褪黑素对丙烯酰胺致睾丸毒性保护作用的机制

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摘　要：【目的】探讨褪黑素(MT)对丙烯酰胺(AA)诱发睾丸毒性的改善作用及机制。【方法】动物随机分为3组：对照组，AA染毒组和AA+MT治疗组，每组各12只大鼠。AA染毒的动物连续4周每日灌胃AA(15 mg/kg)。从染毒3周后开始，治疗组动物先每日腹腔注射MT 10 mg/kg(连续1周)，30 min后再进行AA染毒。对照组动物仅注射同等剂量的生理盐水。随后分别进行线粒体膜电位、TUNEL、免疫印迹和电镜检测。【结果】研究结果表明AA可使睾丸组织线粒体膜电位下降($P < 0.05$)，且MT可令其恢复($P < 0.05$)。和对照组相比，MT可下调睾丸组织中Bax的表达；上调Bcl-2的表达。TUNEL结果进一步证实MT可减缓解睾丸组织中细胞凋亡现象。免疫印迹结果表明AA染毒组Bcl-2/Bax和Bcl-xL/Bak的比值较对照组降低(P皆< 0.05)，而MT治疗组中它们的比值升高(P皆< 0.05)。AA染毒组中Cyt-c，Casp-3，p53和NF-κB的表达水平较对照组明显升高(P皆< 0.05)，较MT治疗组明显下降(P皆< 0.05)。电镜结果证实MT可减轻AA造成的睾丸曲细精管中线粒体结构受损。【结论】MT可能通过和线粒体通路相关的抗凋亡功能，从而缓解AA造成的睾丸毒性。

关键词：褪黑素；丙烯酰胺；睾丸毒性；凋亡；氧化损伤
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Protective Roles of Melatonin against Acrylamide-induced Testicular Toxicity in Rats

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Abstract: 【Objective】The present study aimed to determine the protective role of Melatonin (MT) against Acrylamide (AA)-induced testicular toxicity and the potential molecular mechanism. 【Methods】The animals were randomly divided into three groups, the control group ($n = 12$), the AA group ($n = 12$) and the AA+MT group ($n = 12$). The rats in the AA and AA + MT group were gavaged with AA at a dose of 15 mg/(kg·day) for 4 consecutive weeks. After 3 weeks of AA treatment, MT was intraperitoneally injected 30 minutes before AA treatment at 10 mg/(kg·day) for 1 week in the AA + MT rats. Subsequently, the mitochondrial membrane potential measurement, TUNEL assay, Western blot and electron microscopic techniques were applied in the present study. 【Results】The results showed that AA could decrease the testis mitochondrial membrane potential ($P < 0.05$) which could be recovered by MT ($P < 0.05$). Moreover, MT induced down-regulation of Bax expression and up-regulation of Bcl-2 expression in the testis, compared with AA rats. The amelioration of testicular apoptosis was further confirmed by the TUNEL labeling. Western blot results suggested that the decreased ratios of Bcl-2/Bax and Bcl-xL/Bak in the AA group (both $P < 0.05$) could be recovered by MT treatment (both $P < 0.05$). The levels of Cyt-c, Casp-3, p53 and NF-κB in AA group were markedly elevated compared with the control (all $P < 0.05$), and reduced in MT treatment group (all $P < 0.05$). MT could relieve abnormal mitochondrial structures in the seminiferous tubule in the electron microscopic level. 【Conclusion】MT may exert productive effect through its anti-apoptotic properties associated with mitochondria.

