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Neonatal methyl parathion exposure affects the growth and functions of the male reproductive system in the adult rat

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Methyl parathion (MP) is a well-known organophosphorus pesticide, to which humans are exposed in fruit and vegetables as residues of 0–2 mg/kg, children being at higher risk of exposure. The present study was planned to investigate the effects on the adult male reproductive functions of MP following neonatal exposure. New born male Wistar rat pups were treated orally with either 0 or 0.5 mg/kg MP from postnatal day (PND) 3 to PND 28 and sacrificed on PND 98 for the purpose of examination of the reproductive system. Methyl parathion lowered the body weights from days 10 to 24 ($p < 0.01$), the weights of the reproductive organs ($p < 0.05$–0.01), the epididymal sperm count ($p < 0.01$) and the homogenisation-resistant testicular spermatid head count ($p < 0.01$) and also decreased acid phosphatase (ACP), cholesterol, uric acid, protein, ascorbic acid, and lactate dehydrogenase ($p < 0.01$) levels in the testis but only ACP and cholesterol in the epididymis. The levels of abnormal sperm and testosterone in the testis were increased ($p < 0.01$), whereas the leutinising hormone level and total number of seminiferous tubules decreased in the testes of treated rats ($p < 0.01$). A few tubules showed exfoliation of epithelium and vacuoles. The incidence of stage XIV tubules and ratios of meiotic figures and elongating spermatids to Sertoli cell nucleoli decreased ($p < 0.01$; Mann-Whitney U test). The present results indicate that MP acts as an endocrine disruptor and consequently affects the postnatal development and growth of the male reproductive organs in the rat. These findings are important to the general public, as there is a chance of children being exposed to this pesticide.

Key words: p-nitrophenol, testis, reproductive system, neonatal exposure, anti-oestrogens, pesticides, xenobiotics, endocrine disruptors

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INTRODUCTION

The supposed deterioration in male reproductive functions and increase in reproductive abnormalities during the last few decades have been linked to exposure to environmental chemicals [7, 31]. Many chemicals, such as pesticides, act as endocrine disruptors during the crucial period of development of the male reproductive system, which is dependent on the activities of testosterone and its metabolite-dihydrotestosterone [12]. Androgen-receptor antagonists such as vinclozolin [19], procymidone or some phthalates inhibit male reproductive functions after perinatal exposure [12]. Some other chemicals, for example polychlorinated biphenyls, on the other hand, increase testis size and daily sperm production after neonatal exposure via a mechanism of hypothyroidism-induced hyper-multiplication of Sertoli cells that consequently enhances germ cell multiplication [9]. In general, but not exclusively, the chemicals that affect the functional control of male reproductive system differentiation and growth are weakly oestrogenic or anti-androgenic in nature [35].

