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N-Acetylcysteine for Gestational Stress-Induced Behavioral Toxicity.

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ABSTRACT

Stress during pregnancy has been implicated in various behavioral and emotional alterations in the offspring. Since N-acetylcysteine (NAC) has been shown to play neuroprotective role in offspring subjected to various manipulations in prenatal environment, we have examined the effects of this antioxidant against prenatal stress induced behavioral toxicity. Pregnant rats were restrained thrice daily for 45 minutes during early and late gestational periods. Other groups received early and late gestational restrain stress combined with NAC treatment throughout the gestational period. Behavioral and emotional alterations in offspring were investigated at different postnatal ages. Offspring of rats exposed late gestational stress exhibited increased anxiety like behavior during early growing period as demonstrated by greater time spent in dark area of dark-bright arena. Increased rearing near the walls of open field and greater number of fecal pellets in the open field was also suggestive of enhanced anxiety. These behavioral alterations were however mitigated by prenatal NAC treatment. Interestingly the anxiety like behavior observed during the early growing period of prenatally stressed rats seemed to have reversed. The exploratory activity and motor development were not affected by prenatal stress. Our results suggest that prenatal stress can give rise to increased anxiety like behavior in offspring during a crucial period in life and the supplementation of thiol antioxidants like NAC may prove useful in managing such behavioral deficits.

Keywords: prenatal stress, N-Acetylcysteine, anxiety, exploratory behavior, motor development.

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INTRODUCTION

Gestational stress induced deleterious cognitive outcome [1] in human infants is of serious concern. Prenatally stressed offspring expressed anxiety like behavior [2], depression [3], and modifications in locomotor activity [4]. Prefrontal cortex associated memory loss as well as impairment of Hippocampal dependent cognitive domains, were observed in offspring, as a result of their mothers being repeatedly subjected to variable stress pattern during last week of pregnancy [5]. Cognitive impairment and neuronal loss could result from oxidative damage [6]. Antioxidant therapy can possibly alleviate impairment in learning and memory associated with increased level of free radicals [7]. Antioxidants that can cross both placental and blood brain barriers would be ideal for providing protection against prenatal stress induced neuronal alterations. A thiol containing amino acid, N acetyl cysteine (NAC) being a source for glutathione synthesis, serves as a potent antioxidant. Earlier studies have shown that NAC facilitated neuroprotection by stabilizing the oligodendroglia and preventing loss of myelination [8], protected the growing brain from teratogenic effects of heavy metals such as mercury [9], and caused suppression of oxidative stress in fetal hippocampus [10].These properties of NAC make it a strong candidate to bring about protection against gestational stress induced behavioral toxicity. Thus, in this study, we aimed to investigate the possible potential neuroprotective role of NAC against such behavioral toxicity. The battery of behavioral tests includes evaluating locomotor and exploratory activity, anxiety like behavior and motor development. These tests were carried out during early growing period as well as at adulthood to evaluate the long lasting effect of prenatal stress.

MATERIALS AND METHODS

Healthy, female and male Albino *Wistar* rats (3-4 months of age) weighing around 250 g, were obtained from institutional animal house for the study. Day light cycle, temperature and humidity control were maintained. Animals were housed in polypropylene cages provided with paddy husk, and were fed with standard food pellet and water. Animal procedures were permitted by Institutional Animal Ethics Committee. Animals were handled humanitarianly throughout the experimental procedures.

Mating of rats

Female rats were allowed to mate with fertile male rat (two female rats with one male rat) for four hours per day, after which, the female rats were subjected to confirmation of pregnancy. Presence of sperms in the vaginal smear was considered as positive for pregnancy, and these rats were separated and allotted for different groups (6 rats in each group) and designated as gestational day 0. Pregnant rats were independently housed in separate cages. After delivery, two healthy pups from each litter (one male and one female) were selected for behavioral studies.

Animal groups

Group 1. (Control (C)) Pups of dams who received normal saline ip, in a dose of 10ml/kg body weight during the entire course of pregnancy.

