In vivo study to compare the effects of choline with fluoxetine and clozapine for the modulation of cognitive behavior (Learning memory and exploratory behavior)

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Abstract: Cognitive behavior is associated with physiological processes that affect the working performance of an individual. Cognitive control is used to override self-serving impulses and behave in socially desirable manner. The objective of the study is to compare the effects of Choline with Fluoxetine and Clozapine for the modulation of cognitive behavior including learning, memory, locomotor, exploratory behavior and anxiety. The study was based on twenty four albino rats divided into four equal groups: (1) Control kept on normal saline (2) Fluoxetine (3) Clozapine (4) Choline. Morris Water Maze (WM) test was used for the psychological assessment based on neural mechanism involved in spatial learning and memory. Open field activity test evaluated locomotor and exploratory behavior. The behavior modulation in WM test and open field activity test was determined at 1st, 3rd, 5th and 7th week. Fluoxetine, Clozapine and Choline were used as drugs and administered to the rat groups mentioned earlier. The modulation of behavior in WM test and Open field activity test was recorded at 1st, 3rd, 5th and 7th week after administering the drugs. Impairment in learning behavior in Fluoxetine treated group was observed at 1st, 3rd, 5th and 7th week and in Clozapine group at 1st and 2nd week when compared to Control (Saline) group. Rise in latency time was observed in Fluoxetine treated group but was not significant. Clozapine and Choline had exhibited beneficial effects in memory retention and prevention of learning impairment. The findings have led to the conclusion that Choline and Clozapine improve the memory retention after continuous administration of 5 and 7 weeks. Moreover, Clozapine has different receptor specificity as compared to Choline. However, both improve the learning capability and enhance the memory in rats. Meanwhile, Fluoxetine did not show any considerable enhancement of memory.

Keywords: Choline, cognition, learning, memory, water maze test, open field activity test.

INTRODUCTION

Cognitive behavior is associated to physiological processes such as memory, reasoning, problems solving, tasking, planning, and execution which empowers thoughts regulation and actions for behavioral goals tracking (Bayer, 2012; Robertson *et al.,* 2015). Evidence in scientific literature reported that diminishing of cognitive and motor skills poses substantial socioeconomic problems (Hedden & Gabrieli, 2015). Acetylcholine (Ach), DA and 5-HT have been reported to be specifically involved in locomotion, mood disorders, learning, memory and other relevant behavioral tasks (Hegazy & Ali, 2011).

Hippocampus is the most vital brain region and numerous neuronal processes are particularly vulnerable to aging process (Clark *et al.,* 2007). Cognitive dysfunction being fundamental for schizophrenia as already demonstrated by various studies (Bleuler, 1950; Kraepelin & Robertson, 1919).Numerous drugs have been used to improve cognition and control diseases like schizophrenia and depression, the earliest being Selective Serotonin Reuptake Inhibitors (SSRI's) by acting as indirect agonists of serotonin (5-HT) receptors (Herva's *et al.,* 2001 and Gartside *et al.,* 2003).

Cognitive behavioral therapy (CBT) is a treatment modality for various psychiatric and psychological problems. Newer atypical antipsychotic drugs, e.g. Clozapine, Quetiapine, Aripiprazole, Risperidone, Olanzapine, and Ziprasidone had demonstrated greater efficacy in improving cognition as compared to typical antipsychotic drugs, e.g. Haloperidol. Both typical and atypical antipsychotic drugs have similar mechanism of action by blocking serotonin $(5\text{-HT})_{2A}$ and dopamine (DA) receptors transmission. Animal studies have reported that atypical antipsychotic drugs were more effective when compared to typical antipsychotic drugs in reversing tasks deficits that involved either working or long-term memory (Grayson *et al.,* 2007).

