INTRODUCTION

The decline of reproductive activity occurs in a way that is specific to species and gender. Reproductive activity in women ends upon menopause, whereas there is no complete cessation of reproductive capacity in men (Tenover, 2003). In men, a combination of morphological and molecular changes in reproductive organs with advanced age may lead to a decline in reproductive activity (Handelsman, 2006). Recent research has shown that mice lacking the transient receptor potential vanilloid receptor-1 (TRPV1) pain receptors have a long-lived and youthful metabolic profile at an older age (Riera et al., 2014).

TRPV1 is a nonselective cation channel that was originally found to be expressed in sensory neurons. TRPV1 is activated by heat, protons, capsaicin, and endovanilloids and functions as a multimodal detector of noxious stimuli (Caterina et al., 1997). TRPV1 knockout (KO) mice show impaired nociception and pain sensation (Caterina et al., 2000). Subsequent studies have shown that TRPV1 is expressed in non-neuronal cells, including male germ cells, and its activation is associated with cell protection...
and apoptosis (Mizrak et al., 2008; Mizrak and van Dissel-Emiliani, 2008; De Toni et al., 2016). TRPV1 KO mice have a testis phenotype that is much more vulnerable to cell death induced by oxidative stress stimuli and massive loss of germ cells from seminiferous tubules compared to wild-type (WT) mice (Mizrak and van Dissel-Emiliani, 2008; Martins et al., 2014).

Aptosis has been found to play an important role in the aging process (Muradian et al., 2002). Apoptosis pathways are considered as key targets for delaying or reducing aging, because cell damage and inappropriate systemic signals accumulated during aging can lead to abnormal cell death and limit body function and lifespan (Shen and Tower, 2009). Although it has been found that the lifespan of TRPV1 KO mice is extended, the degree of aging, particularly in the testes, has not been addressed. Aging processes, regulated by genetic and environmental factors, can affect the lifespan. Programmed cell death that occurs during aging could also be associated with a shortened lifespan. This study was performed to investigate the relationship between TRPV1 expression and testicular apoptosis using 32-month-old WT and TRPV1 KO mice.

**MATERIALS AND METHODS**

**Chemicals**

All components of the medium and other chemicals were tissue culture grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

**Animals and testis isolation**

WT and TRPV1 KO male mice (C57BL/6j, six-week-old) were obtained from Central Lab. Animal Inc. (Seoul, Korea) and professor Dong Kun Lee (Gyeongsang National University, dklee@gnu.ac.kr), respectively. The mice were housed in a pathogen-free area under a 12-h light/dark cycle, with free access to food and water. The testes were isolated from mice that died at 32 months of age. Animal experiments were performed in accordance with the guidelines of the Gyeongsang National University Animal Care and Use Committee (GNU-161219-M0072). Individuals were monitored daily and weighed monthly but were otherwise undisturbed until they died. Survival curves were built using known birth and death dates.

**Genotyping**

To identify transgenes, before the experiment, TRPV1 KO mice were genotyped according to the protocol provided by Jackson Laboratories (Bar Harbor, ME, USA). DNA from the tail was extracted using the Phire Tissue Direct PCR Master Mix (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), and the concentration was determined using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, USA). To detect TRPV1 gene truncation in TRPV1 KO mice, forward primers (Jackson Laboratory: #19922, 5’-TGGCTCATATTTGCTTTCAAG-3’ for WT and #oIMR1627, 5’-TAAAGCGCATGCCTCAGACT-3’ for TRPV1 KO) and a common reverse primer (Jackson Laboratory, #19923; 5’ CAGCCCTTAGAGTTGATGGA 3’) were used to produce gene products.

