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Effect of 4-Nitrophenol on Male Mice Reproductive Function

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Abstract: 4-Nitrophenol (PNP) has deleterious effects on mammalian physiological functions. The objective of the study is to identify the effect of 4-Nitrophenol on male mice reproductive function. About 50 adult Kunming male mice (25-30 g) were randomly divided into 5 groups, administrated (0.1, 10 and 100 mg $kgwt^{-1}$) orally for 35 days. Semen quality, histological structure of testis and ultrastructure of primary spermatocytes were detected to analysis effect of PNP on the spermatogenesis function. Furthermore, the functions of liver and kidney were determined by testing the blood of biological indicators. The results showed that 0.1 mg $kgwt^{-1}$ PNP decreased slightly in body weight ($p>0.05$) but significantly reduced and rogen hormone-sensitive organ weights ($p<0.05$). However, semen quality (total sperm count, survival, linear motion and acrosome integrity rate), liver function (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total protein and plasma albumin ($p>0.05$) and renal function (urea, uric acid and creatinine) had no significant changes ($p>0.05$); 10 and 100 mg $kgwt^{-1}$ PNP reduced not only significantly body weight, androgen hormone-sensitive organ weights ($p<0.05$) but also significantly reduced semen quality, liver and renal function and the effect of 100 mg $kgwt^{-1}$ PNP was stronger than that of 10 mg $kgwt^{-1}$ PNP ($p<0.05$); three doses of PNP on the testicular structure and ultra structure of primary spermatocytes had injury effect. With increasing concentrations of PNP, the damage was enhanced. About 100 mg $kgwt^{-1}$ PNP significantly disrupted testicular structure and ultra structure of the primary spermatocytes. This shows that PNP affects the liver and kidney detoxification function which reduced the detoxification ability of body and damages the process of spermatogenesis.

Key words: Adult male mice, 4-Nitro phenol, testes, reproductive function, spermatogenesis, biological indicators

INTRODUCTION

4-Nitrophenol (4-Nitrophenol, PNP) is not only an important component of the particulate matter in diesel exhaust emissions (Diesel Exhaust Particles, DEP) but also metabolites of parathion ester of nifedipine, parathion and other pesticides. 4-nitrophenol was found in air, rainwater, soil, seabed and a variety of food (Rubio *et al*., 2012). PNP could enter human body through respiration or skin which lead to lung cancer, asthma, bronchitis and other respiratory diseases, hypotension and other cardiovascular diseases (Ichinose *et al*., 1997; Miyabara *et al*., 1998; Takafuji *et al*., 1987; Toda *et al*., 2001). PNP could also increase uterine weight in ovariectomized rats, reduce the weight of male rats prostate and seminal vesicles (Li *et al*., 2006b, c) PNMC [3-methyl-4-Nitrophenol (4-Nitro-m-Cresol), PNMC], a PNP homologue reduced the concentration of rat plasma testosterone and adrenal cortex hormones, testicular withering of the quail which showed toxic effects on the reproductive system (Li *et al*., 2006a, 2007). These sresults suggested that the PNP could be used as an endocrine-disrupting agent which produced reproductive toxicity. However, whether PNP could directly affect mammalian spermatogenic functiont is still unclear. In this study, mice were used as experimental animals to analysis different concentrations of PNP on male reproductive function which lays the foundation for further study of PNP reproductive toxicity.

MATERIALS AND METHODS

Materials: PNP (purity>97%) was obtained from Shanghai Yuanye Chem Ltd. (Shanghai, China). All other reagents used in the analyses were of analytical grade and were obtained locally.

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Animals and experimental design: Kunbai male mice (outbred strain) weighing about 30-35 g were provided by a local veterinary research institute. All animal treatment procedures were approved by the Animal Care Committee of Southwest University. These animals were adapted to the laboratory conditions before experiments and were housed, according to the method of Wang *et al.* (2009). About 50 adult SPF Kunming male mice (25-30 g) were randomly divided into 5 groups (n = 10) including: control group; blank (salad oil) group, 0.1 mg kgwt^{-1}. DPNP group, 10 mg kgwt^{-1} dPNP group and 100 mg kgwt^{-1}. DPNP group, all animals were fed administration of 35 days.