Group 2. (NAC) Pups of dams who received NAC alone during the entire course of pregnancy

Group 3. (G1-10) Pups of dams subjected to restrain stress from day 1 to 10 of pregnancy.

Group 4. (G11-DEL) Pups of dams subjected to restrain stress from day 11 of pregnancy till delivery.

Group 5. (G1-10+NAC) Pups of dams subjected to restrain stress from day 1 to 10 of pregnancy along with NAC treatment throughout pregnancy.

Group 6. (G11-DEL+NAC) Pups of dams subjected to restrain stress from day 11 of pregnancy till delivery along with NAC treatment throughout pregnancy.

All pregnant rats delivered at around 21^{st} -24^{th} day of gestation. Pups of of the all groups were brought up by their biological mothers till 21 days after birth and were then weaned.

Stressing procedure

Wire mesh restrainers were made use of for subjecting the pregnant dams to restrain stress procedure [11]. This procedure was regularly performed for 45 min, three times in a day. To avoid familiarization of animals to the regular procedure, restraint timings were shifted randomly within certain time periods. The restrainer was made of a wooden base, a stainless steel wire mesh hinged to the base and a pad padlock with clasp. Restrainers with two different dimensions were prepared, one for stressing rats during early pregnancy (11 cm (L) x 6cm (B) x 6 cm (H)) and the other for stressing rats during late pregnancy (11cm (L) x 8 cm (B) x 8 cm (H)). Immobilization in a restrainer is considered as one of the best known models of stress as it represents emotional as well as physical aspects of stress. Furthermore this type of stress as it does not involve pain, is analogous to physiological stress.

Chemicals

Chemicals and reagents used were HPLC or analytical grade (Sigma, St. Louis, Mo.). N-acetyl cysteine was purchased from Lobo chemicals and procured locally from Sri Durga laboratories, Mangalore.

N-acetyl cysteine treatment

The dose of N-acetyl cysteine (NAC) was selected based on earlier study by Basyigit et al (2007) [12] where 10mg/kg dose (intraperitoneal) was given continuously for 14 days in pregnant rats. The acute oral toxicity of N-acetyl cysteine is low e.g. LD 50> 10,000mg/kg body weight in adult rats. In rats, administered with 250mg NAC/kg body weight per day for 20 weeks, all NAC-related effects observed were marginal. Hence in the present study, 10 mg/kg dose of N-acetyl cysteine dissolved in physiological saline was administered intraperitoneally.

Behavioral testing

Open field exploration

Open-field is a commonly used method to measure the locomotor and exploratory behavior as well as emotionality of rodents in novel environment [13]. The open field apparatus consisted of a box made with dimensions of 100x100x40 cms, brightly and uniformly illuminated from top. The floor consisted of 25 equal squares. The behavioral aspects assessed were the number of squares crossed by locomotion in peripheral (close to wall) and the central zone of the arena, number of rearing and grooming events and number of fecal pellets excreted. Each animal was assessed for a total period of 5 min. The open field exploration was assessed in offspring at 23, 48 and 56 days of postnatal life to assess both short and long term effects.

Dark bright Arena test

The dark bright arena is made of a wooden box, open at the top. One portion (66X34X58 cms) of the box was poorly lit and painted black. The other portion (66X48X58 cms) was brightly lit and pained white. The black portion was separated from the white area with a partition, which was provided by an opening at its base for free access to the animals from one portion to another. Floor area was marked into 9 cm squares. Each rat was placed at the center and the number of lines crossed and time spent in dark and bright areas were assessed for duration of 5 minutes, for measuring the level of anxiety. Increased activity in bright area of the arena indicated anxiolytic action. when a dark environment is simultaneously available. Anxiolytic activity is .characterized by increased activity in the bright section of the test box [13]. Offspring were assessed on day 28 and day 56 of postnatal life.

Rota-rod test

Muscle tone and strength was tested in a rota-rod apparatus [14]. This consists of rotating (15 rpm) wooden rods of different diameter for rats of different age (3.5 cm for post weaning rats). Each rat was made to hold the rotating rod, till it failed to hold and fell down. Total time it could hold was measured. This was taken as a measure of its muscle strength. Each rat was given 4 trials with 15 minutes interval between each trial. The Rota rod test was conducted on days 22 and 46.

