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KEAP1–NRF2 system regulates age-related spermatogenesis dysfunction

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Abstract

Purpose: The average fatherhood age has been consistently increasing in developed countries. Aging has been identified as a risk factor for male infertility. However, its impact on various mechanisms remains unclear. This study focused on the KEAP1–NRF2 oxidative stress response system, by investigating the relationship between the KEAP1–NRF2 system and age-related changes in spermatogenesis.

Methods: For examination of age-related changes, we used 10-, 30-, 60-, and 90-week-old mice to compare sperm count, sperm motility, and protein expression. For assessment of Keap1 inhibition, 85-week-old C57BL/6J mice were randomly assigned to the following groups: control and bardoxolone methyl (KEAP1 inhibitor). Whole-exome sequencing of a Japanese cohort of patients with non-obstructive azoospermia was performed for evaluating.

Results: Sperm count decreased significantly with aging. Oxidative stress and KEAP1 expression in the testes were elevated. Inhibition of KEAP1 in aging mice significantly increased sperm count compared with that in the control group. In the human study, the frequency of a missense-type SNP (rs181294188) causing changes in NFE2L2 (NRF2) activity was significantly higher in patients with non-obstructive azoospermia than in healthy control group.

Conclusions: The KEAP1–NRF2 system, an oxidative stress response system, is associated with age-related spermatogenesis dysfunction.

KEYWORDS
aging, KEAP1–NRF2, male infertility, oxidative stress

1 | INTRODUCTION

Infertility affects every one in five or six couples trying to conceive,[1] and male infertility accounts for 50% of all such cases.[2] The average parents' age at first child-birth is consistently increasing in developed countries.[3] Aging is a known risk factor for women in pregnancy.[4] Likewise, in recent years, research on the relationship between aging and male infertility has increased considerably, and aging has been identified as a risk factor for male infertility.[5] Age-related changes in the testes are attributed to the aging of spermatogonial stem cells[6]

and mitochondrial dysfunction.[7] However, the exact mechanism underlying age-related male infertility remains unexplored.

RNA sequencing has revealed lower expressions of antioxidant genes in the testes and cerebrum, which have an organ–blood barrier, than in other organs, and these organs are known to be vulnerable to age-related changes caused by the accumulation of oxidative stress.[8] The oxidative stress response system comprises enzymes involved in the removal of reactive oxygen species and mechanisms that regulate their expression.[9,10] The Kelch-like ECH-associated protein 1 (KEAP1)–NF-E2-related factor 2 (NRF2) system is one such regulatory system; dysregulation of this system causes diseases associated with age-related changes such as Alzheimer's disease, age-related hearing loss, and loss of salivary gland function.[11–13] It well known that oxidative stress increases with age, partly due to a decrease in KEAP1-NRF2 activity[14]; Nrf2 KO mice are known to have low tolerance to oxidative stress, and spermatogenesis is also known to decrease with age.[15] On the other hand, Keap1 KO mice are known to die after birth, which is a disadvantage of NRF2 overactivity.[16] Although several studies have been conducted on the KEAP1–NRF2 system and its age-related functional impairment in various organs, the relationship between these impaired functions and male infertility still remains unclear.

Therefore, in this study, we investigated the relationship between the age-related changes in the KEAP1–NRF2 system and spermatogenesis. We also determined the therapeutic potential of bardoxolone methyl (BD), a KEAP1 inhibitor, in treating age-related male infertility.

Furthermore, we analyzed single nucleotide polymorphism (SNP) data from whole-exome sequencing to examine the association between the KEAP1–NRF2 system and male infertility in humans. Studies on the relationship between oxidative stress and male infertility have been previously conducted,[17,18] and SNPs in certain genes have been associated with male infertility.[19,20] A few studies have shown an association between SNPs in the NRF2 promoter region and male infertility[21,22]; however, none of the studies had been performed using whole-exome sequencing data. To the best of our knowledge, this is the first study to examine the role of the KEAP1–NRF2 system in male infertility, in both experimental animals and humans.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Osaka University (IACUC approval no.: J007559-005). C57BL/6J mice were procured from Japan SLC (Shizuoka, Japan) and The Jackson Laboratory Japan (Kanagawa, Japan). All animals were kept under standard environmental conditions (22°C, 12/12 h light/dark cycle). For examination of age-related changes, 10-, 30-, 60-, and 90-week-old mice were used ($n = 5$ per group). For assessment of Keap1 inhibition, 85-week-old C57BL/6J mice were randomly assigned to the following groups ($n = 5$ per group): control and bardoxolone methyl (BD) (HY-13324, MedChemExpress, NJ, USA). Based on a previous study, BD was administered to mice at 10 mg/kg by supplementing it in drinking water.[23,24] The mice were sacrificed after 5 weeks of treatment (control and BD groups were alternately sacrificed to exclude confounding by order).

