

Asiatic acid attenuates high-fat diet-induced impaired spermatogenesis

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Abstract. Testicular cell apoptosis is associated with impaired spermatogenesis. It has been reported that Asiatic acid (AA) may suppress apoptosis. However, little is known about the effect of AA on high-fat diet (HFD)-induced impairment of spermatogenesis. The aim of the present study was to determine whether AA protects against HFD-induced impairment of spermatogenesis. Sprague-Dawley rats were randomly divided into three groups: Control group, HFD group and AA (50 mg/kg) + HFD group. Rats fed an HFD were orally administered with AA (50 mg/kg) daily for 12 weeks, and blood samples, testis and epididymis were harvested for further analysis. Sex hormones were detected and hematoxylin and eosin staining was performed to examine the morphological changes of the testis. Semen samples were collected to evaluate sperm quality and apoptosis was determined. The results indicate that AA treatment significantly increased testis weight, testis/body weight, spermatogonia, Leydig cells and Sertoli cells in the testis of obese mice ($P<0.05$). AA treatment also attenuated HFD-induced histological change. AA treatment prevented HFD-induced decrease of sex hormones and the quality of semen samples ($P<0.05$). Furthermore, HFD-induced apoptosis was significantly attenuated by AA treatment ($P<0.05$). In conclusion, the results suggest that AA is able to ameliorate HFD-induced impaired spermatogenesis via inhibiting apoptosis in Sprague-Dawley rats. AA may have therapeutic value in the treatment of obesity-related impairment of spermatogenesis.

Introduction

Infertility is defined as the inability to conceive following >1 year of regular unprotected sexual intercourse and now

affects 10-15% of couples of reproductive age (1,2). Defective sperm function is a common contributing factor, accounting for 30-40% of couples attending infertility clinics (3). Obesity, which is the sixth most important risk factor contributing to the overall burden of infertility worldwide (4), has been reported to impair male infertility (5,6). Accumulating evidence suggests that male infertility is regulated by an orchestrated network comprising numerous pathological changes (7,8). The mechanisms underlying obesity-associated infertility are complex and remain unclear; however, previous studies have indicated that excessive cell apoptosis serves an important role (7,9-11).

Apoptosis is essential for cellular homeostasis and male germ cell development (12,13). Increased apoptosis has been observed in the spermatozoa of male patients with infertility, as well as in the sperm of infertile mice (14,15). High-fat diet (HFD) has been demonstrated to induce apoptosis in rodents, which in turn promotes the progression of infertility (16). Conversely, inhibiting excessive apoptosis attenuates HFD-induced impairment of spermatogenesis (17). Therefore, pharmacological agents that are able to inhibit testicular cell apoptosis are of great therapeutic interest.

Asiatic acid (AA), which is a pentacyclic triterpene isolated from *Centella asiatica*, has been demonstrated to possess a number of pharmacological activities (18). AA is able to protect against cardiac hypertrophy (19), reduce islet fibrosis in animal models of diabetes (20) and ameliorate hepatic lipid accumulation (21). Furthermore, previous studies have indicated that AA attenuates glutamate-induced apoptosis in SH-SY5Y cells (22) and inhibits apoptosis in the striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice (23). However, the effect of AA on obesity-induced impaired spermatogenesis has not yet been reported. The aim of the present study was to investigate whether AA is able to protect against HFD-induced defective spermatogenesis function.

Materials and methods

Reagents. AA (purity, 97%; cat. no. 546712) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rabbit anti-B-cell lymphoma (Bcl)-x1 antibody (cat. no. ab32370), rabbit anti-Fas antibody (cat. no. ab82419, and anti-GAPDH antibody (cat. no. ab8245) were obtained from Abcam (Cambridge, UK). Anti-Bcl-2 antibody (cat. no. 2870) and anti-Bcl-2-associated X protein (Bax) antibody (cat. no. 2722) were purchased from

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Table I. Effects of AA on testis weight, germ cell count and sperm quality.

