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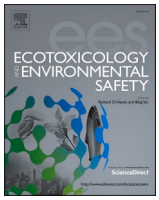

Alleviating 3-MCPD-induced male reproductive toxicity: Mechanistic insights and resveratrol intervention

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ABSTRACT

3-Monochloropropane-1, 2-diol (3-MCPD), a food-borne contaminant, is widely regarded as the primary cause of male infertility. At present, identifying a method to improve/reduce the male reproductive toxicity caused by 3-MCPD is important. In our study, we explored the potential application of resveratrol (RSV) in mitigating the adverse effects of 3-MCPD. Using 7-week-old Sprague-Dawley (SD) rats as animal models, we investigated the impacts and underlying mechanisms of 3-MCPD and RSV on reproductive function. The administration of 3-MCPD led to significant reductions in testicular and epididymal weights, as well as disruptions in spermatogenesis and histological abnormalities. However, co-treatment with RSV and 3-MCPD mitigated these adverse effects. *In vitro* study, RSV exhibited the ability to reverse the decline in Leydig and Sertoli cell populations inflicted by 3-MCPD treatment. Mechanistically, RSV reduced endoplasmic reticulum stress (PARP), inflammasome activation (NLRP3), and autophagy-mediated lysosome dysfunction (p62 and LC3BII) induced by 3-MCPD. In addition, 3-MCPD treatment increased the expression level of steroidogenesis-related proteins, steroidogenic acute regulatory (StAR) and CYP11A1, but RSV normalized StAR expression. Moreover, 3-MCPD-induced pro-inflammatory responses were counteracted by RSV treatment, with the cytokine reduction and modulation of CD206 expression, a marker of macrophage activation. These findings indicate that RSV attenuates 3-MCPD-induced reproductive toxicity, highlighting its application potential as an adjuvant agent for male reproductive health.

1. Introduction

3-Monochloropropane-1,2-diol (3-MCPD), a food-borne contaminant, primarily originates from food processed at high temperatures or treated with acids. 3-MCPD enters the human body through dietary consumption or environmental exposure *via* air and water sources (Chung et al., 2013a). 3-MCPD is primarily found in the manufacture of fats, sauces, cookies, and other processed foods (Lee and Khor, 2015; World Health, 2017). Notably, approximately 73 % of commercially available products (comprising 290 items) have been found to contain 3-MCPD (Chung et al., 2013b; Jedrkiewicz et al., 2016). The global concern revolves around the necessity to minimize 3-MCPD levels in processed foods, thereby ensuring food safety and safeguarding public health (Kim et al., 2015). However, exploring methods to reduce the adverse health effects associated with 3-MCPD exposure remains a challenge among researchers.

The toxicity of 3-MCPD has been extensively investigated since the 1970 s. At present, the primary targets of 3-MCPD include the kidneys and reproductive system, which might be associated with the inhibition of glycolysis (Fattore et al., 2023). Furthermore, 3-MCPD has received

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attention because of its potential classification as a carcinogen (Group 2B) (php, 2012). Moreover, the high levels of 3-MCPD in refined vegetable oil have raised concerns about its potential harmful effects on individuals of different age groups.

Increasing evidence suggests that 3-MCPD may disrupt the male and female reproductive systems. In a male Sprague-Dawley rat model, the administration of 3-MCPD at doses of 36 and 72 mg/kg b.w./day for 4 weeks has been shown to impair spermatogenesis and disturb testosterone paracrine modulation, indicating a potential interference with testicular function (Xing et al., 2022). This inhibitory effect on testosterone production primarily results from the downregulation of key enzymes involved in the rate-determining StAR, and cytochrome P450 side-chain cleavage enzyme (Sun et al., 2014). Moreover, the inhibitory effect of 3-MCPD on spermatogenesis remains irreversibly unchanged even after 7 weeks of exposure, although sex hormone levels returned to normal (Xing et al., 2019). In females, 3-MCPD may induce ovarian inflammation and fibrosis through inflammation and elevation of oxidative stress (Cai et al., 2023; He et al., 2023). Therefore, reducing or preventing the reproductive toxicity of 3-MCPD has always been an important issue.

Addressing these concerns, resveratrol (3,5,4′-trihydroxytrans-stilbene, RSV), a non-flavonoid polyphenolic compound mainly found in more than 70 types of plants and fruits (Jang et al., 1997; Tian and Liu, 2019), has emerged as a potential solution. RSV possesses antioxidant, anti-inflammatory, anti-hyperglycemic, cardioprotective, and neuroprotective properties (Cheng et al., 2019; Duta-Bratu et al., 2023; Moore et al., 2018; Tian and Liu, 2019; Wicinski et al., 2018). RSV is predominantly found in grapes and red wine, and this compound has beneficial effects on the reproductive system (Novakovic et al., 2022), making it a promising candidate for addressing 3-MCPD-induced reproductive toxicity.

RSV has been regarded as an efficacious agent for promoting ovarian function and addressing prevalent reproductive health issues among women such as endometriosis and polycystic ovary syndrome (Novakovic et al., 2022). We have also revealed that RSV can alleviate dysmenorrhea and inhibit uterine fibroid growth (Chen et al., 2019; Hsia et al., 2011). RSV also plays an important role in male fertility preservation because of its antioxidative capacity and ability to activate the AMPK signal pathway (Novakovic et al., 2022). Cui et al. also demonstrated that RSV has a therapeutic effect on improving and preventing obesity-induced semen damage (Cui et al., 2016). RSV reduces testicular damage induced by 2,5-hexanedione and restores spermatogenesis (Jiang et al., 2008). RSV (60 mg/kg) also prevents alterations caused by aflatoxin B1 (7.5 μg/200 g), including changes in seminiferous tubule morphology, decreased sperm count, and reduced sperm motility. This effect is associated with the antioxidative properties of RSV and its ability to reduce apoptosis (Omur et al., 2019). In addition, RSV (20 mg/kg) ameliorates oxidative damage and reduces spermatogenesis induced by polyvinyl chloride (Archana et al., 2018). These studies collectively demonstrate that RSV is a potential chemical that can improve reproductive health, at least partly by ameliorating oxidative stress. Despite these promising findings, whether RSV can improve the reproductive toxicity caused by 3-MCPD remains unclear.

Understanding the mechanism underlying the potential application of RSV in mitigating 3-MCPD-induced reproductive toxicity is important to harness its therapeutic potential. In this study, we aimed to elucidate the potential mechanism underlying RSV's ability to counteract 3-MCPD-induced reproductive toxicity. We determined the treatment dosage of 3-MCPD based on past animal studies and published levels of 3-MCPD esters in US infant formula products (Leigh and MacMahon, 2017; Xing et al., 2022). Considering previous studies that emphasize RSV's antioxidant efficacy and the oxidative stress effects of 3-MCPD, this interplay is identified as a potential mechanism through which RSV acts on 3-MCPD. By delving into the effects of RSV on 3-MCPD-induced male reproductive toxicity, we aim to provide valuable insights into the therapeutic potential of RSV in mitigating the hazards associated with 3-MCPD exposure.