2.2 | Sperm analysis

Spermatozoa obtained from the cauda epididymis were incubated in 200 μL of Toyoda, Yokoyama, and Hoshi (TYH) medium (LSI Medience, Tokyo, Japan). Following incubation for 30 min in TYH medium (total 600 μL), spermatozoa were collected from the top of the drops and analyzed using a CEROS II sperm analysis system (software version 1.4; Hamilton Thorne Biosciences, MA, USA) with 20-micron 4chamber slide (GK Med Systems, PA, USA).

2.3 | Histological examination

Testes were harvested and fixed in 10% formalin. They were embedded in paraffin wax, sliced into 4-μm-thick sections, stained with hematoxylin and eosin (H&E), and examined under a BZ-X700 microscope (Keyence Co., Osaka, Japan). Abnormality was defined as seminiferous tubules showing disorganization of the lumen of the seminiferous tubules, vacuolization, and absence of germ cells. Abnormality rate was defined as (at ×40 magnification):

$$\text{Abnormality rate} = \frac{\text{Number of abnormal seminiferous tubules per field of view}}{\text{Total number of seminiferous tubules}}.$$

2.4 | Immunohistochemistry

For immunohistochemical analysis, formalin-fixed, paraffin-embedded tissue sections were first de-paraffinized. Antigen retrieval was performed in citrate buffer (pH 6) using a pressure cooker, and endogenous peroxidase was blocked using Dako REAL Peroxidase-Blocking Solution (Agilent, CA, USA). The sections were incubated overnight with primary antibodies diluted in Dako Antibody Diluent (Agilent) at 4°C. For staining, the Dako EnVision+ System- HRP-Labeled Polymer Anti-rabbit (Agilent) and Dako Liquid DAB+ Substrate Chromogen System (Agilent) were used according to the manufacturer's instructions, and the sections were counterstained with hematoxylin. Immunofluorescence was examined using a BZ-X700 microscope. The following primary antibodies were used rabbit anti-4-hydroxy-2-nonenal (anti-4HNE; 1:300; # bs-6313R, Bioss Antibodies Inc., MA, USA) and rabbit anti-Ki67 (1:300; # ab16667; Abcam, MA, USA).

Ki67-positive cells were defined as those with stained nuclei, and the number of positive cells in 20 seminiferous tubules was counted.

The intensity of the stain was graded from 0 to 2 as follows: 0, no staining; 1, mild staining (cytoplasm and nuclei in seminiferous tubule faintly stained); 2, strong staining (cytoplasm and nuclei in

seminiferous tubule strongly stained). The 4HNE index was defined as the sum of the scores for 20 seminiferous tubules.

2.5 | Immunofluorescence

Immunofluorescence was performed on 4-μm-thick serial sections of formalin-fixed paraffin-embedded tissues; the tissue sections were de-paraffinized and subjected to antigen retrieval in citrate buffer (pH 6) using a pressure cooker. Primary antibodies were added to each section and incubated overnight at 4°C. The slides were incubated with secondary antibodies for 60 min at room temperature. After washing with Tris-buffered saline containing Tween 20 (Merck, Darmstadt, Germany), the slides were counterstained with Vectashield Hardset Mounting Medium (Vector Laboratories, CA, USA). Immunofluorescence analysis was performed using a BZ-X700 microscope. The primary antibody used was rabbit anti-NRF2 (1:300; #ab31163; Abcam), while anti-rabbit antibody with Alexa Fluor 488 (1:300; #A11034; Thermo Fisher Scientific, MA, USA) was used as the secondary antibody.

2.6 | Immunoblotting

Proteins were extracted from whole testes and homogenized in RIPA buffer (Nacalai Tesque, Kyoto, Japan) with protease inhibitor (Nacalai Tesque, Kyoto, Japan), and whole lysates were collected. The protein concentrations were measured using Lowry's method.[25] The extracted proteins were stored at −80°C until immunoblotting. Twenty micrograms of protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed using immunoblotting. Membranes were blocked with Bullet Blocking One for Western Blotting (Nacalai Tesque, Kyoto, Japan) and incubated overnight with primary antibodies in Tris-buffered saline at 4°C. Anti-rabbit IgG, Horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology, MA, USA), and Chemi-Lumi One (Nacalai Tesque) were used for detection. A ChemiDoc XRS Plus imaging system (Bio-Rad, CA, USA) was used to obtain images of the blots. The ImageJ software (National Institutes of Health, MD, USA) was used to quantify the bands. The primary antibodies used were as follows: rabbit anti-KEAP1 (1:1000; #8047S; Cell Signaling Technology), rabbit anti-Ho-1 (1:1000; #86806S; Cell Signaling Technology), rabbit anti-NQO-1 (1:1000; # ab80588; Abcam), rabbit anti-NRF2 (1:1000; #12721S; Cell Signaling Technology), and rabbit anti-ACTB (1:1000; #4967S; Cell Signaling Technology). Anti-rabbit IgG HRP antibody (1:5000; #7074, Cell Signaling Technology) was used as the secondary antibody.

