Reproductive Effects of 3-Monochloropropane-1, 2-diol on Mice Sperm Function and Early Embryonic Development *In Vitro*

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Abstract 3-Monochloropropane-1, 2-diol (3-MCPD) is a well-known chloropropanol that is synthesized during food processing contamination. While evaluating sperm quality, sperm penetration into oocytes and early embryonic development, the present study investigated the effects of 3-MCPD on micereproduction *in vitro*. During their *in vitro* fertilization (IVF) process, zygotes and 2-cell embryos were incubated with 3-MCPD-supplemented medium until 8-cell embryo formation to evaluate the reproductive toxicity of 3-MCPD. Our data showed that compared with the control group, the fertilization rate and cleavage rate remarkably decreased in groups treated with 1.802 and 3.160 mM 3-MCPD. The percentage of 2-cell embryo and 4-cell embryo formation also significantly decreased ($P < 0.05$). Moreover, 8-cell embryos were not observed in any of the treated groups. Taken together, these results suggest that 3-MCPD exposure has hazardous effects on the micesperm fertilization ability as well as embryonic development.

Keywords: 3-Monochloropropane-1, 2-diol; mouse sperm function; early embryonic development

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1. Introduction

3-MCPD, which belongs to a group of compounds known as chloropropanol, is a well-known food-processing contaminant. It was first identified in soy sauce prepared using acid-hydrolyzed vegetable proteins (acid-HVP) [1,2] and has also been detected in various heat-processed foods [3,4]. Over the past few years, free 3-MCPD has been found to be released from 3-MCPD esters in the gastrointestinal tract [5]. The 3-MCPD esters presented in refined oil at a concentration of 0.3-10 mg/kg was significantly higher compared to those present in soy sauce [6,7,8,9].

In addition, 3-MCPD can cross the blood-brain barrier and blood-testis barrier, which results in a wide distribution of 3-MCPD in body fluids [10]. A renal tubule hyperplasia study determined the contaminant's lowest observed adverse effect level at 1.1 mg/kg body weight per day [11]. This finding was based on a maximum tolerable daily intake of 3-MCPD, which was recommended at 2 µg/kg body weight by the Scientific Committee on Food of the European Union.

3-MCPD has also been reported to exhibit reproductive toxicity in rats [12], monkeys [13] cats and cheetahs [14].

Low dose exposure to 3-MCPD has been reported to cause temporary sterility, while high dose exposure to 3-MCPD induced spermatocoele, which resulted in blockade of the sperm channel in the testis and further caused permanent infertility over time [15]. A 7-day repeated oral dose of 3-MCPD in rats showed that 3-MCPD elicited spermatotoxicity in the epididymis at ≥ 10 mg/kg/day [16]. Moreover, several studies have shown that 3-MCPD affected the energy metabolic pathways of epididymal sperm, resulting in a decrease in mating frequency, birth index and number of alive newborns [17,18,19,20].

Various toxicity studies have been performed to investigate the toxicity of 3-MCPD during the development of the embryo and reproductive organs. 3-MCPD acts both as an epididymal toxicant and as a toxicant that is capable of directly affecting sperm metabolism and sperm motility [21,22]. However, the effects of 3-MCPD on fertilization and early embryonic development have not yet been determined. The aim of this study was to evaluate the effects of 3-MCPD on fertilization and early embryonic development, which was examined by comparing the number of fertilized oocytes and the cleavage of fertilized oocytes in control and 3-MCPD-treated groups. Validating these effects would be of great importance in the evaluation of reproductive and developmental toxicology of 3-MCPD.

2. Materials and Methods

2.1. Chemicals

3-MCPD was obtained from Aladdin (Shanghai, China). Based on a dose-response curve study performed in our previous study, the IC₂₅, IC₅₀, and IC₇₅ of 3-MCPD were 1.027, 1.802, and 3.160 mM, respectively [23]. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) were purchased from Ningbo second hormone factory (Ningbo, Zhejiang, China). Human tubal fluid (HTF) medium and cleavage medium were purchased from SAGE (Louisiana, America) and supplemented with 10 % fetal bovine serum (FBS). FBS was acquired from Gibco (California, America), and stored at -20°C after immersion in a water bath at 56°C for 30 min. Paraffin oil was acquired from Sigma (Santa Clara, California, America).

2.2. Animals

In this study, 6-8 weeks old female and male Kunming mice were obtained from Guangdong Medical Laboratory Animal Center and used as oocyte and semen donors, respectively. All mice were housed under controlled light conditions of a 12-h light-dark cycle in the experimental animal room at room temperature with free access to food and water.

2.3. Collection of Oocytes

Superovulation and oocyte collection were performed as previously described, with slight modifications [24]. Eight-10 week old female mice were injected with 10 IU PMSG in 100 µL of saline at 21:00 (GMT+8), and 10 IU HCG in 100 µL of saline 48 h later. Both fallopian tubes were isolated 14-16 h after HCG administration and maintained in 1 mL of HTF medium, which was previously equilibrated in a humidified atmosphere of 5% CO₂ in air at 37°C. Next, cumulus oocyte complexes (COCs) were collected from the removed oviducts and washed in 10% FBS supplemented with HTF medium droplet 6 times.