Hematological biochemical analysis: Blood samples were taken from the eye sockets of mice under anesthesia using an 1 mL syringe before they were sacrificed (Wang *et al.*, 2009). Blood samples were centrifuged at 5,000 rpm for 4 min and the serum samples were stored at 4°C for hematological biochemical analysis. The activities of Aspartate Transaminase (AST), Alanine Transaminase (ALT) and Alkaline Phosphatase (ALP) were detected according to Manna *et al.* (2004) described the method using the automatic biochemical analyzer (Olympus AU400) were determined.

Sperm collection and analysis of sperm output: The procedure of sperm collection was performed according to Wang *et al.* (2009), sperm were collected by centrifugation with Saline Merthiolate Triton (SMT). The number of sperm was measured using a hemacytometer, according to the method of Elbetieha *et al.* (2001). Epididymal sperm counts were expressed as number of sperms per gram of epididymis. About 1000 sperm per each epididymis were assayed for viability and malformation. Sperm viability was assessed by eosin Y stain and the motility of sperm was assayed by the number of sperm which can move in line. The percent of viable sperm and the motility of sperm were calculated according to method of Wang *et al.* (2009). Sperm malformation rate was assayed by the motility of sperm and intact nature of the acrosome (Krzanowska *et al.*, 1997; Yildiz *et al.*, 2008). The integrity of the acrosome was assessed using Wright-Giemsa stain.

Ultrastructure of primary spermatocyte and histological structure of testes: Samples of testes were immersion-fixed in Bouin's solution for histopathology and embedded in paraffin. Serial sections (5 μm thick) were prepared from paraffin blocks, stained with haematoxylin and eosin, mounted with Dextran Plasticiser Xylene (DPX) and examined using light microscopy according to the method of Tae *et al.* (2005).

For ultrastructure analysis of primary spermatocyte, some testes samples were cut into 2 mm thick slices and fixed in ice-cold fixative consisting of 4% paraformaldehyde, 0.25% glutaraldehyde and 0.15 M Hepes-KOH buffer (pH 7.4) for 30 min. Samples were post-fixed in 2% osmium tetroxide, dehydrated and embedded in Araldite 502. Ultra-thin sections (70-90 nm thick) of the blocks were picked up on copper grids; sections were stained with uranyl acetate and lead citrate and were analyzed under TEM at 80 kV.

Statistical analysis: All data are reported as means SEM. Statistical analyses were performed using SPSS (Version 16.0; SPSS Inc, Chicago, IL, USA). All percentage data were subjected to arc-sine transformation before statistical analysis. Data were analyzed by one-way ANOVA and the Fisher's Least Significant Difference (LSD) method to determine treatment differences. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of PNP on the body weight of mice and androgen-sensitive organ weights: Different concentrations of PNP decreased body weight but 0.1 mg kgwt^{-1} PNP had not significant difference compared to control ($p>0.05$). All concentrations of PNP reduced the ratio of (testis+epididymis)/body weight, (prostate+seminal vesicle weight)/body weight were significantly reduced ($p<0.05$); 100 mg kgwt^{-1}, PNP had most obvious effect on these parameters compared to the control which reduced by 26.91, 55.73 and 44.27%, respectively (for all, $p<0.05$, Table 1).

