The time-dependent adverse effects of a high-fat diet on sperm parameters

Xiangyu Qi[D,1–4], Qingbo Guan[E,1–4], Wenjing Zhang[B,1–4], Xinshuang Huang[C,1–5], Chunxiao Yu[F,1–4]

[1] Department of Endocrinology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China
[2] Shandong Key Laboratory of Endocrinology and Lipid Metabolism, Jinan, China
[3] Shandong Institute of Endocrine and Metabolic Diseases, Jinan, China
[4] Shandong Engineering Laboratory of Prevention and Control for Endocrine and Metabolic Diseases, Jinan, China
[5] Shandong University of Traditional Chinese Medicine, Jinan, China

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Address for correspondence
Qingbo Guan
E-mail: doctorguanqingbo@163.com

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Abstract

Background. Studies indicate a relationship between a high-fat diet (HFD) and sperm quality. However, the time-dependent adverse effects of a HFD on sperm parameters and the underlying mechanisms remain unclear.

Objectives. The present study was designed to determine the effects of a HFD on sperm quality at various time points in order to assess whether a HFD causes cumulative damage to sperm.

Materials and methods. Male C57BL/6 mice were fed a normal diet (the ND group) or a HFD (the HFD group) for 16, 30 or 42 weeks (n = 6 for each group). Body weight, lipid profile, sperm parameters, testicular morphology, and testicular oxidative stress levels were evaluated alongside the proliferation, DNA damage and rate of germ cell apoptosis.

Results. Sperm quality was reduced in HFD-fed animals in a time-dependent manner, which was demonstrated by a decline in sperm density, motility and progressive motility. Further analysis showed a progressive deterioration of the testicular histoarchitecture of HFD-fed mice, which was accompanied by a decrease in DEAD-box helicase 4 (*DDX4*) expression and superoxide dismutase (SOD) levels, increased malondialdehyde (MDA) levels and gamma-H2A histone family member X (*γ-H2AX*) expression, and increased apoptosis of germ cells.

Conclusions. These findings demonstrate that a HFD exerted adverse effects on sperm quality, and the deteriorating effect was progressive with long-term feeding. The inhibited proliferation and apoptosis of germ cells, and the increased oxidative stress levels and DNA damage may be the underlying mechanisms.

Key words: adverse effects, high-fat diet, sperm parameters, time-dependent manner

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Background

With the rapid development of society, an unhealthy high-fat diet (HFD) has become a common dietary problem.[1] In both clinical and animal studies, HFD leads to the occurrence and development of obesity and a series of diseases, including diabetes and cardiovascular disease (CVD).[2–4] Moreover, the adverse effects of a HFD on the quality of male sperm are gradually being recognized.[5–7] Numerous studies have demonstrated that HFD results in a reduced sperm quality.[8] Furthermore, a clinical study showed that a diet containing palmitic acid (PA) may contribute to asthenozoospermia in males.[9] Animal studies have reported a decreasing trend in sperm motility in animals fed a HFD.[10,11] However, whether the HFD has time-dependent adverse effects on sperm quality remains controversial. Fernandez et al. found that consuming a HFD for 16 or 45 weeks did not impair sperm quality, but only reduced the number of ejaculations.[12] Moreover, recent studies found an increase in sperm concentration in mice treated with a HFD for 30 weeks.[11,12] These conflicting findings suggest that a dynamic analysis of the cumulative effects of a HFD is required to understand the impact of a HFD on sperm quality.

The molecular mechanisms of HFD-induced sperm alterations remain uncharted to a large extent. Numerous studies have demonstrated a link between the inhibition of the proliferation of germ cells and the cessation of spermatogenesis. It has also been reported that DEAD-box helicase 4 (*DDX4*) has a vital role in the proliferation of germ cells and that the expression of *DDX4* decreased in the testes of mice and rats.[13,14] In addition, a previous report provided evidence that a HFD may decrease fertility through oxidative stress.[13] A recent study reported that oxidative stress led to DNA double-strand breaks (DSBs), which can be detected as a phosphorylation of gamma-H2A histone family member X (*γ-H2AX*) foci in cells.[15,16] If DSBs are not correctly repaired, *γ-H2AX* will be expressed continuously and germ cells will gradually undergo apoptosis.[17,18] However, the effects of a HFD on the expression of *γ-H2AX* and the proliferation of germ cells remain unclear.

Objectives

The aim of the study was to assess whether a HFD alters sperm quality, and whether a HFD could be the cause of cumulative sperm damage, as well as to pinpoint the underlying mechanisms. The present study investigated alterations in murine sperm quality induced by a HFD over various timeframes.

Materials and methods

Animals and diets

This study was approved by the Animal Ethics Committee of Shandong Provincial Hospital (Jinan, China; approval No. NSFC: NO.2019-243 issued on February 22, 2019). Male 8-week-old C57BL/6 mice (n = 32) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The animals were adapted to a 12-hour light/dark cycle at 22–25°C. They were randomly split into 2 groups (n = 24 per group) and were fed a normal diet (ND) containing 10 kcal% fat (D12450B; Research Diets, New Brunswick, USA)[19] or a HFD containing 60 kcal% fat (D12492; Research Diets)[20] for 16, 30 or 42 weeks (n = 6 for each group at each different time). Mice from each group were euthanized at the end of 16, 30 and 42 weeks of diet intervention.

After 8-hour fasting, all mice were anesthetized with pentobarbital sodium and weighed. Then, sperm was collected immediately from the epididymal cauda. Blood samples were drawn from the retro-orbital vein and stored at –80°C, and the testes and epididymal fat were separated and weighed. The testis coefficient was calculated by dividing the weight of the testis by body weight. The testes were rapidly preserved in liquid nitrogen for Oil Red O staining and protein analysis, or in modified Davidson's fluid (mDF) for morphological analysis.[21,22]

Measurement of lipid profile

The serum levels of glucose (GLU), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglycerides (TG) were evaluated using Mindray Automatic biochemical analyzer BS-830 (Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).[21,22]

Detection of sperm parameters

The epididymal cauda of the mice were transferred to a Petri dish containing HyClone™ Medium 199 (Cytiva, Marlborough, USA) and 0.5% bovine serum albumin (BSA). Using sharp surgical scissors, multiple incisions were made in the epididymal cauda to facilitate sperm suspension in the culture medium. Suspensions were then placed in an incubator (37°C, 5% CO_2) for 5 min to maximize sperm drainage. Subsequently, 10 µL of diluted sperm suspension (1:20) was transferred to a sperm counting plate of the computer-assisted semen analyzer (CASA) IVOS II (Hamilton-Thorne Bioscience, Beverly, USA) for the automated analysis.[22] This analysis included sperm concentration and progressive and nonprogressive motility, as well as characterizing movement as rapid, moderate or static. Sperm velocity was also assessed, including average path velocity (VAP), linear velocity (VSL) and curvilinear velocity (VCL).

