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Original Research Article

NEUROCHEMICAL, HEMATOLOGICAL AND BEHAVIORAL ALTERATIONS RELATED TO ESZOPICLONE ADMINISTRATION IN RATS

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Abstract: This study aimed to shed light on the effect of eszopiclone (ESZ) administration once daily for 30 consecutive days at night time on some neurochemical, behavioral and hematological criteria. A total of 27 male Wister albino rats were assigned to one of three drug treatment groups, vehicle, Eszopiclone (3mg/kg) and (6mg/kg). After 30 days of Eszopiclone administration, the neurochemical analysis revealed a significant reduction in serotonin and glutamate (306.44 ng/ml, 4.33 nmol /µl, respectively) in 6mg/kg treated animals, furthermore dopamine levels were significantly higher in rats treated with Eszopiclone (3mg/kg or 6mg/kg) in compare to control group. Reduced glutathione, superoxide dismutase and catalase levels revealed a significant decrease (0.15 µmol /gm tissue, 25.24 µmol /gm tissue, 1.93 Unit/gmtissue, respectively), while the malondialdehyde levels (15.79 nmol /gm tissue) demonstrated a significant increase in animals treated with 6 mg of ezopiclone. Behavioral assessment was carried out 3 times throughout the study (once/2 weeks) by video recording. It was recorded in 5 tests, including open field, the hole-board, inclined plain, grip and tail suspension tests. There were no changes between the rats in 3 mg/kg of ezopiclone and those in the control group. The rats in 6 mg/kg of ezopiclone showed less response in all behavioral observations, with significant decreases in inclined plain angle (23.35), exploratory time (17 second) and exploratory frequency (2 frequencies) in compare to other groups. The results support the concept that the administration of eszpiclone more than 3 mg/kg may lead to the behavioral changes. There were no serious adverse events regarding hematological indices. It is concluded that eszopiclone administration causes an imbalance between different neurotransmitters in the cerebrum. A marked decrease in antioxidant scavenging capacity with a behavioral alteration in 6mg/kg treated animals. The maximum safe dose of eszopiclone was 3mg/kg and more than this dose could lead to a deleterious reactions as evidenced in this study.

Key words: eszopiclone; neurotransmitters; behavior; hematology

Introduction

Eszopiclone is a soporific, mesmerizing agent that is used for treatment of difficulty maintaining sleep during the night and early morning. It is the S (+) enantiomer of the dextrorotatory form of zopiclone, a cyclo-pyrrolone with no structural likeness to the

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mesmeric drugs zolpidem and zaleplon, or to the benzodiazepines (BZs) and barbiturates (1). Eszopiclone, along with zaleplon and zolpidem, are member of grade of therapies notorious as nonbenzodiazepine benzo-diazepine receptor agonists. These drugs tie up to sites on the gamma aminobutyric (GABAA) receptor agonists that are familiar to or overlap with the sites at which BZs take action (2).

Attic BZs bind equally to GABAA receptors involving all the α-subunit isoforms with exclusion of α4 and α6 (3-5). Recently, the use of BZs has been signified to have a number of damaging undesirable effects, for example, anterograde amnesia, and discontinuation pathologies (6-9). Recognition of these side effects of BZs led to the development of a brand new cohort of non-benzodiazepine hypnotics, incorporating zolpidem and eszopiclone. Zolpidem has an elevated affinity for GABAA receptors containing the α1 subunit, low resemblance for α2 and α3 containing receptors, and no remarkable affinity for α5 encompassing receptors (2,4,5,10). Conversely, eszopiclone evinces significant activity at GABAA receptors containing α1, α2, α3 and α5 subunits (5, 10-13).

Thalamus has a mandatory responsibility in sleep act and rhythmicity, where two well defined subtypes of synaptic GABAA receptors; α1β2γ2 and α3β3γ2 are articulate in thalamocortical relay neurons and in interneurons of the reticular thalamic nucleus, so impairment to the thalamus stand in the way of normal sleep (14). Behavioural studies are crucial not only for increasing the knowledge on the status of animal but also for assessing the animal's response to its environment (15). There were some of behavioral tests that used to assess fear and anxiety in animals, and therefore attained the status of one of the most widely used instruments in animal psychology (16). These tests primarily involve placing the subject animal in a novel “open” space from which escape is prevented by a surrounding wall and then measure the elicited behaviors of the subject in this test situation. In these tests the most commonly used parameters to assess an animal’s state of anxiety are behaviour (body movements; mainly general level of activity and exploration such as movement, ambulation, rearing and freezing) and defecation (17), although other forms of behaviour such as grooming and vocalization, and other parameters such as physiological parameters (heart and respiration rate, electromyogram recording and plasma glucocorticoids levels) have also been included (16). It is generally thought, in the open field tests, that animals which show a high level of activity (all forms of locomotion) and have low defecation scores are considered to be less emotional (18).

