Biochemical and Biophysical Research Communications 456 (2015) 74–79

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

QCM-4, a 5-HT$_3$ receptor antagonist ameliorates plasma HPA axis hyperactivity, leptin resistance and brain oxidative stress in depression and anxiety-like behavior in obese mice

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ARTICLE INFO

Article history:
Received 29 October 2014
Available online 21 November 2014

Keywords:
Depression
Anxiety
Obesity
Leptin resistance
HPA-axis hyperactivity
Oxidative stress

ABSTRACT

Several preclinical studies have revealed antidepressant and anxiolytic-like effect of 5-HT$_3$ receptor antagonists. In our earlier study, we have reported the antidepressive-like effect of 3-methoxy-*N*-*p*-tolylquinoxalin-2-carboxamide (QCM-4) in obese mice subjected to chronic stress. The present study deals with the biochemical mechanisms associated with depression co-morbid with obesity. Mice were fed with high fat diet (HFD) for 14 weeks, further subjected for treatment with QCM-4 (1 and 2 mg/kg p.o.) and standard antidepressant escitalopram (ESC) (10 mg/kg p.o.) for 28 days. Behavioral assays for depression such as sucrose preference test (SPT), forced swim test (FST) and for anxiety such as light and dark test (LDT) and hole board test (HBT) were performed in obese mice. Biochemical assessments including plasma leptin and corticosterone concentration followed by brain oxidative stress parameters malonaldehyde (MDA) and reduced glutathione (GSH) were performed. Results confirmed that QCM-4 exhibits antidepressive effect by increasing the sucrose consumption in SPT, reducing immobility time in FST and anxiolytic effect by increasing transitions and time in light chamber in LDT, increasing head dip and crossing score in HBT. Furthermore, QCM-4 attenuated the hypothalamic–pituitary–adrenal (HPA) axis hyperactivity by reducing the plasma corticosterone, reversing altered plasma leptin, restoring the imbalance of brain MDA and GSH concentration. In conclusion, QCM-4 showed antidepressive and anxiolytic effect by reversing the behavioral alterations that were supported by biochemical estimations in obese mice.

1. Introduction

Depression is known as one of the most fetal disorder leading to high mortality rate in developed countries across the world [1]. Several studies have evident the co-morbid association of anxiety with depression [2,3]. Obesity is another disease burden globally leading to severe cardiovascular and metabolic complication along with premature deaths [4]. Obesity has found to increase the risk of depression where, obese individuals are twice suspected to be prone to develop depression than non-obese persons [5]. One of the preclinical studies has described the depressive behavior in high fat diet (HFD) induced obese mice through alteration in the neurotransmitters pathways [6]. The biological mechanisms for this co-morbid disorder are still not clearly defined.

Several mechanisms play a central role in depression associated with obesity. Hypothamic pituitary adrenal (HPA)-axis hyperactivity in obesity leads to secretion of excess corticosterone that leads to the development of insulin resistance, altered plasma glucose, hyperlipidemia [7], that further triggers the development of severe depression in obese individuals. In our earlier studies we have dealed with the investigation of altered plasma glucose and lipids involved in depression co-morbid with obesity [8,9].

Leptin secreted by adipose cells is well known anti-obesity hormone. Earlier studies have mentioned the important role of leptin in energy homeostasis, reproduction and cognition [10]. Animal model of depression showed reduced leptin level and treatment with leptin reversed the depressive symptoms, thus acting as anti-depressant [11]. Obesity is characterized by leptin resistance as like insulin resistance in diabetic population due to the alterations and defects in leptin transport across the blood brain barrier, leptin receptors in hypothalamus and the associated signaling mechanisms [12]. Leptin increases the serotonin in the fore brain region and reverses the elevated corticosterone, thus regulating the HPA axis hyperactivity that support the antidepressant effect of leptin [13].

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http://dx.doi.org/10.1016/j.bbrc.2014.11.036
0006-291X/

Obesity is characterized by increased level of several inflammatory cytokines [14]. These inflammatory cytokines causes oxidative stress secreting reactive oxygen species [ROS]. Oxidative stress in obesity alters the glucose utilization in fat and skeletal muscles further decreasing the synthesis of insulin from pancreas, thus causing altered plasma glucose [15].