Effect of PNP on the indicators of liver enzymes: PNP (0.1 mg kgwt^{-1}) increased the activity of Alanine Transaminase (ALT), Aspartate Transaminase (AST) and Alkaline Phosphatase (ALP) but Compared to control, differences was not significant ($p>0.05$, for all). With increasing concentrations of PNP, these activities in the blood continued to increase. PNP (100 mg kgwt^{-1}) increased the activity of ALT, AST by approximately 2.28, 1.25 and 1.22 times, respectively as compared

Table 1: Effect of PNP on mean (±SEM) body weight and the weight of testosterone-sensitive organs in mice (n = 10/group)

Groups	Body weight	(Testis and epididymidis) /body weight*1000	Seminal vesicles and rostate/body weight*1000
Control	38.36±1.38^{a}	5.24±0.90^{a}	6.89±1.46^{a}
Blank	36.10±2.71^{ab}	4.53±0.74^{ab}	6.60±0.93^{ab}
0.1 mg kgwt^{-1}	34.80±2.26^{ab}	3.94±0.49^{b}	5.28±0.31^{b}
10 mg kgwt^{-1}	32.56±2.09^{b}	3.54±0.29^{b}	5.26±0.48^{b}
100 mg kgwt^{-1}	27.00±1.00^{c}	2.32±0.57^{c}	3.84±1.46^{c}

Within a column, different letter represents significant difference ($p<0.05$)

to the control. Blank (peanut oil) had no effect on the activity of ALT, AST and ALP ($p>0.05$; Table 2).

Effect of PNP on hepatic synthetic function: PNP (0.1 mg kgwt^{-1}) decreased the level of serum Total Protein (TP) and ALBumin (ALB) but compared to control, differences was not significant ($p>0.05$, for all). With the increase of concentration of PNP, these activites in the blood continued to decrease. PNP (100 mg kgwt^{-1}) decreased the level of TP, ALB by approximately 36.23 and 34.07%, respectively as compared to the control. Blank (peanut oil) had no effect on the activity of ALT, AST and ALP ($p>0.05$; Table 3).

Effect of PNP on renal biochemical indicators: Compared to the control, different concentrations of PNP had no significant effect the concentration of UREA in the blood ($p>0.05$). PNP (0.1 and 10 mg kgwt^{-1}) significantly reduced the level of UA (uric acid) when PNP concentrationwas 100 mg kgwt^{-1}, UA levels reduced 19.15% compared to the control ($p<0.05$). When the concentration of PNP was 0.1 mg kgwt^{-1}, PNP improved the plasma concentration of CRE (creatinine) but the difference was not significant ($p>0.05$) with the increase concentration of PNP, concentration of CRE continues to rise ($p>0.05$) and PNP (100 mg kgwt^{-1}) increased the concentration of CRE by 0.99 times compared with the control ($p<0.05$). Blank (peanut oil) had no effect on the activity of UA and CRE ($p>0.05$; Table 4).

Table 2: Effect of PNP on liver enzymes (±SEM) in mice (n = 10/group)

Groups	ALT (u L^{-1})	AST (u L^{-1})	ALP (u L^{-1})
Control	7.49±2.05^{c}	25.95±2.830^{bc}	14.65±1.82^{c}
Blank	7.55±1.88^{c}	26.50±4.250^{bc}	15.24±3.92^{c}
0.1 mg kgwt^{-1}	7.60±1.60^{c}	30.94±6.680^{c}	22.56±3.97^{bc}
10 mg kgwt^{-1}	16.68±2.43^{b}	43.35±7.830^{b}	23.32±4.21^{b}
100 mg kgwt^{-1}	24.57±5.74^{a}	58.32±11.07^{a}	32.53±6.00^{a}

Table 3: Effect of PNP on hepatic synthetic function (±SEM) in mice (n = 10/group)

Groups	Tp (g L^{-1})	ALB (g L^{-1})
Control	14.96±2.16^{a}	8.13±1.57^{a}
Blank	14.83±2.49^{a}	8.11±1.20^{a}
0.1 mg kgwt^{-1}	14.29±1.82^{a}	7.73±1.26^{a}
10 mg kgwt^{-1}	12.70±0.62^{b}	7.37±0.94^{ab}
100 mg kgwt^{-1}	9.54±0.47^{c}	5.36±1.00^{b}

Within a column, different letter represents significant difference ($p<0.05$)

PNP negatively affects sperm output and quality: Compared to control, different concentrations of PNP decreased sperm count, viability and sperm motility, respectively. PNP (0.1 mg kgwt^{-1}) decreased parameters but compared to control, differences was not significant ($p>0.05$ for all). With the increase of concentration of PNP, sperm output and quality continued to reduce ($p<0.05$). PNP (100 mg kgwt^{-1}) sperm count, sperm motility, sperm rate of linear motion and intact acrosome rates decreased by 56.11, 49.78, 49.33 and 41.97% (for all $p<0.05$). Compared to control, blank (peanut oil) had no effect on sperm count, viability, sperm motility and intact acrosome rate ($p>0.05$; Table 5).

