Ascorbic Acid Ameliorates Nicotine Exposure Induced Impaired Spatial Memory Performance in Rats
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ABSTRACT

Introduction: The long lasting behavioural and cognitive impairments in offspring prenatally exposed to nicotine have been confirmed in animal models. In the present study, we investigated the effect of ascorbic acid on prenatal nicotine exposure induced behavioural deficits in male offspring of rats.

Methods: The pregnant Wistar dams were divided into four groups of six rats: control, vehicle control, nicotine and nicotine+ascorbic acid groups. The nicotine group received daily dose of subcutaneous injections of 0.96 mg/kg body weight (bw) nicotine free base throughout gestation. Pregnant dams in nicotine+ascorbic acid group were first given nicotine free base (0.96 mg/kg bw/day; subcutaneous route) followed by ascorbic acid (50 mg/kg bw/day, orally) daily throughout gestation. The cognitive function of male offspring of all the experimental groups was studied using Morris water maze test at postnatal day 40.

Results: Prenatal nicotine exposure altered spatial learning and memory in male offspring. However, treatment with ascorbic acid ameliorated these changes in rats.

Conclusion: Ascorbic acid supplementation was found to be effective in preventing the prenatal nicotine exposure induced cognitive deficits in rat offspring to some extent.

Keywords: Ascorbic acid, learning, memory, nicotine

El Ácido Ascórbico Mejora el Mal Funcionamiento de la Memoria Espacial Afectada por Exposición a la Nicotina en Ratas
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RESUMEN

Introducción: Se han confirmado afectaciones cognitivas y conductuales de larga duración en la cría prenatalmente expuesta a la nicotina en modelos animales. En el presente estudio, se investigó el efecto del ácido ascórbico en las deficiencias conductuales provocadas por exposición prenatal a la nicotina en las crías de ratas machos.

Métodos: Las ratas Wistar preñadas fueron divididas en cuatro grupos de seis ratas – control, control con vehículo, nicotina, y grupos de nicotina + ácido ascórbico. El grupo de nicotina recibió una dosis diaria de inyecciones subcutáneas de una base libre de nicotina de peso corporal (pc) 0.96 mg/kg durante la gestación. Las ratas preñadas en el grupo de nicotina + ácido ascórbico recibieron primero una base libre de nicotina (0.96 mg/kg pc/día; vía subcutánea) seguida por el ácido ascórbico (50 mg/kg pc/día,vía oral) diariamente a lo largo de la gestación. La función cognitiva de las crías de ratas machos de todos los grupos experimentales, se estudió mediante la prueba de laberinto de agua de Morris en el día postnatal 40 (PD-40).

Resultados: La exposición prenatal a la nicotina alteró el aprendizaje espacial y la memoria en las crías de machos. Sin embargo, el tratamiento con ácido ascórbico mejoró estos cambios en las ratas.

Conclusion: Se halló que la suplementación con ácido ascórbico era eficaz en la prevención de los déficits cognitivos por exposición a la nicotina prenatal en crías de rata en cierta medida.
INTRODUCTION
For centuries, tobacco was consumed by humans, either chewed or smoked. It is a common sight to see people of all ages or gender smoking and this does not exclude pregnant mothers. Although cigarette smoking during pregnancy is associated with adverse fetal, obstetrical and developmental outcomes, 15–20% of women were found to be smoking throughout the duration of pregnancy (1–3).

Cigarette smoking during pregnancy is associated with adverse reproductive outcomes such as increased infant mortality, morbidity, reduced birthweight, enhanced susceptibility to respiratory diseases and changes in the immune system (4–7). Nicotine, the major component of tobacco, is proven to be a neuroteratogen (8–10). Evidence from animal studies has confirmed that nicotine can cause deficits in learning and memory (11–14). After nicotine exposure in utero, attention and memory deficits were observed in adult offspring of rats and mice (13, 15).

Furthermore, epidemiological studies have also linked cognitive deficits to prenatal cigarette smoke exposure in humans. Deficits in learning, memory and problem solving skills were also observed in the children who were exposed to nicotine during gestation (16). It has been shown that nico-tine halts neuronal cell replication, leading to a decrease in total cell count and subsequent deficits in synaptic connectivity and neurochemical activity (17). It is well known that nicotine contributes a major proportion of the net oxidative stress imposed by tobacco use. Nicotine induced oxidative stress in rat brain tissue has also been reported (18). Additionally, nicotine exposure significantly increased lipid peroxidation in various tissues of rats (19).