5-HT_3 receptor the only ion channel type in serotonergic receptors family is highly expressed in the hippocampus, amygdala and area postrema [16]. Several pre-clinical studies have reported the potential antidepressant and anxiolytic activity of serotonergic type 3 receptor blockers [17,18]. Selective serotonin reuptake inhibitors (SSRIs) acts as functional antagonists of 5-HT_3 receptors while exhibiting there antidepressant effect [19]. In our earlier study the antidepressant effect of 3-methoxy-*N*-*p*-tolylquinoxalin-2-carboxamide (QCM-4) in obese mice subjected to chronic stress was studied. [8,9]. The present study was designed to investigate the mechanisms of QCM-4 for antidepressant and anxiolytic effect in obese mice using preliminary behavioral paradigms such as sucrose preference test (SPT), forced swim test (FST), light and dark test (LDT) and hole board test (HBT) and biochemical investigations such as plasma corticosterone, leptin estimations and brain malonaldehyde (MDA) and decreased glutathione (GSH) respectively.

2. Materials and methods

2.1. Experimental animals

Male Swiss albino mice (20–25 g) were procured from Choudhary Charan Singh Haryana Agricultural University Hissar, India (Reg. No. 417/01/a/CPCSEA). The mice were properly housed and maintained under standard laboratory conditions (temperature 22 ± 2 °C and room humidity 60 ± 10%), 12:12 h of light/dark cycle and provided with food and water ad libitum. All the experimental protocols followed were in compliance with Institutional Animal Ethics Committee (IAEC) of Birla Institute of Technology & Science, Pilani, India (Protocol No. IAEC/RES/18/09).

2.2. Experimental high fat diet (HFD)

All the animals were fed for 14 weeks with HFD for induction of obesity [20].

2.3. Drugs and chemicals

QCM-4 was synthesized in-house by the medicinal chemistry group of BITS Pilani, India. Escitalopram (ESC) was obtained from Ranbaxy Research Laboratory (Gurgoan, India) as a generous gift sample. ELISA kit for leptin was purchased from Aviscera Bioscience Inc, USA. All the chemicals and reagents used in various estimations were of laboratory grade and standard.

2.4. Experimental design

Forty-eight mice were randomly divided into 8 different groups (n = 6/group). Group I-Normal pellet diet (NPD) control, group II-NPD + QCM-4 (1 mg/kg p.o.), group III-NPD + QCM-4 (2 mg/kg p.o.), group IV-NPD + ESC (10 mg/kg p.o.), group V-HFD control, group VI-HFD + QCM-4 (1 mg/kg p.o.), group VII-HFD + QCM-4 (2 mg/kg p.o.) and group VIII-HFD + ESC (10 mg/kg p.o.). QCM-4 was prepared in 0.25% sodium carboxymethyl cellulose and standard escitalopram in distilled water freshly every day and were administered orally by using oral gavage (p.o.) once daily for 28 days (see Table 1).

2.5. Behavioral assays for depression

2.5.1. Sucrose preference test (SPT)

SPT was performed as per the method described earlier [21]. Mice were give training by placing two bottles of sucrose solution (1%, w/v) for 24 h followed by replacing one bottle with water for next 24 h. After overnight fasting two bottles with sucrose and water respectively were placed on cages and volume consumed after 24 h was measured and percent sucrose preference was calculated.

2.5.2. Forced swim test (FST)

A method of Porsolt et al. [22] was adopted to perform FST, were animals were allowed to swim in glass cylinder (diameter: 22.5 cm, height: 30 cm) filled with water (23 ± 2 °C) to 15 cm height of 15 cm for 6 min having initial 2 min for adjustment and recording the immobility time.

2.6. Behavioral assays for anxiety

2.6.1. Light and dark test (LDT)

LDT was performed by suing the method mentioned earlier [23]. Mice were subjected for 5 min to apparatus with light and dark chambers respectively and the transition count and time in light chamber were measured.

2.6.2. Hole board test (HBT)

Method of Nolan et al. [24] was used to perform HBT. A gray plexiglas chamber (40 cm × 40cm) elevated to 15 cm from floor having 16 equidistant holes of 3 cm diameter was used where head dip and crossing score were recorded for 5 min.