Key words: melatonin; acrylamide; testicular toxicity; apoptosis; oxidative damage
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Acrylamide (AA) is a highly water-soluble micromolecular organics that widely used in both industries and laboratories[1]. Individuals can be exposed to AA through skin contact and breathing. Therefore AA is regarded as an importantly toxic chemical compound for occupational exposure[2-3]. Besides, non-occupational population exposure to AA from foods and drinking water in the daily life has attracted considerable attentions. It was reported that the formation of AA is related to high temperature (more than 120 ℃) treatment for certain carbohydrate-rich foods and low-protein diets[4-5]. Also, AA is one product of burning tobacco[6]. Previous studies showed that AA is a potential neurotoxicant[7], rodent carcinogen and possible human carcinogen[8-10]. In addition, male reproductive toxicity of AA has been reported in our previous study[11] and other studies[12-13], which mainly including testicular damages such as vacuolation and swelling of the spermatids, DNA breakage in germ cells, and abnormal morphology of the head and tail of sperms. Melatonin (MT), a neuroendocrine hormone synthesized and secreted mainly by the pineal gland in mammals, possesses extensive physiological functions including regulating circadian rhythm, maintaining function of sex gland, serving as free radicals scavenging and antioxidant as well as antinociceptive agent[14-21]. Several researches suggested that a reduction of melatonin was related to various neurodegenerative diseases as well as ischemic brain injury[22-24]. The biosynthesis of MT is largely associated with human age, that is, MT is secreted with the highest level by 3 ~ 5 years old young children, and gradually decreased along with age increase[25]. It has been reported that MT with concentration of 0.6 ~ 5.0 pg/mL exists in the seminal fluid of normal person[26]. Weaver indicated that MT exerts important effects through modulating the antioxidation and immuno-regulation process in maintainability of male reproductive function[27]. The aforementioned studies imply a potential of MT to be a novel therapeutic agent for reproductive toxicity induced by AA. Mitochondrial membrane potential measurement, TUNEL assay, Western blot and electron microscopic techniques were conducted in this study which demonstrated a protective role of MT against AA-induced testicular toxicity, as well as the potential molecular mechanism. The results are conducive for further understanding the physiological role of MT and provide a theoretical direction in preventing and relieving the testicular toxicity induced by AA or other analogous chemicals.

1 Materials and methods

1.1 Experimental animals

Thirty-six three-week-old postweaning male Sprague-Dawley rats weighing 52 ~ 60 g at the beginning of experiment were supplied by Animal Experimental Center of Guangdong Province. The experimental procedures were approved by the Ethics Committee for Animals, Guangdong Pharmaceutical University and strictly conformed to the National Institutes of Health Guide for the Care and Use of Animals in Research. The animals had ad libitum access to water and standard rat diet, which were housed in an animal room under 12h/12h light-dark cycle.

This experiment was performed according to ethical rules.

1.2 Study design and treatment

The animals were randomly divided into three groups, i.e. the control group (n = 12), the AA group (n = 12) and the AA + MT group (n = 12). In each group, four rats were used for mitochondrial membrane potential measurement, four were used for TUNEL assay and electronic microscopic detection, and the remaining four were used for Western blot.

The rats in the AA and AA + MT group were gavaged with AA (dissolved in 0.9% saline, Sigma) at a dose of 15 mg/kg each day for 4 consecutive weeks. After 3 weeks of AA treatment, MT(dissolved in 5% ethanol/saline solution, Sigma) was intraperitoneally injected 30 minutes before AA treatment at 10 mg/kg each day for 1 week in the AA + MT rats. The dose of MT was chosen based on previous

studies[28-31]. The rats in the normal group were given 0.9% saline of the same volume.

1.3 JC-1 mitochondrial membrane potential measurement

Four rats per group were sacrificed under pentobarbital anesthesia (50 mg/kg), testis was extracted, stored at -80 ℃ until using. JC-1 was used to measure the mitochondrial membrane potential changes of testis in the different groups according to the manufacturer's direction (Beyotime). The suspension was incubated with JC-1 staining solution (5 μg/mL) at 37 ℃ for 30 min and rinsed for three times. The JC-1 dye exhibits potential-dependent accumulation in mitochondria and aggregates formation. The mitochondrial JC-1 monomers (Excitation in 488 nm; Emission in 590 nm) and aggregates (Excitation in 530 nm; Emission in 590 nm) were detected using a multifunctional microplate reader (Spectra Max M5, Molecular Devices). The membrane potential in each group was calculated as the ratio of red (aggregates)/green (monomers) fluorescent intensity value.