**Reverse transcription–polymerase chain reaction (RT–PCR)**

Total RNA was isolated from mice testes using TRlzo™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, the tissues were incubated in the isopropanol and TRlzo™ Reagent for lysis. The tissue lysate was precipitated by centrifugation for 10 min at 12,000×g at 4°C. The RNA pellets were washed in 75% ethanol, air-dried, and solubilized in RNase-free water. The isolated total RNA (3 µg) was used to synthesize the first-strand cDNA using a reverse transcriptase kit (DiaStart™ RT kit; SolGent, Daejeon, Korea). PCR amplification was performed by using first-strand cDNA, Taq polymerase (G-Taq, Cosmo Genetech, Seoul, Korea), and specific primers for mouse TRPV1 (#NM_001001445.2, forward: 5’-TCACCGTACAGCTCTGTTGTC-3’ and reverse: AAGAAGTCCCCGTTAGCAGC). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH, #NM_017008, forward: 5’-CTACCGTCAGCTCTGTTGTC-3’ and reverse: AAGAAGTCCCCGTTAGCAGC) was used as a loading control. The PCR conditions included initial denaturation at 94°C for 5 min, then 32 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel to verify the product size. The images of DNA fragments were directly captured with a gel imaging system using a digital camera (Canon, Tokyo, Japan) and an ultraviolet transilluminator (Vilber Lourmat, Marne La Vallee, France). The DNA fragments were directly sequenced with the ABI PRISM® 3100-Avant Genetic
Analyzer (Applied Biosystems, CA, USA).

**Hematoxylin and eosin (H&E) staining**

Histological changes in testes were analyzed by staining with hematoxylin and eosin (H&E) solution. The tissues were fixed in 4% paraformaldehyde solution overnight at 4°C, washed in 0.1 M PBS, embedded in paraffin, and cut into 5 μm-thick sections. The sections were air-dried on gelatin-coated slides, deparaffinized, and washed with tap water for 5 min. After washing, the sections were immersed in hematoxylin solution for 5 min. The degree of hematoxylin staining was verified in tap water, followed by eosin staining for 1 min. The sections were dehydrated through a graded series of alcohols (70% to 100% ethanol, 3 min each), cleared in xylene, and mounted with permount mounting medium (Fisher Chemical, Geel, Belgium). The stained sections were observed and photographed using a BX61VS microscope (Olympus, Tokyo, Japan). Five sections from each sample were evaluated.

**Immunohistochemistry (IHC)**

To identify the expression and localization of TRPV1, the testis sections were processed by immunostaining. Deparaffinized tissue sections were permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After three washes in PBS, the sections were incubated with blocking buffer (10% normal goat serum in 0.1 M PBS) for 60 min at room temperature. Without washing, the sections were then incubated with rabbit polyclonal anti-TRPV1 (1:200 dilutions, Alomone Lab, Jerusalem, Israel) primary antibody overnight at 4°C. After three washes in PBS, the sections were incubated in the dark for 1.5 h with FITC-conjugated anti-rabbit IgG secondary antibody (Abcam, Cambridge, UK) diluted at 1:400 in PBS. Finally, the sections were washed three times in PBS and stained with propidium iodide (PI) for nuclei staining. The stained sections were wet-mounted with Gel/Mount™ (Biomeda Corp., Foster City, CA, USA) and observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

**TUNEL staining**

Apoptotic signal in the testes was assessed using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Briefly, paraffin-embedded testis sections were deparaffinized, washed, rehydrated, and washed again. The tissue slides were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, washed three times in PBS, and permeabilized with 20 μg/mL proteinase K solution for 10 min at room temperature. After three washes in PBS, the slides were refixed in 4% paraformaldehyde for 5 min at room temperature. The slides were washed in PBS for 5 min and equilibrated in an equilibration buffer for 10 min. The tissues were labeled with TdT reaction mix for 60 min at 37°C in a dark humidified chamber. The reaction was stopped with 2 X SSC solution, followed by

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**Fig. 1.** Prolonged lifespan of mice lacking TRPV1 gene (A) PCR products obtained from genotyped WT and TRPV1 KO mice were 289 bp and 176 bp, respectively. (B) Kaplan-Meyer survival curves for male WT and TRPV1 KO mice are shown. (C) Box plot showing the survival data. WT and TRPV1-/- represent wild-type and TRPV1 KO, respectively.
washing three time in PBS. Counter staining was carried out by incubating with 5 μg/mL PI for 10 min at room temperature in the dark. TUNEL-positive cells were observed using a confocal laser scanning microscope (Olympus).

