 mg/kg)	43.67 \pm 3.584*	113.8 \pm 3.135*	345.5 \pm 31.48*
UCMS+CANA (30 mg/kg)	40.00 \pm 1.390*	102.0 \pm 3.642*	407.3 \pm 11.21*
UCMS+REMO (10 mg/kg)	43.67 \pm 4.080*	111.8 \pm 2.496*	419.2 \pm 20.83*
UCMS+REMO (30 mg/kg)	39.50 \pm 2.778*	99.83 \pm 2.949*	449.2 \pm 12.34*

signifies a significant difference compared to the normal control group ($\#p < 0.05$) and the disease control group ($*p < 0.05$). There are six animals in each group. Mean \pm SEM is used to express the values, (NC = Normal control group, UCMS = Unpredictable chronic mild stress, UCMS + Fluoxetine (FLU) treated 10 mg/kg, UCMS + Canagliflozin (CANA) treated with 15, 30 mg/kg, UCMS + Remogliflozin (REMO) treated with 10, 30 mg/kg.

RESULTS

Effect on Body Weight by SGLT2 Inhibitor Drugs

The normal control group, compared to the animals' weight, increases significantly ($\#p < 0.05$) throughout the disease group of UCMS. The reduction of body weight of mice was observed, which was significant ($*p < 0.05$) after the commencement of treatment in comparison with the disease group of UCMS (Fig. 1).

Effect on Behavioral Parameters by SGLT2 Inhibitor Drugs

Force swim test

The disease group of UCMS had a significantly longer period of immobility than a group of normal control ($\#p < 0.05$). Comparing the disease groups to the treatment groups receiving canagliflozin, remogliflozin, and a standard control group of fluoxetine, a significant reduction in immobility time was observed ($p < 0.05$).

Tail suspension test

Animals' reduced range of motion indicates their immobility. In the current study, the disease group's unpredictable chronic mild stress (UCMS) immobility time increased in a significant manner as compared to the group of normal control ($\#p < 0.05$). Treatment groups of canagliflozin, remogliflozin, and Standard control

groups of fluoxetine have significantly ($*p < 0.05$) declined immobility times than the disease groups of UCMS.

Locomotor activity

In comparing the disease group's UCMS to the group of normal control, a declined locomotor activity was observed in a significant manner for the disease control group ($\#p < 0.05$). The locomotor activity was increased in a significant manner ($*p < 0.05$) for all the treatment groups (canagliflozin, remogliflozin and standard fluoxetine) in comparison with the disease control group (Table 4).

Effect on Neurotransmitters Level by SGLT2 Inhibitor Drugs

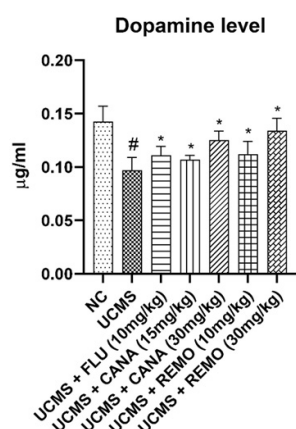
Dopamine level

Dopamine levels ($\mu\text{g/dl}$) in the UCMS group were significantly lower than in the normal control group ($\#p < 0.05$). In comparing the groups receiving treatment (canagliflozin, remogliflozin and standard fluoxetine), with the UCMS group the level of dopamine was increased in a significant manner ($*p < 0.05$) (Fig. 2).

Serotonin level

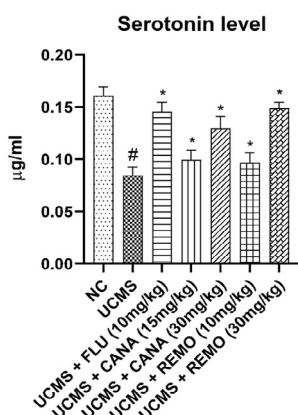
Serotonin levels ($\mu\text{g/dl}$) in the UCMS were significantly decreased as compared to the normal control group ($\#p < 0.05$). In comparing the groups receiving treatment (canagliflozin, remogliflozin and Standard fluoxetine),





signifies a significant difference compared to the normal control group ($\#p < 0.05$) and the disease control group ($*p < 0.05$). There are six animals in each group. Mean \pm SEM is used to express the values, (NC = Normal control group, UCMS = Unpredictable chronic mild stress, UCMS + Fluoxetine (FLU) treated 10 mg/kg, UCMS + Canagliflozin (CANA) treated with 15, 30 mg/kg, UCMS + Remogliflozin (REMO) treated with 10, 30 mg/kg).