2.7. Biochemical estimations

2.7.1. Collection of blood

Animals were rested for 2 days after the behavioral assays and mice bleeded (0.2 ml) in a tube containing 20 µl of EDTA solution (10%, 100 µl/ml of blood) by retro-orbital puncture and centrifuged at 10,000 rpm for 15 min and plasma was collected and stored properly.

2.7.2. Leptin estimation

Leptin was estimated in duplex by using the ELISA kit from Aviscera Bioscience, Inc. (Catalog No. SK00050-08; Lot No. 201111107).

2.7.3. Corticosterone estimation

A modified method by Katyare and Pandya [25] was used for leptin estimation. Briefly, 0.2 ml plasma sample was mixed with 0.2 ml of chloroform: methanol mixture followed by addition of 3 ml chloroform and centrifuged. Chloroform layer was treated with sodium hydroxide (0.1 N), followed by 30 N sulfuric acid. Sulfuric acid layer was separated and kept in dark room for 30–60 min and fluorescence was measured using SL-174-spectrofluorometer with 472 nm excitation and 523 nm emission wavelengths, respectively. Plasma corticosterone content was measured as percentage of with respect to NPD control (considering NPD control as 100%).

2.7.4. Measurement of brain malonaldehyde (MDA)

MDA was measured using a method of Ohkawa et al. [26]. Sodium dodecyl sulfate (8%), glacial acetic acid (20%) pH 3.5 and thiobarbituric acid (0.8%) were treated with brain homogenate and incubated for 60 min at 90 °C and centrifuged at 1000 rpm for 10 min. Absorbance was measured at 532 nm in the supernatant using Perkin Elmer lambda 20 spectrophotometer (Shimadzu,

Table 1
Schematic representation of study protocol.

Days	14 weeks	Day 0–28th day	29–42nd day				46th day	47th day onwards
Study protocol	HFD feeding	QCM-4/ESC/vehicle treatment once daily for 28 days	Behavioral assays 29–33 Sucrose preference test	36 FST	39 LDT	42 HBT	Collection of blood and brain samples Plasma glucose, leptin and corticosteroids, Brain MDA and GSH	Biochemical assessment

Kyoto, Japan) and MDA content was expressed as microgram (μg) per mg protein.

2.7.5. Measurement of brain reduced glutathione (GSH)

A method of Ellman [27] was used for estimation GSH in brain homogenate. Brain homogenate was mixed with (5%) sulfasalicylic acid (1:1) and centrifuged at 12,000 rpm for 10 min. The supernatant was treated with 150 μl (pH 7.4) phosphate buffer, and 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent) was added 3 times. After 10 min and the absorbance was measured using Perkin Elmer lambda 20 spectrophotometer (Shimadzu, Kyoto, Japan) at 412 nm and expressed as μg of GSH per mg of protein.

2.7.6. Protein estimation

Protein was measured in brain homogenate using a method described by Lowry et al. [28] with bovine serum albumin (BSA) (1 mg/ml) as a standard (data not presented).

2.8. Statistical analysis

Graph Pad PRISM software version 2.01 (GraphPad Software, La Jolla, USA) was used for data analysis. All the values were expressed as mean ± standard error of the mean (S.E.M.). Differences between various groups for behavioral and biochemical assays were analyzed using two way analysis of variance (ANOVA) followed by Bonferroni post test and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of QCM-4 on SPT in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and ESC (10 mg/kg p.o.) significantly [$f(7,40) = 43.22$; $p < 0.01$] increased the sucrose consumption in obese mice (Fig. 1).

3.2. Effect of QCM-4 on immobility score in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and ESC (10 mg/kg p.o.) significantly [$f(7,40) = 40.23$; $p < 0.01$] reduced the immobility time in obese animals (Fig. 2).

3.3. Effect of QCM-4 on LDT in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and standard ESC (10 mg/kg p.o.) significantly reduced the latency for first entry in dark chamber [$f(7,40) = 118.1$; $p < 0.01$], increased the transition score [$f(7,40) = 139.0$; $p < 0.01$] and time spent in light chamber [$f(7,40) = 6.75$; $p < 0.01$] in obese animals (Table 2).

3.4. Effect of QCM-4 on HBT in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and standard reference drug ESC (10 mg/kg p.o.) significantly reduced the latency for first head dip [$f(7,40) = 82.27$; $p < 0.01$] and increased the head dip score [$f(7,40) = 79.02$; $p < 0.01$] and crossing score [$f(7,40) = 115.4$; $p < 0.01$] in obese animals (Table 3).

