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Protective effects of *Coridius chinensis* extracts on rat reproductive damage induced by manganese

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Abstract

Manganese (Mn^{2+}) is an environmental pollutant, and testis is one of the main target organs. *Coridius chinensis* (*C. chinensis*), a traditional Chinese medicine, has been shown widely used in treating various kinds of pain, nephropathy and erectile dysfunction. In our recent study, we found that Mn^{2+} exposure caused testicular injury could be rescued in part by the antioxidant activity of *C. chinensis* extracts (CcE). However, there is dearth of extensive knowledge on the therapeutic effects of *C. chinensis* on manganese-induced reproductive toxicity. In the present study, Sprague-Dawley (SD) rats were administered manganese chloride alone or co-treated with CcE for 30 consecutive days. Results indicated that *C. chinensis* mediated suppression of spermatogenic dysfunction, and the number of apoptotic cells was significantly decreased in CcE-treated groups. Furthermore, the disintegrated testicular ultrastructural structure caused by Mn^{2+} was partially repaired in CcE-treated groups. *C. chinensis* significantly inhibited Mn^{2+}-induced decline in biomarkers of blood–testis barrier (BTB) including occludin, claudin1, zonula occludens-1 and junctional adhesion molecule 1, whereas it decreased the expression of focal adhesion kinase (FAK) and c-Src. This study demonstrated that c-Src and FAK might be involved in the repair of Mn^{2+}-induced testicular injury by *C. chinensis*, but further research is needed.

KEYWORDS
apoptosis, blood-testis barrier, *Coridius chinensis*, HPLC, manganese

1 | INTRODUCTION

Manganese (Mn^{2+}) is found in very small amounts in the body, but it plays an important role in maintaining normal physiological condition and involves in many cellular biological processes. It also involves the composition of many enzymes and affects the enzyme activity. It is also required for other aspects, such as blood glucose regulation, development of bone and reproduction, and brain (Aschner & Aschner, 2005; Greger, 1999). Although an adequate intake of Mn^{2+} is necessary for the human body, the excessive Mn^{2+} exposure can lead to male fertility decline. Numerous studies proved that environmental toxicants, manganese (Mohammed et al., 2018; Wirth et al., 2007), cadmium (El-Neweshy et al., 2013; Koriem et al., 2013) and mercury (Ernst & Lauritsen, 1991; Slivkova et al., 2010), have negative effects on spermatogenesis in rodents and humans. Further research has been reported that the major target site of environmental toxicants in testes was blood–testis barrier (BTB). The environmental toxicants are accumulated in the Sertoli cells by

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The first three authors contributed equally to this work.

junction-associated 'pores' and/or drug transporters, which in turn lead to decreased sperm count and motility, and eventually cause spermatogenic dysfunction (Cheng, 2014; Cheng & Mruk, 2012; Gao et al., 2015; Siu, Mruk, et al., 2009; Su et al., 2012). Several other studies have demonstrated that environmental toxicants exposure also caused decreased activity of superoxide dismutase and catalase, and increased content of nitric oxide, malondialdehyde (MDA) and reactive oxygen species (ROS; Dkhil et al., 2016; Kong et al., 2019; Koriem et al., 2013; Liu et al., 2019). These studies suggested that environmental toxicants were important factors in oxidative stress, and oxidative stress may be an important factor in spermatogenic cell apoptosis.

Phosphatidylinositol 3-kinase (PI3K) is localized at the apical ectoplasmic specialization (ES) and basal ES in the seminiferous epithelium (Siu et al., 2005). Fine particles caused reproductive dysfunction in male rats by overactivation of the PI3K/Akt signalling pathway (Cao et al., 2015). c-Src, a nonreceptor protein kinase, is a sarcoma-inducing gene of a member of rous sarcoma virus (Src) kinase family, detected primarily in apical ES (Lee & Cheng, 2005). Following jugular vein administration of c-Src inhibitor PP1 (C16H19N5, Mr 281.4) induced the loss of germ cells in adult rats (Hanke et al., 1996). Focal adhesion kinase (FAK) displays a stage-specific expressing pattern in BTB (Siu et al., 2003). The physiological level of FAK was surging during cadmium-induced BTB disruption (Siu, Mruk, et al., 2009). Previous studies demonstrated that FAK was one of the downstream targets of c-Src in epithelial cells, and FAK-c-Src dual kinase complex is involved in multiple cellular events under physiological and pathological conditions (Bolós et al., 2010; Brunton & Frame, 2008). The FAK can be activated by the phosphorylation mediated by PI3K and c-Src activated by oxidative stress (Wong & Cheng, 2011). It has been reported that PI3K/c-Src/FAK signalling pathway involved in damage of intestinal epithelial cell tight junctions (TJ) caused by oxidative stress and acceleration of Caco-2 cell migration in vitro (Basuroy et al., 2010).

Coridius chinensis is a traditional Chinese medicine. It is widely used in treating various kinds of pain, nephropathy and erectile dysfunction (Luo et al., 2012; Yan et al., 2014). Previous studies demonstrated that *C. chinensis* exerted positive effects in various experimental models with anti-microbial (Li et al., 2015), anti-carcinogenic property (Hou et al., 2012; Qin, 2011) and anti-inflammatory activity (Shi et al., 2014). It also improved reproductive damage caused by environmental pollutants (Wang et al., 2017). Our previous study found that sperm count and serum testosterone level were increased, and sexual capacity was also improved after treatment with *C. chinensis* in rats exposed to Mn^{2+} (He et al., 2016). Furthermore, our recent study found that *C. chinensis* can effectively improve the activity of antioxidant enzymes and reduced the product of lipid peroxidation, MDA (Liu et al., 2019). We also found that *C. chinensis* can exert an antioxidant capacity by inhibiting oxidative stress and cell apoptosis caused by Mn^{2+} exposure in testes. These results indicated that *C. chinensis* can effectively improve reproductive damage caused by environmental toxicants. Additionally, an increasing number of studies have to pay attention

to explore what is the effective component of the *C. chinensis* (Tan, Tian, Cai, Luo, et al., 2019; Tan, Tian, Cai, Yi, et al., 2019). However, the potential regulatory mechanisms remain to be explored. In the present research, we found that c-Src and FAK might be involved in the repair of Mn^{2+}-induced testicular injury by *C. chinensis*, and N-acetylnoradrenaline was found to be one of the effective compounds in *C. chinensis*, but further research is needed.

