Heat stress disrupts spermatogenesis via modulation of sperm-specific calcium channels in rats

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Abstract

Heat is a detrimental environmental stressor that disrupts spermatogenesis and results in male infertility. Previous investigations have shown that heat stress reduces the motility, number, and fertilization ability of living spermatozoa. Sperm hyperactivation, capacitation, acrosomal reaction, and chemotaxis towards the ova are regulated by the cation channel of sperm (CatSper). This sperm-specific ion channel triggers the influx of calcium ions into sperm cells. The aim of this study in rats was to investigate whether heat treatment affected the expression levels of CatSper-1 and -2, together with the sperm parameters, testicular histology and weight. The rats were exposed to heat stress for 6 days and the cauda epididymis and testis were collected 1, 14, and 35 days after heat treatment to measure sperm parameters, gene and protein expression, testicular weight, and histology. Interestingly, we found that heat treatment caused a notable downregulation of CatSper-1 and -2 expression at all three time points. In addition, there were significant reductions in sperm motility and number and an increase in the percentage of abnormal sperm at 1 and 14 days, with cessation of sperm production at 35 days. Furthermore, expression of the steroidogenesis regulator, 3 beta-hydroxysteroid dehydrogenase (3β-HSD) was upregulated in the 1-, 14- and 35-day samples. Heat treatment also upregulated the expression of the apoptosis regulator, BCL2-associated X protein (BAX), decreased testicular weight, and altered testicular histology. Therefore, our data showed for the first time that heat stress downregulated CatSper-1 and -2 in the rat testis, and that this may be a mechanism involved in heat stress-induced impairment of spermatogenesis.

Keywords CatSper, Heat stress, Rats, Spermatogenesis

1. Introduction

Infertility is a health issue that affects approximately 15% of couples who have regular unprotected intercourse (Speroff and Fritz, 2005). The latest World Health Organization (WHO) statistics indicate that approximately 50–80 million people suffer from infertility worldwide (Briceag et al., 2015; Kumar and Singh, 2015). According to recent studies, 20–70% of infertility cases are attributable to male-related factors (Agarwal et al., 2015; Qi et al., 2016). According to the International Committee for Monitoring Assisted Reproductive Technology and the WHO, infertility is defined as the inability to conceive after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009).

The mammalian testis must be maintained in the temperature range of 2–8°C below the core body temperature for normal spermatogenesis and good testicular function to occur (Ritzén, 2008). A rise in testicular temperature may be harmful to spermatogenesis and as a result can cause infertility (Lin et al., 2015; Shadmehr et al., 2018). Infertility is now becoming more prevalent because of occupational or lifestyle factors such as prolonged driving or sitting, sauna use, and wearing tight-fitting underwear (Barazani et al., 2014; Kim et al., 2013). The testis exhibits a range of changes in response to heat stress, including heat shock, oxidative stress, and apoptosis (Lin et al., 2015; Zhang et al., 2015). Scrotal heat stress compromises the thermoregulatory system in the testis by exacerbating mitochondrial dysfunction, leading to a rise in the production of reactive oxygen species (ROS), a decline in the production of antioxidant enzymes and heat shock proteins, and increased apoptosis and cell death (Rasooli et al., 2010; Kumar et al., 2016). In addition, heat stress reduces the sperm count and motility and causes an increase in the percentage of abnormal sperm (Modaresi et al., 2018). Heat stress also induces apoptosis of the

spermatogonial germ cells in the seminiferous tubules, resulting in lower sperm density, altered testicular morphology (Rasooli et al., 2010; Wang et al., 2014), and consequently poor fertilization capacity both in vivo and in vitro.

Mammalian spermatozoa must proceed through a variety of challenges after ejaculation before fertilizing an egg (Darszon et al., 2011). These processes are usually stimulated by activation of ion channels expressed on the sperm membrane (Lishko et al., 2012; Singh and Rajender, 2015), such as the cation channel of sperm (CatSper) complex, which consists of four pore-forming subunits (CatSper1–4) and five accessory subunits (β, δ, ε, γ, and ζ) (Chung et al., 2017). CatSper-1 and -2 are considered vital for flagellar beat and hyperactivated motility of sperm (Carlson et al., 2005; Marquez et al., 2007), whereas CatSper-3 and -4 are involved in the acrosome reaction and penetration of the egg coat (Jin et al., 2007; Jin et al., 2005). Any disruption of the CatSper subunits impairs the CatSper channel and results in male infertility (Chung et al., 2017; Hwang et al., 2019).

Previous studies have demonstrated downregulated expression of CatSper genes in mice who received oral administration of bisphenol A (Wang et al., 2016), cadmium (Wang et al., 2017), and dutasteride or nifedipine (Srivastav et al., 2018). CatSper expression was also suppressed after oral administration of methamphetamine in rats (Jahromi et al., 2022), spinal cord injury in a mouse model (Rezaian et al., 2009), and experimental varicocele in rats (Soleimani et al., 2018). However, there have been no studies on the relationship between heat stress and CatSper expression. The current study in heat-stressed rats investigated the changes at different time points in mRNA and protein levels of CatSper-1, CatSper-2, 3 beta-hydroxysteroid dehydrogenase (3β-HSD) as a steroidogenesis marker,

BCL2-associated X protein (BAX) as an apoptotic marker, sperm parameters (motility, number, and abnormal sperm percentage), and testicular weight and histology.

2. Materials and methods

2.1. Chemicals and animals

Chloral hydrate, 1% eosin Y solution, and 10% neutral buffered formalin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other reagents used in the study were of the highest grade commercially available. Eight-week-old male Wistar rats (180–200 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All the animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University (approval number/ T2022-64).

2.2. Experimental design

Twenty-four male Wistar rats were divided randomly into two groups, a control group (12 rats) and a heat-treated group (12 rats), with 4 animals in each group investigated at 3 different time points. The rats in the heat-treated group were used to generate a heat stress model based on the methods described by Yamamoto et al. 2000; Rockett et al. 2001 and Vera et al. 2005. In brief, rats in the heat-treated group were anesthetized by intraperitoneal administration of chloral hydrate (400 mg/kg), and their testes immersed in a thermostatically controlled water bath at 43°C for 30 min once daily for 6 days. The rats in the control group were treated in the same way, except their testes were immersed in a water bath maintained at 22°C. The rats were then sacrificed by cervical dislocation at either 1, 14, or 35 days after treatment according to Kanter et al. (2013), with the cauda epididymis and testis collected to determine sperm parameters, testicular weight, and the expression of CatSper-1, CatSper-2, 3β-HSD, and BAX.

2.3. Measurement of testes weight

The weight of the right and left testes of the heat-treated rats was measured using an electronic balance and compared to those of the control group (Yadav et al. 2017).

2.4. Histological examination of the rat testes

The left testis was excised and embedded in OCT compound, followed by freezing with dry ice/ethanol. Frozen sections of the testis were cut into 10 μm thick sections using a cryostat (CM3050S; Leica Biosystems, Tokyo, Japan), followed by hematoxylin-eosin (H&E) staining to observe testicular histology. Briefly, the sections were stained with Mayer's hematoxylin solution for 10 min at room temperature, washed with distilled water, and then stained with 1% eosin Y solution for 1 min at room temperature. The sections were dehydrated in 80–100% ethanol, cleared with xylene, mounted in hydrophobic mounting medium (Entellan New® 21), and observed using a fluorescence phase contrast microscope (BZ-9000, Keyence, Osaka, Japan).

2.5. Sperm collection and analysis

The cauda epididymis was removed and placed in a sterilized petri dish containing 2 mL of normal saline prewarmed to 37°C and then macerated to release the epididymal contents in a suspension that was investigated using the same methods as for semen (Hafez, 1970).

2.5.1. Sperm motility analysis

Sperm motility was analyzed according to the procedure of Slott et al. (1991). A drop of the epididymal suspension was placed on a prewarmed glass slide, covered with a

prewarmed glass cover slide, and then inspected under a light microscope (Axio Vert.A1, Carl Zeiss, Germany) to assess individual sperm motility. Several microscopic fields were examined to evaluate the percentage of motile spermatozoa.

2.5.2. Sperm count using a Neubauer hemocytometer

The sperm concentration is the number of sperm in one milliliter of semen, while the sperm count is the total number of sperm in a sample (one ejaculate). The sperm cell concentration per milliliter of semen was determined as described by Robb et al. (1978). Semen was further diluted with normal saline and a few drops of formalin added to kill the spermatozoa. The sperm were counted using an improved Neubauer hemocytometer counting chamber (Matsunami Glass Ind, Ltd, Osaka, Japan).