Hematoxylin and eosin staining

Testis samples that had been fixed in mDF for 24 h were embedded in paraffin, and then sectioned at a thickness of 5 µm. Sections were deparaffinized, rehydrated, stained with hematoxylin and eosin (H&E), and then scanned with the Aperio VERSA light microscopy scanning system (Leica Biosystems, Richmond, USA).[22] Damage to seminiferous tubules and spermatogenesis were evaluated according to the Cosentino's scoring system: 1. Normal testicular architecture with an orderly arrangement of germinal cells; 2. Injury showed less orderly, non-cohesive germinal cells and closely packed seminiferous tubules; 3. Injury exhibited disordered sloughed germinal cells, with reduced size of pyknotic nuclei and less distinct seminiferous tubule borders; 4. Injury exhibited seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells.

Oil Red O staining

Frozen testis sections (7-µm thick) were sunk in 95% alcohol for 15 s, stained with Oil Red O (Beyotime Biotechnology, Shanghai, China) for 15 min in the dark, and counterstained with hematoxylin for 3 min.[22] The specimens were then imaged using the Aperio VERSA light microscopy scanning system (Leica Biosystems).

Malondialdehyde and superoxide dismutase measurements

The malondialdehyde (MDA) content (STA-330; Cell Biolabs Inc., San Diego, USA) and superoxide dismutase (SOD) levels (S0087; Beyotime Biotechnology) of testes were evaluated using commercially available kits (MDA: STA-330; Cell Biolabs Inc.; SOD: S0087; Beyotime Biotechnology) according to the manufacturer's instructions.

Immunofluorescence

Paraffin-embedded testis sections (5-µm thick) were deparaffinized, immersed in citrate buffer antigen retrieval solution and heated in a microwave oven for 20 min. Then, they were cooled for 60 min and incubated in 5% BSA for 60 min at room temperature. Afterward, sections were incubated overnight at 4°C with rabbit polyclonal anti-*DDX4* (ab13840, 1:200) or recombinant anti-*γ-H2AX* (ab81299, 1:200) primary antibodies (Abcam, Cambridge, UK). The negative control was incubated without a primary antibody. On the next day, sections were incubated with a secondary antibody (1:1000; Thermo Fisher Scientific, Waltham, USA). The nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI).[21] Images were acquired with a Nikon AX confocal microscope (Nikon Corp., Tokyo, Japan), equipped with a ×40 objective. Images were merged and processed using Nikon confocal analysis software. For descriptive analyses, each sample was examined at least 3 times.

Terminal deoxynucleotidyl transferase dUTP nick end labeling

Paraffin-embedded testis sections (5-µm thick) were stained using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (KGA704-1; Keygen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. The cell nuclei were stained with DAPI and the sections were observed using the Aperio VERSA light microscopy scanning system (Leica Biosystems). Using ImageJ software (National Institutes of Health, Bethesda, USA), the number of TUNEL-positive (TUNEL+) cells/high power field (HPF) was calculated. Each sample was assessed at least 3 times.

Statistical analyses

Outliers were removed by the robust regression followed by outlier identification (ROUT) method, using GraphPad Prism 9 (GraphPad Software Inc., San Diego, USA). Data were analyzed using two-way analysis of variance (ANOVA) followed by the Tukey's post hoc test for the between-diet per period and between-period per diet. A value of $p < 0.05$ was considered statistically significant.

Results

Characterization of mice fed a high-fat diet

To evaluate the effect of a HFD on body weight and metabolic parameters, the relevant values were examined. Throughout the course of the study, body weight, testis weight, testis coefficient, epididymal adipose weight, and TC were significantly higher in the mice fed a HFD compared to the mice fed a ND, at all timepoints (16 weeks (w): $p < 0.05$; 30 w: $p < 0.05$; 42 w: $p < 0.05$). Glucose levels were similar at 16 and 42 weeks and increased at 30 weeks ($p < 0.05$; Fig. 1). Total TG and LDL-C levels were similar between the groups at 16 weeks but increased at 30 weeks ($p < 0.05$) and 42 weeks ($p < 0.05$). Furthermore, the body weight (16 w compared to 42 w: $p < 0.05$), testis coefficient (16 w compared to 42 w: $p < 0.05$), GLU (16 w compared to 30 w: $p < 0.05$; 16 w compared to 42 w: $p < 0.05$; 30 w compared to 42 w: $p < 0.05$), TC (16 w compared to 30 w: $p < 0.05$; 16 w compared to 42 w: $p < 0.05$), and LDL-C (16 w compared to 42 w: $p < 0.05$; 30 w compared to 42 w: $p < 0.05$) were elevated significantly in a time-dependent manner in mice fed a HFD, while only GLU (16 w compared to 42 w: $p < 0.05$; 30 w compared to 42 w: $p < 0.05$) and TC (30 w compared to 42 w: $p < 0.05$) were elevated over time in mice fed ND. These results indicated that