2 | MATERIALS AND METHODS

2.1 | Animals

Fifty male Sprague-Dawley rats of 8 weeks of age were purchased from the Third Military Medical University, China. Rats were supplied with standard pellet feed and maintained at constant temperature ($24 \pm 2°C$) and a 12/12-h light/dark cycle. Before the experiment, animals were given adjustable feeding for 1 week. Animal care and the experimental protocol were reviewed and approved by the Animal Research and Ethics Committees of Zunyi Medical University.

2.2 | Drugs extraction and chemicals

Coridius chinensis was purchased from Baiyi Pharmaceutical Co., Ltd. The dried sample of *C. chinensis* was crushed to powder, and the latter (1000 g) was extracted with seven times the volume of petroleum ether (PE), then overnight cold soaking and vacuum recovery. Finally, the paste of PE fraction was diluted into solution by 0.5% carboxymethylcellulose sodium solution, which was used in this study. $MnCl_2 \cdot 4H_2O$ (Kemiou) was used for intraperitoneal (I.P) injection, and it was of highest available purity.

2.3 | Experimental groups and treatment regime

Rats were randomized into five different groups (ten rats/group). Normal control group: rats received I.P injection of normal saline (0.5 ml/day) for 30 days. Manganese model group: rats received $MnCl_2$, 15 mg/kg BW by I.P for 30 days. Manganese + CcE-treated group: rats received CcE (50, 100 or 200 mg/kg BW) by intragastric administration for 30 days, and CcE were given after $MnCl_2$ administration as in Mn-group. Body weight was recorded daily, and 24 h after the last treatment, the final body weight was determined. Both testes and epididymides were excised, weighed and used in succedent experimental research.

2.4 | Organ coefficient and epididymal sperm concentration

The relative weight of testis and epididymis was the ratio of testis and epididymis (g) to BW (100 g). Spermatozoon was extruded from

the cauda of left epididymis and incubated in phosphate buffered saline (PBS) for 15 min at 37°C. The incubated spermatozoon was made free sperm suspension and subsequently diluted 1:10. Finally, the sperm quantity was counted by the haemocytometer.

2.5 | Testicular histopathological and immunohistochemical analysis

Testes were fixed in 4% (v/v) paraformaldehyde, dehydrated in ethanol and embedded in paraffin, followed by preparing 5μm thickness sections and mounting on slides for haematoxylin and eosin. The immunohistochemical procedures were conducted according to Liu et al. (2019). The sections were dewaxed, hydrated and performed using a Vectastain ABC kit (Burlingame, CA) as recommended. MVH (1:1000; Abcam) and SOX9 (1:500; Millipore) were the primary antibodies. Then, the slides were rinsed three times with PBS, incubated with HRP-conjugated secondary antibody and finally stained with 3,3′-diaminobenzidin. The images were taken with a Nikon light microscope with a charge coupled device (CCD) camera.

2.6 | Apoptosis analysis

Apoptotic cells in testicular tissue were performed with DeadEnd™ Fluorometric TUNEL System (Promega). The paraffin-embedded sections of testis were assayed by TUNEL method to detect internucleosomal DNA fragmentation that is characteristic of apoptosis. The apoptotic cells were labelled with green fluorescence signal in a blue background by a Nikon light microscope, and the images were captured by the Nikon DS-Ri1 CCD camera.

2.7 | Transmission electron microscope examination

Testicular tissues were fixed in 2.5% glutaraldehyde fixation fluid for about 30 min until testicular tissues hardened. After rinsed in 0.1 M PBS for three times, osmium acid was used for secondary fixation and staining. After washing with deionized water, 1% uranium dioxide acetate was dyed overnight at 4°C. Then, tissues were dehydrated in increasing concentrations of ethanol and graded acetone solutions and then fixed in epoxy resin. Ultrathin sections (60 nm) were made by an Ultratome (Leica, Reichert Ultracuts). Finally, ultrathin sections were examined using a Hitachi-7500 transmission electron microscope (HITACHI) at the Electron Microscope Laboratory of the Chongqing Medical University.

2.8 | Western blotting

Tissues (about 100 mg) were extracted in radioimmunoprecipitation assay buffer mixed with phenylmethylsulfonyl fluoride and protease and phosphatase inhibitors cocktail (Solarbio, R0010). Protein lysates (approximately 30 μg) were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane with constant current of 200 mA for 90 min. Next, PVDF membrane was incubated in skim milk for 2 h and then labelled with primary antibodies overnight at 4°C. After washing three times, PVDF membrane was probed with secondary antibodies for 2 h. Finally, the target proteins were detected using an enhanced chemiluminescence substrate (FD bio-Pico).

2.9 | Component analysis by high-performance liquid chromatography

The sample was extracted with PE, and then, the sample (13.19 mg) and standard (1.79 mg) were dissolved with 4 and 10 ml of acetonitrile respectively. Ultrasound (power 250 W, frequency 50 kHz) was carried out for 30 min and filtered with 0.22 μm microporous membrane. Before loading the standard solution, it was diluted 20 times. The filtrate was used for content determination on a Agilent Eclipse Plus column (C18, 150 mm × 4.6 mm, 5 μm) with a mobile phase of acetonitrile-water (3:2, v/v) at the flow rate of 0.8 ml/min. The detection wavelength was 203 nm, and the column temperature was 30°C. The volume of N-acetylnoradrenaline standards was 2, 5, 10, 15 and 20 μl. The calibration curve of N-acetylnoradrenaline standard was drawn, and then, the N-acetylnoradrenaline concentration of the sample was calculated based on the calibration curve. The X represents the volume of N-acetylnoradrenaline standard and Y represents absorption peak area (mAU).

