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Mitochondrial OXPHOS is involved in the protective effects of L-arginine against heat-induced low sperm motility of boar

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ABSTRACT

The present study aimed to analyze the time- and temperature-responses of boar sperm and clarify the mechanism underlying the protective effects of L-arginine on heat-induced low sperm motility. Mature boar sperm was used to evaluate the effects of temperature, exposure time, L-arginine level and their interactions on sperm motility, respectively. Results showed increasing exposure time resulted in the decreased total motility and rate of rapid progressive sperm, and the increased rates of the immotile sperm and the sperm shaking in place at 38 and 39 °C, respectively ($P < 0.05$). L-arginine supplementation at the dose of 1.0 mM increased total motility and decreased rate of immotile sperm ($P < 0.05$). Heat at 39 °C decreased total motile and rate of rapid progressive sperm ($P < 0.05$), increased the level of sperm reactive oxygen species (ROS) ($P < 0.05$), reduced mitochondrial membrane potential ($\Delta\Psi m$), ATP content and the activities of mitochondrial respiratory chain complexes (MRCC) III and V ($P < 0.05$), which were attenuated by L-arginine supplementation. There were significant increases in the relative mRNA expression of *nuclear respiratory factor 1* and *peroxisome proliferator-activated receptor gamma coactivator-1 alpha* in heat-exposed group without L-arginine supplementation. In conclusion, the rising temperatures impacted boar sperm motility in a time-dependent manner. *In vitro* addition of L-arginine to boar semen had a dose-dependent effect on sperm motility and sperm incubated with 1.0 mM L-arginine showed elevated motility. L-arginine supplementation can ameliorate heat-induced increase in ROS level and decreases in MRCC activities, which further maintain mitochondrial oxidative phosphorylation function, ATP synthesis and boar sperm motility.

1. Introduction

L-arginine plays a key role in sperm physiology and has been shown to maintain sperm motility. Seasonal male infertility occurs during hot summer months and the elevated scrotal temperature have been reported to cause poor semen quality in bulls (Hansen, 2009), boars (Li et al., 2015) and men (Shiraishi et al., 2010). Our recent study found dietary L-arginine evidently improved boar semen quality in summer, which might be attributed to the enhanced mitochondrial function and antioxidative capacity in sperm (Chen et al., 2018). Previous studies reported semen cryopreserved with L-arginine showed high sperm motility and low lipid peroxidation (de Andrade et al., 2018; Özer Kaya et al., 2018). Nitric oxide (NO) was a short-lived free radical and can be synthesized from L-arginine by enzyme in spermatozoa, and expected to reduce lipid peroxidation by eliminating reactive oxygen species (ROS) (Srivastava et al., 2006; Jovicić et al., 2018). These findings indicated L-arginine had a protective effect on boar sperm motility and the possible mechanism was related to sperm ROS scavenging.

Mitochondrial oxidative phosphorylation (OXPHOS) is a highly efficient pathway of producing large amounts of ATP *via* five mitochondrial respiratory chain complexes (MRCC I to V) of the electron transport chain (ETC) in inner membrane of mitochondria (Smith et al., 2012). During the process for ATP synthesis, electron leakage from the mitochondrial ETC led to mitochondrial ROS generation, which was considered to be the major source of ROS and negative correlation with sperm motility (Koppers et al., 2008). Recent study found mitochondrial OXPHOS was fully functional under temperature more than 10 °C warmer than human body core temperature, suggesting mitochondria were optimized to nearly 50 °C and might be more sensitive to the extra or excess heat (Chretien et al., 2018). Previous study reported excess heat increased oxygen consumption, basal proton leak, and ROS production in mitochondria (Mujahid and Akiba, 2009). Moreover, excess

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heat can also decrease mitochondrial membrane potential ($\Delta\Psi m$), MRCC activities and ATP production in mitochondria of boar sperm, leading to poor sperm motility (Gong et al., 2017). These results suggested mitochondrial OXPHOS was involved in heat-induced ROS overproduction and poor sperm motility.

Spermatozoa contains a complex repertoire of mRNA, which can be used for estimating male reproductive potential (Ostermeier et al., 2002; Rogenhofer et al., 2013). Previous study reported mammalian sperm including human, bovine, mouse, and rat can independently translate nuclear-encoded proteins by mitochondrial ribosomes and inhibition of protein translation significantly reduced sperm motility, capacitation and *in vitro* fertilization rate (Gur and Breitbart, 2006, 2007, 2008). In boar sperm, a total of 18 mRNAs were found to have higher abundance signals in summer than those in winter, which were related with metabolic process and cellular biosynthetic process (Yang et al., 2010). Sperm mRNA abundance was related with sperm capacitation in boars (Hwang et al., 2013), implying boar sperm mRNA might play an important role in new synthesized proteins and mitochondrial OXPHOS as swimming in sow reproductive tracts.

Sperm generally prefer swimming towards warmer temperatures and can respond to the ascending temperature gradient, which has been proposed to be one of the processes guiding sperm to the fertilization site of oviduct (Bahat et al., 2012). There were increasing temperature gradients with a range of 37.0-38.9 °C in oviduct lumens of normally-cyclic gilts (Hunter and Nichol, 1986) and sow oviduct fluid contained many types of amino acids including arginine (Guerin et al., 1995), indicating arginine might play an important role in sperm transport in sow oviduct. The present study was conducted to elucidate the *in vitro* effect of L-arginine on boar sperm motility and mitochondrial OXPHOS under high temperatures. Time- and temperature-responses analysis was used to evaluate the heat-sensitivity of boar sperm and five additional L-arginine levels were designed to determine the optimal concentration of L-arginine supplementation. Boar semen were added with 1.0 mM L-arginine at 39 °C for 1 h and sperm were collected to measure sperm mitochondrial $\Delta\Psi m$, MRCC activities and ATP content as well as the relative mRNA expression of OXPHOS-related genes. The results may provide new sights in understanding the mechanism underlying the positive effect of L-arginine on boar sperm motility.