2.5.3. Total sperm abnormalities (%)

The number of abnormal sperm was measured using the method of Filler (1993). Briefly, one drop of semen was mixed with one drop of eosin stain. A smear was prepared, air-dried, and examined under a light microscope. Abnormal sperm was recorded as the percentage (%) of the number of abnormal spermatozoa relative to the total sperm concentration.

2.6. RNA purification from testicular tissue

Total RNA was extracted from the testis and purified using NucleoSpin® RNA (Macherey-Nagel, Germany). At first, a homogenizer pestle was used to homogenize 30 mg of testis in 350 μL of lysis buffer (RA1) to form a lysate, which was then passed through a

NucleoSpin® filter. Approximately 350 μL of 70% ethanol were added to the homogenized lysate and mixed several times by pipetting. The mixture was placed in a NucleoSpin® RNA column and centrifuged for 30 s at 11,000 x g, with the flow fraction then discarded. Next, 350 μL of MDB was added to the column as the membrane desalting buffer and the column was centrifuged at 11,000 x g for 1 min to dry the membrane and remove the flowthrough. The membrane was then washed three times: the first time by adding 200 μL of RAW2 buffer and centrifuging for 30 s at 11,000 x g to discard the flowthrough; the second time by adding 600 μL of buffer RA3 and centrifuging for 30 s at 11,000 x g; and the third time by adding 250 μL of buffer RA3 and centrifuging for 2 min at 11,000 x g to dry the membrane. Finally, the RNA was eluted using 60 μL of RNase-free water, followed by centrifugation at 11,000 x g for 1 min. The total RNA concentration was measured using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Rockland, USA) (Vershinin et al., 2021).

2.7. mRNA quantification using real-time RT-PCR

cDNA was synthesized from 200 ng of total RNA with PrimeScript RT Master Mix (Perfect Real Time, Takara Bio, Otsu, Japan) on a MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA). The reverse transcription reaction was performed at 37°C for 15 min, followed by 85°C for 5 s. Real-time RT-PCR was performed using TB Green™ Premix Ex TaqTM II (Tli RNaseH Plus, Takara Bio) and the Thermal Cycler Dice Real-Time System III (Takara Bio). To analyze the mRNA expression levels of CatSper-1, CatSper-2, 3β-HSD, BAX, and β-actin, the cDNA was denatured at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s to produce amplification. The primer

sequences used are shown in Table 1. By analyzing the Ct value for β-actin (Supplementary Table 1), we confirmed that heat treatment did not affect the level of β-actin mRNA. Therefore, we considered β-actin as the internal control for this experiment. The CatSper-1, CatSper-2, 3β-HSD, and BAX mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin.

2.8. Immunohistochemical analysis of CatSper-1, CatSper-2, 3β-HSD, and BAX in testicular cross-sections following heat treatment

A cryostat was used to prepare 10 μm frozen cross-sections of the collected testicles. The sections were then rinsed twice with PBS for 5 min, after which they were blocked in PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton-X-100 for 1 hr at room temperature. Following three consecutive PBS washing steps that each lasted two min, the cross-sections were incubated with either rabbit anti-rat CatSper-1, rabbit anti-rat CatSper-2, rabbit anti-rat 3β-HSD, or rabbit anti-rat BAX in 3% BSA/PBS at dilutions indicated by the manufacturer. Following an overnight incubation at 4°C, the cross-sections were treated with an Alexa Fluor 488 conjugated anti-rabbit IgG antibody in 3% BSA/PBS for 1 hr at room temperature as directed by the manufacturer. Next, the sections were washed with PBS and then observed with CLSM. The fluorescence intensity of the antibody staining was quantified using ImageJ software. The following primary antibodies were used: anti-CatSper 1 rabbit polyclonal antibody (MBS2520520; MyBioSource, CA, USA), anti-CatSper 2 rabbit polyclonal antibody (AP54271; Abcepta, CA, USA), anti-3β-HSD rabbit polyclonal antibody (PA5-106895; ThermoFisher Scientific, MS, USA), and anti-BAX rabbit polyclonal antibody (#2772; Cell Signaling Technology, MS, USA).

2.9. Statistical analysis

Student's t-test was used to determine the statistical significance of the comparison between two groups, while one-way ANOVA was used to compare multiple groups. p values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Effects of heat treatment on testicular weight

We first examined the effect of heat treatment on testis weight at various time intervals (Fig. 1). The treated rats exhibited a significant decrease in testis weight one day after heat stress compared with that observed in the control rats, with this decrease not improving after 14 and 35 days.

3.2. Morphological changes in the testis following heat treatment

Next, we examined the effect of heat treatment on testicular histology at various time intervals (Fig. 2). The testis of the control rats showed normal architecture, with the seminiferous tubules filled with germ cells at different stages of spermatogenesis and abundant spermatozoa in the lumen. The seminiferous tubules of the rats in the recovery group after one day of heat stress exposure were shrunken with a decreased diameter and were empty of spermatogenic cells. In addition, the lumen was increased in diameter in the hyperthermia group compared to that in the control group due to the removal of spermatogenic cells. This damage did not show improvement even at days 14 and 35.

3.3. Changes in sperm parameters after heat treatment

We then investigated the effect of heat treatment on sperm parameters at the three time points (Fig. 3). Heat treatment was associated with a significant decrease in sperm motility in the heat treatment group at 1 and 14 days compared with that observed in the control group (Fig. 3A). The sperm count was also decreased markedly after heat treatment at 1 and 14 days compared with that in the control group (Fig. 3B). Furthermore, there was a

significant decrease in the percentage of normal sperm (Fig. 4A) and an obvious increase in the percentage of abnormal sperm (Figs. 4B–F) in the heat-treatment rats compared with that in the control rats at days 1 and 14 (Fig. 3C). We also noted a cessation in sperm production (azoospermia) 35 days after heat treatment.

3.4. Alterations in the CatSper-1 and -2 mRNA and protein level following heat treatment

We then evaluated the influence of heat treatment on mRNA and protein levels of CatSper-1 and-2 in rat testes at various time points (Fig. 5 and Fig. 6). We used the expression of the β-actin gene as the internal control for this experiment. For the first time, we found a notable downregulation in CatSper-1 mRNA expression levels in the heat-treatment group compared with that in the control group on day 1, with this downregulation remaining at 14 and 35 days after heat stress (Fig. 5A). We also observed a significant reduction in CatSper-2 mRNA expression in the heat-stressed rats compared with that observed in the control rats (Fig. 6A). In addition, significant reductions in the expression of the CatSper-2 gene were observed on days 14 and 35 compared to that on day 1 after heat stress, suggesting that downregulation of CatSper-2 occurred in a time-dependent manner (Fig. 6A). Moreover, consistent with the RT-PCR data, the immunohistochemical analysis confirmed that the CatSper-1 and -2 protein level were reduced in the testicular tissue of the rats on days 1, 14, and 35 days after heat treatment compared with that observed in the control rats (Figures 5B-5E and 6B-6E).

3.5. Alterations in 3β-HSD and BAX mRNA and protein levels following heat treatment

Finally, we examined the effect of heat treatment on mRNA and protein levels of the steroidogenesis and apoptosis markers. We found that heat treatment resulted in a significant increase in 3β-HSD mRNA and protein levels on day 1 compared with those in the control group, with this expression also increased significantly on days 14 and 35 (Fig. 7). Expression of the BAX gene was increased in rats exposed to heat stress as soon as day 1 compared with that measured in the control rats, with this increase being maintained on days 14 and 35 (Fig. 8A). In addition, we showed a significant increase in BAX protein level in the heat stressed rats on days 1, 14, and 35 compared with that in the control rats, and considerable upregulation of BAX protein on day 1 compared to that observed on days 14 and 35 (Fig. 8B-8E).

4. Discussion

In this study, we first examined the effects of heat stress on testis weight and sperm characteristics including motility, number, and abnormalities. Testicular weight was reduced significantly in the heat-stressed rats compared with that measured in control rats at 1, 14, and 35 days. This finding is consistent with that of Paul et al. (2008) who observed that mice exposed to local heating at 42°C for 30 min showed a significant reduction in testis weight by 40% and 60% of the control level at 7 and 14 days following post-exposure, respectively. In addition, Ziaeipour et al. (2019) reported that mice subjected to hyperthermia at 43°C for 20 min every second day for 5 wk had a significant decrease in testis weight 35 days following exposure. The decrease in testicular weight we observed in our study may have occurred due to the damage in testicular histology observed by H&E staining, which was characterized by shrunken and small seminiferous tubules and a loss of spermatogenic cells. According to Aldahhan et al. (2019), the reduction in testicular weight after heat treatment may be due to germ cell apoptosis and loss of pachytene spermatocytes and round spermatids. Furthermore, Rasooli et al. (2010) reported that heat stress increased apoptosis of spermatozoa, which led to a reduction in testis and epididymis weights.

