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Research Paper

LONGITUDINAL STUDY ON THE EFFECT OF RESTRAINT STRESS DURING PREWEANING PERIOD ON POSTNATAL GROWTH OF BODY AND CONSTITUENT PARTS IN ALBINO MICE

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Stress is the phenomenal; any stimulus which displaces the state of normal physiological function can cause stress and has its effect in various forms. Somatic growth and development are also affected by stress affecting growth releasing hormone. This study was conducted to observe growth pattern in normal and stressed condition in growing mice. Experiments were conducted to investigate the effect of restraint stress applied at different times of the light-dark cycle on growth rates (body and tail length and body weight of rats). BALB/ C strain of Albino rats were restrained for 6 hours daily from birth to end of three weeks (21 days) 6 hourly from birth till postnatal day 21 (21 days stress) and from postnatal day 16 to 21 day (5 day stress) in a restraining device, which consisted of a wooden platform to which a wire-mesh was attached. Body weight (BW), tail length (TL) and body length (BL) was recorded at the beginning and at the end of stress both in control and experimental groups. Recorded parameters were subjected to statistical analysis between the groups. Results of the study revealed that stress affects body weight significantly and longitudinal growth of body and tail lengths of albino mice.

Keywords: Albino mice, Restraint stress, Body measurements

INTRODUCTION

Stress is a highly individualized response of an organism to external or internal challenges which individual cannot control or can control with difficulty. Any stimulus that displaces the state of normal physiological function can cause 'stress'. Brief early life experiences, pre or postnatal, can cause significant changes in the stress response system and emotionality that persist into adulthood (e.g., susceptibility to disease, Barker, 1996; depression, Phillips, 2002; anxiety-type behavior, Heim and Nemeroff, 2001; hyper-responsiveness to stressors, Ladd *et al*., 2000).The current interest in early life experience stems from the observation that specific early events can apparently programme the' set-point"

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of the hypothalamic–pituitary–adrenal (HPA) axis by altering the effective feedback. Ganong (1995) defines " stress" as those stimuli which increases ACTH is an intricate chain which begins with the inputs at the higher centre, that triggers the hypothalamus. In response to stress full stimuli hypothalamus releases corticotrophin releasing hormone (CRH) to stimulate the pituitary, which inturn releases ACTH. Thus, adrenal cortex is stimulated for release of cortisol and androgen precursors. ACTH and cortisol are secreted in episodic manner in response to stress.

Observations at birth and thereafter, day by day progress in growth of body, head and tail in the postnatal life of man and animals are amazing experience. Enormous studies on birth and growth phenomenon in the postnatal life of animas have been compiled over the years (Aremario *et al.*, 1984; Alario *et al.*, 1987; Avishai-Ephner *et al* 1995; Bruno *et al.*, 1990). However, most of the investigators followed the traditional cross sectional method of study in which groups of animals generally mice or rats were sacrificed periodically (Tanner, 1951; and Tanner, 1962). Different strains of laboratory rats have been used for various types of investigations (Asling and Frank, 1963; Harel, 1995).

Over the decades, many longitudinal studies were reported, e.g. Saxton and Silberg (1947), Hughes and Tanner (1970) and Williams *et al.* (1974). Any such studies were related to development and growth (Morgan and Naismith, 1982).Specifically related with postnatal development and growth, some researchers have shown proclivity to study certain skeletal elements (Groot, 1963; Harkness and Trotter, 1980). Growth and development manifest by increment in size of the different parts of the body like head length, body length and tail length (Hughes, 1970). In the present study two parameters were considered for investigation, (i) growth of normal albino mice, (ii) growth of albino mice reared under stress. As far for the stress is concerned restraint stress was inflicted.