Due to the adverse effects of benzodiazepines, the uses of non-benzodiazepines were more popular in last few years. However, non-benzodiazepines receptor agonists had adverse effects, including dementia, delirium, sleepwalking, serious injuries and fractures (19-23), furthermore, it is associated with an increase in hospitalizations and motor vehicle accidents. Insomnia may be predisposing factor to develop anxiety disorders (24,25).

Therefore, the objective of this study was to quantify changes in some neurotransmitters and behavioral profile following the administration of Eszopiclone (3mg and 6mg). Another objective was to determine oxidative stress related to Eszopiclone administration in addition to the assessment of alterations in hematological profile.

Material and methods

The ethical approval was taken from the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Drug

Eszopiclone (Sepracor Inc., Marlberough, MA) was dissolved in 50 ml of acetate buffer and the dose of the drug was 3 mg/kg and 6 mg/kg of body weight, according to Huang et al. (26).

Animals

A total of 27 male Wister rats aged 7-8 weeks and weighed 180-200 gm. The experimental procedures were carried out in research unit, Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were

assigned randomly into 3 groups (9 rats in each one) and each group was subdivided into 3 replicates (3 rats in each/ cage). The rats were housed in stainless-steel cages (40 cm L*25 cm W*20 cm H), maintained in a climate-controlled environment at 23±2ºC; 60±10% of humidity with a 12/12 dark/light cycle (6 am: 6 pm).

Experimental strategy

Rats were randomized to 3 groups, each group holding 9 rats. Group 1 received 1 ml of saline (control animals). Group 2 was given 3mg/kg eszopiclone. Group 3 received 6mg/kg eszopiclone. All treatments were given orally once daily for 30 days at night time (10 pm).

Blood samples

Samples were taken from retro-orbital sinus (27) and Sodium salt of EDTA was used as an anticoagulant for estimation of some hematological frameworks.

Analysis of tissue samples

The brain of each rat was pulled out and cleaned from the stuck tissues and weighted. It was homogenized in 0.05 ml potassium phosphate buffer (pH 7.4) using Electronic Homogenizer and was kept at -80 °C until estimation of dopamine, serotonin, glutamate, glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels.

Dopamine estimation

Whole brain was dissected out, wet tissue was homogenized in HCL-butanol for about 1 minute (1:10 ratio), centrifuged for 10 minutes at 3000 rpm, and one ml of supernatant was taken after centrifugation for dopamine estimation as described by Shyamjith et al. (28).

Serotonin and Glutamate estimation

The content of brain from serotonin was estimated by the method mentioned by Schlumfjf et al. (29), while the glutamate levels were measured by multiple development paper chromatography (30).

GSH and SOD estimation

The levels of GSH and SOD in the brain were measured as mentioned by Davies et al. (31) and Ohkawa et al. (32) respectively.

Estimation of CAT and MDA

CAT was estimated by the decomposition of H2O2 after adding 1 ml of H2O2 to 2 ml of the prepared sample (0.1 ml of supernatant +1.9 ml of phosphate buffer). While, malondialdehyde levels were estimated by thio-barbituric acid reaction (32).

Hematological studies

Total RBCs count and WBCs were scrutinized according to the method illustrated by Dacie and Lewis (33) while hemoglobin concentration was estimated colorimetrically following method of Wintrobe (34). The packed cell volume was determined using the microhematocrite method according to Darcourt et al. (35).

Behavioural observation

The behavioural assessment was carried out in an experimental room under the same environmental conditions. The experimenter was blind to the treatments of the animals. It was carried out 3 times (once/2 weeks) during the light phase of the light–dark cycle. The behaviour of the rat was recorded using a video camera fixed over the experimental cage and a focal sampling technique was used to record the behavior from the electronic compact disc.

Open field test

The rats were held from the tails and placed in one corner of the open field arena (70 L*70 W*40 H cm), which was divided into 16 equal squares. Behaviours were recorded by video recording for 5 minutes after 2 minutes setting the rat in the apparatus (for acclimation) and the arena was cleaned with 5% ethanol before the next rat. The behavioural parameters recorded were ambulation or locomotion frequency (the number of floor sections entered with two feet), rearing frequency (the number of times the animal stood on its hind legs), stereotype counts (the number of grooming movements), and immobility (freezing) duration (total time in

seconds without spontaneous movements). The movement of rats in squares reflected the level of anxiety-like behavior (36).