Sperm density and mobility are crucial factors for male fertility (Kobeasyab et al., 2015). Our results showed decreases in sperm number and motility associated with an increase in the percentage of abnormal sperm in rats exposed to high temperatures after 1 and 14 days, with a cessation of sperm production at 35 days. These findings are in accordance with those of Mahdivand et al. (2019) who observed that heat stress resulted in significant decreases in sperm concentration and viability, a lower rate of fertility, and an increase in

chromatin abnormalities. However, in the current study we used a subjective method for determining sperm motility, similar to that used by other researchers (Banavath et al. 2021 and Oliveira et al. 2015). In future studies we plan to use the more accurate CASA system to confirm sperm motility after heat stress. Mice exposed to scrotal hyperthermia at 40°C for 20 min were reported to have a significant reduction in total and progressive motility of approximately 40% and a higher percentage of morphologically abnormal sperm on days 14 and 21 after heat treatment (Rizzoto et al. 2020). Rams subjected to scrotal insulation were also shown to have reduced sperm motility and an increased number of sperm with head or midpiece abnormalities 2 weeks after the initiation of insulation that lasted for 3 weeks (Kastelic et al., 2017). This decrease in sperm quality could be due to damage of the sperm plasma membrane caused by overproduction of reactive oxygen species stimulated by heat stress. This possibility is supported by evidence that spermatozoa are vulnerable to ROS due to the high level of polyunsaturated fatty acids in their plasma membrane (Agarwal et al., 2014).

We next investigated the effect of heat stress on mRNA and protein expression levels of the CatSper-1 and -2 genes in the rat testis. In both mice and humans, the CatSper gene family is expressed mainly in the testis and plays a key role in sperm motility and male fertility (Nikpoor et al. 2004; Ren et al. 2001). Reduced CatSper channel expression in sperm may impede sperm motility and hyperactivity, leading to male infertility (Sun et al. 2017). The CatSper genes are therefore crucial for mammalian fertilization. Based on our real time PCR and immunohistochemical results, CatSper-1 and -2 mRNA and protein expression levels were downregulated in testicular tissue at 1, 14, and 35 days after heat stress. Furthermore, our findings are consistent with a prior study in a rat model in which

the use of oral methamphetamine was shown to reduce the expression of these genes (Jahromi et al. 2022). Rezaian et al. (2009) also reported that gene expression of CatSper-1 and -2, but not CatSper-3 or -4, was decreased 2 weeks in a mouse model of spinal cord injury. These authors hypothesized that one of the causes of reduced sperm motility in this spinal cord injury model was downregulation of CatSper-1 and -2 expression. Infertile men with asthenozoospermia exhibit similar reductions in CatSper1–4 gene expression as well as CatSper-1 and -2 protein expression (Jin et al. 2021). Conversely, CatSper-1 or -2 null mouse spermatozoa have been shown to have impaired sperm motility, aberrant flagellar beating, and lack of hyperactivity and acrosome reaction, that culminate in overall infertility (Ho et al. 2009; Ren et al. 2001; Wang et al. 2009). Taken together, these data suggest that heat treatment may cause immobility of sperm by blocking the CatSper-specific calcium channel.

We also examined the effect of heat stress on 3β-HSD and BAX mRNA and protein levels in rat testis as markers of steroidogenesis and apoptosis, respectively. Synthesis of testosterone in the testis is essential for normal spermatogenesis (Ruwanpura et al. 2010). The expression of highly regulated genes, such as 17β-HSD3 (17β-hydroxysteroid dehydrogenase 3) and 3β-HSD has been shown to be critical for precise testosterone synthesis (Lin et al. 2015). Our study showed a significant increase in mRNA and protein expression of 3β-HSD at days 1, 14, and 35 in heat-stressed rats compared with that in control rats. These results are consistent with those of Shiraishi et al. (2010) who reported that elevated scrotal temperature upregulated testosterone in patients, possibly to reduce testicular oxidative stress-mediated apoptosis. Furthermore, there is evidence in mice that testosterone levels are highest four days after heat stress, which indicates the hypothalamic-

pituitary-Leydig cell axis may be stimulated to protect germ cells from heat damage (Li et al. 2013).

We observed a significant increase in the expression levels of mRNA and protein of BAX at 1, 14, and 35 days after heat exposure. These results are similar to those of Delkhosh et al. (2021) who reported an increase in the apoptosis index and mRNA expression of caspase 3 and BAX in testicular tissue of rats immersed in a water bath at 43°C for 20 min every second day for 8 wk. Our results also showed a marked elevation in the heat treated groups in the expression of BAX protein on day 1 compared to that measured on days 14, and 35. These findings are in line with those of Matsushita et al. (2016) who found that the testis of rats subjected to scrotal hyperthermia at 43°C for 15 min experienced a peak in their apoptotic index 1 day after the heat treatment, followed by a time-dependent decline. Collectively, these results suggested that heat stress-mediated scrotum apoptosis was triggered intensely in the rat testis within 1 day and may have been caused by protein and DNA damage induced in the testicles by the heat treatment associated with enhanced rates of cellular injury and apoptosis (Kanter et al., 2013). However, a more moderate heat stressed model based on a real scenario that occurs in the environment would be useful for obtaining a better understanding of the mechanisms underlying male infertility caused by heat stress.

5. Conclusion

This study showed that heat stress impairs sperm characteristics and testicular histology. Our data also showed that expression of CatSper-1 and -2 are downregulated following heat exposure. This latter finding sheds light on one of the mechanisms underlying male infertility caused by heat stress and suggests new concepts for further therapeutic strategies.

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Data availability

The data will be made available on request.

References

Agarwal, A., Durairajanayagam, D., Halabi, J., Peng, J., Vazquez-Levin, M., 2014. Proteomics, oxidative stress and male infertility. Reprod. Biomed. Online. 29, 32-58.

Agarwal, A., Mulgund, A., Hamada, A., Chyatte, M.R., 2015. A unique view on male infertility around the globe. Reprod. Biol. and Endocrinol. 13, 1-9.

Aldahhan, R.A., Stanton, P.G., Ludlow, H., de Kretser, D.M., Hedger, M.P., 2019. Acute heat-treatment disrupts inhibin-related protein production and gene expression in the adult rat testis. Molecular and cellular endocrinology 498, 110546.

Banavath, A. N., Srinivasa, S. N., 2021. Ameliorative effect of Punica granatum on sperm parameters in rats exposed to mobile radioelectromagnetic radiation. Asian Pac. J. Reprod. 10(5), 225.

Barazani, Y., Katz, B.F., Nagler, H.M., Stember, D.S., 2014. Lifestyle, environment, and male reproductive health. Urol. Clin. 41, 55-66.

Briceag, I., Costache, A., Purcarea, V.L., Cergan, R., Dumitru, M., Briceag, I., Sajin, M., Ispas, A.T., 2015. Fallopian tubes--literature review of anatomy and etiology in female infertility. J Med Life. 8(2), 129-31.

Carlson, A.E., Quill, T.A., Westenbroek, R.E., Schuh, S.M., Hille, B., Babcock, D.F., 2005. Identical phenotypes of CatSper1 and CatSper2 null sperm. Journal of Biological Chemistry 280, 32238-32244.

Chung, J.J., Miki, K., Kim, D., Shim, S.H., Shi, H.F., Hwang, J.Y., Cai, X., Iseri, Y., Zhuang, X., Clapham, D.E., 2017. CatSperζ regulates the structural continuity of sperm Ca^{2+} signaling domains and is required for normal fertility. Elife 6.

Darszon, A., Nishigaki, T., Beltran, C., Treviño, C.L., 2011. Calcium channels in the development, maturation, and function of spermatozoa. Physiol. Rev. 91, 1305-1355.

Delkhosh, A., Shoorei, H., Niazi, V., Delashoub, M., Gharamaleki, M.N., Ahani-Nahayati, M., Dehaghi, Y.K., Raza, S., Taheri, M.H., Mohaqiq, M., 2021. Coenzyme Q10 ameliorates inflammation, oxidative stress, and testicular histopathology in rats exposed to heat stress. Hum. and Exp. Toxicol. 40, 3-15.

Filler, R., 1993. Methods for evaluation of rat epididymal sperm morphology. Methods in Toxicol. 3, 334-343.

Hafez, E., 1970. Rabbits. Reproduction and breeding techniques for laboratory animals, 273-315.

