Section A-Research paper

Anti-Diabetic and Anti-Depressant Potential of Canagliflozin, Remogliflozin and Teneligliptin in UCMS model of Zebrafish: Behavioral and Physiological Responses

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Introduction

Diabetes is a chronic and progressive syndrome commonly associated with several neuropsychiatric comorbities, of which depression is the most studied. The prevalence of depression is about two or three times higher in diabetic patients compared to the general population. It is believed that the diabetes - depression relation is bidirectional, i.e., the depression can lead to diabetes and conversely diabetes could facilitate the emergence of depression. Depression is one of the most neglected symptoms in diabetic patients and is directly linked with lowering of quality of life. The treatment of depression in these patients is still quite ineffective and in many cases treatment-refractory.[1, 2] Furthermore, some of the first choice drugs used to treat the depression affect the blood glucose control, aggravating the hyperglycemic state. These issues underscore the urgency in studies searching for new pharmacological targets for the treatment of depression associated with diabetes. For this, a better understanding of the pathophysiology that relates this comorbidity becomes critical. In this respect, this review will focus on some hypotheses that have been proposed to explain the mechanisms underlying depression associated with diabetes, highlighting the treatment options currently available and their limitations.[3] Among these hypotheses, we will point out the hyperglycemia as a primary metabolic cause of the depression development, the involvement of the dysregulation of hypothalamic pituitary-adrenal (HPA) axis and of neurotransmitter systems, especially monoaminergic system.Besides, the role of oxidative stress, neuro-inflammation and cell death, especially in hippocampus and prefrontal cortex, brain areas important for the mediation and modulation of emotional behavior will also be discussed. Finally, we will bring up the influence of the epigenetic regulation with respect to neuropsychiatric disorders. Emerging evidence suggests that these outcomes may be important targets for future depression research in T2DM[4].

Role of AMPK in diabetes mellitus & Depression

AMPK is a serine/threonine protein kinase that regulates cellular energy homeostasis by mainly activating glucose and fatty acid absorption, as well as oxidation when cellular energy is low. It is made up of three subunits that work together to produce pharmacological effect[(5)]. It stimulates hepatic fatty acid oxidation, ketogenesis, glucose uptake, skeletal muscle stimulation, suppression of cholesterol synthesis, triglycerides synthesis, and lipogenesis by binding to adenosine diphosphate (ADP) and adenosine monophosphate (AMP).[(6)] The allosteric activation of AMPK by AMP results in two types of effects. The first is negative regulation, while the second is positive regulation. Lipogenesis, glycogenesis, and protein synthesis are examples of negative regulation, whereas glucose uptake and glycolysis, fatty acid oxidation, and autophagy are examples of positive regulation.[(7)] According to many findings, AMPK is downregulated in depressive states. Neurons and estrocytes are both damaged at this lower level. The ULKI pathway activates the AMPK pathway, which then activates the mTOR pathway.[(8)] In neurons, aberrant and harmful proteins accumulate during cellular stress. The m-TOR pathway can be activated in a variety of ways. ULKI appears to play a role in autophagy control in depressive states, according to a variety of clinical and preclinical findings.[(9)] In depression, autophagy regulates catabolic processes and is important for neuronal growth regulation. AMPK activators are critical for autophagy activation. AMPK is transiently unregulated during stress, which is accompanied by a shift in the cellular ratio of AMP to ATP.[(10)] AMPK activation maintains the AMP/ATP ratio and contributes to protein synthesis and neuronal development. AMPK has an anti-diabetic effect through regulating lipid and protein metabolism, which activates the mTOR, DAG, and other pathways. This results in insulin resistance as well as the mutation and proliferation of the stimulating environment. The anti-diabetic effect of AMPK is achieved via activating the PI3K/AKT pathway, which activates the mTOR and ULK-1 pathways. AMPK is activated by an increase in the intracellular AMP: ATP ratio, as well as phosphorylation of Thr172 on the -subunit's "activation loop"7 by one of its three upstream kinases. Multiple tissues are affected by this activation. Its activation increases or stimulates glucose uptake, GLUT 4 translocation, fatty acid oxidation, and inhibits glycogen and protein synthesis in skeletal muscles.[(11)] It stimulates glucose absorption and fatty acid oxidation in the liver, but inhibits gluconeogenesis and cholesterol production. It stimulates the same in adipose tissue, but it slows insulin release from pancreatic -cells, and the signal provided by insulin increases food intake in the hypothalamus.[(12)]