Effect of PNP on histological structure of testes: Testes from the control group were in various stages of spermatogenesis; Leydig cells were abundant in the interstitum (Fig. 1a). Blank (peanut oil) had no effect as compared to control (Fig. 1b). In the PNP (0.1 mg kgwt^{-1}) group, interstitial vein was congested but structures of spermatogenic cells, Leydig cells had be affected (Fig. 1c). In the PNP (10 mg kgwt^{-1}) group, shape of sperm cells in the seminiferous tubules changed from circular to rhombus (Fig. 1d). PNP (100 mg kgwt^{-1}) made spermatogonia vacuolization, increase the number of sperm cells in the seminiferous tubules from circular to rhombus and reduce the number of cells in the seminiferous tubules significantly (Fig. 1e).

Effect of the PNP on the ultrastructure of primary spermatocytes: In the control group, primary spermatocytes had normal Endoplasmic Reticulum (ER) and mitochondria profiles, cytoplasmic organelles was abundant, chromatin distribution was normal, the structure of chromatospherite and the boundary of the nuclear membrane were clear (Fig. 2a). Blank (peanut oil) had no effect on the ultrastructure of Leydig cells (Fig. 2b). In the PNP (0.1 mg kgwt^{-1}) group, cytoplasm of

Table 4: Effect of PNP on renal biochemical indicators (±SEM) in mice (n = 10/group)

Groups	Urea (mmol L^{-1})	UA 440 (umol L^{-1})	CRE (umol L^{-1})
Control	1.74±0.38^{a}	99.82±21.93^{a}	10.40±2.57^{b}
Blank	1.79±038^{a}	95.56±13.45^{a}	10.61±1.72^{b}
0.1 mg kgwt^{-1}	1.72±0.47^{a}	87.73±10.02^{b}	11.16±1.62^{b}
10 mg kgwt^{-1}	1.77±0.24^{a}	87.32±9.980^{b}	11.68±1.55^{b}
100 mg kgwt^{-1}	1.75±0.66^{a}	80.70±21.22^{c}	20.65±2.50^{a}

Within a column, different letter represents significant difference ($p<0.05$)

Table 5: Effect of PNP on sperm output and quality

Groups	Sperm count (108 g^{-1} epididymidis)	Viability (%)	Sperm motility (%)	Intact acrosome rate (%)
Control	6.22±0.35^{a}	84.35±4.93^{a}	68.37±6.45^{a}	84.98±7.38^{a}
Blank	6.00±0.38^{a}	83.65±5.25^{a}	69.37±7.24^{a}	81.47±6.95^{a}
0.1 mg kgwt^{-1}	5.80±0.41^{a}	79.54±4.87^{a}	65.58±6.34^{a}	79.36±7.21^{a}
10 mg kgwt^{-1}	4.98±0.46^{b}	68.36±3.94^{b}	54.36±5.88^{b}	72.15±6.98^{b}
100 mg kgwt^{-1}	2.73±0.26^{c}	42.36±2.46^{c}	34.64±4.97^{c}	49.31±5.25^{c}