Fig. 2: Effect of SGLT2 inhibitor drugs on dopamine level in UCMS model



signifies a significant difference compared to the normal control group ($\#p < 0.05$) and the disease control group ($*p < 0.05$). There are six animals in each group. Mean \pm SEM is used to express the values, (NC = Normal control group, UCMS = Unpredictable chronic mild stress, UCMS + Fluoxetine (FLU) treated 10 mg/kg, UCMS + Canagliflozin (CANA) treated with 15, 30 mg/kg, UCMS + Remogliflozin (REMO) treated with 10, 30 mg/kg).

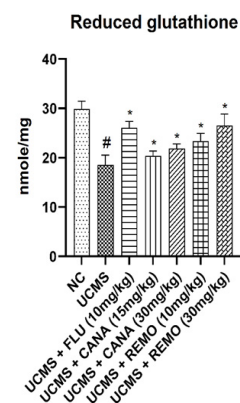
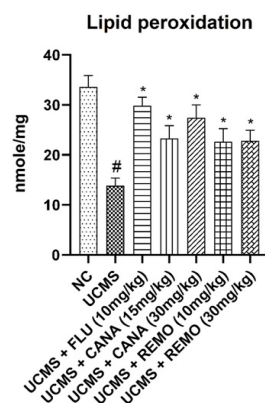
Fig. 3: Effect of SGLT2 inhibitor drugs on serotonin level in UCMS model

with the UCMS group the level of serotonin was increased in a significant manner ($*p < 0.05$) (Fig. 3).

Effect on Parameters of Oxidative Stress by SGLT2 Inhibitor Drugs

Level of lipid peroxidation and reduced glutathione

In comparison to the UCMS and group of normal control, a significant declined in the levels of lipid peroxidation



signifies a significant difference compared to the normal control group ($\#p < 0.05$) and the disease control group ($*p < 0.05$). There are six animals in each group. Mean \pm SEM is used to express the values, (NC = Normal control group, UCMS = Unpredictable chronic mild stress, UCMS + Fluoxetine (FLU) treated 10 mg/kg, UCMS + Canagliflozin (CANA) treated with 15, 30 mg/kg, UCMS + Remogliflozin (REMO) treated with 10, 30 mg/kg).

Fig. 4: Effect of SGLT2 inhibitor drugs on lipid peroxidation and reduced glutathione level in UCMS Model

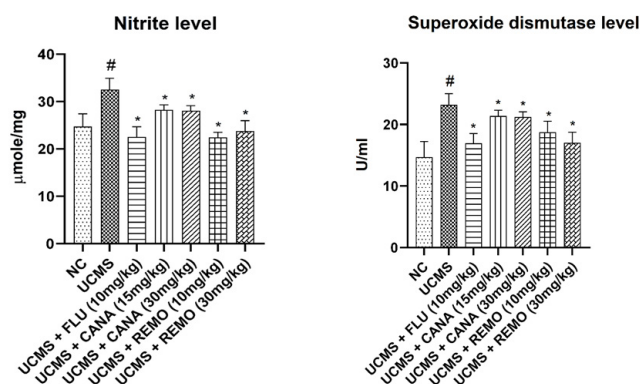
and reduced glutathione was observed. Comparing the groups receiving treatment (canagliflozin, remogliflozin and standard fluoxetine), with the UCMS group the level of lipid peroxidation and reduced glutathione was increased in a significant manner ($*p < 0.05$) (Fig. 4).

Nitrite and superoxide dismutase levels

In comparison to the UCMS and group of normal control, a significant increase in the levels of nitrite and superoxide dismutase was observed. Comparing the groups receiving treatment (canagliflozin, remogliflozin and Standard fluoxetine), with the UCMS group, the level of nitrite and superoxide dismutase was decreased in a significant manner ($*p < 0.05$) (Fig. 5).