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Fluoxetine an SSRI was first synthesized in 1971 disclosed in 1974 and was approved by Food and Drug Administration (FDA) in 1987 (Wenthur *et al.,* 2014). It absorbed almost completely after oral administration (70 to 90%), had high central nervous system penetration and largest distributive volume among any SSRI (Wenthur *et al.,* 2014). It had a long half-life; 1-3 days for acute dosing and 4-6 days on chronic dosing. It is metabolized in the liver by cytochrome P_{450} enzyme system (Wenthur *et al.,* 2014). Recent evidence reported that chronic Fluoxetine treatment prevents fear generalization and enhances subsequent extinction (Pedraza *et al.,* 2019). Moreover, chronic treatment of Fluoxetine enhances excitatory and synaptic transmission in the hippocampus (Van Dyke *et al.,* 2019). Another study reported improved spatial memory among female Wistar rats upon single and chronic administration of Fluoxetine (Kus *et al.,* 2017).

Choline being an essential nutrient for various biological functions is a precursor to acetylcholine, sphingomyelin and phosphatidylcholine and methyl donor betaine for converting homocysteine to methionine. Neurons obtained choline from serum choline derived from dietary intake or de novo synthesis. Adequacy in choline concentration in brain is protective against cognitive decline due to aging and different dementia types also including Alzheimer's disease (Amenta *et al.,* 2001).

A recent review (Leermarkers *et al.,* 2015) had made clear conclusion regarding the beneficial effects of choline. A recent study regarding Choline supplementation in healthy adults reported improved vasomotor performance and decreased pupil size after ingestion of choline (Naber *et al., 2015*), while other studies reported no beneficial effects of choline supplementation on memory function (Lippelt *et al.,* 2016; Nagrecha *et al.,* 2013). Among animal models, prenatal choline supplementation had shown improvement in long term memory in rats (Meck & William, 2003). Other studies had also demonstrated choline supplementation in rats with cognitive impairment had resulted in attenuation of cognitive deficits. However, the effects of choline supplementation on cognitive performance and motor function in healthy rats have not yet been studied systemically. This study was conducted with an objective to assess the cognitive behavioral modulation by Choline as compared to Fluoxetine and Clozapine.

MATERIAL AND METHODS

Animals

The study was initiated after permission was granted by Board of Advance and Research Committee, University of Karachi. Animals for experimentation were handled strictly following the guidelines provided by the Institutional Animal Ethics Committee (IAEC) and National Advisory Committee for Laboratory Animals Research (NACLAR). The study was approved by Advance Studies and Research Board (BASR) with study protocol number (BASR No/ 03034/ Pharm). The study was conducted at the Department of Pharmacology, University of Karachi from August, 2018 till January, 2019).

Twenty four healthy male albino rats weighing 180 to 200 grams used in this study were provided by animal house, International Centre for Chemical and Biological Studies (ICCBS), University of Karachi. Rats were caged in pair under a 12hr cycle of light/dark cycle with room temperature being controlled at (24 ± 2^0C). Free tap water access and standard rodent diet was provided for three days before the start of experimentation. The stress effects were minimized by following the standard handling protocols.

Drug and dose administration

The rats were divided into four groups and designated as treatment group being treated with Choline. The dose was adjusted according to the weight of rats. The Fluoxetine was used in powdered form and stock solution was prepared by adding in 10 ml 0.9% NaCl. Then calculated amount of suspension 2mg, 5mg and 8mg/kg/day was administrated with the feeding tubes to respective groups. Control group was given normal saline as 2.0 ml/kg/day. All the drugs were freshly prepared seven days prior to the administration to observe the cognitive behavior modulation.

Water maze test

The water maze (WM) apparatus used in the current study consisted of transparent glass tank of rectangular shape with dimensions 60 cm diameter and 30 cm height water filled at room temperature and powder milk was used to make water opaque. A wooden platform of dimension 15 by 13cm was placed in affix location hidden 2cm beneath the water surface.

Procedure

Water maze (WM) test assessed performance of the effect on spatial memory as demonstrated by Holscher and Mara, 1997. The rats were trained initially. Each rat was placed in water tank with wall facing and was given cut-off time of 2 minutes for climbing and locating the submerged wooden platform. In case if the rat succeeded, 10 seconds was the time allowed to stay on platform (Morris 1984). If the rat failed to reach the platform within the allocated time of 2 minute, it was gently guided to the wooden platform. The retention latency memory function for rats were tested and recorded. The retention latency is the time taken by each rat for locating the hidden wooden platform; one hour (short term) following training. For each session the cut-off time was set as 2 minute.