2. Materials and methods

2.1. Animals

7-week-old male Sprague-Dawley rats were procured from Lasco Co., Ltd., and adapted for 1 week by housing in a temperature-controlled room (22 °C ± 1 °C) with 12 h of artificial illumination daily (0800–2000 h) with *ad libitum* access to water and food. Thereafter, the rats were randomly divided into five groups (6 rats/group). The SD rats were treated with 3-MCPD (36 or 72 mg/kg BW) and RSV (5 and 20 mg/kg BW) alone or in combination for 6 weeks. All animal procedures conducted in this study were in accordance with a protocol approved by the IACUC (Institutional Animal Care and Use Committee; LAC-2020–0530) of Taipei Medical University. After sacrificing the rats, their testis and epididymides were collected, weighed, and then stained with Hematoxylin and eosin (H&E). The testes were fixed in 10 % buffered formalin for 24 h. Thereafter, the testes were embedded in paraffin, sectioned, hydrated, H&E stained, and observed under a light microscope.

2.2. Reagent

Resveratrol was acquired from TCI (Tokyo, Japan). 3-MCPD was sourced from Sigma-Aldrich (St. Louis, MO, USA). Bafilomycin A1 (BaFA1) was obtained from Cayman chemical (Ann Arbor, MI, USA). DMEM F12 was obtained from CAISSON (Smithfield, UT, USA). Fetal bovine serum (FBS) was purchased from CORNING (Manassas, VA, USA), and horse serum was procured from Gibco (Life Technologies, Carlsbad, CA, USA).

2.2.1. Cell culture

The Leydig (TM3) and Sertoli (TM4) cell lines, obtained from ATCC, were cultured at 37 °C in a 5 % CO_2 environment. Both cell lines are cultured in a DMEM-F12 medium containing 5 % horse serum and 2.5 % FBS, with the addition of 1.2 g/L of sodium bicarbonate and 15 mM HEPES. The TM3 cell culture medium also requires the addition of 2.5 mM L-glutamine and 0.5 mM sodium pyruvate. The cell lines were analyzed after treatment with 3-MCPD (at a maximum concentration of 100 μM) and RSV (at a maximum concentration of 10 μM), either individually or in combination. The chemical doses were indicated as the ultimate molar concentrations employed within the flask.

2.2.2. Cell viability analysis

Cell viability was assessed using the MTT assay (MedChemExpress, Monmouth Junction, NJ, USA). Cells were exposed to 3-MCPD and RSV, either alone or in combination, for 24 and 48 h. Subsequently, they were treated with an MTT solution (0.1 mg/mL) for 4 h. The resulting formazan product was dissolved in DMSO, and its absorbance was measured at 570 nm using a spectrophotometer. Cell numbers were determined through trypan blue staining. After treatment, cells were harvested using a trypsin-EDTA solution, and their numbers were calculated using a hemocytometer.

2.2.3. Western blot

Cells and testis samples were lysed with a lysis buffer supplied with protease and phosphatase inhibitors (Roche Diagnostics, Rotkreuz, Switzerland). The protein samples were separated by using 10–15 % SDS-PAGE and transferred to a polyvinylidene fluoride membrane. In evaluating proteins associated with endoplasmic reticulum stress/apoptosis (PARP, Cell Signaling, Boston, MA, USA), inflammation activation (NLRP-3, Novus Biologicals, Centennial, CO, USA), CD206 (Proteintech, Rehovot, Israel), and autophagy (p62 and LC3BII, Cell Signaling), the protein expression of critical enzymes involved in steroidogenesis, StAR (Cell Signaling), and CYP11A1 (Proteintech) was

also examined. GAPDH (Proteintech) was used as the loading control for all samples. The eBlot Touch Imager (eBlot Photoelectric Technology, Shanghai, China) was used for signal visualization, and the signals were analyzed using the Image-J software program (NIH, Bethesda, MD, USA).

2.2.4. RT-PCR

RNA was isolated from testis tissues by using TRIzol (Bioman, Taiwan) and extracted by using the Direct-zol RNA Miniprep kit (Zymo Research Corp., Irvine, CA, USA). RNA was reverse transcribed by using the PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan), and RT-qPCR was operated by QuantStudio-1 Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA) using SYBR Green Master Mix (Applied Biosystems). The following primer sequences were used for star (forward: 5′-AGCTCCAAATGCCACTACCT-3′, reverse: 5′-TGGCCTTTTA-CAGAGGAGCA-3′), cyp11a1 (forward: 5′-ACCCCATCTCTGTGACCTTG-3′, reverse: 5′-TCGACCCATGGCATAGCTAG-3′), and gapdh (forward: 5′-CAGTCTTCTGGGTGGCAGTGAT-3′, reverse: 5′-TGAGGCCGGTGCT-GAGTATGT-3′). The samples were normalized using gapdh with the delta CT approach for quantitative analysis.

2.2.5. ELISA assay

The levels of inflammatory cytokines in serum, including TNF-α and IL-6, were assessed using an ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) with a sensitivity of 10 pg/mL and intra-assay coefficients of variation (CV) at 4.1 % and interassay CV at 4.7 %. All procedures were carried out in accordance with the kit's protocol.

2.2.6. Hematoxylin and eosin staining

The testis were fixed in 10 % buffered formalin for 24 h. Thereafter, the testes were embedded in paraffin, sectioned, hydrated, H&E stained, and observed under a light microscope. H&E staining was performed to assess the histological changes in testicular tissues. Morphological alterations in the testes were examined under a light microscope.

2.2.7. Statistical analysis

The experimental results were presented as mean ± SD. Initial data analysis will involve analysis of variance. If significance is observed, then *post hoc* analysis will be conducted using Duncan's multiple-range test (Steel and Torrie, 1980).

3. Results

3.1. RSV ameliorated 3-MCPD that caused reproductive toxicity: impact on testicular and epididymal parameters

In our experiments, treatment with 3-MCPD (at doses of 36 and 72 mg/kg) led to a significant reduction in testicular and epididymal weights ($p < 0.001$). However, reproductive toxicity induced by 3-MCPD was mitigated after 6 weeks of co-treatment with 3-MCPD and RSV (at doses of 5 and 20 mg/kg, Fig. 1). The morphology of the testicles and accessory testicles is depicted in Supplementary Figure 1. Furthermore, histological analysis of the testis through H&E staining revealed that spermatogenesis was suppressed in the testes treated with 3-MCPD, with disruptions observed within and outside the seminiferous tubules. By contrast, combination treatment with RSV and 3-MCPD significantly restored the reproductive toxicity caused by 3-MCPD.