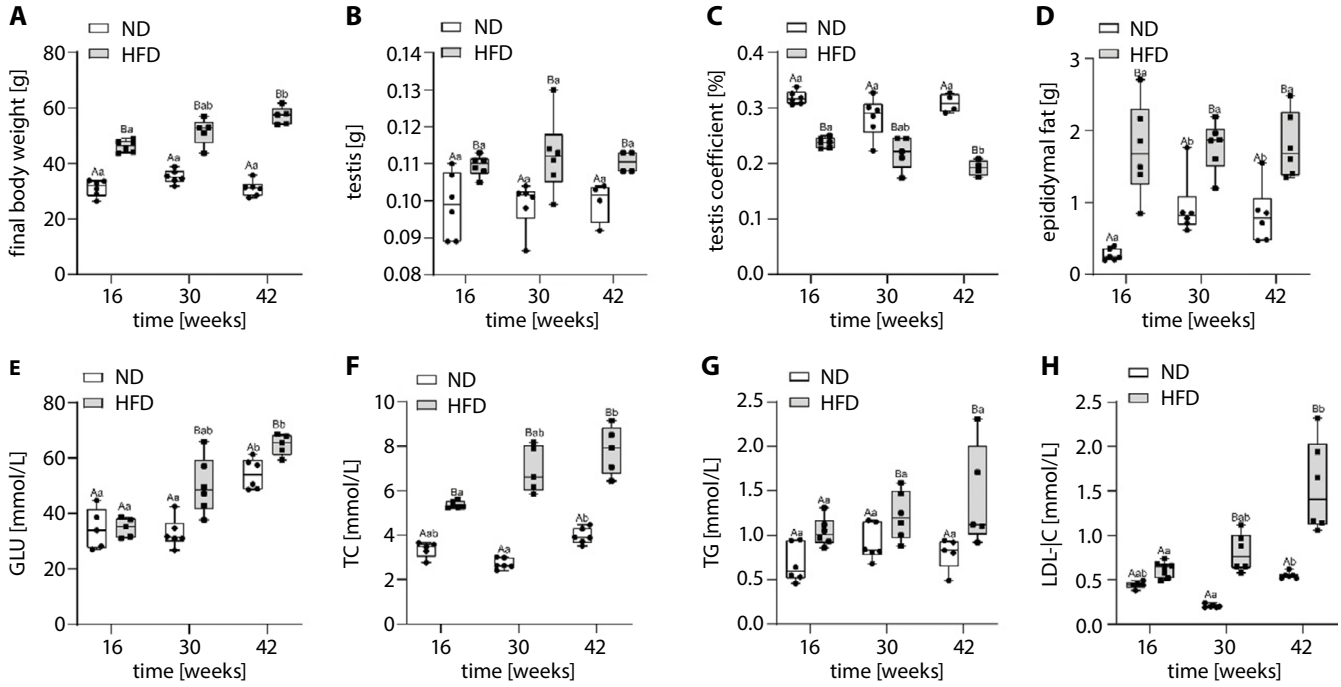

Fig. 1. General conditions induced by a high-fat diet (HFD). Comparison of body weight between the normal diet (ND) and HFD groups (A). Testicular weight (B), testis coefficient (C) and epididymal fat (D) in the ND group and the HFD group (n = 6 for each group). Comparison of the levels of serum glucose (GLU) (E), total serum cholesterol (TC) (F), triglycerides (TG) (G), and low-density lipoprotein cholesterol (LDL-C) (H), between the ND and HFD groups (n = 6 for each group). The capital letters refer to the comparison between the ND and HFD groups at the same feeding time, while lowercase letters refer to the comparisons between different feeding times. Different letters indicate a significant difference ($p < 0.05$)

the high-fat model was successfully established. The high-fat model tended to have more metabolic disorders the longer the mice were fed a HFD.

Alteration of sperm parameters induced by a high-fat diet

To confirm the effects of a HFD on sperm quality, sperm parameters in the epididymal cauda were evaluated with a CASA after different feeding intervals. The CASA evaluation revealed that striking changes occurred in the sperm of all of the HFD groups compared to the control groups (Fig. 2). It was found that 16 weeks on a HFD did not cause any significant change in the sperm parameters, although sperm concentration was reduced by approx. 20%. Continuous feeding with a HFD for 30 and 42 weeks caused increasing sperm dysfunction, including a lowered sperm concentration and decreased motility. Indeed, there was a significant decrease in the progressive motility of sperm at 42 weeks ($p < 0.05$). Furthermore, when animals fed the HFD for different periods were compared, a time-dependent impairment was found for all parameters except for VAP, VSL and VCL, and all of the damaging changes were statistically significant (sperm density: 16 w compared to 42 w: $p < 0.05$; static sperm: 30 w compared to 42 w: $p < 0.05$; sperm motility, rapid sperm and moderate sperm: 16 w compared to 42 w: $p < 0.05$; sperm progressive motility: 16 w compared to 30 w: $p < 0.05$, and 16 w compared to 42 w: $p < 0.05$). Conversely, there was only a time-dependent difference in sperm concentration in the ND groups (16 w compared to 42 w: $p < 0.05$; 30 w compared to 42 w: $p < 0.05$), and there were no differences in sperm parameters related to motility. These results demonstrate that sperm parameters were adversely affected by a HFD and these adverse effects were most severe in the mice fed a HFD for 42 weeks.

Alterations of testicular histoarchitecture induced by a high-fat diet

To investigate changes in the seminiferous tubules after HFD exposure, testicular morphology was analyzed using H&E staining for the 3 feeding intervals (Fig. 3A). Normal histological features were observed in all 3 ND groups (Fig. 3A). Conversely, seminiferous tubules were loosely arranged in the mice fed a HFD for 16 weeks. The changes in histoarchitecture were more evident in the 30-week group, with distinct structural disorganization and increased vacuolization in both seminiferous tubules and interstitial spaces. Drastic structural alterations, characterized by obvious tubular degeneration and prominent vacuolization, were noticed after 42 weeks. Figure 3B shows the Cosentino's scores obtained after the histopathological examination of each group. There were significant increases in both scores in the HFD mice after 30 and 42 weeks compared to the ND group (30 w: $p < 0.05$; 42 w: $p < 0.05$). Furthermore, the scores