Statistical analysis
The data are represented as the mean ± S.D. Significant differences between groups were evaluated using paired Student’s t-test and the log-rank test for lifespan (OriginPro 8, Northampton, MA, USA). A value of $p < 0.05$ was considered to be significant.

RESULTS

Long-lived TRPV1 KO mice
To determine whether TRPV1 is associated with longevity, WT and TRPV1 KO mice were housed until death in a pathogen-free animal area. TRPV1 genotypes were analyzed using specific primers with sequences provided by Jackson Laboratory. Male mice identified as homozygous TRPV1 KO were used in this study. Homozygous TRPV1 KO and WT mice yielded product sizes of 176 bp and 289 bp, respectively (Fig. 1A). TRPV1 KO mice were significantly longer lived, which is in line with a previous study, as shown by their survival curves compared to WT mice ($p = 0.023$, Fig. 1B). The lifespan was increased by 11.8% in male TRPV1 KO mice compared to WT mice. TRPV1 KO males lived approximately 100 days longer than WT mice, on average, and the maximum lifespan was markedly extended in TRPV1 KO mice (Fig. 1C). The TRPV1 KO mice were developmentally normal and fertile.

Expression of TRPV1 in mouse testis
The levels of TRPV1 mRNA and protein expression in the testes were determined by RT-PCR and IHC, respectively. Protein expression levels were compared between young (seven-week-old) and aged (32-month-old) mice. The testes expressed TRPV1 mRNA (Fig. 2A). Immunohistochemistry data showed that the expression of TRPV1 protein was increased in testes of older mice compared to young mice. TRPV1 was predominantly expressed in the Leydig cells of young mice, but a different expression pattern appeared in old testes. TRPV1 was expressed in the entire region of the older mice testes, and its expression was localized to the perinucleus area and the plasma membrane of testicular cells, including germ, Sertoli, and Leydig cells (Fig. 2B). As shown in Fig. 2C, the levels of TRPV1 protein expression were increased by approximately three-fold in testes obtained from aged mice compared to young mice.

![Image](image.jpg)

**Fig. 2.** Increased TRPV1 expression in old mice testes. (A) RT-PCR products for TRPV1 in testes. GAPDH was used as a loading control. (B) TRPV1 immunostaining in testes of young and old mice. Green and red indicate FITC fluorescence and PI, respectively. Scale bar, 30 μm. (C) Normalized fluorescence intensity of TRPV1 protein expression in testes of young and old mice. The fluorescence intensity was normalized to that of each background fluorescence intensity. *$p < 0.05$ compared to the young mice. FI and au represent fluorescence intensity and arbitrary unit, respectively.
Reduction of apoptosis in testicular cells obtained from TRPV1 KO mice

H&E staining showed that spermatogenic cells were orderly arranged, and no pathological damage was observed in the seminiferous tubules of young mice. However, older mice showed a decrease in the number of testicular cells in the lumen of the seminiferous tubules compared to young mice (Fig. 3A). There were no histological changes between old WT and TRPV1 KO mice. Apoptotic cells determined by TUNEL assay were abundant in the old WT testes (Fig. 3B). Apoptotic cells were mainly found in the spermatocytes and Sertoli cells of old WT mice, whereas DNase-treated apoptosis (positive control, PC) was higher in the seminiferous epithelium. Quantitative analysis showed a significant reduction in old TRPV1 KO compared to WT mice (Fig. 3C, p < 0.01).