2. Materials and methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Certification No. SYXK(Su)2011-0036).

2.1. Experimental design

Twelve mature Duroc boars, aged from 15 to 28 months, were used to collect semen samples for *in vitro* experimental design, using the gloved-hand technique. Ejaculates with > 80% total motility were pooled and purified with prewarmed Ham's F10 medium. The pooled semen were then incubated at 37 °C under 5% CO_2 atmosphere until further experimental designs. The present study included 3 experimental designs as follows: (1) Pooled semen were diluted to 12 groups (5 replicates per group) to analyze time- and temperature-responses of boar sperm. The groups were arranged as a 3 × 4 factorial design, which included 3 temperature levels (37, 38, 39 °C) and 4 levels of incubated time (0, 1, 3, 5 h). (2) Five concentrations of L-arginine with 0, 0.25, 0.5, 1.0 and 2.0 mM were designed to detect the optimal level, which had protective effect on heat-induced poor sperm motility. Boar semen were treated with 5 levels of L-arginine and incubated at 39 °C for 1 h, respectively. (3) In order to determine the protective effect of L-arginine on sperm motility under high temperature, pooled semen were diluted to 4 groups (5 replicates per group). The groups were arranged as a 2 × 2 factorial design, which had 2 temperature levels (37, 39 °C) and 2 levels of L-arginine (0, 1.0 mM). Immediately following the 1-h incubation, sperm motility was assessed and sperm were collected to detect mitochondrial function.

2.2. Sperm motility

Sperm motility parameters were assessed using HST computer-assisted sperm analysis (CASA) with specific analysis software (NatureGene Device Co. Ltd., New Jersey, USA). Ten μL of each sample was transferred to a warm glass slide at 37 °C and 5 random microscopic fields in the same glass slide were analyzed to investigate sperm motility parameters including the percentages of total sperm motility (velocity of average path ≥ 4 μm/s), sperm with rapid progressive motility (velocity of average path ≥ 25 μm/s), immotile sperm and the sperm shaking in place.

2.3. Mitochondrial $\Delta\Psi m$ analysis

The variations of $\Delta\Psi m$ were evaluated using the specific probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzymidazolyl carbocyanine iodide) kits (G009, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The lipophilic cationic fluorochrome JC-1 is present as protomeric aggregates in mitochondria with high $\Delta\Psi m$ and present as monomers in mitochondria with low $\Delta\Psi m$. The protomeric aggregates form can be measured at excitation wavelength of 549 nm and emission wave length of 590 nm, while the monomer form can be measured at excitation wavelength of 488 nm and emission wave length of 530 nm by flow cytometer, respectively. Briefly, semen samples were diluted into isotonic buffer with JC-1 and incubated under 37 °C for 30 min. Samples were well-mixed before analysis and 10,000 events were acquired on flow cytometer.

2.4. ROS (DCFH-DA)

Intracellular ROS of sperm was measured using the sensitive probe DCFH-DA (2,7-dichlorofluorescin diacetate) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent DCF (2′,7′-dichlorofluorescin), which can be measured at excitation wavelength of 500 nm and emission wave length of 530 nm. Semen samples were diluted (10×10^6 cells/mL) and incubated with DCFH-DA under 37 °C for 15 min. Spermatozoa were centrifuged and the resuspended in 1 mL PBS. Non-sperm-specific events were gated out and 10,000 cells were examined per independent sample using a flow cytometer.

2.5. Sperm ATP concentration

Sperm were collected and lysed by 300 μL of extraction buffer. The suspension was centrifuged at 12,000 *g* for 5 min, and supernatant was maintained at 4 °C until analysis. ATP concentration was determined using the ATP determination kit according to the manufacturer's instruction. Samples were analyzed in a Glomax 96 microplate luminometer (Promega Corporation, Madison, USA). An ATP stock was used as standard to make serial dilutions. Each standard concentration was analyzed in triplicate for standard curve construction. The linearity of the relationship between ATP concentration and bioluminescence was tested in the range of 0.01-10 μM.

2.6. Activities of MRCC III and V

The activities of MRCC III was determined using kits by colorimetric method according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Sperm protein was extracted and corrected to the same concentration. Briefly, sperm were collected, suspended with lysed buffer, frozen at −70 °C and thawed at 37 °C three times to extract the mitochondrial proteins. BCA protein assay kit was used to measure protein concentration and absorbance was

determined on a Smartspec™ Plus spectrophotometer. MRCC III-linked ubiquinol cytochrome c reductase activity was measured by monitoring the reduction of cytochrome c at 550 nm. The activity was measured with or without antimycin A, a specific inhibitor of ubiquinol cytochrome c reductase. The specific activity of MRCC III was calculated by subtracting the antimycin A-nonsensitive activity from the total activity and is expressed as μM $CoQH_2$ mg/min. MRCC V activity was determined using a 96-Well Plate Reader M200 to measure the absorption at 636 nm by following the ATP increases using an ATP synthase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. RNA extraction

Sperm RNA was extracted from the purified sperm according to previous study (Savadi-Shiraz et al., 2015). Briefly, sperm were washed twice with sterile PBS and the pellet was resuspended in 700 μL of RNeasy (TNA Bee, BioSite, Täby, Sweden). Sperm were homogenized on ice for 5–10 min and then incubated on ice for 10-15 min to obtain phase separation. The upper phase was saved to a new tube and incubated at - 20 °C overnight to precipitate the RNA. The RNA was pelleted from the solution at 20,000 *g* for 12 min at 4 °C. The total RNA concentration and purity were determined by a spectrophotometer (SMOIF, Shanghai, China). For each sample, 5 μg of total RNA was reverse transcribed to cDNA with M-MLV reverse transcriptase (TaKaRa, Dalian, China) and oligonucleotide primers.

2.8. Quantitative real-time PCR

Targets genes and the housekeeping gene *beta actin* (*β-actin*) were quantified by real-time PCR on an ABI 7300 system using a commercial kit (SYBR Premix Ex Taq, TaKaRa, Dalian, China). Primers were designed with Primer 5.0 to produce an amplification product according to the gene sequence of pig (http://www.ncbi.nlm.nih.gov/pubmed). The primer sets used were shown in Table 1. PCR reactions (consisting of SYBR Premix Ex Taq, ROX Reference Dye, 200 nM primer, and 100 ng cDNA template) were run in triplicates in a 20-μl total reaction volume. The amplification conditions were as follows: DNA polymerase activation at 95 °C for 30 s, followed by 42 amplification cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The specificity of the PCR product was verified with a melting curve and by agarose gel electrophoresis. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All samples were measured in triplicate. The values were normalized using *β-actin* as the endogenous standard.

