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Relationship between memory improvement and brain acetylcholine following chronic choline supplementation in rats

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Abstract: Central cholinergic neurotransmission has an essential part in the learning and memory pathways. Being the precursor of acetylcholine (ACh), choline is particularly associated with attention, learning and memory functions. The production and release of ACh in brain can be facilitated via increased availability of free choline. Therefore, the current study was aimed to determine the correlation between choline supplementation-induced memory improvement and brain acetylcholine content. Two groups of rats (n=6) were orally administered with tap water (Control Group) and aqueous solution of choline bitartarate (Test group) respectively for a period of five (5) weeks. Cognitive performance was assessed by using behavioral tests include Novel object recognition test, Morris water maze test and Passive avoidance test to evaluate the changes in learning and memory performances. After behavioral analysis rats were decapitated and their brain samples were collected for estimation of acetylcholine levels. Following chronic administration of choline bitartarate powder improvements in recognition, associative and spatial memory performance was observed as compared to control group. Levels of acetylcholine were also increased in brain following choline supplementation. Improvements in learning and memory performances along with neurochemical alterations following chronic administration of choline bitartarate suggest that choline significantly improved the cognitive performances via enhancing the brain acetylcholine levels so it might be used as an effective therapy for neurological disorders affecting learning and memory performance.

Keywords: Choline bitartarate, Recognition memory, Spatial memory, Associative memory, Acetylcholine
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INTRODUCTION

In memory and other cognitive capacities, cholinergic neurotransmission plays a crucial part that has been perceived since 1970s [1-3]. Studies revealed the association of central cholinergic system in the processes of learning and memory in both invertebrates[4] and vertebrates[5]. This cholinergic neurotransmission has been supposed to be associated with variations in information retrieval, extinction and also in the processes of acquisition, consolidation and reconsolidation[6, 7] and any disturbance or dysfunction in cholinergic neurotransmission causing memory loss and assumed to have a critical role in Alzheimer's disease (AD) [2, 3]. Evidence revealed that in AD several markers of cholinergic activity (both pre and post synaptic) are declined [7, 8], most important of which is choline and acetylcholine (ACh) [7].

Choline is a vital dietary constituent [9, 10] that is required for cell membrane-associated phospholipid synthesis, methyl metabolism, transmembrane signaling, transportation and metabolism of cholesterol and more importantly in cholinergic neurotransmission [11] as it is the precursor of an essential neurotransmitter, ACh which is particularly associated with attention, learning and memory functions[10]. Choline is particularly a α7nicotininc cholinergic receptor agonist [7, 12] that might be imperative in numerous psychological processes and in brain development [10, 13] as it helps in the formation of tissues inside the nervous system that involves in the growth and development of brain [14].

Studies showed that choline is also involved in enhancement of nerve signaling capacity, maintenance of their structural integrity and protection of dynamic neuronal membranes[15]. Deficiency of choline can bring about reduction in memory and concentration leading to mood variations and other cognitive deficits during aging[15]. Since ACh is synthesized via combination of acetate and choline, so when there is a deficiency of choline, the ACh cannot be appropriately produced and delivered as a result function of brain can suffer[15]. Due to this reason choline has been the center of various studies to decipher that it can facilitate memory performance or not[3]. It is believed that the rate of synthesis and release of ACh can be facilitated via increased availability of free choline [3, 16]. Besides this, some studies reported that choline supplementation enhances brain choline availability but is unable to enhance ACh synthesis or release so it might be inadequate to relieve cognitive disturbances of AD [10, 17]. So, the current study was intended to find out the correlation between choline-supplementation-induced memory impairment and brain acetylcholine content. We have investigated that whether ACh levels are positively correlated with improvement in various forms of memory following choline intake.

MATERIALS AND METHODS

Animals

For this study twelve locally bred Albino-Wistar rats were bought from Dow University of Health Sciences, OJHA campus, Karachi, Pakistan. Animals were caged individually with ad libitum access to cubes of standard rodent diet and tap water under a 12:12 h light/dark cycle (lights on at 7:00 am) at room temperature (22±2 °C). In order to rule out the environment-induced psychological affliction rats were exposed to acclimation period and to several handling procedures seven days prior to the experiment to overcome the handling stress and uniqueness. The methods and procedures used in this study were permitted by the institutional ethics and animal care committee and executed in strict accordance with National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised1985). To avoid the order and time effect all the treatments and behavioral procedures were performed in a balanced design.