The essential nutrient ‘vitamin C’ or ascorbic acid (AA) has been shown to scavenge free radicals and it also forms a strong line of defence against the reactive oxygen species (ROS) induced cellular damage (20). Since nicotine induced developmental neurotoxicity is potentially related to the oxidative stress (21), the antioxidant properties of AA is thought to have protective effect against neurotoxicity induced by prenatal exposure to nicotine. In vitro studies have demonstrated that AA alone or in combination with vitamin E can effectively protect the human placenta against the deleterious effects of oxidative stress induced by nicotine (20). It has also been shown that AA can prevent the adverse effects of prenatal nicotine exposure on offspring pulmonary function (22). However, there are studies which state that AA only protects the caudate part of the brain (which is most susceptible to oxidative stress) leaving the other regions exposed and more susceptible to the effects of nicotine (23).

In the present study, we investigated the effect of ascorbic acid on prenatal nicotine exposure induced behavioral deficits in male offspring of Wistar rats.

SUBJECTS AND METHODS
The present study was conducted in accordance with the guidelines of Institutional Animal Ethics Committee. Wistar rats weighing ~ 225 g were used for the study. They were housed in sanitized polypropylene cages (41 cm × 28 cm × 14 cm) containing sterile paddy husk as bedding. The animals were maintained under controlled conditions of temperature (23 ± 2 °C), and a 12-hour light-dark cycle. All animals had free access to water and were fed on a commercially available standard diet (ad libitum). The female Wistar rats were mated with male rats at 3:1 ratio. After the confirmation of pregnancy, pregnant rats were subjected to various experimental conditions.

Experimental design
After confirmation of pregnancy, 10-week old female Wistar rats were designated into four groups of six rats each. Group A (control): left untreated in the home cage; Group B (vehicle control): received normal saline (0.9% NaCl, orally) throughout the gestation; Group C (nicotine): received daily dose of subcutaneous injections of 0.96 mg/kg body weight (bw) of nicotine free base, whereas Group D (nicotine+AA) were given both nicotine free base (0.96 mg/kg bw/day; subcutaneous route) and AA (50 mg/kg bw/day, orally) daily throughout gestation. After weaning, six male pups from each group were collected randomly and maintained in separate cages. They were subjected to spatial memory performance using Morris water maze test on postnatal day 40 (PD-40). The dose of various drugs used in the current study is in accordance with earlier reports (24, 25).

Analysis of spatial memory performance
Spatial memory was evaluated using the Morris water maze test. The water maze apparatus consisted of a water tank of 1.80 m in diameter and 75 cm in depth, which was filled with water 50 cm deep (24 ± 1 °C). Four points on the rim of the pool were designated as north (N), south (S), east (E) and west (W), thus dividing the pool into four quadrants. There was a 10 cm × 10 cm size platform placed 2 cm below the water surface in one of the quadrants: the target quadrant. Nontoxic white paint powder was added to the water just before the experiment to make the water opaque. To provide extra maze cues for allowing the rats to develop a spatial map strategy, a black and white picture was hung on the wall. Position of the cue was kept unchanged throughout the experimental period.
Acquisition phase (spatial learning phase)
The rats were trained in the maze in seven sessions over four consecutive days as described by Narayanan et al (26) with modifications. Two sessions were done on each day except on the first day in which only one session was given. Each session consisted of four trials. During the training trial, each of the rats was placed into the water so that they faced the wall of the pool. Subsequent trials were initiated from different positions in the maze. For each rat, the point of immersion into the pool varied between N, S, E and W but the quadrant in which the platform was located remained constant. Thereby, the rat was unable to predict the platform location from the location at which it was placed into the pool.