Effect on Evaluation of Brain Histopathology by SGLT2 Inhibitor Drugs

Analyses of the brain's histology reveal that while the disease group- unpredictable chronic mild stress (UCMS) treated with SGLT2 inhibitor drugs significantly lessened the damage to the cells, the disease control group's neurons were destroyed. Neuronal cell architecture in the disease treated with canagliflozin and remogliflozin was somewhat similar to that of the normal control group, suggesting less cell damage in comparison to the disease control groups. Thus, this result points to the beneficial effects of the SGLT2 inhibitor drugs on brain histology. Normal nerve cells are shown by green arrows. Pyknotic cells are irregular, shrunken, and have a damaged nucleus. Pyknosis is a condition that affects senescent (old) leukocytes and is caused by programmed cell death



signifies a significant difference compared to the normal control group ($\#p < 0.05$) and the disease control group ($*p < 0.05$). There are six animals in each group. Mean \pm SEM is used to express the values, (NC = Normal control group, UCMS = Unpredictable chronic mild stress, UCMS + Fluoxetine (FLU) treated 10 mg/kg, UCMS + Canagliflozin (CANA) treated with 15, 30 mg/kg, UCMS + Remogliflozin (REMO) treated with 10, 30 mg/kg).

Fig. 5: Effect of SGLT2 inhibitor drugs on nitrite and superoxide dismutase level in UCMS Model

(apoptosis). Pyknosis is characterized by a thick, compact nucleus that fragments (karyorrhexis), resulting in the appearance of dark-staining nuclear chromatin spheres, indicated by red arrows (H and E, $\times 10$) (Fig. 6).

Effect on the Expression of mRNA levels (AMPK, Tumor Necrosis Factor- α , mTOR and BDNF) in the Hypothalamic Region of the Brain (HTB) in Mice by SGLT2 Inhibitor Drugs

The comparative gene expression profiling of pro-inflammatory cytokines such as TNF- α and AMPK, BDNF, and mTOR in the hypothalamus region was quantified using the Ct value acquired from qPCR in brain tissue. The disease group - unpredictable chronic mild stress (UCMS) had significantly lower levels of AMPK, BDNF, and mTOR mRNA expression compared to the treatment group of canagliflozin (15 and 30 mg/kg), remogliflozin (10 and 30 mg/kg) significantly raised the expression of mRNA levels specifically AMPK, BDNF in addition mTOR in HTB ($*p < 0.05$). There was a notable rise or overexpression in the mRNA expression levels of TNF- α in the disease group of unpredictable chronic mild stress (UCMS) that had after-treatment groups with significant decrease or downregulation of TNF- α mRNA expression levels perceived in HTB ($*p < 0.05$) (Fig. 7, 8).

DISCUSSION

Depression is a crippling psychiatric condition that significantly impacts patients' quality of life, affecting millions worldwide. A challenge in treating depression is the absence of a specific target that provides effective long-term antidepressant effects. Depression is associated with the dysregulation of HPA axis due to varied types