3.2. RSV attenuated 3-MCPD-induced reproductive cell death: insights from cell viability and growth analysis

In vitro experiments using TM3 and TM4 cell lines were conducted to investigate the toxic effects of 3-MCPD on the male reproductive system. The cytotoxic concentration of 3-MCPD for both cell types (Fig. 2A and B) was initially determined by treating them with varying concentrations of 3-MCPD. Subsequently, we utilized these concentrations (60 and 100 μM) to assess the application potential of RSV in mitigating 3-MCPD toxicity (Fig. 2C and D). The cell number and cell viability were evaluated using the MTT assay and trypan blue assay, respectively. The results revealed that 3-MCPD significantly inhibited the growth and viability of TM3 and TM4 cells. However, combination treatment with 3-MCPD and RSV demonstrated a dose-dependent improvement in the reduction of cell numbers and viability induced by 3-MCPD (Fig. 2).

3.3. RSV improved 3-MCPD-induced endoplasmic reticulum stress, inflammation, and autophagy

We utilized various markers, including PARP (an indicator of endoplasmic reticulum stress, Fig. 3A), NLRP3 (a marker for inflammasome activation, Fig. 3B), p62, and LC3BII (markers of autophagy, Fig. 3C), to obtain a comprehensive understanding of the mechanism underlying the reproductive toxicity of 3-MCPD and to assess the mechanism by which RSV mitigates this toxicity. The experimental results showed that treating TM3 and TM4 cells with 3-MCPD led to an increase in PARP cleavage. However, when compared with the group treated with 3-MCPD, co-treatment with RSV at concentrations of 0.1 and 0.5 μM resulted in a reduction in PARP cleavage. Similar trends were observed in the protein expression of NLRP3. Furthermore, the induction of p62, along with the presence of LC3BII, indicated lysosomal dysfunction caused by 3-MCPD, and this effect was alleviated by RSV. After using bafilomycin A1 (BaFA1), we evaluated the role of lysosome fusion in the functions of 3-MCPD and RSV in male reproduction. The blockade of lysosome fusion inhibited the beneficial effects of RSV in TM3 cells while causing impairment in TM4 cells and affecting the toxicity of 3-MCPD (see Supplementary Figure 2). This reveals the importance of autophagy in male infertility. These findings collectively provide strong evidence that RSV can restore the 3-MCPD-induced stress, inflammatory responses, and autophagy in the male reproductive system.

3.4. RSV reversed 3-MCPD-induced steroidogenesis disorder

Considering the toxicity of 3-MCPD on Leydig cells, we also evaluated its impact on testosterone biosynthesis in these cells. We assessed the testicular protein expression and mRNA levels of StAR and CYP11A1, which are crucial regulators controlling the rate-limiting step in steroidogenesis (Fig. 4). The result showed that 3-MCPD treatment increased the expression level of StAR and CYP11A1 proteins in contrast to the control group. However, combination treatment with RSV and 3-MCPD resulted in the expression level of StAR returning to baseline. Similar trends were observed for CYP11A1, indicating a restoration to baseline levels.

3.5. RSV reduced 3-MCPD-induced inflammatory reactions

The level of serum cytokines, including TNF-α and IL-6, was also assessed to confirm the pro-inflammatory effects of 3-MCPD (as indicated by increased NLRP3 protein expression level *in vitro*, Fig. 5). TNF-α induces fever and inflammation, while IL-6 promotes inflammation by facilitating the infiltration, migration, and activation of immune cells, as well as local vasodilation (Białas et al., 2009). The results indicated that the group treated with 3-MCPD (at a dose of 72 mg/kg) exhibited elevated levels of TNF-α and IL-6 ($p < 0.001$) in the serum. However, combination treatment with RSV (at doses of 5 and 20 mg/kg BW) and 3-MCPD resulted in the restoration of these cytokine levels to baseline. Therefore, RSV can counteract the inflammation induced by 3-MCPD as evidenced by the normalization of serum TNF-α and IL-6 levels. Macrophages play a crucial role in the activation of inflammatory reactions (Nicolas et al., 2017). We examined the expression of CD206 as an indicator of macrophage activation. The results indicated that CD206 was activated after 3-MCPD induction, but this activation was significantly regulated by RSV treatment.