2.7 | Single-cell analysis

A single-cell transcriptome data of murine testis were obtained from the previous report.[26] The expression of Keap1 and Nrf2 was re-analyzed by Loupe Browser software (10× Genomics, CA, USA). The cell types of each cluster were identified manually by the expression of characteristic marker genes used in the previous study.[26]

2.8 | Testosterone analysis

Testosterone was measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS), which was performed by Asuka Pharmaceutical.

2.9 | SNP analysis

Fifty patients with non-obstructive azoospermia (especially maturation arrest) who visited Osaka University Hospital between 2000 and 2018 were enrolled for this study. For whole-exome sequencing, genomic DNA was extracted from peripheral blood using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific), according to manufacturer's instruction. Library was prepared using the SureSelect Clinical Research Exome V2 (Agilent Technologies, CA, USA) according to the manufacturer's protocol. Sequencing was performed on an Illumina HiSeq 3000 (Illumina, CA, USA) platform in 100 bp paired-end format. Germline mutations were identified using the Genomon pipeline (https://github.com/Genomon-Project/genomon-docs/tree/v2.0). GRCh37 was used as the reference genome.

Among the SNPs extracted from patients with non-obstructive azoospermia, we searched for the presence of KEAP1 and NRF2 (NFE2L2) SNPs, and compared the incidence of these SNPs with that in a healthy cohort. As control cohorts, we used the publicly available databases, namely, GEM Japan Whole Genome Aggregation (GEM-J WGA), Tohoku Medical Megabank Organization (ToMMo) database, Human Genetic Variation Database (HGVD), and Genome Aggregation Database (GenomAD).

This study was approved by the Institutional Review Board of Osaka University Hospital (IRB no. 668-2), and written informed consent was obtained from all study participants.

2.10 | Statistical analysis

All data were analyzed using JMP® 15 (SAS Institute Inc., Cary, NC, USA) and are presented as mean ± standard deviation (SD). p-Values <0.05 were considered statistically significant. Two-tailed Student's t-test for experiments with two groups, and the Tukey–Kramer method for experiments including ≥3 groups were used for analysis, as appropriate. Pearson's chi-square test was used to evaluate differences in the proportions of SNPs between patients with non-obstructive azoospermia (our data) and the control cohort (GEM-J WGA). $^*p<0.05$.

3 | RESULTS

3.1 | Age-related changes in sperm count, testicular pathology, and Ki67-positive cell count

In 90-week-old mice, sperm motility remained unchanged, but the sperm count was lower than that in 10-, 30-, and 60-week-old mice ($n=5$ per group; Figure 1A,B). Furthermore, analysis of sperm motility showed that only linearity (LIN) tended to increase with age (Figure 1C and Figure S1). Testicular weight increased at 30, 60, and 90 weeks of age compared to 10 weeks of age (Figure 1D), but there was no difference when corrected for body weight; rather, testicular weight decreased at 60 and 90 weeks of age compared to 30 weeks of age (Figure 1E). The percentage of abnormalities in the seminiferous tubules increased with advancing age from 10 to 90 weeks (Figure 1F,G). The number of Ki67-positive cells was significantly lower at 90 weeks, suggesting a reduction in sperm concentration (Figure 1H,I). In addition, pathological images of the epididymis showed vacuolation between epithelium cells in 60- and 90-week-old mice, which was not observed in 10-week-old mice (Figure 1J).

3.2 | Changes in oxidative stress and KEAP1 expression in the testes with aging

The oxidative stress marker 4-HNE (4-hydroxy-2-nonenal) showed staining in germ cells as well as in Sertoli cell. The 4-HNE index in the testes increased with age in weeks, indicating that oxidative stress in the seminiferous tubules increased with aging (Figure 1K,L). The expression of KEAP1 also increased with age (Figure 1M). Based on previous single-cell genome analysis data, KEAP1 is expressed not only in somatic cells but also in germ cells, and its expression was also observed in both NRF2 (Figure S2). Both *Keap1* and *Nrf2* were expressed in type A spermatogonia in the germ cells. Therefore, we decided to investigate whether inhibition of Keap1 improves spermatogenesis.