Open field activity in novel environment

Open field activity test was used for the assessment of locomotor activity. In this test animals are exposed to an open arena, entirely new environment in which animals were not given an aresive or appetitive stimuli and were given opportunity to explore freely for fixed time duration. Open field consisted of wooden board with dimensions as 76 cm length and 76cm width, with wall height as 42 cm. The board is divided into 25 squares of equal sizes. Box was being purposely built with washable material.

Procedure

Open field test provides simultaneous measurement of locomotion, exploration and anxiety. High frequency behavior indicates lower level of anxiety. Rat entries in number of central squares and time duration spent in the central square were measure for exploratory behavior and decreased level of anxiety (Walsh and Cummins, 1997).

A quite room with white light was used to perform experiment. Animals were taken out from cage and were placed at the central square in the open field. All four paws crossing the number of squares and movement latency were counted for time duration of 5 minutes (Ikram *et al.* 2007). In the novel environment, at 1st, 3rd, 5th and 7th week the activity of Choline, Fluoxetine, Clozapine and Saline treated rats were monitored. The balance design was used for scoring purpose for avoidance of order effect of rats open field behaviors. Rats were placed in centre or one of the four corners of the field for behavioral scoring (Brown *et al.*, 1999). High frequency / duration of rat behavior indicated raised exploratory behavior and reduced anxiety level.

The frequency with which rats crossed one of the great lines with all the four paws determined locomotor activity, while the time spent in the central square by rats showed exploratory activity.

STATISTICAL ANALYSIS

The data was analyzed using SPSS version 20 (IBM). Data was entered and validated twice in the statistical software to correct for incorrect entries. Quantitative variables were presented as mean ± Standard deviation (SD). Test of normality, *Kolmogorov–Smirnov test* was performed. As the data across experimental groups were normally distributed, *One Way Analysis of Variance (ANOVA)* test with post-hoc comparison using *Turkey's test* was used to compare the effect of the drugs. For inferential statistics purpose, the p-value<0.05 was considered significant.

RESULTS

Table 1shows the comparison of latency time in seconds among the four groups (Saline, Choline, Fluoxetine and Clozapine) on water maze test. There was no significant difference as p-value > 0.05 in learning behavior impairment as indicated by increased latency time i.e. reaching the platform at 1st and 3rd week. Significant difference in latency time was observed at 5th and 7th week as p-value <0.05. Choline with mean latency time of 6.5 sec and Clozapine as mean latency time of 6 sec were significantly lower as compared to the mean latency time for Saline as 10.8 seconds and Fluoxetine as 11 seconds at 5th week. Moreover, difference in mean latency time was also found significant at 7th week, as the mean latency time for Choline and Clozapine was 4 seconds and 5.16 seconds, respectively; significantly lower as compared to Saline and Fluoxetine mean latency time which were 11.33 sec and 11.8 seconds. Result showed that rats treated with Choline showed improved learning behavior reached to that particular place to save them in less time as compared to antidepressant used in this study. fig. 1 gives the overall performance of male albino rats on water maze test.