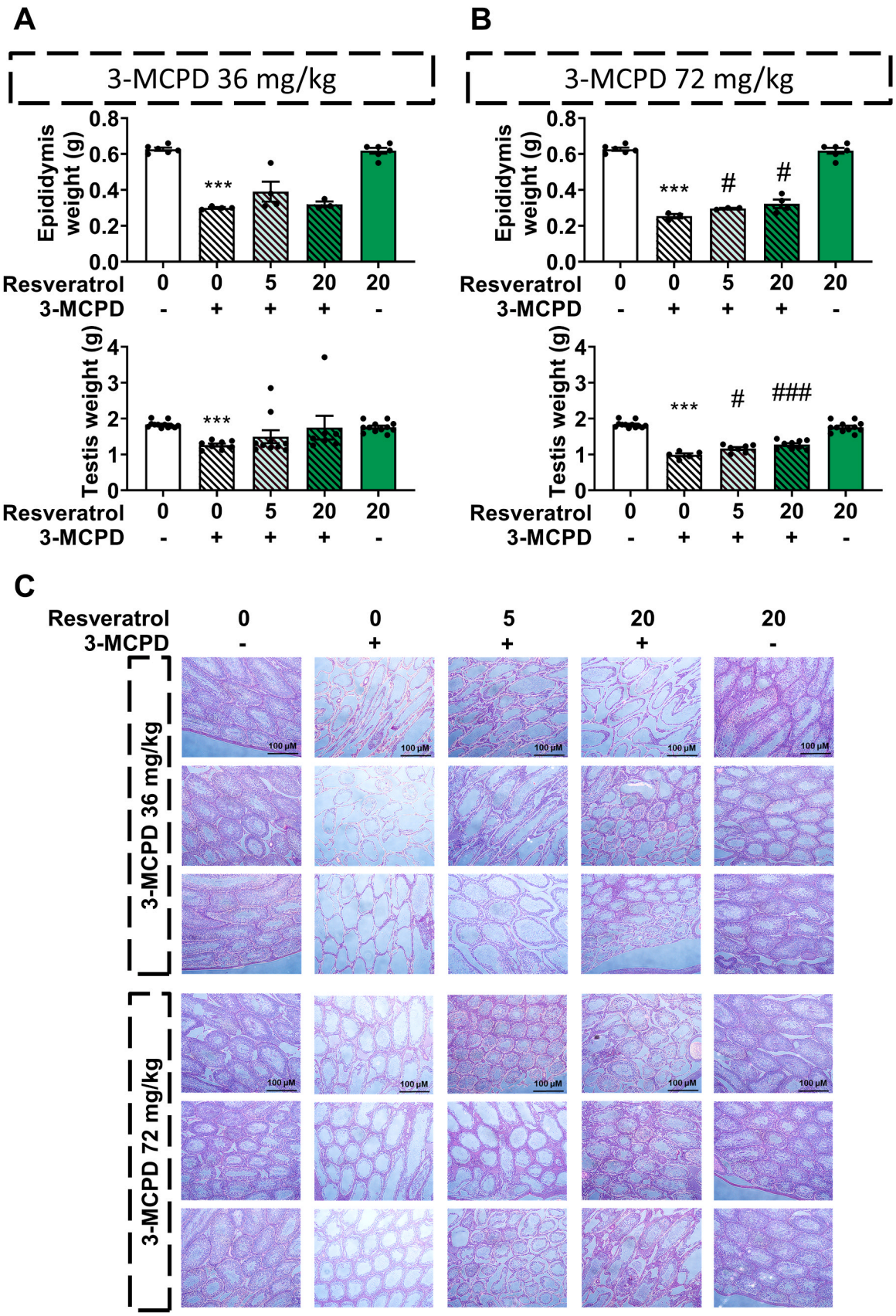

Fig. 1. Resveratrol reduces the reproductive toxicity induced by 3-MCPD in SD rats. Seven-week-old SD rats were treated with 3-MCPD (36 or 72 mg/kg BW) and resveratrol (5 and 20 mg/kg BW) alone or in combination for 6 weeks. The weight of the testis and epididymis was determined after being sacrificed. (C) Hematoxylin and eosin (H&E) staining was performed on testis sections for histological analysis. ***, $p < 0.001$ compared with the control group. #, $p < 0.05$; ###, $p < 0.001$ compared with the 3-MCPD-induced group. The solid dot represents the measured value. Data are presented as the mean ± SD of six rats/group.

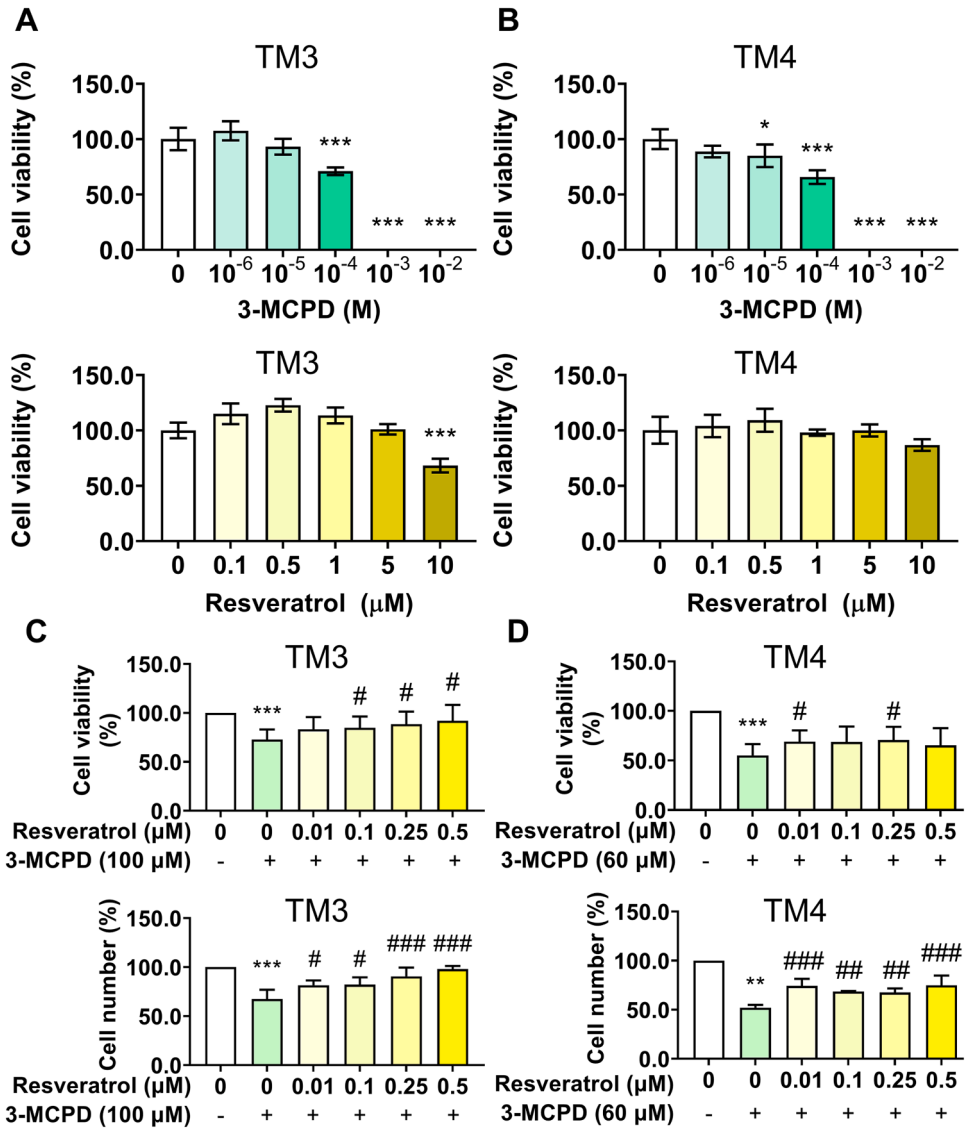

Fig. 2. Resveratrol reduces the reproductive toxicity induced by 3-MCPD in TM3 and TM4 cells. (A) TM3 and (B) TM4 cells were treated with 3-MCPD (100 or 60 μM) and resveratrol (0.01 to 0.5 μM), and cell viability was assessed using the MTT assay. (C) Cells were treated with RSV and 3-MCPD in combination for 48 h using the MTT assay, and (D) cell counting was used for proliferation analysis. ###, $p < 0.001$ compared with the control group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the 3-MCPD induced group. Data are presented as the mean ± SD. Similar results were repeated three times.

