Journal of Infertility and Reproductive Biology

Journal of Infertility and Reproductive Biology, 2013, Volume 1, Issue 2, Pages: 26-30

Restraint stress and lead-induced reduction in progeny output by male rats

P. Hari Priya[1], B.P. Girish[1,2], P. Sreenivasula Reddy[2*]

1. Department of Biotechnology, Sri Venkateswara University, Tirupati – 517502, India
2. Department of Zoology, Sri Venkateswara University, Tirupati – 517502, India

Abstract

We investigated whether restraint stress interferes with lead-induced suppressed fertility in rats. Male Wister rats (weighing 200 ± 10 g in the beginning of the experiment) were subjected to either restraint stress (5 hours/day) or exposed to lead (0.15%) or both for 60 days. To assess the fertility, control and experimental males were cohabited with sexually mature normal females. No significant changes ($p>0.05$) were observed in the body weight gain of experimental males. Similarly, no changes ($p>0.05$) were observed in the mating index. In contrast, there was a significant decrease ($p<0.05$) in fertility rate, measured by counting live foetuses in the uterus of normal females mated with males exposed to either restraint stress or lead. In rats mated to males subjected to both restraint stress and lead treatment, a significant decrease ($p<0.05$) in live fetuses was observed as compared to rats mated with lead alone exposed males.These data demonstrate that adult male rats exposed to either restraint stress or lead significantly decreased fertilization capacity and stress *potentiates* the reproductive toxicity of lead.

Key words: Lead, Restraint stress, Fertility, Implantation loss, Rat.

1. Introduction

Lead (Pb) is a highly toxic heavy metal and a major environmental contaminant which is released into the environment by several routes, but principally by industrial, mining and human activities (1). The Pb toxicity is associated with severe damage to various organs, particularly the testes, in both humans and animals (2-5). A significant reduction in testicular weights associated with decreased androgen secretion as a result of exposure to Pb was observed in men indicating that testicular endocrine function had been compromised (6-8). Recently, using rat model, we have reported a significant decrease in spermatogenesis and steroidogenesis after exposure to Pb (9). Although it is well known that Pb is associated with adverse effects on male reproduction, very few studies are available related to fertility alterations caused by low doses of Pb exposure. Several studies have focused the relationship between immobilization stress and sexual behavior in male rats (10). Restraint stress results in a significant decrease in testis weight, germ cell degeneration, and decline semen quality, sperm concentration, morphology, and fertility potential of males (11-14).Though the inhibitory effect of chronic immobilization on plasma testosterone has been a common finding in adult rats (15-19), very few studies have focused on fertility output. Therefore, it becomes important to study the effect of Pb on fertility under the influence of stress. Thus, the present study was undertaken to evaluate the influence of restraint stress on Pb-induced alterations in fertilization capacity of male Wistar rat.

[*]**Corresponding Address:** Department of Zoology, Sri Venkateswara University, Tirupati- 517 502. Tel: +91-877-2249320 Email: Reddy_1955@yahoo.co.in

2. Material and methods

2.1 Chemicals and reagents

Lead acetate of analytical reagent grade (purity>99.5%) was procured from E-Merck, Bombay, India. All other chemicals used in study were of the highest purity available and purchased from SSR Scientific Company, Tirupati, India.

2.2 Animals and treatment

Adult male (n=40) (body weight 200 ± 10 g) and virgin female (n=40) (body weight 180 ± 10 g) Wistar rats were purchased from authorized vendor (M/S Raghavendra Enterprises, Bengaluru, India). The animals were fed on pellet diet (HLL Animal feed, Bengaluru, India) and water *ad libitum*,

maintained under standard laboratory conditions (temperature, 22–25°C; light:dark cycle, 12:12 hr). The rats were acclimatised for 10 days before experimentation. All animal experimentation was conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (20). This experiment was also reviewed and approved by the Institutional Animal Ethical Committee at S.V. University, Tirupati, India (vide No. IAEC/No.438/01/a/CPSEA).