Parameter	Control	HFD	AA + HFD
Testis weight (g)	3.32±0.13	2.64±0.09 ^a	2.86±0.09 ^b
Testis weight/body weight (g/kg)	8.11±0.39	5.52±0.14 ^a	6.54±0.21 ^b
Spermatogonia (number/field)	25.26±2.18	15.52±1.79 ^a	23.10±1.39 ^b
Leydig cells (number/field)	8.21±0.15	4.53±0.33 ^a	5.21±0.08 ^b
Sertoli cells (number/field)	9.21±0.14	5.34±0.24 ^a	7.16±0.13 ^b
Sperm concentration (x10 ⁶ /ml)	57.61±4.21	45.36±3.17 ^a	53.11±2.54 ^b
Sperm viability (%)	93.39±2.65	85.31±1.70 ^a	92.02±1.33 ^b
Sperm motility (%)	75.07±1.60	66.28±1.88 ^a	71.10±1.48 ^b

Data are expressed as the mean ± standard deviation. ^aP<0.05 vs. control and ^bP<0.05 vs. HFD. AA, Asiatic acid; HFD, high fat diet.

Cell Signaling Technology, Inc. (Danvers, MA, USA). TUNEL kits (cat. no. 11684817910) were purchased from Roche Applied Science (Penzberg, Germany).

Animal treatment. All experiments in the present study were performed in compliance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of Puai Hospital of Huazhong University of Science and Technology (Wuhan, China). A total of 24 adult male Sprague-Dawley rats (180–200 g, 8–9 weeks) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). All rats were housed with a 12-h light/dark cycle at 20–25°C and 50±5% humidity, with *ad libitum* access to food and water. Rats were randomly divided into three groups: Control group (n=8), HFD group (n=8) and the AA + HFD (n=8) group. Rats in the control group were fed with a normal diet, whereas the other rats were fed with an HFD (protein, 18.1%; fat, 61.6%; carbohydrates, 20.3%) for 12 weeks to induce obesity. AA was dissolved in 1% CMC-Na as a vehicle for *in vivo* experiments. Rats in the AA + HFD group were orally administered with 50 mg/kg AA once per day for 12 weeks, and the other two groups received the same volume of vehicle as control. At the end of the study period, rats were euthanized with an overdose of sodium pentobarbital (200 mg/kg, Sigma-Aldrich; Merck KGaA) and blood samples were harvested from the abdominal aorta for further analysis. Finally, rats were sacrificed via cervical dislocation and testes and adipose tissues were harvested.

Histological analysis. Testis samples were fixed in 4% paraformaldehyde at room temperature for 24 h, dehydrated and embedded in paraffin. Tissues were cut into 5-μm sections for further analysis. The sections were stained with hematoxylin and eosin at room temperature and observed under a light microscope (magnification, x400; E100; Nikon Corporation, Tokyo, Japan). In each group, 30 fields in 6 rats were randomly selected and the number of spermatogonia, Leydig cells and Sertoli cells were calculated using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Blood pressure. Prior to sacrifice, rats were anesthetized using 0.5% isoflurane (Sigma-Aldrich; Merck KGaA), and a microtip

catheter transducer (SPR-839; Millar, Inc., Houston, TX) was inserted into the right carotid artery and left ventricle to detect the systolic blood pressure according to the manufacturer's protocol.

Hormone detection. Serum was collected from the tail vein of animals and fasting insulin was determined using a rat insulin ELISA Kit (cat. no. EZRMI-13K, EMD Millipore, Billerica, MA, USA) 3 days prior to sacrifice. Sex hormones were detected using kits for estradiol (E2; cat. no. E-EL-R0065c), testosterone (T; cat. no. E-EL-R0072c), follicle stimulating hormone (FSH; cat. no. E-EL-R0391c) and luteinizing hormone (LH; cat. no. E-EL-R0026c) purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China) according to the manufacturer's protocol. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as previously described (24).