1.4 TUNEL assay

Four rats per group were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffered saline (PBS, pH 7.4, 4℃). Testes were removed and post-fixed overnight at 4℃. Sections were cut at 30 μm using a vibratome, stored at 4℃ until use. To examine whether testicular cells underwent apoptosis following AA treatment, TUNEL assay was performed by an in situ cell death detection kit (POD, Roche), followed by Hoechst 33342 staining. Sections were applied to TUNEL labeling according to the manufacturer's instructions. All images were captured using a fluorescence microscope (Olympus).

1.5 Western blot

Four rats of each group were decapitated after being anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the testes were extracted, and homogenized in a freshly prepared lysis buffer with protease inhibitors. The homogenate was centrifuged at 12 000 r/min for 30 min (r = 12 cm), and the protein concentration was determined using BioRad DC protein assay (BioRad, Laboratories). Samples were separated on an SDS-PAGE gel (10% gradient gel) and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% skim milk for 2-4 h at room temperature and incubated overnight at 4℃ with one of the following primary antibodies: rabbit anti-Bcl-2 (1∶100, Abcam), rabbit anti-Bcl-xL (1∶1000, Abcam), rabbit anti-Bax (1∶1000, Abcam), mouse anti-Bak (1∶500, Abcam), rabbit anti-Cyt-c (Cytochrome-c) (1∶1000, Cell Signaling Technology), rabbit anti-Casp-3 (Caspase-3) (1∶1000, Millipore), rabbit anti-p53 (1∶1000, Abcam), rabbit anti-NF-κB p65 (Nuclear factor kappa B) (1∶2000, Abcam), and mouse anti-β-actin (1∶1000, Millipore). After washed with TBST for 4 times, membranes were incubated with homologous horseradish peroxidase (HRP)-conjugated secondary antibodies (1∶3000, Amersham Biosciences) for 2 h at room temperature. The membranes were washed with TBST for 4 times. Blots were visualized in enhanced chemiluminescence (ECL) kit (Pierce) for 5 min and exposed to hyperfilms (Kodak) for 1-10 min. Western blots were made in triplicate. The optical density of each labeled band was measured using Image J software.

1.6 Electron microscopic detection for testis

Pieces of tissue were cut from the testis of four rats per group, postfixed for 1 h in 1% osmium tetroxide (OSO4), dehydrated in a graded series of ethyl alcohols, impregnated with 1% uranyl acetate in 100% alcohol, flat embedded in 100% Epon 812 resin, and then mounted on microscopic slides. The ultrathin sections were cut from these specimens with a Leica ultramicrotome at 70 ~ 80 nm thick. These sections were mounted on collodion-coated mesh grids, stained with 0.4% lead citrate and 4% uranyl acetate, and finally examined and captured with an electron microscope (FEI).

1.7 Statistical analysis

All experimental data are presented as mean ± SD (standard deviation) in this study. The software SPSS 16.0 was used for all statistical analyses. Com-

parisons among multiple groups involved one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be significant.

2 Results

2.1 The measurement of mitochondrial membrane potential in the testis of different experimental rats

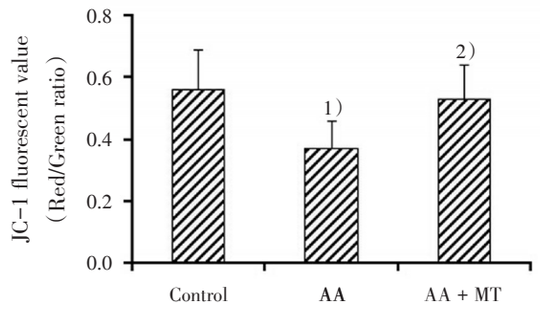
To determine the toxic effects of AA as well as the potential protective role of MT on testicular mitochondrial function, membrane potential as an indicator of mitochondrial activity in all experimental groups was evaluated. The results showed that the membrane potential in the testis of AA group was significantly decreased compared to the control (F = 101.16, P = 0.019, Fig.1), suggesting that mitochondrial function was dysregulated by AA exposure. Importantly, after MT treatment, the testis of AA + MT rats exhibited higher membrane potential in comparison with that of AA rats (F = 85.53, P = 0.026, Fig. 1).