2.4. Collection of Spermatozoa

The collection of spermatozoa and the IVF procedure were performed according to a previously described method, with minor modifications [25]. Eight -10 weeks old male mice were sacrificed 14 h after HCG administration of female mice. Each caudaepididymidis was isolated aseptically from the mice and placed in petri dish with 1 mL of HTF supplemented with 10 % FBS. The caudaepididymidis was excised using ophthalmic scissors, and a dense mass of sperm was squeezed out and subsequently incubated in 1 mL of HTF supplemented with 10 % FBS. Next, the caudaepididymidis and medium in the petri dish were placed into a 1.5-mL centrifuge tube and placed in an incubator at 37°C for 60-90 min to obtain the capacitated spermatozoa.

2.5. In Vitro Fertilization

One day prior to the experiment, the HTF medium and cleavage medium were equilibrated in a 37°C in a 5 % CO₂ incubator. On the next day, mature oocytes were added to a drop of 100 µL of HTF (with 10% FBS)

medium containing 3-MCPD at different concentrations, and the capacitated sperm suspension was added to the droplet to achieve a final sperm concentration of 1×10⁶ /ml. Fertilized oocytes were examined 6 h after fertilization by extrusion of the second polar body and the presence of two pronuclei. The number of fertilized oocytes was recorded.

2.6. In vitro Early Embryonic Development Procedure

When fertilization was completed, the fertilized oocytes were washed in HTF medium droplet supplemented with 10 % FBS 6 times to remove the unfertilized oocytes and spermatozoa. Next, the fertilized oocytes were moved to cleavage-FBS medium containing 3-MCPD at different concentrations and observed using an inverted microscope. The number of 2-cell embryos, 4-cell embryos and 8-cell embryos were recorded.

2.7. Statistical Analysis

The values were expressed as the mean of triplicate measurements for all experiments. SPSS 19.0 and GraphPad Prism 5 were used for statistical analysis. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of Chemical Exposure on the Fertilization Rate

Oocytes were observed for fertilization 6 h after IVF using an inverted microscope. The fertilized oocytes were evaluated to be normal by the appearance of two pronuclei or two polar bodies. However, some of the fertilized oocytes appeared with only one polar body, which may be potentially due to it located in the back of the oocyte and being hidden by another polar body (Figure 1). When fertilization was performed with spermatozoa pretreated with 3-MCPD at the concentration of IC₂₅, IC₅₀ or IC₇₅, the fertilization rate and fracture rate significantly decreased with an increase in the 3-MCPD concentration ($P < 0.01$ and $P < 0.05$, respectively) (Table 1). During *in vitro* fertilization, 3-MCPD induced a decrease in the fertilization rate, particularly at high concentrations of IC₅₀ and IC₇₅, which resulted in a greater than 73% decrease compared with the control group. Moreover, the fracture rate of oocytes in the IC₅₀ and IC₇₅ group was higher compared to the control group, in which the fracture rate of oocytes in the IC₇₅ group was 46.15 %. In addition, when 3-MCPD-treated fertilized oocytes were moved to normal cleavage medium after fertilization, no 2-cell embryos were observed (data not shown). Thus, 3-MCPD could affect fertilized oocyte development and subsequent 2-cell formation.

Table 1. Effect of 3-MCPD on mouse oocyte *in vitro* fertilization

Category	No. of oocytes	No. of fertilized oocytes (%)	No. of fractured oocytes (%)
Control	101	92(90.52±15.41)	0
IC ₂₅	99	77(77.00±12.79)	10(10.14±3.82)*
IC ₅₀	108	20(17.86±10.50)**	42(37.96±8.63)**
IC ₇₅	104	16(13.24±6.38)**	46(46.15±6.94)**

Mean ± SD, n = 3. * $p < 0.05$ vs. control. ** $p < 0.01$ vs. control.

Figure 1. *In vitro* fertilization and early embryonic development in mouse. (A) Epididymal. (B) Oviductal magnum. (C) Cumulus-oocyte complexes. (D) Fertilization (E) Fertilization egg (F) Fertilization egg ($\times 400$) (G) 2-cell embryos (H) 4-cell embryos (I) 8-cell embryos

3.2. Effects of 3-MCPD Exposure on Early Embryonic Development In Vitro

Fertilized oocytes were moved into cleavage medium (with 10 % FBS) after normal fertilization and observed using an inverted microscope on the third day (Figure 2). As shown in Table 2, the number of 4-cell embryos and 8-cell embryos was remarkably less compared to the number of 2-cell embryos in both the control and 3-MCPD-treated

groups, indicating a developmental block due to the mouse embryo itself. 3-MCPD induced a significant decrease in the percentage of embryos at 2- and 4-cell stage formation ($P < 0.05$). The 4-cell formation rate of the 3-MCPD-treated group significantly decreased, indicating that 3-MCPD induced a developmental block in 2-cell embryos. In addition, 8-cell embryos were not found in any of the treated groups indicating a remarkable inhibition of 3-MCPD on the formation of 8-cell embryos.