Semen analysis. Isolated epididymides were immediately placed in Ringer's solution (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) and cut into pieces. The concentration, viability and motility of sperm were determined as previously described (25). The sperm gradually left the epididymis and semen samples were carefully collected. The number of sperm was counted using a hemocytometer (AMQAX1000, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the concentrations were calculated according to the manufacturer's protocol. Eosin-nigrosin staining solution was used to determine sperm viability at room temperature for 5 min, and light microscopy (magnification, x400) was used to observe the spermatozoa. Using this staining live spermatozoa are white in color, whereas dead spermatozoa are pink or red (26). Sperm motility was detected by computer-assisted sperm analysis (CASA). Sperm was incubated in Ringer's solution at room temperature for 30 min and subsequently placed in CASA assay chambers (Hamilton Thorne Research, Beverly, MA, USA). Sperm tracks (1.5 sec, 30 frames) were captured (frequency, 60 Hz) and further analyzed by HTM-IVOS Sperm Analyzer software (version 12.2L; Hamilton Thorne Research) (27).

Immunohistochemistry and TUNEL staining. Immunohistochemistry was performed to detect the expression

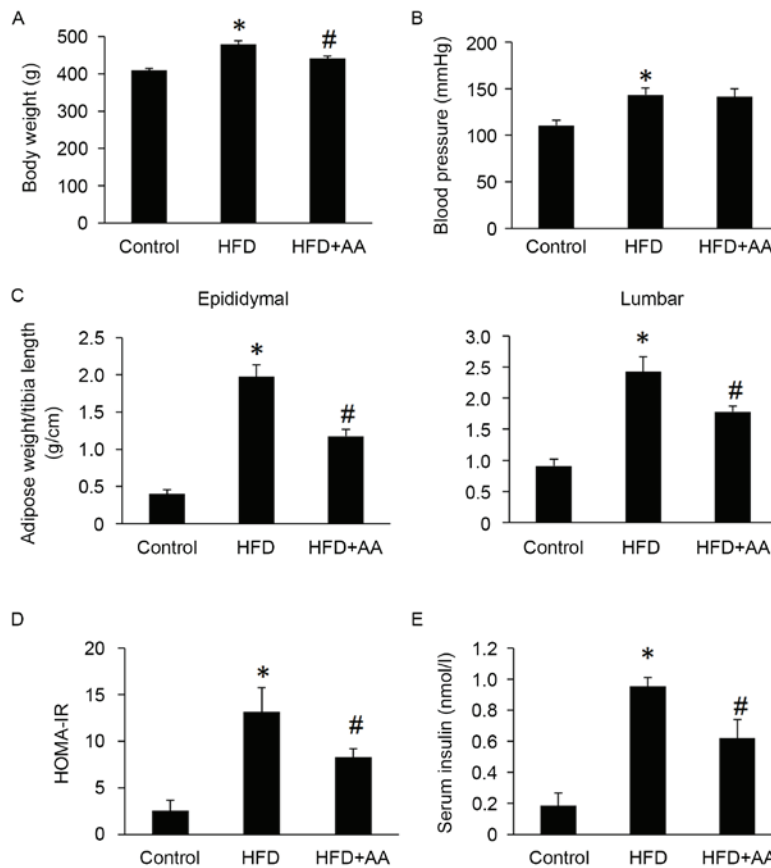


Figure 1. AA improves the cardiometabolic profile in rats fed with an HFD (n=8). (A) Body weight, (B) blood pressure, (C) epididymal and lumbar adipose weight/tibia length, (D) HOMA-IR and (E) serum insulin levels. *P<0.05 vs. control and #P<0.05 vs. HFD. AA, Asiatic acid; HFD, high fat diet; HOMA-IR, homeostasis model assessment of insulin resistance.

of Fas and Bcl-xl. Testis tissue sections were deparaffinized and boiled in sodium citrate buffer (pH=7.0, 5 min, MXB Biotechnologies, Fuzhou, China) for antigen retrieval after rehydration in a descending alcohol series. Sections were subsequently incubated with primary antibodies (anti-Fas, 1:1,000; anti-Bcl-xl, 1:500) at 4°C overnight after eliminating the internal peroxidase activity using 3% hydrogen peroxide incubation at room temperature for 20 min. Sections were subsequently incubated with the secondary antibody (EnVision™+HRP reagent; 1:100; cat. no. GK500610A, Gene Technology Co., Ltd., Shanghai, China) at 37°C for 30 min. Sections were incubated with diaminobenzidine at room temperature for 2 min and observed under a light microscope (magnification, x100 and x400; E100; Nikon Corporation). A total of 30 fields were randomly selected in 6 rats from each group and the expression of Fas and Bcl-xl were identified using integrated optical density. Apoptosis was detected using the TUNEL kit according to the manufacturer's protocol. A total of 100 cells were randomly selected in each group and the number of positive cells was calculated manually.