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Data analysis

All results represent mean ± S.E.M. The data were analyzed using one way analysis of Variance (ANOVA) test followed by Bonferroni's multiple comparison test. unpaired "t" test was used to compare data between male and female groups. P values < 0.05 were considered significant.

RESULTS

There was no significant difference in any of the parameters studied, between male and female animals; therefore the mean values of both genders were collapsed.

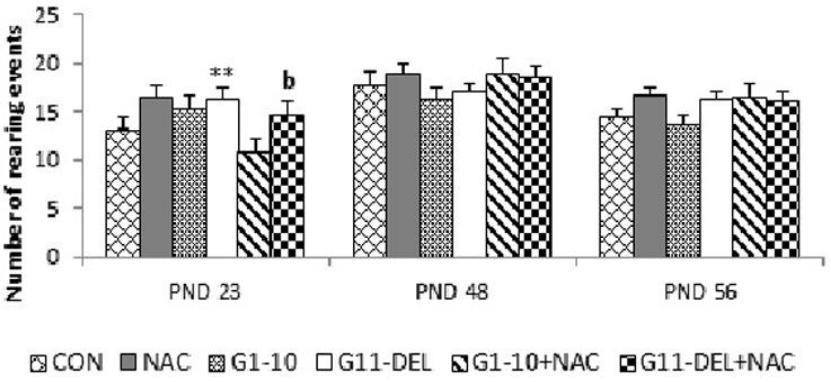
Behavioral tests

Effect of prenatal stress and NAC on Open field exploration

On post natal day (PND) 23:

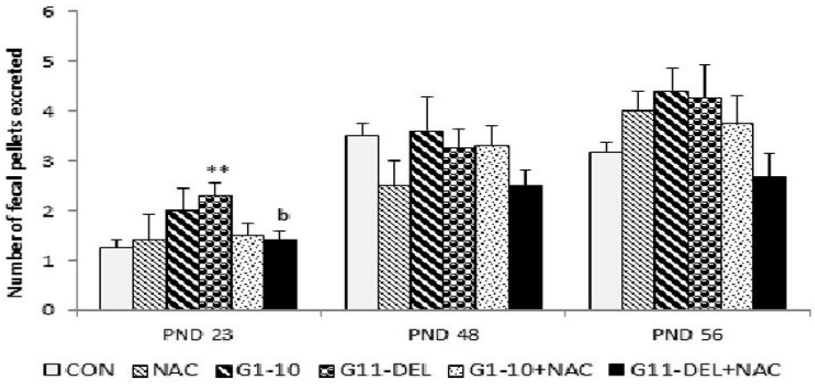
There was no significant difference between any group, in the number of peripheral squares or central squares entered, however the number of rearing as well as number of fecal boli excreted were significantly increased ($p<0.01$) in pups exposed to late gestational stress (group 4) when compared to control group. The number of rearing and number of fecal pellets were seen to be lesser ($p<0.05$) in group 5 pups (late gestational stress +NAC) as compared to group 4. (Fig.1 and Fig.2).

Figure 1: Effect of prenatal stress and NAC on number of rearing events in an Open field arena (PND23, 48 and 56).



Data are expressed as mean±SEM. n=12. (One way ANOVA, Bonferroni's test)
Con Vs G11-Del, ** p<0.01; G11-Del+NAC Vs G11-Delb p<0.05

Figure 2: Effect of prenatal stress and NAC on number of boli of fecal pellets excreted in an Open field arena (PND23, 48 and 56).



Data are expressed as mean±SEM. n=12. (One way ANOVA, Bonferroni's test)
Con Vs G11-Del, ** p<0.01; G11-Del+NAC Vs G11-Delb p<0.05

On PND 48:

No significant variation was observed in number of central squares/ peripheral squares crossed, number of rearing/grooming events, and number of fecal pellets excreted in any of the groups.

On PND 56:

No significant alteration was observed in number of central squares/ peripheral squares crossed, number of rearing/grooming events, and number of fecal pellets excreted in any of the groups.