Restraint stress has been proposed as an animal model of depression and anorexia nervosa, as many investigators have shown that stress suppresses food intake and body weight gain in rats (Ganong WF 1995, Kennett GA *et al*, 1986). The stress-induced reduction in food intake has been demonstrated both as maintained decrease in 24-h food intake during and after repeated daily restraint stress, (Ganong, 1995; and Kratin *et al.*, 1990) and as an acute response in stress has ended, restraint rats fail to return to the body weight of control animals (Ganong 1995, Kennett *et al.*, 1986, Kratin *et al.*, 1990). In the rat, repeated separation from the mother for 3 h. has been shown to have potentially deleterious effects. Separated rat pups showed reduced hippocampal glucocorticoid receptors (GR), elevated basal plasma glucocorticoids (Plotsky and Meaney, 1993), and became hyper responsive to stressors during behavioral development (Ladd *et al* 2000). There are reports that maternal separation of 3 hrs. can lead to adult rats having reduced cognitive performance (Ladd *et al.,* 2000). The mechanism underlying this hyper-responsiveness appears to be a reduction in the negative feedback to the HPA axis provided by the hippocampal brain region. Separated rat pups have a reduced density of glucocorticoid receptors in the hippocampus and consequently reduced capacity to inhibit the responsiveness of the HPA axis. In contrast, rat pups exposed to a positive post-natal experience (i.e., born to mothers that shows higher levels of maternal grooming) shows positive effects of this

experience. Pups born to more maternal mothers have increased hippocampal GR expression and reduced hypothalamic corticotropin-releasing hormone messenger ribo-nucleic acid (CRH mRNA) expression, suggesting that these pups had a less responsive HPA axis (Liu *et al* 1997). These pups also showed changes in the neural circuitry controlling fear behavior (Caldji *et al* 1998), suggesting that they should be less behaviorally and physiologically responsive to fear and stress-inducing stimuli. Other work where pups were removed from the mother for a short period (15 min) and then returned (a procedure now known to cause an increase in maternal grooming(Macri *et al*., 2003), resulted in pups which were more easily handled, and showed reduced ACTH and glucocorticoid responses to an open-field test (Nunez *et al*., 1996).

Since the parameters are precise but extensive, literature analyzed were substantial (Armario *et al*., 1985a and b; Alario *et al*., 1987; Hennessy *et al*., 1989; Avishai-Ephner *et al*., 1995). Thus the present work consists of growth of body length, tail length, day of eye opening of albino mice observed from birth to 3 weeks postnatal age and from postnatal age 16 to postnatal day 21, the study was conducted in normal health of growing albino rats and stress like restraint stress. To the best of our knowledge and endeavor we could not come across any single study which describes the effect of chronic restraint stress on albino rats.

MATERIALS AND METHODS

Animals

In the present study albino mice of BALB/C strain of both sexes were used. Control and experimental of both sexes consisting of twelve albino mice in each group were form edrandomly.

Environment

The mice were maintained in well-ventilated room. Temperature ranged between 27±3^{0}C. Doors and windows were closed during morning, evening and night hour to prevent them from colds. They were kept in natural source of light which was 12:12 hour L: D cycle. Size of the cages for housing the mice were 40 X 25 X 16cm. Cages contained paddy husk (Sterilized), which were changed on every 2nd day.

Diet Regimen

The animals were fed with synthetic food pellets and tap water adlibitum. The food pellets contained mainly what and 22.05 crude proteins, 3.99% crude oil, 2.62% crude fibre, 1.34% sand silica and 7.81% ash. Drinking water was acidified with hydrochloric acid to give a pH of 2.0 -2.5 this was achieved by adding 2 ml of hydrochloric acid to 3 liters of tap water. The purpose of adding acid to water is to prevent massive bacterial proliferation in the water bottle.

Stress Regimen

The new born pups (P_0) were divided into two subgroups (a) Control (C) (b) Restraint stress (RS)

Control Group

New born pups in this group remained undisturbed with their mothers till the postnatal day 21.

b) Restraint Stress Group (RS)

Pups in this group were grouped into two sub groups

(i) 5 days stress group: Pups were stressed in a wire mesh restrainer, for 5 days (6h/day) from

P_{16}-P_{21} (postnatal day 16-21) in restrainer No-3 having dimensions –4.8 cm (L) x 2.2 cm (B) x 2.4 cm (H).

(ii) 21 days stress group: pups in this subgroup were stressed 6h/day for 21 days in the restrainer No-1 (P_0-P_7) having dimensions (2.5cm (L) x 1.4cm (B) x 1.1cm (H), (Figure 1) restrainer No-2 (P_8-P_{14}) having dimensions (3.5 cm (L) X1.6 cm (B) x 1.2 cm (H) Figure 1 and restrainer No-3 (P_{15}-P_{21}) having dimensions (4.8 cm (L) x 2.2 cm (B) x 2.4 cm (H) (Figure 2).