Western blot analysis and reverse transcription-quantitative polymerase chain analysis (RT-qPCR). Total proteins from fresh testis tissues were isolated using radioimmunoprecipitation assay lysis buffer (Wuhan Servicebio Technology Co., Ltd.). Protein concentrations were determined using a bicinchoninic acid assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.).

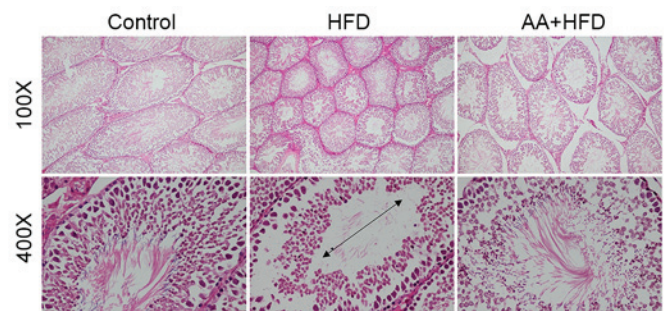


Figure 2. Hematoxylin and eosin staining of testis tissue in rats fed with an HFD (n=6). Arrowheads indicates the diameter of seminiferous tubules. HFD, high fat diet; AA, Asiatic acid.

Proteins (50 µg) were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk at room temperature for 1 h, and subsequently incubated overnight at 4°C with the following primary antibodies: Anti-GAPDH antibody, anti-Bax antibody and anti-Bcl-2 antibody (all 1:1,000). The membrane was subsequently incubated with IRDye 800CW-conjugated secondary antibody (1:10,000, cat. no. LI 926-32211; LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 1 h. Finally, the membrane was scanned using a two-color infrared imaging system (Odyssey; LI-COR Biosciences) and protein expression levels were normalized to GAPDH.

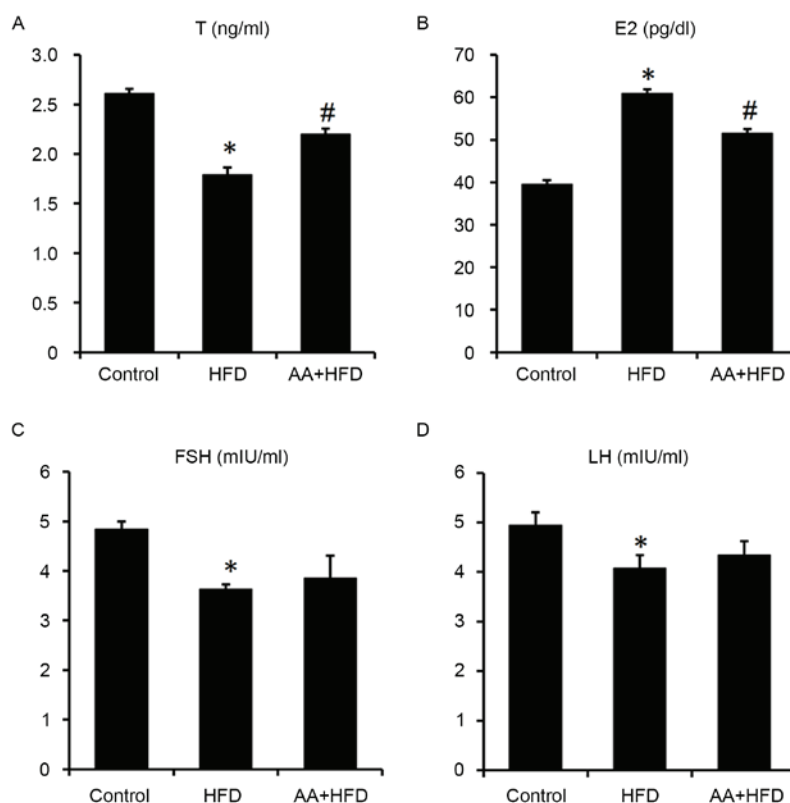


Figure 3. AA treatment improves abnormal serum hormone levels of (A) T, (B) E2, (C) FSH and (D) LH induced by HFD (n=8). *P<0.05 vs. control and #P<0.05 vs. HFD. AA, Asiatic acid; T, testosterone; E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; HFD, high fat diet.