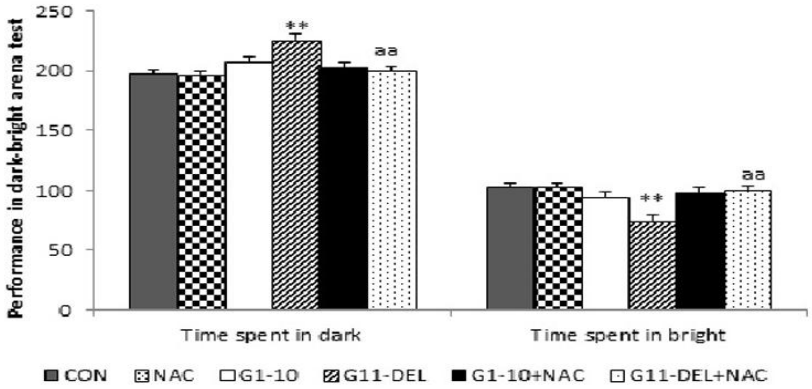
Dark-bright arena test

Effect of prenatal stress and NAC on anxiolytic activity.

On PND 28:

There was no statistically significant difference in the number of lines crossed in dark and bright areas, between any of the groups.However, the offspring exposed to late gestational stress (G11-Del) spend significantly ($p<0.01$) longer time in dark compartment and shorter time in the bright area ($p<0.01$) when compared to control group. G11-Del pups treated with NAC showed statistically significant difference in time spent in these two compartments when compared to G11-Del group ($p<0.01$) (Fig.3).

Figure 3: Performance in the dark and bright arena test of 28 days old rat pups exposed to gestational stress and prenatal NAC: Time spent in dark and bright areas.



Data are expressed as mean±SEM. Animal groups: (n=12). One way ANOVA, Bonferroni's test)
Con Vs G11-Del, ** $p<0.01$; G11-Del+NAC Vs G11-Delaa $p<0.01$

On PND 56:

There was no statistically significant difference either in the number of lines crossed in dark and bright areas, or in the time spent in dark and bright compartments between any of the groups.

Rotating rod holding time

There was no statistically significant difference in the time the animals were able to hold on to the rotating rod between any group when tested either on PND 22 or on PND 46.

DISCUSSION

This study was designed to investigate the mitigating influence of N-acetyl cysteine on behavioral alterations induced by prenatal stress. We examined the prenatal stress induced exploratory activity, locomotion and emotional aspects in the open field task, level of anxiety in dark bright arena as well as motor development in the rota rod apparatus.

Our study showed no significant difference in open field exploratory activity in response to early or late gestational stress, in postnatal age 23, 48 or 56 days. However, in line with our findings, lately, Bustamante et al, 2013[15] did not find alteration in locomotor behavior in CF-1 mice in response to late gestational stress, when tested on PND 23. Activity of rodents in an open field setting is usually referred to degree of exploratory behavior when an animal is subjected to a novel environment; yet, this behavioral activity may be confounded by presence of anxiety. Nebulous findings are reported by earlier studies with respect to prenatal stress induced alterations in locomotion. Diminished locomotion in open field environment was reported in 16days old offspring of Long–Evans rats exposed to late gestational restrain stress [16] and in female offspring of Wistar rats [17], after exposure to early gestational stress, however no such impairment in basal locomotion was seen by other studies involving late gestational stress [18]. On the other hand an increased locomotor behavior was detected in male rats exposed to late gestational restrain stress every day [19].

However, interestingly, we observed an increased number of rearing near the peripheral walls of the open field, and also increase in the number of fecal pellets, indicative of anxiety, in 23 days old offspring exposed to late gestational stress alone. Increase in the rearing events close to the walls of open field arena, though not cited in earlier reports may be an expression of escape tendency that is related to anxiety. In line with our finding, prenatal stress resulted in increased anxiety like behavior in rat offspring exposed to late gestational stress [20].In contrast to our finding, Fujioka et al (2001) [21]observed a reduction in rearing behavior in male offspring of dams exposed to mild restrain stress during last week of gestation. In dark bright arena, we found no deficits in behavioral patterns in offspring exposed to early gestational restrain stress. However, on PND 23 but not on PND 56, offspring exposed to late gestational stress spent more time in dark compartment of dark bright arena, demonstrating increased anxiety. Rotating rod holding time in rotarod apparatus was also not significantly effected suggesting that prenatal stress had no influence on motor development.