2.10 | Statistical analysis

Data were considered to be statistical differences were analysed by one-way ANOVA using the repeated measures model followed by post hoc multiple comparisons tests and presented as mean ± SD. Statistical analysis was performed by the Graphpad Prism 8 software. Differences were evaluated significance at $p < 0.05$.

3 | RESULTS

3.1 | Organ coefficient and epididymal sperm concentration

In this study, we tested the sperm concentration and coefficient of testes and epididymides in treatment and control groups. As we can see in the Table 1, the development of testes was normal in control group, but testis was seriously deteriorated in Mn^{2+}-exposure group (Table 1). Compared with Mn^{2+}-exposure group, the testes coefficient in different doses of CcE-treated groups was faintly improved,
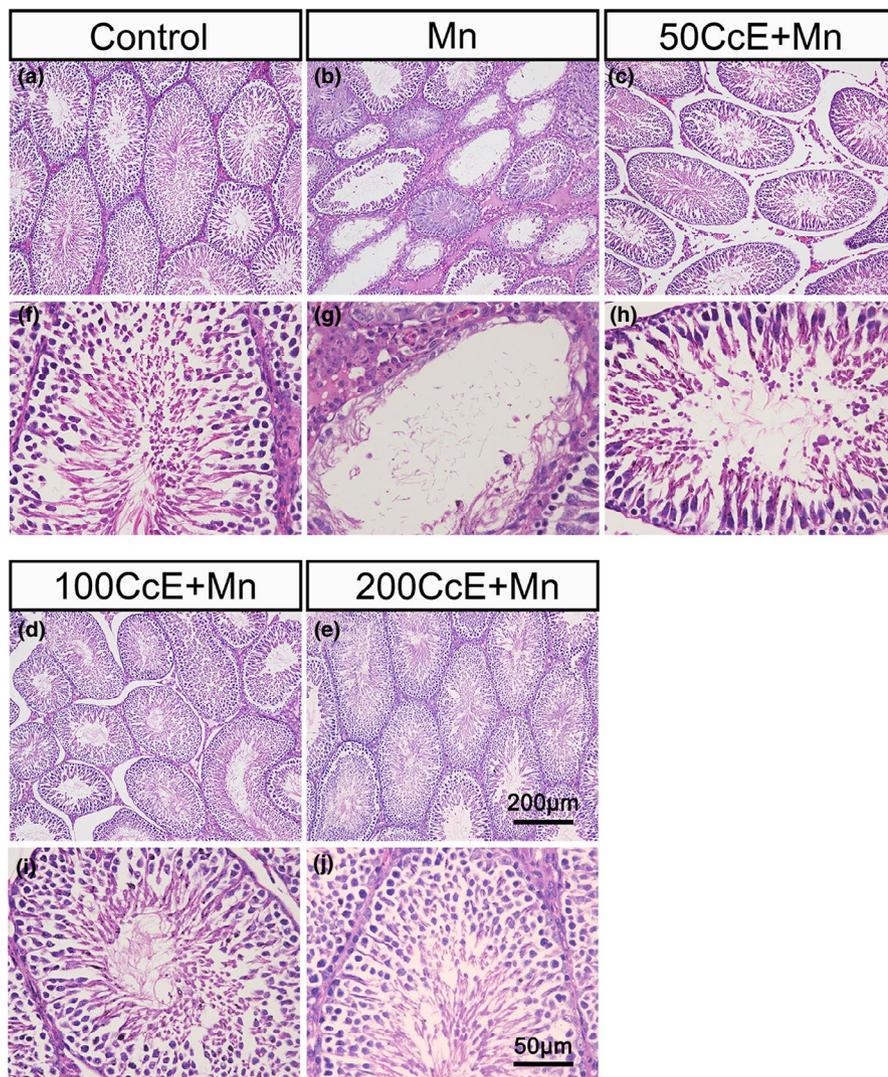
Group	Organ coefficient (g/100 g)		Sperm concentration ($\times 10^7$ cells/ml)
	Testes	Epididymis	
Control	1.40 ± 0.05	0.42 ± 0.01	4.09 ± 0.28
Mn^{2+}	$1.19 \pm 0.04^*$	$0.35 \pm 0.02^*$	$2.48 \pm 0.38^*$
50 CcE + Mn^{2+}	1.25 ± 0.05	0.36 ± 0.02	2.96 ± 0.29
100 CcE + Mn^{2+}	1.29 ± 0.03	0.38 ± 0.00	$3.39 \pm 0.12^\#$
200 CcE + Mn^{2+}	1.28 ± 0.03	0.36 ± 0.01	2.99 ± 0.06

TABLE 1 Sperm concentration and organ coefficient of control and treatment male SD rats

Note: Data were expressed as Mean $\pm$ SD ($n = 10$).

Abbreviation: CcE, *Coridius chinensis* extracts; Mn^{2+}, the model group.

*Significant differences ($p < 0.05$) compared with the control group.

#Significant differences ($p < 0.05$) compared with the model group.

FIGURE 1 Effects of CcE on the morphological structure of testicular tissue in male rats exposed to Mn^{2+}. (a) F control; (b) G Mn^{2+}; (c) H 50CcE + Mn^{2+}; (d) I 100CcE + Mn^{2+}; (e) J 200CcE + Mn^{2+}. CcE, *Coridius chinensis* extracts; Mn^{2+}, the model group

but no significant difference. In consistent with the coefficient of testes, the epididymal sperm concentration in Mn^{2+}-exposure group was remarkably lessened than control. Furthermore, the epididymal sperm concentration was increased in different doses of CcE-treated groups, and there was a significant difference in 100 CcE + Mn^{2+} group (Table 1).