Figure 2. Effect of 3-MCPD on fracture in mouse oocytes and mouse embryos

Table 2. Effects of 3-MCPD on the development of mouse IVF embryos

Category	No. of oocytes	No. of 2-cell embryos (%)	No. of 4-cell embryos (%)	No. of 8-cell embryos (%)
Control	116	107(90.01±8.52)	28(24.41±8.3)	8(7.04±3.10)
IC ₂₅	105	78(72.96±12.27) *	7(6.73±3.95) *	0*
IC ₅₀	119	72(60.76±12.68) *	6(5.63±3.74) *	0*
IC ₇₅	98	60(61.27±14.68) *	2(0.10±0.10) *	0*

Mean ± SD, n = 3. *p < 0.05 vs. control.

4. Discussion

The content of 3-MCPD in food is critical for health [26]. In this context, the reproductive toxicity of 3-MCPD was evaluated regarding its effect on fertilization and preimplantation embryonic development.

Kunming mice were selected due to their short reproductive cycle, rapid cleavage, and easily manipulated growing environment. PMSG and HCG were injected to induce ovulation prior to COC collection. Importantly, the time of exposure of oocytes and spermatozoa in air during their collection should be as short as possible, and the temperature, light and pH of medium should be appropriately maintained.

Sperm motility is important in male fertility and early embryonic development [27], and it is also a quick and sensitive index for reproductive toxicity evaluation [28]. However, there is still no consensus on whether the exposure of sperm to 3-MCPD induces sperm motility. Jelks found that the parameters of sperm motility were not affected at 10 mg/kg 3-MCPD in Sprague Dawley rats but that the ATP levels were significantly decreased [21]. In contrast, Xie et al. detected a significant increase in the percentage of abnormal sperm at a 10 mg/kg 3-MCPD in Sprague Dawley rats, which may be attributed to a deficiency in ATP [29]. However, other reports have also shown a decrease in sperm motility upon exposure to 3-MCPD [14,30]. Our results showed that sperm motility was not statistically significant between the 3-MCPD-treated and control groups. Thus, 3-MCD did not induce any toxicity in sperm motility within a short time period. Because 3-MCPD could trigger a significant increase in the fracture rate, we proposed that the effect of fertility by 3-MCPD could be revealed using oocytes instead of sperm pathway *in vitro*. 3-MCPD combined with glutathione *in vivo* forms oxalate [31], which results in a decrease in GSH levels, which serves as an indicator of cytoplasmic maturation in oocytes [32,33].

During *in vitro* fertilization, 3-MCPD induced a decrease in the fertilization rate and an increase in the oocyte fracture rate, particularly at high concentrations of IC₅₀ and IC₇₅. 3-MCPD has also been reported to inhibit the expression of genes affecting glycogen synthetase activity and to generate a decrease in the sperm's storage capacity, resulting in dysfunctional sperm-oocyte binding and a subsequent decrease in fertility [21].

Early embryonic developmental procedures, including the formation of 2-cell embryos, 4-cell embryos and 8-cell embryos, were adversely affected by 3-MCPD treatment. In this study, 3-MCPD significantly decreased the formation rates of 4-cell embryos. Cleavage terminated after the formation of 4-cell embryos; that is, no 8-cell embryos formed in the IC₇₅ group. Taken together, these results suggested that early embryonic development was remarkably inhibited by 3-MCPD.

Developmental blockade, which normally occurs during embryonic development *in vitro*, was observed at the 2-cell stage in the present study. A lack of some components in the culture medium and/or inappropriate environmental conditions for embryonic development could result in a developmental block of early embryos [34], which often occurs in 2-cell embryos in mice [35]. A family of genes known as Bcl-2 or nuclear fragmentation may be the cause of this blockade phenomenon [36], and further studies are warranted.

5. Conclusions

Our results indicated that 3-MCPD resulted in a decrease in the fertilization rate and an increase in oocyte fracture rate. 3-MCPD exposure has hazardous effects on the mice embryonic development by reducing the percentage of 2-cell embryo and 4-cell embryo formation. Moreover, a developmental block at the two-cell stage exists in mouse embryos.

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Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

3-Monochloropropane-1, 2-diol, 3-MCPD; *In vitro* fertilization, IVF; Acid-hydrolyzed vegetable proteins, acid-HV; Pregnant mare serum gonadotropin, PMSG; Human chorionic gonadotropin, HCG; Human tubal fluid, HTF; Fetal bovine serum, FBS; Cumulus oocyte complexes, COCs; Inhibition Concentration, IC.

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