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RESEARCH ARTICLE

Induction of Micronuclei in Hippocampal Neurons Exposed To Chronic Prenatal Restraint Stress in Male and Female Wistar Rat Pups

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Abstract

With a view to assess the effect of chronic maternal stress on genotoxicity in hippocampal neurons of neonatal rat brain, pregnant Wistar rats were subjected to restraint stress from embryonic day 11 till delivery. The newborn rat pups were sacrificed and hippocampal cells were isolated out and stained with Giemsa stain. Results were compared with rats of the same age and sex born to control mothers which were not stressed. The results showed that prenatal restraint stress induces genotoxicity in hippocampal neurons. Genotoxicity induced by maternal restraint stress was evident by the increased number of micronuclei in the hippocampal neurons of rats born to stressed mothers. This chromosomal damage may be due to the result of stress on the developing hippocampus, a structure of brain concerned with learning and memory. These results suggested that prolonged prenatal stress leads to DNA damage which can result in long lasting malfunction of hippocampus which extends to and is manifested in adulthood.

INTRODUCTION

Behavioral development is shaped by a highly complex process involving the interplay of complex biological and environmental factors (1). All sorts of early environmental influences can leave indelible imprints and influence the development of offspring. A vast number of factors such as genetic makeup (2, 3) epigenetic factors like nutrition (4), environmental toxins (5, 6) and stressful condition (7, 8, and 9) play a major role on normal growth in the intrauterine development.

In most of the cases, affects of such insults will be carried to the young age or even to the whole life span of the individual (10, 11). Though any system of the body is the target of flawed development, nervous system becomes the main target of faulty development.

There is growing evidence that prenatal adversities could be implicated in foetal programming of adult chronic diseases (12). Chronic or repeated stress during human fetal development has been associated with various learning, behavioral or mood disorders including depression in later life (13). In rats, the offspring of dams subjected to various gestational stressors (eg. immobilization, foot shock or overcrowding) display physiological and behavioral alterations that persist into adulthood (14). It has been shown that enhanced production of stress hormones by the mother during critical periods of fetal brain development provokes a longer corticosterone response to stress

(15). In males, prenatal stress alters analgesic sensitivity, taste preference and sexually dimorphic behaviors like sex behavior and partner preference (16, 17). Many studies of
prenatal stress have shown effects on the HPA axis in adult rats, such as elevated basal or stress- induced plasma ACTH and corticosterone (CORT) levels (18,19).
Micronucleus is formed when chromosomal fragments behave independently of remaining chromosomes during the division of cells damaged by genotoxic agents, and the frequency of micronucleus is considered to reflect genotoxic damage to cells (20). This endogenous source of DNA damage results from cellular metabolism or routine errors in DNA replication and recombination. Since normal cells also present micronuclei due to various environmental factors, it is necessary to compare the exposed group with this control group.
Various studies have reported the induction of micronuclei in leukocytes, erythrocytes, splenocytes etc (21, 22, and 23). Genotoxic studies on tobacco smoke has shown that cigarette smoke condensate induces sister chromatid exchanges and micronuclei in bone marrow and lung cells (24). Studies on hydrocarbons have shown that it affects rapidly dividing cells and may have long term consequences for occupational exposures (25). Miyakosh et al suggests that micronuclei test using new born rat atrocities could be used as a screening test of environmental and occupational geotaxis chemicals in the CNS cells (26). Studies on pups suggest that fenarimol can act on cell DNA through direct exposure of litter via milk (27). Recent intriguing reports have shown that SO2 derivatives cause oxidative damage in multiple organs of male and female mice (28).
The interest in the present study stemmed from the lacuna in the literature. There are no previous studies which report the genotoxic effects of prenatal stress on hippocampal neurons. Hence such a study was designed to investigate the genotoxic effects of prenatal stress on hippocampal neurons of dentate gyrus.

2. Methods

2.1. Animals

In bred adult female rats of Wistar strain were housed in the presence of a male rat. The animals had free access to food and water under a constant light-dark cycle (12:12 hr) with controlled temperature (22±3ºC) and humidity (approximately 50±10%) in an air conditioned animal house. Institutional Animal Ethical Committee (I.A.E.C) approval (IAEC/KMC/06/2005-2006) was obtained before the conduct of the experiment and
care was taken to handle the rats in humane manner.