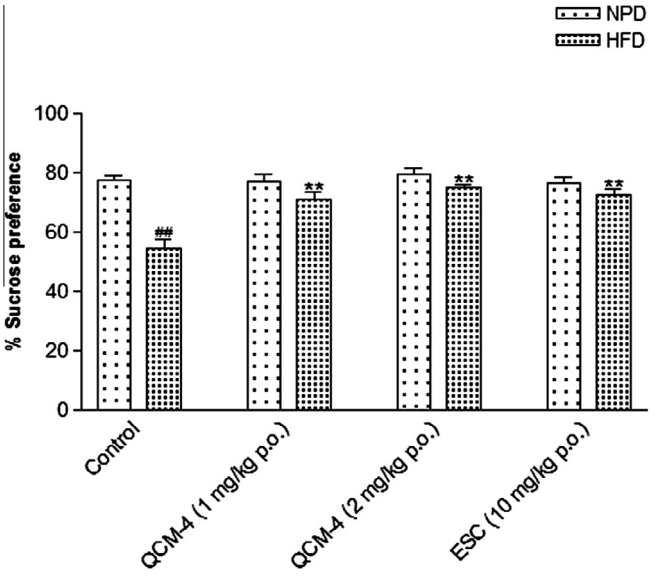

Fig. 1. Effect of QCM-4 (1 and 2 mg/kg p.o.) treatment on sucrose preference test in obese mice. Values represents mean ± S.E.M., $^{\#\#}p < 0.01$ as compared to NPD control group, $^{**}p < 0.01$, as compared to HFD control group, $n = 6$/group.

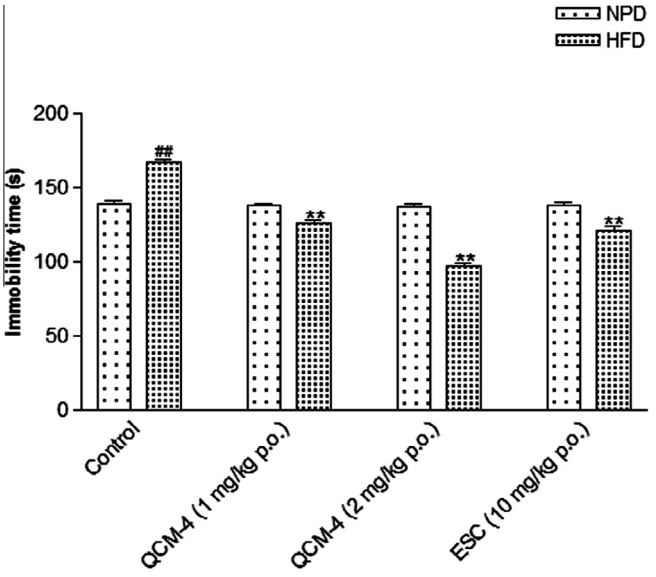

Fig. 2. Effect of QCM-4 (1 and 2 mg/kg p.o.) treatment on the immobility time on FST in obese mice. Values represents mean ± S.E.M., $^{\#\#}p < 0.01$ as compared to NPD control group, $^{**}p < 0.01$ as compared to HFD control group, $n = 6$/group.

Table 2
Effect of QCM-4 on LDT in obese mice.

Groups	1st Light chamber entry (s)	Number of transitions	Time in light chamber (s)
NPD control	30.50 ± 2.06	18.33 ± 1.82	29.50 ± 3.13
NPD + QCM-4 (1 mg/kg p.o.)	31.33 ± 2.56	16.67 ± 1.93	28.83 ± 4.45
NPD + QCM-4 (2 mg/kg p.o.)	30.37 ± 2.44	17.33 ± 1.69	29.33 ± 3.85
NPD + ESC (10 mg/kg p.o.)	29.83 ± 2.43	19.00 ± 2.59	29.33 ± 2.49
HFD control	49.17 ± 3.17$^{\#\#}$	6.83 ± 1.08$^{\#\#}$	13.50 ± 1.77$^{\#\#}$
HFD + QCM-4 (1 mg/kg p.o.)	37.17 ± 2.40**	11.00 ± 0.77**	29.83 ± 3.77**
HFD + QCM-4 (2 mg/kg p.o.)	34.50 ± 3.33**	15.83 ± 1.78**	43.50 ± 4.15**
HFD + ESC (10 mg/kg p.o.)	35.67 ± 3.13**	14.00 ± 1.63**	40.33 ± 2.29**

Values represents mean ± S.E.M., $^{\#\#}p < 0.01$ vs NPD control, $^{**}p < 0.01$ vs HFD control, $n = 6$/group.