3.6. Effect of RSV on autophagy impairment caused by 3-MCPD

Recent studies have suggested that autophagic cell death could contribute to testicular damage and infertility (Ali et al., 2022). Therefore, the impact of 3-MCPD on autophagy in the male reproductive system was assessed *in vivo* (Fig. 6). The experimental results revealed that the rats treated with 3-MCPD (at doses of 36 and 72 mg/kg) for 6 weeks exhibited an increase in p62 expression. As shown in Fig. 6A, combination treatment with RSV and 3-MCPD effectively restored p62 levels ($p < 0.01$ and $p < 0.001$). In addition, we evaluated LC3BII expression, a protein in an autophagy-active form, and found that 3-MCPD treatment significantly reduced LC3BII/LC3BI ratios (<0.001, Fig. 6B). However, combination treatment with 3-MCPD and RSV did not reverse this effect. The inconsistent expression of LC3BII and p62 indicates that the autophagic process is disrupted or impaired, thereby causing adverse effects on reproduction.

4. Discussion

Our experiments confirmed the successful establishment of our animal model by observing reduced testicular and epididymal weights, impaired spermatogenesis, and damaged seminiferous tubules in SD rats exposed to 3-MCPD. These results are consistent with previous research (Xing et al., 2022). In addition, our cell culture experiments confirmed that the model has successfully demonstrated a decrease in cell count following exposure to 3-MCPD. Notably, Sertoli cells exhibited greater sensitivity to 3-MCPD compared with Leydig cells. Furthermore, the detrimental effects of 3-MCPD on the male reproductive system include increased endoplasmic reticulum stress, inflammatory responses, and induction of cellular autophagy *in vivo* and *in vitro*.

Previous research has confirmed that approximately 1 in every 6 to 7 couples faces the risk of infertility (te Velde et al., 2000; Thoma et al., 2013). Sperm count has declined by 50 % to 60 %, and a gradual decrease in sperm morphology and motility has been reported from 1973 to 2011 (Auger et al., 1995; Centola et al., 2016; Levine et al., 2017; Romero-Otero et al., 2015; Slama et al., 2004). Conversely, human exposure to 3-MCPD has increased because of changes in food manufacturing processes and consumption, which may result in daily lifelong 3-MCPD exposure (Crews et al., 2002; Hamlet et al., 2002; Jedrkiewicz et al., 2016). Therefore, male infertility may be associated

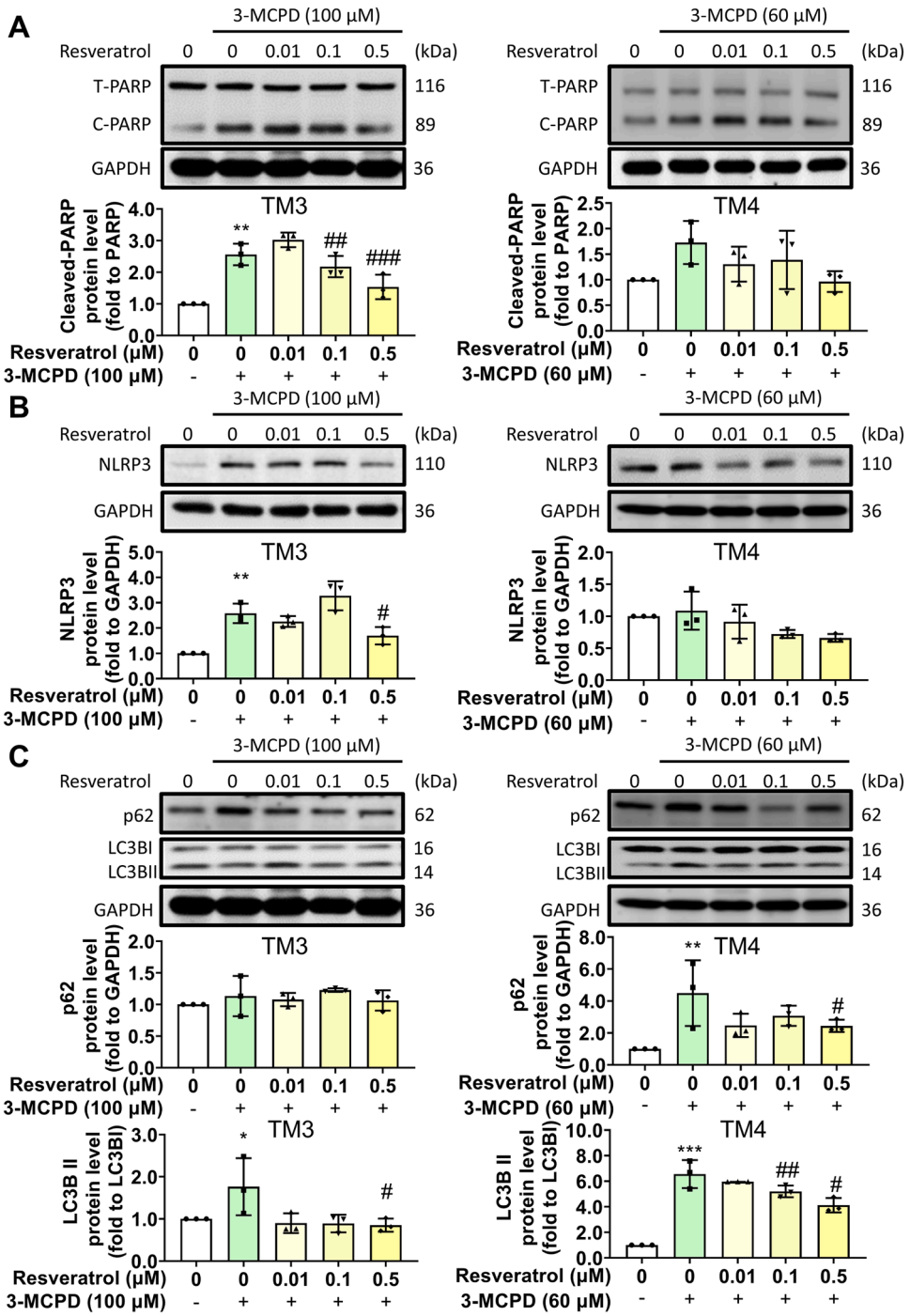

Fig. 3. Resveratrol improved 3-MCPD-induced cellular stress, inflammation, and autophagy. TM3 and TM4 cells were treated with varying concentrations of 3-MCPD (100 or 60 μM) and resveratrol (ranging from 0.01 to 0.5 μM) either alone or in combination for 48 h. The expression levels of (A) PARP, (B) NLRP3, and (C) p62 and LC3B were assessed *via* Western blot analysis and quantified using ImageJ. GAPDH protein levels were determined and used as an internal control. These results were consistently replicated in three separate experiments. The solid dot represents the measured value. Data are presented as the mean ± SD. Statistical significance is denoted as follows: *, $p < 0.05$; **, $p < 0.01$ compared with the control group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with the 3-MCPD-induced group.

with 3-MCPD exposure. Thus, determining methods to ameliorate the toxicity of 3-MCPD on the male reproductive system is of great importance.