2.9. Data analysis

The effects of different temperatures and exposure time and their interactions were analyzed by two-way ANOVA followed by Bonferroni post-tests using the GraphPad Prism Version 5.0 soft-ware program (GraphPad Software, San Diego, USA). Effects of different levels of L-arginine on sperm motility were analyzed by one-way ANOVA and the interactive effects between L-arginine and heat exposure were analyzed by two-way ANOVA. Data are expressed as the mean ± SD.

3. Results

3.1. Effect of different temperatures on sperm motility

Time-response curves of different temperatures were illustrated in Fig. 1. Increasing exposure time resulted in the decreased total motility and rate of rapid progressive sperm, and caused the increased rates of the immotile sperm and the sperm shaking in place at 38 and 39 °C, respectively. Compared with sperm at 37 °C, sperm motility decreased evidently after 1-h, 3-h, and 5-h exposure, respectively. After the 1-h heat exposure at 38 and 39 °C, there were significantly decreases in total motility and rate of rapid progressive sperm as compared with 0-h heat exposure, respectively. At the 1-h time point, the rate of rapid progressive sperm was lower at 39 °C than that at 38 °C ($P < 0.05$) (Fig. 1B). There are significant Te × Ti interactions on 4 parameters of sperm motility, respectively.

3.2. Effect of different levels of L-arginine on sperm motility

In Fig. 2, the rate of rapid progressive sperm was increased by L-arginine supplementation at the doses of 0.25, 0.5 and 1.0 mM ($P < 0.05$), and decreased nearly to the control value at the dose of 2.0 mM after the 1-h exposure at 39 °C ($P < 0.05$). Groups with 0.25 and 1.0 mM L-arginine had higher total motility and lower rate of immotile sperm than the group without L-arginine supplementation ($P < 0.05$), respectively. L-arginine supplementation at dose of 1.0 mM was considered as the optimal concentration for positive addition because the values of total motility and the rate of rapid progressive sperm peaked at the 1.0 mM points.

3.3. L-arginine ameliorated heat-induced poor sperm motility

Based on two-way ANOVA analysis, total motile and rate of rapid progressive sperm decreased in groups without L-arginine after the 1-h exposure at 39 °C ($P < 0.05$), while there was no significant difference in these two parameters between two temperature-exposed groups

Table 1
Primers used in PCR.

Gene name	GenBank ID	Location	Primer sequence	Product size
NRF1	100517203	Nucleus	F: AGTGAGCCAGACTGAACACA R: CATGGACCTGCTGTACTTGC	71
PGC-1α	397013	Nucleus	F: GACATGTGCAACCAGGACTC R: AAGATCTGGGCAAAGAGGCT	84
COX5B	492822	Nucleus	F: TCTGAAGACGTACTGCACTC R: CGTCCTGGGATAGCATCTGT	68
ATP-5B	100157156	Nucleus	F: TTCATGCTGAGGCTCCTGAA R: CCACCATGAGCTTTGGCTAC	100
ATP-5O	733678	Nucleus	F: ACCCAAAGTGGCTGCTTCTA R: CTTTGCTGTCATGTCGCTCA	82
β-actin	414396	Nucleus	F: CAACACAAACGGTTCCCAGT R: GACCACATGTTTGCCATCCA	70
COX1	808503	Mitochondria	F: AAAGACATCGGCACCCTGTA R: AGCGCGAATTAGTAGGCTCA	81

β-actin, beta actin; *NRF1*, *nuclear respiratory factor 1*; *PGC-1α*, *peroxisome proliferator-activated receptor gamma coactivator-1 alpha*; *COX1*, *cytochrome c oxidase subunit 1*; *COX5B*, cytochrome c oxidase subunit 5B; *ATP-5B*, *ATP synthase F1 subunit beta*; *ATP-5O*, *ATP synthase peripheral stalk subnunit OSCP*.

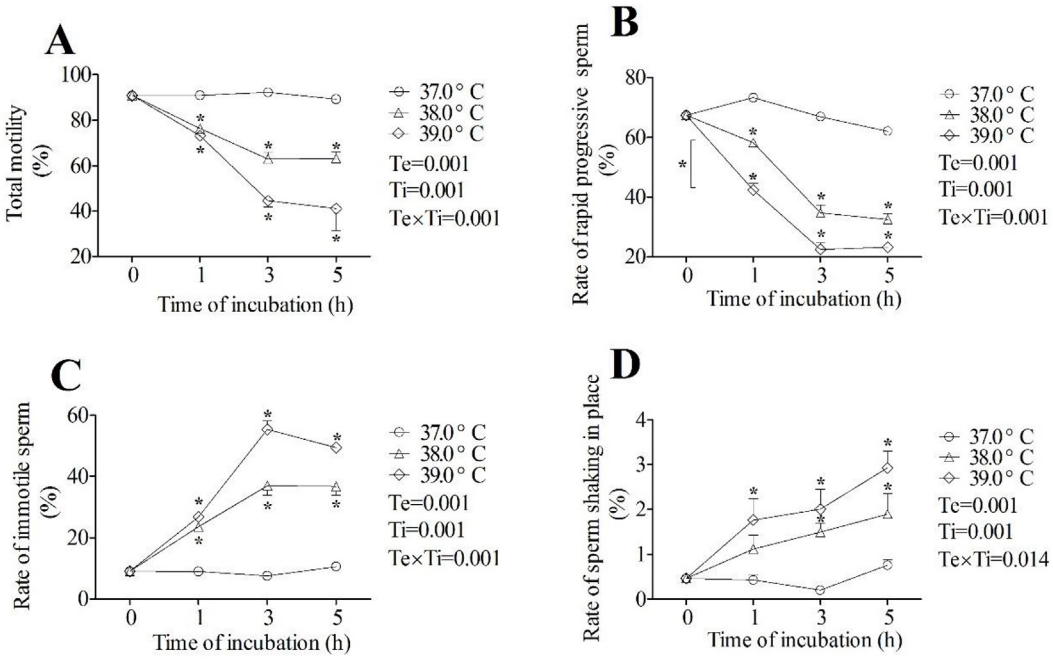

Fig. 1. Effect of different temperatures on boar sperm motility. A. Total sperm motility; B. Sperm with rapid progressive motility (velocity of average path ≥ 25 μm/s, Asterisk left beside half bracket indicates significant difference between 38 °C and 39 °C at the 1-h point, *, $P < 0.05$); C. Immotile sperm; D. Sperm shaking in place. Ti, time of incubation (0, 1, 3, 5 h); Te, temperatures (37.0, 38.0, 39.0 °C); Two-way ANOVA was used to analyze the Ti × Te interaction effects of time and temperature and followed by Bonferroni post test. Data are shown as mean ± SD, n = 5. Asterisks above bars or left beside half bracket indicate significant difference from the 0-h points (*, $P < 0.05$).

