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Toxic Mechanisms of 3-Monochloropropane-1,2-Diol on Progesterone Production in R2C Rat Leydig Cells

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ABSTRACT: 3-Monochloropropane-1,2-diol (3-MCPD) is a well-known food processing contaminant that has been shown to impede the male reproductive function. However, its mechanism of action remains to be elucidated. In this study, the effects of 3-MCPD on progesterone production were investigated using R2C Leydig cells. 3-MCPD caused concentration-dependent inhibition of cell viability at the IC_{25}, IC_{50}, and IC_{75} levels of 1.027, 1.802, and 3.160 mM, respectively. Single cell gel/comet assay and atomic force microscopy assay showed that 3-MCPD significantly induced early apoptosis. In addition, 3-MCPD significantly reduced progesterone production by reducing the expression of cytochrome P450 side-chain cleavage enzyme, steroidogenic acute regulatory protein, and 3β-hydroxysteroid dehydrogenase in R2C cells. The change in steroidogenic acute regulatory protein expression was highly consistent with progesterone production. Furthermore, the mitochondrial membrane potential and cAMP significantly decreased.

KEYWORDS: *3-MCPD, R2C cells, progesterone, early apoptosis, mitochondrial membrane potential, cAMP*

INTRODUCTION

3-Monochloropropane-1,2-diol (3-MCPD) was first found in soy sauce during hydrochloric acid hydrolysis.[1] It has also been detected in bread,[2] cereals,[3] coffee,[4] meat,[5,6] and drinking water.[7] 3-MCPD can cross the blood−testis barrier and the blood−brain barrier and is widely distributed in body fluids.[8,9] Based on the lowest observed adverse effect level for renal tubule hyperplasia study (1.1 mg/kg body weight per day),[10] a maximum tolerable daily intake of 3-MCPD is up to 2 μg/kg body weight recommended by the Scientific Committee on Food of the European Union. In recent years, it was found that free 3-MCPD could be released from 3-MCPD esters in the gastrointestinal tract.[15] 3-MCPD esters were often present in refined oil at 1−10 mg/kg level, which is significantly higher than that of 3-MCPD in soy sauce.[11−14]

A number of animal toxicity studies on free 3-MCPD have shown that it exhibits reproductive toxicity in most in vivo assays.[16] In the past few decades, 3-MCPD has been considered a potential male contraceptive due to its rapid and reversible action on the post-testicular maturation of spermatozoa. However, research funding is elusive because of the unacceptable side effects of 3-MCPD observed in primates.[17,18] Recent findings suggest that 3-MCPD inhibits protein tyrosine phosphorylation by blocking the cAMP/PKA pathway in sperm, resulting in animal infertility.[19] Further research demonstrated that 3-MCPD could induce apoptosis in testicular cells,[20] affect testicular lipid metabolism in vivo, glucose metabolism, and oxygen utilization, as well as decrease testosterone secretion in cultured rat Leydig cells in vitro.[7]

Leydig cells are the primary source of testosterone in males and their differentiation in the testes is an important event in the reproductive system development of male. With differentiation, Leydig cells develop their capacity to synthesize and secrete different androgens.[21,22] Progesterone production is a process of testosterone biosynthesis. Steroid hormone synthesis is initiated with the steroidogenic acute regulatory (StAR) protein, a key factor in the transfer of cholesterol from the cytoplasm into the inner membrane of mitochondria. In the mitochondrial inner membrane, cholesterol is converted to pregnenolone by cytochrome P450 side-chain cleavage enzyme (P450scc). Pregnenolone is then transported to the smooth endoplasmic reticulum and sequentially converted into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD).[23]

Although several investigators have reported that 3-MCPD did not affect spermatogenesis or induce hormonal changes in the blood and testes of adult male rats,[17] the effects of 3-MCPD on immature mammalian reproduction and the pathway of 3-MCPD reproductive toxicity have yet to be determined.

The aims of this study were to evaluate the effects of 3-MCPD on progesterone production activity and to elucidate the cellular mechanism involved. Validating these effects can help establish the acceptable daily intake of 3-MCPD in humans and its concentration limits in food, thereby potentially lowering the risk of the resultant disease.