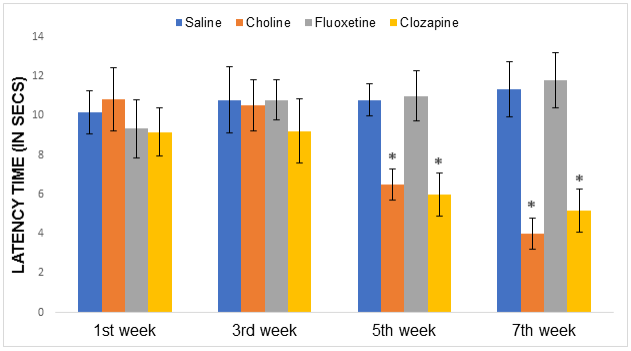

Fig. 1: Overall performance of the albino rats in water maze test

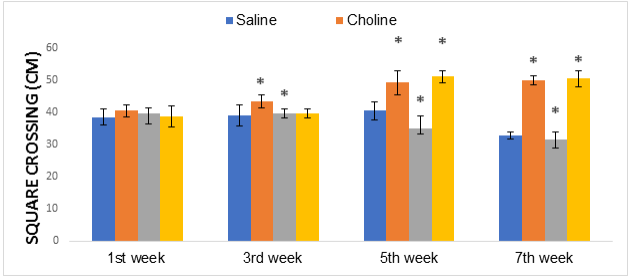

Fig. 2: Overall performance of the male albino rats in open field activity test

Table 2 shows result for square crossing exhibited by four groups (Saline, Choline, Fluoxetine and Clozapine) in open field activity test. No significant difference was observed in mean square crossing among four groups at 1st week (P-value >0.05). Significant difference in mean square crossing among four groups was observed at 3rd, 5th and 7th week (P-value <0.05). At 3rd week significant difference in mean square crossing was found between Choline and Fluoxetine (43.30 vs. 39.60; p-value <0.05). Moreover at 5th week, significant difference in mean square crossing was observed between Choline and

Fluoxetine (49.3 vs. 35.1; p-value< 0.05), Choline and Clozapine (49.3 vs. 51.1; p-value< 0.05) and Fluoxetine and Clozapine (5.1 vs. 51.1; p-value < 0.05). Furthermore at 7th week, significant difference in mean square crossing was found between Choline and Fluoxetine (50.0 vs. 31.5; p-value <0.05), Choline and Clozapine (31.5 vs. 50.5; p-value <0.05) and Fluoxetine and Clozapine (31.5 vs. 50.5; p-value <0.05).

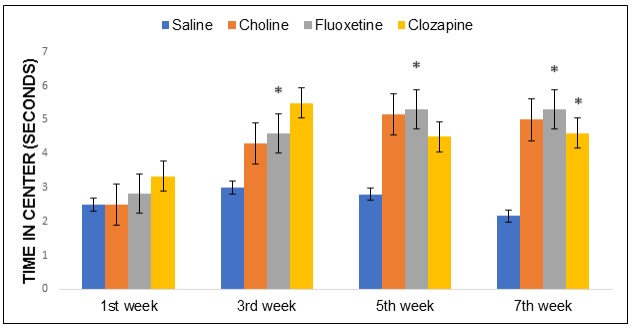

Fig. 3: Time spent in centre by albino rats in open field activity test

Fig. 2 represents similar details where increase in locomotor and exploratory behavior was exhibited by Choline in 3rd to 7th week. Thus Choline is reported an increased behavior which is a measure of decreased anxiety level. Fluoxetine showed a trend of decrease in Mean square crossing from 1st to 7th week. Clozapine exhibited significant increase in locomotor activity in 5th and 7th week, thus an enhanced behavior is reported as a measure of reduced level of anxiety.

The table 3 shows details of time spent in centre by male albino rats in four groups (Saline, Choline, Fluoxetine and Clozapine) at time period 1st week, 3rd week, 5th week, and 7th week. No significant difference was observed in mean time spent in centre among four groups at 1st week (P-value>0.05). Significant difference in mean time spent in centre four groups was observed at 3rd, 5th and 7th week (P-value<0.05). Time spent in the centre exhibited by Choline and Fluoxetine as compared to Saline was significant (p-value <0.05) from 3rd to 7th week, whereas Clozapine showed significant (p-value<0.05) time spent in centre in the 7th week. Central location is considered as the anxiety provoking space with low level of anxiety allows the animals to spend more time in the centre. fig. 3 also give the time spent in centre of the albino rats in open field activity test.