Hole-board test

This test included a board (80 cm L* 80 cm W), which had 4 equal holes distributed in four quarters of board. The exploratory behavior (time and frequency) was recorded (37) through head entrance of rat in holes of this apparatus.

Inclined plain test

According to the procedures followed by Abou-Donia et al. (38), rats were placed in the horizontal position on a flat plane and the angle of falling rat from flat plane was calculated.

Grip strength test

The strength of rat's forepaw was recorded by hanging in wood dowel (5mm diameter) (39). Time of grip in wood dowel was recorded in seconds to assess forepaw strength.

Tail suspension test (TST)

TST has been adopted as a behavior model to predict antidepressant effect of drugs and chemicals in rodents (40). In this test, the rats were suspended 50 cm above the floor and throughout a 5-minute, the total duration of mobility and struggling was recorded.

Statistical analysis

Data were statistically analyzed using SAS statistical system Package (41), where it was analyzed by one-way ANOVA using the SPSS 16.0 computer program. Duncan's Multiple Range test was performed to compare means value between experimental groups. The results were presented (mean ± SE) with normal distribution and significantly differed at $P<0.05$.

Results

The neurochemical analysis presented a marked reduction in serotonin and glutamate with a noticeable elevation in dopamine levels in brain homogenate, alterations that were more observable in 6mg/kg treated animals (Table 1). Reduced glutathione, superoxide dismutase and catalase levels revealed a significant decrease (0.15 µmol /gm tissue, 25.24 µmol /gm tissue, 1.93 Unit/gmtissue, respectively), while the malondialdehyde levels (15.79 nmol /gm tissue) evoked a significant increase in the same group (Table 2). There were no serious adverse events regarding hematological indices (Table 3). There were no clear changes between the first and second group, while the rats in group 3 had less response to all behavioral observations, with significant differences in inclined plain (23.35) and exploratory test (17 second and 2 frequencies) (Table 4,5).

Table 1: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on serum levels of serotonin, glutamate and dopamine in male Wistar rats

Groups	Serotenin (ng / ml)	Glutamin (nmol /µl)	Dopamin (µmol /gm tissue)
Control	488.29±5.40^{a}	8.03±0.59^{a}	50.94±2.39^{b}
3 mg/kg Ezopiclone	428.49±14.96^{b}	5.64±0.35^{b}	60.66±1.68^{a}
6 mg/kg Ezopiclone	306.44±6.88^{c}	4.33±0.38^{b}	59.28±2.18^{a}

^{abc} Means within the same column having different superscripts are significantly different at $P \leq 0.05$).
ng= Nanogram; ml=Milligram; nmol=Nanomole; µl=Micromilly; µmol=Micromole; gm=Gram and kg=Kilogram

Table 2: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on antioxidant parameters (MDA, SOD, CAT and GSH) in male Wister rats

Groups	MDA (nmol /gm tissue)	SOD (µmol /gm tissue)	CAT (Unit/gm tissue)	GSH (µmol /gm tissue)
Control	6.28±0.28[c]	54.16±1.93[a]	5.79±0.17[a]	0.344±0.016[a]
3 mg/kg Ezopiclone	13.05±0.45[b]	41.69±1.38[b]	3.03±0.18[b]	0.257±0.010[b]
6 mg/kg Ezopiclone	15.79±0.62[a]	25.24±1.30[c]	1.93±0.10[c]	0.15±0.007[c]

[abc] Means within the same column having different superscripts are significantly different at P ≤ 0.05).
nmol=Nanomole gm=Gram µmol=Micromole mg=Milgram kg=Kilogram

Table 3: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on some hematological parameters in male Wister rats

Groups	RBCS (10^6/ µl)	HB (gm/dl)	PCV (%)
Control	7.24±0.10	10.7±0.43	40.29±0.15
3 mg/kg Ezopiclone	6.98±0.13	10.72±0.32	40.28±0.67
6 mg/kg Ezopiclone	7±0.19	11.06±0.08	40.56±0.69

µl=Micromilly; gm=Gram; dl=Deciliter; mg=Milgram and kg=Kilogram

Table 4: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on the open field test in male Wister rats

Groups	Latency time (S.)	Grooming frequency	Rearing frequency	Ambulation frequency
Control	2.33±0.15 [b]	2±0.20 [a]	6±0.29 [a]	34.67±0.49 [a]
3 mg/kg Ezopiclone	3±0.16 [a]	1.33±0.07 [b]	6.33±0.27 [a]	33±0.64 [a]
6 mg/kg Ezopiclone	0.33±0.02 [c]	0.33±0.03 [c]	3.33±0.14 [b]	23±0.56 [b]

[abc] Means within the same column having different superscripts are significantly different at P ≤ 0.05).
mg=Milgram; S.=Second and kg=Kilogram.