MATERIALS AND METHODS

Cell Culture and Treatments. Rat Leydig R2C cells (ATCC, Manassas, VA, USA) were cultured in F12 medium (Gibco, Rockville, MD, USA) supplemented with sodium pyruvate, $NaHCO_3$, 15% horse serum, 2.5% FBS, and a 1% penicillin/streptomycin mixture, and

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maintained at 37 °C in a 5% CO_2 incubator.[24] For stimulation, cells were treated with 3-MCPD ranging from 0.5 to 6 mM, or fresh F12 medium as the solvent control, for 4 or 24 h. The culture media were removed and centrifuged at 400*g* for 5 min at 4 °C. The supernatants were stored at −20 °C for the progesterone assay.

Cytotoxicity Assay and Treatments. MTT assay was performed to determine the effects of 3-MCPD (Aladdin, Shanghai, China) on R2C cell viability.[25] Briefly, 4×10^3 cells per well were seeded in a 96-well flat-bottomed plate (Costar, Cambridge, MA, USA), cultured at 37 °C for 24 h, and then incubated with different concentrations of 3-MCPD (0,0.5, 1, 2, 4, and 6 mM) for 48 h. Next, 20 μL of MTT (5 mg/mL in PBS) was added to each well and cells were incubated at 37 °C for 4 h. After the medium was removed at the end of incubation, 150 μL of DMSO (dimethylsulfoxide) was added to each well, and the mixture was shaken at ambient temperature for 10 min until the absorbance was measured at 570 nm using a microplate reader (Thermo Scientific, Chantilly, VA, USA). The 25% (IC_{25}), 50% (IC_{50}), and 75% (IC_{75}) maximal inhibitory concentrations of 3-MCPD for reducing the cell number after 48 h culture were subsequently calculated.

Atomic Force Microscopy (AFM) Measurements. For AFM measurement, R2C cells were cultured in six-well plates and incubated with 3-MCPD for 24 h. The cells were rinsed twice in phosphate-buffered saline solution for 5 min before being fixed by immersion in 4% paraformaldehyde. The cells were fixed for 30 min, rinsed in PBS, and then stored at room temperature. The effects of 3-MCPD on the morphology of R2C cells were investigated with Bioscope Catalyst Nanoscope-V (Veeco Instruments, Santa Barbara, CA, USA).[26] The AFM images were collected in tapping mode using silicon probes with a spring constant of 5 N/m and a resonant frequency of 150 kHz (Budget Sensors). The scan areas were dependent on the size of R2C cells. Image processing and data analysis were performed with Veeco NanoScope Analysis Software.

Single Cell Gel/Comet Assay. The single cell gel/comet assay was essentially performed as described previously.[27] Briefly, R2C cells treated with 3-MCPD were harvested and embedded in 0.8% low-melting agarose on slides precoated with normal melting point agarose. Slides collected from the above assay procedures were placed in a lysis solution (2.5 mM NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 10% DMSO, and 1% Triton ×100) for 1 h at approximately 5 °C. DNA was allowed to unwind in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13.6) for 20 min. The slides were then placed in a horizontal electrophoresis tank (Bio-Rad, Hercules, CA, USA), exposed to 25 V for 15 min, washed twice with neutralization buffer (0.4 M Tris, pH 7.5), and dehydrated in ethanol for 5 min. After ethidium bromide staining, 20 randomly selected cells per coded slide were visualized under a fluorescent microscope and submitted to image analysis using CASP software. Both Olive tail moment (OTM), defined as the product of the distance between the barycenters of the head and tail and the proportion of DNA in the tail, and percentage of DNA in the comet tail were used to evaluate the extent of DNA damage in individual cells.