Drugs

The Choline tablet powder, purchased from Powder City, New York, USA was used in the experiment. All chemicals were of analytical grade. All the reagents were freshly prepared before the start of the procedure. Drug solution was made freshly each day for administration. Controls received equal volume of tap water.

Experimental Protocol

The animals (n=12) (weight, 150-200 g) were randomly assigned into two experimental groups (n=6); Control and Test (Choline). Control rats received tap water daily via oral route in a volume of 0.2 ml/150g body weight to each rat. Test rats received aqueous solution of choline bitartarate tablet powder via oral route at the dose of 50mg/kg body weight daily in a volume of 0.2 ml/150g body weight to each rat for the duration of 5 weeks. The dose selection is equivalent to the recommended tablet dose for humans mentioned by manufacturing company and previous literature reports. At the end of 4 weeks treatment schedule, the behavior studies were carried out during which the drug administration was continued. Behavioral tests performed includes Novel Object Recognition test (NORT) to assess recognition ability, Morris Water Maze Test (MWM) and Passive Avoidance (PA) Test for the assessment of spatial and associative learning and memory performance. Later rats were decapitated, and their brains were dissected out from the skull within 30 s after decapitation. By using fine forceps, the membrane covering the brain, was removed.

Behavioral Protocols

Object Recognition Task

The novel object recognition test, established by Ennaceur and Delacour in 1988[18] , is the test for determining the cognitive ability of animals. It was based on the rat's capability to differentiate a novel (new) object in a familiar environment [18] and is used to evaluate memory for interactions with novel objects. The procedure used in this test was basically the same as that Okuda et al 2004 with minor modifications [19]. The apparatus consists of square box made of gray painted wood having dimensions of 45x45x45cm^3. In order to saturate it with olfactory stimuli, cleaning of box was not allowed throughout the experiment. The objects to be distinguished were two similar transparent glasses filled with white cement (used as familiar objects, A1 and A2) in order to make them heavy enough so that rats could not be able to move them, and a metallic container of same size filled with white cement (used as a novel object, B). The size of the objects was 2.5 times the size of the rat so that the rat could easily sniff it. The test consists of three phases; habituation, training, and test phase. Habituation phase is performed on day 1 during which rats were exposed to the arena for 10 minutes in order to familiarize them to the testing chamber. Then, 24 hrs. after pre-exposure rats training phase was done in which rats was placed inside the box with two alike objects (A1 and A2) and allowed to explore them for 10 minutes. After 10 min, the rats were taken out from the box and returned back to its home cage. Similar Objects A1 and A2 were also then removed from the box. The test phase was performed twenty minutes after the training phase, in which rat was taken out from their home cage and placed back into the object recognition box, where it is exposed to one new object (B) and one of the old object for 3 minutes. The parameters monitored during this phase includes the sniffing time for the novel and familiar object. Object exploration was explained as when the rat directed its nose toward the object at a distance of <2cm. It has been reported that when there is no difference in exploration for two

objects at the test phase then it can be interpreted as a cognitive deficit [20].

Morris Water Maze Test

The Morris water maze (MWM) test is the test of spatial learning and memory [21]. The apparatus of test consists of a circular pool of water with a diameter of 45 cm, height of 37cm, and depth of 12cm. The pool is a metal cylinder painted white on the inner surface and is filled with water (23±2 °C) which was made opaque with milk in order to obscure the platform to allow proficient tracking of the swim paths of the rats [22]. The escape platform is also made of metal cylinder with flat metallic top having a surface diameter of 8 cm, and it is placed 2 cm below the surface of water during training phase. The parameters monitored in our experiment, includes the reference (long-term) memory and working (short-term) memory assessed in terms of latency to locate the escape platform. The method used in the present study was consists of two phases: training and testing. For each phase the cut off time was 2 min. Firstly, the training phase was performed during which each rat was placed into the water pool in such a way that their face was towards the wall of the pool. After placing the animal 120 seconds was given to each animal to find and mount onto the hidden platform. Once the rat found the platform it was permitted to stay on it for 10 s. But if it failed to locate the platform during the assigned time, then it was directed gently towards/onto the platform [20]. The test phase consists of two sub-trials: STM (short-term memory) and LTM (long-term memory) trial. In both sessions the memory performance of rat was assessed via recording the retention latency. STM was determined 60 min after training phase, and LTM was assessed after 24h of training [23].