Results of open field task and dark bright arena tests essentially prove that late gestational period is a sensitive period, and any stressful events during this period can lead to undesirable behavioral changes with respect to emotionality and anxiety in the offspring. Our findings were supported by findings of other studies. Children exposed to prenatal anxiety during late gestational period were more likely to undergo emotional alterations [22] as this is the period when rapid brain growth takes place.

Additionally our results indicate that the rat offspring exposed to late gestational stress showed anxiety like behavior only on early post natal age, but this difference was diminished when tested later on day 48 and 56 in an open field task or on day 56 in dark bright arena. In support to this finding, one month old male offspring exposed to unpredictable restrain stress during late prenatal period displayed impaired spatial learning and memory tasks, which did not prevail at 3 month of age, suggesting a recovery of these prenatal stress induced behavioral parameters [23], furthermore reduced locomotion was observed at PND 16 but not on PND 60 in rats subjected restrain during late gestational period [16]. Batuev and coworkers (1996) [24] reported late gestational stress induced higher anxiety and lower locomotion during one month of age the effect which was absent when tested later during four months of age.

It is important to point out here that, although behavioral changes in response to late gestational stress took place in offspring only during early growing period, and were later found to be reversed, this growth period in rodents is related to an age of 11 to 16 years of age in humans. Undoubtedly this is an age when emotions and anxiety plays a very crucial role in an individual's life, which can hinder the quality of life at a latter age. Protection against such behavioral changes therefore indubitably requires attention.

Among the various neuroprotective interventions aiming at ameliorating brain injury, only few have been proved safe for clinical use. N Acetyl cysteine is one such antioxidant which is proved to be safe during pregnancy. Moreover, having known the fact through several studies that, being able to cross the placental as well as the blood brain barriers, and improve brain GSH concentration in experimental animals [25], NAC can be considered as a promising remedy that may possibly mitigate the neurotoxic modifications brought about by prenatal restrain stress.

Several studies have demonstrated the neuroprotective role of NAC. It prevented loss of spatial learning in GSH-deficient rats [26]. It has been reported by Paintlia et al, 2004[8] that prenatal administration

of N-acetyl cysteine preserved oligodendroglial precursor cell numbers and myelination against endotoxin challenge. Recently N-acetyl cysteine (NAC) is being considered as a beneficial agent in the management of psychiatric disorders. Sufficient number of clinical studies has proved NAC favorable in bringing about neuroprotection in humans. Human volunteers suffering from Alzheimer's disease showed benefit from some but not all cognitive dysfunctions after being treated daily with 50 mg/kg/day for six month duration [27]. However, the protective role of NAC against prenatal restrain stress induced behavioral alterations has not been well established.

Our results point out that NAC offered protection against behavioral changes in offspring exposed to late gestational stress. Increase in anxiety that resulted from prenatal exposure to restrain stress was reversed by prenatal treatment with NAC.

The mechanism by which NAC brought about protection against behavioral toxicity is not fully clear. It is well-known that NAC is a potent antioxidant, provides cysteine, and replenishes GSH. These actions may be responsible for its protective role against prenatal stress induced behavioral toxicity.

CONCLUSION

In summary, we can conclude from the pattern that emerges from these results that chronic restrain stress during late gestational period can cause undesirable effects on anxiety and emotionality of the offspring at early growing period. N-acetyl cysteine, an antioxidant that is identified as safe during pregnancy may offer significant protection against this behavioral toxicity, thus preventing any adverse effect on the quality of life at latter age of an individual.