2.2 Timed Pregnancy in rats

For breeding, virgin female rats were placed with adult males overnight during whole estrus cycle. A vaginal smear was examined on the next day morning. The presence of
sperms in the smear will confirm the mating and that day is taken as day zero of pregnancy for further counting the days. Each pregnant female was separated and kept in an individual cage and fed with standard feed. Pregnant females were assigned randomly into control mothers (n=6) and prenatal stressed mothers (n=6) groups.

2.3 Prenatal stress protocol

Pregnant rats in the stressed group were stressed daily from embryonic day 11 till delivery. They were exposed to a regimen of restraint stress by placing in a wire mesh restrainer for 6 hours per day. The wire mesh restrainer has a wooden base and stainless steel wire mesh restrainer hinged to the base. The restrainer is of 11cm (L) × 6cm (B) × 6cm (H) dimensions. This type of restrainer will restrict the animal's movement only without any pain or suffocation. All dams delivered at term (21-22 days of gestation). Control mothers were left undisturbed in the home cage for the duration of their pregnancies. All dams (both control and stressed group) delivered at term (21-22 days of gestation).

2.4 Micronuclei Assay

To assess the prenatal stress induced genotoxicity, the new born rat pups were sacrificed, hippocampal dentate gyrus was dissected out and cells were isolated and stained with Giemsa stain.

2.5 Hippocampal dissection

Rat pups were sacrificed; head was separated and transferred to phosphate buffer solution. Later it was dissected under water. Midline section of the skull was taken; hippocampus was separated and transferred to a watch glass with saline. Tissue was passed through 20, 24 and 26 gauge needles in order to make a cell suspension. Cells were smeared on a glass slide and stained with working solution of Giemsa.

2.6 Preparation of Giemsa stain

2gm of Giemsa powder was added to 66ml of glycerin and incubated at 60ºC in water bath with constant stirring for 2 hours. The mixture was cooled to room temperature and 66ml of methanol was added to it and mixed thoroughly. The stock solution thus prepared was filtered through Whatmann No: 1 filter paper and stored at 4ºC. To produce 50 ml of working solution, 3ml of stock solution was added to 47ml of phosphate buffer to get a final concentration of 6%. Slides were stained using Giemsa stain for ten minutes, washed in distilled water and then air dried. Later they

were observed under oil immersion and the micronuclei were counted in each field and then the number was compared with that of normal rats.

3. Statistical analysis

Data was presented as Mean ± SEM. Two tailed student t-test was used to compare the statistical significance of the data using Graph pad in stat software. Probability (P) value less than 0.05 was considered statistically significant.

4. Results

The rats born to the control mothers and stressed mothers were healthy. There was no visible abnormality in the rats born to stressed mothers.

4.1 Assessment of Micronuclei in Male rats

The results of assessment of chromosomal damage showed that the normal males showed 3% (3.333± 0.33) and the stressed males showed 9 %(9.833± 0.87) of micronuclei. (n=6; NC vs S, ****P<0.0001, Unpaired, two tailed Student's t-test, t = 6.960, DF- 10, Fig.1, and Table: 1)

4.2 Assessment of Micronuclei in Female Rats

The results of assessment of genotoxicity in female rats showed that the normal females showed 1 %(1.667±0.42) and stressed females showed 11% (11.17 ± 0.47) of micronuclei (n=6; NC vs. S, ****P<0.0001, Unpaired, two tailed Student's t-test, t = 14.92, DF- 10, Fig.1 and Table: 1)