Figure 1: Restrainers With Mice A. Restrainer No. 1-Used to Stress the Mice Below 7 Days Of Age, B. Restrainer No. 2- Used to Stress the Mice Between 8 and 14 Days Of Age

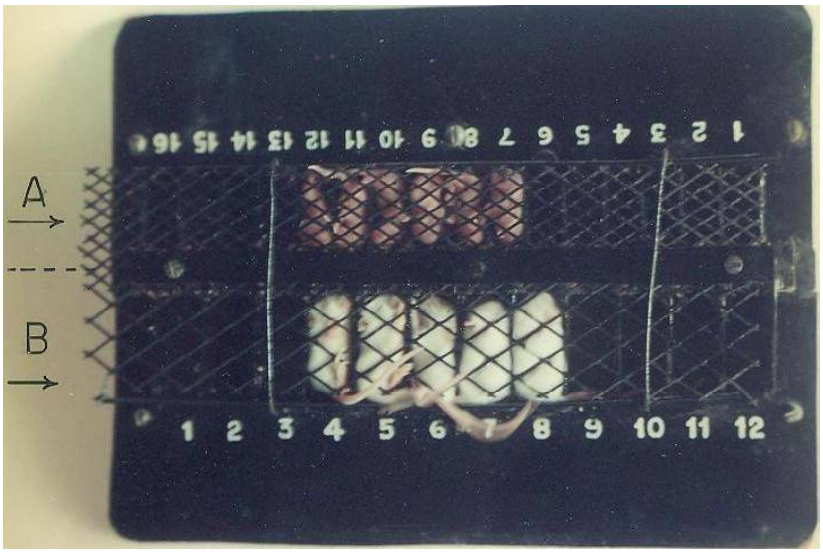

Figure 2: Restrainers With Mice A. Restrainer No. 3-Used to Stress the Mice Between 15 and 30 Days of Age

All the pups in the restraint stress group remained with their mother except when they were subjected to stress. Control mice pups remained unseparated from the mothers. The food and water were withdrawn from experimental group during stress presentation. After stress presentation mice were returned to their respective cages.

As the animals were allowed to grow and subjected to stress their growth was assessed including parameters like (i) Body weight gain, (ii) Tail length gain, (iii) Body length gain and (iv) Eye opening day. At the time of birth and at postnatal day all the growth parameters were observed and recorded.

DATA COLLECTION

Body Weight: The body weights were measured using an electronic balance at the commencement of stress exposure and at the end of stress exposure. Body weight gain is defined as difference in pre-stress and post-stress body weights. In case of controls, body weights were measured at an age corresponding to beginning and end of stress exposure in stressed groups.

Body Length: The body length, i.e., from tip of snout to base of tail was measured at birth (P_0) or at 16th postnatal day and at postnatal day 21 (P_{21}) in the preweaning mice. The body length gain i.e., body length at P_{21}- body length at P_0/P_{16} was calculated for each mice.

Tail length: In growing preweaning mice tail length i.e., from base to tip, was measured using a measuring scale at the commencement and last day of stress exposure and also in corresponding age matched controls. Tail length gain i.e., tail length at P_{21}-tail length at P_0/P_{16} was calculated for each mice.

Eye opening: In the growing mice day of eye opening was taken as a growth parameter. Eye is said to be open when there appears visible slit between the upper and lower eyelids. Day of eye opening is noted in control and stressed mice.

Data presentation: The body weight gain in gram, tail length and body length in centimeter has been tabulated as mean and standard deviation. Unpaired '*t*' test was used for comparison of data between two groups and a '*p*' value less than 0.05 was considered as significant.

RESULTS

Results were made on the observations of growths of normal and stressed mices on body weight (BW), body length (BL) and tail length (TL).

Growths of Mices Under Normal Condition

Body weight gain

The body weight gain was significantly decreased in 21 days restraint stressed (from birth to postnatal day 21) mice compared to controls. (6.32 ± 1.74 g in control vs 4.14 ± 0.68 g in restraint stressed, P < 0.001). There was no change in 5 days stress group (Table 1).