3.2 | Protective effect of *C. chinensis* on the histomorphology of testis

To further explore the effects of *C. chinensis* on Mn^{2+} exposure caused testicular injury, we examined the histomorphology of testis by haematoxylin–eosin staining. A larger number of developing

spermatocytes were regularly marked in the spermatogenic epithelium of seminiferous tubules in control group (Figure 1a,f). Compared with control group, however, the layers of spermatogenic cell were obviously reduced and numerous empty patches were observed in seminiferous tubules in Mn^{2+} group. What is more, empty seminiferous tubules were also noted in Mn^{2+} group (Figure 1b,g). By contrast, the layers of spermatogenic cells were remarkably increased and only a few vacuoles existed in CcE + Mn^{2+} groups.

3.3 | Protective effect of *C. chinensis* on Mn^{2+}-induced spermatogenic dysfunction

We also examined the therapeutic effects of CcE in testes exposed to Mn^{2+} by analysing the expression of germ cell-specific marker MVH and Sertoli cell-specific marker SOX9 by immunohistochemistry. Massive multi-layered and regularly arranged MVH-positive germ cells were noted in control group (Figure 2a). By contrast, only scattered MVH-positive cells were observed in Mn^{2+} group, and the spermatogenic cell layers were significantly reduced (Figure 2b). After CcE treatment, however, the number and layers of

MVH-positive germ cells were increased significantly (Figure 2c–e). In addition, in the seminiferous tubules of control group, the SOX9-positive signal was well organized and located near the basement membrane (Figure 2f). Compared with the control group (Figure 2f), there was no significant difference in the signal of SOX9-positive cells in CcE-treated group (Figure 2h–j), whereas some dislocated Sertoli cells were observed in Mn^{2+} group (Figure 2g), suggesting that the normal functions of Sertoli cells were affected with Mn^{2+} treatment and *C. chinensis* had a positive role in suppressing the Mn^{2+}-induced spermatogenic dysfunction.

3.4 | Protective effect of *C. chinensis* on Mn^{2+}-induced cell apoptosis

Previous studies have suggested that apoptosis was enhanced in testicular tissue following oxidative stress induced by environmental toxicants, and it could be rescued by antioxidant in some extent [6, 15]. In the seminiferous tubules of control group (Figure 3a), a small number of apoptotic cells were noted. However, we found the number of TUNEL-positive apoptotic cells (Figure 3b, white arrowheads) was notably increased in Mn^{2+}-group. The signal of apoptosis was
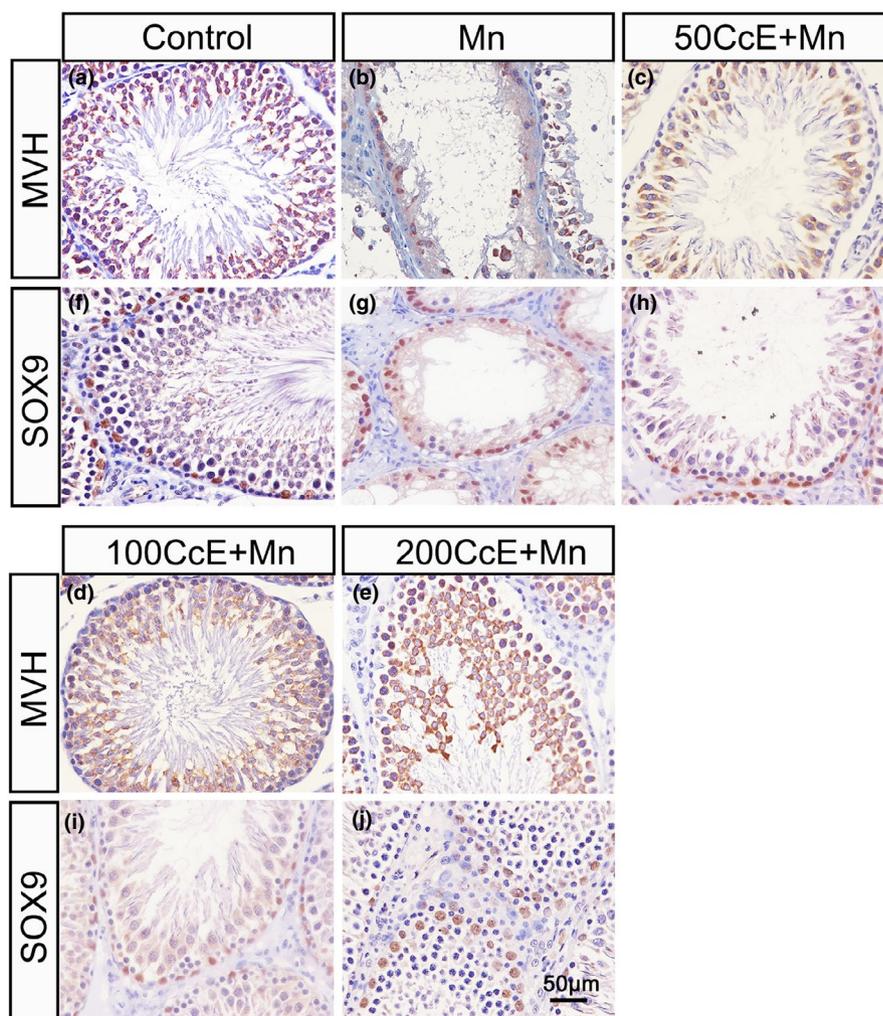