Table 5: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on sensorimotors tests (grip, tail suspension and inclined plain) and exploratory behaviour in male Wister rats

Groups	Grip time (S.)	Tail suspension time (S.)	Inclined plain angle	Exploratory test	
				Time (S.)	Frequency
Control	3.67±0.11[a]	8.67±0.14[a]	43.33±3.04[a]	35.67±95[a]	5.33±0.12[a]
3 mg/kg Ezopiclone	2.5±0.09[b]	7.67±0.12[b]	43.12±3.05[a]	29±0.62[b]	4.33±0.12[b]
6 mg/kg Ezopiclone	1.5±0.1[c]	4.66±0.13[c]	23.35±1.82[b]	17±0.63[c]	2±0.08[c]

[abc] Means within the same column having different superscripts are significantly different at P ≤ 0.05).
S=Second; mg=Milgram and kg=Kilogram.

Discussion

Eszopiclone among others like zolpidem, zopiclone and zaleplone are medications which commonly referred to as non-benzodiazepine agonists. In 1980, the sedative molecules were developed to overcome the deleterious aspects of benzodiazepine therapy (1). This cyclopyrro-

lone derivative was classified as a potentiator of GABAA receptors, binds to all subtypes inducing sedation and hypnosis for removal of the sleep disorders (35,42). However, Buxton et al. (43) had recently found that eszopiclone in a dose of 3mg for two months treatment did not significantly influence sleep.

GABA is the major inhibitory neurotransmitter in the brain, and activation of GABAA receptors causes neuronal inhibition by increasing chloride ion conductance. GABAA receptors exist as multiple subtypes and these subtypes are differently located throughout the brain. Winsky (44) mentioned that the different GABAA receptor subtypes may cause differences in clinical effects caused by various benzodiazepine and non-benzodiazepine. Still, it is important to evaluate other neurotransmitters if there are any alteration in their levels could be attributed to eszopiclone nightly treatment for 30 consecutive days.

Neurochemically, it is inevitable that eszopiclone acts through modulation of GABAA receptor sites, despite a research by Kumar et al. (45) demonstrated that ESZ sleep induction doesn't require activation of GABAergic sleep-regulatory neurons in the preoptic hypothalamus. Our findings revealed that eszopiclone led to a significant decrease in serotonin levels in brain tissue homogenate, which was more pronounced in the 6mg/kg treated rats (306.44 ng / ml). Dopamine levels showed a marked elevation in eszopiclone 3 mg (60.66 μmol /gm tissue) and 6mg (59.28 μmol /gm tissue) treated rats, respectively, while glutamate values displayed a marked reduction in both treated groups (5.64 nmol /μl and 4.33 nmol /μl, respectively). The identified correlations between eszopiclone and other neurotransmitters than GABAA are unknown. Literature is small since inadequate work had been done on this matter, however, ESZ was found to suppress serotonergic and non-serotonergic neurons (45). Interestingly, Russell et al. (46) found that ESZ 3mg/kg for 18 successive days besides alleviated social defeat stress in mice, its neurochemical findings were elusive.

The decrease in serotonin level may be due to anxiety, obesity, insomnia, and fibromyalgia (47). This might explain the behavioral changes in rats treated with ESZ for 30 consecutive days, especially the group received 6mg/kg body weight. There were certain neurons in brain had the ability to synthesize, store, and released the serotonin. Serotonin in human being plays the important role in the regulation of a variety of processes within the brain, including, depression, mood, emotions, aggression and sleep (48,49).

To our knowledge, non-benzodiazepines have been scarcely investigated from the point of view of dopamine, glutamate, and other neurotransmitters estimation. Apart from potential confounding variables, what might be the reason for the association between eszopiclone treatment and neurotransmitters, neurotransmitter activity is delicately balanced, a shift in an inhibitory neurotransmitter alters other excitatory ones and vice versa. The findings of this paper declared a marked elevation in dopamine levels and a decrease in the glutamate levels in groups 2 and 3 in comparison to control group.

Despite the fact that activation of dopamenirgic receptors in the DRN increases the activity of serotoninergic neurons (50), the increase in dopamine levels in our findings did not necessarily elevate the serotonin levels as we presented a significant decrease in serotonin values after eszopiclone nightly treatment for 30 days suggesting the direct effect of this non-benzodiazepine on serotoninergic receptors, necessitating the need for more research in this area.