Ho, K., Wolff, C.A., Suarez, S.S., 2009. CatSper-null mutant spermatozoa are unable to ascend beyond the oviductal reservoir. Reproduction, Fertility and Development. 21, 345-350.

Hwang, J.Y., Mannowetz, N., Zhang, Y., Everley, R.A., Gygi, S.P., Bewersdorf, J., Lishko, P.V., Chung, J.J., 2019. Dual sensing of physiologic pH and calcium by EFCAB9 regulates sperm motility. Cell 177, 1480-1494. e1419.

Jahromi, Z.A., Meshkibaf, M.H., Naghdi, M., Vahdati, A., Makoolati, Z., 2022. Methamphetamine Downregulates the Sperm-Specific Calcium Channels Involved in Sperm Motility in Rats. ACS Omega. 7, 5190.

Jin, J., Jin, N., Zheng, H., Ro, S., Tafolla, D., Sanders, K.M., Yan, W., 2007. Catsper3 and Catsper4 are essential for sperm hyperactivated motility and male fertility in the mouse. Biol. of Reprod. 77, 37-44.

Jin, J.L., O'Doherty, A.M., Wang, S., Zheng, H., Sanders, K.M., Yan, W., 2005. Catsper3 and catsper4 encode two cation channel-like proteins exclusively expressed in the testis. Biol. of Reprod. 73, 1235-1242.

Jin, Z.R., Fang, D., Liu, B.H., Cai, J., Tang, W.H., Jiang, H., Xing, G.G., 2021. Roles of CatSper channels in the pathogenesis of asthenozoospermia and the therapeutic effects of acupuncture-like treatment on asthenozoospermia. Theranostics. 11, 2822.

Kanter, M., Aktas, C., Erboga, M., 2013. Heat stress decreases testicular germ cell proliferation and increases apoptosis in short term: an immunohistochemical and ultrastructural study. Toxicol. and Ind. Health. 29, 99-113.

Kastelic, J., Wilde, R., Rizzoto, G., Thundathil, J., 2017. Hyperthermia and not hypoxia may reduce sperm motility and morphology following testicular hyperthermia. Veterinární Medicína. 62, 437-442.

Kim, B., Park, K., Rhee, K., 2013. Heat stress response of male germ cells. Cell. and Mol. Life Sci. 70, 2623-2636.

Kobeasyab, M., El-Naggarac, A., Abdallahd, A., 2015. A novel methods for protective role against reproductive toxicity of carbofuren in male rats using palm pollen grains and vanadyl (II) folate as a new compound. J. of Chem. and Pharm. Res. 7, 1142-1148.

Kumar Roy, V., Marak, T.R., Gurusubramanian, G., 2016. Alleviating effect of Mallotus roxburghianus in heat-induced testicular dysfunction in Wistar rats. Pharmaceutical biology 54, 905-918.

Kumar, N., Singh, A.K., 2015. Trends of male factor infertility, an important cause of infertility: A review of literature. J. of Hum. Reprod. sci. 8, 191.

Li, Y., Huang, Y., Piao, Y., Nagaoka, K., Watanabe, G., Taya, K., Li, C., 2013. Protective effects of nuclear factor erythroid 2-related factor 2 on whole body heat stress-induced oxidative damage in the mouse testis. Reprod. Biol. and Endocrinol. 11, 1-10.

Lin, C., Shin, D.-G., Park, S.G., Chu, S.B., Gwon, L.W., Lee, J.-G., Yon, J.-M., Baek, I.-J., Nam, S.-Y., 2015. Curcumin dose-dependently improves spermatogenic disorders induced by scrotal heat stress in mice. Food and Function. 6, 3770-3777.

Lishko, P.V., Kirichok, Y., Ren, D., Navarro, B., Chung, J.-J., Clapham, D.E., 2012. The control of male fertility by spermatozoan ion channels. Ann. Rev. of Physiol. 74, 453-475.

Mahdivand, N., Najafi, G., Nejati, V., Shalizar-Jalali, A., Rahmani, F., 2019. Royal jelly protects male rats from heat stress-induced reproductive failure. Andrologia 51, e13213.

Marquez, B., Ignotz, G., Suarez, S.S., 2007. Contributions of extracellular and intracellular Ca^{2+} to regulation of sperm motility: release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. Developmental biology 303, 214-221.

Matsushita, K., H. Miyake., K. Chiba., and M. Fujisawa., 2016. Clusterin produced by S ertoli cells inhibits heat stress-induced apoptosis in the rat testis. Andrologia 48, no. 1 (2016): 11-19.

Modaresi, M., Emadi, M., 2018. The effects of rosemary extract on spermatogenesis and sexual hormones of mice under heat stress. Trends Journal of Sciences Research 3, 69-74.

Nikpoor, P., Mowla, S.J., Movahedin, M., Ziaee, S.A.M., Tiraihi, T., 2004. CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility. Hum. Reprod. 19, 124-128.

Oliveira, N. N. P. M., Félix, M. A. R., Pereira, T. C. S., Rocha, L. G. P., Miranda, J. R., Zangeronimo, M. G. and Sousa, R. V. D., 2015. Sperm quality and testicular histomorphometry of wistar rats supplemented with extract and fractions of fruit of Tribulus terrestris L. Braz. Arch. Biol. Technol. 58, 891-897.

Paul, C., Murray, A.A., Spears, N., Saunders, P.T., 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. Reproduction 136, 73.

Qi, X., Wang, K., Zhou, G., Xu, Z., Yu, J., Zhang, W., 2016. The role of testicular artery in laparoscopic varicocelectomy: a systematic review and meta-analysis. Inter. Urol.and Nephrol. 48, 955-965.

Rasooli, A., Jalali, M.T., Nouri, M., Mohammadian, B., Barati, F., 2010. Effects of chronic heat stress on testicular structures, serum testosterone and cortisol concentrations in developing lambs. Anim. Reprod. Sci. 117, 55-59.

Ren, D., Navarro, B., Perez, G., Jackson, A.C., Hsu, S., Shi, Q., Tilly, J.L., Clapham, D.E., 2001. A sperm ion channel required for sperm motility and male fertility. Nature. 413, 603-609.

Rezaian, J., Movahedin, M., Mowla, S.J., 2009. CatSper genes expression, semen characteristics and histology of testes in the contusive spinal cord-injured mice model. Spinal Cord. 47, 76-81.

Ritzén, E.M., 2008. Undescended testes: a consensus on management. Eur J Endocrinol 159, S87-90.

Rizzoto, G., Boe-Hansen, G., Klein, C., Thundathil, J., Kastelic, J., 2020. Acute mild heat stress alters gene expression in testes and reduces sperm quality in mice. Theriogenology 158, 375-381.

Robb, G., Amann, R., Killian, G., 1978. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. Reprod. 54, 103-107.

Rockett, J.C., Mapp, F.L., Garges, J.B., Luft, J.C., Mori, C., Dix, D.J., 2001. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. Biol Reprod. 65, 229–239.

Ruwanpura, S.M., McLachlan, R.I., Meachem, S.J., 2010. Hormonal regulation of male germ cell development. The J. of Endocrinol. 205, 117-131.

Shadmehr, S., Tabatabaei, S.R.F., Hosseinifar, S., Tabandeh, M.R., Amiri, A., 2018. Attenuation of heat stress-induced spermatogenesis complications by betaine in mice. Theriogenol. 106, 117-126.

Shiraishi, K., Takihara, H., Matsuyama, H., 2010. Elevated scrotal temperature, but not varicocele grade, reflects testicular oxidative stress-mediated apoptosis. World j. of Urol. 28, 359-364.

Singh, A.P., Rajender, S., 2015. CatSper channel, sperm function and male fertility. Reprod. Biomed. Online. 30, 28-38.

Slott, V.L., Suarez, J.D., Perreault, S.D., 1991. Rat sperm motility analysis: methodologic considerations. Reprod. Toxicol. 5, 449-458.

Soleimani, M.Z., Mashayekhi, F.J., Hasanzade, M.M., Baazm, M., 2018. Alteration in CatSper1 and 2 genes expression, sperm parameters and testis histology in varicocelized rats. Inter. J. of Reprod. Biomed. 16, 183.

Speroff, L., Fritz, M.A., 2005. Clinical gynecologic endocrinology and infertility. lippincott Williams & wilkins.

Srivastav, A., Changkija, B., Sharan, K., Nagar, G.K., Bansode, F.W., 2018. Influence of antifertility agents Dutasteride and Nifedipine on CatSper gene level in epididymis during sperm maturation in BALB/c mice. Reprod. 155, 347-359.