3.3 | Keap1 inhibitor increases sperm counts in aging mice

Treatment of aging mice with the KEAP1 inhibitor (BD) did not affect the sperm motility (Figure 2A), but significantly increased the sperm count ($p=0.0001$; Figure 2B). Furthermore, analysis of sperm motility showed an increase in curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) and a decrease in LIN (Figure 2C–E and Figure S3). No change in testicular weight was observed after treatment with BD treatment (Figure 2F). Since KEAP1 is expressed in Leydig cells, the effect on testosterone was also considered and measured, but no significant difference was observed (Figure S3f). No significant changes were also observed in the epididymis (Figure S3g). The expression of KEAP1 decreased in the BD-treated group ($p=0.0022$; Figure 2G). As nuclear translocation of NRF2 is important for its function, we evaluated it using immunofluorescence. The percentage of cells with nuclei positive for NRF2 significantly increased in the BD group ($p=0.0002$; Figure 2H,I). In addition, the expression of antioxidant genes (*Hmox1* and *Nqo-1*) increased in the BD-treated group ($p=0.0432$ and 0.0194, respectively; Figure 2J), and the 4HNE index, a marker of oxidative stress, decreased ($p=0.0140$; Figure 2K,L). The BD-treated group also showed a lower rate of abnormalities in the seminiferous tubules ($p=0.0027$; Figure 2M,N) and an increased rate of Ki67-positive staining compared to the control group ($p=0.0004$; Figure 2O,P).

3.4 | Investigation of SNPs in *KEAP1* and *NRF2* in infertile men

We searched the whole-exome sequencing data of male infertility patients for SNPs in *KEAP1* and *NFE2L2* (gene encoding NRF2). Patient characteristics are shown Table.1. The Johnsen's score was 2 for the lowest and 7 for the highest. SNPs were found in *KEAP1* and *NFE2L2* (rs187892356 (1/50; 2%) and rs181294188 (2/50; 4%), respectively). Rs187892356 is classified as a missense variant that converts arginine to cysteine, while rs181294188 is also a missense variant that converts alanine to valine. The frequency of rs187892356 in male infertile patients was not significantly high, but showed a general increasing trend (Table S1, $p=0.1817$). Rs181294188 was significantly more frequent in infertile males than in the healthy Japanese cohorts (Table 2, $p<0.0001$). These two SNPs were more common in Asian patients compared with the global cohort, although they were even more common in our patients with non-obstructive azoospermia.

4 | DISCUSSION

In humans, spermatological tests of 16- to 72-year old individuals revealed a decrease in spermatogenesis after the age of 34 years[27]; furthermore, in vitro fertilization, implantation, pregnancy, and fertility rates decline with each additional year of age in males.[28] However, a few studies have focused on the effect of aging on spermatogenesis in laboratory animals such as mice. We show that spermatogenesis decreased at 90 weeks of age in C57BL/6J mice. Age-related changes in the testes are believed to be caused by the aging of sperm6atogonial stem cells[6] and abnormal mitochondrial function[7]; however, the detailed mechanisms remain unclear with regard to several aspects. The relationship between NRF2 and spermatogenesis has received much attention in recent years.[29,30] Nrf2-knockout mice show no problems in spermatogenesis at 2 months of age, but at 6 months, sperm counts are reduced compared with those in wild-type mice[15]; similarly, age-related changes in the gonads of *Drosophila* are associated with NRF2 activity.[31] In contrast, there have been no studies focusing on KEAP1, which regulates NRF2, and age-related changes in the testes. Here, we show that besides NRF2, KEAP1 is also essential in regulating age-related changes in the testes.