Methyl parathion (MP; o, o-dimethyl o-p-nitrophenyl phosphorothioate), popularly known as "cotton poison", is an organophosphate insecticide licensed only for agricultural use in many countries, although there are instances of its misuse as a domestic spray to contain insects, as happened in the USA in the 1990s [29]. It is most commonly used on cotton but also on other crops such as corn, peaches, wheat, barley, rice, vegetables and fruit [16]. The insecticidal properties of MP reside in its ability to inhibit acetylcholinesterase activity, thus enhancing endogenous acetylcholine accumulation [8], and this property seems to be responsible for many, if not all, of its adverse effects [29]. Methyl parathion significantly inhibited acetylcholinesterase activities in the brain and plasma of both foetal and adult rats when given to pregnant rats at a 10 mg/kg dose-level [2]. Placental transfer of MP has been demonstrated in rats, indicating that exposure may be dangerous to pregnant women, which might affect the pregnancy outcome [1]. MP showed some teratogenic effects on chick embryos involving the musculoskeletal system and inducing abdominal hernias and haemorrhagic spots in the brain and upper part of the body [20]. MP also induced an irregular oestrous cycle and modified its duration and phases, pro-oestrous and di-oestrous being significantly affected at 2.5–3 mg/kg dose-levels in rats, although this did not affect fertility [33]. At 5 mg/kg (i.p. for 15 days) dose-levels MP decreased the compensatory weight gain of the ovaries and decreased the healthy follicles in the ovaries of chemi-castrated rats [5, 11]. At dose-levels of 1–2 mg/kg MP affected the structure of the placenta, in which vascular congestion, degeneration of trophoblast giant cells and alterations to the foetal-maternal interface have been observed [22]. Neonatal exposure to MP caused inhibition of brain acetylcholinesterase, more than that in adults [28]. *In utero* exposure to MP at 1–1.5 mg/kg resulted in decreased acetylcholinesterase activity and increased choline acetyltransferase activity in all brain regions during the developmental period in rat foetuses [13]. Maternal exposure to MP at these dose-levels also decreased the foetal protein synthesis in a dose-dependent pattern [14]. On the other hand, very few studies have been conducted on the effects of MP on the male reproductive system. In adult mice very high doses of MP induced abnormal sperm formation but did not affect the sperm count [24]. In white-throated munias (*Lonchura malabarica*), MP decreased the activity of acetylcholinesterase in the testis as well as causing structural changes in the seminiferous tubules and the authors concluded that the testicular damage was mediated via the altered activities of this enzyme [23].

Although not unequivocal, the results of a study conducted in the USA to evaluate the neurobehavioural health of children who have been exposed to MP indicate that this pesticide affected the short-term memory and attention and caused behavioural and motor-skill problems [30]. Children are highly vulnerable not only to MP but to all pesticide exposure, as they accompany their parents to farms and play [16], due to hand-to-mouth behaviour and unusual dietary patterns [21]. However, there are no studies which focus on the effects of MP on the adult male reproductive system following neonatal exposure. The results of the present study show that neonatal MP exposure at human exposure dose-levels affects the growth and functions of the reproductive system in the male rat.

MATERIAL AND METHODS

Animals

Virgin female Wistar rats were mated with male rats. The pregnant rats were then housed separately in plastic cages with paddy husk bedding. The newborn pups were sexed on the basis of anogenital distance and maintained with their mothers up to postnatal day (PND) 28, at which day they were housed separately. The male pups were adjusted to two groups of five animals each on PND 2. All animals

had access to food and water *ad libitum*. All the experimental procedures employed in this study and the maintenance of the animals was strictly under the guidance of the Institutional Animal Ethical Committee and Federal Laws.

Methyl parathion and treatment

Methyl parathion (Metacid 50) was procured from Bayer India Ltd., Mumbai. This is an emulsifiable insecticide containing 50% w/w of MP and remaining as an emulsifier. Although the exact human exposure levels are equivocal, several studies report that 0–2 mg/kg may be exposed as residues in fruit and vegetables and by other means [4, 16]. Thus a dose-level of 0.5 mg/kg was selected and administered to one group of newborn male pups orally once daily, starting on PND 3 and continuing to PND 28. Another group received water and served as a control.

Body and organ weights

Both treated and control animals were weighed before, during and after treatment. On PND 98 all 10 animals were anaesthetised (pentobarbital sodium, 45 mg/kg), and when the rats were in deep sleep a lethal dose of anaesthesia was given to sacrifice them. Following this laparotomy was conducted and the reproductive tract exposed. All reproductive organs were removed and weighed.

Sperm analysis

The right epididymis was minced in 1 mL phosphate buffered saline (PBS; pH 7.4) and filtered through 80 μ nylon mesh to obtain a filtrate. The sperm count was conducted as per the standard procedure in a Neubauer counting chamber [26, 37]. One drop of eosin Y was added to the remaining filtrate and smears were prepared on clean glass slides and dried. For each animal 500 sperm were screened and classified as normal and abnormal sperm according to the standard procedure [27].

Homogenisation-resistant testicular spermatid head count (HRSC)

The right testis from each animal was homogenised in 5 mL of PBS and the homogenisation-resistant spermatid heads were counted in the Neubauer chamber and expressed as HRSC/testis.