Sun, X.-h., Zhu, Y.-y., Wang, L., Liu, H.-l., Ling, Y., Li, Z.-l., Sun, L.-b., 2017. The Catsper channel and its roles in male fertility: a systematic review. Reprod. Biol. and Endocrinol. 15, 1-12.

Vera, Y., Rodriguez, S., Castanares, M., Lue, Y., Atienza, V., Wang, C., Swerdloff, R.S., Sinha Hikim, A.P., 2005. Functional role of caspases in heat-induced testicular germ cell apoptosis. Biol Reprod. 72, 516–522.

Vershinin, Zlata., Michal Feldman., Thilo Werner., Lital Estrella Weil., Margarita Kublanovsky., Elina Abaev-Schneiderman., Menachem Sklarz., (2021). BRD4 methylation by the methyltransferase SETD6 regulates selective transcription to control mRNA translation. Science advances 7, 22, eabf5374.

Wang, H., Liu, J., Cho, K.-H., Ren, D., 2009. A novel, single, transmembrane protein CATSPERG is associated with CATSPER1 channel protein. Biol. of Reprod. 81, 539-544.

Wang, H.F., Chang, M., Peng, T.T., Yang, Y., Li, N., Luo, T., Cheng, Y.M., Zhou, M.Z., Zeng, X.H., Zheng, L.P., 2017. Exposure to cadmium impairs sperm functions by reducing CatSper in mice. Cell. Physiol. and Biochem. 42, 44-54.

Wang, H.F., Liu, M., Li, N., Luo, T., Zheng, L.P., Zeng, X.H., 2016. Bisphenol a impairs mature sperm functions by a CatSper-relevant mechanism. Toxicol. Sci. 152, 145-154.

Wang, P., Li, Y., Hu, X.c., Cai, X.L., Hou, L.P., Wang, Y.F., Hu, J.H., Li, Q.W., Suo, L.J., Fan, Z.G., 2014. Cryoprotective effects of low-density lipoproteins, trehalose and soybean lecithin on murine spermatogonial stem cells. Zygote. 22, 158-163.

Yadav, G., Awasthi, J., Pandey, N., Shrestha, S., Jha, C., 2017. Effect of vitamin E against heat stress induced testicular damage in wistar albino rats. Int J Anat Res 5, 3800-3804.

Yamamoto, C.M., Sinha Hikim, A.P., Huynh, P.N., Shapiro, B., Lue, Y.H., Salameh, W.A., Wang, C., Swerdloff, R.S., 2000. Redistribution of Bax is an early step in an apoptotic pathway leading to germ cell death in rats, triggered by mild testicular hyperthermia. Biol Reprod. 6, 1683–1690.

Zegers-Hochschild, F., Adamson, G.D., De Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., Van der Poel, S., 2009. The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology, 2009. Human reproduction 24, 2683-2687.

Zhang, M.H., Shi, Z.D., Yu, J.C., Zhang, Y.P., Wang, L.G., Qiu, Y., 2015. Scrotal heat stress causes sperm chromatin damage and cysteinyl aspartate-spicific proteinases 3 changes in fertile men. Journal of assisted reproduction and genetics 32, 747-755.

Ziaeipour, S., Rezaei, F., Piryaei, A., Abdi, S., Moradi, A., Ghasemi, A., Azad, N., Abdollahifar, M.A., 2019. Hyperthermia versus busulfan: Finding the effective method in animal model of azoospermia induction. Andrologia 51, e13438.

Figures legends

Fig. 1 Effect of heat treatment on testicular weight

The white and black columns indicate the testicular weight of the control and heat-treated rats, respectively. The data are expressed as the mean ± SD of four rats from each group. #, $p < 0.001$ vs. control group (t-test).

Fig. 2 Effect of heat treatment on testicular histology by H&E staining

L indicates lumen, SC indicates spermatogenic cells and black arrow indicates seminiferous tubules. Scale bar = 200 µm.

Fig. 3 Effect of heat treatment on sperm parameters

A) Sperm motility (%), (B) sperm number ($\times 10^6$ sperm/mL), and (C) abnormal sperm (%). The white and black columns indicate the sperm parameters of the control and heat-treated rats, respectively. The data are expressed as the mean ± SD of four rats from each group. #, $p < 0.001$ vs. control group (t-test). NM, no sperm motility; NS, no sperm.

Fig. 4 Microphotographs of morphologically normal sperm and of various sperm abnormalities

A) Normal rat sperm, (B) acephalic sperm, (C) detached head, (D) bent neck, (E) no tail, and (F) coiled tail.

Fig. 5 The effect of heat treatment on the CatSper1 mRNA and protein level in the rat testis

(A) CatSper1 mRNA level (B-D) Green fluorescence represents CatSper1 protein in the testicular tissue (E) Quantification of fluorescence intensity by ImageJ. The data are expressed as the mean ± SD of four rats from each group. #, $p<0.001$ and ##, $p<0.01$ vs. day-1 control group; ¥ , $p < 0.001$ vs. day-14 control; *, $p<0.05$ (One-way ANOVA followed by Tukey's post-hoc test). Scale bar = 20 µm.

Fig. 6 The effect of heat treatment on the CatSper2 mRNA and protein level in the rat testis (A) CatSper2 mRNA level (B-D) Green fluorescence represents CatSper2 protein in the testicular tissue (E) Quantification of fluorescence intensity by ImageJ. The data are expressed as the mean ± SD of four rats from each group. #, $p<0.001$ vs. day-1 control group; *, $p<0.05$ vs day-14 control; $ < 0.001 vs. day-35 control; +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$ (One-way ANOVA followed by Tukey's post-hoc test). Scale bar = 20 µm.

Fig. 7 The effect of heat treatment on the 3β-HSD mRNA and protein level in the rat testis (A) 3β-HSD mRNA level (B-D) Green fluorescence represents 3β-HSD protein in the testicular tissue (E) Quantification of fluorescence intensity by ImageJ. The data are expressed as the mean ± SD of four rats from each group. #, $p<0.001$ vs. day-1 control group; ¥ , $p < 0.001$ vs. day-14 control; $ < 0.001 vs. day-35 control; €, $p < 0.001$; *, $p<0.05$ (One-way ANOVA followed by Tukey's post-hoc test). Scale bar = 20 µm.

Fig. 8 The effect of heat treatment on the BAX mRNA and protein level in the rat testis

(A) BAX mRNA level (B-D) Green fluorescence represents BAX protein in the testicular tissue (E) Quantification of fluorescence intensity by ImageJ. The data are expressed as the mean ± SD of four rats from each group. #, $p<0.001$ and ##, $p<0.01$ vs. day-1 control group; ¥ , $p < 0.001$ vs. day-14 control; $ < 0.001 vs. day-35 control; €, $p < 0.001$; *, $p<0.05$; ***, $p<0.001$ (One-way ANOVA followed by Tukey's post-hoc test). Scale bar = 20 µm.

Table 1: Primers for the genes analyzed by real time PCR.

Genes	Primer	Sequence (5′ -3′)
β-actin	Forward	ACTATCGGCAATGAGCGGTTCC
	Reverse	CTGTGTTGGCATAGAGGTCTTTACG
CatSper1	Forward	TCTTGGAGCGATGAGGAC
	Reverse	GACGATTGTGTCAGGCA
CatSper2	Forward	TGGTTGTTGCTTGGT
	Reverse	TTCCTTGACTGGTTCCTCT
3β-HSD	Forward	TCCCCAGTGTATGTAGGCAATGTGGC
	Reverse	CCATTCCTTGCTCAGGGTGC
BAX	Forward	CGCGTGGTTGCCCTCTTCTACTTT
	Reverse	CAAGCAGCCGCTCAACGGAGGA

Figure 1.

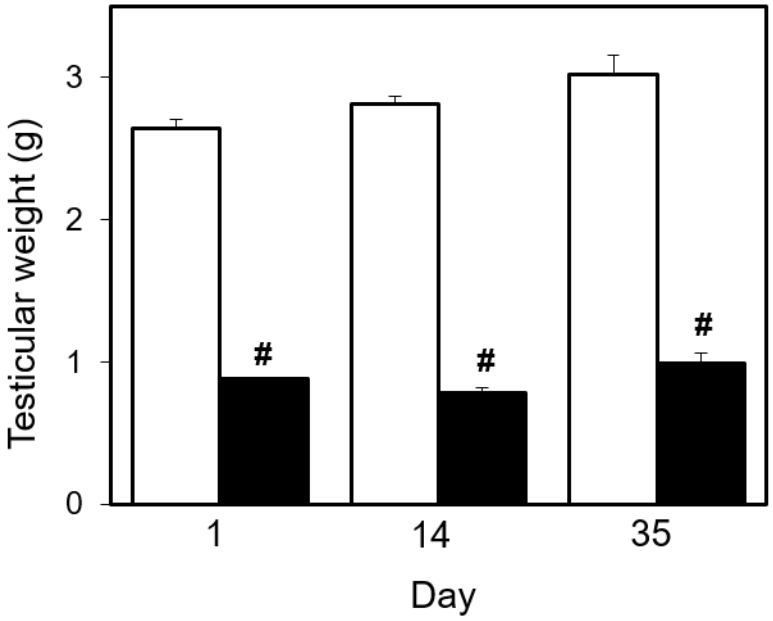


Figure 2.

