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3-Monochloropropane-1, 2-diol causes irreversible damage to reproductive ability independent of hormone changes in adult male rats

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ABSTRACT

3-MCPD, a contaminant frequently detected in foodstuffs, has been reported to damage human kidneys and testes. Previous studies can be used to evaluate the risk to humans of exposure to excessive 3-MCPD for a short period. However, the effects of withdrawal after 3-MCPD exposure have rarely been studied. Adult male SD rats were orally administered 0, 36 and 72 mg 3-MCPD/kg b.w./day for 4 weeks, followed by a 7-week recovery period. 3-MCPD significantly reduced RBC, HGB and HCT levels, indicating a phenotype of anemia, which returned to normal after the recovery period. 3-MCPD induced dysfunction in the liver and kidneys, which were characterized by hepatomegaly and elevated serum ALT, TBIL levels, and nephromegaly and elevated serum urea, UA contents. These effects were also restored to normal after the recovery period. Although the abnormal levels of testosterone and progesterone returned to normal, 3-MCPD-induced atrophy in testes, decreased sperm concentration and motility, and an increased rate of teratosperm still existed after the recovery period. 3-MCPD can induce restorable anemia and dysfunction in liver and kidney but irreversibly damage the reproductive system with normal sex hormone levels. This study may provide a novel perspective for characterizing the ongoing risk of exposure to 3-MCPD.

1. Introduction

3-Monochloro-1, 2-propanediol (3-MCPD) is a common member of a group of food processing contaminants, the chloropropanols, which has been frequently found in foods in its free form (Baer et al., 2010; Jedrkiewicz et al., 2016), in its ester form with different fatty acids, or in both forms (Bakhiya et al., 2011; Habermeyer et al., 2011). The free form of 3-MCPD has mainly been found in acid-hydrolyzed vegetable protein soy sauce, according to data collected in a survey of 2035 food samples from 10 countries, with levels of 3-MCPD up to 1779 mg/kg (Lee and Khor, 2015). In many other foodstuffs, such as cereal products, coffee, baby formula, edible oil and fats, 3-MCPD has most commonly been found in the form of mono- or di-esters with fatty acids (Bakhiya et al., 2011; Habermeyer et al., 2011; Schilter et al., 2011). 3-MCPD esters can be rapidly hydrolyzed to the free form of 3-MCPD in the gastro-intestinal tract (Abraham et al., 2013). According to the scientific opinion of the European Food Safety Authority (2016), the highest levels of 3-MCPD from esters (> 5210 μg/kg) were found in palm oil and fat, but most vegetable oils and fats contained substantial quantities. These surveys have shown that consumers may occasionally be exposed to high levels of 3-MCPD for a short time.

3-MCPD can cross the blood-testis and blood-brain barriers to become widely distributed in a variety of organs (Yamada et al., 2010), causing adverse effects such as hepatotoxicity, nephrotoxicity, and male reproductive toxicity (Lynch et al., 1998; Lee and Khor, 2015; Lee et al., 2015). It has been found that exposure to 37 mg/kg b.w. of 3-MCPD (ca. 1/4 of the median lethal dose, LD_{50}) led to nephromegaly (JECFA, 2002; Cho et al., 2008b), hepatomegaly and acute renal tubular necrosis (Onami et al., 2014). When the exposure dose was increased to 100 mg/kg b.w. (ca. 2/3 LD_{50}), 3-MCPD also caused glomerular nephritis (Jones et al., 1978) and renal dysfunction with proteinuria and glucosuria (Morris and Williams, 1980). Exposure to 3-MCPD at a dose of 36.97 mg/kg b.w. (ca. 1/4 LD_{50}) also reduced sperm concentration and motility, and caused germinal epithelium degeneration (Cho et al.,

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2008b). These results were obtained at high doses in a range of 1/4–2/3 LD_{50}. However, it would be impossible for a host to be continually exposed to such a high dose. Therefore, it is necessary to understand the effects of 3-MCPD when the stimulus has been withdrawn after an acute exposure. Therefore, the present study aims to evaluate the toxic effects of 3-MCPD on male Sprague–Dawley (SD) rats under acute exposure conditions and after the withdrawal of the stimulus. The results will be useful for gaining a better understanding of the toxicity risk characteristics of 3-MCPD.

2. Materials and methods

2.1. Chemicals and reagents

3-MCPD (3-chloro-1, 2-propanediol, purity > 99%, Cas No. 96-24-02) was purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). The stock solution was prepared every two weeks by dissolving 3-MCPD in distilled water at a concentration of 100 g/L then further diluted to a suitable concentration with distilled water for gavage every day. The solution was stored in glass vessels at 4 °C and protected from the light.