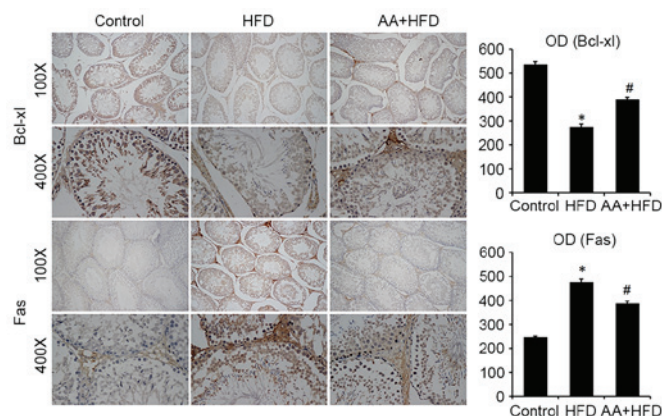


Figure 4. Immunohistochemistry revealed that AA increases the expression of Bcl-xl and decreases the expression of Fas compared with the HFD group (n=8). *P<0.05 vs. control and #P<0.05 vs. HFD. AA, Asiatic acid; Bcl-xl, B-cell lymphoma extra large; HFD, high fat diet; OD, optical density.

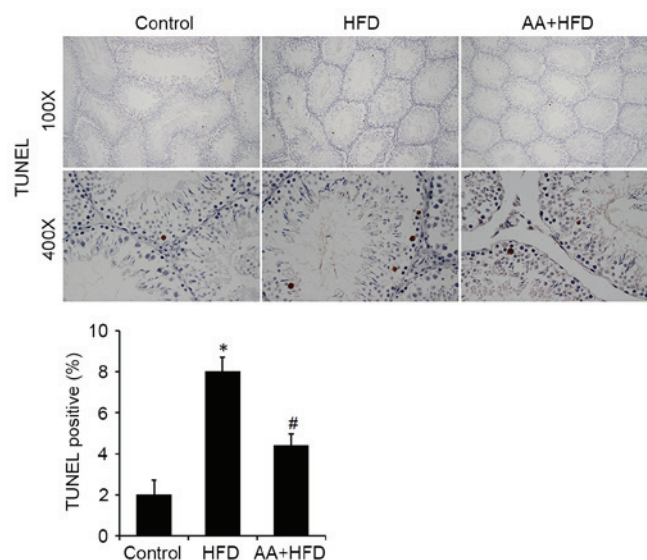


Figure 5. AA treatment reduces HFD-induced apoptosis as detected by TUNEL staining (n=8). *P<0.05 vs. control and #P<0.05 vs. HFD. AA, Asiatic acid; HFD, high fat diet.

RNA was isolated from tissues using an RNeasy mini kit (Qiagen AB, Sollentuna, Sweden) and RT-qPCR was performed with a Bio-Rad iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using PrimeScript™ RT reagent kit with gDNA Eraser (RR047A, Takara Bio, Inc., Otsu, Japan). SYBR Premix Ex Taq™ II was obtained from Takara Bio, Inc. (DRR820A). The temperature protocol for reverse transcription was: 37°C for 15 min, 85°C for 5 sec. The thermocycling conditions for PCR were: Initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 45 sec; dissociation at 95°C for

15 sec and 60°C for 30 sec. The primers used were as follows: Bax, forward 5'-ATCCAGGATCGAGCAGGGAGGATG G-3' and reverse, 5'-TGCCCGCCTACTTCAACGA-3'; Bcl-2, forward 5'-CTTCCAGCCTGAGAGCAACC-3' and reverse 5'-CATCCCAGCCTCCGTTATCC-3'; GAPDH, forward 5'-GACATGCCGCCTGGAGAAAC-3' and reverse 5'-AGCC CAGGATGCCCTTTAGT-3'. Relative mRNA expression