Rats were randomly divided into four groups consisting of ten animals in each group. The animals of group I were maintained on normal tap water while rats of group II were maintained on 0.15% lead solution (equivalent to 819 mg/L lead) for 60 days. Rats in group III were subjected to immobilization by placing them inside plastic tubes (dimensioned 6 cm in diameter and 45 cm in long) without promoting pain for 5 hr a day during light phase beginning from 9.00 am. Undesirable stress was avoided as much as possible by gentle handling and noiselessness throughout the experiment. Animals in group IV were subjected to restraint stress as in group III and maintained on 0.15 % lead solution. A stock solution was prepared freshly prior to use by dissolving 1% (w/v) lead acetate in distilled water and diluted with filtered tap water to the desired concentration. Rats were observed daily for clinical signs of toxicity (salivation, rhinorrhoea, lachrymation, ptosis, squinted eyes, convulsions, stupor, tremors), postural changes (erection of fur, exophthalmia) and non-sexual behavior (such as cleaning of face, self grooming, climbing in the cage) from the first day of treatment. The body weight of the animals was recorded on the day of initiation of the treatment and also on the day of cohabitation.

2.3 Analysis of progeny outcome parameters

After completion of treatment, each male from the experimental and control groups (n=10) was transferred to a mating cage and cohabited with untreated female rats in proestrus on a 1:1 basis in the home cage of the male. The maximum duration of pairing was 5 days. Female rats were checked for the presence of copulatory plugs, and vaginal washings were examined for the presence of spermatozoa each morning during cohabitation to determine whether copulation had occurred (21). The day on which evidence of copulation identified was defined day zero of gestation. The conception time, the interval between the first day of cohabitation and the day of vaginal plug/or sperm in vaginal smear, was recorded for each female. The number of pregnant rats with each experimental group or the control group was recorded for determination of fertility index. All confirmed-pregnant females were euthanized on gestation day 20 and then cesarian-sectioned. Both uteri were dissected and inspected for implantation and resorption sites, and the numbers of live and resorbed foetuses (by employing 2N NaOH solution) and implantations were recorded. In addition, both ovaries were removed and the numbers of corpora lutea (representing the numbers of ovulated oocytes) were recorded. Fertility index and, pre- and post-implantation loss was calculated. Foetuses were individually weighed and crown rump length was recorded, and also examined for gross external malformations.

2.4 Statistical analysis

The data were statistically analysed using analysis of variance (one-way ANOVA) followed by Dunnet's test. Differences were considered to be significant at $p>0.05$. Data were presented as mean ± standard deviation (S.D). All statistical tests were performed using statistical package for social sciences (SPSS Inc., Chertsey, U.K.).

3. Results and discussion

All the animals were apparently normal and no unusual behaviors (viz. head flicking, head searching, facial movements, head and forelimb clonuses, biting, licking, self-mutilation, circling, tremors, and convulsions, ataxia, grooming and walking backwards) were observed in any of the rats. None of the animals were excluded from the research. No changes in fur appearance, skin colour, lacrimation, urination, respiration, salivation, vocalization, postural, or gait abnormalities were observed in any of the control and treated rats. The mean body weight gain in the control and after exposure to restraint stress or lead alone or in combination was not significantly different (data not shown).

None of the control or experimental rats died or was excluded from the experiment. Generally, all control or experimental rats exhibited sings of sexual motivation as soon as female was introduced, including grooming, licking the genitalia, chasing the female, and passing under each other's body. Though visual observations on male sexual behaviour are not quantified, they made several attempts to mount the female.