2.2. Animal husbandry and maintenance

Sixty 6-week-old male SD rats were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The animals were housed in polycarbonate cages (four per cage) with soft wood shavings for bedding in a specific pathogen-free room, controlled at a temperature of 24 ± 2 °C and a relative humidity of 55 ± 5% with lighting on a 12-h light/dark cycle. All animals were fed rat chow and water *ad libitum*. The animal experiments were performed humanely according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of China Agricultural University (No. CAU20170109-3).

2.3. Experimental design

After one week of acclimation, 60 rats with a body weight of about 225 g were randomly divided into 3 groups as follows: i) control group, ii) low dose group and iii) high dose group. The doses were set at 36 (ca. 1/4 LD_{50}, low dose group) and 72 mg/kg b.w. (ca. 1/2 LD_{50}, high dose group), according to the LD_{50} value for 3-MCPD (152 mg/kg b.w.) for rats (Ericsson and Baker, 1970). The rats were administered 3-MCPD by gavage for 4 weeks, at a volume of 0.5 mL per 100 g b.w. of rats. The control group rats were given the same volume of ultrapure water. After 4-weeks of exposure, 12 animals per group were sacrificed. To investigate the reversibility or possible delayed toxic effects of 3-MCPD, the remaining eight rats per group underwent a recovery period then were sacrificed. The recovery period lasted seven weeks after 3-MCPD exposure had ceased (Fig. 1). The rats were sacrificed by cervical dislocation after being anesthetized by ethyl ether inhalation. The blood was collected from the orbital sinus for hematological and biochemical tests. The liver, kidneys (paired), spleen, testes (paired) and epididymis (paired) were carefully dissected out then weighed to calculate the relative organ weight using the following formula:

Relative organ weight (%) = [organ weight / final body weight] × 100

2.4. Hematological examination

Blood samples were collected from the orbital sinus of all the rats under diethyl ether for hematology. The white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT) and mean platelet volume (MPV) were analyzed using an RT-7200 automatic hematology analyzer (Rayto Life and Analytical Sciences Co. Ltd., Shenzhen, China).

2.5. Serum biochemical and hormone examination

The blood samples were centrifuged at 1200 × *g* for 20 min at 4 °C. The collected serum was stored at −80 °C for further blood biochemistry examinations. The blood urea nitrogen (BUN), uric acid (UA), creatinine (CREA), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL) and direct bilirubin (DBIL) levels were measured using a fully automatic biochemical analyzer (BS-420, Mindray Co., Shenzhen, China). The serum testosterone and progesterone levels were assayed by radioimmunoassay (Beijing Sino-UK Institute of Biological Technology, Beijing, China) according to the manufacturer's instructions.

2.6. Sperm concentration, motility and rate of teratosperm

The sperm count and motility of the sperm samples were determined by computer-assisted sperm analysis (CASA) according to a slightly modified procedure reported previously (Zhou et al., 2008). Briefly, once the flesh epididymis had been isolated from the testes, it was immediately transferred to a clean dish with 5 mL Gibco M199 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 3% bovine serum albumin at 37 °C. Subsequently, five deep cuts were made in the epididymis to allow the spermatozoa to diffuse into the medium for 10 min. Ten 10 μL of the sperm suspension were then placed into a sample sink for analysis. For the motility and sperm count analysis, a total of approximately 400 spermatozoa were analyzed in each sample, and thirty frames were acquired at a frame rate of 60 Hz. During analysis, the playback feature was used to check

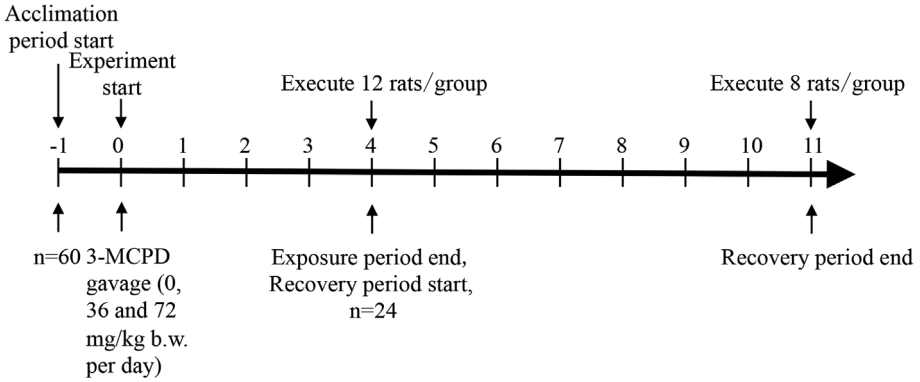

Fig. 1. Experimental scheme. After one-week of acclimation, adult male SD rats were divided into 3 groups then treated with 0 (control), 36 (low), 72 (high) mg 3-MCPD/kg b.w. for a 4-week exposure period followed by a 7-week recovery period.