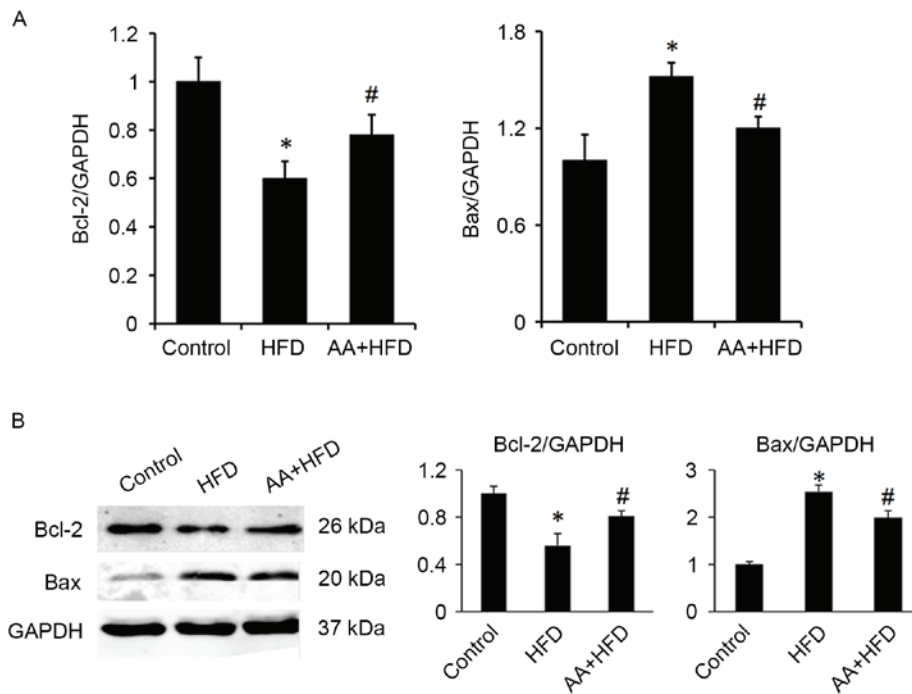


Figure 6. AA increases levels of Bcl-2 and decreases levels of Bax in rats fed with an HFD (n=6). (A) mRNA and (B) protein levels of Bax and Bcl-2. *P<0.05 vs. control and #P<0.05 vs. HFD. AA, Asiatic acid; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; HFD, high fat diet.

levels were analyzed using the $2^{-\Delta\Delta C_q}$ method (28). The mRNA levels were normalized to GAPDH.

Data analysis. Results in each group are expressed as the mean + standard deviation. All statistical tests were conducted using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Multiple group comparisons were made using one-way ANOVA followed by a post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

AA improves cardiometabolic profile in rats subjected to HFD. Body weight, HOMA-IR, serum insulin, epididymal and lumbar adipose tissues weights were all significantly increased in rats with HFD compared with control rats (P<0.05; Fig. 1). However, AA treatment significantly attenuated these HFD-induced increases (P<0.05; Fig. 1). Rats in the HFD also had significantly increased systolic blood pressure compared with the control group (P<0.05); however, no significant difference was observed between the HFD and AA + HFD group (Fig. 1).

AA treatment improves pathological changes of testes induced by HFD. Following 12 weeks of HFD, rats in the HFD group had significantly decreased testis weight and testis weight/body weight compared with the control group (P<0.05; Table I), which was also confirmed by histological analysis (Fig. 2). Atrophic seminiferous tubules with smaller diameters were also observed in the testis of rats with HFD (Fig. 2). HFD also resulted in a significant reduction in spermatogonia, Leydig cells and Sertoli cells compared with the control group (P<0.05; Table I). Sperm concentration, sperm viability and motility were significantly decreased in rats

subjected to an HFD diet compared with control rats (P<0.05; Table I). AA treatment significantly attenuated the decreased testis weight, testis/body weight, spermatogonia, Leydig cells and Sertoli cells (P<0.05; Table I), and markedly improved the HFD-induced atrophy of seminiferous tubules (Fig. 2).