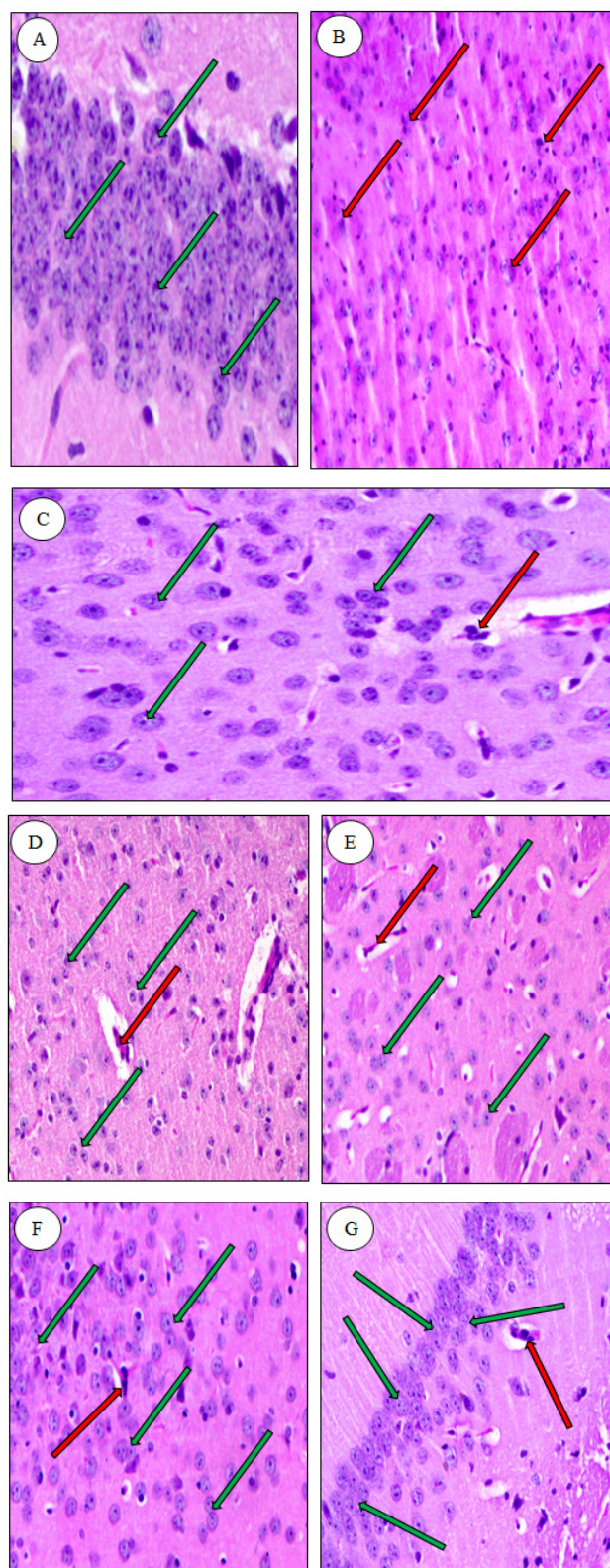


Fig. 6: Effect of SGLT2 inhibitor drugs on evaluation of brain histopathology: A - Normal control group, B- Disease group (UCMS), C- Standard control group (FLU), D- Treatment control group (CANA - 15 mg/kg), E- Treatment control group (CANA - 30 mg/kg), F- Treatment control group (REMO - 10 mg/kg), G- Treatment control group (REMO - 30 mg/kg)



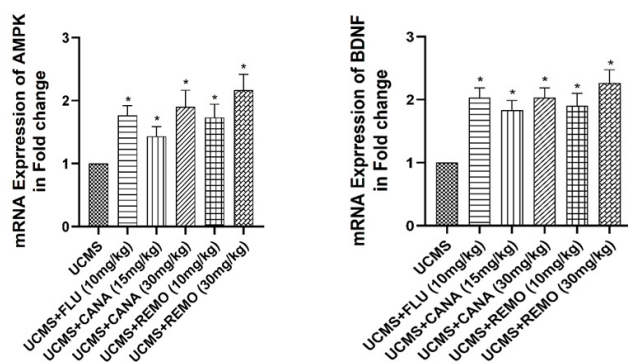


Fig. 7: Effect of SGLT2 inhibitor drugs on mRNA expression levels of AMPK, BDNF in the hypothalamic region of the brain (HTB) in mice analyzed by using qPCR. The treated group compared to the disease group of unpredictable chronic mild stress (UCMS) group is shown by the bars. The values ($n = 6$) are expressed as Mean \pm S.E.M., (* $p < 0.05$)

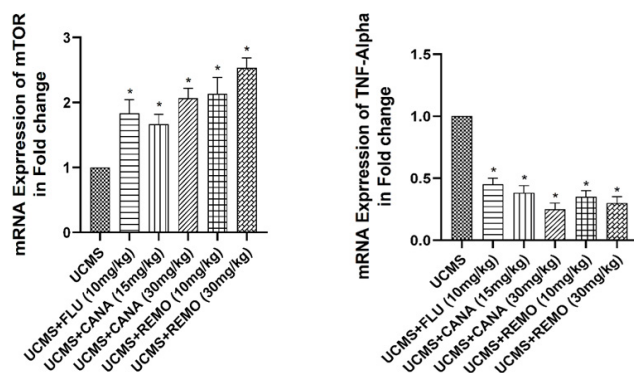


Fig. 8: Effect of SGLT2 inhibitor drugs on mRNA expression levels of mTOR and TNF- α in the hypothalamic region of the brain (HTB) in mice analyzed by using qPCR. The treated group compared to the disease group of unpredictable chronic mild stress (UCMS) group is shown by the bars. The values ($n = 6$) are expressed as Mean \pm S.E.M., (* $p < 0.05$)