Passive Avoidance Test

The Passive Avoidance task (PAT) is a fear-aggravated test used to evaluate associative memory performance in rodents. Passive avoidance is an operant conditioning task where an animal learns to suppress movement in order to avoid an aversive stimulus. This memory testing in this test is based on the association formed between an aversive stimulus such as a mild foot shock and a particular environmental context. During this test, animals learn to avoid an environment in which an aversive stimulus (such as a foot-shock) was supplied previously. This test is also beneficial to assess the effect of novel chemical entities on learning and memory performance as well as to study the pathways involved in memory retention. The paradigm for PAT comprises of a box contains two separate compartments one as an illuminated ‘safe’ and the other is a dark 'punishable' one, that are connected together with a door to allow free crossing from one compartment to another. Both compartments have a grid floor consisting of rods having a diameter of 5 mm positioned with the distance of 0.5 cm between them. The Passive avoidance task was comprised of two sessions, training and testing. During the training phase, rat was placed in an illuminated box and was allow to explore the box. When the rat prompted into the dark compartment by its instinct stepped with its four paws, the door was closed immediately and a foot-shock (1.5 mA) was delivered through the grid floor to its paws for 5 seconds. Afterwards, it was allowed to stay in this compartment for 10 sec then the door was opened and it immediately returns back to illuminated safe compartment. In the training session the initial step-through latency to enter the dark compartment was noted with the help of stop watch. In the test phase, rats were placed in the illuminated safe compartment again after training session with cutoff time of 3 minutes. The test session was further divided into two phases; acquisition and retention phase. In the acquisition phase, the rat was placed in the illuminated safe compartment again after 60 minutes (1hr.) of last learning trial. While during the retention phase, the rat was placed in the illuminated safe compartment after 24 hr. (1 day) of last learning trial. In both phases the step-through latency (the time taken by the rat to enter the shock-paired dark compartment) was recorded with the help of stop watch[24]. The decline in step-through latencies (retention or recall latency) to enter the dark compartment in the test phase were considered as a measure of “amnestic” effects[25].

Assay of Brain Acetylcholine Content

The concentration of acetylcholine in brain tissue was determined by the procedure of Hestrin, 1949 [26] as explained by Augustinson, 1957 [27] and Batool et al. 2016 [28]. The brain tissue was boiled for enzyme inactivation and to discharge the bounded acetylcholine which then reacted with ferric chloride to develop a brown color and then absorbance was read at 540 nm against the reagent

blank. The acetylcholine content was presented as µmol/g of brain tissue.

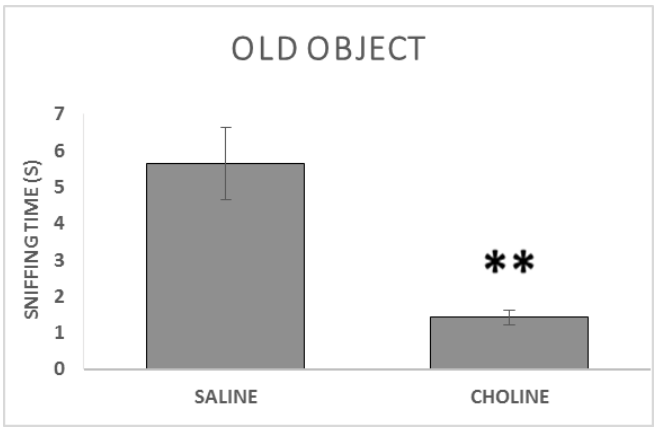
Statistical Analysis

The data of all experiments is presented as mean ± S.D. The statistical analysis was done by SPSS software version 20.0 for windows program on a computer. All the parameters were tested with Shapiro-Wilks test and were found to be normally distributed ($p<0.05$); therefore, in instances of multiple mean comparisons, the results of behavioral and neurochemical assessment were evaluated by Student's *t*-test for the two experimental groups for each parameter. The significance level for all comparisons was $p\leq0.05$.