Table 3
Effect of QCM-4 on HBT in obese mice.

Groups	1st head dip latency (s)	Head dip score	Number of crossings
NPD control	32.67 ± 4.33	27.00 ± 3.60	34.50 ± 3.43
NPD + QCM-4 (1 mg/kg p.o.)	32.33 ± 2.80	27.33 ± 3.00	33.33 ± 3.58
NPD + QCM-4 (2 mg/kg p.o.)	33.50 ± 3.06	27.00 ± 2.77	35.38 ± 3.04
NPD + ESC (10 mg/kg p.o.)	32.33 ± 2.32	27.17 ± 3.28	35.00 ± 3.73
HFD control	17.00 ± 2.59$^{\#\#}$	14.00 ± 2.63$^{\#\#}$	15.67 ± 2.70$^{\#\#}$
HFD + QCM-4 (1 mg/kg p.o.)	25.17 ± 2.21**	16.67 ± 2.99	22.17 ± 3.06
HFD + QCM-4 (2 mg/kg p.o.)	30.83 ± 3.55**	23.67 ± 4.72**	30.00 ± 4.62**
HFD + ESC (10 mg/kg p.o.)	27.17 ± 1.74**	21.33 ± 1.82*	28.17 ± 2.98**

Values represents mean ± S.E.M., $^{\#\#}p < 0.01$ vs NPD control, $^{*}p < 0.05$; $^{**}p < 0.01$ vs HFD control, $n = 6$/group.

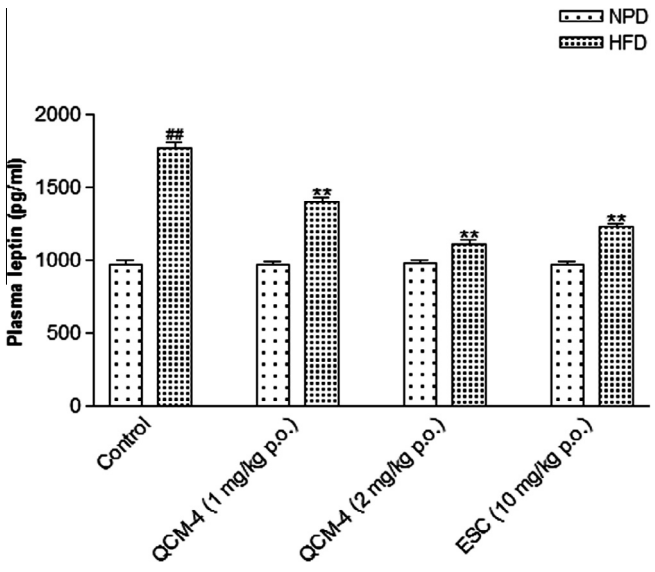

Fig. 3. Effect of QCM-4 (1 and 2 mg/kg p.o.) treatment on plasma leptin level in obese mice. Values represents mean ± S.E.M., $^{\#\#}p < 0.01$ as compared to NPD control group, $^{**}p < 0.01$ as compared to HFD control group, $n = 6$/group.