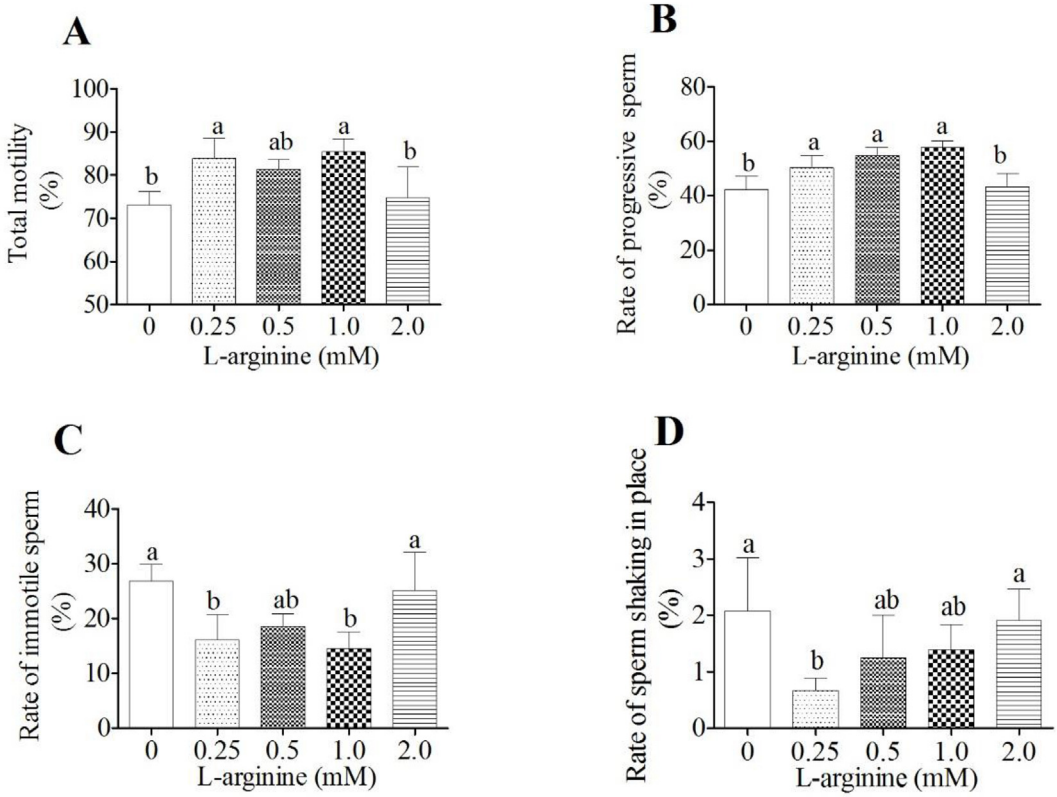

Fig. 2. *In vitro* effect of increasing L-arginine concentrations on boar sperm motility after 1-h heat exposure at 39 °C. A. Total sperm motility; B. Sperm with rapid progressive motility (velocity of average path ≥ 25 μm/s); C. Immotile sperm; D. Sperm shaking in place. Data are shown as mean ± SD, n = 5. Statistical analysis by one-way ANOVA and different letters above the bars indicated significant differences from each other ($P < 0.05$).

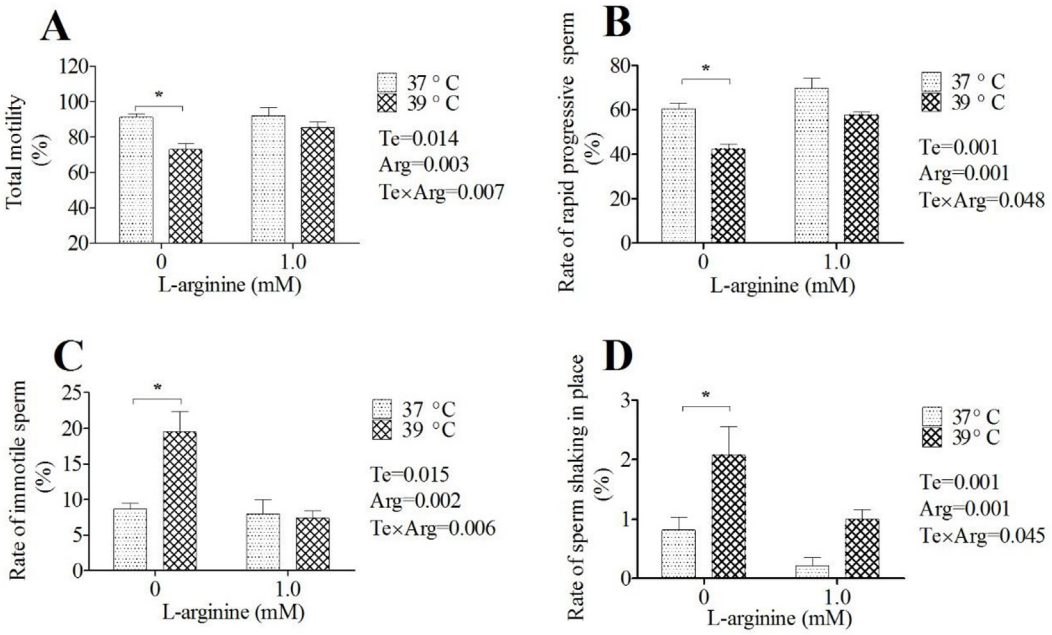

Fig. 3. L-arginine supplementation ameliorated heat-induced poor motility of boar sperm. A. Total sperm motility; B. Sperm with rapid progressive motility (velocity of average path ≥ 25 μm); C. Immotile sperm; D. Sperm shaking in place. Te, temperatures (37.0, 39.0 °C); Arg, L-arginine (0, 1.0 mM); Two-way ANOVA was used to analyze the Te × Arg interaction effects of temperature and L-arginine and followed by Bonferroni post test. Data are shown as mean ± SD, n = 5. Asterisks above the bars indicated significant differences from each other (*, $P < 0.05$).