This study showed that RSV effectively improves testicular damage caused by 3-MCPD *in vivo* and *in vitro*. In SD rats, RSV (5 and 20 mg/kg BW) co-treatment significantly improved 3-MCPD (72 mg/kg BW)-induced testicular and epididymal weights loss. This compound also ameliorates impaired spermatogenesis and damaged seminiferous tubules in SD rats exposed to 3-MCPD (Fig. 1). Extrapolating the RSV concentration of 20 mg/kg BW in SD rats using "a simple practice guide for dose conversion between animals and human" would approximate to an effective human dose of 0.56 mg/kg (Nair and Jacob, 2016). Previous studies have demonstrated that 3-MCPD stimulated ROS production (Buhrke et al., 2018; Nazari et al., 2020). In this study, 3-MCPD-induced ROS production might be mediated by increased endoplasmic reticulum stress, which is supported by elevated levels of PARP cleavage (Fig. 3A).

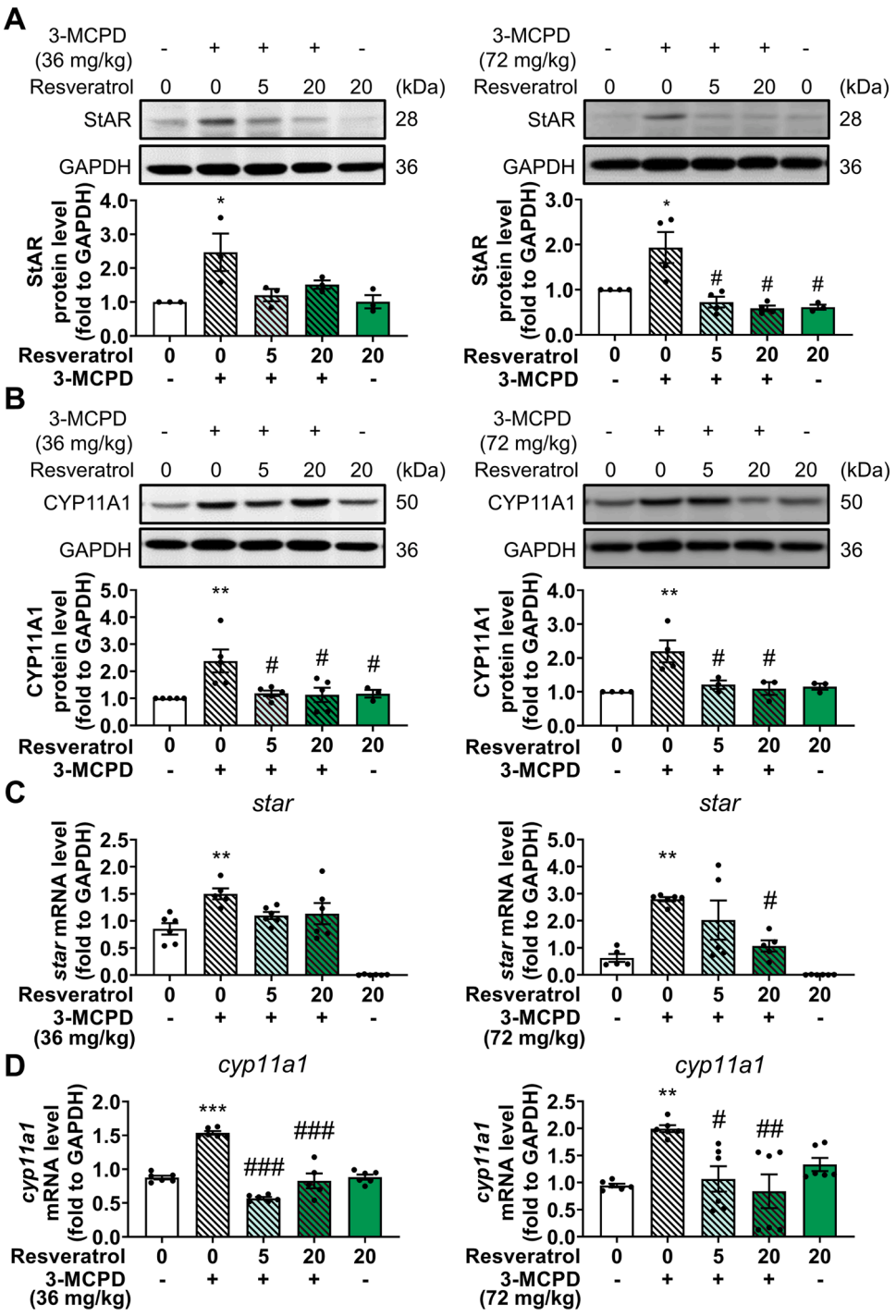

Fig. 4. Effects of resveratrol and 3-MCPD on the expression of StAR and CYP11A1 in SD Rats. Seven-week-old SD rats were treated with 3-MCPD (36 and 72 mg/kg BW) and resveratrol (5 and 20 mg/kg BW) alone or in combination for 6 weeks. The protein levels of (A) StAR and (B) CYP11A1 and the mRNA levels of (C) *star* and (D) *cyp11a1* were evaluated for homogeneity from the rat testis. Statistical significance is denoted as follows: $*p < 0.05$; $***p < 0.001$ compared with the control group. #, $p < 0.05$; ###, $p < 0.001$ compared with the 3-MCPD-induced group. The solid dot represents the measured value. Data are presented as the mean ± SD of three rats/group.

RSV might reduce 3-MCPD toxicity through its antioxidative ability (Banerjee et al., 2019; Banerjee et al., 2016), thereby decreasing cell death and inflammatory responses (Fig. 2 and Fig. 5).

In addition, the male reproductive cell toxicity induced by 3-MCPD is associated with the promotion of an inflammatory response. Important mechanistic insights into 3-MCPD-induced reproductive toxicity are also provided in this study by inducing NLRP3 inflammasome activation in Leydig and Sertoli cells (Fig. 3B). Moreover, after 3-MCPD treatment, an increase in TNF-α and IL-6 concentrations is observed in SD rat testis, which are significantly reduced when co-treated with 3-MCPD and RSV (Fig. 5). The inflammatory cytokine IL-6 is associated with germ cell apoptosis and male infertility (Rival et al., 2006), indicating that the ability of RSV to ameliorate 3-MCPD-induced reproductive toxicity may be related to its ability to reduce IL-6. Thus, RSV exerts protective effects on 3-MCPD-induced testicular injury by acting on the anti-inflammatory, NLRP3 signaling pathway.