Biochemical analysis in the testis and epididymis

The remaining homogenate of the testis and the filtrate of the epididymis were used for biochemical analysis. The activities of acid phosphatase (ACP; Kind and King method), cholesterol (Zak's method), uric acid (only in the testis; Caraway method), and total protein (Biuret method) [36] and ascorbic acid level (2, 4-dinitrophenol-2 indophenol method) [18] were quantified colorimetrically. The activity of lactate dehydrogenase (LDH; only in the testis) was estimated by a UV Kinetic IFCC kit (Bayer Diagnostics India Ltd., Gujarat) in a HITACHI 902 auto-analyser according to the manufacturers' instructions.

Hormone analysis

The testicular levels of testosterone and leutinising hormone (LH) were analysed in the homogenate by electrochemiluminescence immunoassay-ECLIA in a Roche Elecsys 1010 immunoassay analyser, Germany (Roche Diagnostics Gmbh D-68298 Mannheim) according to the manufacturers' instructions.

Histopathological analysis of the testis

The left testis was processed for paraffin embedding after fixing in Bouin's fluid. Five micron sections of the testis were obtained and stained with haematoxylin and eosin or periodic acid-Schiff's reaction. The control and treated testes were evaluated for any structural changes. The number of tubular cross-sections was counted in sections taken from the middle part of the testis. The seminiferous tubule diameter (STD), the epithelial height (SE) and the luminal diameter of the seminiferous tubules were measured using an eyepiece micrometer calibrated with a stage micrometer. For STD and luminal area, two measurements were taken in such a way that they were perpendicular to each other; the average of these then served as the diameter. For the SE two measurements were taken in each tubule opposite each other and their average was then taken. All these measurements were taken in 10 transversely cut tubules per testis and the average of these 10 values served as the final value for that animal. From the luminal diameter, the luminal area was calculated by fitting the data into πr^2, where r was the radius of the lumen. In each testis 100 randomly encountered tubules were analysed for the incidence of exfoliation (sloughing) of seminiferous epithelium and such tubules expressed as a percentage incidence. In each testis five transversely cut stage VII tubules were selected and the diameters of the nuclei of different germ cells, with the exception of elongated spermatids, were measured. The diameter of each nucleus was the average of two measurements, one at right angles to the other. From this the nuclear volumes

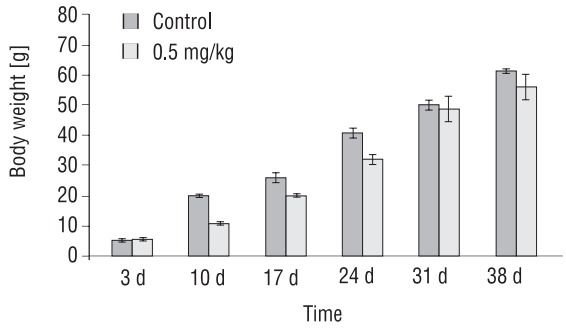

Figure 1. Effect of neonatal exposure to MP on body weight of rats. Data are represented as means ± SD from 5 animals/ /group. Decrease in body weights in treated group was significant from PND 10 to PND 24 ($p < 0.01$). Days shown in X-axis represent the postnatal age.

were calculated by the formula $4/3\pi r^3$, where r was the radius of the nucleus. Similarly, five transversely cut stage XIV tubules were selected in each testis, in which Sertoli cell nucleoli, meiotic figures, and elongating spermatids (step 14) were counted and expressed as per Sertoli cell nucleoli incidence. A total of 100 randomly encountered tubules were analysed to obtain the incidence of stage XIV tubules.

Statistical analysis

The data were expressed as means ±SD from 5 animals per group. The differences between control and treated groups were compared for statistical significance by the Mann-Whitney U test with the level of significance set at $p<0.05$.