Upregulation or increased phosphorylation of these pathways, which have a role in cell death and neurotransmitter release, has been suggested in several studies. These circuits are down regulated in depressive behaviour. As a result, Canagliflozin & Remogliflozin, Teneligliptin may be helpful in the treatment of depression. As a result, we focused on the anti-depressive role of particular anti-diabetic medications that work through AMPK while researching new therapy options for depression. As a result, the goal of our research was to look into the facts of an anti-diabetic medicine in a UCMS-induced depression model as well as possible molecular mechanisms.

MATERIAL AND METHODS

Drugs and chemicals

Canagliflozin - Dr. Reddy's Laboratories Hyderabad, India, Remogliflozin- Glenmark Pharmaceuticals Limited Mumbai, India, Tenegliptin- Ami Lifesciences Pvt. Ltd Gujarat Vadodara, Fluoxetine- Palam Pharma Pvt. Ltd Ahmedabad, Gujarat, Metformin- Atom Pharma, Surat, Gujarat, was obtained as gift sample. All other reagents and chemical used in the experiment were of analytical grade purchased from SD Fine Chemical, Mumbai, India.

Animals and housing

A total of 80 adult (~50:50 males: female ratio) "wild type" (short fin) zebrafish (D. rerio) were obtained from a commercial supplier (Rajkot, Gujarat). All fish were acclimated for at least two weeks in the experimental room and housed in groups of 8 fish in 10, heated (28± 2 °C) tanks with constant aerated water. Fish were kept on a 14– 10 h day/night cycle and fed three times a day with commercial flakes and supplemented. All protocols were approved by the Institutional Animal Ethics Committee (IAEC) and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Proposal number: RKCP/COI/Re/21/122.

Experimental design and treatments

The zebrafish was divided in to eight group, Normal control, Diabetes control, Diabetes + UCMS control, Diabetes + UCMS + Canagliflozin & Remogliflozin and Diabetes + UCMS + Teneligliptin, Diabetes +UCMS + Fluoxetine and Diabetes +UCMS + Metformin.

Diabetes was induced by the fish were placed in fish tanks (n=10) for a period of 14 days; the tanks contained a 111 mM glucose solution[13, 14]. At the end of the induction of diabetes, fish were taken at random from each batch to corroborate their diabetic status, which was maintained throughout the experiment. On 15oth day of induction Started stressor fish were submitted twice a day to one of the following stressors [15] (Table 1): restraint stress, consisting of maintaining each animal for 90 min inside a small 2 ml micro centrifuge tube open in both ends to allow water flow; heating tank water up to 33 °C for 30 min; social isolation, maintaining animals alone for 45 min in a 250 ml beaker; cooling tank water up to 23 °C for 30 min; crowding of 10 animals for 50 min in a 250 ml beaker; exposition to predator (Archocentrus nigrofasciatus fish) in close proximity for 50 min but avoiding direct contact; low water level on housing tanks until animals' dorsal body wall were exposed for 2 min; tank water replacement, three consecutive times with animals inside; tank change, three consecutive times; and chasing animals for 8 min with a net. Aeration and temperature were controlled during each stressor presentation (except during heating and cooling stress). To prevent habituation and maintain unpredictability, time and sequence of stressors' presentation were changed daily. A non-stressed control group remained in the same room during the equivalent period. Despite the stressful conditions intermittently presented to the fish, no extreme suffering was caused nor abnormal number of deaths observed. At the end of the induction of diabetes and depression, fish were taken at random from each batch to corroborate their diabetic status, which was maintained throughout the experiment.[16] After completion of induction, treatment of Canagliflozin & Remogliflozin