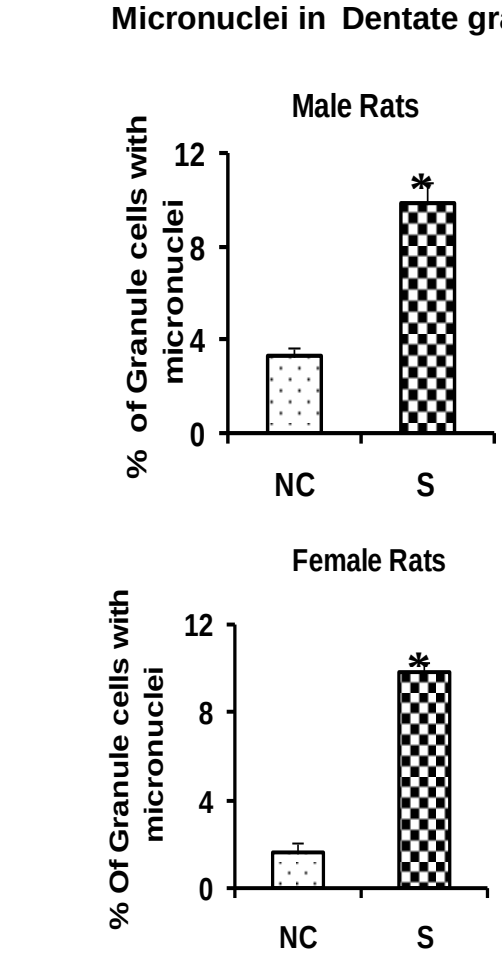

Fig 1

Figure-1: Percentage of granule cells in dentate gyrus of hippocampus with micronuclei in male and female rats pups born to normal control (NC, n=6) and pups born to stressed mothers (S, n=6).Note there is a significant increase in the % of granule cells having micronuclei. NC Vs S **** P<0.0001, Unpaired student's t-test.

	Male (n=6)	Female (n=6)
NC	3.333 ± 0.33	1.667 ± 0.42
Stressed	9.833 ± 0.87	11.17 ± 0.47
t =	6.960	14.92
P value	P < 0.0001 ***	P < 0.0001 ***
Df	10	10

Table:1: **Percentage of micronuclei present in dentate gyrus cells in male and female rats. Each value represent Mean ± SEM**

Table 1: Percentage of micronuclei present in dentate gyrus cells in male and female rats. Each value represents Mean±SEM

5. Discussion

Chronic prenatal stress is known to impair learning and memory (29). For our knowledge, this is the first report to establish a relation between the effects of prenatal restraint stress and increased number of micronuclei in the dentate gyrus of hippocampal neurons.Exposure of rat pups to maternal restraint stress resulted in DNA damage in the hippocampal neurons. This effect of prenatal stress on chromosomal damage has not been reported hitherto.

The method of stress used in the present study is one of the well known methods of stress. Different methods of stress procedures have been used by earlier workers such as forced immersion in cold water, social stress by exposing the rats to cat, electric foot shock (30.31, 32). Stress by restrainer method used in this study is convenient, and animals will not suffocate, at the same time restrained. Stress by this method is known to increase the adrenal gland weight and glucocorticoid hormone level (33).

Micronuclei are generated from nuclear material during cell division due to chromosomal damage (during interphase or early mitosis) and can be observed only after mitosis. The spontaneously occurring micronuclei can have entire chromosome (34). Micronuclei assay can be done in plant cells, bone marrow erythrocytes, lymphocytes, germ cells, hepatocytes, epithelial cells and normal as well as tumor cells. Since normal (control) cells also present micronuclei due to various environmental factors, it is necessary to compare the exposed (treated with chemical) group with this control group. Accumulated evidence indicates that acute periods of prenatal stress have profound effects on hypothalamo- pituitary – adrenal (HPA) function and behaviour (11).

The hippocampus is one of the targets for glucocorticoid hormone, and it is highly vulnerable regions of the brain. Dentate gyrus is a part of hippocampal formation. It is thought to contribute to new memories as well as other

functional roles. It is notable as one of a select few brain structures currently known to have high rates of neurogenesis.

It has been reported that chronic repeated psychosocial, restraint stress, chronic treatment with corticosterone or adrenal steroids cause apical dendritic atrophy in CA3 pyramidal neurons of the hippocampus and cause specific cognitive deficits in spatial learning and memory (35).

Chronic restraint stress has been reported to increase corticosterone levels in pregnant dams. Glucocorticoids are very liposoluble and cross placental and blood- brain barriers (36).Corticosteroids secreted by the adrenal cortex readily enter the brain where they coordinate, together with other components of the stress system, the organism's ability to cope up with stress (37). Therefore, it could be concluded that exposures to excess glucocorticoids during critical windows of neuroendocrine development might have led to these effects on chromosomal damage which ultimately resulted in the increased number of micronuclei. Taken together, these results suggest that prenatal stress is a genotoxic agent.

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