DISCUSSION

Cognitive behavioral impairment is effective on daily function and are disabling for the persons with different etiology requires the integration of Pharmacological and psychosocial intervention (Kopel & Liberman, 1995; *Moeibius et al.,* 1999). Learning and memory was assessed through Morris water maze test, widely used for behavioral testing as it measures the psychological processes as well as neural mechanisms involved for spatial learning. Anti-depressants used in this study, Fluoxetine one of the most commonly used antidepressants had caused impairment in learning and memory by increasing the latency time to reach the platform though not significant as compared to Saline. The study findings showed relevance with the findings of Gramle Amol *et al., (2003)* that reported Fluoxetine causes more learning impairment as compared to Amitriptyline but was reversed by Citicoline having different mechanism of action. Learning and memory are the significant components of cognition, so learning and memory function impairment can also be regarded as impaired cognition. Another study reported that repetitive but low dose administration of Fluoxetine had detrimental effects on long term memory in adult naïve rats however learning remained unaffected (Elizabath *et al.*, 2013). The reported findings are consistent with our study results.

It was also reported by another animal study (Kumar *et al.*, 1996) conducted on mice that the effects of anti-depressant drugs (Imipramine and Fluoxetine) on cognitive functions was being impaired by the muscarinic antagonists. The augmentation of learning and memory tasks studied by employing passive avoidance paradigms Amitriptyline and Imipramine showed significant impairment of memory, whereas Fluoxetine reversed the scopolamine induced memory impairment.

The consequences of anti-depressant drugs on cognitive function among diseased subjects (human and laboratory animals) facilitated evaluation of drugs effects on cognition without any disease complexities. Depressive episodes among depressed individuals may be inclined by several factors that included the basic neuropathology as well as the frequency/ severity (Amol *et al.*, 2015).

The hallmark findings of the current study was that Fluoxetine increased the latency time to reach the platform which was not significant therefore it concluded that learning was neither impaired nor enhanced by anti-depressant, i.e., Fluoxetine. However, recent evidence argues that the anti-depressants target glutamatergic system suggesting that the re-organization of glutamatergic networks could lead to adverse memory outcome. Thus, cognition enhanced that act on glutamatergic synapses might be effective to overcome impairment of memory with long term administration of Fluoxetine (Cheung & NY, 2011) which is being reported in the current study.

Clozapine being an atypical anti-psychotic (Naheed & Green, 2001) drug has a wide range of receptor activity, including antagonism of dopamine D4, H1, alpha-1-adrenergic, and muscarinic receptors. The drug may prove to be a safer alternative compared to Fluoxetine in this

Table 1: Latency time (sec) exhibited by each group in water maze test

	Saline Mean ± SD (n = 6)	Choline Mean ± SD (n = 6)	Fluoxetine Mean ± SD (n = 6)	Clozapine Mean ± SD (n = 6)
1st week	10.16±1.10	10.83±1.60	9.33±1.47	9.16±1.21
3rd week	10.8±1.70	10.50±1.30	10.8±1.04	9.20±1.63
5th week	10.8±0.80	6.50± 0.80*	11.00±1.26	6.00±1.10*
7th week	11.33±1.40	4.00±0.80*	11.80±1.41	5.16 ±1.10*

Table 2: Square crossing exhibited by each group in open field activity test

	Saline Mean ± SD (n = 6)	Choline Mean ± SD (n = 6)	Fluoxetine Mean ± SD (n = 6)	Clozapine Mean ± SD (n = 6)
1st week	38.50±2.42	40.50±1.87	39.60±1.67	38.80±3.31
3rd week	39.10±3.18	43.30±2.06*	39.60±1.36*	39.60±1.36
5th week	40.50±2.88	49.30± 3.77*	35.10±3.81*	51.10±1.83*
7th week	32.80±1.09	50.00±1.41*	31.50±2.34*	50.50 ±2.51*