RESULTS

Neonatal treatment at a human exposure dose-level of 0.5 mg/kg for 25 days decreased the body weight of pups from PND 10 to PND 24 (Fig. 1; $p < 0.01$). Recovery was observed on day 4 after the cessation of treatment (PND 31) although the rats still suffered from decreased body weight. The weights of all reproductive organs decreased ($p < 0.05$–0.01) and the reduction was approximately 64% and 48% of the control values for the seminal vesicle and prostate respectively, compared to between 14% and 29% for other organs (Table 1). The effects on sperm count were significant ($p < 0.01$) and the decrease was about 62% against control value (Fig. 2). The homogenisation-resistant spermatid head count was also decreased ($p < 0.01$) to 49% against the control value (Fig. 3). The incidence of all types of abnormal sperm increased significantly ($p < 0.05$–0.01). The abnormalities noticed were mainly of the tail and these accounted for more than a threefold rise in the total incidence of abnormality (Table 2).

Reductions of approximately 31% in ACP, 49% in cholesterol, 39% in uric acid, 30% in total protein, 38% in ascorbic acid and 18% in LDH levels

Table 1. Effect of neonatal exposure to methyl parathion on organ weights (in g) on PND 98

Organ	Control	0.5 mg/kg
Testis	1.70 ± 0.13	1.46 ± 0.12*
Epididymis	0.44 ± 0.02	0.35 ± 0.03*
Ductus deferens	0.17 ± 0.01	0.11 ± 0.01*
Seminal vesicle	0.74 ± 0.17	0.27 ± 0.16**
Prostate	0.44 ± 0.03	0.23 ± 0.05**

Data are represented as mean ± SD from 5 animals/group; *$p < 0.05$, **$p < 0.01$, control versus treated group

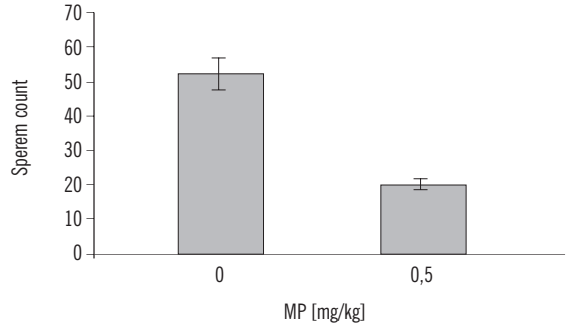

Figure 2. Effect of neonatal MP exposure on sperm count ($\times 10^6$) on PND 98. Data are represented as means ± SD from 5 rats/group; $p < 0.01$, control versus treated group.

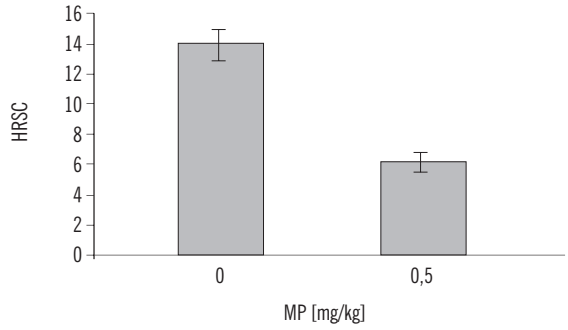

Figure 3. Effect of neonatal MP exposure on homogenisation-resistant spermatid head count ($\times 10^6$) (HRSC) on PND 98. Data are represented as means ± SD from 5 rats/group; $p < 0.01$, control versus treated group.

Table 2. Effect of neonatal MP exposure on sperm morphology in the rat on PND 98

Parameter	Control	0.5 mg/kg
Head abnormalities	1.00 ± 1.00	3.20 ± 1.10**
Tail abnormalities	49.60 ± 4.93	170.60 ± 22.59**
Cephalocaudal junction defects	0.20 ± 0.45	3.40 ± 1.14*
Total abnormalities	50.80 ± 4.21	177.20 ± 23.37**

Data are represented as means ± SD from 5 animals/group; *p < 0.05, **p < 0.01, control versus treated group

Table 3. Effect of neonatal MP exposure on biochemical and hormonal parameters of the testis on PND 98