of stress. The hypothalamic pituitary adrenal axis (HPA axis) is impacted by AMPK activation as well. The HPA axis decreased stress tolerance. The hormone responsible for the release of corticotrophin is secreted from the hypothalamus and binds to corticotrophin-releasing hormone receptors in the pituitary gland to trigger the HPA axis response. When this receptor interacts with the ACTH receptor on the adrenal gland, adrenocorticotrophic hormone is released. When this receptor is activated, cortisol is released, and cortisol regulates blood sugar, metabolism, memory formation, inflammation, and regulation. Numerous studies indicate that the AMPK activator is important in the pathophysiology of depression.^[5,6] The body's master regulator, AMPK, is made up of regulatory β - and gamma-subunits as well as catalytic alpha-subunits. Two isoforms of the alpha-subunit exist: alpha-1 and alpha-2. Gamma-subunits have three gamma-1, 2, and 3 isoforms, while regulatory β has two

β -1, 2 isoforms.^[7,8] Each of these isoforms has a distinct job and function. Prior research has demonstrated that AMPK is involved in inflammatory responses, metabolism of lipids and glucose, synthesis of specific proteins, redox control, autophagy, and biogenesis of mitochondria. It affects how fat and protein are metabolized in diabetes mellitus (DM), which is closely related to mTOR activity and insulin resistance. Inhibiting the oxidation of fatty acids, which prevents the consumption of energy and the production of proteins, AMPK is the downstream substrate that restores energy levels. Alpha-subunit's Thr172 on the activation loop at number 7 is phosphorylated by one of the three AMPK's kinetic processes when the protein is activated. This boosts the intracellular AMP:ATP ratio. By increasing glucose absorption, translocation of GLUT 4, oxidation of fatty acid and downregulating the synthesis of glycogen as well as proteins, this activation directly impacts skeletal muscle. As it inhibits gluconeogenesis and cholesterol, it operates in the liver by promoting the absorption of glucose and fatty acid oxidation.^[9,10]

AMPK activator phosphorylates AMPK through an upstream route. By phosphorylating Bcl2, AMPK phosphorylation triggers the PI3 K/Akt pathway, which promotes survival and inhibits apoptosis. Additionally, mTOR, a regulator of the autophagy process, is modulated by PI3K/Akt. AMPK activation on the alpha-subunit causes the phosphorylation of the ULK1 pathway, and an increase in AMP/ATP levels causes the autophagy process to be stimulated. XPC and TSC1/2 pathways are activated by AMPK activation on the γ subunit, and TSC1/2 promotes mTOR to promote protein synthesis and cell growth while reducing symptoms of depression.^[11-13]

An increasing amount of research indicates a connection between diabetes and stress progression. In laboratories, animal models such as UCMS and the isolation of social development are frequently employed to simulate the onset of human depression.

Using a UCMS model, mice were given one week of different stressors.^[14,15,17] Numerous studies have shown that chronic stress produces weight gains because it increases food intake. Additionally, UCMS groups have been shown to further increase food intake. The body weight of the disease groups of mice in the current study increased significantly during disease induction, but it decreased significantly following treatment groups and the standard control group.

To assess the depression-like behavior in mice, three parameters, viz. the forced swim test (FST), tail suspension test (TST) and locomotor activity as well were used, which show behavioral despair and social fear in experimental animals. These tests were used to confirm depression in the UCMS group, which showed a significant increase in immobility time in FST and TST, further decreasing locomotor count. Following treatment groups, in this investigation, the groups treated with canagliflozin, remogliflozin, and standard fluoxetine showed a significant

reduction of immobility time in FST and TST, as well as a significant increase in locomotor activity in comparison to the disease control group. The study's behavioral characteristics showed that both canagliflozin and remogliflozin were as effective as a standard control group of fluoxetine at reducing depressive symptoms.^[16]