2.2 The results of TUNEL assay in the testis of different experimental rats

Hoechst 33342 and TUNEL fluorescent double-labeling was used to detect the testicular apoptosis induced by AA and the potential protection of MT. The results showed that the positive cells labeled by TUNEL in the AA group was more than that in the control group, while these positive cells in the AA + MT group was less than that in the AA group (Fig.2). Similar results were also observed in the double-labeled cells, suggesting a protective role of MT against the testicular apoptosis induced by AA.

2.3 The expression of Bax, Bak, Bcl-2, Bcl-xL, Cyt-c, Casp-3, p53 and NF-κB in the testis of different experimental rats

Western Blot was applied to compare the expression levels of Bax, Bak, Bcl-2, Bcl-xL, Cyt-c, Casp-3, p53 and NF-κB proteins related to apoptosis pathway in the testis of different experimental groups. The results revealed that in the AA group, the expression levels of Bax and Bak were elevated



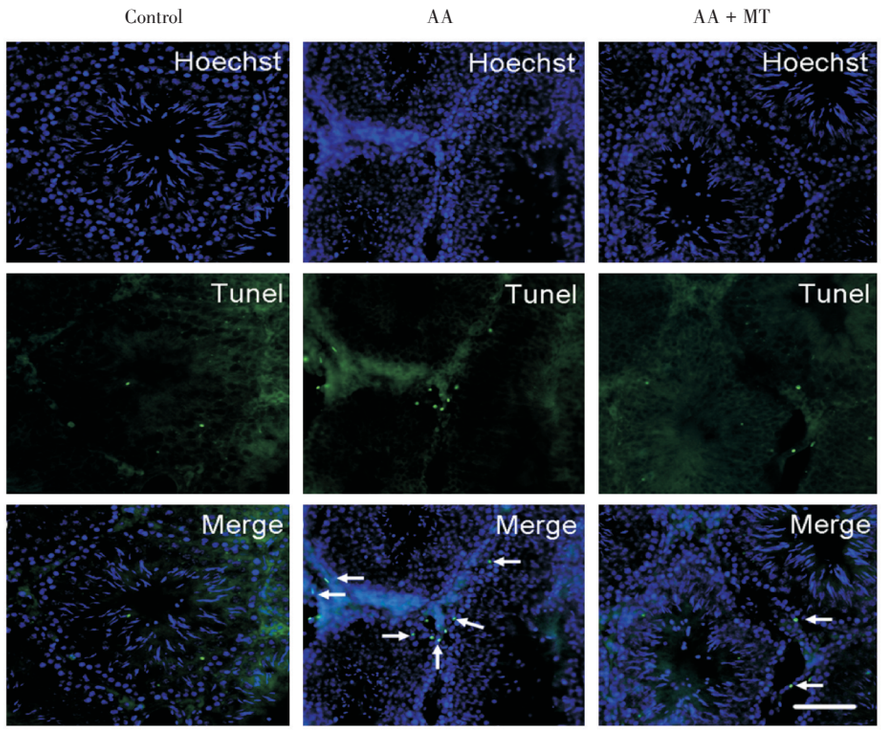
JC-1 red/green fluorescent intensity value was calculated in the testis of different experimental rats. AA is short for acrylamide, MT is short for melatonin. ANOVA, 1) $P < 0.05$, compared with the control; 2) $P < 0.05$, compared with the AA group. Each bar represents the mean ± SD of 4 rats.

Fig.1 The measurement of mitochondrial membrane potential

while those of Bcl-2 and Bcl-xL were reduced compared with the control (all $P < 0.05$, Fig 3A and 3B). However, MT treatment could up-regulate the expression of Bcl-2 and Bcl-xL, and down-regulate the expression of Bax and Bak (all $P < 0.05$, Fig.3A and 3B). In addition, the ratios of Bcl-2/Bax (F = 90.21, P = 0.014) and Bcl-xL/Bak (F = 66.68, P = 0.021) in the AA group were obviously decreased in comparison with the control (Fig. 3C). However, the ratios of these values were increased after MT treatment compared with the AA group (Bcl-2/Bax: F = 77.93, P = 0.019; Bcl-xL/Bak: F = 48.01, P = 0.023, Fig.3C). Moreover, the expression levels of Cyt-c, Casp-3, p53 and NF-κB in the AA group were markedly elevated with different degrees compared with the control (F = 92.71, P = 0.017; F = 157.32, P = 0.013; F = 37.82, P = 0.024; F = 74.73, P = 0.018, successively). However, MT treatment could down-regulate the expression of those proteins compared with the AA group (F = 92.72, P = 0.015; F = 28.02, P = 0.028; F = 57.32, P = 0.016; F = 49.74, P = 0.027, successively, Fig. 3D and 3E).