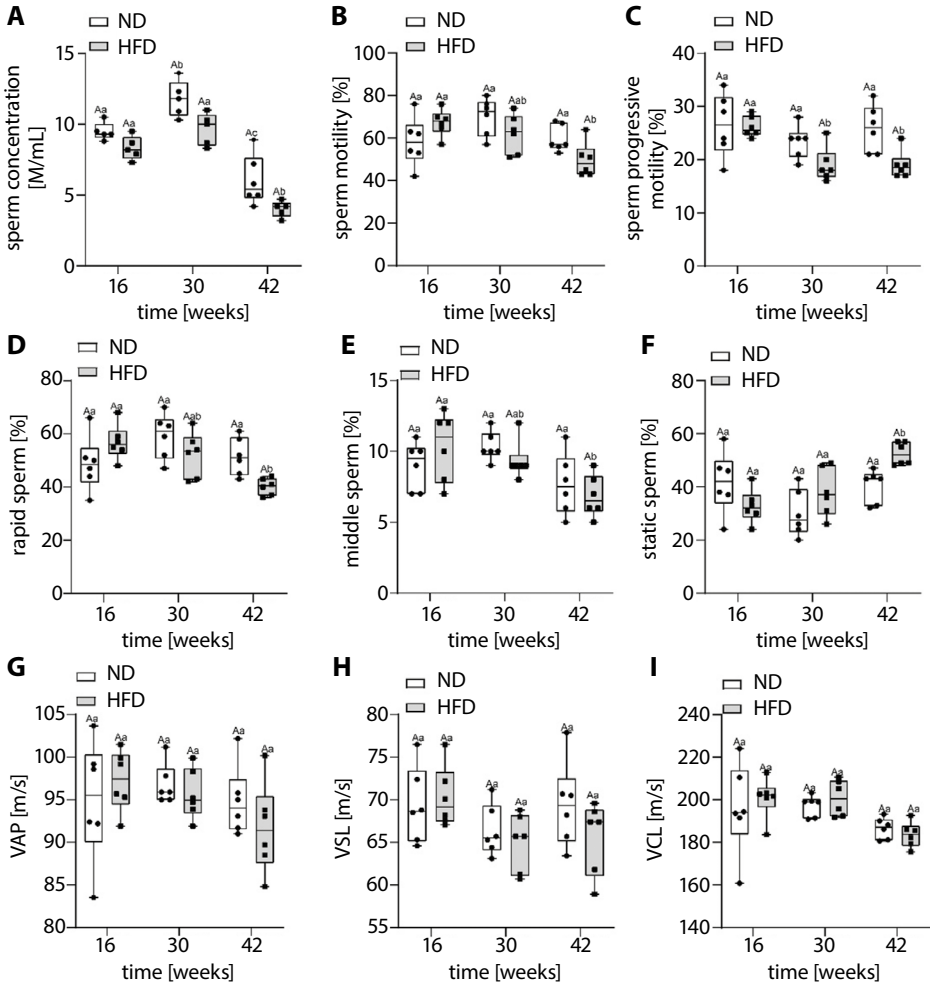

Fig. 2. Sperm parameter changes induced by a high-fat diet (HFD). Comparison of sperm concentration (A), sperm motility (B), sperm progressive motility (C), rapid sperm (D), moderate sperm (E), static sperm (F), average path velocity (VAP) (G), linear velocity (VSL) (H), and curvilinear velocity (VCL) (I) between the normal diet (ND) and HFD groups (n = 6 for each group). The capital letters refer to comparisons between ND and HFD groups at the same feeding time, while lowercase letters refer to comparisons between different feeding times. Different letters indicate a significant difference ($p < 0.05$)

increased in a time-dependent manner in mice fed a HFD (16 w compared to 30 w: $p < 0.05$; 16 w compared to 42 w: $p < 0.05$).

To evaluate ectopic lipid deposition in the testes of mice fed a HFD, testicular morphology was analyzed using Oil Red O staining at 16, 30 and 42 weeks (Fig. 3C). Ectopic lipid deposition was increased in all HFD groups compared with the ND groups (Fig. 3B). Interestingly, while frozen sections from the group fed a ND exhibited progressive ectopic lipid deposition in the testes with time, a more obvious progressive deposition was observed with prolonged time in the testes of the mice fed a HFD. These results indicate that the short-term influence of a HFD on testicular histoarchitecture may not be obvious; however, long-term effects may mean distinct damage.

High-fat diet inhibited the proliferation of germ cells

While conventional sperm analysis can determine the sperm quality to a certain extent, it may not display potential defects such as the inhibition of proliferation. To evaluate the proliferation of germ cells, *DDX4* was detected in mouse testes by means of immunofluorescent staining (Fig. 4). Compared with the corresponding ND groups, a considerable decrease in the number of *DDX4*-positive cells was observed in all of the HFD groups. Interestingly, in the HFD groups, *DDX4* decreased with time and reached a minimum expression at 42 weeks. These findings highlight the deleterious role a HFD has in testicular injury, which most likely occurred through the inhibition of the proliferation of germ cells, and this inhibition worsened with time.

High-fat diet exposure induced oxidative stress in testes

To evaluate whether a HFD influences sperm quality by aggravating oxidative stress, the testicular oxidative stress-related index was assessed, and the levels of the antioxidative enzyme SOD and pro-oxidative by-product MDA were determined (Fig. 5A,B). At the end of the experimental procedure, the MDA concentration tended to increase while the SOD concentration tended to decrease in all HFD groups. However, none of the changes were significantly different, except for the MDA concentration at 42 weeks ($p < 0.05$). The MDA concentration