its accuracy. Twenty visual fields for each sample were randomly selected for the calculation of crude sperm density using TOX IVOS software V.12.0 (Hamilton Thorne Inc., Danvers, MA, USA). The rate of teratosperm was calculated using the following formula:

Rate of teratosperm (%) = [the counts of teratosperm / total counts] × 100

2.7. Histopathology, morphometry and immunofluorescence

After macroscopic study, the organs (liver, kidneys, spleen, testes and epididymis) from each animal were fixed in a 10% buffered formalin solution. The prepared sections were processed in paraffin wax, sectioned then stained with hematoxylin-eosin (HE) for histopathological observations. For quantifying spermatogenesis, the diameter and epithelial height of the seminiferous tubules were evaluated according to Qiu et al. (Qiu et al., 2013). Briefly, 20 randomly-selected round seminiferous tubules in each cross-section were measured using an optical microscope. Each tubule was measured twice in two perpendicular directions. For the same tubule, the height of the seminiferous epithelium was measured from the membrane base to the tubular lumen in two different regions.

For immunofluorescence, sections were washed with PBS three times then blocked with blocking buffer (1% BSA, 0.1% Triton, 1 × PBS) at 37 °C for 30 min. The sections were then incubated with the primary anti-SOX9 antibody (Abcam, Cambridge, UK) at 4 °C overnight. After rinsing with PBS, a secondary antibody (Beyotime, Shanghai, China) was applied for 1 h at 37 °C. The sections were then washed with PBS and counterstained for nuclei using 4,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) following the manufacturer's instructions.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation. One-way analysis of variance followed by Dunnett's multiple test was used to compare mean values from the high or low dose group with the control group using IBM SPSS Statistics software (Version 21, IBM Corp., Armonk, NY, USA). Differences between mean values were considered significant if $p \leq 0.05$.

3. Results

3.1. 3-MCPD induced bodyweight loss in adult male rats

The variation in the bodyweight and bodyweight gain of the rats during the experiment is shown in Fig. 2. Exposure to 3-MCPD led to lower increases in the bodyweight of rats compared with those of the control group, especially for the high dose group (Fig. 2A) ($p < 0.05$). The body weight gains were found to be lower in 3-MCPD treatment groups during the exposure period, and recovered to a similar level as the controls during the recovery period when 3-MCPD had been withdrawn (Fig. 2B).

3.2. 3-MCPD induced restorable anemia

The effects of 3-MCPD on the hematological parameters are shown in Table 1. 3-MCPD significantly decreased the levels of RBC, HGB and HCT ($p < 0.05$), indicating an anemia phenotype but left the levels of WBC, MCV and MCH unchanged ($p > 0.05$). However, RBC, HGB and HCT returned to normal levels after the 7-week recovery period.

3.3. 3-MCPD induced restorable injury in liver and kidney

The relative organ weights for the liver and kidneys, histological

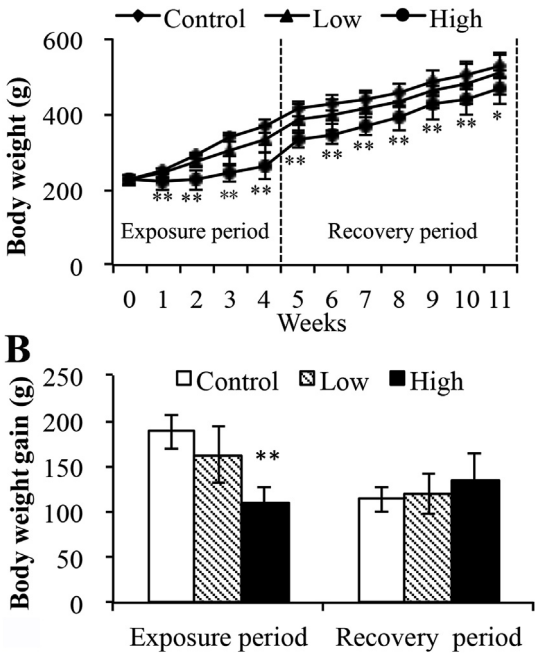

Fig. 2. The mean (A) bodyweights and (B) bodyweight gains of SD rats during the experiment with a 4-week exposure to 3-MCPD followed by a 7-week recovery period. Data are presented as means ± SD (n = 8–12). * and ** indicate significant differences from the control group at $p < 0.05$ and $p < 0.01$, respectively.