After being released into water, each rat was given three minutes to find the hidden platform. Once the rat located the platform, it was permitted to remain on it for 15 seconds. In each training session, the latency (time in seconds) to escape onto the hidden platform was recorded. If the rat was unable to find the platform within the specified time, it was guided to the platform and allowed to remain on it for 15 seconds. After each trial, the rat was removed from the maze, dried using a towel and put back to their home cage for an inter-trial interval of five minutes before the next. Each animal had eight trials/day and a total of 28 trials before the probe trial. Following the completion of learning sessions (in four consecutive days), one day (on fifth day) rest was given to all the animals. The probe trial was conducted on the sixth day as described below.

Probe trial (memory retention phase)
Each rat was subjected to a single probe trial for a duration of 30 seconds in which there was no hidden platform kept in the maze. Before starting the probe trial, the maze water was made opaque as described above. Followed by this, the maze was divided (using a thread) into four quadrants by joining N and S and E and W. A video camera (Sony) was fixed on a tripod stand facing straight to the maze for recording the activity of each rat in the maze. During the probe trial, each rat was placed into the water as mentioned in the acquisition phase. As a measure of spatial working memory, the time taken to reach the target quadrant, the time spent in the target quadrant and number of platform crossing (searching the former platform) was measured. All events were recorded and these video files were used to calculate the above said parameters for each rat.

Statistical analysis
The results were expressed as mean ± SE. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test (post-hoc) using GraphPad Prism software (version 5.1). P < 0.05 was considered as statistically significant.

RESULTS
Spatial learning
Analysis of the results of water maze learning sessions revealed deficits in progressive learning in rats subjected to nicotine exposure prenatally. However, other group animals began to identify the submerged platform as the trials increased. Their escape latencies were found to be reduced, compared to the nicotine exposed group. This was clearly evident in the fourth day of acquisition performance. The results showed an enhanced progressive learning in normal control/vehicle/nicot ine+AA treated groups compared to nicotine exposed group and this difference was statistically different (Fig. 1). The nicotine exposed rats took a longer time to identify and reach the hidden platform, indicating the learning impairment. This was significantly attenuated by the administration of ascorbic acid as demonstrated by the decreased escape latency of nicotine+AA rats to reach the hidden platform.

![Fig. 1: Water maze acquisition performance. Prenatal nicotine exposure affected water maze acquisition task in rats as indicated by their longer escape latency to reach the hidden platform. This was significantly attenuated by the administration of ascorbic acid as demonstrated by their decreased escape latency to reach hidden platform on the final day trials. δδp < 0.01, Ṗp < 0.05. One way ANOVA and Tukey tests.](image-url)
Spatial memory retention

Latency to reach the target quadrant: Rats exposed to nicotine prenatally took more time to reach the target quadrant during the memory retention test. The mean value was ~12 seconds in the probe trial. Analysis of variance test revealed a statistically significant difference in the mean values of the nicotine exposed group compared to other groups [control vs nicotine; **p < 0.01, vehicle control vs nicotine; δδp < 0.01; one-way ANOVA and Tukey tests] (Fig. 2). Additionally, AA administration along with nicotine ameliorated nicotine induced cognitive impairment in rat offspring (nicotine vs nicotine+AA; ϕϕp < 0.01; one-way ANOVA and Tukey tests).

Time spent in the target quadrant: Analysis of time spent in the target quadrant during the probe trial revealed that animals exposed to nicotine prenatally spent significantly less time in the target quadrant, where the platform was positioned during the training sessions [control vs nicotine; **p < 0.01, vehicle control vs nicotine; δp < 0.05; one-way ANOVA and Tukey tests] (Fig. 3). Rats exposed to nicotine + AA swam more time in the target quadrant and thereby spent more time in the target quadrant; and this difference was statistically significant (nicotine vs nicotine+AA; ϕϕp < 0.01; one-way ANOVA and Tukey tests).