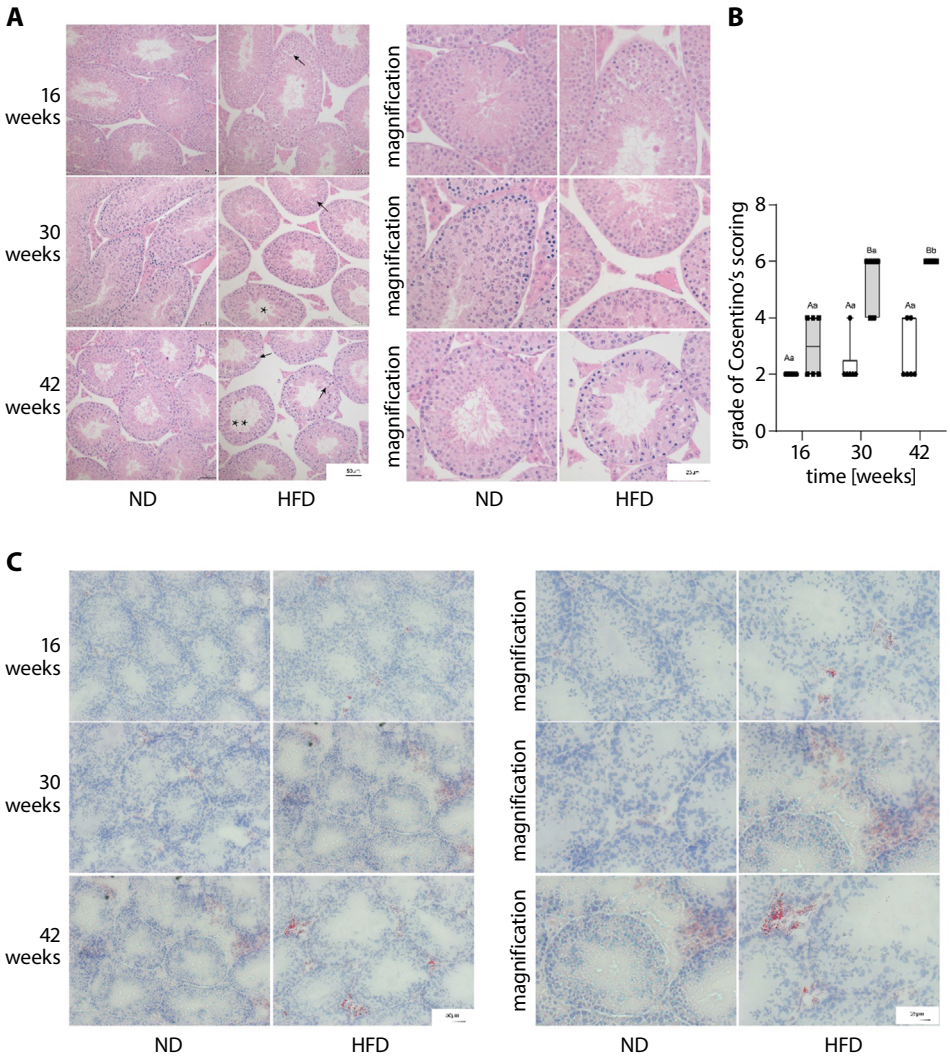

Fig. 3. Testicular morphological changes and lipid deposition induced by a high-fat diet (HFD). A. Representative pictures of testicular hematoxylin and eosin (H&E) staining (n = 3 for each group); B. Grade of Cosentino's scoring (n = 3 for each group, each sample was assessed at least 2 times); C. Representative pictures of testicular Oil Red O staining (n = 3 for each group). The capital letters refer to comparisons between normal diet (ND) and HFD groups at the same feeding time, while lowercase letters refer to comparisons between different feeding times. Different letters indicate a significant difference ($p < 0.05$)

* partial atrophy; ** complete atrophy; ← vacuolization.

increased significantly with time (16 w compared to 42 w: $p < 0.05$), and the SOD concentration tended to decrease over time in the HFD group. The results indicate that the level of testicular oxidative stress in obese mice increased, which may result in a reduced sperm quality.

High-fat diet exposure induced the apoptosis of germ cells

To further assess whether the decreased epididymal sperm quality was a result of germ cell apoptosis, TUNEL staining was performed on the mouse testes (Fig. 5C,D). A considerable increase in the number of TUNEL+ cells was observed at 30 and 42 weeks (30 w: $p < 0.05$; 42 w: $p < 0.05$). When animals fed the HFD for different periods were compared, a time-dependent increase in the number of TUNEL+ cells was found (16 w compared to 30 w: $p < 0.05$; 16 w compared to 42 w: $p < 0.05$). These findings demonstrate the deleterious role of a HFD in inducing injury to the testes through the initiation of apoptosis in germ cells, and this activation increases with time.

High-fat diet exposure induced DNA damage in mouse testes

To detect DNA damage in the testes, the expression of *γ-H2AX* was evaluated using immunofluorescence. As shown in Fig. 6, the HFD groups had a significantly higher *γ-H2AX* expression in comparison with the ND groups. More interestingly, the expression of *γ-H2AX* increased in a time-dependent manner for both the ND groups and the HFD groups. These results indicate that DNA damage was induced by the HFD, which may be an underlying mechanism of the decreased sperm parameters.

Discussion

The relationship between a HFD and HFD-related disorders such as obesity, CVD and cancers has been recognized for a long time.[14] Recently, a great deal of evidence has come to light showing that a HFD can also exert a profound impact on sperm quality. However, the cumulative effects