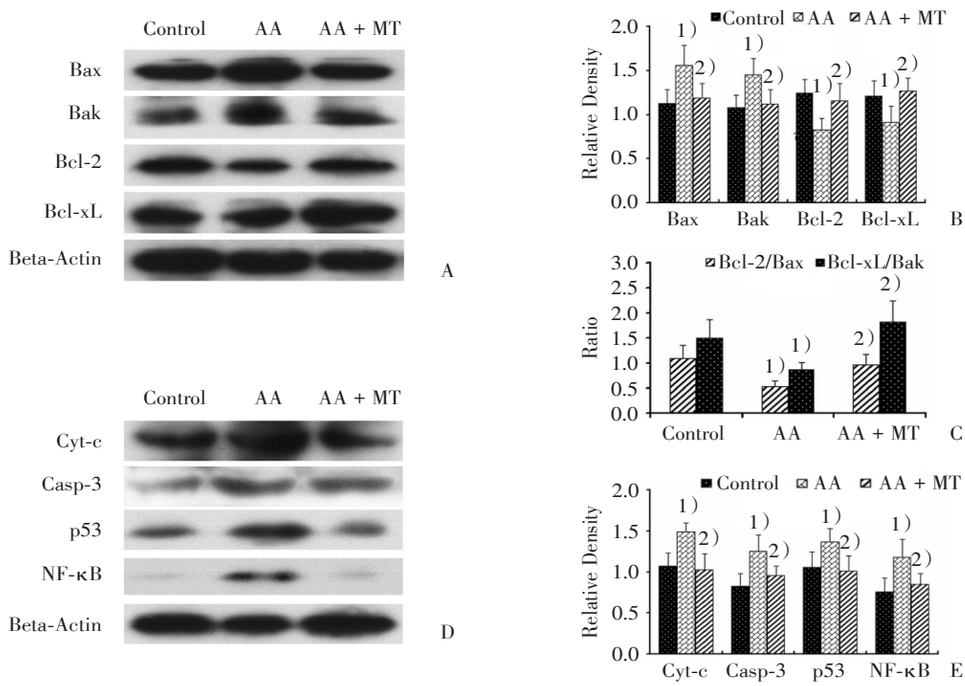
2.4 The examination of tesicular ultrastructure in different experimental rats

Electron microscope was performed to further illustrate the aforementioned dysfunction of the mitochondria occur in the testis of AA rats. The ultrastruc-



The images showed Hoechst 33342 and TUNEL double-labeled cells (as shown in white arrows) in the testis of the control group (n = 4), the AA group (n = 4) and the AA + MT group (n = 4), respectively. AA is short for acrylamide, MT is short for melatonin. All images are the same magnification, and scale bar=100 μm