Glutamate is a group of endogenous amino acids which act as excitatory neurotransmitters for learning and memory. As neurotoxins, they are believed to be involved in the pathogenesis of a variety of neurodegenerative disorders in which cognition is impaired. McEntee and Crook (51) found that cerebral structures may be particularly vulnerable to the neurotoxic actions of these excitatory amino acids, especially in the elderly who are most susceptible to impairments of mnemonic function. The affinities of (S)-DMZ and N-oxide zopiclone (two major metabolites) to the central BZD receptor site were > 20-fold and >250-fold lower than (S)-zopiclone. (S)-DMZ

was shown to enhance GABAA current by increasing the affinity of receptors for GABA but dose dependently inhibited agonist evoked currents at both N-methyl-D-aspartate receptor NMDA (glutamate) and nACh (acetylcholine) receptors, an effect that was mimicked by zopiclone. Inhibitory actions at NMDA and nACh receptors are also evident at a 20 μM (52).

Rats in group 2 (3mg of Eszopiclone/kg) showed lower GSH, SOD, CAT activities compared to rats in group 3 (6 mg). While, MDA level was significantly higher in group 2 than group 3. These values were more pronounced in animals treated with ESZ (6mg/kg), indicating that the oxidative stress could be gained on brain tissue after administration of ESZ.

Brain tissue is uniquely susceptible to oxidative stress as cellular residents of brain demonstrating markers of oxidative damage in major depressive disorders, insomnia and bipolar disorders (53). Eszopiclone showed deviations from reasonable antidepressants outcome treating oxidative stress accompanying brain disorders, administration of ESZ increased lipid peroxidation and suppressed antioxidant enzymatic activity. In contrast to our findings, Szebeni et al. (53) found that cellular residents of brain white matter revealed markers of oxidative damage in major depressive disorder (MDD), medications that interfere with oxidative damage or pathways activated by oxidative damage have potential to improve treatment for MDD.

One of the most possible explanations of the presented oxidative stress is the reduction of serotonin level in the brain caused by eszopiclone treatment. Serotonin acts as a scavenger of hypochlorous acid (HOCl) in the cerebrum. Serotonin was shown to inhibit the oxidation of 2-thio-5-nitrobenzoate by HOCl in a biphasic fashion. Serotonin might act to decrease chlorinative tension in the brain (54).

There were no serious adverse events during the study regarding hematological parameters. On the other hand, Lovett et al. (55) suggested that over dose of ESZ (30 times normal dose) evoked a marked hemolytic anemia and methemoglobinemia in adult women.

The data of behavior as shown in Table 4 and 5 revealed that rats in group 2 and 3 showed behavioural alterations as compared to control group. The changes in behavior are considered as a marker of emotional condition of the living organism (56), representing in grooming frequency and freezing time. Furthermore, frequencies of rearing and ambulation in this test help in the measurement of hyperactivity (57), locomotory activity (58) and anxiety-like behavior (59). In the current study, animals receiving 6 mg/kg of Eszopiclone had an increase in rearing frequency and freezing time, while frequencies of ambulation and grooming were the highest in control and rats treated with 3 mg/kg of eszopiclone.

In the current finding, the exploratory time and frequency were significantly higher in control and rats treated with 3 mg/kg, while it was the lowest in rats treated with 6 mg/kg. These results were consistent with locomotory behavior in open field test. These results may be due to the positive effect of Eszopiclone in treatment of insomnia (60). In this regard, the durations of immobility in tail suspension were higher in rats treated with 6 mg/kg. Moreover, grip strength delineated the motor neurotoxicology (61).

There was difference in grip strength among experimental groups, where it was higher in control and rats treated with 3 mg/kg, respectively than 6 mg/kg. Also, the marked depletion of the neurotransmitter here may be responsible for this neurobehavioral alterations especially lack of exploration and depression (62). The most of behavioural changes may be due to histopathological alteration of brain, as mentioned before by Khalil et al. (61), who cited that neuronal degeneration and cerebral oedema led to behavioural alteration.

Conclusion

In the present study, Eszopiclone administration caused an imbalance between different neurotransmitters in cerebrum presented by an elevation in dopamine and a marked reduction in serotonin and glutamate levels. Therefore, there are potentials suggesting cross-talking between GABAergic, serotoninergic, dopaminergic and NMDA

systems when eszopiclone is used. Behavioral activity highlighted side effects of 6mg/kg ESZ and oxidative stress was more conspicuous in 6mg/kg ESZ than in 3mg/kg treatment as substantiated by the decrease in antioxidant enzymes and the increase in lipid peroxidation.

Conflict of interest

None of the authors have any conflict of interest to declare

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