All the females that cohabited with control males and males exposed to restraint stress or lead had plugs and produced pups. In contrast, plugs were present in 10 of 10 females cohabited with males exposed to combined stresses; but only 8 of these

females retained 5-6 pups each.The number of corpora lutea was almost similar ($p>0.05$) in all the groups (Table 1). Exposure to restraint stress resulted in a 6-fold increase in pre-implantation loss compared to control ($p<0.05$); pre-implantation loss remained elevated in rats sired by males exposed to lead or both stress. No significant post-implantation loss ($p>0.05$) was observed in rats mated with control and restraint stress exposed males; whereas, post-implantation loss increased in the rats mated with males exposed to lead (Table 1). As a consequence of the increases in pre- and post-implatation loss, a significant ($p<0.05$) reduction in the number of pups per rat was observed in experimental groups compared with the controls. The mean weight and crown rump length of foetuses were not affected in rats mated with control and rats exposed to restraint stress, nor do the stresses induce an increase in the incidence of external malformations in the foetuses (data not shown). Foetal crump rump length and foetal weight significantly ($p<0.05$) decreased in rats mated with males exposed to either lead or both lead and restraint stress (Table 1). Bioavailability of testosterone is not only important for the maintenance of structural integrity of testis and accessory sex organs and maintenance of spermatogenesis (22) but also essential for expression of secondary sex characters (23). Significant decrease in circulatory testosterone levels were observed in rats after exposure to either restraint stress or lead (24). The inhibitory effect of chronic immobilization on plasma testosterone has been a common finding in adult rats (15-19, 25). It was also suggested that restraint stress may induce Leydig cell hyposensitivity to gonadotropin leading to blockade of testosterone biosynthesis at normal LH levels (26). It was also reported that rats subjected to chronic immobilization exhibited decreased spermatid production and spermatozoon concentration in cauda epididymis (14). Decrease in daily sperm production, epididymal spermatozoon concentration and plasma testosterone level was also observed in rats after chronic Pb exposure (9). Whether the reduced fertility observed in rats exposed to both stresses resulted from low sperm numbers, decreased motile and viable sperm or depressed sexual desire cannot determined from

Table 1: Effect of restraint stress and exposure to lead alone or in combination on reproductive performance in male rats

Parameter	Control	Restraint Stress (RS)	Pb	RS+Pb
Pregnant/mated	10/10	10/10	10/10	8/10
Conception time (days)	$1.21^{a} \pm 0.22$	$2.13^{b} \pm 0.29$ (76.03)	$2.54^{b} \pm 0.23$ (109.92)	$3.75^{c} \pm 0.46$ (209.92)
Mating index (%)	10	100	100	80
Fertility index (%)	100 (10/10)	100 (10/10)	100 (10/10)	80 (8/10)
No. of corpora lutea/rat	$13.83^{a} \pm 1.16$	$13.75^{a} \pm 1.26$ (-0.58)	$13.87^{a} \pm 1.15$ (0.29)	$13.59^{a,\#} \pm 1.91$ (-1.74)
No. of implantations/rat	$13.24^{a} \pm 0.52$	$10.12^{b} \pm 1.41$ (-23.57)	$10.02^{b} \pm 1.63$ (-24.32)	$8.67^{b,\#} \pm 1.53$ (-34.52)
Implantation rate (%)	95.73	73.59	72.24	63.79
Pre-implantation loss (%)	4.27	26.39	27.76	36.20
No. of embryos resorbed	-	-	01	01
No. of fetuses/rat	$13.16^{a,\$} \pm 0.75$	$9.97^{b,\$} \pm 1.13$ (-24.24)	$8.01^{c,\$} \pm 1.03$ (-39.13)	$5.14^{d} \pm 1.02$ (-60.94)
Post-implantation loss (%)	0.61	1.48	20.06	40.72
Foetal crown rump length (cm)	$3.92^{a,\$} \pm 0.36$	$3.89^{a,\$} \pm 0.41$ (-0.77)	$2.81^{b,\$} \pm 0.33$ (-28.32)	$2.14^{b,*} \pm 0.32$ (-45.41)
Foetal weight (g)	$3.67^{a,\$} \pm 0.29$	$3.71^{a,\$} \pm 0.31$ (1.09)	$2.07^{b,\$} \pm 0.36$ (-43.60)	$2.02^{b,*} \pm 0.43$ (-44.96)