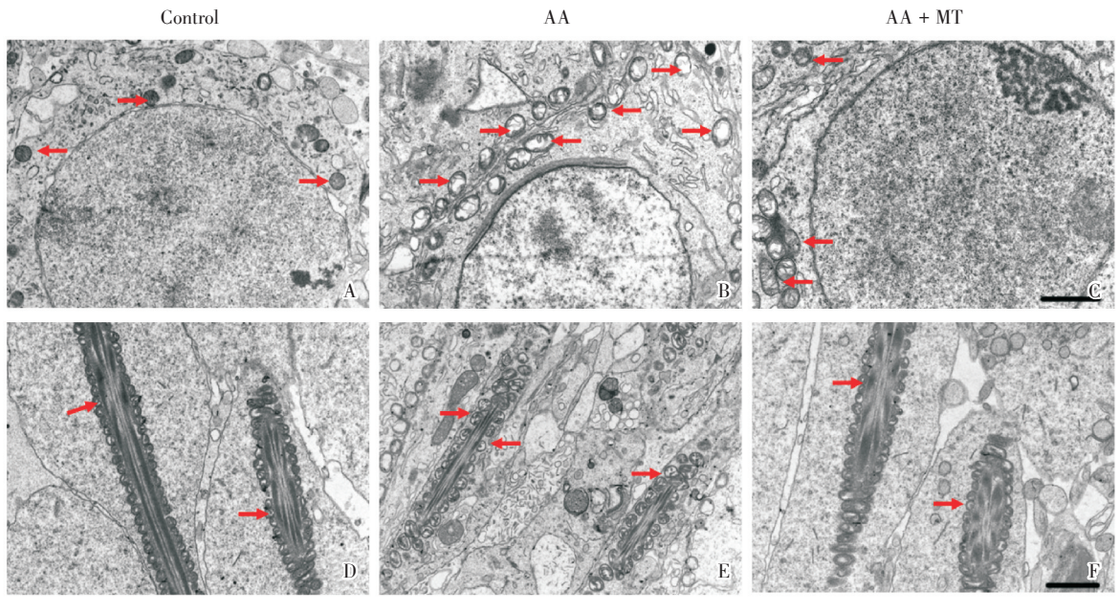
Fig.2　The apoptosis detection of the testis using TUNEL labeling



The Western blot results showed the expression levels of different proteins in the control group, the AA group and the AA + MT group, respectively. AA is short for acrylamide, MT is short for melatonin. ANOVA, 1) $P < 0.05$, compared with the control; 2) $P < 0.05$, compared with the AA group. Each bar represents the mean ± SD of 4 rats.

Fig.3　Comparison of protein expression of apoptotic-relative factors in the testis among different groups

ture of testicular seminiferous tubules in all experimental groups can be observed clearly under electron microscope. The results showed that in the AA group, the mitochondrial structures appeared swelling and showed different degrees of vacuolization, especially disruption of the mitochondrial crista in the seminiferous tubule of rat testis. Besides, their mitochondrial sheaths underwent hydroncus and deformation in the middle segment of sperm tails (Fig. 4B and E). After MT treatment, swollen or disrupted mitochondrias and th qeir sheaths had improvement to some extent (Fig. 4C and 4F).



The images A–C showed the ultrastructure of the seminiferous tubules, while D–F showed the ultrastructures of longitudinal section of the middle segment of sperm tails in the testis of the control group ($n = 4$), the AA group ($n = 4$) and the AA + MT group ($n = 4$), respectively. Notably, the mitochondrial structures (A–C) and mitochondrial sheath (D–F) were indicated by red arrows in all images. AA is short for acrylamide, MT is short for melatonin. A and D: Control; B and E: the AA group; C and F: the AA + MT group. Images A–C are the same magnification, and scale bar = 1 μm; images D–F are the same magnification, and scale bar = 0.5 μm.

Fig.4 The electron microscopic detection of rat testis in the control and the experimental groups

3 Discussion

The JC–1 staining results showed that the exposure to AA could induce decreased mitochondrial membrane potential in the testis. However, after MT treatment, the testis of these rats exhibited higher membrane potential value in comparison with that of AA rats. The decreased mitochondrial membrane potential in the testis is a symbolic event for the early stage of cell apoptosis. The AA–induced swelling and vacuolization of mitochondrial structures in the seminiferous tubule and deformation of the mitochondrial sheaths in the middle segment of sperm tails were observed with electron microscopic detection. Importantly, MT could effectively relieve these disrupted symptoms about mitochondrial function of the testis. The results further suggest that MT could effectively alleviate AA–induced testicular histopathological damage and dysregulation of the mitochondrial function through its anti–apoptotic effects. And, mitochondria may be the target site where MT exerts its anti–apoptotic action.