Parameter	Control	0.5 mg/kg
ACP [IU/L]	129.42± 2.01	89.42± 7.92**
Cholesterol [mg/dL]	224.90± 9.16	116.00± 20.88**
Ascorbic acid [mg/dL]	97.72± 4.46	60.36± 10.94**
Total protein [g/dL]	3.60± 0.12	2.50± 0.41**
Uric acid [mg/dL]	25.78± 7.84	15.66± 3.78*
LDH [IU/L]	2129.60± 123.34	1730.00± 120.42**
Testosterone [ng/g testis]	20.06± 2.74	167.01± 31.79**
LH [mIU/L]	5.02± 0.12	< 0.1

Data are represented as means ± SD from 5 animals/group; *p < 0.05, **p < 0.01, control versus treated group

were observed. The increase in testosterone level was around 730% of the control level. LH level was below the detection limit (Table 3). However, in the epididymis MP reduced only ACP and cholesterol levels significantly (Table 4).

Qualitatively, the testis did not show any degenerating tubules, but in all animals, mild epithelial sloughing was seen, although there were no effects on STD and SE. The number of tubules showing exfoliation of the seminiferous epithelium increased ($p < 0.01$), although the rise was not very great (Table 5). MP decreased the incidence of stage XIV tubules and in these the meiotic figures and elongating spermatids were also decreased. A decrease of about 13% was observed in the number of tubular cross-sections per testis in the group treated. The luminal area of seminiferous tubules was also decreased in the treated rats (Table 5). No effect on nuclear volumes was seen in the treated rats (Table 6).

Table 5. Effect of neonatal exposure to MP on seminiferous tubules and epithelium in the rat on PND 98

Parameters	Control	0.5 mg/kg
STD [μm]	328.01 ± 15.93	315.55± 30.40
SE [μm]	70.59 ± 5.78	76.12± 6.92
Luminal area [μm^2]	22542.12± 1062.11	17591.18± ± 1718.13**
Tubular cross--sections/testis	472.05 ± 2.01	412. 01± 2.05**
Tubules with sloughing [%]	0.20 ± 0.45	4.80± 1.30*
Stage XIV tubules [%]	6.60 ± 0.89	4.00± 1.80*
MF/SN	2.91 ± 0.51	1.98± 0.36*
ES/SN	7.82 ± 0.82	3.99± 0.13*

Data are represented as means ± SD from 5 animals/group. STD — seminiferous tubule diameter, SE — seminiferous epithelial height, MF/SN — number of meiotic figures per Sertoli cell nucleolus, ES/SN — number of elongating spermatids per Sertoli cell nucleolus/ stage XIV tubule; *p < 0.05, **p < 0.01, control versus treated group

DISCUSSION

Previous studies have shown that exposure to oestrogenic or anti-androgenic compounds impair the male reproductive functions [6, 10]. To the best

Table 4. Effect of neonatal exposure to MP on biochemical parameters of the epididymis on PND 98

Parameters	Control	0.5 mg/kg
ACP [IU/L]	132.76 ± 3.95	106.51± 15.8*
Cholesterol [mg/dL]	76.12 ± 9.5	45.64± 11.72**
Ascorbic acid [mg/dL]	8.39 ± 1.59	6.92± 1.90
Total protein [g/dL]	4.30 ± 0.68	4.38± 0.74

Data are represented as means ± SD from 5 animals/group; *p < 0.05, **p < 0.01, control versus treated group

Table 6. Effect of neonatal MP exposure on nuclear volume (μm^3) of germ cells on PND 98

Germ cells	Control	0.5 mg/kg
Spermatogonia	66.93 ± 18.71	76.24± 33.20
Primary spermatocytes	448.78± 111.73	504.49± 135.23
Round spermatids	189.52± 2.19	190.50± 10.93