(Fig. 3 A and B). In groups without L-arginine supplementation, heat exposure at 39 °C had higher rates of immotile sperm and sperm shaking in place than those at 37 °C ($P < 0.05$), and there was no significant difference after L-arginine supplementation (Fig. 3 C and D). There were significantly Te × Arg interactions in all 4 parameters.

3.4. Mitochondrial function

The mitochondrial probe JC-1 was used to assess $\Delta\Psi m$ with flow cytometer and the percentage of high $\Delta\Psi m$ decreased significantly as exposed to heat at 39 °C (Fig. 4 A and B). The superoxide fluorescent probe DCFH-DA was applied to detect sperm ROS level and heat at 39 °C significantly increased sperm ROS level (Fig. 4D). As shown in Fig. 4C, sperm ATP level was also lower in sperm at 39 °C than that at 37 °C ($P < 0.05$). Meanwhile, the enzyme activities of MRCC III and V significantly decreased in sperm at 39 °C comparing with those in sperm at 37 °C (Fig. 4 E and F). In groups with L-arginine supplementation, there was no significant difference in sperm ROS level, the percentage of high $\Delta\Psi m$, ATP content and MRCC III activity between two groups at 37 and 39 °C. There were significantly Te × Arg interactions in sperm ROS level, the percentage of high $\Delta\Psi m$, and MRCC V activity.

3.5. Mitochondrial ATP synthesis-related genes

In order to clarify the role of sperm mRNA on mitochondrial OXPHOS during heat exposure at 39 °C, this study selected mitochondrial ATP synthesis-related genes such as *ATP synthase F1 subunit beta* (*ATP-5B*), *ATP synthase peripheral stalk subunit OSCP* (*ATP-5O*), *cytochrome c oxidase 1* (*COX 1*) and *COX5B* (Table 1). Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) can up-regulate the mRNA levels of *COX1*, while the co-activation of PGC-1α, nuclear respiratory factor 1 (NRF-1) and NRF-2 directly up-regulates the mRNA levels of *COX5B*, *ATP-5B* and *ATP-5O* (Sharma et al., 2015). Thus, the mRNA levels of *PGC-1α* and *NRF-1* genes were also studied. As Fig. 5 shows, in the groups without L-arginine, there were obvious increases in the relative mRNA levels of *NRF1* ($P < 0.05$), *PGC-1α* ($P < 0.05$), *COX1* ($P < 0.05$), *COX5B* ($P > 0.05$), *ATP5B* ($P > 0.05$) and *ATP5O* ($P > 0.05$) as sperm exposed to the heat at 39 °C. In the groups with L-arginine, there were no remarkable difference in the mRNA levels of these six genes. The *P* values of the Te × Arg interactions were 0.012 for *NRF1* (Figs. 5 A), 0.020 for *COX1* (Figs. 5 C), 0.029 for *COX5B* (Figs. 5 D), and 0.020 for *ATP5B* (Fig. 5 E).

4. Discussion

Spermatogenesis is high sensitive to the elevated temperature and the collected fresh semen typically are kept under lower temperature during *in vitro* storage. *In vivo* effect of short-term heat exposure caused the damaged germ cells and poor semen quality, which cannot recover until several weeks later, due to the duration of an entire spermatogenic cycle in boar testes (Li et al., 2015). Sperm quality of fresh boar semen can be preserved well at 20 °C, and spermatozoa viability would decrease to 1.6% at 39 °C for 48 h compared with the value of 46.9% at 20 °C (Zou and Yang, 2000). Within a wide temperature range (20–41 °C), sperm incubated for a short-term time (10–20 min) had higher motility as the temperature increased (Bahat et al., 2012; Bonato et al., 2012), while sperm motility and longevity tended to decrease as the increasing exposure time (2–48 h) (Gong et al., 2017; de Oliveira Carvalho et al., 2018). Higher temperature enhanced sperm motility by promoting sperm metabolic activity and increased energy consumption and by-product formation (Rodríguez-Gil and Bonet, 2016). Boar semen stored at 39 °C for 48 h had significant lower percentage of acrosome-intact spermatozoa than those stored at 20 °C, indicating the enhanced sperm motility was accompanied with the degenerated function and the decreased longevity (Zou and Yang, 2000). In the present study, time-response analysis revealed that total motility and the rate of rapid progressive sperm markedly decreased with the increased exposure time at 38 and 39 °C, implying higher temperatures induced the degenerated function of boar sperm. Sperm consequent movement relied on continuously synthesized energy, which further caused the by-product accumulation such as electron leakage and ROS generation (Koppers et al., 2008). Meanwhile, higher temperature at 39 °C sharply decreased sperm motility and increased the percentage of immotile sperm, resulting in the reduced sperm longevity. It suggested that the