RESULTS

The objective of current study is to compare the effects of choline supplementation on learning and memory performance along with determining changes in content of brain acetylcholine which may help in future to use choline for treating cognitive disorders. Effect of choline on spatial and non-spatial learning and memory performance and cognitive functions was assessed during this study using memory specific behavioral tasks that are NORT, MWM and PA followed by assessment of brain acetylcholine content.

Recognition memory was determined by NORT in terms of sniffing time for novel and familiar (Old) objects presented in fig. 1 (a & b). It was observed that recognition memory was improved in choline treated group evident by increased sniffing time for new object and decreased sniffing time for old object. Data analysis of sniffing time for old object by Student's *t*-test revealed significant (t (10) = 8.995, $p<0.0001$) reduction in sniffing time for old object in choline-supplemented group in comparison to control group as presented in figure 1a. While sniffing time for novel object was found to be significantly (t (10) = 9.84, $p<0.0001$) enhanced in choline-supplemented group as compared to control group (figure 1b).



(a)

(b)

Figure 1: Effect of choline on recognition memory was evaluated by Novel object recognition task in terms of sniffing time for familiar (old) (a) and novel object (b) during 3 min choice phase. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by Student's t-test and expressed as *=$p<0.05$, **=$p<0.01$, compared to control (Saline).

Effect on spatial cognitive abilities of animals was determined by using MWM task, in which rats learn to escape the submerged platform in a pool via using distal visual cues. It was found that there is a significant improvement in STM (t (10) = 8.98, $p<0.0001$) (see figure 2a) and LTM (t (10) = 7.27, $p<0.0001$) (see figure 2b) in choline-treated rats evident by significant ($p<0.01$) increment in latency time in comparison to control rats (fig. 2 a & b).

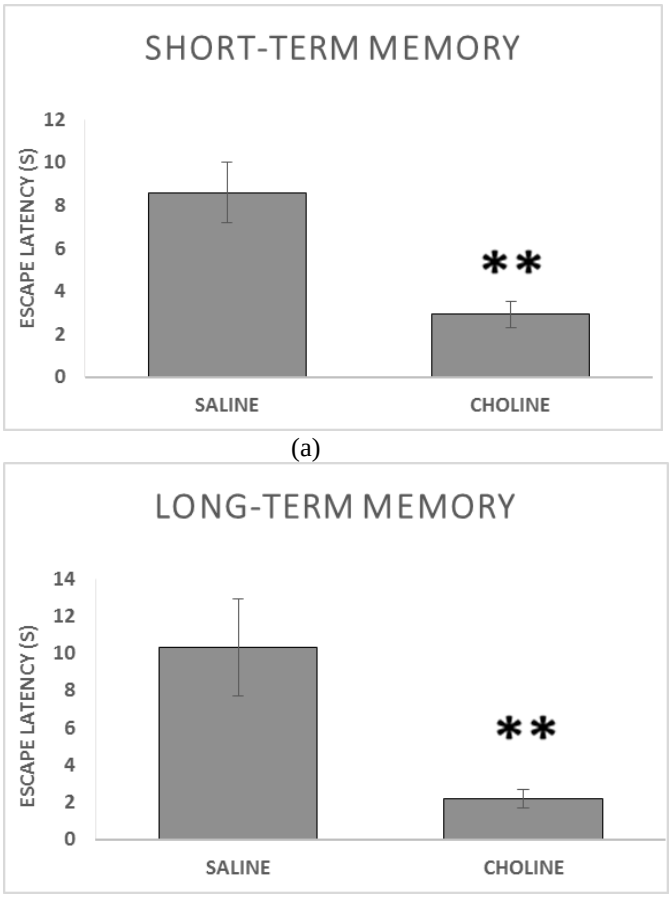


Figure 2: Effect of choline on spatial memory performance of rats was determined in a spatial memory task [Morris Water Maze (MWM)] as STM (a) and LTM (b). For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by Student's t-test and expressed as *=p<0.05, **=p<0.01, compared to control (Saline).