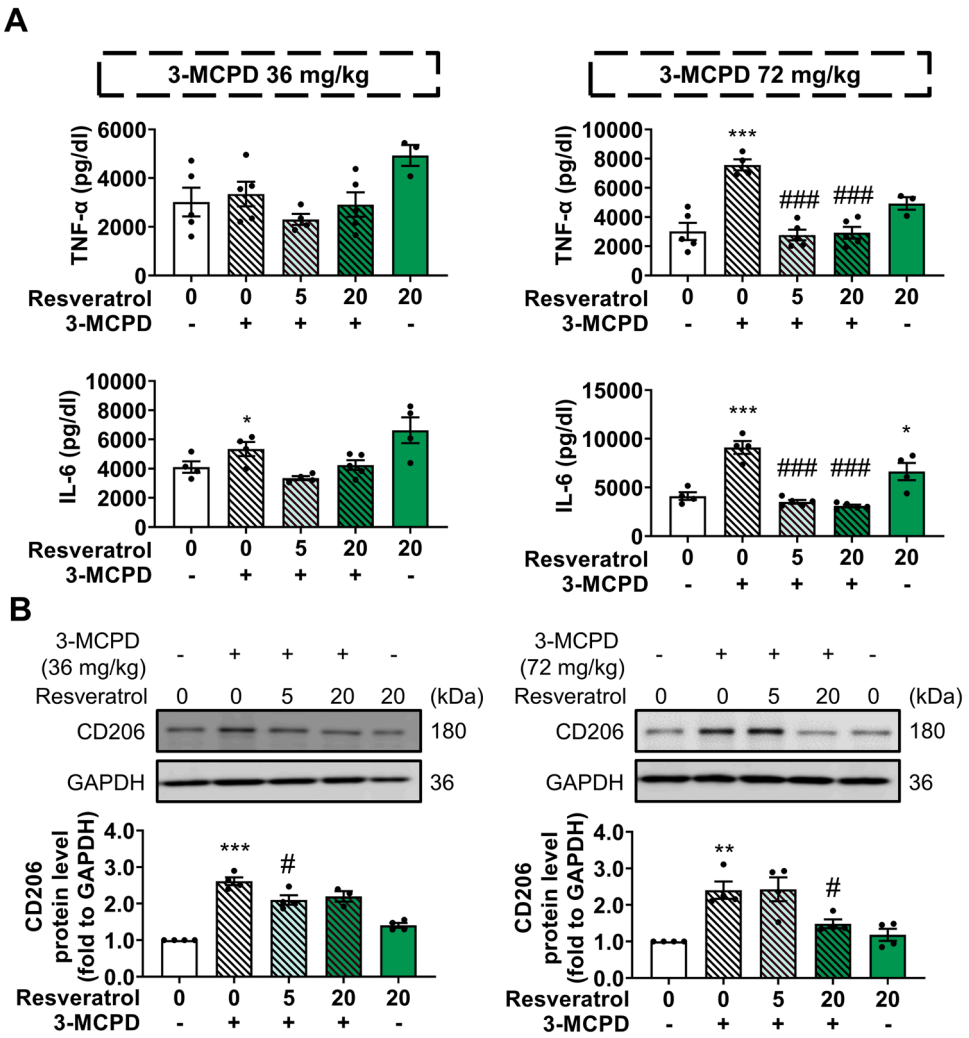

Fig. 5. Resveratrol reduces the inflammatory response induced by 3-MCPD in SD rats. Seven-week-old SD rats were treated with 3-MCPD (36 and 72 mg/kg BW) and resveratrol (5 and 20 mg/kg BW) alone or in combination for 6 weeks. (A) Inflammatory cytokines, including TNF-α and IL-6, were assessed in serum. (B) RSV intervention mitigated CD206 induced by 3-MCPD. Statistical significance is represented as follows: **, $p < 0.01$; ***, $p < 0.001$ compared with the control group. ###, $p < 0.001$ compared with the 3-MCPD-induced group. The solid dot represents the measured value. Data are presented as the mean ± SD for each group, with each group consisting of 3 to 5 rats.

In this study, 3-MCPD treatment increases ER stress (Fig. 3A), which can trigger cellular autophagy when it surpasses the capacity of cells as further validated by increased p62 protein levels in our experiments (Fig. 3C and Fig. 6A). In our animal experiments, divergent outcomes were noted: 3-MCPD treatment elevated p62 levels while substantially decreasing the LC3BII/I ratio (Fig. 6). This result implies that 3-MCPD treatment inhibits autophagy, and this effect may be associated with reduced LC3 expression. In general, p62, whose elimination depends on autophagy, can bind to abnormal proteins, facilitating their degradation by interacting with LC3 to activate autophagy. Maintaining the physiological p62 levels *via* autophagy activation is important for disease prevention and control (Islam et al., 2018; Mu et al., 2017). Hence, autophagy inhibition and disruption caused by 3-MCPD may be related to the onset of diseases.

Spermatogenesis refers to the process through which sperm developed from spermatogonia within the seminiferous tubules. This process typically takes about 60 to 72 days (Shiraishi and Matsuyama, 2017). This process primarily relies on the secretion of testosterone by Leydig cells, and it is supported and protected by Sertoli cells (Barrett et al., 2012; Payne et al., 1985). In this study, we observed that 3-MCPD induced cell death in Leydig and Sertoli cells. In addition, spermatogenesis affected the expression of the Zonula Occludens-1 (ZO-1) protein in TM4 cells (Fig. S2), which is crucial for maintaining the structural integrity of the blood–testis barrier. Our findings reveal that 3-MCPD increases PARP cleavage, thereby elevating endoplasmic reticulum stress and ROS levels, which primarily contribute to the reduction of ZO-1 protein levels (Shen et al., 2018) in Sertoli cells. Therefore, the reduction in ZO-1 protein levels following 3-MCPD treatment appears to be a reasonable consequence. Moreover, the use of chemicals such as RSV with antioxidant properties to ameliorate 3-MCPD-impaired spermatogenesis shows great application potential for future treatments. However, an increase in the protein expression level of StAR and CYP11A1 in rat testes following 3-MCPD treatment was observed (Fig. 4). The stimulatory effects of 3-MCPD on StAR and CYP11A1 protein levels can be reversed in the co-treatment group.

Although this study successfully elucidates the mechanism through which RSV mitigates 3-MCPD-induced damage to the male reproductive system using *in vivo* and *in vitro* methods, certain limitations should be noted. The concurrent administration of RSV and 3-MCPD hinders the determination of whether RSV can effectively alleviate reproductive toxicity after prior exposure to 3-MCPD. The study's 6-week duration may not capture the effects of prolonged drug exposure, and an extensive panel of indicators could enhance mechanistic insights. Moreover, the impact of RSV on pups or during pregnancy remains unexplored.