(10 uM) , Teneligliptin and Fluoxetine & Metformin (10 uM) given to zebrafish for 7 days. Table no.1:

Weeks	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	9:00 am Restraint stress 2:00 pm Heating	10:00 am Social isolation 4:00 pm Cooling	10:30 am Crowding 1:30 pm Predator	9:00 am Low water level 3:00 pm Tank change	8:00 am Cooling 2:00 pm Crowding	11:00 am Tank change 5:30 pm Chasing	8:00 am Heating 12:00 pm Social isolation
Week 2	10:00 am Tank change 4:00 pm Tank change	11:00 am Predator 2:30 pm Heating	10:30 am Low water level 3:00 pm Chasing	8:00 am Tank change 1:00 pm Crowding	9:30 am Restraint stress 5:00 pm Low water level	8:30 am Social isolation 1:00 pm Cooling	9:00 am Tank change 5:30 pm Chasing

1) Behavioral apparatus:

One week after stress, all fish were retested in the behavior apparatus with the aim of evaluating the potential residual effect of stress in the fish.[15, 17]

1.1) Locomotion

Locomotor activity was used as a general index of behavioral excitation/inhibition. Activity was evaluated by comparison to control group using the following scores: 1 — virtually immobile; 2 — slower than normal; 3 — normal; 4 — increased locomotion; 5 — intense locomotion.

1.2) Color

Zebrafish change their color in response to certain stimuli. Fish that exhibit signs of fear (e.g. freezing or erratic movement) quickly become pale, especially when the background is light. When fish are more excited or aggressive, they become more chatoyant. Fish color was rated visually by comparing to control group and scored as follows: 1 — pale; 2 — lighter than normal but not pale; 3 — normal; 4 — darker than normal but not chatoyant with dark-blue stripes; 5 — chatoyant with darkblue stripes.

1.3) Shoal cohesion.

Zebrafish prefer swimming in groups and group aggregation is termed shoal cohesion. This behavioral strategy is thought to be effective against predators in several fish species. In contrast to other studies using only one fish during experiments, placing three in the test tank allows

maintenance of their natural shoaling behavior. Shoal cohesion was measured by comparison to control group according to the following scores: 1 — complete lack of group cohesion or fish interaction; 2 — loose or partial shoaling behavior; 3 — normal distance and shoaling behavior; 4 — increased shoal cohesion.

Anesthesia and Sacrifice

The fish were placed in cold water at 4 ◦C to induce hypothermia; signs of having reached stage III of anesthesia were monitored when loss of balance, loss of operculum movements and loss of reactivity were observed. Later, the sacrificing of the fish was performed using a scalpel by making a cross-section cut in the back of the fish (tail) to obtain blood, which was used immediately to carry out the various analyses[18, 19].

Analysis of Blood Glucose:

To measure the different biochemical parameters, the fish in each batch were fasted for 12 h. These were subsequently transferred to fish tanks with glucose-free water for a period of 15 min. Next, the fish in each batch were anesthetized and sacrificed (as shown in the anesthesia and sacrifice section) to obtain blood, which was used to determine the Glucose levels were measured by means of a glucometer (Accu-Chek, Roche Diabetes Care India Pvt Ltd.[14, 20, 21]