FIGURE 2 Effects of CcE on the expression of MVH and SOX9 in male rats exposed to Mn^{2+}. (a) F control; (b) G Mn^{2+}; (c) H 50CcE + Mn^{2+}; (d) I 100CcE + Mn^{2+}; (e) J 200CcE + Mn^{2+}. CcE, *Coridius chinensis* extracts; Mn^{2+}, the model group
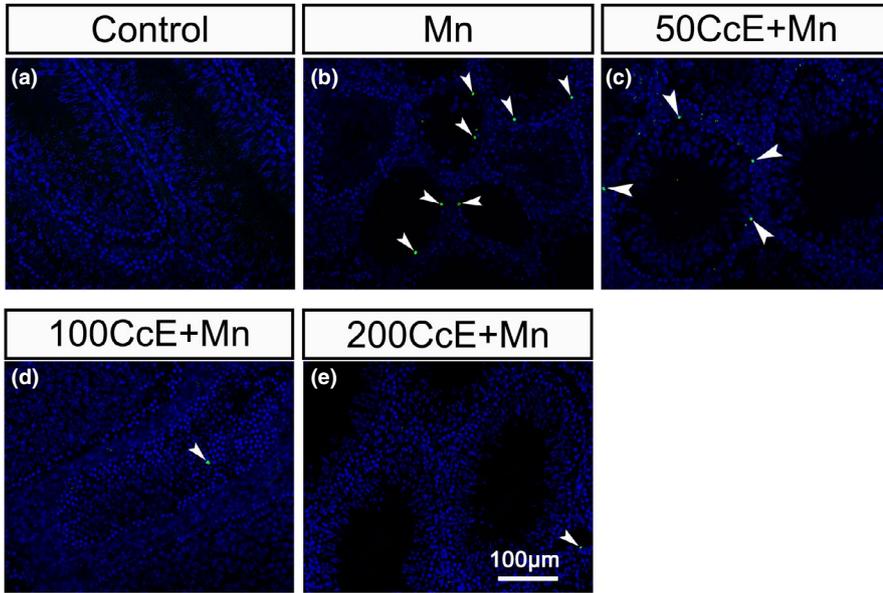
FIGURE 3 Effects of CcE on the cell apoptosis in male rats exposed to Mn^{2+}. (a) control; (b) Mn^{2+}; (c) 50CcE + Mn^{2+}; (d) 100CcE + Mn^{2+}; (e) 200CcE + Mn^{2+}. The apoptotic cells (green) were indicated by white arrowheads; the cell nucleus (blue) were stained with 4′, 6-diamidino-2-phenylindole (DAPI). CcE, *Coridius chinensis* extracts; Mn^{2+}, the model group
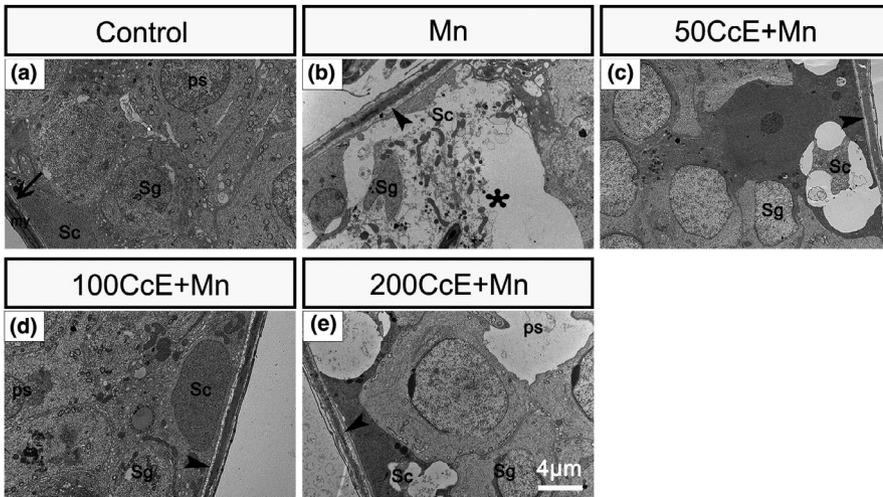
FIGURE 4 Effects of CcE on the ultrastructural changes of testicular tissues in male rats exposed to Mn^{2+}. (a) control; (b) Mn^{2+}; (c) 50CcE + Mn^{2+}; (d) 100CcE + Mn^{2+}; (e) 200CcE + Mn^{2+}. Sg, Spermatogonia; ps, primary spermatocytes; my, myoid cell; basal lamina (arrow), massive plasmarrhexis (asterisks), and swollen between myoid cell and basal lamina (arrowhead). CcE, *Coridius chinensis* extracts; Mn^{2+}, the model group
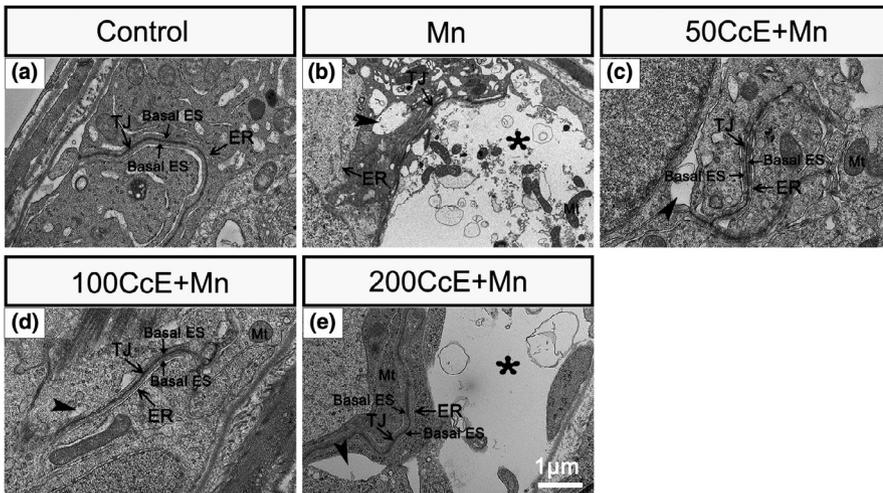
FIGURE 5 Effects of CcE on the ultrastructural features of BTB in male rats exposed to Mn^{2+}. A, control; B, Mn^{2+}; C, 50CcE + Mn^{2+}; D, 100CcE + Mn^{2+}; E, 200CcE + Mn^{2+}.TJ, tight junctions; ER, endoplasmic reticulum; Basal ES, basal ectoplasmic specialization; Mt, mitochondria; cytoplasmic vacuolations (arrowhead) and massive plasmarrhexis (asterisks). CcE, *Coridius chinensis* extracts; Mn^{2+}, the model group