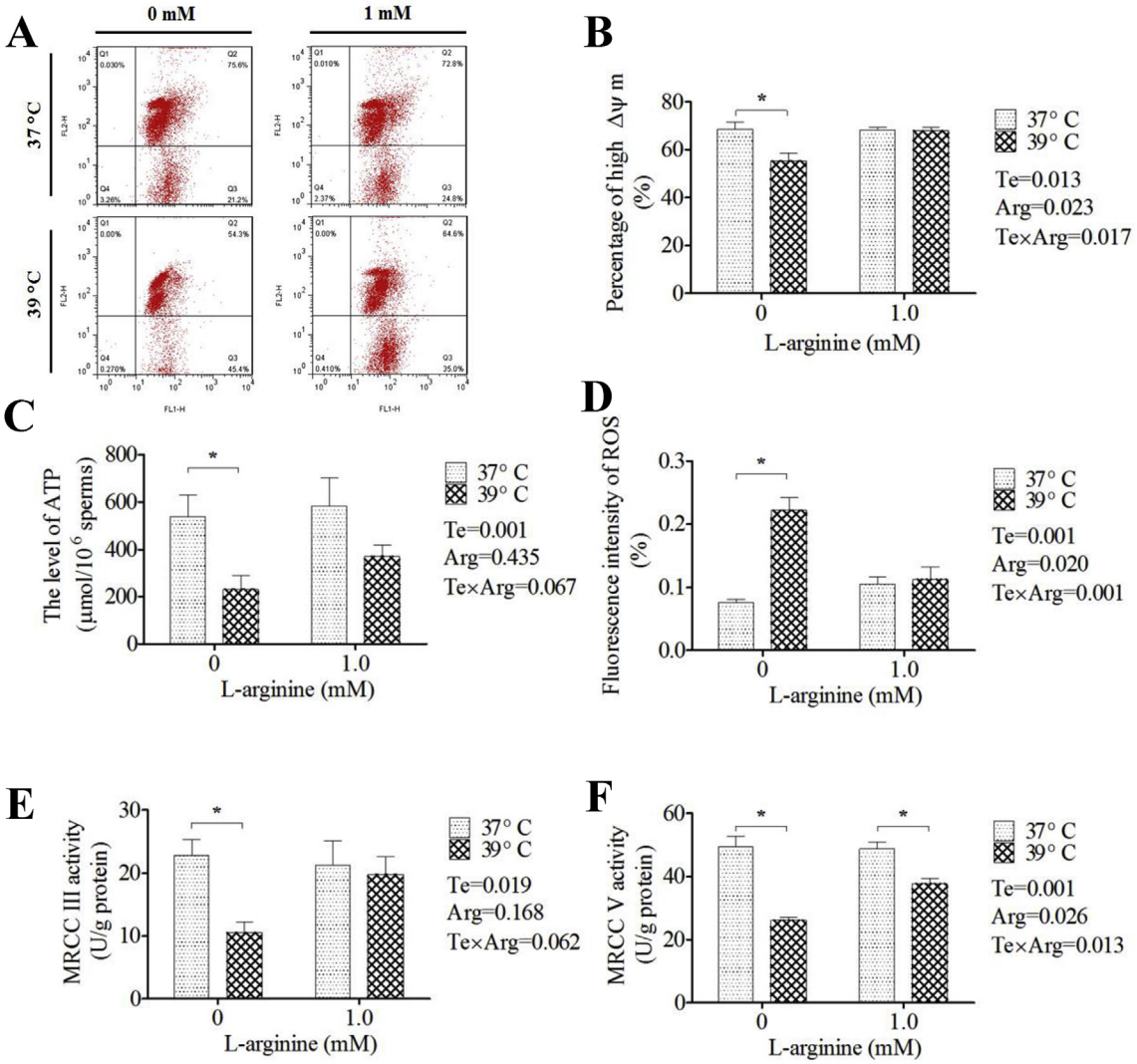

Fig. 4. Protective effect of L-arginine supplementation on heat-induced mitochondrial OXPHOS dysfunction in boar sperm. A-B. Mitochondrial staining with JC-1 using flow cytometric analysis to evaluate the ΔΨm of sperm cultured with or without 1.0 mM arginine under different temperatures. C. Sperm ROS level using the DCFH-DA staining. D. Sperm ATP level. The enzymatic activities of mitochondrial respiratory chain complex III (E) and V (F). Te, temperatures (37.0, 39.0 °C); Arg, L-arginine (0, 1.0 mM); Two-way ANOVA was used to analyze the Te × Arg interaction effects of temperature and L-arginine and followed by Bonferroni post test. Data are shown as mean ± SD, n = 5. Asterisks above the bars indicated significant differences from each other (*, $P < 0.05$).

overwhelmed ROS might not be cleared effectively and led to the degenerated function and poor sperm motility.

L-arginine confers a protective effect on sperm motility. However, high level of L-arginine can have adverse effect on sperm motility and there were differences in the optimal dose of L-arginine among different species. During equilibration of ram semen, 10 mM L-arginine supplementation significantly decreased sperm motility, but 5 mM significantly increased sperm membrane integrity and arginase activity (Özer Kaya et al., 2018). Bovine semen added with 1.0 mM L-arginine increased *ΔΨm* and membrane integrity in post-thawed sperm, accompanying with 11 up-regulated proteins and 29 down-regulated proteins (Maciel et al., 2018). Boar sperm showed the increased viability in the presence of L-arginine at the doses from 0 to 2.0 mM and reached the high values at doses of 1.0 and 2.0 mM after 2-h exposure at 39 °C (Funahashi, 2002). In the present study, *in vitro* addition of L-arginine to boar semen had a dose-dependent effect on sperm motility and 1.0 mM L-arginine exhibited the optimal effect on sperm motility after 1-h exposure at 39 °C, indicating it can increase or decrease sperm motility depending on its concentration and the underlying mechanism might be related with the changed proteome and mitochondrial function.

Mitochondria are major actors in thermogenic process and a substantial proportion of energy is released as heat to maintain stable body temperature, but part of the energy drives ATP synthesis by MRCC V (Birceanu, 2018; Lane, 2018). Generally, superoxide (O^{2-}) is generated in the intermembrane space by MRCC III and in the matrix by MRCC I and III (Fig. 6), and eventually converted into H_2O by endogenous antioxidative enzymes (Murphy, 2009; Sanderson et al., 2013). Activation of ROS generation at MRCC III led to the rapid release of hydrogen peroxide into sperm cytoplasm, but the induction of ROS on matrix side at MRCC I can only be detected once matrix antioxidant protection has been overwhelmed (Koppers et al., 2008). It suggested MRCC III was the major source of intracellular ROS in sperm. Recent study reported MRCC activities were maximal at or slightly above 50 °C and mitochondria were more than 10 °C warmer when the RC was fully functional in human cells at an external temperature of 38 °C (Chretien et al., 2018). However, the released energy as heat impacted MRCC activities when the external temperature was above or higher than 39 °C and caused inhibition of heat release and the temperature rise in mitochondria. A 6-h exposure to 42 °C induced mitochondria dysfunction characterized by the decreased of *ΔΨm* and ATP content in mitochondria of boar sperm, leading to poor sperm motility (Gong et al.,