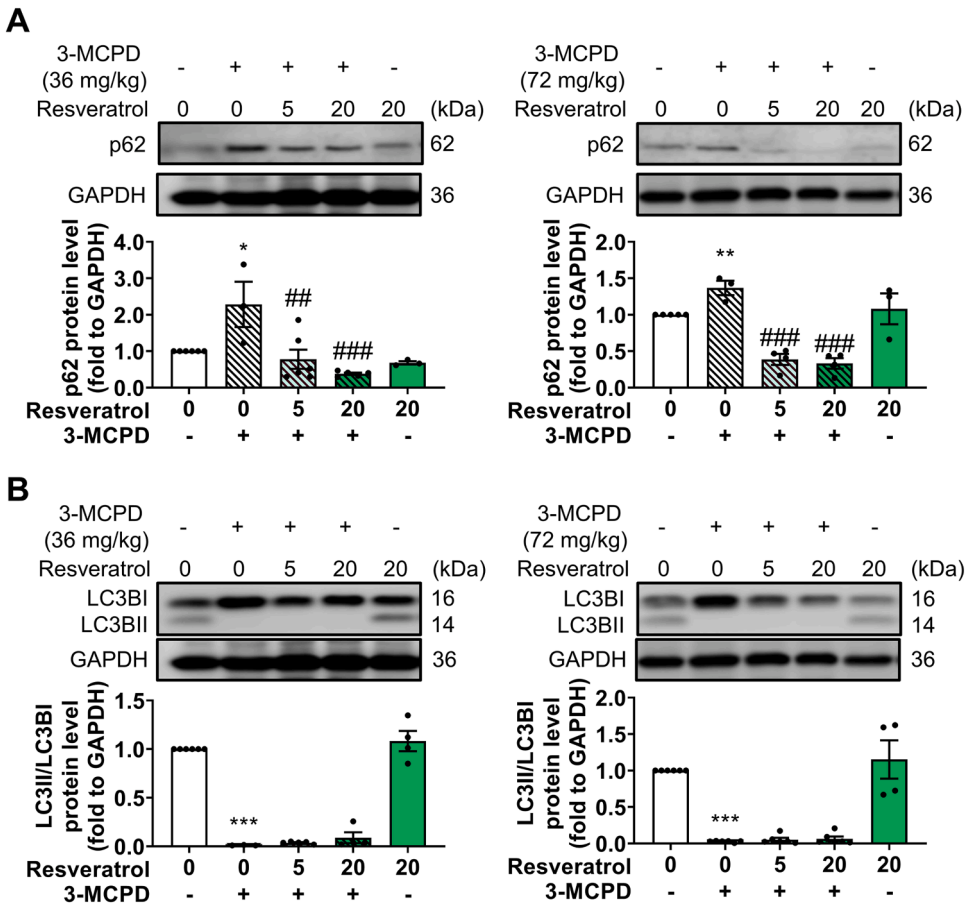

Fig. 6. Resveratrol alters the autophagy response induced by 3-MCPD in SD rats. Seven-week-old SD rats were treated with 3-MCPD (36 and 72 mg/kg BW) and resveratrol (5 and 20 mg/kg BW) either alone or in combination for 6 weeks. (A) The expression levels of p62 and (B) LC3BII were assessed *via* Western blot analysis. Statistical significance is denoted as follows: $^{*}p < 0.05$; $^{***}p < 0.001$ compared with the control group. ## $p < 0.01$; ### $p < 0.001$ compared with the 3-MCPD-induced group. The solid dot represents the measured value. Data are presented as the mean ± SD for each group, with each group consisting of 3 to 5 rats.

Furthermore, clinical studies, such as assessing 3-MCPD metabolite levels in male patients with infertility and evaluating RSV efficacy, would provide valuable insights into the scope of this cellular and animal-based research.

5. Conclusion

RSV can ameliorate 3-MCPD-induced male reproductive toxicity through various pathways, including anti-inflammatory effects, reduction of endoplasmic reticulum stress, and decreased cell death, *in vivo* and *in vitro*. Furthermore, RSV exhibits significant improvements at a concentration of 20 mg/kg (equivalent to a human-equivalent dose of approximately 0.56 mg/kg/day). Thus, further research is warranted to elucidate the action mechanism and potential application of RSV (Fig. 7).

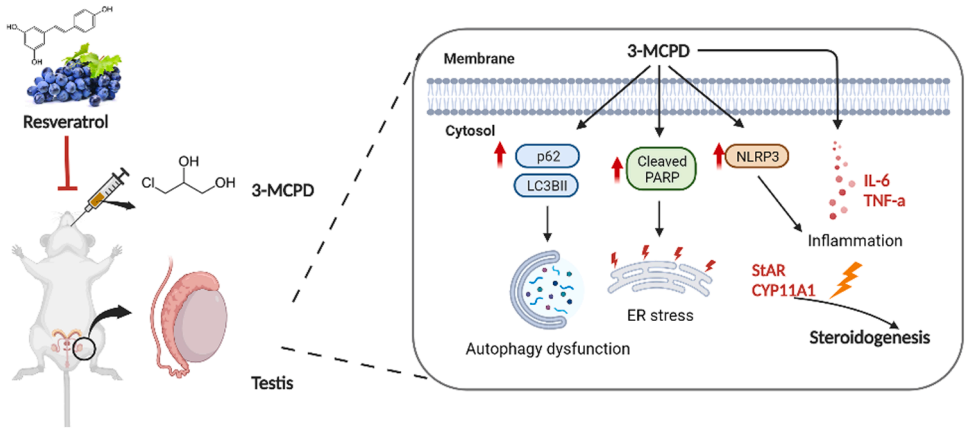

Fig. 7. Resveratrol induces protective effect against 3-MCPD-induced infertility through autophagy and inflammation modulation. Resveratrol effectively alleviates 3-MCPD-induced male reproductive toxicity. This effect is associated with the modulation of cellular autophagy (elevated p62 and LC3BII levels), endoplasmic reticulum stress (increased PARP cleavage), inflammation (upregulation of NLRP3, IL-6, and TNF-α), and steroidogenesis (involving StAR and CYP11A1).

CRediT authorship contribution statement

Ko-Chieh Huang: Formal analysis, Methodology. **Kai-Lee Wang:** Investigation, Writing – original draft. **Chiang Yi-Fen:** Investigation, Writing – original draft. **Shih-Min Hsia:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Hsin-Yuan Chen:** Methodology. **Mohamed Ali:** Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.115978.

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