Synapses between neurons and target cells are facilitated by endogenous chemicals called neurotransmitters. Major excitatory neurotransmitters in the brain and spinal cord are dopamine and serotonin; these specific neurotransmitters are chiefly responsible for a multiplicity of diseases, which include anxiety, epilepsy, schizophrenia and other common neurological disorders. Dopamine regulates the actions of other monoamines and serves as a neurotransmitter and neuromodulator inside the brain. Dopamine levels fall together with dopamine transporter levels in depressed conditions. Anxiety, mood, appetite, temperature, eating habits, sexual behavior, and movement are all influenced by the monoamine neurotransmitter serotonin. Furthermore, it has been shown that insulin resistance causes behavioral disorders by changing the way serotonin is converted in the brain. In the current investigation, the UCMS group significantly decreased dopamine and serotonin levels. After the treatment, it was observed that dopamine and serotonin levels were increased in a significant manner. These findings suggest that the treatments reduce dysfunction related to dopamine and serotonin turnover in the brain.^[18]

The brain's antioxidant activity has been demonstrated to decline under conditions of constraint stress, according to numerous research. When the hypothalamic-pituitary-adrenal (HPA) axis is triggered by stress, leading to the production of reactive oxygen species, the adrenal gland releases glucocorticoids. Because endogenous antioxidants are known to express themselves rather poorly in neurons, oxidative stress can harm them greatly. Between the production of healthy oxidants and harmful oxidative stress, antioxidants establish an equilibrium. The observations reveal that the levels of lipid peroxidation and reduced glutathione antioxidant enzymes are decreased significantly in the disease group (UCMS). The treatment groups of remogliflozin, canagliflozin and standard fluoxetine ameliorate the decreased levels of antioxidant enzymes. In addition, the nitrite and superoxide dismutase levels were increased in diseased groups, and after being treated with remogliflozin, canagliflozin and standard fluoxetine showed a significant reduction in the nitrite and superoxide dismutase levels. These findings indicate that therapies are keeping the redox state in balance.^[18,19]

Important brain regions for learning and memory processes are the prefrontal cortex, the hypothalamus, and the hippocampal regions. Small nuclei in the hypothalamus carry out a variety of tasks. Through the pituitary gland, it connects the endocrine and neurological systems. The hormones secreted by the pituitary gland are either

stimulated or inhibited by the hypothalamus hormones. According to earlier research, depression modifies the morphology of the hippocampus, which changes how the structure operates. The brain's hypothalamus region (HTB) histopathological analysis indicates that the disease group's UCMS of brain histopathology shows some morphological alterations and neurons were destroyed. Normal nerve cells are shown by green arrows. Pyknotic cells are irregular, shrunken, and have a damaged nucleus. Pyknosis is a condition that affects senescent (old) leukocytes and is caused by programmed cell death (apoptosis). Pyknosis is characterized by a thick, compact nucleus that fragments (karyorrhexis), resulting in the appearance of dark-staining nuclear chromatin spheres, indicated by red arrows (H and E, $\times 10$). The majority of the histopathological data point to the beneficial effects of remogliflozin and canagliflozin treatment doses. Our research revealed that canagliflozin and remogliflozin enhance brain function in the regions such as the hippocampus, hypothalamus as well as prefrontal cortex.^[19,20] An imbalance in the synaptic structural plasticity of all the given specific three regions of the brain has been associated with depression in animal models and human patients. The above given three regions of the brain in depressive patients possess atrophy significantly due to reasons such as reduced dendritic branches and their length, as well as spinal density of hippocampus neurons.^[13,15] Depressed people tend to have smaller hippocampus size, which is linked with the harshness of their depression. Previous animal studies reveal that persistent stress induces prefrontal cortex and hippocampal dendritic shrinkage and neuronal death, causing depression-like behaviors.