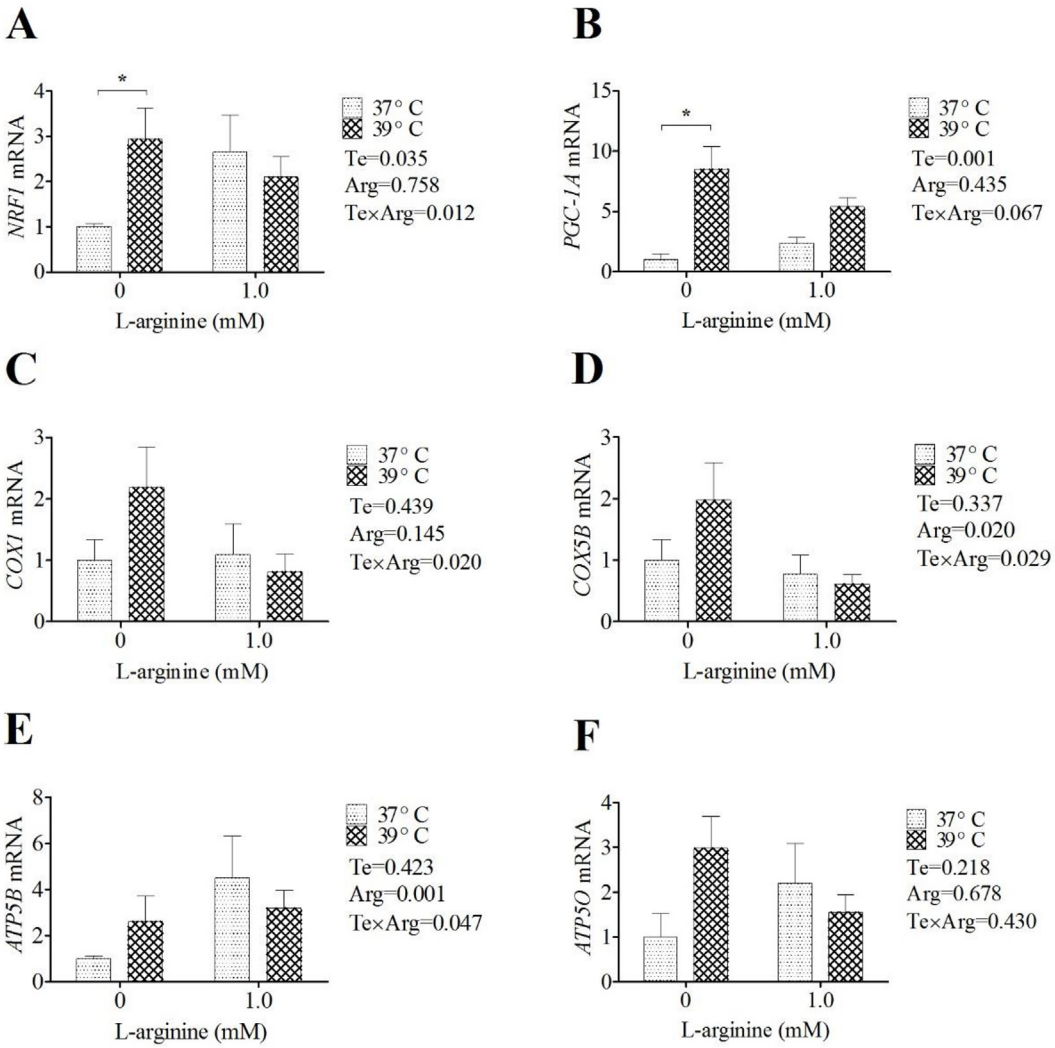

Fig. 5. Effect of L-arginine supplementation on the mitochondrial mRNA expression in boar sperm under high temperature. The relative mRNA expression of *NRF1* (A), *PGC-1A* (B), *COX1* (C), *COX5B* (D), *ATP5B* (E), *ATP5O* (F) as measured by real-time PCR. Te, temperatures (37.0, 39.0 °C); Arg, L-arginine (0, 1.0 mM); Two-way ANOVA was used to analyze the Te × Arg interaction effects of temperature and L-arginine and followed by Bonferroni post test. Data are shown as mean ± SD, n = 5. Asterisks above the bars indicated significant differences from each other (*, $P < 0.05$).

2017). In this study, sperm exposed to 39 °C for 1 h showed the decreased of $\Delta\Psi m$, MRCC III and V activities and ATP content, indicating the elevated external temperature changed mitochondrial OXPHOS and ATP synthesis in boar sperm. Considering that intracellular ROS of sperm was mainly generated at MRCC III and MRCC V was responsible for ATP synthesis, the increased ROS and decreased ATP might be resulted from the heat-induced negative effects on MRCC III and V activities after 1-h heat exposure at 39 °C.

Normal L-arginine uptake plays a key role in mitochondrial redox balance and its deficiency can induce ROS accumulation. Abnormal mitochondrial L-arginine transport caused oxidative stress following mitochondria stress and the increasing mitochondrial L-arginine availability can improve $\Delta\Psi m$, ATP turnover together with the significantly decreased ROS production and cell death (Williams et al., 2014). Mitochondrial arginine metabolism supported bioenergetics in asthma (Xu et al., 2016), and promoted the cellular survival capacity (Geiger et al., 2016). In the present study, heat induced the increased ROS level and decreased of $\Delta\Psi m$, ATP content and MRCC activities, while L-arginine supplementation inhibited ROS accumulation and maintaining sperm motility. Considering that increasing antioxidant effects can increase MRCC activities and ameliorated mitochondrial injury (Zhou et al., 2016), L-arginine supplementation might maintain boar sperm motility *via* reducing mitochondrial ROS production and protecting mitochondrial OXPHOS from heat-induced stress. The temperatures in oviduct lumens of sows were close to 39 °C (Hunter and Nichol, 1986) and sow oviduct fluid contained arginine (Guerin et al., 1995), implying L-arginine might confer a protective role as boar sperm is swimming in the oviduct lumens of sow.