Table 3: Time in centre exhibited by each group in open field activity test

	Saline Mean ± SD (n = 6)	Choline Mean ± SD (n = 6)	Fluoxetine Mean ± SD (n = 6)	Clozapine Mean ± SD (n = 6)
1st week	2.50±0.54	2.50±1.04	2.83±1.75	3.33±0.51
3rd week	3.00±3.18	4.31±0.51*	4.60±0.51*	5.50±0.54
5th week	2.83±1.16	5.16± 0.40*	5.30±0.81*	4.50±0.54
7th week	2.16±1.72	5.00±0.06*	5.30±1.21*	4.60 ±0.51*

*P<0.05, **P<00.1

subset of patients as impairment was relatively less as per the study conducted. Clozapine enhanced learning and memory on 5th and 7th week which was significant, as the drug had high affinity for dopamine D4 receptor, the mechanism by which D4 blockade could improve cognition is not fully known (Jentch *et al.*, 1999). Some of atypical anti-psychotic, can enhance the release to the acetylelcholine in the prefrontal cortex possibly making contribution for cognitive enhancement (Ichikawa *et al.*, 2002) but was reversed by Choline, which has a novel mechanism of action. The effect of atypical anti-psychotic drugs on memory needs further investigation as Fluoxetine had failed to produce significant enhancement of memory but Clozapine and Choline improved learning and memory on 5th and 7th week as compared to Saline. Furthermore, it could be hypothesized that Choline and Clozapine improves learning and memory behavior as compare to Fluoxetine.

Open filed activity test allowed general activity level assessment in large arena by square crossing with simultaneous evaluation of anxiety like characteristics, exploratory and locomotor behaviors. The current study results showed that Choline and Fluoxetine treated groups showed increased square crossing in 3rd, 5th and 7th week which was significant (P-value < 0.05) as compared to Saline whereas Clozapine showed significant increase in (P-value < 0.05) locomotor activity on 3rd, 5th and 7th week, whereas time spent in centre was found significant only in 7th week by Clozapine as compared to saline. The enhanced behavior was shown by Choline and Fluoxetine the 3rd, 5th and 7th week showed low level of anxiety assessed by increase locomotor and exploratory activity but Clozapine treated group showed only significant results on 5th and 7th week.

Chronic SSRIs treatments have proven effective in depressed or anxious patients and also in animal models (Barr *et al.*, 1997). Fluoxetine though devoid of serotonin receptor affinity (Beasly *et al.*, 1992) but acts as an indirect agonist stimulating multiples 5HT receptors (Hoyer *et al.*, 1994). Serotonergic mechanism plays a significant role in locomotor activity modulation at number of levels in the neuroaxis, i.e. spinal cord, limbic structures, basal ganglia and frontal cortex (Borcco *et al.*, 2002). Clozapine possess anxiolytic properties in certain experimental models in rodents and other species (Winley *et al.*, 1993). This is consistent with current study findings showing highly significant square crossing (locomotor) and time spent in the centre (exploratory) exhibited by rats treated with Clozapine in 5th and 7th week. These high behaviors were indicative of low level of anxiety. The result complies with the argument that Clozapine reduced anxiety related behaviors (Fie *et al.*, 2002).

Recent studies had also reported the beneficial effects of choline. A study reported that after choline intake the locomotor performance, cognition and also motor

function was significantly improved among rats (Naber *at al.,* 2015). Another study further reported that choline administration improved cognitive and locomotor function (Tabassum *et al.,* 2017). However, it has been reported that the effect of choline is dependent on dosage as small doses could be non-productive while high dosage could be toxic thus moderate dose is recommended (Borges *et al.,* 2015). Moreover, another study reported that adult rats, aged at least 6 months having received 10 weeks dietary choline supplementation showed enhanced declarative memory performance (*Moreno et al.,* 2018). However, the study by Tayebati *et al.,* (2017) reported that choline administration did not affect the expression of inflammatory markers or stimulation of inflammatory process in the cerebral areas of the brain.

CONCLUSION

Study findings could help us to draw conclusion that Choline alone can be used to improve cognitive behaviors or along with other potent anti-depressant and atypical anti-psychotic. Furthermore, future research in this regard is desirable to be conducted among a larger number of subjects both animal and human to build more profound and robust evidence about effect of these drugs on learning, memory and anxiety.

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