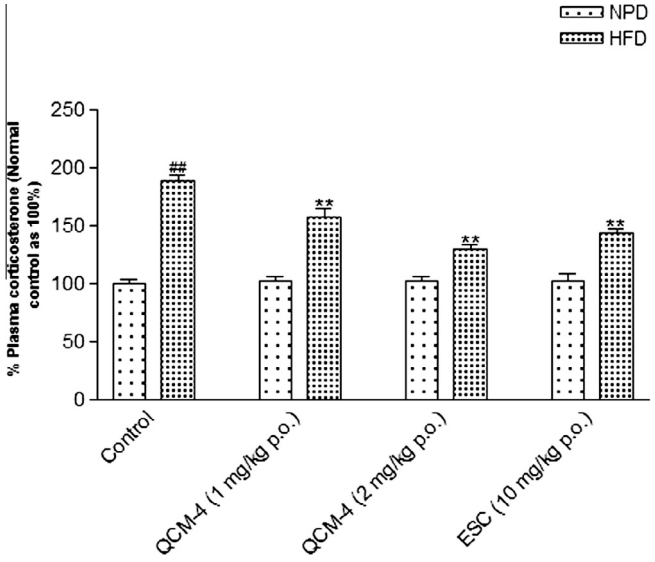

Fig. 4. Effect of QCM-4 (1 and 2 mg/kg p.o.) treatment on plasma corticosteroids in obese mice (NPD group considered as 100%). Values represents mean ± S.E.M., $^{\#\#}p < 0.01$ as compared to NPD control group, $^{**}p < 0.01$ as compared to HFD control group, $n = 6$/group.

3.5. Effect of QCM-4 on plasma leptin in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and ESC (10 mg/kg p.o.) significantly [$f(7,40) = 435.2$; $p < 0.01$] reversed the elevated plasma leptin concentration in obese mice (Fig. 3) thus, showing that QCM-4 improves the leptin sensitivity in obese mice.

3.6. Effect of QCM-4 on plasma corticosterone in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and ESC (10 mg/kg p.o.) significantly [$f(7,40) = 279.2$; $p < 0.01$] decreased the plasma corticosterone level in obese mice (Fig. 4).

3.7. Effect of QCM-4 on brain MDA concentration in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and ESC (10 mg/kg p.o.) significantly [$f(7,40) = 120.6$; $p < 0.01$] reversed the elevated brain MDA in obese mice (Table 4).

3.8. Effect of QCM-4 on brain GSH concentration in obese mice

QCM-4 (1 and 2 mg/kg p.o.) and standard ESC (10 mg/kg p.o.) significantly [$f(7,40) = 442.9$; $p < 0.01$] increased the brain GSH concentration in HFD obese mice (Table 4).

Table 4
Effect of QCM-4 on brain MDA and GSH in obese mice.

Groups	Brain MDA μg/mg protein	Brain GSH μg/mg protein
NPD control	1.71 ± 0.19	0.62 ± 0.07
NPD + QCM-4 (1 mg/kg p.o.)	1.77 ± 0.16	0.63 ± 0.08
NPD + QCM-4 (2 mg/kg p.o.)	1.75 ± 0.17	0.62 ± 0.10
NPD + ESC (10 mg/kg p.o.)	1.71 ± 0.14	0.54 ± 0.05
HFD control	3.01 ± 0.49$^{\#\#}$	0.07 ± 0.01$^{\#\#}$
HFD + QCM-4 (1 mg/kg p.o.)	2.71 ± 0.41	0.19 ± 0.03
HFD + QCM-4 (2 mg/kg p.o.)	2.49 ± 0.41*	0.36 ± 0.07**
HFD + ESC (10 mg/kg p.o.)	2.66 ± 0.38*	0.27 ± 0.04**

Values represents mean ± S.E.M., $^{\#\#}p < 0.01$ vs NPD control, $^{*}p < 0.5$, $^{**}p < 0.01$ vs HFD control, $n = 6$/group.

4. Discussion

Obesity is a stressful abnormal condition and in stress situation clinically lack of interest is observed whereas in rodents it is reflected in terms of reduced interest to consume sweet solution [29]. QCM-4 treatment significantly increased the sucrose consumption in obese animals thus showing anti-anhedonic effect corresponding to antidepressant activity. FST has high predictive validity that indicates the similar mechanism of the antidepressant in depressed patient and animal models of depression tested with newer chemical agents [30]. As FST shows more influence on monoamines alterations it signifies an important model in studying the neurobiological and genetic mechanisms involved in the antidepressant effect of standard drugs or new chemical entities (NCE's) [31]. Chronic treatment with QCM-4 reduced the immobility time and increased the swimming behavior in obese mice that was in compliance with our earlier study [9].