morphology and the corresponding serum biochemistry indices in rats exposed to 3-MCPD for 4 weeks followed by recovery for 7 weeks are shown in Fig. 3. 3-MCPD significantly increased the relative weights of the liver (Fig. 3A) and kidneys (Fig. 3B), which then returned to the control levels after the recovery period. Although 3-MCPD did not induce significant changes in CREA (Supplementary Fig. S1) and the histomorphology of kidney, such as necrosis or mononuclear cell aggregation (Supplementary Fig. S1), significant increases in urea (Fig. 3C) and UA (Fig. 3D) contents were found in rats treated with a high dose of 3-MCPD. As with the changes in weight, the content of urea and UA in the serum decreased to the control levels after the 7-week recovery period (Fig. 3C–D). Hypertrophy of the centrilobular hepatocyte was also observed in the liver of 3-MCPD-treated rats (Fig. 3E–G), especially in the high dose group (Fig. 3G). There was no obvious change in the levels of serum ALP and DBIL (Supplementary Fig. S1), but a significant increase in levels of ALT (Fig. 3K) and TBIL (Fig. 3L). These changes in serum biochemicals returned to normal after the 7-week recovery period. Similarly, the injuries induced in the liver by 3-MCPD disappeared (Fig. 3H–J).

3.4. 3-MCPD induced irreversible damage in reproductive system and the toxicity was independent of sex hormones

The relative weight of the testes and epididymis, the total sperm concentration, the rate of motile sperm, progressive sperm, and the teratosperm in semen extracted from the cauda epididymis are shown in Fig. 4. 3-MCPD induced a significant decrease in the weight of the testes (Fig. 4A) and epididymis (Fig. 4B), and in the total sperm concentration (Fig. 4C) at the high dose of 3-MCPD ($p < 0.01$). Significant decreases in the rate of motile sperm (Fig. 4D), progressive sperm (Fig. 4E) and a significant increase in the rate of teratosperm (Fig. 4F) were found in male rats exposed to both doses of 3-MCPD for 4 weeks ($p < 0.01$). After recovery for 7 weeks, the reproductive toxicity of 3-MCPD did not disappear or lessen: instead, the damage became more severe, especially the effects on sperm (Fig. 4A–F).

The 4-week exposure to 3-MCPD also led to a significant decrease in

Table 1
Effect of 3-MCPD exposure for 4 weeks followed by withdrawal and a 7-week recovery period on the hematological parameters of male SD rats.

	Exposure period			Recovery period		
	Control	Low	High	Control	Low	High
WBC (109/L)	7.5 ± 1.5	9.9 ± 4.7	8.2 ± 3.3	9.3 ± 1.8	8.4 ± 1.9	9.1 ± 1.6
RBC (1012/L)	6.27 ± 0.54	5.51 ± 0.54**	5.46 ± 0.6**	6.81 ± 0.37	6.36 ± 0.45	6.77 ± 0.51
HGB (g/L)	138.2 ± 5.8	120.1 ± 8.3**	121.6 ± 7.5**	136 ± 5	129.8 ± 11.2	133.5 ± 9.2
HCT %	39 ± 3.4	33.9 ± 2.9**	34.3 ± 3.4**	40.1 ± 1.9	38.2 ± 3.4	40.2 ± 3.1
MCV (fL)	62.3 ± 1.6	61.6 ± 2.2	62.9 ± 1.6	59 ± 0.9	60 ± 2.8	59.4 ± 1.2
MCH (pg)	22.2 ± 1.5	21.9 ± 1.4	22.4 ± 1.8	20 ± 0.6	20.36 ± 0.96	19.75 ± 0.45

Note: Data are presented as means ± SD (n = 8–12). * and ** indicate a significant difference from the control group at $p < 0.05$ and $p < 0.01$, respectively.