Statistical analysis

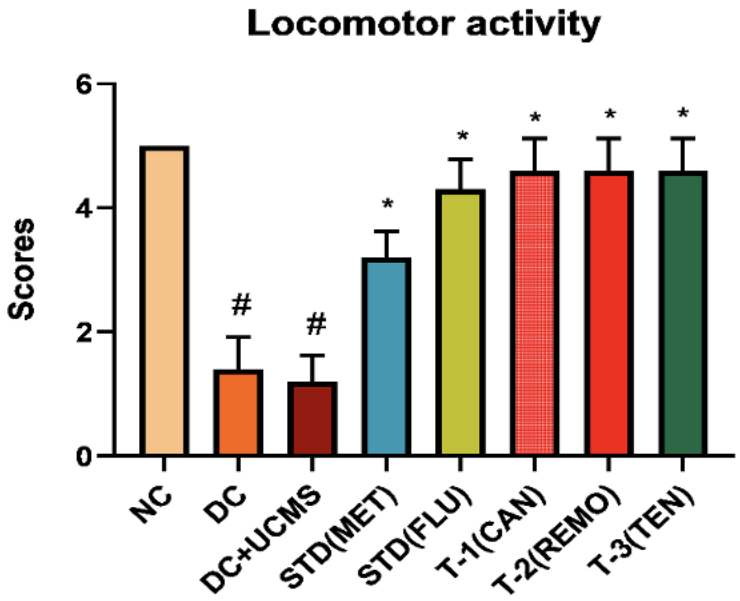
All the values are stated as mean ± SEM. Control group and treatment groups are statistically tested using one-way ANOVA followed by post hoc Bonferroni multiple comparisons in Prism 5, GraphPad Software, Inc. The significance level was set at $P < 0.05$ OR $P<0.001$ for all tests.

Result:

1. Behavioral apparatus

a) Locomotion activity:

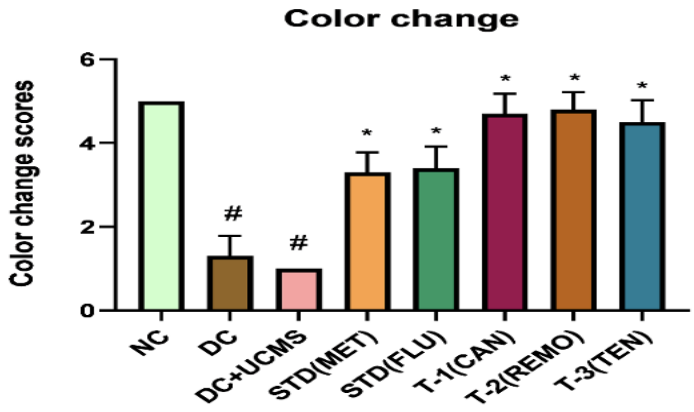
Locomotor activity was used as a general index of behavioral excitation/inhibition. Activity was evaluated by comparing to ‘internal control’ fish, using the following scores: 1— virtually immobile; 2— slower than normal; 3—normal; 4—increased locomotion; and 5— intense locomotion. This score has a 0.50 correlation (Spearman test) with an objective measure using crossing counts (frontal area of the tank divided in 9 rectangles) performed by separate and independent observers blinded to each other’s' results (n= 10). Although this correlation level is only moderate, it is acceptable to detect gross changes in locomotion.



significantly different from normal control group. * significantly different from disease control group. #: p ***<0.001. Each group consists of n = 10. Values are expressed as Mean ± SEM, (NC = normal control group, DM = Diabetes mellitus, DM+UCMS = Disease control, DM+ UCMS = Diabetic treated with Remogliflozin, Canagliflozin, Teneligliptin, (10 uM) UCMS+DM+MET = Disease control treated with metformin, UCMS+DM+FLU = Disease treated with fluoxetine (10 uM). A significant decrease activity was found in disease control group as compared to normal control group. While significantly increase activity in disease treated with Remogliflozin, Canagliflozin, Teneligliptin compared to the disease control group.

b) Color change:

Zebrafish change their color in response to certain stimuli. Fish that exhibit signs of fear (e.g., freezing or erratic movement) quickly become pale, especially when the background is light. When fish are more excited or aggressive, they become more chatoyant. Fish color was rated visually by comparing to 'internal control' fish and scored as follows: 1—pale; 2—lighter than normal but not pale; 3—normal; 4—darker than normal but not chatoyant with dark-blue stripes; and 5—chatoyant with dark-blue stripes.