Tail length gain

The tail length also significantly decreased in 21 days restraint stressed mice compared to controls (4.45 ±0.40 cm in control vs 3.37± 0.45 cm in restraint stressed, P<0.001). There was no change in 5 days group (Table 2).

Growth in body length

Like body weight and tail length gain, body length also significantly decreased in 21 days restraint stressed mice compared to control (3.08± 0.52 cms in control vs 2.62± 0.36 cms.

Table 1: Body Weight (Mean ± SD, N = 12 rat/group) of Albino Mice in Control and Under Restraint Stress

Groups	Body Weight Gain (gm)	
	Stress Duration	
	5 days	21 days
Control (C) (n)	6.32 ± 1.74 (12)	6.32 ± 1.74 (12)
Restraint Stress (RS) (n)	5.56 ± 0.59 (12)	4.14*** ± 0.68 (12)

Note: $p < 0.001$ in comparison to control by students't' (two tailed), n=number of animals.

Table 2: Tail Length (Mean± SD, N=12 rat/Group) in Control and Stress Mice

Groups	Tail Length Gain (cm)	
	Stress Duration	
	5 days	21 days
Control (C) (n)	4.45 ± 0.40 (12)	4.45 ± 0.40 (12)
Restraint Stress (RS) (n)	4.40 ± 0.24 (12)	3.37*** ± 0.45 (12)

Note: $p < 0.001$ in comparison to control by students't' (two tailed), n=number of animals.

Inrestraint stressed, P< 0.001). There was no change in 5 days group (Table 3).

iv. Eye opening Day

Eye opening day was delayed in 21 days restraint stressed group but not in 5 days stressed group.

Figure 3: photographs of mice.A.16 days old control mouse, B-16 days old mouse exposed to restraint stress from birth onwards. Note: Mouse in A opened its eyes on postnatal day13, whereas mouse in B, did not open its eyes even on postnatal day 16.

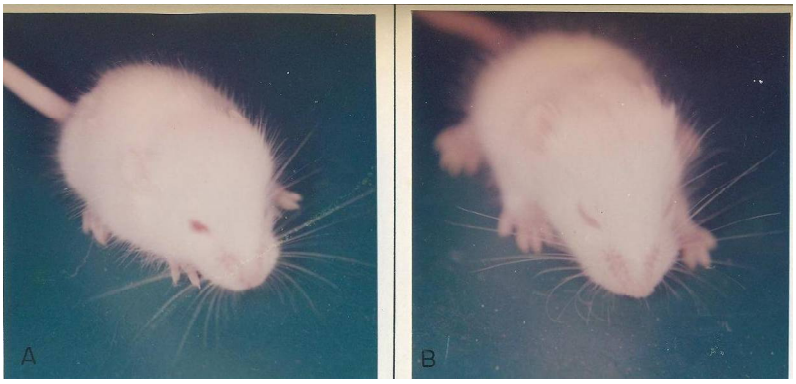

Table 3: Body Length (Mean ± SD, N=12 rat/Group) in Control and Stress Mice

Groups	Body Length Gain (cm)	
	Stress Duration	
	5 days	21 days
Control (C) (n)	3.08 ± 0.52 (12)	3.08 ± 0.52 (12)
Restraint Stress (RS) (n)	2.93 ± 0.46 (12)	2.62*** ± 0.36 (12)

Note: $p < 0.001$ in comparison to control bystudents't' (two tailed), n=number of animals.

Table 4: Preweaning age-Day of Eye Opening Each Value Represents Number of Pups Which Opened Their Eyes on that Particular Postnatal Day, N=Number of Animals

Groups			5 days stress				
	n	12th d	13thd	14th d	15thd	16thd	17thd
Control	12	–	4	8	–	–	–
Restraint stress	12	–	6	6	–	–	–
Groups			21 days stress				
	n	12th d	13thd	14th d	15thd	16thd	17thd
Control	12	–	4	8	–	–	–
Restraint stress	12	–	–	1	6	2	3

Control mice opened their eyes on 13(n=4) or 14th (n=8) postnatal day (Figure 3) whereas mice restraint stressed from birth onwards opened their eyes between 15-17 postnatal day. (Figure 3) (Postnatal day 15: n=5, Postnatal day 16; n=5 and postnatal day 17=2) (Table 4).