Furthermore, the anxiolytic profile of QCM-4 in obese animals was studied. In LDT the new environment and light acts as a mild stressor and the exploratory behavior of rodents is analyzed with respect to their innate aversion response to the light area [32]. HBT is another behavioral paradigm for evaluating anti-anxiety activity, emotionality or response to stress in rodents to an abnormal environment at laboratory level [33]. Head dip score is major parameter that shows the exploratory behavior of animal and displays the sensitivity for changes in the emotional state of the test animal [24]. Chronic treatment with QCM-4 showed anxiolytic effect in obese mice in LDT and HBT.

Dysregulation of HPA axis is well characterized by abnormal production of cortisol and the inflammatory response has great impact in depressed patients [34]. The raised level of corticosteroids in pre-clinical as well as clinical studies of depression and anxiety is reported earlier in several literatures [35]. Exogenous administration of corticosteroids results in metabolic abnormalities, mostly those reflected with obesity such as hyperinsulinemia, insulin resistance, and altered plasma glucose [36]. Elevated cortisol in obese patients suggests the hyperactivity of HPA axis which is related to the higher body mass and altered cortisol binding globulin [37]. Collectively depression and obesity reflects higher production of corticosterone in the blood that was reversed by chronic treatment with QCM-4 in obese mice.

Leptin is a peptide secreted by adipocytes acts as an anti-obesity hormone regulating the energy homeostasis by negative feedback mechanism [38]. Leptin receptors are present in the hippocampus and amygdala regulating mood [39]. HPA axis dysregulation is an important factor in the pathogenesis of depression that leads to elevated corticosterone in blood and leptin has shown inhibitory action on the excess plasma corticosterone in animal models thus, showing antidepressant effect and inverse relationship with corticosteroids [40]. In obesity leptin resistance occurs in similar way as like insulin resistance and treatment with leptin in obesity is ineffective in regulation of food intake and energy homeostasis [41]. Leptin resistance in obesity is resulted due to the abnormal functions of leptin receptors, irregular leptin signaling pathways and defects across the blood brain barrier [12]. Our results with chronic treatment with QCM-4 has suggested the reversal of significantly higher leptin level in obese mice and thus exhibiting antidepressant effect.

The imbalance between oxygen free radicals and antioxidant defense system leads to oxidative stress [42]. ROS consists of singlet oxygen, peroxynitrite and superoxide free radicals whereas antioxidant system is composed of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase [43]. Obesity is characterized by elevated ROS that increases the oxidative stress and neurodegenration [44]. In obesity the production of lipid peroxidation is resulted due to the interaction of polyunsaturated fatty acids and ROS in brain [45]. Malonaldehyde is index of lipid peroxidation that was raised abnormally in obese mice [46]. On the other hand the antioxidant system enzyme GSH was decreased significantly in obese mice [47]. Several pre-clinical studies of depression have well evident the abnormal increase and decrease in brain MDA and GSH levels, respectively [48]. Chronic treatment with QCM-4 reversed the abnormal production of brain MDA and elevated the brain GSH level in obese mice, thus exhibiting the antidepressant effect.

Several studies in animal models have suggested the antidepressant and anxiolytic activity of 5-HT_3 receptor antagonists [17,18]. The exact mechanism still remains unclear, but the probable mechanism suggests that 5-HT_3 receptor antagonists such as ondansetron and QCM-4 acts by antagonising the postsynaptic receptors upon which they increases the synaptic transmission of serotonin in various regions of brain [49]. Therefore, QCM-4 possibly acts by allosteric modulation of serotonergic system and increasing serotonergic neurotransmission for antidepressant and anxiolytic effect.

Serotonin plays a significant role in the pathogenesis depression and obesity and regulates the mood, appetite and sleep [50]. Serotonin is co-localized in pancreatic cells in association of insulin and regulation of plasma glucose controlling the insulin release [51]. Moreover serotonin plays crucial role in the modulation of HPA axis in stress associated depression [52]. Serotonin is involved in the functioning of leptin that acts as antidepressant [53]. Overall, it is quite clear that serotonin holds the key in regulation of depression and anxiety associated with obesity. QCM-4 acts allosterically by increasing the neurotransmission of serotonin in different regions of brain and shows antidepressant and anxiolytic effect in obese mice.

Acknowledgments

We are thankful to Birla Institute of Technology and Science (BITS), Pilani, India and University Grant Commission (UGC), Government of India for providing support and research facilities to pursue this work.

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