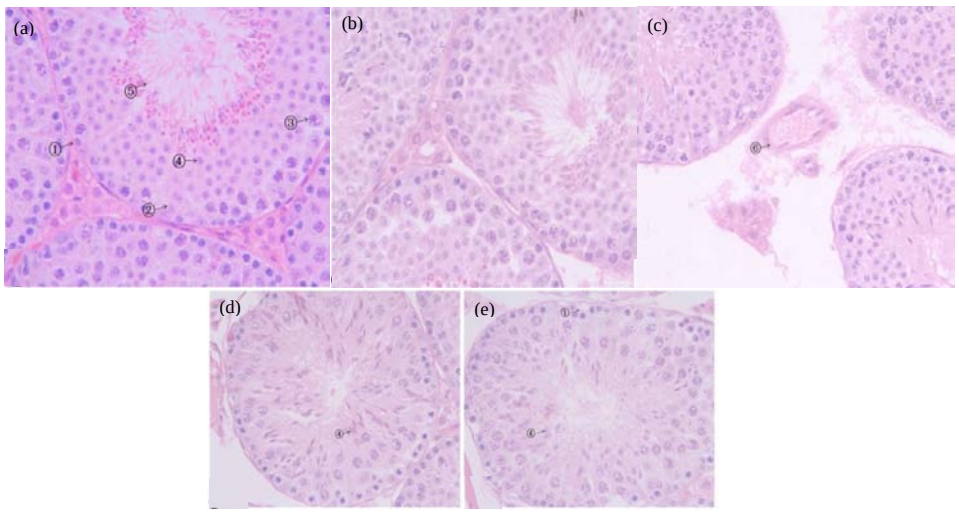

Fig. 1: Effect of PNP on histological structure of testes (×400): a) control; b) blank; c) 0.1 mg kgwt^{-1}; d) 10 mg kgwt^{-1}; e) 100 mg kgwt^{-1}; 1) Spermatogonium; 2) Sertoli cell; 3) Primary spermatocytes; 4) Sperm cell; 5) Sperm; 6) Vein

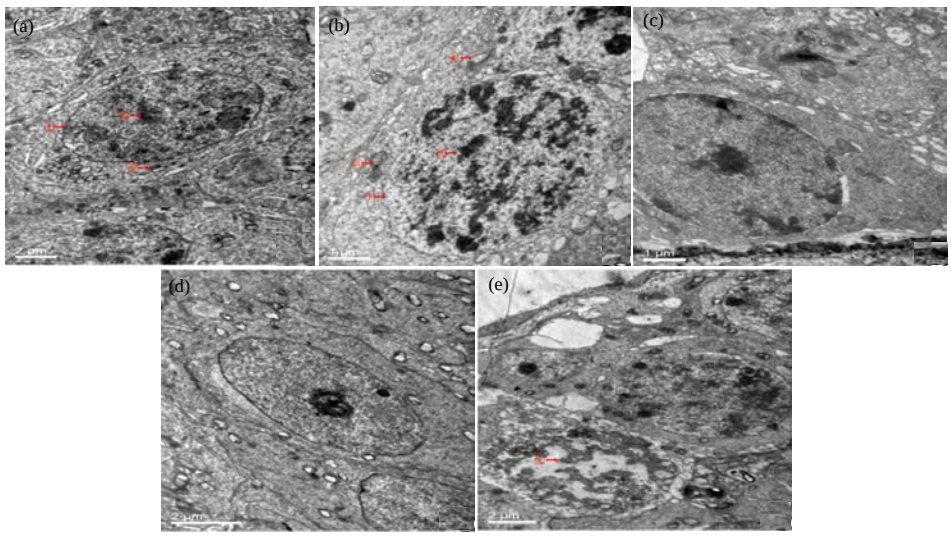

Fig. 2: Effect of of the PNP on the ultrastructure of primary spermatocytes: a) control (×6000); b) blank (×12000); c) 0.1 mg kgwt^{-1} (×12000); d) 0 mg kgwt^{-1} (×10000); e) 100 mg kgwt^{-1} (×7000); 1) Nuclaer; 2) Chromatin; 3) Mitochondria; 4) Endoplasmic reticulum

primary spermatocytes was reduced and the number of mitochondria endoplasmic reticulum were reduced (Fig. 2c). In the PNP (10 mg kgwt^{-1}) group, cytoplasm of primary spermatocytes and the number of mitochondria endoplasmic reticulum continues to be reduced and the vacuole could be found in the cytoplasm (Fig. 2d). PNP (100 mg kgwt^{-1}) made chromatin arrangement confusion in the nucleus, reduced interstitial space in chromatin and increased organelle fragmentation, vacuoles in the cells (Fig. 2e).