Values are mean ± SD; n=10 (unless mentioned) #n=8; $n=95; *=40
Values in parentheses are % change from control
Values with same superscript in a row do not differ significantly from each other by Dunnet's test

the present data. The presence of copulatory plugs in all the females may indicate sexual desire is not depressed in males. The observed pre-implantation loss may represent unfertilized, ovulated oocytes or the death of early embryos prior to implantation. We have reported earlier deterioration in spermatozoa quality and epididymal sperm density in rats exposed to restraint stress and lead. Exposure to lead also resulted in development of sperm with malformed head. The reduced fertilization capacity of stressed male is probably due to decrease in sperm quality, such as motility, viability and morphology. Moreover, we have also observed that the rate of pre- and post-implantation loss in female rats mated with stressed male rats significantly increased. The cause of this manifestation is probably due to fertilization of oocytes with damaged spermatozoa. Additional in-depth studies are needed involving artificial insemination using a fixed number of sperm from the cauda of epididymis. These studies will help us determine the ability of sperm fertility at a given time point.

Based on our results, it is clear that exposure to either lead or chronic restraint stress has adverse effects on progeny outcome in rats. Further, these findings also indicate the possible role of restraint stress, characterized by decreased number of fetuses per rat, in potentiating the reproductive toxic effect of Pb. Since stressed life style and environmental pollution are phenomena in modern World, sedentary life which includes some aspects of restraint stress is bio-medically imperative in male reproductive failure in modern society.

Authors' Contribution Statement

PSR conceived the idea, participated in its design, supervised the work, provided the grants for the study, evaluated the data, and coordinated the study. PHP and BPG participated in designing the study, carried out the treatment of animals, and performed fertility studies. All authors drafted the manuscript for publication, read, and approved the final manuscript.

Acknowledgements

The authors are thankful to the Head, Department of Zoology, S.V. University, Tirupati for providing the laboratory and animal facilities. PSR is thankful to University Grant Commission, New Delhi for providing financial assistance in the form of research grant. BPG is thankful to CSIR, New Delhi for financial support in the form of Junior Research Fellowship.

Conflict of interest statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

1. Fisher, I. J., Pain D. J., Thomas V. G. (2006). A review of lead poisoning from ammunition sources in terrestrial birds. *Biological Conservation*, 131, 421-432.
2. Buerger, T. T., Mirarchi, R. E., Lisano, M. E. (1986). Effects of lead shot ingestion on captive mourning dove survivability and reproduction. *Journal of Wildlife Management*, 50, 1-8.
3. Ramo, C., Sańchez, C., Hernańdez, S. A. (1992). Lead poisoning of greater flamingos *Phoenicopterus ruber*. *Wildfowl*, 43, 220-222.
4. Fair, J. M., Ricklefs, R. E. (2002). Physiological, growth, and immune responses of Japanese quail, chicks to the multiple stressors of immunological challenge and lead shot. *Archives of Environmental Contamination Toxicology*, 42, 77-87.
5. Snoeijs, T., Van de Casteele, T., Adriaensen, F., Matthysen, E., Eens, M. A. (2004). Strong association between immune responsiveness and natal dispersal in a song bird. *Proceedings of Royal Society B: Biological Sciences*, 271, 199-201.
6. Naha, N., Bhar, R. B., Mukherjee, A., Chowdhury, A. R. (2005). Structural alteration of spermatozoa in the persons employed in lead acid battery factory. *Indian Journal of Physiology and Pharmacology*, 49, 153-162.
7. Xu, H. H., Chen, Z. P., Shen, Y. (2006). Meta analysis for effect of lead on male productive function. *Journal of Industrial Hygiene Occupational Disease*, 24, 634-636.
8. Kasperczyk, A., Kasperczyk, S., Horak, S., Ostalowska, A., Grucka-Mamczar, E., Romuk, E., Olejek, A., Birkner, E. (2008). Assessment of semen function and lipid peroxidation among lead exposed men. *Toxicology and Applied Pharmacology*, 228, 378-384.
9. Reshma Anjum, M., Sainath, S. B., Suneetha, Y., Reddy, P. S. (2011). Lead acetate induced reproductive and paternal mediated developmental toxicity in rats. *Ecotoxicology and Environmental Safety*, 74, 973-978.
10. Almeida, S. A., Kempinas, W. G., Lamano-Carvalho, T. L. (2000). Sexual behavior and fertility of male rats submitted to prolonged immobilization induced stress. *Brazilian Journal of Medical Biology Research*, 33, 1105-1109.
11. Zemjanis, R., Gondos, B., Adey, W. R., Cockett, A. T. (1970). Testicular degeneration in *Macaca*

nemestrina induced by immobilization. *Fertility and Sterility*, 21, 335-340.