Associative memory of all experimental groups was assessed by passive avoidance test via monitoring the step-through latency to enter the dark compartment in both training and test phase. Results have shown that associative memory was improved following treatment with choline as evident by significant increment in step-through latencies in choline supplemented rats during acquisition (t (10) = 12.33, p<0.0001) and retention (t (10) = 26.19, p<0.0001) phases compared to controls (figure 3 a & b). These results suggest that associative memory was improved following intake of choline.

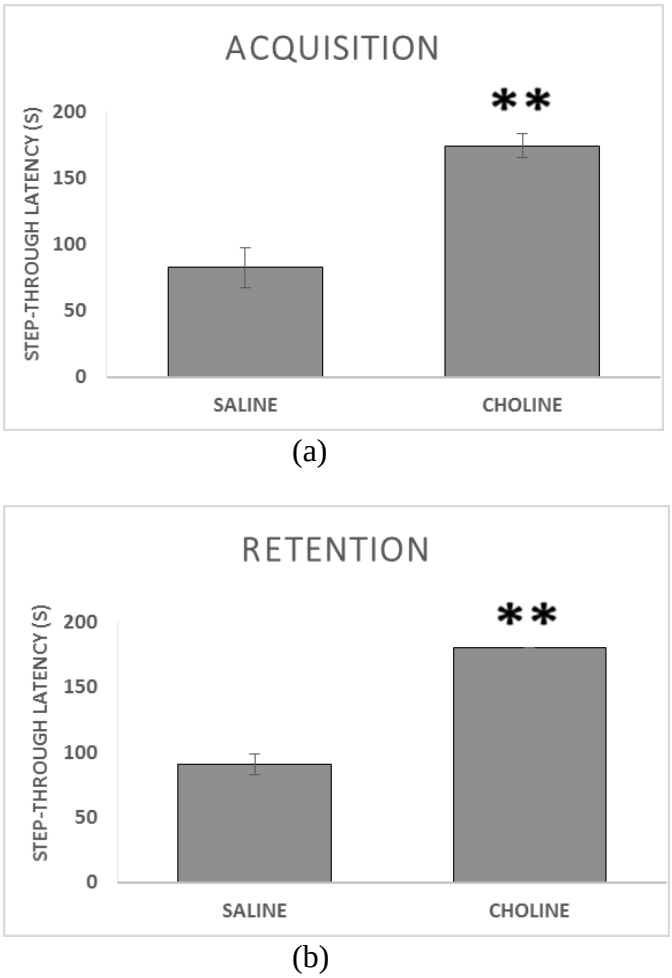


Figure 3: Effect of choline on avoidance memory performance of rats was assessed in Passive avoidance test (PA) for duration of 5min by recording the step-through latencies during acquisition phase (a) performed after 60 minutes of initial training trial and during retention phase (b) performed after 24hr of initial training trial. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by Student's t-test and expressed as *=p<0.05, **=p<0.01, compared to control (Saline).

Acetylcholine content in brain of experimental groups was determined by spectrophotometric analysis. It was observed that acetylcholine content was significantly enhanced (t (10) = 5.79, p<0.0001) following choline treatment (figure 4).