Radioimmunoassay. Progesterone is the major steroid produced by R2C cells. Concentrations of progesterone in the media were measured using a radioimmunoassay kit (Beijing North Institute of Biological Technology, Beijing, China) according to the manufacturer's instructions.[28]

Quantitative RT-PCR Analysis. Quantitative real-time polymerase chain reaction (PCR) was conducted to validate the altered gene expressions of StAR, P450scc, and 3β-HSD. Total RNA was extracted from cells using an E.Z.N.A. Total RNA Kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's instructions. The quality of the RNA was assessed by measuring the A_{260}/A_{280} ratio in a microplate reader (Thermo Scientific, Chantilly, VA, USA). A two-step reverse transcription system was used for real time RT-PCR. First-strand cDNA was synthesized using an M-MLV First-Strand Synthesis Kit (Omega Biotek, Norcross, GA, USA). PCR was performed in a CFX96 System (Bio-Rad, Hercules, CA, USA). Each sample was conducted in triplicate, and each reaction mixture was prepared using SYBR Premix Ex Taq (TaKaRa, Kusatsu, Japan) in a total volume of 20 μL. Amplifications were performed at 95 °C for 30 s and then at 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The fluorescence signal was observed. Data were analyzed using Bio-Rad CFX Manager. The PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis to confirm amplification specificity. The mRNA expressions of StAR, P450scc, and 3β-HSD were normalized to the mRNA expression of RPS16, and the adjusted expressions in the control group were taken as references (relative quantification = 1) using the $2^{-\Delta\Delta Ct}$ method as previously described. The following primer sequences were used: StAR, forward (5′→3′) CCCAAATGTCAAGGAAATCA and reverse (5′→3′) AGGCATCTCCCCAAAGTG; P450scc, forward (5′→3′) AAGTATCCGTGATGTGGG and reverse (5′→3′) TCATACAGTGTCGCCTTTTCT; 3β-HSD, forward (5′→3′) CCCTGCTCTACTGGCTTGC and reverse (5′→3′) TCTGCTTGGCTTCCTCCC; RPS16, forward (5′→3′) AAGTCTTCGGACGCAAGAAA and reverse (5′→3′) TGCCCAGAAGCAGAACAG.[29]

Western Blot Analysis. Briefly, for protein isolation, cells were lysed in RIPA lysis buffer (Cell Signaling, Beverly, MA, USA) on ice, insoluble material was removed by centrifugation at 12 000*g* for 15 min at 4 °C, and the supernatants were collected. Protein concentration was determined by BCA assay. Samples were electrophoresed in 12% SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1−2 h at room temperature and then incubated at 4 °C overnight using relative primary antibodies: rabbit antirat StAR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000, rabbit antirat 3β-HSD antibody (Santa Cruz Biotechnology) at 1:500, and rabbit antirat GAPDH antibody (Cell Signal Technology, Beverly, MA, USA) at 1:1000. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, the membranes were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol.

Measurement of Mitochondrial Membrane Potential (MMP). The disruption of the mitochondrial electron transport chain, MMP ($\Delta\Psi_m$), and ATP synthesis has been associated with reduced Leydig cell steroidogenesis.[30] MMP was measured to evaluate the mitochondrial damage induced by 3-MCPD. The fluorescent, lipophilic, and cationic probe JC-1 (Beyotime Biotech, Nantong, China) was used to measure the $\Delta\Psi_m$ of R2C cells according to the manufacturer's instructions. Briefly, after different treatments, cells were cultured in six-well plates and incubated with JC-1 staining solution (5 μg/mL) for 20 min at 37 °C. Cells were then rinsed twice with JC-1 staining buffer and then detected with FCM (BD FACSCalibur, San Jose, CA, USA). The $\Delta\Psi_m$ of R2C cells in each treatment group was calculated as the fluorescence ratio of red (i.e., aggregates) to green (i.e., monomers). Qualitative assessment of JC-1 uptake by mitochondria was performed using a confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

cAMP Accumulation. cAMP quantitative determination was performed using a cAMP-Glo assay kit (Promega, Madison, WI, USA), which provides a homogeneous, bioluminescent, and high-throughput assay to measure cAMP levels in cells.[31] Briefly, R2C cells were plated in complete medium in 96-well dishes at a concentration of 1×10^4 cells/well 24 h before test compound exposure. Cells were stimulated with various concentrations of 3-MCPD for 4 h. After 20 min of treatment with induction buffer (PBS containing the phosphodiesterase inhibitors IBMX (0.5 mM) and Ro 20-1724 (0.1 mM); Sigma-Aldrich, St. Louis, MO, USA), the reaction was terminated by the addition of 20 μL of cAMP-Glo Lysis Buffer. As per the manufacturer's instructions, 40 μL of cAMP Detection Solution (containing protein kinase A) was added to each well after 15 min at room temperature, and 80 μL of Kinase-Glo Reagent was added after another 20 min at room temperature. The plates were then incubated for 10 min at room temperature, and luminescence was measured using a plate-reading luminometer (Biotek, Synergy HT, VT, USA).