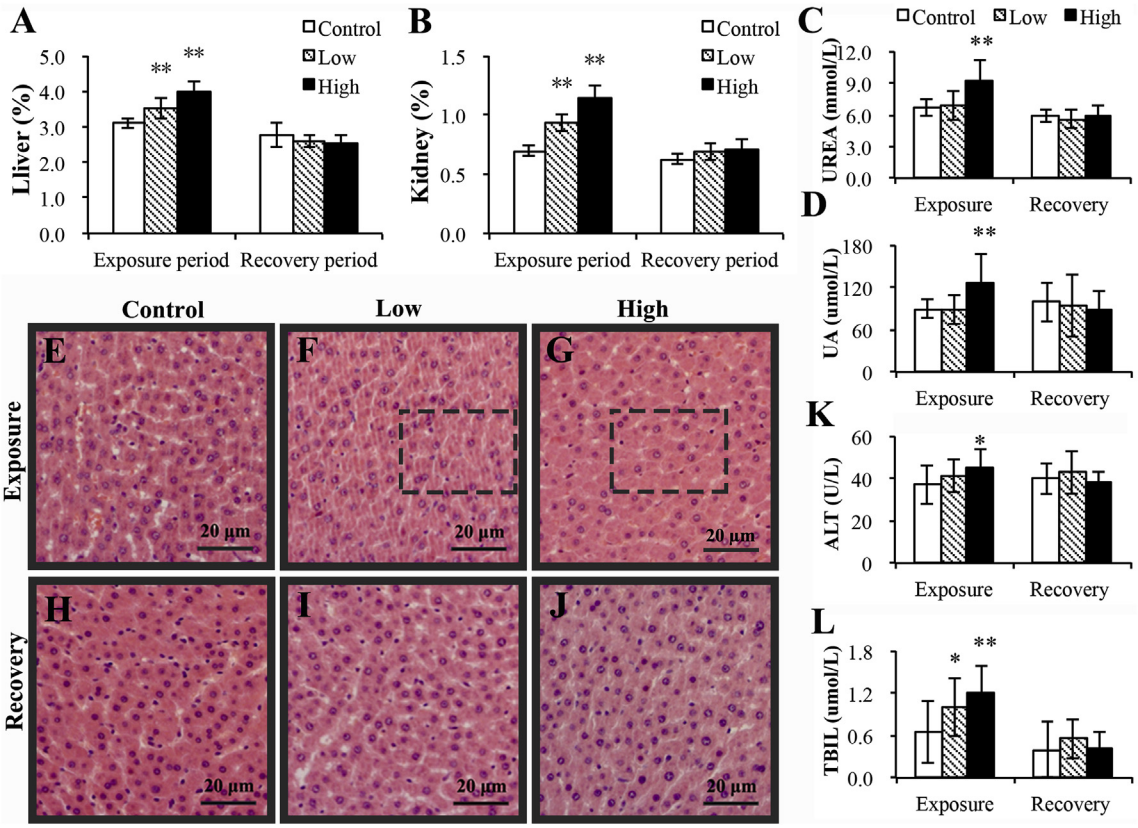

Fig. 3. Effect of 3-MCPD on: the relative weight of (A) liver and (B) kidney, (C, D) serum biochemistry index of kidney function, (E–J) histomorphology of liver, and (K, L) serum biochemistry index of liver function in male SD rats treated for a 4-week exposure period followed by a 7-week recovery period. The rectangle in the histomorphology indicates the hypertrophy hepatocyte. Data are presented as means ± SD (n = 8–12). * and ** indicate significant differences from the control group at $p < 0.05$ and $p < 0.01$, respectively.

the serum testosterone level ($p < 0.01$, Fig. 5A) and a significant increase in the content of progesterone ($p < 0.05$, Fig. 5B). 3-MCPD did not affect the level of gonadotropin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) ($p > 0.05$, Supplementary Fig. S2). Unlike its effects on the testes, epididymis and sperm, after the 7-week recovery, both hormones returned to the control levels ($p > 0.05$, Fig. 5A–B). The HE staining (Fig. 5C–H) showed that 3-MCPD induced germinal epithelial atrophy in the testes with vacuolation and many fewer germ cells in the seminiferous tubules of rats exposed to 3-MCPD (Fig. 5D–E). Immunofluorescence suggested no reduction in the number of $SOX9^+$ Sertoli cells (Fig. 5I–K) for both doses of 3-MCPD. The calculations of the diameter of the seminiferous tubule (Fig. 5L) and the epithelial height (Fig. 5M) from the pathological sections from each group showed that 3-MCPD significantly decreased their values ($p < 0.01$, Fig. 5I–J). Overall, these results suggested that 3-MCPD led to the atrophy of the testes (Fig. 5D–E), which did not recover after its withdrawal (Fig. 5G–H).