depleted when treated with *C. chinensis* (Figure 3c–e, white arrow-heads). These results showed that *C. chinensis* has the protective effect on Mn^{2+}-induced cell apoptosis.

3.5 | Protective effect of *C. chinensis* on Mn^{2+}-induced testicular ultrastructural damage

Next, we examine the ultrastructural damage and improved effect of testicular tissues caused by Mn^{2+} exposure and *C. chinensis* treatment. As shown in Figure 4, compared with control group, abnormal germinal epithelium was observed in the testicular ultrastructure of Mn^{2+} group. Damaged spermatogenic cells adhesion, degenerated Sertoli cells, swollen between myoid cell and basal lamina (arrowhead), and massive plasmarrhexis were observed in Mn^{2+} group (Figure 4b). However, the testes of the CcE-treated rats showed moderately swollen between myoid cell and basal lamina (arrowhead) and no distinctly massive plasmarrhexis were noted (Figure 4c–e). In 50CcE + Mn^{2+} group (Figure 4c) and 200CcE + Mn^{2+} group (Figure 4e), few degenerated Sertoli cells were observed. The degenerated primary spermatocytes also appeared in 200CcE + Mn^{2+} group (Figure 4e).

3.6 | Protective effect of *C. chinensis* on Mn^{2+}-induced testicular structure damage

Numerous studies have confirmed that the major target site of environmental toxicants was Sertoli cells BTB in the testes, which led to germ cell exfoliation and BTB disruption (Hew et al., 1993; Wiebe et al., 2000). We examined the ultrastructural changes of the BTB. As shown in Figure 5, massive plasmarrhexis and numerous cytoplasmic vacuolations were observed in Mn^{2+} group (Figure 5b). However, no obviously abnormal structure of BTB was noted in CcE-treated groups (Figure 5c–e). Besides, basal ES was dissolved in Mn^{2+}-exposed testis, and no dissolved ER was found in CcE-treated groups (Figure 5c–e).

3.7 | Protective effect of *C. chinensis* on Mn^{2+}-induced testicular damage may be related to c-Src and FAK

In order to study the detailed knowledge of *C. chinensis* on testicular structure damage caused by Mn^{2+} exposure, the expression of BTB associated proteins was analysed by Western blotting. The phosphorylation status of occludin was conferred by FAK at the Sertoli cell BTB, and which also determines the integrity of the occludin-zonula occludens-1 (ZO-1) complex. In our experiments, the Western blotting results suggested that phosphorylation level of p-FAK was remarkably increased and p-Src was slightly increased in Mn^{2+} group (Figure 6a,b). After treated with *C. chinensis*, however, the level of p-FAK was notably reduced and the level of p-Src was also

slightly decline (Figure 6a,b). Moreover, in Mn^{2+}-exposure group, the expression of occludin and claudin1 occurred considerably up-regulated than control (Figure 6c,d). By contrast, the expression of occludin and claudin1 in 100 CcE + Mn^{2+} group was markedly increased when treated with medium concentration of CcE and that of other CcE-treated groups were also increased with different extent (Figure 6c,d). In addition, compared with control, the expression of ZO-1 and junctional adhesion molecule 1 (JAM-A) in Mn^{2+}-exposure group was remarkably diminished, whereas the expression of JAM-A was obviously increased and ZO-1 was slightly increased following CcE treatment (Figure 6e,f). These results showed that the possible protective role of *C. chinensis* on Mn2+-induced testicular structure damage may be related to the down-regulation of c-Src and FAK.

3.8 | N-acetylnoradrenaline from the insect *C. chinensis*

For the purpose of exploring the effective components of *C. chinensis*, CcE was analysed by high-performance liquid chromatography (HPLC). As shown in Figure 7, the HPLC chromatogram of *C. chinensis* sample (Figure 7b), which has a relatively symmetric peak (Figure 7b, red circle) at the retention time of 1.761 min, compared with standard (Figure 7a, red circle). The linear regression equation is $Y = 4141.2X + 9.1932$ ($R^2 = 0.9911$), and the calibration curves of N-acetylnoradrenaline had good linearity over the range of 0.0179–0.179 µg. The peak area of the sample was 137.2628 mAU*S, and the content of N-acetylnoradrenaline was 3.3411%, which was calculated according to the linear regression equation.