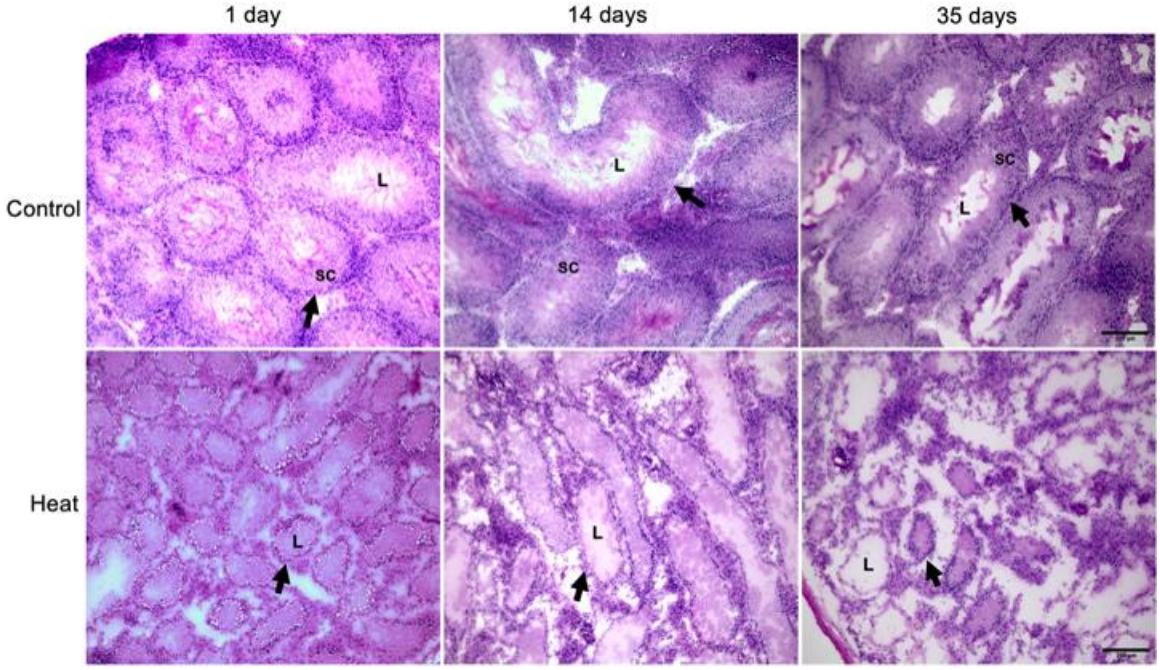


Figure 3.

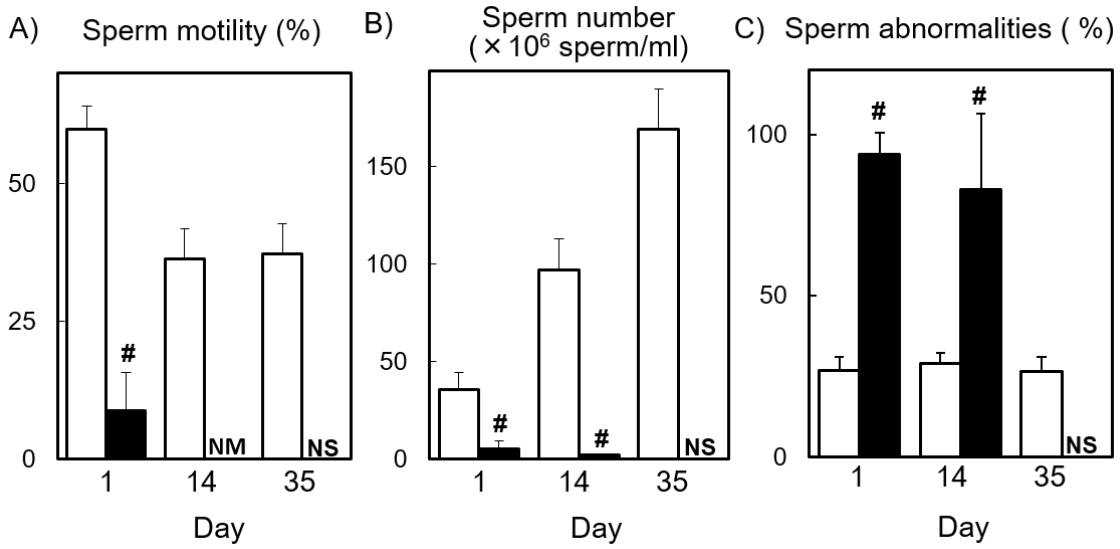


Figure 4.

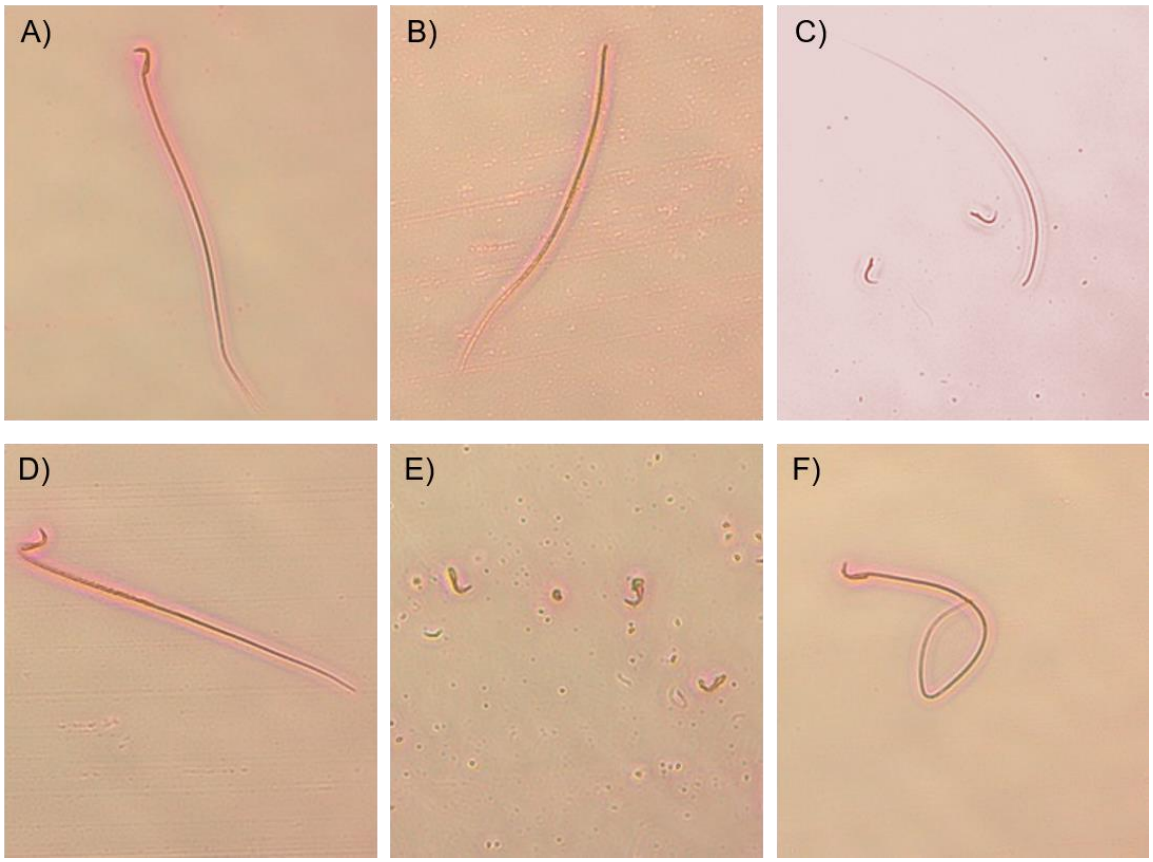


Figure 5.