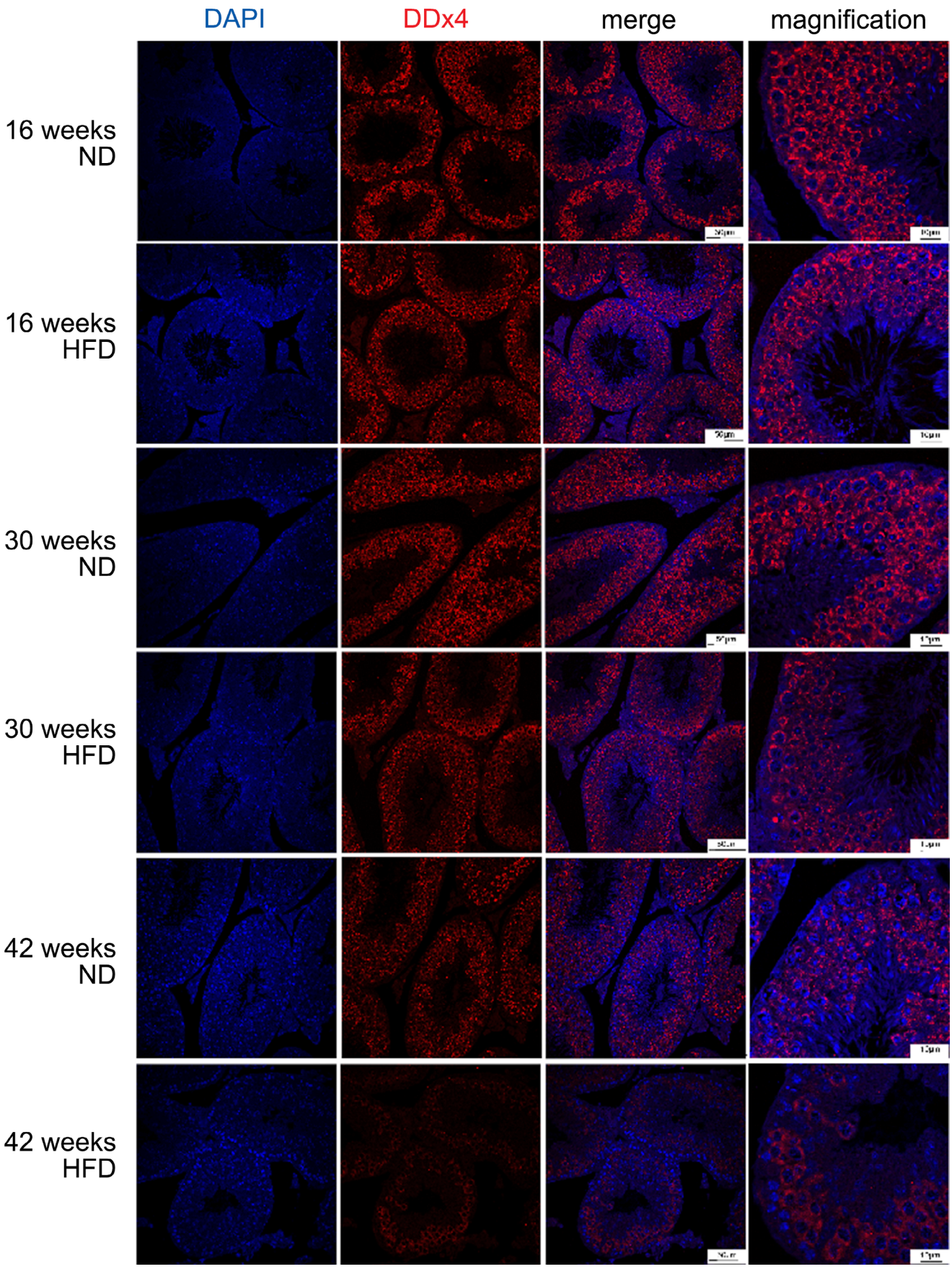

Fig. 4. Inhibition of germ cell proliferation induced with a HFD. Representative images of DEAD-box helicase 4 (*DDX4*) staining in the testes of mice fed a HFD for 8, 16 or 42 weeks (n = 3 for each group)

ND – normal diet; HFD – high-fat diet.

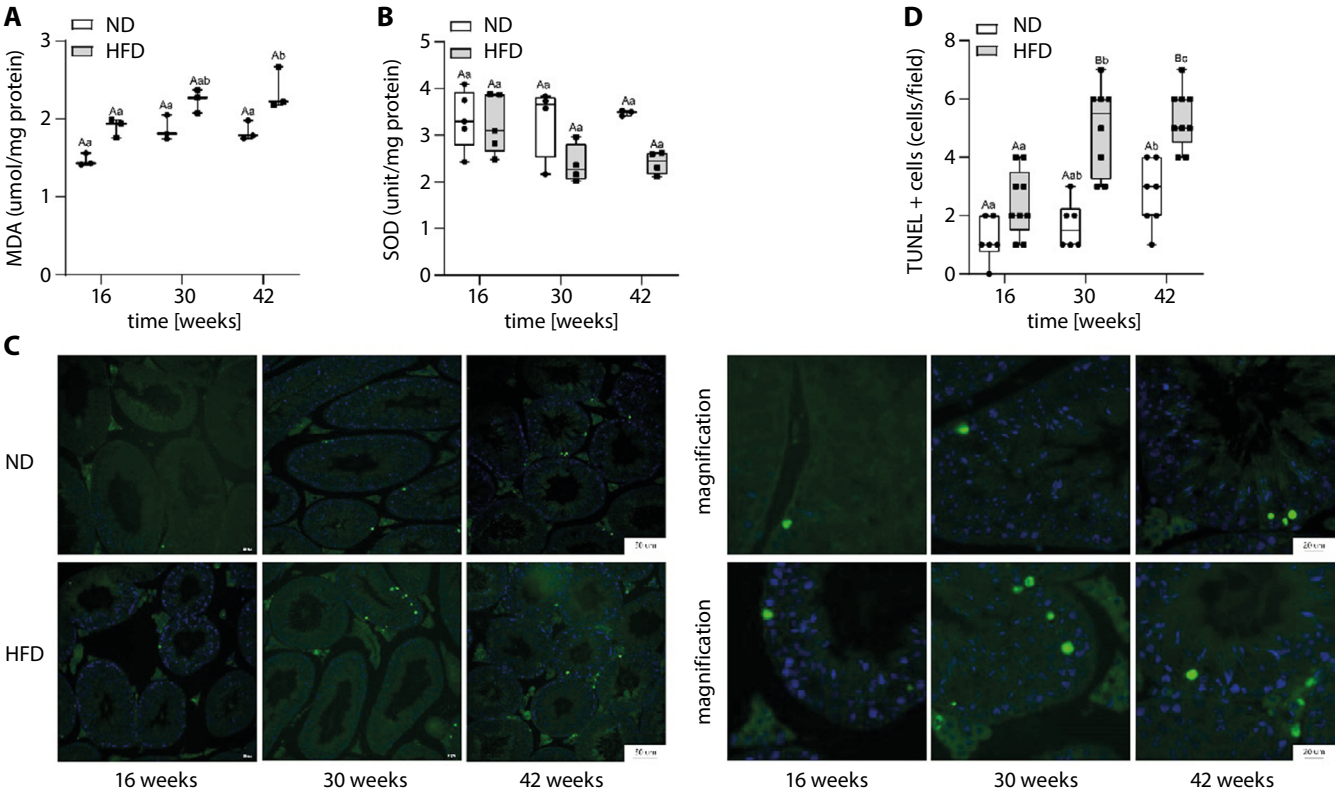

Fig. 5. Oxidative stress and apoptosis induced with a high-fat diet (HFD). Malondialdehyde (MDA) concentration (A) and testicular superoxide dismutase (SOD) concentration (B) (n = 3/4 for each group); C. Representative pictures of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining; D. TUNEL-positive germ cells/high power field (n = 3 for each group, each sample was assessed at least 2 times). The capital letters refer to comparisons between normal diet (ND) and HFD groups at the same feeding time, while lowercase letters refer to comparisons between different feeding times. Different letters indicate a significant difference ($p < 0.05$)

of a HFD on sperm quality are controversial and there is currently a lack of in-depth investigations in this area. In this study, by developing a HFD-related model, it was demonstrated that a HFD reduced sperm quality. Also, HFD-induced sperm damage may be cumulative and occur in a time-dependent manner. The inhibited proliferation of germ cells, increased oxidative stress levels in the testes, increased DNA damage, and the promotion of apoptosis may be the underlying mechanisms.

In this study, mice exposed to a HFD for 16, 30 or 42 weeks experienced weight gain, testicular weight gain and epididymal adipose weight gain. Abnormal lipid and GLU metabolism were also observed at different intervals in the HFD group, including increased GLU, TC, TG, and LDL-C. These results demonstrate that the HFD-related model was successfully established. Moreover, body weight, GLU, TC, and LDL-C increased in a time-dependent manner in the HFD groups, while only epididymal fat deposits and GLU increased in a time-dependent manner in the ND groups.