Statistical Analysis. Values are means of triplicate measurements for all experiments. Statistically significant differences among the control and 3-MCPD-treated groups were examined using one-way ANOVA. All statistical significance was based on $p < 0.05$.

RESULTS

Determining the Inhibitory Concentrations of 3-MCPD in R2C cells. MTT cytotoxicity assay was conducted to determine the concentrations to be used in the following assay. 3-MCPD caused a concentration-dependent inhibition of cell viability, which decreased from 5.99% to 96.87%. Based on the dose−response curve, the IC_{25}, IC_{50}, and IC_{75} of 3-MCPD were 1.027, 1.802, and 3.160 mM, respectively.

AFM Images and Morphological Changes. Untreated R2C cells were divided into sectors and measured 32.13 μm in diameter (Figure 1A). The nucleus was clear and brightly

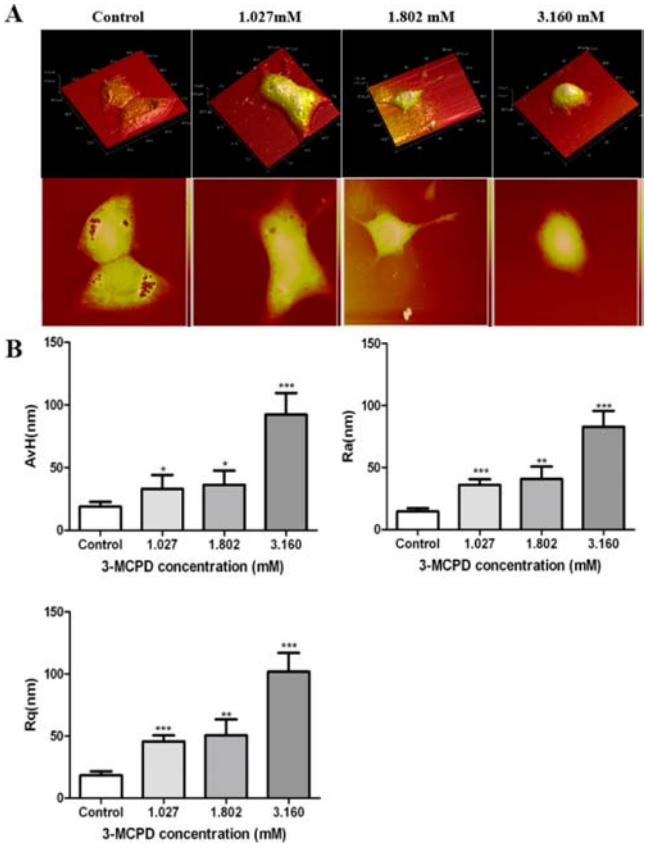

Figure 1. Representative AFM images of untreated R2C cells and R2C cells treated with 3-MCPD for 24 h. (A) Three-dimensional images of the entire cell are shown in the first row, whereas topographic images are shown in the second. (B) Ra (average roughness), Rq (root-mean-square roughness), and AvH (average height) were compared between untreated and 3-MCPD-treated cells. Mean ± SD, $n = 10$. *$p < 0.05$ vs control. **$p < 0.01$ vs control. ***$p < 0.001$ vs control.

colored. The average height (AvH) and roughness (Ra/Rq) of cells were determined to be 18.48 and 14.7/18.48 nm, respectively (Figure 1B). Studies suggested that a distinct set of morphological changes including loss of focal adhesions, the formation of cell membrane buds or blebs, and a decrease in total cell volume were associated with apoptosis.[32] In our experiment, after treatment with 3-MCPD, R2C cells showed morphological changes: the cells became oval, and their surface became extremely rough. As the nuclei of the treated cells had exploded, and indicated by the large area in the bright region, the height of treated cells was greater than that of untreated cells. Our result showed that 3-MCPD could induce R2C cells apoptosis.