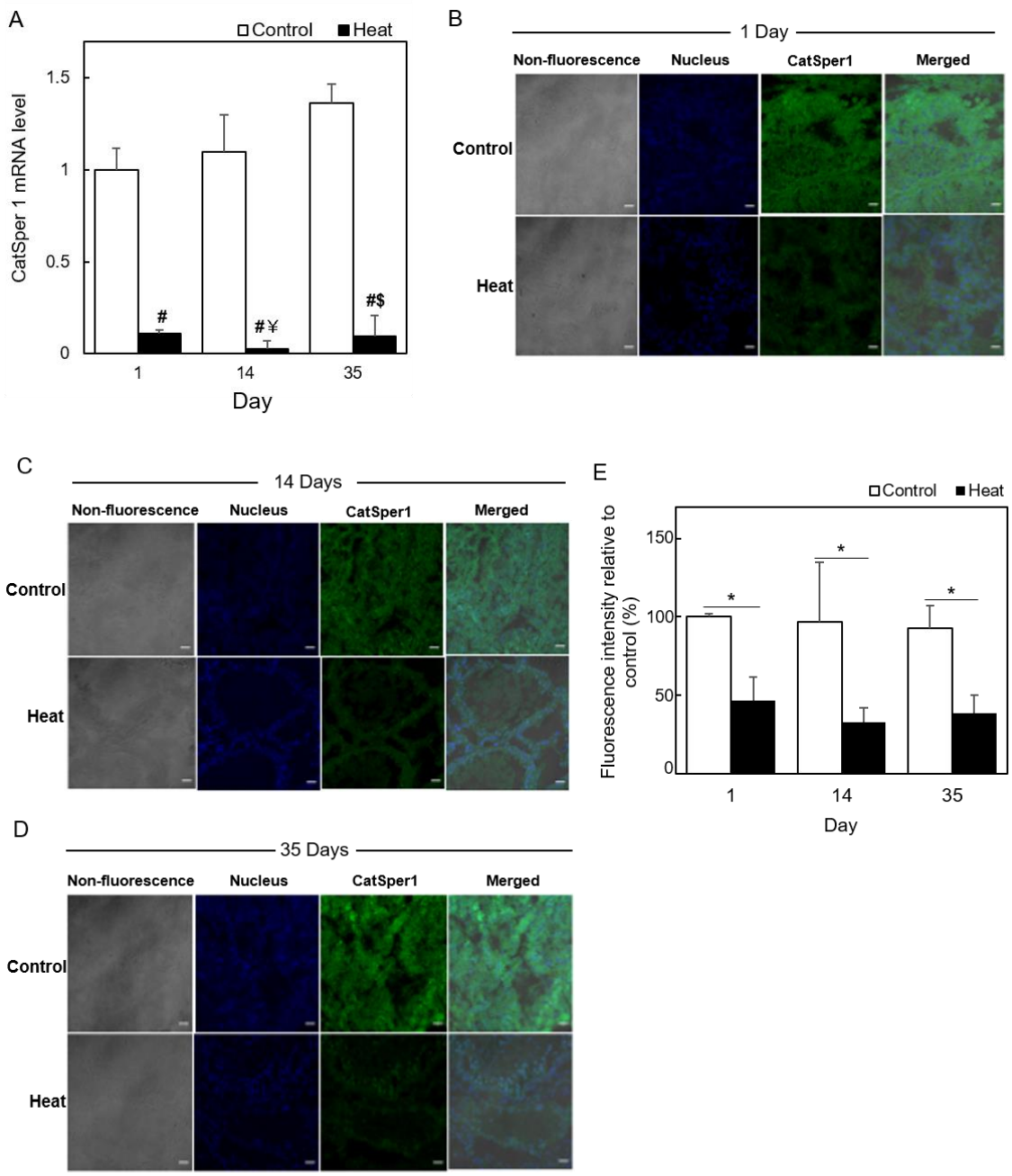

A
Control
Heat
CatSper 1 mRNA level
1.5
1
0.5
0
#
#¥
#$
1
14
35
Day
B
1 Day
Non-fluorescence
Nucleus
CatSper1
Merged
Control
Heat
C
14 Days
Non-fluorescence
Nucleus
CatSper1
Merged
Control
Heat
D
35 Days
Non-fluorescence
Nucleus
CatSper1
Merged
Control
Heat
E
Control
Heat
Fluorescence intensity relative to control (%)
150
100
50
0
*
*
*
1
14
35
Day

Figure 6.

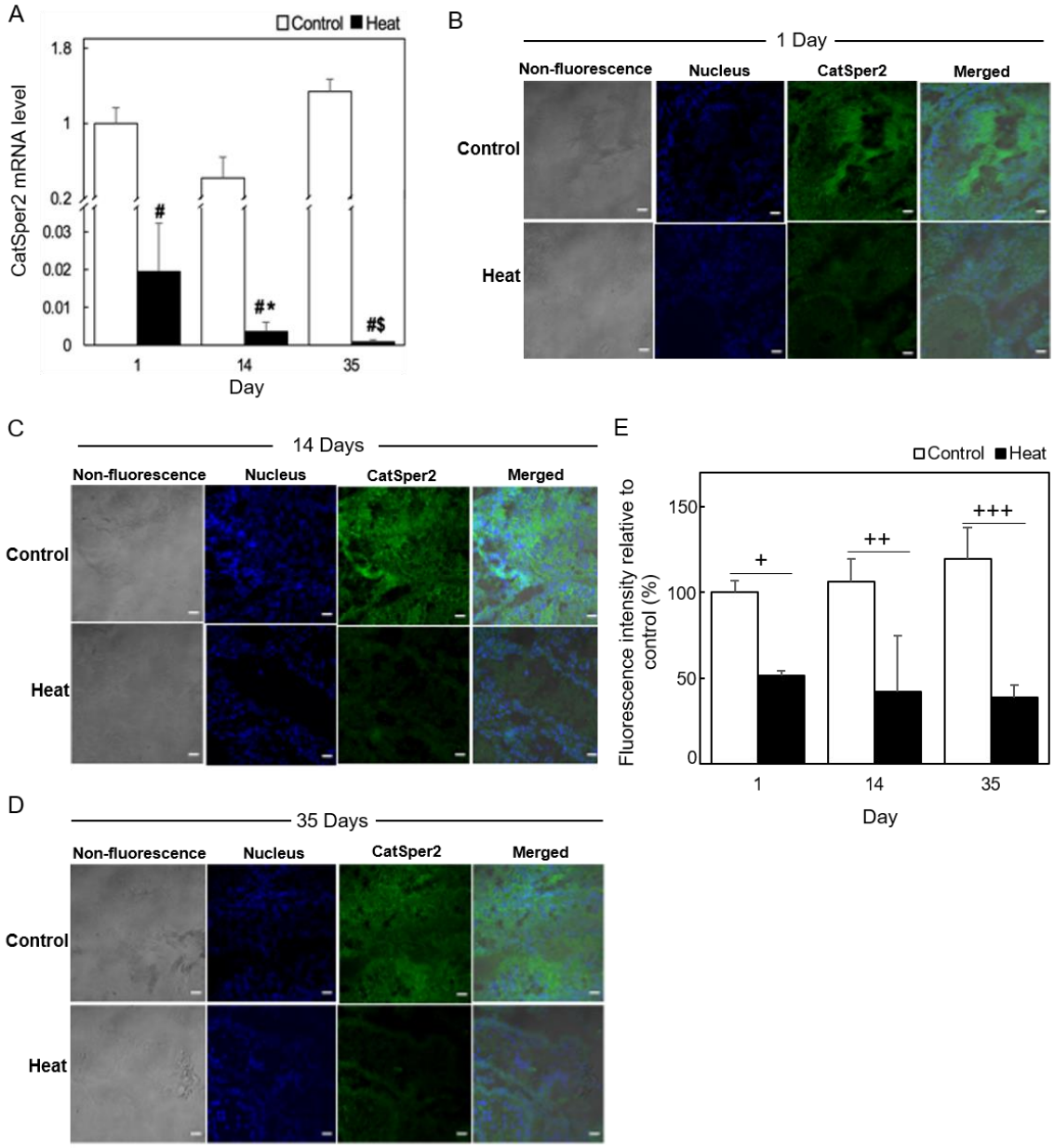

A
Control
Heat
CatSper2 mRNA level
1.8
1
0.2
0.03
0.02
0.01
0
#
#*
#$
1
14
35
Day
B
1 Day
Non-fluorescence
Nucleus
CatSper2
Merged
Control
Heat
C
14 Days
Non-fluorescence
Nucleus
CatSper2
Merged
Control
Heat
D
35 Days
Non-fluorescence
Nucleus
CatSper2
Merged
Control
Heat
E
Control
Heat
Fluorescence intensity relative to control (%)
150
100
50
0
+
++
+++
1
14
35
Day