4. Discussion

In the present study, adult male SD rats were exposed to 3-MCPD at doses of 36 and 72 mg/kg b.w., corresponding to approximately 1/4 and 1/2 of LD_{50}, respectively. The effects of 3-MCPD on the bodyweight, hematology indices, structure and functions of the liver (ALT, TBIL), kidney (urea, UA) and reproductive system (testes, epididymis, sperm) were evaluated. To investigate whether the damage caused by 3-MCPD might be alleviated or restored after ceasing exposure to 3-MCPD, the same measurements were carried out on rats exposed to 3-MCPD after a recovery period of 7 weeks. After considering the significant toxicity to the reproductive system, we chose 7 weeks as the recovery period, which has been reported to be long enough for mature spermatozoa to form from germline stem cells because the total cycle of rat spermatogenesis lasts between 48 and 53 days (Hess, 1990).

The significant decreases in RBC, HGB and HCT levels (Table 1) indicated that 3-MCPD had induced anemia (Uthman, 2009) Other

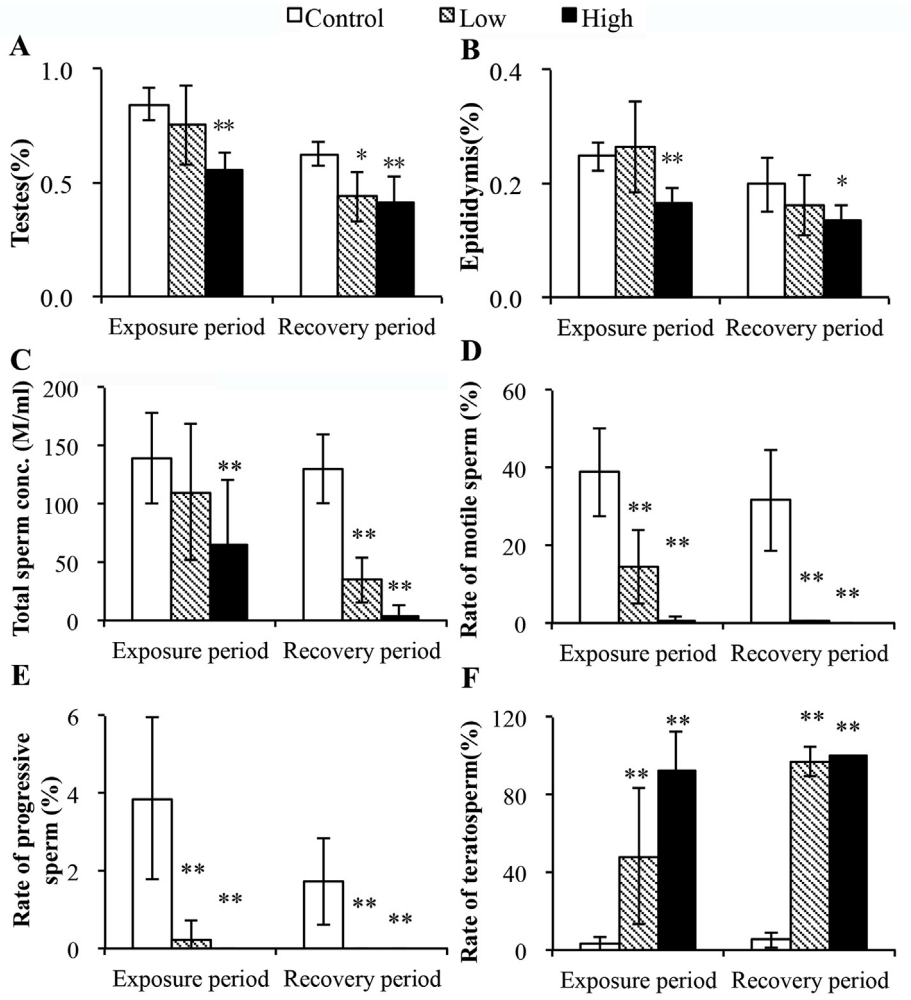

Fig. 4. The relative weight of (A) testes and (B) epididymis; (C) total sperm concentration; and the rates of (D) motile sperm, (E) progressive sperm and |(F) teratosperm in male SD rats exposed to 0 (control), 36 (low), 72 (high) mg 3-MCPD/kg b.w. for 4 weeks followed by a 7-week recovery period. Data are presented as means ± SD (n = 8–12). * and ** indicate significant differences from the control group at $p < 0.05$ and $p < 0.01$, respectively.

studies have also verified this anemia-inducing effect of 3-MCPD and 3-MCPD esters (Kirton et al., 1970). It was found that the anemia was normochromic indicated by the unchanged MCH level possibly associated with the splenomegaly induced by 3-MCPD (a significant increase in spleen weight, Supplementary Fig. S3). Splenomegaly is the characteristic of hypersplenism, which may be a cause of normochromic anemia (Brill and Baumgardner, 2000), or secondary to anemia (Lv et al., 2016). This may also be the reason for the recovery 7 weeks after the withdrawal of 3-MCPD.