**DISCUSSION**

Our study showed a significant increase in lifespan when lacking the TRPV1 gene. TRPV1 KO male mice lived 12% longer than their WT counterparts. The lifespan and longevity of TRPV1 KO mice examined in our study were similar to the findings of Riera et al. (2014), who demonstrated that TRPV1 KO mice showed a youthful metabolic profile when they are older compared to WT mice of the same age. The KO mice exhibited improvements in special memory and motor coordination skills that decreased with age. This report suggests that TRPV1 KO mice may have a slower progression of aging compared to WT. Currently, we do not know exactly how TRPV1 regulates aging and lifespan. It is hard to believe that a single gene regulates lifespan. It seems likely that TRPV1 controls many other molecules that affect cell proliferation and apoptosis in the body. TRPV1 is expressed in the testes (Barnes et al., 1998; Mizrak and van Dissel-Emiliani, 2008; De Toni et al., 2016), and TRPV1 activation induces apoptosis of many types of cells (Ramirez-Barrantes et al., 2016; Hou et al., 2019). Our study focused on testicular aging of TRPV1 KO mice by investigating cytological and histological changes.

Various testicular changes occur in old mice, including the reduction of testicular volume (Gosden et al., 1982; Wolf et al., 2000), accumulation of lipofuscin (age pigment) in Leydig cells (Miquel et al., 1978), multiple alterations in the Sertoli cell population (Cummins et al., 1994), loss of germ cells, and molecular alterations (Santiago et al., 2019). Our study showed decreases in the diameter and volume of seminiferous tubules and the number of Sertoli and germ cells in old mice. However, there was no changes in the number of Leydig cells between young and older mice. The expression of TRPV1 increased in the testes of older mice. Older mice showed an increased number of apoptotic cells in the seminiferous tubules compared to young mice. Interestingly, there were no significant differences in the number of testicular cells between old WT and TRPV1 KO mice. However, old TRPV1 KO mice showed lower apoptosis in the testes than WT mice. Therefore, TRPV1 was identified as a molecule that is changed in the testes of old mice. It is thought that
other factors such as low androgen levels, mitochondrial dysfunction, high oxidative stress and inflammatory factors may still contribute to testicular degeneration in TRPV1 KO mice.

What does it mean to reduce apoptosis in the testes of TRPV1 KO mice? Apoptosis is known to be a major cause of germ cell loss (Gunes et al., 2016) and aging (Shen and Tower, 2009). Barnes et al. (1998) reported that the percentage of seminiferous tubules with apoptotic cells was not significantly altered with age (Barnes et al., 1998). Jara et al. (2004) proved that not all organs in mice have the same response to aging. Apoptosis occurred in the epididymis and ventral prostate, but the seminal vesicles and other prostatic lobes were not affected (Jara et al., 2004).

The role of apoptosis is controversial in aging. Apoptosis protects tissues by removing inappropriate and damaged cells when cells are exposed to stressful environments. However, constant stress, such as aging, can increase apoptotic resistance, resulting in the improper survival of cells that cannot function normally (Pollack and Leeuwrenburgh, 2001). Aging is associated with decreased apoptosis in several cell types (Tower, 2015). On the other hand, aging increases apoptotic signaling and the testicular apoptosis that is responsible for the decline of testicular function (Jara et al., 2004; Yang et al., 2015). Based on our results, we believe that apoptosis is associated with testicular aging, and high expression levels of TRPV1 in old mice induce testicular apoptosis. TRPV1 may not be directly related to the loss of testicular cells.

A limitation of this study is that specific markers to identify Sertoli, Leydig, and germ cells were not applied. Further study is needed to investigate the associations between TRPV1, testosterone, and Leydig and Sertoli cells. In addition, the role of TRPV1 in testicular aging should be identified in more detail. In conclusion, we confirmed that TRPV1 is associated with testicular apoptosis. In testes, TRPV1 expression levels were highly increased in old mice. In addition, TRPV1 KO old mice showed a longer lifespan and a lower level of apoptosis in the testes compared with those in WT mice. Our results suggest that TRPV1 may be associated with testicular aging.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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