REFERENCES

[1] Bergman K, Sarkar P, Glover V, O'Connor TG. Biol Psychiatr 2010; 67(11): 1026-1032.
[2] Miyagawa K, Tsuji M, Fujimori K, Saito Y, Takeda H. Neurosci Res 2011; 70(1):111-117.
[3] Van den Bergh BR, Van Calster B, Smits T, Van Huffel S, Lagae L. Neuropsychopharmacol 2007; 33(3): 536-545.
[4] Clinton S, Miller S, Watson SJ, Akil H. Psychoneuroendocrinol 2008; 33(2):162-177.
[5] Julie A. Markham, Adam R. Taylor, Sara B. Taylor, Dana B. Bell, James, Koenig. Frontiers Behav Neurosci 2010; 4: 173
[6] Fukui K, Onodera K, Shinkai T, Suzuki S, and Urano S. Ann NY Acad Sci 2001; 928: 168–175,
[7] Bickford PC, Gould T, Briederick L, Chadman K, Pollock A, Young D. et al. Brain Res 2000; 866: 211–217
[8] Paintlia MK, Paintlia AS, Barbosa E, Singh I, Singh AK. J Neurosci Res 2004; 78(3): 347-361.
[9] Falluel-Morel, Anthony, Lulu Lin, Katie Sokolowski, Elizabeth McCandlish, Brian Buckley, Emanuel DiCicco-Bloom. J Neurosci Res 2012; 90(4): 743-750.
[10] Lante F, Meunier J, Guiramand J, Maurice T, Cavalier M, de Jesus Ferreira MC, Aimar R, Cohen-Solal C, Vignes M, Barbanel G. Free Radic Biol Med 2007; 42:1231–1245.
[11] Madhyastha SK, Prabhu LV, Nayak S, Rai R, Pai MM, Madhyastha PS. Iranian J Pharmacol Ther 2008; 7(1): 71-77.
[12] Basyigit I, Tugay M, Dilioglugil MO, Yildiz F, Maral H, Sozubir S. Human Exp Toxicol2007; 26(2): 99-103.
[13] Madhyastha S, Somayaji SN, Nalini K, Nayak BS, Bairy KL. Indian J Pharmacol 2002; 34(5): 350-355.
[14] Hamm RJ, Pike BR, O'Dell DM, Lyeth BG, Jenkins LW. J Neurotrauma 1994; 11: 187-196.
[15] Bustamante C, Henríquez R, Medina F, Reinoso C, Vargas R, Pascual R. Int J Develop Neurosci 2013; 31(4):267-273.
[16] Zimmerberg B, Blaskey LG. Pharmacol Biochem Behav 1998; 59(4):819–27.
[17] Lehmann J, Sto¨hr T, Feldon J. Behav Brain Res 2000; 107(1–2):133–44.
[18] Ward HE, Johnson EA, Salm AK, Birkle DL. Physiol Behav 2000; 70:359–66.
[19] Weller A, Glaubman H, Yehuda S, Caspy T, Ben-Uria Y. Physiol Behav 1988; 43:139–43.
[20] Vallée M, Mayo W, Dellu F, Le Moal M, Simon H, Maccari S. The J Neurosci 1997; 17(7): 2626-2636.
[21] Fujioka T, Fujioka A, Tan N, Chowdhury GMI, Mouri H, Sakata Y, Nakamura S. Neurosci 2001; 103(2):301–7.
[22] O'Connor TG, Heron J, Glover V. J American Acad Child Adol Psychiatr 2002; 41(12):1470–1477.
[23] Wu J, Song TB, Li YJ, He KS, Ge L, Wang LR. Brain Res 2007; 1141: 205-213.

[24] Batuev AS., Vinogradova EP, Polyakova ON. Zhurnal Vysshei Nervnoi Deyatelnosti Imeni I P Pavlova 1996; 46:558–563
[25] Dean O, van den Buuse M, Copolov D, et al. Int J Neuropsychopharmacol 2004; 7(S1):262.
[26] Choy KH, Dean O, Berk M, Bush AI, van den Buuse M. Eur J Pharmacol 2010; 649: 224–228.
[27] Adair JC, Knoefel JE, Morgan N. Neurol 2001; 57:1515–1517.