DISCUSSION

In this study, different concentrations of PNP had an effect on the body weight of mice and androgen-sensitive organs, semen quality and with the increase of the concentration of PNP, the effect was the stronger ($p<0.05$). Low concentration of PNP had no significant on mouse liver and kidney function ($p>0.05$). With the increase of concentration of PNP, detoxification and synthetic functions of liver and kidney function has been

impacted significantly. In addition, the study also found that different concentrations of PNP affected the testicular structure and ultra structure of primary spermatocytes. With the increase of concentrations of PNP, the damage to testicular structure and ultrastructure of primary spermatocytes had been enhanced. PNP (100 mg $kgwt^{-1}$) significantly undermined testicular structure and ultrastructure of primary spermatocytes.

Sakai-Kato *et al.* (2004) found that PNP could reduce liver detoxification function by inhibiting liver uridine diphosphate glucuronic acid transferase (Uridine diphosphate Glucuronosyl Transferase; UGT) activity. Mi *et al.* (2010) also found that the PNP can increase the MDA (malondialdehyde) levels, lower GSH-Px and SOD activities and the antioxidant suppressed this effect. This experiment found that diffence doses of PNP increased Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) enzyme activity in liver while reducing the level of serum Total Protein (TP), Albumin (ALB) and affecting kidney function which suggested that high concentrations of PNP slows down non-toxic conversion rate so that the PNP could damage the function of other organs of the body. This phenomenon may be due to the fact that PNP play a role in the liver detoxification when liver cells are damaged, the stability of the cell membrane was damaged, ALT, AST and ALP released into the blood, increase the activity of these enzymes in the serum, kidney UA and the increase of the CRE. On the other hand, researchers also found that the synthetic function of liver cells decreased and the growth of mice have been affected. These results inferred the ability of depredating PNP in the liver declined and other organs functions were impacted (Mi *et al.*, 2010).

Different concentrations of PNP reduced the weight of the body weight and androgen-sensitive organs and this role is enhanced with increasing PNP concentration. Li found that PNP could increase uterine weight in ovariectomized rats and reduce male the weight of the rat prostate and seminal vesicles (Toda *et al.*, 2001) consistent with the results. Here, 1, 10 or 100 mg $kgwt^{-1}$ PNMC muscle injected into the hen's body, 5 days later, body weight, egg weight and hatching rate were not significantly affected but the 100 mg $kgwt^{-1}$, PNMC make plasma LH, estradiol, progesterone levels rise (Li *et al.*, 2008) and found PNMC not only reduced the concentration of plasma testosterone and adrenal hormones in rats (Li *et al.*, 2007), Leydig cells *in vitro* and could reduce the time and dose-dependent manner the ability to secrete testosterone (Li *et al.*, 2006a) the effect of PNP on body weight and androgen-sensitive organ could result from reducing the ability of the testicular synthesis of testosterone results.

PNP reduced not only significantly the weight of male mice and androgen-sensitive organ weights and also reduced total sperm count, sperm viability, sperm motility and acrosome integrity and the PNP damaged seminiferous fine tube structure causing the ultrastructural changes of the primary spermatocytes suggesting that PNP may directly affect sperm production. Mi *et al.* (2010) found that PNP could make embryonic chicken testicular cells to produce nuclear condensation, cytoplasmic vacuolization and reduce the number of spermatogenic cells, consistent with this role of the PNP. PNP as a small molecular, could pass through the blood-testis barrier and affected the primary spermatocytes development but the specific mechanism needs further study.

CONCLUSION

This study shows that PNP affeced the liver and kidney function so that the body's detoxification capacity decreased and produced damage to sperm production and thus decreased reproductive capacity in mice.

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