Arginine acts as NO precursor and its administration results in the increased NO production and can potentially have therapeutic utility in mitochondrial disorder (El-Hattab et al., 2014). NO can affect the functionality of spermatozoa through free radical scavenging, deactivating and inhibiting the production of superoxide anions (de Andrade et al., 2018). Our recent study revealed boars fed with dietary L-arginine significantly increased serum NO levels during hot summer month, and the ejaculated sperm had remarkably high ATP content, low malondialdehyde level and increased sperm motility (Chen et al., 2018). In the present study, boar sperm incubated at 39 °C for 1 h had high ROS level and low ATP content, which were ameliorated by L-arginine supplementation. Malondialdehyde level was a product of lipid peroxidation and up-regulated as ROS level increased (Lushchak, 2014). These results showed both *in vivo* and *in vitro* L-arginine supplementation can attenuate heat-induced poor sperm motility and sperm ROS level, implying L-arginine might maintain normal sperm motility

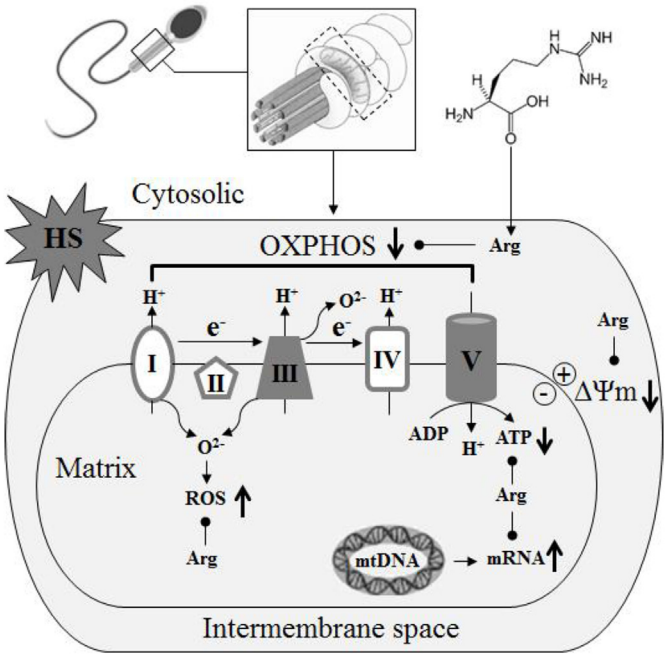

Fig. 6. Schema showing how L-arginine supplementation may ameliorate heat-induced mitochondrial OXPHOS dysfunction in boar sperm. ADP, adenosine diphophate; ATP, adenosine triphophate; Arg, L-arginine; OXPHOS, oxidative phosphorylation; HS, heat stress; I, NADH dehydrogenase (Mitochondrial respiratory chain complex, MRCC I); II, succinate dehydrogenase/fumarate reductase (MRCC II); III, cytochrome c reductase (MRCC III); IV, cytochrome c oxidase (MRCC IV); V, ATP synthase (MRCC V); ROS, reactive oxygen species; ΔΨm, mitochondria membrane potential; mtDNA, mitochondrial DNA; ↓, decrease; ↑, increase; ⏊, inhibition.

through scavenging ROS by the NO-related pathway during high ambient temperature.

Mitochondria provide sperm energy in form of ATP produced by ATP synthase while MRRC IV as the terminal ETC enzyme directly impact the amount of ATP production. The synthesized PGC-1α can be transported into mitochondria and up-regulate the mRNA levels of mitochondrial DNA (mtDNA) *COX1*, which is the biggest subunit of MRRC IV and takes center stage in complex assembly (Dennerlein and Rehling, 2015). ROS had been shown to strongly induce the expression of PGC-1α and its overexpression obviously increased MRCC IV activity, indicating PGC-1α might promote MRRC IV assembly *via* up-regulating down-stream targeted genes such as mtDNA *COX1* and nuclear DNA (nuDNA) *COX5B* (St-Pierre et al., 2006; Srivastava et al., 2007). Quercetin has been reported to possess ROS scavenging and can attenuate chronic oxidative damage by the co-activation of PGC-1α, NRF-1 and NRF-2, which directly up-regulated the mRNA levels of mtDNA and nuDNA genes such as *ATPase 6*, *COX1* and *COX5B* (Sharma et al., 2015). The findings that sperm can independently activate translation of the stored mRNA by mitochondria implied it's possible for translation of nuDNA genes including *PGC-1α*, *NRF-1*, *NRF-2*, *ATP 5-B*, *ATP 5-O and COX5B* in boar sperm (Gur and Breitbart, 2006, 2007, 2008). In the present study, boar sperm exposed to 39 °C for 1 h exhibited increased mitochondrial mRNA level of *COX1*, which might be attributed to heat-triggered PGC-1α signaling pathway and transcription of *COX 1* in mitochondria. Boar sperm exposed to 42 °C for 6 h decreased mitochondrial protein content including COX1 protein (Gong et al., 2017). This was because of heat exposure at 39 °C for 1 h induced mild oxidative stress, which might promote MRCC assembly *via* activating mitochondrial PCC-1α/COX 1 pathway. Compared with the amelioration of L-arginine addition on heat stress at 39 °C for 1 h, heat exposure at 42 °C for 6 h might lead to the strong oxidative damage and overwhelmed sperm capacity of oxidative stress-defense. Given that sperm RNA is unlikely to be transcribed from nuDNA due to the changed chromatin structure during sperm DNA compaction, the increased mRNA levels of nuDNA genes after 1-h heat exposure at 39 °C might result from the different mechanism that need to be defined in future research.

In summary, Fig. 6 shows a possible mechanism underlying that how L-arginine supplementation may ameliorate heat-induced mitochondrial OXPHOS dysfunction in boar sperm. OXPHOS is carried out in the inner mitochondrial membrane by five enzymatic complexes (I to V) of the ETC. Electrons were transferred along the ETC, thereby releasing protons into the intermembrane space. This accumulation of cations comprises the *ΔΨm*, which is required for ATP production by MRCC V. This process naturally generates low level of ROS as a by-product, which can be metabolized by endogenous catabolic enzymes. However, high temperature exposure can induce mitochondrial OXPHOS dysfunction, leading to the increased ROS level, the decreased of MRCC activities, *ΔΨm* and ATP production (Fig. 6). L-arginine supplementation can attenuate heat-induced increase in ROS level and decreases in *ΔΨm* and MRCC activities, which might maintain the ATP synthesis and sperm motility of boar.

5. Conclusion

1. Heat temperature at 39 °C sharply decreased sperm motility and increased the percentage of immotile sperm with the increased exposure time, resulting in the reduced sperm longevity.
2. *In vitro* addition of L-arginine to boar semen had a dose-dependent effect on sperm motility and sperm exposed to 39 °C for 1 h showed the increased sperm ROS level, the decreased of *ΔΨm*, MRCC activities and ATP content, which can be attenuated by 1.0 mM L-arginine supplementation.

Conflicts of interest

The authors declare that there is no conflict of interest.

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