4 | DISCUSSION

In the mammalian, the BTB is a layer of barrier structure between spermatogenic tubules and capillary blood, which includes interstitial capillary endothelium, basement membrane, connective tissue, spermatogenic epithelial basement membrane and the tight connection between supporting cells. It can prevent certain substances from entering the sperm epithelium to form and maintain a microenvironment that is conducive to spermatogenesis. It can also prevent sperm antigen substances from escaping outside the seminiferous tubules and causing autoimmune reactions. The BTB is known to be composed of structural protein complexes (e.g. occludin/ZO-1, JAM-A/ZO-1 and claudins/ZO-1) and regulatory factors (e.g. FAK, c-Src, c-Yes and tumour necrosis factor-α; Wong & Cheng, 2005). It has been reported that knockdown of FAK rendered FAK-silenced insensitive to cadmium, which revealed FAK plays an important role in cadmium caused the reproductive injury (Siu, Mruk, et al., 2009). Furthermore, the assembly and maintenance of the BTB also need appropriate relative ratio of FAK to p-FAK-Tyr397 in vitro (Siu, Mruk, et al., 2009). However, it remains unknown whether FAK mediates Mn^{2+}-induced dysfunction of testicular BTB in vivo. In the present study, the results revealed that the ultrastructural features of BTB
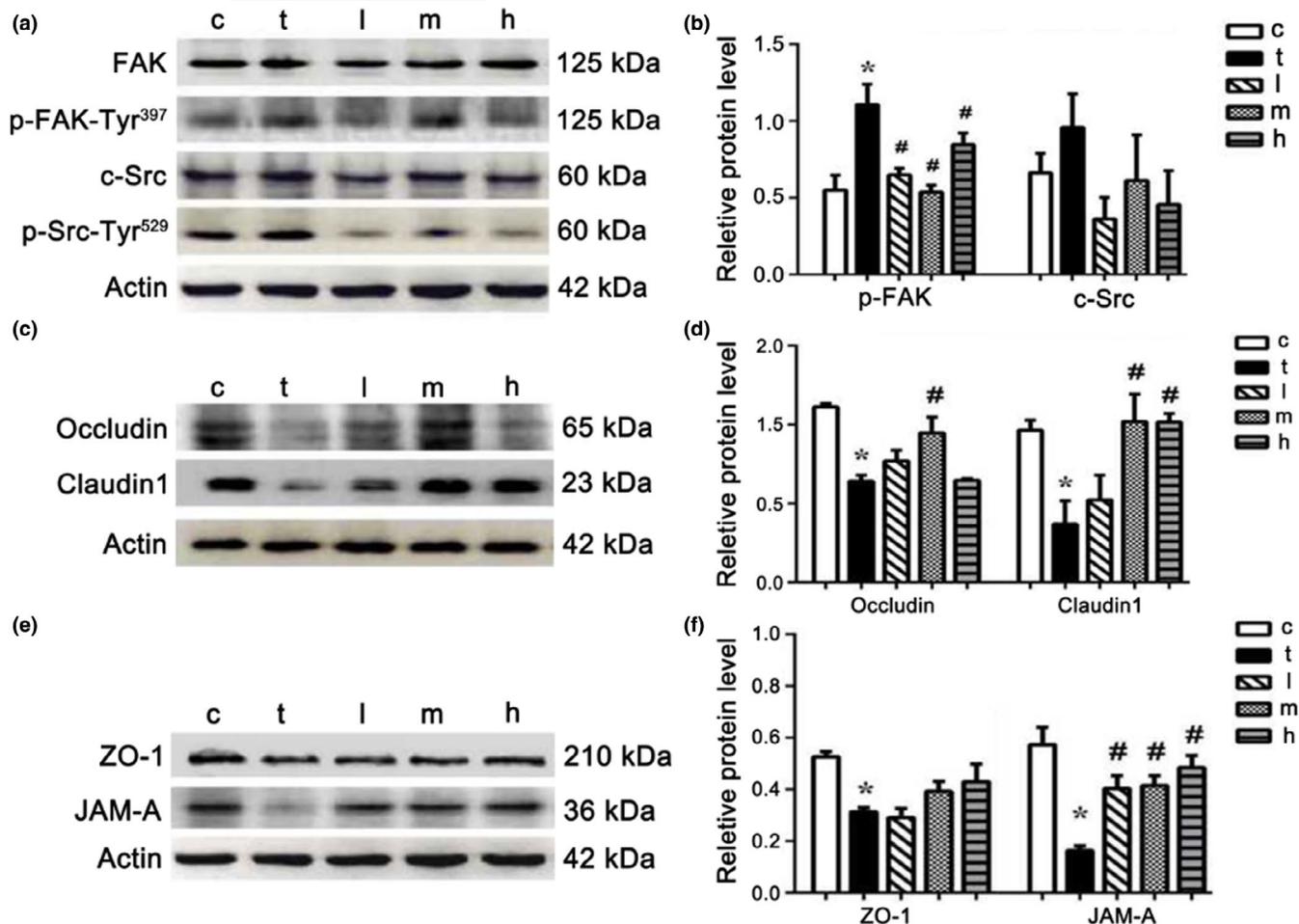
FIGURE 6 Effects of CcE on the expression of BTB associated proteins in male rats exposed to Mn^{2+}. c, control group; t, model group; l, 50 CcE + Mn^{2+}; m, 100 CcE + Mn^{2+}; h, 200 CcE + Mn^{2+}. (a) Western blot gel image detecting p-FAK and p-Src expression. (c) Western blot gel image detecting occludin and claudin1. (e) Western blot gel image detecting ZO-1 and JAM-A expression. (b, d, f) The graph shows the quantification of Western blot results. Data are presented as Mean ± SD. BTB, blood–testis barrier; CcE, *Coridius chinensis* extracts; Mn^{2+}, the model group. *Significant differences ($p < 0.05$) compared with control group; #Significant differences ($p < 0.05$) compared with model group

appeared dissolution and broke when exposed to Mn^{2+}. In addition, to correlate electron microscopy examination with BTB, permeability alteration would be better to clarify the BTB damage in future research. Moreover, the level of p-FAK and p-Src was increased significantly, while the level of occludin, claudin1, ZO-1 and JAM-A was reduced significantly in Mn^{2+} group. In short, environmental toxins such as Mn^{2+} induce testicular dysfunction through the initial impact on the ultrastructure of the testis, thereby destroying germ cell adhesion, leading to the shedding and loss of germ cells, reducing the number of spermatozoon, and ultimately leading to male subfertility or infertility.