There are well-documented studies on the toxic effect of 3-MCPD on the liver and kidney (Cho et al., 2008a; Barocelli et al., 2011; Lee et al., 2015). In the present study, 3-MCPD significantly increased the relative weight of the liver (Fig. 3A) and induced obvious hypertrophy in the centrilobular hepatocytes (Fig. 3F–G) indicating damage to the structure of the liver (Sellers et al., 2007). This histological results revealed that the hepatomegaly (indicated by the significant increase in relative liver weight) was most likely to have resulted from hepatocellular hypertrophy, not from other alternative causes such as hyperplasia, fibrosis and neoplasia (Hall et al., 2012). The increased ALT and TBIL levels in the serum (Fig. 3K–L), as unspecific markers for hepatocyte injury, also revealed liver damage (Haschek and Rousseaux, 2013), and when the stimulus was withdrawn, both ALT and TBIL levels returned to their normal levels and the liver recovered from the toxic effect of 3-MCPD. 3-MCPD also induced a significant increase in kidney weight (Fig. 3B), although there were no significant histopathological changes (Supplementary Fig. S1). Like the increased levels of urea and UA, the toxic effect of 3-MCPD on the kidneys agreed with previous studies (Jeong et al., 2010). Although the kidney was one of the targeted organs as well as the reproductive system (Jedrkiewicz et al., 2016), the present study found only a mild and reversible injury. Severe renal toxicity induced by 3-MCPD has been found in studies using an even higher dose (Jones et al., 1978; Morris and Williams, 1980) or exposed to 3-MCPD for a longer period (Cho et al., 2008b).

3-MPCD has been reported to reduce male fertility in several mammalian species, such as rats, hamsters, guinea-pigs, dogs, rams, and rhesus monkeys (Jones, 1983). Previous studies on the mechanism of the reproductive toxicity of 3-MCPD have mainly focused on the decrease in the motility of the sperm (Kwack et al., 2004; Cho et al., 2008b) caused by inhibiting the production of energy (Miki et al., 2004). In the present study, it was found that 3-MCPD not only inhibited sperm motility (Fig. 4D) but also dramatically decreased the sperm counts (Fig. 4C) and increased the rate of teratosperm (Fig. 4F). There was an obvious decrease in the number of cells in the seminiferous tubules (Fig. 5D–E, 5G-5H). We further measured Sertoli cell numbers by immunofluorescence analysis for SOX-9, a Sertoli cell-specific transcript in seminiferous tubules (Kilcoyne et al., 2014; Rebourcet et al., 2018). Considering the unchanged SOX-9$^+$ Sertoli cells analyzed by immunofluorescence (Fig. 5I–K), those cells lost were mainly spermatogenic. 3-MCPD has also been found to induce the failure of spermatogenesis in rats (Dixit and Agrawal, 1980) and fruit