Ozen et al. reported that cell apoptosis accompanied with lipid peroxidation occurs in the testis of rats following formaldehyde treatment, while MT could relieve the oxidative damage and cell apoptosis in the testis[32]. The Bcl–2 family proteins govern the mitochondria–dependent pathway in apoptosis and can regulate apoptosis by forming hetero and homodi-

mers in the mitochondrial membrane. The ratio of anti-apoptotic factor (Bcl-2 or Bcl-xL) to pro-apoptotic factor (Bax or Bak) is often used in determining apoptosis[33]. In this study, we compared the expressions of Bax and Bcl-2 as well as cell apoptosis in the testis of the AA rats and the AA + MT rats. Western blot results showed that MT could down-regulate Bax expression and up-regulate Bcl-2 expression in the testis compared with AA rats. Furthermore, decreased expression of Bcl-2, Bcl-xL and increased expression of Bax, Bak resulted in marked decreases in Bcl-2/Bax and Bcl-xL/Bak ratios of AA rat testis. In contrast, the ratios of Bcl-2/Bax and Bcl-xL/Bak the testis were recovered after MT treatment. The changes of Bcl-2/Bax and Bcl-xL/Bak ratios might lead to impaired permeability and lower potential of the mitochondrial membrane in testis. Moreover, the number of positive cells labeled by TUNEL in the AA group was detected, while these positive cells in the AA + MT group were obviously decreased under light microscopy. These results suggest that cell apoptosis occurs in the testis of the AA rats which can be recovered or relieved by MT treatment.

It has been reported that the up-regulation of the pro-apoptotic protein Bax could lead to the over-expression of casp-3[24], which as an executioner to initiate apoptosis. Thus, subsequently, several relevant apoptotic factors such as Cyt-c, Casp-3, p53 and NF-κB were detected in the present study. It is well known that Cyt-c is associated with the inner mitochondrial membrane and serves as an essential component in the electron transfer chain. The increased mitochondrial permeability can release some proteins located on the inner mitochondrial membrane to cytosol, including Cyt-c and apoptosis-inducing factor (AIF) which can activate caspase and caspase-dependent apoptosis pathway[34-36]. Casp-3 has normally been considered as an executioner caspase[37]. In this study, the expression of Cyt-c and Casp-3 were markedly elevated in the testis of AA rats. MT treatment could suppress the activation of Casp-3 in association with decreased Cyt-c expression. The tumor suppressor p53 can induce cell-suicide process or the imposition of reversible growth inhibition phenomenon[38]. Several researches suggested that p53 expression could down-regulate the expression of anti-apoptotic factor Bcl-2[39], and in turn higher expression of Bcl-2 could block the apoptosis induced by p53[40]. In addition, the pro-apoptotic factor Bax is a direct transcriptional target of p53[41]. The restoration of p53 in the murine leukaemia cell M1 was associated with increased expression of Bax[40-42]. Our results were conformed to those studies that the expression level of p53 was markedly elevated accompanied with increased Bax and decreased Bcl-2 in the testis of AA rats. However, MT treatment could down-regulate the expression of p53 and Bax and up-regulate the expression of Bcl-2. NF-κB is a transcription factor that as a candidate for the regulation of spermatogenic cellular apoptosis. It is well known that a variety of extracellular signals could activate the expression of NF-κB, such as interleukin-1 (IL-1), tumor necrosis factor α (TNFα), active oxygen and free radicals. The activation of NF-κB could up-regulate the expression of pro-apoptotic factor Bcl-xs in hippocampal neurons of cerebral ischemia animals[43]. Our results showed that MT treatment could down-regulate the relative high NF-κB expression in the testis induced by AA. One possible mechanism is that it depends on NF-κB activate either pro-apoptotic factor or anti-apoptotic factor in this model as a result of Bcl-2 family proteins as the target gene of NF-κB.

In conclusion, the results of the present study indicate that the up-regulation of relevant apoptotic factors induced by AA treatment may result in the formation of pores in the mitochondrial membranes, and lead to increased mitochondrial permeability and mitochondrial dysfunction in the testis of AA rats. We suppose that MT can perform an effectively protection against AA-induced testicular toxicity in rats, which may exert productive effect through its anti-apoptotic properties associated with mitochondria. But the relevant mechanisms still need further investigations. In addition, another experimental study will be designed to evaluate whether melatonin improves the quality of sperm in adult male AA-rats.

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