The ability of sperm to fertilize the ovum partly depends on its concentration and kinematics, both of which affect the fertility of males from a wide range of animals, such as fish, birds and mammals.[23,24] Therefore, the concentration of sperm present and the kinematic parameters, including sperm motility, sperm progressive motility, and the VCL, VSL and VAP in the epididymal cauda are vital indicators of sperm quality. Furthermore, a series of studies have shown that sperm motility and sperm progressive motility signify the function of mitochondria and energy status, which have been associated with fertility.[25,26] Studies have also shown that the VCL reflects the high-energy state of sperm and hyperactivated motility, which are essential for successful fertilization.[27,28] However, to a large extent, the short-term and cumulative effects of a HFD on these sperm parameters are controversial. A previous study found that sperm concentration tended to reduce after 8 weeks of a HFD,[29] while another study reported that sperm quality was not impaired in mice fed a HFD for 16 or 45 weeks. Furthermore, a recent study demonstrated that feeding a HFD from 12 to 24 weeks did not influence any of the motility parameters or the concentration of sperm in the epididymal cauda.[30]

The current study aimed to reveal the short-term and cumulative effects of a HFD on sperm parameters. There was a trend of decreased sperm concentration after 42 weeks of exposure to a HFD, which is consistent with our previous work. When sperm motility parameters were compared between different time points, the differences were found in the mice fed a HFD but not in the mice fed a ND. This may not only be due to the aging process but also due to the reproductive toxicity induced by the HFD. These

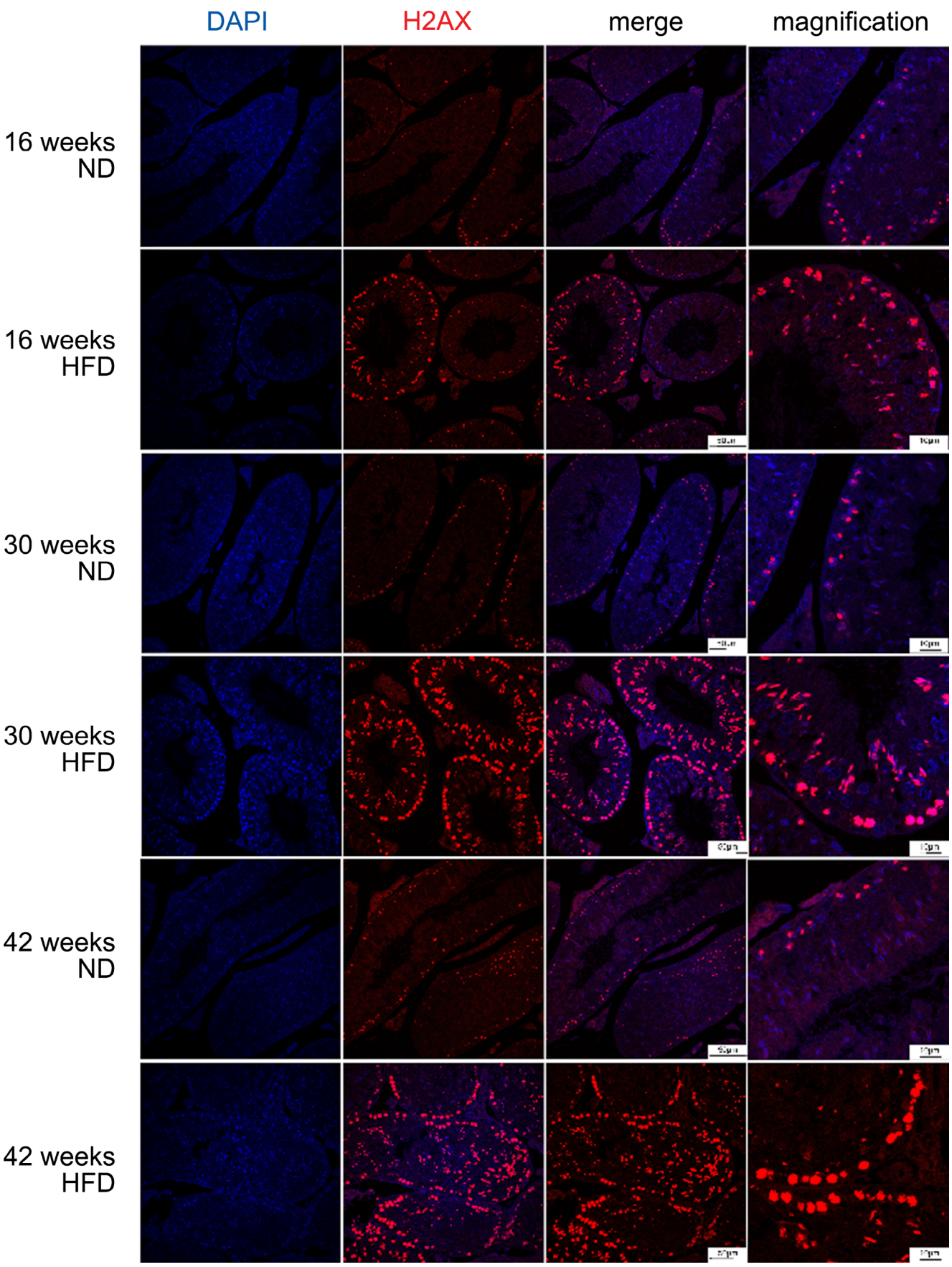

Fig. 6. Increased DNA damage induced with HFD. Representative images of gamma-H2A histone family member X (*γ-H2AX*) staining in the testes of mice fed a HFD for 8, 16 or 42 weeks (n = 3 for each group)

ND – normal diet; HFD – high-fat diet.

results showed that sperm parameters were adversely affected by a HFD and tended to be more severe over time.

Spermatogenesis is a complex process that produces sperm in the seminiferous tubules of the mammalian testes. When the structure of the seminiferous tubules is damaged, spermatogenesis is impaired.[31–33] Numerous studies, including those by Fan et al.[34] and Hammami et al.[11] have shown that the seminiferous epithelia become atrophied in mice fed a HFD. In contrast, Gómez-Elías et al. showed normal histological results in mice fed a HFD.[35] Although the histological results were varied, the histological analysis could explain the variations in the sperm parameters in all cases. In this study, the alterations in the relative sperm parameters in mice fed a HFD were also supported by histological changes. Indeed, distinct structural disorganization, loose seminiferous tubules and a decreased number of mature sperm provide distinct evidence that spermatogenesis was impaired and restricted in mice fed a HFD. Furthermore, the tissue damage induced by a HFD was shown to occur in a time-dependent manner, and included partial to complete atrophy of tubules and increased vacuolization within tubules.