Detection of DNA Damage in R2C Cells After In Vitro Treatment with 3-MCPD. The levels of DNA damage in R2C cells exposed to different doses of 3-MCPD for 4 h are shown in Figure 2. Damage was expressed as the median OTM. Using

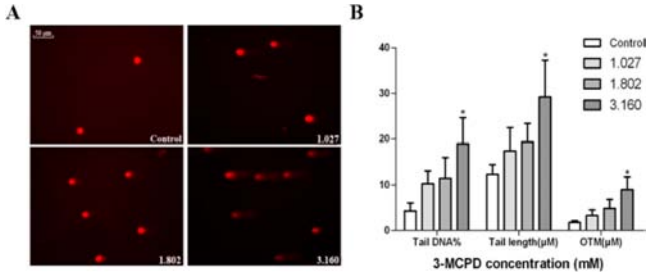

Figure 2. (A) Representative comet images of R2C cells under control and those incubated with 3-MCPD. (B) Effects of 3-MCPD on head DNA (%), tail length, and OTM in R2C cells. Mean ± SD, $n = 3$. *$p < 0.05$ vs control.

the percentage of DNA in the comet tail and tail length instead of OTM gave similar profiles. Compared with those in the control group, the levels of tail DNA (%), tail length, and OTM increased by 342.6%, 137.4%, and 409.7%, respectively, whereas a statistically significant increase in DNA damage was observed at the IC_{75} of 3-MCPD.

Effects of 3-MCPD on Progesterone Secretion and the Expressions of Steroidogenic Enzymes in R2C Cells. The time- and dose-dependent progesterone levels of Leydig cells after exposure to 3-MCPD are shown in Figure 3. When the

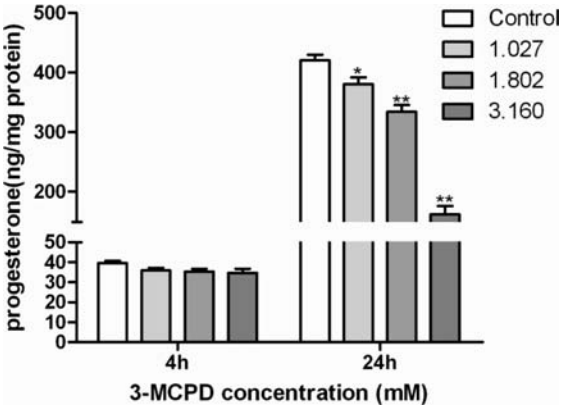

Figure 3. Effects of 3-MCPD on progesterone production of R2C cells. Mean ± SD, $n = 3$. *$p < 0.05$ vs control. **$p < 0.01$ vs control.

cells were simulated by 3-MCPD for 24 h, progesterone production decreased by 9.5%, 20.5%, and 61.2% at IC_{25}, IC_{50}, and IC_{75}, respectively. To investigate the effects of 3-MCPD on progesterone production, we assessed changes in the expressions of steroidogenic enzyme genes due to 3-MCPD treatment at the mRNA and protein levels. As depicted in Figure 4A, the effects of 3-MCPD on StAR, P450scc, and 3β-HSD expression levels were analyzed via quantitative real-time PCR analysis. 3-MCPD induced a time-dependent decrease in StAR and P450scc expressions as well as a dose-dependent decrease in 3β-HSD expression. Western blot analysis (Figure

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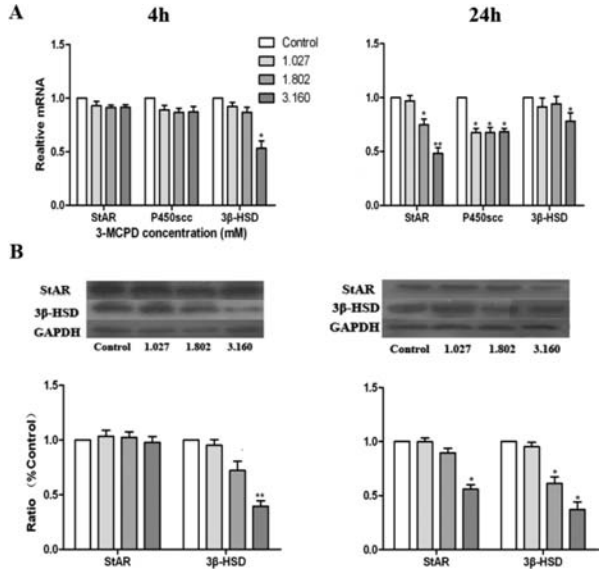

Figure 4. Effects of StAR, P450scc, and 3β-HSD expressions by real-time PCR (A) and Western blot analysis (B). Mean ± SD, $n = 3$. *$p <$ 0.05 vs control.