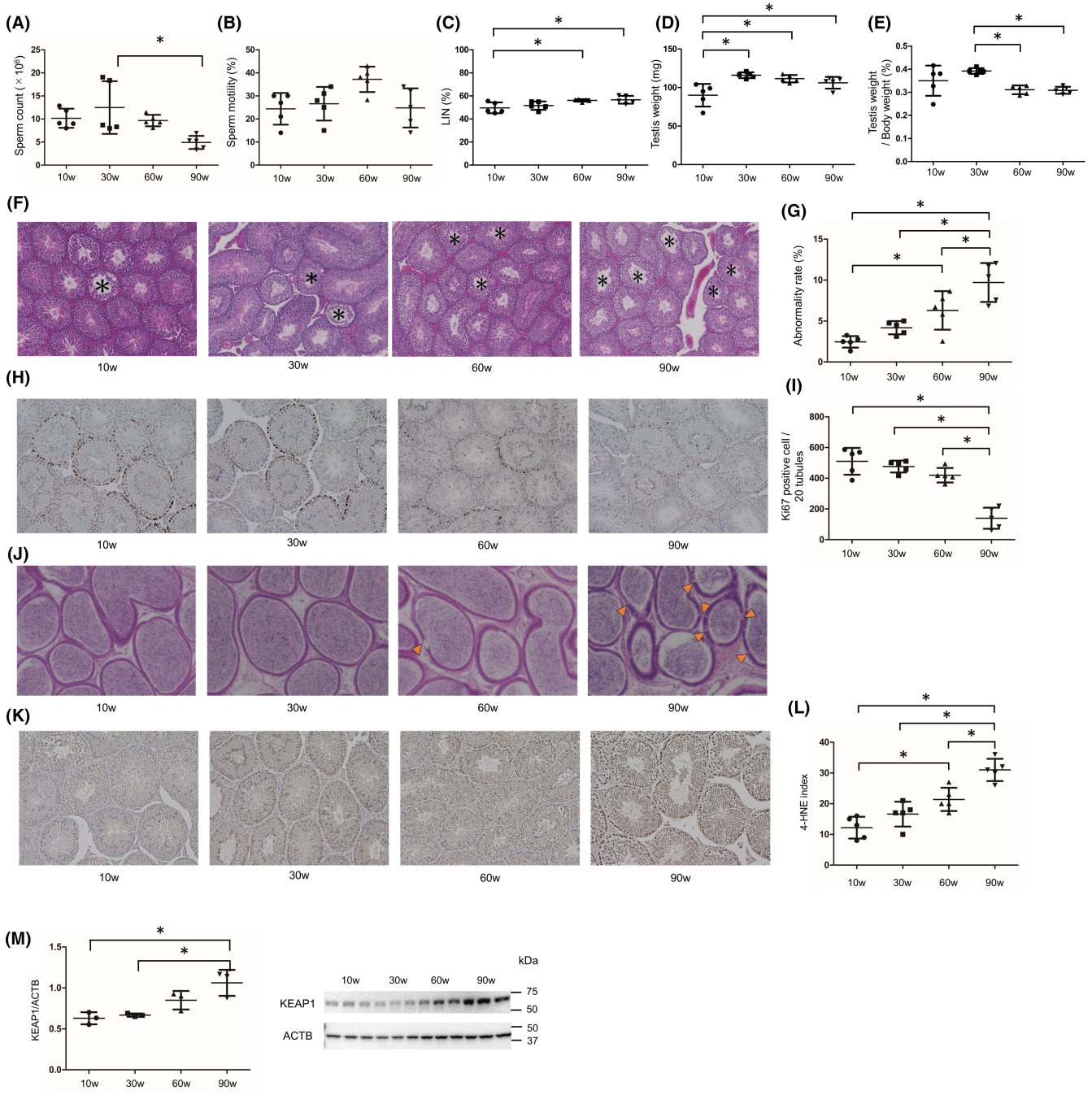

FIGURE 1 Age-related changes in spermatogenesis. (A) Sperm count, (B) Sperm motility, (C) LIN, (D) testis weight, and (E) testis weight/body weight in 10-, 30-, 60-, and 90-week-old mice ($n = 5$ per group). (F) Hematoxylin and eosin staining of the testes in 10-, 30-, 60-, and 90-week-old mice; (*) shows abnormal seminiferous tubules. (G) Age-related changes in the abnormality rate of seminiferous tubules ($n = 5$ per group). (H) Ki-67 immunohistochemistry of the testes, and (I) Ki-67-positivity rate of 20 seminiferous tubules ($n = 5$ per group) of 10-, 30-, 60-, and 90-week-old mice. (J) Hematoxylin and eosin staining of the epididymis in 10-, 30-, 60-, and 90-week-old mice; triangle shows vacuolation. (K) 4-HNE immunohistochemistry of the testes, and (L) 4-HNE index in 10-, 30-, 60-, and 90-week-old mice ($n = 5$ per group). The intensity of the staining was graded from 0 to 2 as follows: 0, no staining; (1) mild (cytoplasm and nuclei in seminiferous tubule faintly stained); (2) strong (cytoplasm and nuclei in seminiferous tubule strongly stained). 4-Hne index was the sum of the scores of 20 individual seminiferous tubules. (M) Representative results of immunoblotting (right) and quantitative analysis (left) of KEAP1 and ACTB in the testis of 10-, 30-, 60-, and 90-week-old mice ($n = 3$ per group). All data are expressed as mean ± SEM. All statistical analysis were performed using Tukey–Kramer method. 4-HNE, 4-hydroxy-2-neonenal; LIN, Linearity.