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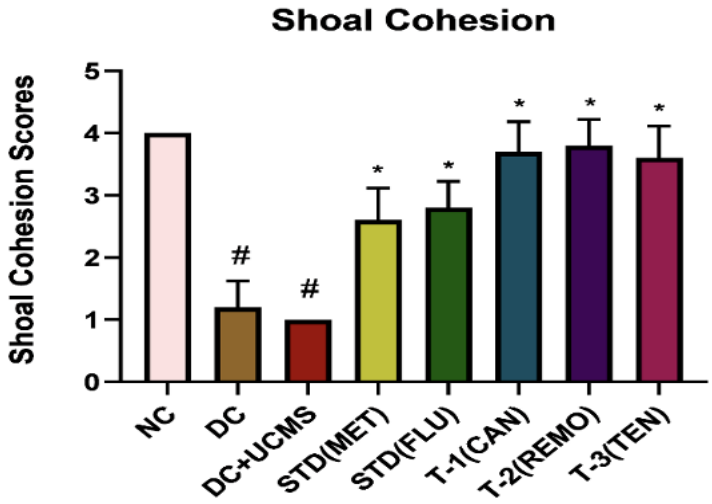
c) Shoal cohesion:

Zebrafish prefer swimming in groups and group aggregation is termed shoal cohesion. This behavioral strategy is thought to be effective against predators in several fish species. In contrast to other studies using only one fish during experiments, placing three in the test tank allows the maintenance of their natural shoal behavior.

Shoal cohesion was measured individually by comparing to 'internal control' fish (i.e., a group of three untreated fish habituated in an independent tank) according to the following scores: 1—complete lack of group cohesion or fish interaction; 2—loose or partial shoaling behavior; 3—normal distance and shoaling behavior com- pared to 'internal control'; and 4—increased shoal cohesion. This score has a −0.81 correlation (Spearman test) with an objective measure of distance between the 3 fish (using Image J software) in pictures extracted from video recordings every 15 s for 5 min. This analysis was performed by separate and independent observers blinded to each other's' results (n= 10).



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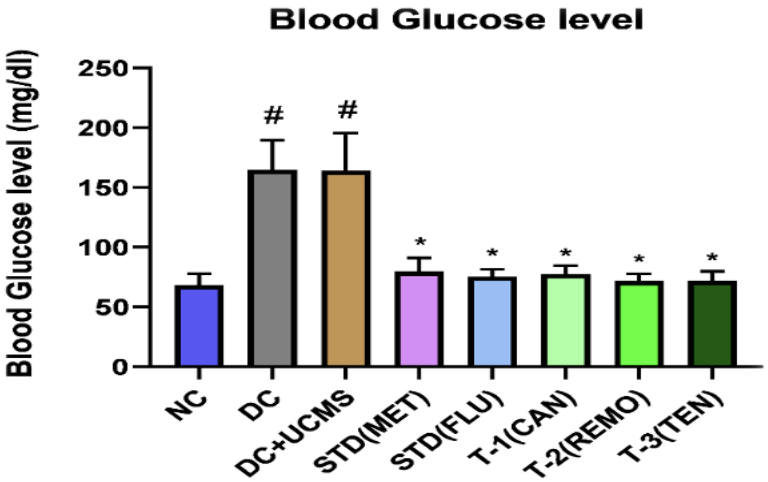
2) Glucose level:

The Glucose levels were measured by means of a glucometer (Accu-Chek, Roche Diabetes Care India Pvt Ltd.



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Discussion:

Diabetes is one of the largest global health issues. According to the American society of diabetes, it is metabolic disorder that produces syndromes associated with anabolic hormones secretion, insulin secretion and action. Chronic stage of diabetes mellitus is associated with organ damage such as kidney, heart, blood vessels etc.[22, 23] depression is psychiatric disorder which is characterized by a loss of interest in activity and accompanied by an inability to carry out daily activities, for at least two weeks. There are many incidences which show that there is a notably association between DM & depression.[24, 25] Depression is associated with 60% risk of DM and DM doubles the incidence of depression. Different type of studies suggests that there are any bidirectional or causal relationship between DM and depression. The presence of depression and anxiety in diabetic patients worsens the prognosis of diabetes, increases the non-compliance to the medical treatment, decreases the quality of life and increases the mortality, many studies in the last decade reported that co-morbidity of diabetes and depression have been triggered tremendously[26, 27].