** Former platform crossing: Platform crossing was found to be more (~4) with control and vehicle control groups during the probe trial. However, it was evident from the results that animals exposed to nicotine prenatally swam significantly fewer times across the former location of the platform in the target quadrant compared to other groups [control vs nicotine; *p < 0.05, vehicle control vs nicotine; δδp < 0.01; one-way ANOVA and Tukey tests] (Fig. 4). Analysis of variance test revealed a significant difference in the platform crossing mean values of nicotine exposed group, compared to nicotine+AA group, suggesting the role of ascorbic acid in attenuating the effect of nicotine on cognitive functions (nicotine vs nicotine+AA; ϕϕp < 0.01; one-way ANOVA and Tukey tests).
DISCUSSION

Prenatal nicotine exposure induced long-lasting cognitive deficits in rat offspring. Animals in all groups learnt the water maze acquisition task over a period of four days but nicotine exposure decreased this ability of rats as described in the results. In the memory retention test, in spite of 28 extensive trial sessions, offspring exposed to nicotine prenatally took a longer time to reach the target quadrant and they were unable to recall the position of the former hidden platform. In contrast to this, pretreatment with AA improved both spatial learning and spatial memory retention, indicating the positive effect of AA on nicotine induced learning and memory impairment in rats. The poor spatial navigation observed in the present study might be due to the direct effect of nicotine on developing pups in utero, as it can cross the placental barrier and affect the developing brain (27, 28).

Prenatal nicotine exposure has resulted in spatial memory deficits in the Morris water maze and radial-arm maze tasks in prepubertal, adolescent, and adult rats (13, 29, 30). It is suggested that these cognitive changes may be the result of long-lasting alterations to the nicotinic and adrenergic neuro-transmitter systems following in utero exposure to nicotine (30). Nicotine exposure toward the end of prenatal development also reduces performance in cognitive tasks (31). The impaired cognitive function observed in prenatal nicotine treated rats can be explained based on the neuro-chemical changes such as disruption of neuronal migration, cell proliferation, and cell differentiation, imbalance in the functional status of the cholinergic and catecholaminergic neurotransmitter systems in the brain (32). It might be due to up-regulation and increased receptor binding of dopamine (DA) and norepinephrine (NE) by which nicotine might slow down the synaptic activity in the brain (30). Eppolito and Smith have demonstrated that prenatal nicotine exposure throughout the gestation and early postnatal period lead to spatial learning memory deficits but not in spatial working memory on the Morris water maze task (26). On the contrary, nicotine exposure during gestation resulted in the impairment of both spatial learning memory and spatial working memory in the current study. This difference might be due to methodological differences.

The mechanism of prenatal nicotine induced memory impairment might be due to its oxidant effects and free radical production followed by cell death in various brain regions responsible for memory formation (21, 23). Nicotine has been shown to produce nitric oxide, which suppresses the mitochondrial oxidative stress scavenger system in different brain regions (18). Antioxidant effects of vitamin E and C have been reported against prenatal nicotine induced oxidative stress effects in rats and in monkeys (18, 22). The positive effect of AA on the behavioral deficits caused by the restraint stress was also reported (33, 34). A report suggests that monosodium glutamate (MSG) induced altered neurobehavioural performance were significantly prevented by the supplementation of ascorbic acid in periadolescent rats (35).

In the current study, pretreatment with AA improved both spatial learning (Fig. 1) and spatial working memory performance (Figs. 2–4). It has been proven that dehydroascorbic acid, the oxidized form of ascorbic acid, enters the brain by means of GLUT-1 transporter present on the endothelial cells of the blood brain barrier, which helps in the transport of glucose and dehydroascorbic acid into the brain (36). In addition, the placental transfer of ascorbic acid has also been demonstrated (22). Thus, oral administration of AA may have resulted in the entry of high levels of dehydroascorbic acid into the fetal rat brain, which could have protected neurons from the deleterious effects of free radicals. Also, reports suggest that as an antioxidant, ascorbic acid indirectly contributes to the fight against free radical damage in cell membrane by rejuvenating vitamin E and thereby reducing the destructive process of lipid peroxidation (37). Recent reports indicate the significant role of ascorbic acid.
acid in cultured cells. The authors found that relatively high concentrations of ascorbic acid in the brain not only promote NGF-induced differentiation of neural precursor cells but also participate in the development of neural network-forming cells (38). Further studies will explain the key mechanism for the improved behavioural effects seen in rats after the administration of ascorbic acid.

CONCLUSION
Chronic nicotine exposure during the intrauterine period induced alterations in hippocampus dependent memory performances in rat offspring. Ascorbic acid supplementation was found to be effective in ameliorating these changes and thus shows the efficacy of this water soluble vitamin against various cognitive deficits.

REFERENCES