Various studies have reported the role of the KEAP1–NRF2 system as an oxidative stress control system.[32,33] Under normal conditions, KEAP1 remains bound to NRF2 in the cytoplasm (Figure 3A), whereas under oxidative stress, this binding is disrupted, and NRF2 is translocated into the nucleus. Nuclear translocation of NRF2 induces the expression of antioxidant genes through its binding to

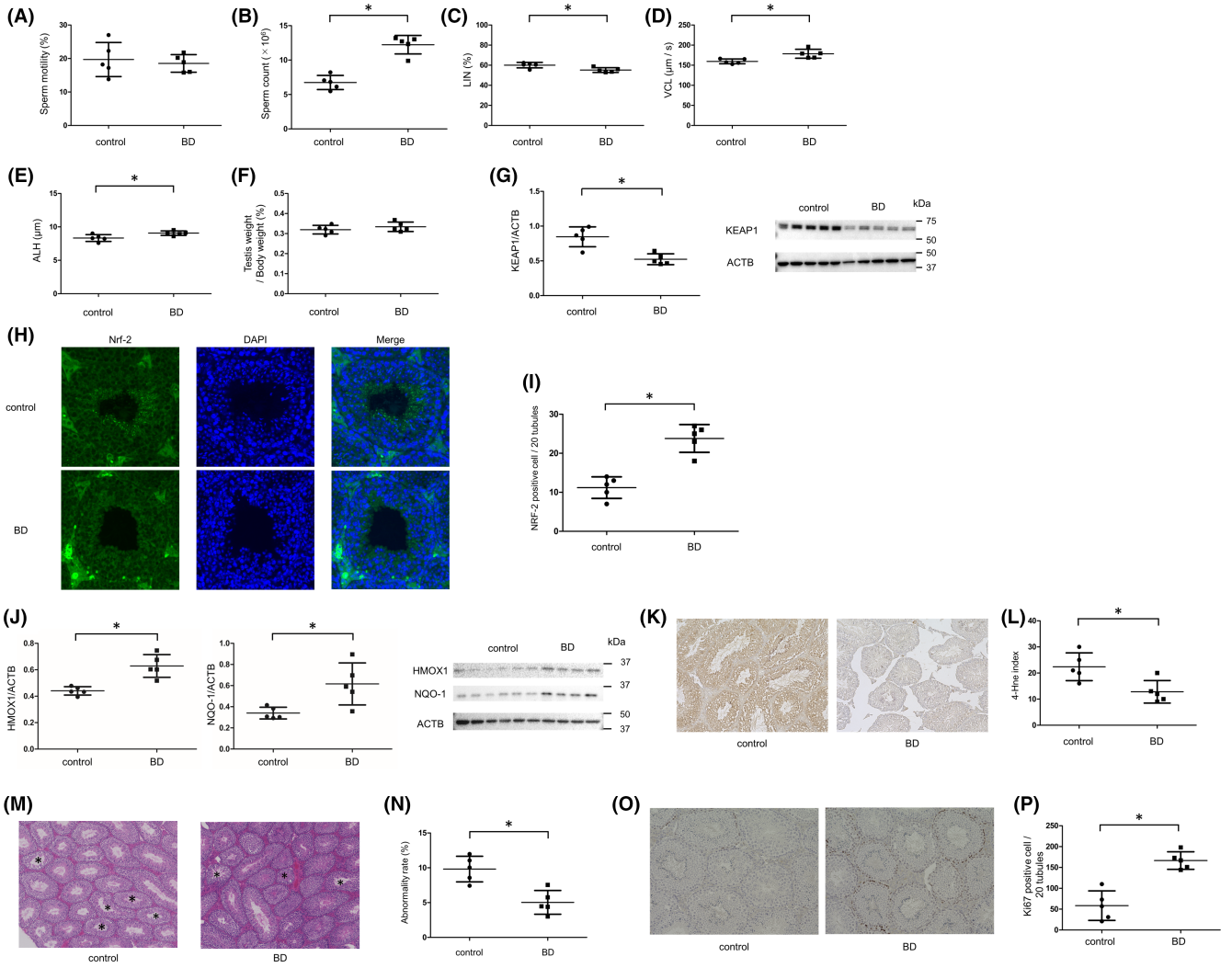

FIGURE 2 Effect of bardoxolone methyl administration. (A) Sperm motility, (B) Sperm count, (C) LIN, (D) VCL, (E) ALH, and (F) testis weight/body weight in the control and bardoxolone methyl (BD) groups ($n = 5$ per group). (G) Representative immunoblotting (right) and quantitative analysis (left) of KEAP1 and ACTB of the testes of mice in the two groups ($n = 5$ per group). (H) Representative immunofluorescence images of NRF2 staining of the testes of mice in the two groups. (I) NRF-2 positive cells in 20 seminiferous tubules in the two groups ($n = 5$ mice per group). (J) Representative immunoblotting (right) and quantitative analysis of HMOX1 (left), NQO-1 (middle) and ACTB in the two groups ($n = 5$ per group). (K) 4-HNE immunohistochemistry of the testes, and (L) 4-Hne index of the mice in the two groups ($n = 5$ per group). (M) Hematoxylin and eosin staining of the testes of mice in the two groups ($n = 5$ per group); (*) shows abnormal seminiferous tubules. (N) Abnormality rate of seminiferous tubules in the two groups ($n = 5$ per group). (O) Ki-67 immunohistochemistry of the testes, and (P) Ki-67 positivity rate in individual 20 seminiferous tubules of mice in the two groups ($n = 5$ per group). Data are expressed as the mean ± SEM. All statistical analyses were performed using the two-tailed Student's *t*-test. 4-HNE, 4-hydroxy-2-neonenal; ALH, amplitude of lateral head displacement; LIN, linearity; VCL, curvilinear velocity.