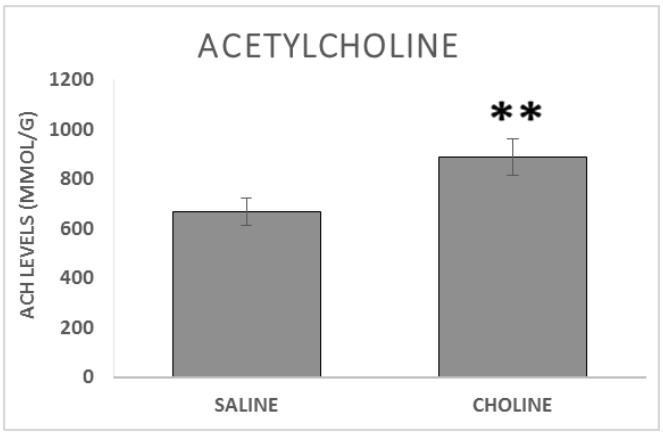
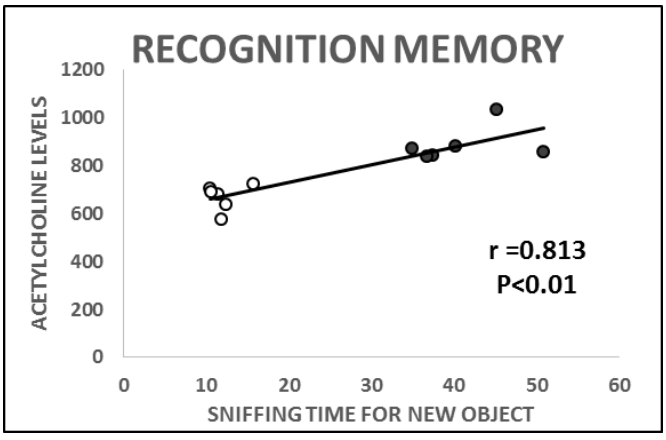
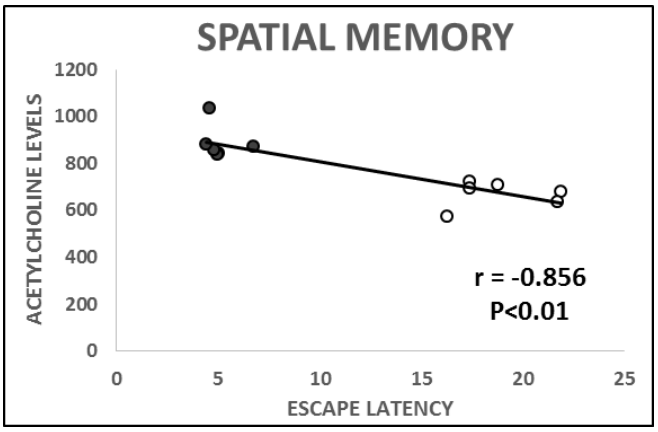


Figure 4: Effect of choline on acetylcholine levels in brain of rats. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by Student's t-test and expressed as *=p<0.05, **=p<0.01, compared to control (Saline).

These results suggest that acetylcholine content of brain was increased following intake of choline. Correlation analysis was done by Pearson correlation test between brain acetylcholine content and improvement in memory function. Pearson correlation analysis revealed that enhancement in recognition memory (r= 0.813, p<0.01) and improvement in associative memory (r = 0.885, p<0.01) was positively correlated with acetylcholine levels in brain (figure 5 a & c) while enhancement in spatial memory was found to be negatively correlated (r =-0.856, p<0.01) with brain acetylcholine content (figure 5 b). These results suggest that as acetylcholine levels are increasing in brain, contributing to better memory performance.



(a)



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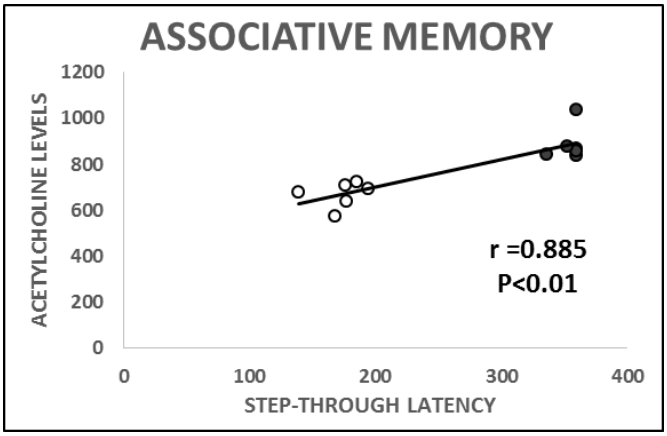

(c)

Figure 5: Correlation analysis between acetylcholine content and improvement in memory function. Pearson Correlation analysis showed significant correlative relationship for recognition (a), spatial (b) and associative (c) memory performance.