AA treatment attenuates the HFD-induced abnormal serum sexual hormone levels. Serum E2 levels were significantly increased and T levels were significantly decreased in the HFD compared with the control group (P<0.05; Fig. 3A and B). However, AA treatment for 12 weeks significantly ameliorated the HFD-induced abnormal serum sexual hormone levels (Fig. 3A and B). Serum FSH and LH were both significantly reduced in HFD rats compared with the control group (P<0.05), whereas no significant difference was observed between the HFD and AA + HFD groups (Fig. 3C and D).

AA suppresses apoptosis in the testis of rats fed with HFD. Immunohistological analysis revealed that the protein level of Bcl-x1 was significantly decreased in the HFD rats compared with the control group (P<0.05) and that AA treatment significantly ameliorated this effect (P<0.05; Fig. 4). Fas was also significantly upregulated in the HFD group compared with the control rats (P<0.05) and this effect was significantly ameliorated with AA treatment (P<0.05; Fig. 4). TUNEL analysis was used to investigate the apoptotic rate of germ cells in the testis. The results revealed that rats in the HFD group had a significantly higher rate of apoptosis compared with the control rats (P<0.05), and that AA significantly inhibited HFD-induced testicular cell apoptosis (P<0.05; Fig. 5). These results were corroborated by subsequent analysis of mRNA and protein levels, which indicated

that HFD induced a significant downregulation in Bcl-2 and a significant increase in Bax expression compared with the control group ($P < 0.05$; Fig. 6). Treatment with AA, however, significantly ameliorated these effects, inducing a significant increase in Bcl-2 and decrease in Bax compared with the HFD group ($P < 0.05$; Fig. 6).

Discussion

The number of worldwide overweight individuals has grown rapidly, resulting in an escalation of obesity-associated health problems including infertility (29). As a result, there is a greater need to develop pharmacological agents for and to explore the novel and specific regulators of obesity-associated infertility. The results of the present study indicate that AA may attenuate HFD-induced impaired spermatogenesis. AA was also demonstrated to ameliorate endocrine disorders and suppress HFD-induced testicular cell apoptosis.

Endocrine disorders are key features of spermatogenesis dysfunction (30). T is able to promote spermatogenesis via intracellular signaling pathways (31). A recent study indicated that metformin-induced T level increases were able to improve reproductive function in obese male rats (17). Consistent with this, the present study demonstrated that AA upregulates the level of T and reduces the level of E2, which suggests that improved sex hormone levels may contribute to the protective effects of AA. AA had no significant effect on FSH and LH levels, which indicates that it does not affect pituitary hormones.

It is known that spermatogenesis is a complex process that relies on coordinated cell proliferation and apoptosis (32). Excessive cell apoptosis is reported to be a prevalent phenomenon in defective spermatogenesis (33); therefore, inhibiting excessive cell apoptosis and reconstructing the balance between cell proliferation and apoptosis may be an effective treatment for defective spermatogenesis. In view of the anti-apoptotic properties of AA (22,23), it was hypothesized that AA may suppress HFD-induced apoptosis in the testes. The results of the present study revealed that AA significantly inhibits testicular cell apoptosis, which suggests that apoptosis may be one of the underlying mechanisms by which AA protects against HFD-induced defective spermatogenesis. Conversely, it has previously been reported that AA induces tumor cell apoptosis (34,35). The reason for these incompatible results may be that apoptosis serves different roles in different pathological processes.

The precise mechanisms that mediate the antiapoptotic effects of AA remain to be elucidated. A recent study indicated that AA is able to activate AMP-activated protein kinase α (19), which has been demonstrated to be a negative regulator of apoptosis (36,37). AA has also been reported to suppress inflammation and oxidative injury in human bronchial epithelial cells (38), which is associated with apoptosis. Further study is required to determine the precise mechanisms underlying the protective effects of AA.

In conclusion, the results of the present study demonstrated that AA is able to attenuate HFD-induced spermatogenesis dysfunction via inhibiting excessive apoptosis. These findings provide theoretical evidence for the use of AA as a treatment for obesity-associated infertility.

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