antioxidant response elements (AREs) (Figure 3B). Induction of antioxidant genes alleviates oxidative stress and prevents the accumulation of oxidative stress and cellular damage, thereby preventing the acceleration of aging.[12,34] We show, for the first time, that the expression of KEAP1 increases with aging in the testes. Furthermore, administration of KEAP1 inhibitors increased the nuclear translocation of NRF2 in seminiferous tubules, increased the expression of the antioxidant genes (*Hmox1* and *Nqo-1*), and reduced oxidative stress. Reduction in oxidative stress increased the number of Ki67-positive cells in seminiferous tubules and improved spermatogenesis. This indicated that oxidative stress improved via the KEAP1–NRF2 system by suppressing the expression of KEAP1, which increased

TABLE 1 Characteristics of patients.

	Patients ($n = 50$)
Age (years)	34.3 ± 5.2
Sperm retrieval rate (%)	40 (18/45)
Testis size (mL)	10.5 ± 5.4
Johnsen's score	4.3 ± 1.4
LH (mIU/mL)	6.6 ± 4.2
FSH (mIU/mL)	18.9 ± 13.4
Testosterone (ng/mL)	4.8 ± 8.2
Estradiol (pg/mL)	26.5 ± 10.2
Prolactin (ng/mL)	10.8 ± 6.3

TABLE 2 Single nucleotide polymorphism of NFE2L2 (rs181294188) observed in this study, compared with open database.

	Population	Alt	Total	Frequency	*p*-Value
Our research	Japanese	2	50	0.04	<0.0001
GEM-J WGA	Japanese	44	15 192	0.00289	
ToMMo	Japanese	50	16 760	0.00298	
HGVD	Japanese	14	2420	0.00579	
GenomAD	East Asian	10	1560	0.00641	
GenomAD	Whole World	32	152 174	0.00021	

Abbreviations: GEM-J WGA, GEM Japan Whole Genome Aggregation; GenomAD, Genome Aggregation Database; HGVD, Human Genetic Variation Database; ToMMo, Tohoku Medical Megabank Organization.