DISCUSSION

The current study was aimed to determine the correlation between choline supplementation-induced memory improvement and brain acetylcholine content. We have found improvement in memory function following chronic choline intake and this improvement is positively correlated with enhancement in brain acetylcholine content. In the present study choline tablet powder was administered orally for a period of 4 weeks to rats in the dose equivalent to adult human dose (500mg/day). After 5 weeks of supplementation various aspects of learning and memory performance were evaluated using specific behavioral tests. Levels of acetylcholine in brains of rats were determined and correlated with memory performance. As it is assumed that choline intake might alter functioning of brain via

acetylcholine formation affecting cholinergic neurotransmission [29].

Choline, a macronutrient, is essential for functioning of liver, movement of muscles, nerve functioning and development of brain, and nerve function, and also for maintaining a normal healthy metabolism. Evidence shows that choline plays a significant part in the neuronal development and functioning [10]. Moreover, being a component of cell membrane and neurotransmitter involved in nerve signaling, choline has a particular part in preservation of memory and in preventing loss of cognitive and memory function. It performs a significant job of maintaining brain elasticity via balancing acetylcholine levels in brain [30]. Findings of present study show significant improvement in learning and memory performance across various aspects. For determining effect on recognition memory NOR test was used which assesses episodic memory that intensely dependent on recognition ability determined by monitoring rodent's exploratory behavior and their extemporaneous preference for novel objects [18]. Results showed a significant enhancement in recognition ability. For determining the effect of choline supplementation on spatial memory performance the MWM task was used[20]. It was found that following chronic choline intake both short-term and long-term spatial memory performance was improved. Moreover, to observe the consequences of choline intake on associative memory passive avoidance task was performed to determine learning and memory performance associated with a stressful stimulus and evaluates emotional memory [31]. Present findings demonstrated a marked increment in associative memory in both acquisition and retention phases. Present results are in agreement with some of earlier reports which state that choline supplementation during pregnancy and development responsible to enhance memory performance later in life [7, 10, 14, 32]. While in contrary other studies reported that choline treatment alone had no effect on short-term memory or attention, choline may potentially facilitate memory function in combination with other compounds [3, 33]. It might be due to saturation of the choline transporters when cholinergic neurons are inactive [3, 16] but present findings are contradictory to these reports propose that chronic choline administration at adequate dose is responsible for enhancement in learning and memory function.

It is reported that choline is the precursor of ACh which is particularly associated with attention, learning and memory functions [10]. It is believed that the rate of synthesis and release of ACh can be facilitated via increasing availability of free choline [16, 3]. Reports showed that reduction in acetylcholine levels might result in decline of cognitive function, including AD and senile dementia [30]. In the current study we have determined that following chronic choline intake acetylcholine concentration of brain was increased suggesting that as availability of free choline increases, acetylcholine levels rise leading to improved memory performance. This increment in acetylcholine might be due to its increased synthesis and release in nerves. Results of correlation analysis also showed a positive correlation between memory performance and acetylcholine levels. Previously studies showed that no positive relationship was found between choline availability and acetylcholine concentration in healthy persons [10, 3, 34]. However, positive correlation observed in current study is consistent with other studies which report that reduced levels of plasma free choline was related with decline in cognitive performance [10]. Studies have also showed that Alzheimer's patients have very low acetylcholine content and the medications used to treat AD are basically mimicking the choline's effect of increasing ACh levels and associated effects [31]. So, in a future study it would be worthwhile to determine whether the same dose of choline could be able to relieve cognitive dysfunction observed in AD.

From the present findings it can be concluded that chronic choline intake may be responsible to improve memory function and cognitive processing via increasing synthesis and releases of acetylcholine in brain. Moreover, a positive relationship is observed between memory improvement and brain content of acetylcholine following chronic choline supplementation. Hence, it can be suggested that choline may be considered as a potential therapeutic agent in AD.

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