AMPK is kinase which is master regulator in our body and it consists of catalytic α – subunit and regulatory β – and γ – subunits. The α- subunit has a two isoforms α1 and α2, regulatory β has two β1, β2 isoforms and γ – subunits has a three γ1, γ2, γ3 isoforms. These all isoforms have its individual function and role. Previous studies have proved the function of AMPK is in the inflammation, lipid metabolism, glucose metabolism, protein synthesis redox regulation and autophagy and mitochondrial biogenesis.[28] In DM it acts on metabolism of lipid and protein which is directly linked with m-TOR function which leads to insulin resistance. The AMPK is downstream substrate to restore energy level by activation of process of oxidation of fatty acid, inhibiting use of energy and synthesis of protein.[29] The activation of AMPK increases the ratio of intracellular AMP: ATP and phosphorylate Thr172 on the "activation loop7" of the α – subunit by one of its three upstream kinesis. This activation directly effects on the skeletal muscle which increase the glucose uptake, GLUT4 translocation, fatty acid oxidation and inhibition of the glycogen and protein synthesis. In liver, it acts by stimulation of the glucose uptake and fatty acid oxidation but inhibit gluconeogenesis and cholesterol[30, 31].

In last decade, Zebrafish have been gaining popularity in behavioural brain research because of having good performance in spatial and non-spatial learning. Classical neurotransmitter systems involved in learning and memory of zebrafish have been identified. Therefore, zebrafish used to evaluate the effect of both developmental and UCMS. Many studies suggested that neurochemical changes in dopaminergic & serotonergic system and behaviour response & social activity cause by stressor.[21]

In present study we have developed model of zebrafish to evaluate anti-depressant effect in prediabetes state. Several studies have suggested that the UCMS induced zebrafish have a depression like symptoms and change in its locomotion and spatial memory. Also reported to have down regulation of AMPK in UCMS induced zebrafish. In present study we have evaluated locomotor activity, color change and shoal cohesion, blood glucose level in zebrafish. There were significant decreased in locomotor, color change, shoal cohesion activity is observed in UCMS controlled group & diabetes controlled group. While it is found to be Significantly increased in disease treated with Canagliflozin (CAN), Remogliflozin, (REMO), Teneligliptin (TEN), and standard –Metformin (MET), Fluoxetine (FLU) controlled groups. And a significant increased blood glucose level (mg/dl) are found in disease control and UCMS controlled group as compared to normal control groups. While it is treated with Canagliflozin (CAN), Remogliflozin, (REMO), Teneligliptin (TEN), and standard –Metformin (MET), Fluoxetine (FLU) controlled groups significantly decreased blood glucose level as compared to disease controlled group & UCMS controlled group.

In zebrafish, the HPI axis presents similar function and structure compared to mammalian HPA axis, coordinating the adaptive responses of an organism to any stressor agent. Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances of survival.[32] The stress response is related with HPA activation through increase of CRF and cortisol release and is controlled by several loops that tend to normalize the time-integrated secretion of cortisol. This response to stress with the resultant activation of the HPA axis is meant to be acute or at least with limited duration.[20, 21]In this case, the response has an adaptive feature, without major damage to the body. However, intense chronic stress over activates, the HPA axis, prompting the development of a state of exhaustion that leads to dysregulation of stress mediators, pathologies and even death, as first proposed by Selye in 1936.

AMPK activator acts on upstream pathway phosphorylation of AMPK cause activation of IP3/Akt pathway which cause survival and inhibition of apoptosis by phosphorylation of Bcl2. PI3/Akt is also modulator of m-TOR which is regulator of autophagic process.[33] Activation of AMPK also acts on hypothalamus pituitary adrenal axis (HPA axis). Function of HPA axis is reducing the stress tolerance. Response of HPA axis is carried by the hypothalamic release of corticotrophin releasing hormone which located in pituitary gland.[34, 35] Activation of this receptor cause release of adrenocorticotrophic hormone and its bind with ACTH receptor on adrenal gland. The activation of this receptor release cortisol and function of cortisol control of blood sugar level, memory formulation, metabolism, regulation and control of inflammation. Several studies suggest the activator of AMPK plays a significant role in depression.

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