DISCUSSION

In life of an animal and man development and growth are almost symphonetic. Studies on rats often begin from before birth, i.e., prenatal development and followed to be observed in the postnatal life. altogether it makes voluminous literature and at the same time makes it beyond comprehension of the researcher. Therefore this paper was limited to postnatal period of albino rats life from day zero to day 21 (3 weeks). It was well recognized for many years that the size of the litter is an important determinant of overall growth of the rats (Gates, 1925). Hughes and Tanner (1970) reported that somatic growth of the laboratory rat is sensitive to litter size. It is directly linked to demand and supply of milk. The more pups in the litter, the lesser milk available for the individual pups, resulting in slower growth rate. In the following year, Park and Nowsieiski (1971) emphasized the importance of genetic pattern and maternal environmental factors. Quality and quantity of food provided to the laboratory animal at any stage has important significance for growth of the animal (Moss, 1954;

Morgan, 1982; Lews, 1989). In our earlier studies, Mukerjee (1987) and Shaligram (1998) we maintained only five pups kept with a healthy dame. It was common to observe that late in third week postnatal the young rat pups a started to nibble the solid food provided to the dame; this can be taken as additional nutrient supplement for the young rat pups. Generally the rats are ominivorous, they prefer fresh food. They instinctively stores excess food by covering them under the paddy husk provided on the floor of the cage. However, unless starvations is threatened, rats avoid stale food and water (Lane-Petter, 1976). Chronic exposure to stressors of certain severity cause anorexia and reduce body weight (Marti *et al*. 1994). In this study it has been observed that chronic stresses like maternal deprivation, restraint stress and electrical foot shock reduce food intake, increase water consumption and cause reduced physical activity by causing longer period of sleep. Rats in the stress group develop fear which is noticed by mere handling before stress presentation. Handling cause more urination and excrement.

In the present study there was a reduction in body weight gain, tail length gain and body length gain in mice undergone 21 days stress. Eye opening was also delayed in the above age group. Body weight gain was reduced in mice subjected to 21 days restraint stress. These results suggest growth retardation due to stress. Similar results were reported by Pullen (1977); Drago *et al*., (1999); Smagin *et al*., (1999) NagarajaHSand Jegannathan (1999) and Santos *et al*., (2000). Retarded body growth may be due to (i) decreased food intake (ii) movement of corticotropin releasing factor (CRF) in the hypothalamic region (Delbende *et al*., 1992). CRF receptors in the hypothalamus mediate acute response to stress that can lead to permanent changes in hormonal or metabolic processes that determine the body weight and composition (Smagin *et al*., 1999). Pathways projecting from limbic areas to hypothalamus could stimulate CRF secretion into pituitary adrenal axis. Increased CRF could mediate this stress induced suppression of food intake thereby reducing body weight (Delbende *et al*., 1992) and also may reduce other growth parameters. It was reported that growth hormone gene expression in the brain, significantly suppressed by exposure to restraint stress (Yashizato *et al*., 1998) which may also cause growth retardation. Circulating growth hormone level is shown to be decreased in animals exposed to stress during neonatal period (Kuhn *et al*., 1978).

Tail length of the rat which at birth is shorter than the body length, increases rapidly due to addition of the newer vertebra and the intervertebral discs by formation of sclerotomic and notochordal elements. In absence of substantial literature of the development of the vertebrae of albino rat knowledge of the development of vertebrae and the intervertebral discs in humans (Muller and O'Rahilly 1986; and O'Rarahilly *et al*., 1990) would explain the sequence of growth in the linear increment of the body size of Wistar rats.

CONCLUSION

In the preweaning group 21 days stress reduced body weight gain, body length gain and tail length gain were reduced in mice to a greater extent than in 5 days stress. Eye opening day was also delayed in the above age group. These results suggests growth retardation due to stress, which may be due to suppression of growth hormone gene expression by stress, at a very early stage of development as reported in the literature.

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