Data are represented as means ± SD from 5 animals/group; p > 0.05, control versus treated group

of our knowledge, this is the first report on the effects of MP on adult male reproductive functions after neonatal exposure. The body weights of treated rats decreased significantly. This might have been due to decreased nutrition, although the rats recovered after the cessation of treatment. Organ weights also decreased generally, but a spectacular effect was observed on the weights of the seminal vesicle and prostate. These changes clearly indicate an alteration in the endocrine control of the postnatal growth of the male reproductive system [25]. The differentiation of derivatives of the Wolfian duct such as the epididymis, ductus deferens and seminal vesicles are controlled by testosterone, whereas the attainment of prostate growth is tightly controlled by dihydrotestosterone [12]. The testicular testosterone level increased dramatically, although this could not stimulate the growth of organs, thus indicating that MP acted like an androgen receptor-antagonist, somewhat similar to flutamide [25]. However, MP did not affect the negative feed-back on LH secretion, as the latter decreased to an undetectable limit. This indicates that the hypophyseal-gonadal axis is unaffected by MP. The decrease in weight of the testis or epididymis or ductus deferens was less compared to that of the seminal vesicle or prostate. The reason may be that MP competes more vigorously with testosterone/dihydrotestosterone to bind to androgen receptors on the seminal vesicle or prostate but not as vigorously on other organs.

Menthyl parathion parathion also affected the sperm and spermatid counts, reducing both of them in a way similar to the effects of vinclozolin or flutamide [38]. Potential endocrine disruptors have been linked with deterioration in semen quality in humans as well as in wildlife [32]. This study shows that MP is one of those agents involved in the process of impairment of the reproductive system. The decreased sperm and spermatid counts were due to the cytotoxicity of MP, which decreased the number of germ cells in stage XIV tubules. Moreover, the progression of previous stage tubules into stage XIV has also been inhibited by MP, indicating cytotoxicity. Furthermore, this change in sperm concentration must also be due to a decrease in the number of tubular cross sections, revealing that MP has affected the growth and coiling of seminiferous tubules.

Another important effect was on sperm morphology and biochemical parameters. MP induced a rise in the total number of sperm abnormalities, to which tail defects mainly contributed. This finding indicates that MP affects the structural components of the tail. Mathew et al. [24] reported that MP at very high dose-levels increased the sperm abnormalities as a result of genetic damage to germ cells in adult mice. The present results obtained after very low-dose treatment indicate that rats are more sensitive to MP than mice [34]. All the biochemical parameters tested in this study, with the exception of testosterone level, showed decreased activity because of a reduced source in the testis, owing to the decreased growth of the latter. The two marker enzymes (ACP and LDH), endogenous ascorbic acid, an antioxidant required for normal testicular function [15], uric acid, which also has a protective role [3], cholesterol (a precursor molecule of testosterone biosynthesis) and total protein all showed decreased activity, indicating an inhibited biochemical milieu in the testis. Increased testosterone accumulation negatively affected the cholesterol level, indicating the over-use of the latter for testosterone synthesis, as well as a decreased LH level, revealing an unaffected negative feed-back loop. The increased level of testosterone must therefore have been due to a competitive binding of MP or its metabolites to androgen receptors. This aspect of MP toxicity, however, requires further examination in different *in vivo* and *in vitro* systems.

Methyl parathion did not affect the structure of the testis significantly except for a few vacuoles in the epithelium, which we think are biologically unimportant. This finding is in contrast to the effects of many other chemicals administered during the development of the male reproductive system, where the testicular structure has been severely affected [12, 17, 25]. One reason for this discrepancy may be late observation on PND 98, by which time recovery from the damage would have taken place. Even if this were the reason, we should have observed at least some remnants of structural changes sufficient to make the judgement that MP affected the structure of the testis after neonatal exposure. However, that was not the case in this study, indicating that the structure of the testis was unaffected by MP. On the other hand, we did not examine the structure of other reproductive organs, a subject which, however, also requires close examination.

On the basis of these findings it can be concluded that MP affects the function of the male reproductive system after neonatal exposure. This finding is of great importance, as children are the group most prone to inadvertent exposure to MP, and this would adversely affect the growth of the reproductive system.

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