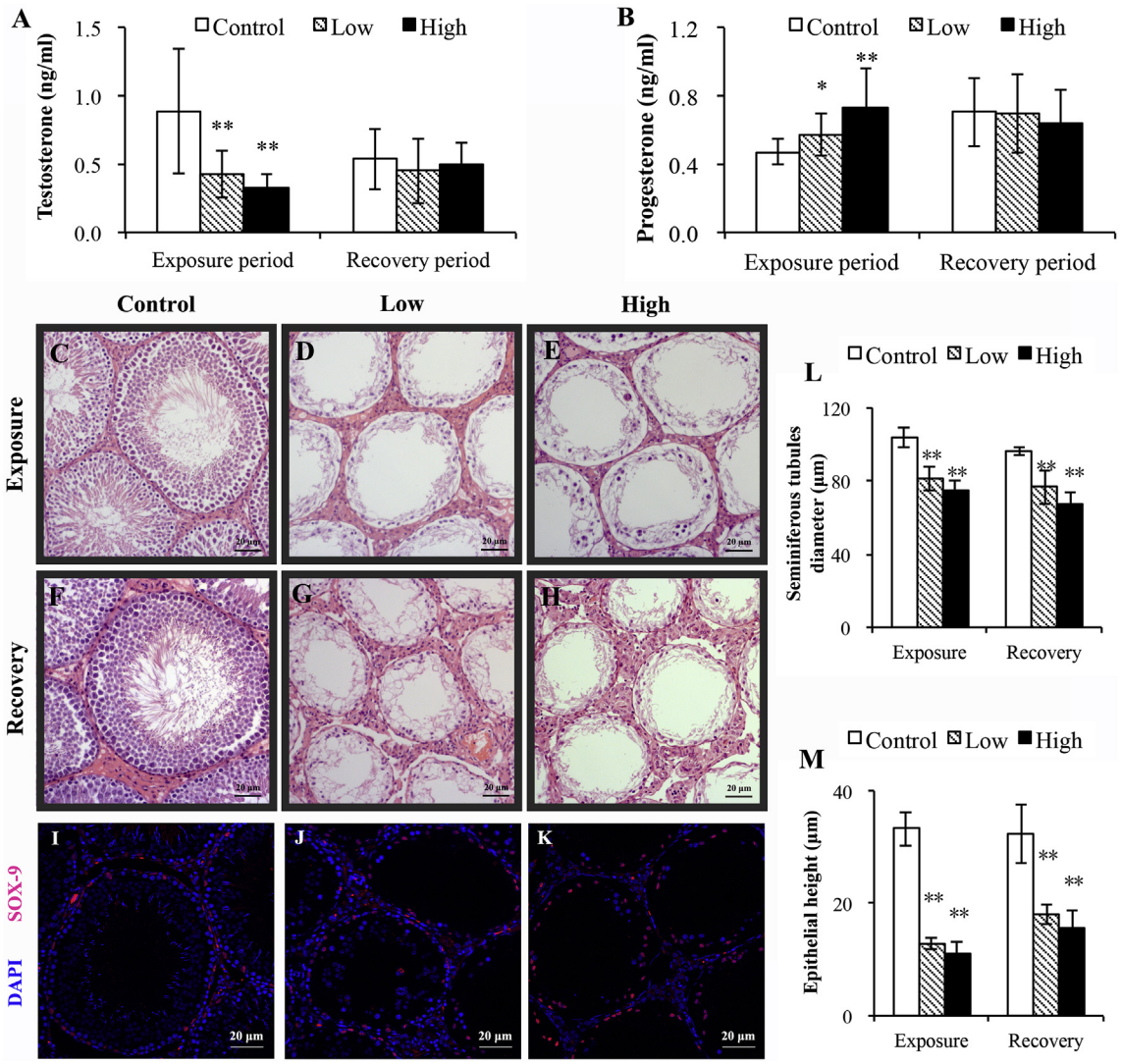

Fig. 5. Effects of 3-MCPD on levels of (A) testosterone, and (B) progesterone in the serum; and on (C–H) the histomorphology; (I–K) the immunofluorescence for SOX-9 positive cells; and (L, M) the morphometry of the testes. The male SD rats were exposed to 0 (control), 36 (low) and 72 (high) mg 3-MCPD/kg b.w. for 4 weeks followed by a 7-week recovery period. Data are presented as means ± SD (n = 8–12). * and ** indicate significant differences from the control group at $p < 0.05$ and $p < 0.01$, respectively.

bats (Mahmoud et al., 2018). This could explain the irrecoverable effects of 3-MCPD on sperm count (Fig. 4C), and the rates of motile sperm (Fig. 4D), progressive sperm (Fig. 4E) and teratosperm (Fig. 4F), even in rats which had recovered for a time sufficient to complete a new spermatogenic cycle. Although testosterone is critical for the ability to reproduce (O'Shaughnessy, 2014), studies on how 3-MCPD decreased serum levels of testosterone have been contradictory (Kwack et al., 2004; El Ramy et al., 2006; Sun et al., 2013). Kwack et al. (2004) found that 3-MCPD significantly reduced sperm motility but did not change the content of hormones both in the blood and testes. As discussed earlier, the impairment of male fertility induced by 3-MPCD was irreversible, and this may be concerned more with spermatogenesis than with hormone levels.

5. Conclusions

3-MCPD-induced normochromic anemia and dysfunctions in the liver and kidney of SD rats, as well as abnormalities in reproductive hormone levels can all be restored after removing the exposure to 3-MCPD. However, 3-MCPD induced damage in the morphology of the testes, which irreversibly decreased the number and abnormality of sperm. Further studies of the effect of 3-MCPD on spermatogenesis may clarify the real mechanism of the reproductive toxicity of 3-MCPD in SD rats.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.023.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.023.

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