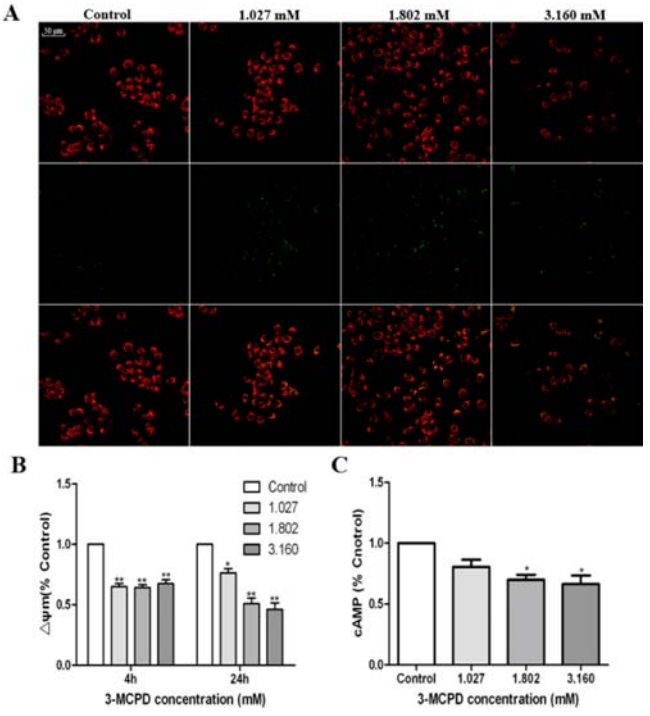

Figure 5. Effects of 3-MCPD on MMP and cAMP levels of R2C cells. (A) Representative photomicrographs of R2C cells with JC-1 under control and those incubated with 3-MCPD for 4 h. (B) Images (top and middle) represent the polarized (red) and depolarized (green) mitochondrial inner membranes. The absolute red/green JC-1 intensity ratio was measured ($\Delta\Psi_m$). (C) Intracellular levels of cAMP after 3-MCPD treatment. Mean ± SD, $n = 3$. *$p < 0.05$ vs control. **$p < 0.01$ vs control.

4B) showed similar effects of 3-MCPD on StAR and 3β-HSD protein levels. These data suggested that 3-MCPD affected the expressions of steroidogenic enzyme genes.

Effects of 3-MCPD on MMP and cAMP Levels. To determine the mitochondrial function in the toxic effect of 3-MCPD in R2C cells, we examined the MMP using JC-1 staining. As shown in Figure 5A, red fluorescence indicated the existence of the aggregate form of JC-1 in mitochondrial membranes at resting potential, whereas green fluorescence indicated the presence of free JC-1 in the depolarized MMP, especially in 3-MCPD-treated cells. Quantitative analysis of mitochondrial membrane depolarization by flow cytometry after JC-1 staining showed that 3-MCPD induced mitochondrial membrane depolarization (green fluorescence), in which significant changes were already observed at 4 h and became more marked at 24 h (Figure 5B). Intracellular cAMP levels in R2C cells followed an identical trend that significantly decreased with inhibition of 3-MCPD (Figure 5C).

■ DISCUSSION

3-MCPD has been found in foods as a result of processing, storage, or migration. The 3-MCPD content is critical for food safety.[5] In adult rats and humans, 3-MCPD could result in temporary sterility as an inhibitor of reproductive function.[33,34] Using R2C cells as immature LC model, this study showed that 3-MCPD could produce inhibitory effects on progesterone production in a time- and dose-dependent fashion (Figure 3). In addition, the characteristics of this suppression indicated that 3-MCPD induced early apoptosis in R2C and decreasd expressions of StAR, P450scc, and 3β-HSD (Figure 4A and B). Data from the present study suggested that 3-MCPD significantly decreased progesterone production by regulating the expression level of StAR mRNA and protein in treatment at IC_{75} for 24 h.