Figure 7

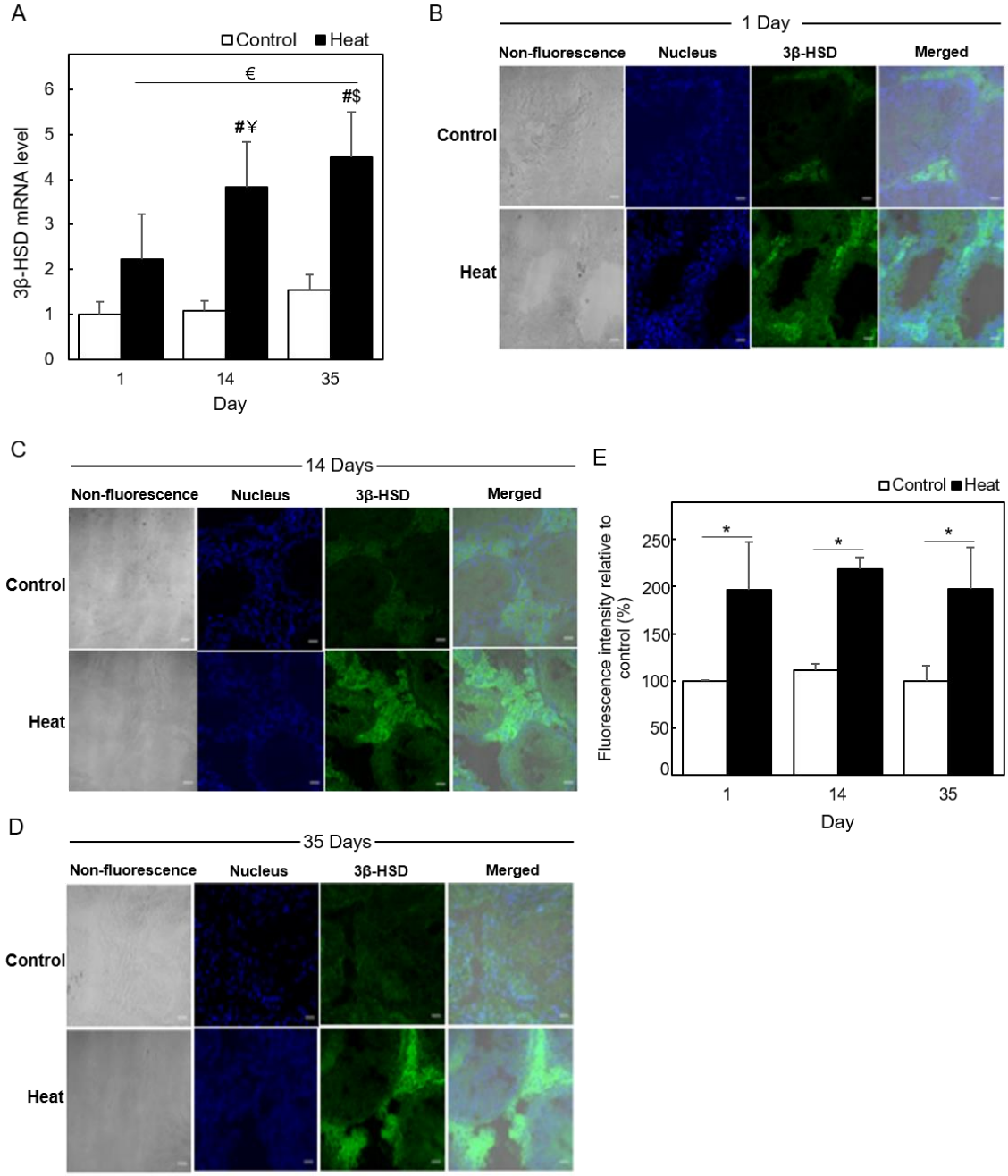
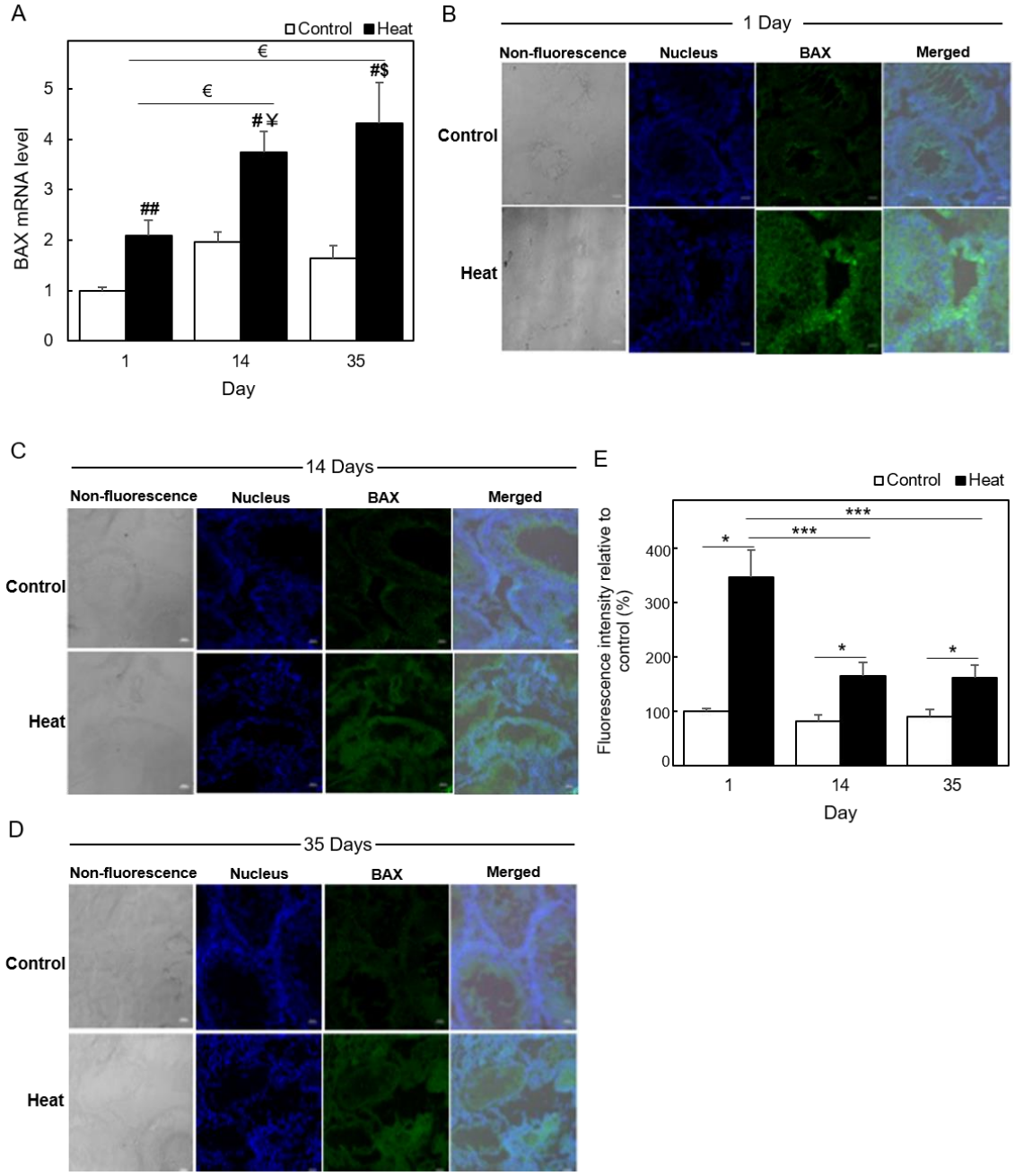

A
Control
Heat
€
#¥
#$
3β-HSD mRNA level
0
1
2
3
4
5
6
1
14
35
Day
B
1 Day
Non-fluorescence
Nucleus
3β-HSD
Merged
Control
Heat
C
14 Days
Non-fluorescence
Nucleus
3β-HSD
Merged
Control
Heat
D
35 Days
Non-fluorescence
Nucleus
3β-HSD
Merged
Control
Heat
E
Control
Heat
Fluorescence intensity relative to control (%)
0
50
100
150
200
250
*
*
*
1
14
35
Day

Figure 8.


A
Control
Heat
BAX mRNA level
Day
1
14
35
##
#¥
#$
€
€
B
1 Day
Non-fluorescence
Nucleus
BAX
Merged
Control
Heat
C
14 Days
Non-fluorescence
Nucleus
BAX
Merged
Control
Heat
D
35 Days
Non-fluorescence
Nucleus
BAX
Merged
Control
Heat
E
Control
Heat
Fluorescence intensity relative to control (%)
Day
*
***
***
*
*