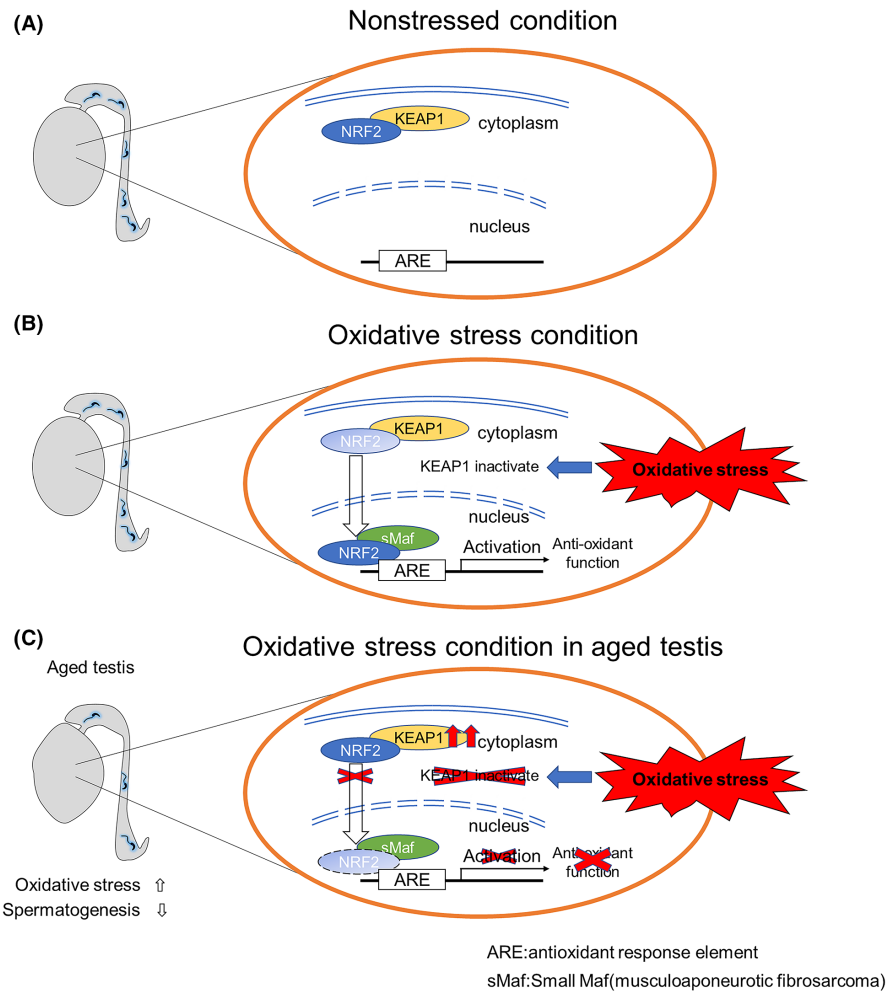

FIGURE 3 Schematic representation of the KEAP1–NRF2 system in the testis. (A) KEAP1–NRF2 system under non-stressed conditions. (B) KEAP1–NRF2 system in the testes under oxidative stress. KEAP1 and NRF2 dissociate and NRF2 is translocated to the nucleus where it exhibits antioxidant activity by activating antioxidant genes. (C) KEAP1–NRF2 system in the testis of aged individual. In an aged testis, excessive KEAP1 inhibits nuclear translocation of NRF2, resulting in the accumulation of oxidative stress.

with aging. Furthermore, the amelioration of oxidative stress in the seminiferous tubules leads to the proliferation of germ cells and increases the sperm count (Figure 3C). This suggests that increased expression of KEAP1 with aging may increase oxidative stress and reduce spermatogenesis. Furthermore, it has been reported that age-related decline in spermatogenesis is due to a decrease in type A spermatogonia[35]; considering the co-localization of KEAP1 and NRF2 in type A spermatogonia in our single-cell analysis, the KEAP1-NRF2 system may be a factor in the age-related decline in spermatogenesis.

We found missense variants of *KEAP1* and *NRF2* (*NFE2L2*) (rs181294188 and rs187892356, respectively) which were frequently expressed in male patients with non-obstructive azoospermia than in healthy cohorts. This suggests that the KEAP1–NRF2 system is not only involved in age-related spermatogenesis dysfunction but may also be the cause of non-obstructive azoospermia. Previous reports underline the relationship between KEAP1, NRF2, and spermatogenesis in humans, suggesting that SNPs in the *NRF2* promoter region are predominant in infertile men.[21,22] When

correlated with our study, the KEAP1–NRF2 system indeed plays an important role in the human spermatogenesis.

Recently, novel therapies targeting the KEAP1–NRF2 system have been developed.[32,36] Dimethyl fumarate, an NRF2 activator, has been clinically used to treat multiple sclerosis[37]; similarly, BD, a KEAP1 inhibitor, has been clinically used in treating the Alport syndrome[38] and has entered phase III clinical trials in the treatment of diabetic nephropathy.[39] BD is known to dissociate the bond between KEAP1 and NRF2 and activate NRF2 by binding to the cysteine residues of KEAP1.[40] Although BD has been reported to be effective against age-related hearing loss,[11] Alzheimer's disease,[13] and osteoarthritis,[23] its use for treating male infertility has not been reported. Administration of BD successfully increased the nuclear translocation of Nrf2, which was associated with decreased oxidative stress, thus improving spermatogenesis. This study highlights a potential novel treatment option for male infertility, which is often difficult to treat.

This study also had some limitations. Although we identified SNPs in the KEAP1–NRF2 system in patients with non-obstructive azoospermia, their relationship with aging needs to be further investigated. Furthermore, we have not been able to examine how missense-type mutations alter protein formation, and we believe that further studies are needed. Moreover, the involvement of oxidative stress pathways other than the KEAP1–NRF2 system in spermatogenesis remains uninvestigated. BD administration inhibits KEAP1 function but does not essentially decrease KEAP1 expression. We consider that the decreased expression of KEAP1 in this study may be the result of decreased oxidative stress in the testes.

In conclusion, this study reveals that the KEAP1–NRF2 system plays an important role in spermatogenesis. Using SNP analysis, we found that missense variants of KEAP1 and NRF2 were more frequently expressed in male patients with non-obstructive azoospermia than in healthy cohorts. Nevertheless, the study also highlights bardoxolone methyl (BD) as a potential therapeutic option for male infertility.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest. Shinichiro Fukuhara is an Editorial Board member of Reproductive Medicine and Biology and a co-author of this article. To minimize bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964. Written informed consent was obtained from all patients included in the study.

ANIMAL STUDIES

All animal procedures were approved by the Institutional Animal Care and Use Committee of Osaka University (J008270-003).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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