Microscopic evaluation of cellular changes in treated cells and the negative control did not show any gross viability changes. In the trypan blue assay, all 3-MCPD doses used maintained cell viability at levels higher than 98% (data not shown). Therefore, the disruptive effects seen were not lethal to the cells and served as the basis for the selection of these concentrations in our experiments.

It is confirmed that mitochondrial membranes have developed a strategy to regulate steroid synthesis in steroidogenic cells. It has been shown that $\Delta\Psi_m$, ATP synthesis, ΔpH, and $[Ca^{2+}]_{mt}$ are all required for steroid biosynthesis and mitochondria is sensitive to oxidative status.[35] Our study demonstrated that 3-MCPD significantly reduced the MMP in a dose-dependent manner (Figure 5A). The significant change in MMP induced by 3-MCPD was confirmed earlier than that in the progesterone production. In addition, the results showed that StAR mRNA and protein expressions depended on $\Delta\Psi_m$ level and that perturbation of the mitochondria suppressed StAR mRNA and protein expressions.

Disruption of progesterone production induced by 3-MCPD is considered to be potentially related to cAMP signal transduction cascade. Our results showed that the decreased cAMP production was significant at the IC_{50} concentration—the same 3-MCPD concentration at which progesterone production was also inhibited. Surprisingly, cAMP production and MMP decreased in R2C cells treated with 3-MCPD for 4 h, whereas no significant reduction was found in progesterone production. As some sites of 3-MCPD in the steroidogenic pathway were located in MMP and cAMP, the effects of 3-MCPD on progesterone production require a certain amount of time in Leydig cells.

AFM is a useful tool for collecting cellular surface information and studying cellular damage and apoptosis.[26] Untreated R2C cells appear as sectors with a complete membrane, relatively regular shape, and smooth surface (Figure

1). However, R2C cells treated with 3-MCPD exhibited a reduced cell membrane and a much rougher surface. The Ra, Rq, and AvH of the treated cells were all higher than those of the untreated cells, indicating that 3-MCPD can induce changes in the morphological structure of R2C cells.

The single cell gel/comet assay is a well-established genotoxicity assay.[36] It is a useful tool to analyze the presence of DNA strand breaks in vitro. Several studies have characterized that DNA damage was induced by reactive oxygen species (ROS) and increasing ROS could significantly decrease leydig cells steroidogenesis.[37,38] Research has reported that 3-MCPD and its metabolites might damage DNA in CHO cells.[39] As the results of genotoxicity studies differ from the expressions of target genes in progesterone production, the present study was designed to investigate if genotoxic mechanisms are involved in the induction of gene expression on progesterone production in R2C cells with 3-MCPD. In addition, the results showed that there was an increase in tail moment value with an increase in the concentration of 3-MCPD (Figure 2). These suggest that 3-MCPD is genotoxic on R2C cells under the present test conditions in vitro. Following the induction of DNA damage, a prominent route of cell inactivation is apoptosis. During the last ten years, specific DNA lesions that trigger apoptosis have been identified. DNA damage induced cells death by apoptosis.[40] Early features of cells undergoing apoptosis also include cell shrinkage and DNA fragmentation. The AFM and SCGE results suggest that 3-MCPD induced early apoptosis in R2C cells and then affected their progesterone production.

In conclusion, we have attempted to determine the factors responsible for the effects of 3-MCPD on progesterone production observed in R2C cells. To our best knowledge, this is the first report showing the involvement of the expressions of steroidogenic enzymes (StAR, P450scc, and 3β-HSD) in immature Leydig cells caused by 3-MCPD. Also, we found the change in StAR expression was highly consistent with progesterone production. The findings suggest that the 3-MCPD may be mediated by MMP and cAMP levels subsequent to a reduction in R2C cells progesterone production. In addition, 3-MCPD induced the morphological changes and DNA damage of Leydig cells, demonstrating that 3-MCPD might have caused early apoptotic cell death.

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Author Contributions

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Notes

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