A neurotrophin called BDNF is essential for the plasticity of synapses and is important for both depression and antidepressant therapy. To better understand how canagliflozin and remogliflozin regulate BDNF expression and potentially improve depressive-like behaviors brought on by stress, we looked at this relationship. Our results indicated that the treatment with canagliflozin and remogliflozin, BDNF mRNA, and protein expression increased extremely significantly and fluoxetine treatment results were also significant. According to previous research, the active CREB pathway engages the histone acetyltransferase CBP and escalates the gene transcription process which includes BDNF, that regulates synaptic plasticity. By facilitating the acetylation process of histones such as H3 and H4, the latter changes the chromatin's structure to a relaxed one, increasing transcription factor accessibility. Thus, we looked into the function of AMPK/CREB signaling to learn more about the mechanistic process behind the raised expression of BDNF. Our results also demonstrated that the phosphorylation of AMPK and CREB was significantly increased in the prefrontal cortex as well as in the hypothalamus and hippocampus,



after the treatment with canagliflozin, remogliflozin, and fluoxetine. A key player in the control of bioenergy metabolism is AMP-activated protein kinase (AMPK). The human body's homeostasis is maintained by AMPK, an essential energy susceptor that controls the cell's network of energy metabolism and raises the capacity of brain cells to withstand inner and outward stress. The findings reveal that in all three specific brain regions, viz. prefrontal cortex, hippocampus, and hypothalamus of mice there was enhanced AMPK phosphorylation and mRNA expression after treatment. Additionally, AMPK activation through our treatment drugs points toward the promising future drug for antidepressant therapy.^[22, 23]

Depression's neuropathophysiology heavily relies on mTOR signaling. Depression has been linked to cytokines and neuroinflammation. In the treatment of mental illnesses, various inflammation-suppressing agents have proved beneficial. It has been shown in multiple investigations that symptoms resembling depression arise as a result of elevated neuroinflammation. Lately, there has been evidence linking the mTOR pathway to serious depression in both experimental animal models and humans. In the current investigation, the route could be a viable target for new antidepressant medication discovery. The activation of the mRNA expression of the mTOR pathway is highly correlated with SGLT-2 inhibitor drugs canagliflozin and remogliflozin. Both the treatment drugs significantly increased the expression of mTOR levels as compared to the disease group. Increased p70S6K phosphorylation in the prefrontal cortex and confirmed activation of the mTOR pathway through AMPK activation were observed in our experiments. These findings of our study suggest that the raised activation of the mTOR pathway gives rise to cell growth and protein synthesis in brain cells. This molecular mechanism activation is beneficial for the future development of antidepressant therapy.^[23, 24] Certain investigations identified that increased levels of TNF- α are found in patients with depressive disorder. Raised TNF- α is directly linked to the decreased generation of neurons in the hippocampus, decreased levels of BDNF and the expression of its receptors, in addition, increased levels of IDO in a depressive state. Functional TNF- α gene polymorphisms, specifically at position-308(A/G), have been linked in certain studies to a hereditary vulnerability to depression.^[25-27] Notably, there exists contradictory data in the literature about TNF- α levels in blood among those afflicted with depression. It was suggested that the hypothalamus-pituitary-adrenal (HPA) axis was more activated, leading to a higher cortisol production that inhibited the release of TNF- α . Our study suggests that the activation of the mRNA expression of the TNF- α in the disease group was significantly reduced with the treatment drugs at different doses. So, the current investigation suggests that in the prefrontal cortex, hippocampus, and hypothalamus region of the brain, there is enhanced AMPK, BDNF, mTOR, and decreased TNF- α

regulation of mRNA expression with the treatment drugs at dose-dependent manner.

CONCLUSION

In conclusion, our results show that the unpredictable chronic mild stress UCMS impairs cortical synaptic functioning and causes anxiety/depression. UCMS inactivates AMPK subunits α , β , γ for energy control. Different stressors of the UCMS paradigm may inactivate the AMPK pathway, which may contribute to depression. SGLT2 inhibitor drugs of canagliflozin and remogliflozin enhanced stimulation of the AMPK signaling pathway of subunits α , β , γ phosphorylation. Specifically, phosphorylation of α -subunits activates ULK1 phosphorylation to increase autophagy, which will be beneficial in anti-depression treatment. γ -subunit phosphorylation causes activation of TSC1/2 phosphorylation to activate the mTOR pathway, which in turn stimulates cell growth and protein synthesis in neuronal cells and also inhibits apoptosis, which proved progressive effects in the management of depression. Our findings state that the use of SGLT2 inhibitor drugs in the therapy of depression works through the possible mechanism of AMPK pathway activation.

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