Apart from the distinct structural disorganization of the testes of male mice, ectopic deposition of lipids was found. In the testes of animals fed a HFD, a large volume of lipid accumulation was deposited in the testicular interstitium, compared with the control animals, and this accumulation reached its maximum with a prolonged HFD. These results suggest that the testes and seminiferous tubules were damaged after the intervention of a HFD, which led to impaired spermatogenesis.

Spermatogenesis is responsible for producing sperm and passing genetic information to the next generation. The germ cell-specific gene, *DDX4*, is crucial for the proliferation and survival of germ cells, both of which play a critical role in spermatogenesis.[13] The expression of *DDX4* has been reported in undifferentiated seminiferous tubule cells (spermatogonia and spermatocytes), while no *DDX4* expression was detected in the sperm.[36] In the present study, immunofluorescence results indicated that the expression of *DDX4* was positive in spermatogonia and spermatocytes, which is consistent with previous studies. Further analysis showed that the level of *DDX4* was higher in mice fed a HFD than in those fed a ND. This indicates that the damaging role of a HFD on the quality of sperm most likely occurred through the inhibition of the germ cell proliferation. Similarly, Khanlarkhani et al. reported that there was a link between the inhibition of the proliferation of germ cells and the cessation of spermatogenesis. There was also a relationship between the arrest of proliferation and sterility.[37] Notably, the inhibition worsened with time in groups consuming a HFD. This indicates that a long-term and continuous HFD has a more serious effect on sperm quality.

Oxidative stress is highly correlated with a wide range of metabolic disease states, including metabolic disorders induced by a HFD.[38,39] Oxidative stress is a process in which free radicals, which are inadequately neutralized by antioxidants, cause cumulative damage, and this damage is aggravated by the reduced activity of antioxidative enzymes, such as SOD.[40] Numerous studies have reported that there are many sources of oxidative stress in animals fed a HFD. Furthermore, strong evidence showing that increased levels of oxidative stress in testes may impact sperm quality was demonstrated in previous studies.[10,41,42] In the present study, the testicular levels of SOD and MDA were evaluated to determine the effects of a HFD on the levels of oxidative stress. It was found that the HFD downregulated SOD and upregulated MDA in testicular tissues, which suggests that the HFD leads to increased levels of oxidative stress in the testes. These results indicate that oxidative stress may be an underlying mechanism responsible for the reduced sperm quality induced by a HFD.

Numerous recent studies have reported that increased oxidative stress along with decreased antioxidant defense may result in sperm DNA damage. One study reported that fluoride exposure generated reactive oxygen species (ROS) followed by H2AX phosphorylation (*γ-H2AX*), which is a marker of DSB and can be used to monitor DNA repair.[15] Another recent study reported that significant negative correlations were found between *γ-H2AX*, sperm concentration and kinematic parameters, such as sperm motility and sperm progressive motility.[16] Consequently, in this study, the number of *γ-H2AX*-positive cells was assessed using immunofluorescence. A higher number of *γ-H2AX*-positive germ cells was observed in the testes of mice fed a HFD, compared with those fed a ND. In the HFD groups, the number of *γ-H2AX*-positive germ cells was higher at 30 and 42 weeks than at 16 weeks. The results demonstrate that *γ-H2AX* is negatively correlated with sperm quality, which is in agreement with the aforementioned study.

It has been demonstrated that the accumulation of *γ-H2AX* indicates that the incidence of unrepaired DNA breaks has increased. Without repair of these DSBs, this condition would be accompanied by the apoptosis of the germ cell.[17,18] In this study, apoptosis was evaluated in testis sections from various groups using TUNEL staining. There was an increase in the number of apoptotic germ cells in all HFD groups compared with the ND groups. A further analysis revealed that there was also a time-dependent increase in TUNEL+ nuclei and seminiferous tubules. Additionally, an obvious increase in the number of *γ-H2AX*-positive germ cells was paralleled by a strong increase in the number of TUNEL+ cells, suggesting that DNA DSBs did not repair appropriately, which resulted in the apoptosis of the germ cells. Studies have shown that apoptosis under physiological conditions can maintain the number of germ cells in a balanced state to ensure adequate production of sperm.[43–45] However, an increased number of apoptotic germ cells is one of the most vital

mechanisms underlying the decreased number of sperm in mice fed a HFD.[46–48] Therefore, the combination of elevated oxidative stress in the testes increased DNA damage, and the initiation of apoptosis of germ cells may have led to a significant decline in sperm quality.

Limitations

Several limitations of the present study should be mentioned. No information was obtained about sex hormones that can affect sperm quality, such as testosterone levels.[49,50] At the same time, conclusions could only be drawn on the association between a HFD and sperm quality, but not the association between a HFD and fertility.[51,52] Further studies are needed to validate this relationship. Moreover, some details, such as data on food and water intake, were not measured. Furthermore, even though the distribution of the data cannot be convincingly determined for very small samples, the authors assume that the observations came from a normal distribution, and agree that if this assumption is not true, the reported p-values and confidence intervals are unreliable, and must be interpreted with caution. Additional research will be carried out to add more details, which will make future studies more rigorous and accurate.

Conclusions

In summary, the results reported in the study demonstrate that not only did a HFD have short-term deteriorative impact on sperm quality but also resulted in a progressive deterioration of sperm quality over an extended period. Mice fed a HFD had histological changes, inhibited proliferation of germ cells, increased testicular oxidative stress levels, increased DNA damage, and an increased number of apoptotic germ cells. These findings reinforce the need for maintaining vigilance against high-fat diets and underscore the importance of improving dietetic habits as soon as possible. As obesity is a growing health problem, more research is needed to investigate the relationship between obesity and male fertility in a greater depth.

ORCID iDs

Xiangyu Qi https://orcid.org/0000-0002-9874-8599
Qingbo Guan https://orcid.org/0000-0002-1549-5304
Wenjing Zhang https://orcid.org/0000-0001-6336-8636
Xinshuang Huang https://orcid.org/0000-0003-1021-0020
Chunxiao Yu https://orcid.org/0000-0003-4605-6010

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