Furthermore, numerous experiments have demonstrated that *C. chinensis*, the traditional Chinese medicine, had good medical care functions (Hou et al., 2012; Li & Li, 2010; Wang et al., 2017). However, the detailed understanding of *C. chinensis* remains unclear. Interestingly, in this study, in the adult rat model administered by Mn^{2+}, the testicular ultrastructural was damaged, but it was rescued appropriately when treated with different doses of CcE in treatment

groups. The damaged ultrastructural features of testis were repaired partially, and the level of p-FAK and p-Src was remarkable decreased, while the level of occludin, claudin1, ZO-1 and JAM-A was significant increase in CcE-treated groups. This may indicate that occludin, claudin1, ZO-1 and JAM-A are substrates of FAK, which is consistent with an earlier report (Siu, Mruk, et al., 2009). Meanwhile, we also found that the relative expression of occludin did not increase gradually with the increase in the dose of *C. chinensis*. The therapeutic results indicate that the improvement effect of CcE may be dose-dependent to a certain extent. It is necessary to further explore the optimal dosage in combination with relevant studies.

In addition, our previous studies have shown that the Mn^{2+} exposure decreased the activity of antioxidants and increased the level of ROS (Liu et al., 2019), which was consistent with other previous studies (Khosravi et al., 2019; Liu et al., 2013). It was also reported that the disruption of paracellular permeability induced by oxidative stress was prevented by tyrosine–kinase inhibitors in vitro (Rao et al., 1997). Subsequent experiments have revealed that the
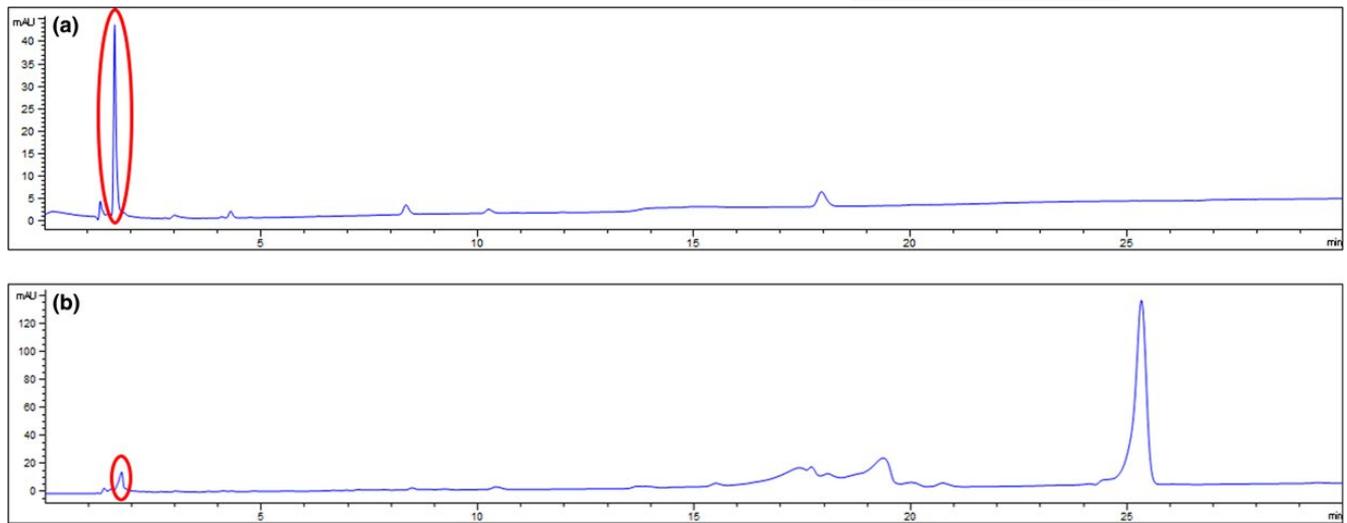
FIGURE 7 High-performance liquid chromatography chromatogram of *Coridius chinensis* and N-acetylnoradrenaline. (a) N-acetylnoradrenaline; (b) *C. chinensis*

phosphorylation level of tyrosine was increased, and the complexes of occluding-ZO-1 and E-cadherin-β-catenin were dynamically reallocated in oxidative stress by tyrosine–kinase-dependent mechanism (Basuroy et al., 2003; Rao et al., 2002). Besides, oxidative stress could cause damage to junction by FAK regulated by PI3K/c-Src (Sen et al., 2006). Thus, it is noteworthy that oxidative stress was induced by Mn^{2+} exposure and which could cause epithelial and endothelial permeability increased, then induced male BTB dysfunction and finally led to germ cells loss. These findings thus imply manganese can cause reproductive systems damage through different signalling pathways.

In recent studies, more and more attention has been given to the isolation of natural products from *C. chinensis* (Luo et al., 2012; Shi et al., 2014). N-acetylnoradrenaline extracted from *C. chinensis* have been identified, but its function in reproductive protection is unclear in in vivo (Xu et al., 2020). Our study in Mn^{2+}-exposed rats further suggested that the N-acetylnoradrenaline extracted from *C. chinensis* may have protective effect on the BTB damage. However, there are still many unknowns on the effects of N-acetylnoradrenaline present in *C. chinensis*. In future studies, we will focus on analysing the role of the isolated components of *C. chinensis* through in vivo and in vitro experiments.

In summary, these results of this study proved that Mn^{2+} exposure in the rats could induce testicular spermatogenesis dysfunction and germ cells exfoliation. However, the abnormal phenotypes were significantly improved by *C. chinensis* intervention. Furthermore, the level of BTB associated proteins was obviously increased and p-FAK was significantly decreased, indicating c-Src and FAK might be involved in the repair of Mn^{2+}-induced testicular injury by *C.chinensis*, but further research is needed.

CONFLICT OF INTEREST

The authors declare to have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been included in the manuscript, and additional information, if required, is available from the corresponding author upon reasonable request.

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