12. Kholkute, S. D., Udupa, K.N. (1979). Effects of immobilization stress on spermatogenesis & accessory sex organs in rats. *Indian Journal of Experimental Biology*, 17, 206-208.
13. Bonde, J. P., Ernst, E., Jensen, T. K., Hjollund, N. H., Kolstad, H., Henriksen, T.B., Scheike, T., Giwercman, A., Olsen, J., Skakkebaek, N.E. (1998). Relation between semen quality and fertility: a population based study of 430 first-pregnancy planners. *Lancet*, 352, 1172-1177.
14. Almeida, S. A., Petenusci, S. O., Anselmo-Franci, J. A., Rosa-e-Silva A, A., Lamano-Carvalho, T, L. (1998). Decreased spermatogenic and androgenic testicular functions in adult rats submitted to immobilization-induced stress from prepuberty. *Brazilian Journal of Medical Biology Research*, 31, 1443-1448.
15. Charpenet, G., Taché, Y., Forest, M. G., Haour, F., Saez, J. M., Bernier, M., Ducharme, J. R., Collu, R. (1981). Effects of chronic intermittent immobilization stress on rat testicular androgenic function. *Endocrinology*, 109, 1254-1258.
16. Collu, R., Gibb, W., Ducharme, J. R. (1984). Role of catecholamines in the inhibitory effect of immobilization stress on testosterone secretion in rats. *Biology of Reproduction*, 30, 416-422.
17. Demura, R., Suzuki, T., Nakamura, S., Komatsu, H., Odagiri, E., Demura, H. (1989). Effect of immobilization stress on testosterone and inhibin in male rats. *Journal of Andrology*, 10, 210-213.
18. López-Calderón, A., Ariznavarreta, C., González-Quijano, M. I., Tresguerres, J. A. F., Calderón, M. D. (1991). Stress induced changes in testis function. *Journal of Steroid Biochemistry and Molecular Biology*, 40, 473-479.
19. Orr, T. E., Mann, D. R. (1992). Role of glucocorticoids in the stress-induced suppression of testicular steroidogenesis in adult male rats. *Hormones and Behaviour*, 26, 350-363.
20. CPCSEA (Committee for the Purpose of Supervision on Experiments on Animals) (2003). CPCSEA guidelines for laboratory and animal facility. *Indian Journal of Pharmacology*, 35, 257-274.
21. Dunnick, J.K., Gupta, B.N., Harris, M.W., Lamb, J.C. (1984). Reproductive toxicity of dimethyl phosphonate in male Fischer rat. *Toxicology and Applied Pharmacology*, 73, 480-490.
22. Mann, T. (1974). Secretory function of the prostate, seminal vesicle, and other male accessory organs of testis. *Journal of Reproduction and Fertility*, 52, 12-17.
23. Steinberger, E. (1971). Hormonal control of mammalian spermatogenesis. *Physiological Reviews*, 51, 1-22.
24. Haripriya, P., Reddy, P. S. (2011). Effect of restraint stress on lead-induced male reproductive toxicity in rats. *Journal of Experimental Zoology*, 317A, 455-465.
25. Tavakoli, P., Ahmadi, R., Maf, M. (2012). Restraint stress is bio-medically important in male reproductive failure. International Conference on Chemical, Biological and Medical Sciences (ICCBMS 2012), August 25-26, Kuala Lumpur (Malaysia), pp 17-19.
26. Charpenet, G., Taché, Y., Bernier, M., Ducharme, J. R., Collu, R. (1982). Stress induced testicular hyposensitivity to gonadotropins in rats: Role of the pituitary gland. *Biology of Reproduction*, 27, 616-623.