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# Role of AMPK in the expression of tight junction proteins in heat-treated porcine Sertoli cells

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ABSTRACT

Hyperthermia can cause dysfunction of the tight junctions (TJs) in testes. Adenosine 5′-monophosphate-activated protein kinase (AMPK) participates in the regulation of TJs in testis. However, whether AMPK regulates the expression of TJ proteins in the response of Sertoli cells to heat treatment remains unknown. We subjected Sertoli cells from 3-week-old piglets to heat treatment (43 °C, 30 min), which decreased cell viability, and increased the early apoptosis rate. These effects were reversible and the cells gradually recovered to normal viability at 48 h post-heat treatment. Expression of TJ proteins (claudin 11, JAMA, occludin, and ZO1) was detected in immature porcine Sertoli cells. The mRNA and protein levels of TJ proteins significantly decreased at 1 h after heat exposure, but recovered with increasing recovery time. Additionally, the expression of claudin 11 in the cytoplasm was also markedly decreased by heat treatment. AMPK phosphorylation, the cellular ATP level, and $Ca^{2+}$/calmodulin-dependent protein kinase kinase B (CaMKKB) level, but not the liver kinase B1 (LKB1) level, were downregulated by heat treatment. More importantly, activation or overexpression of AMPK, which is a regulator of the assembly of TJs, partially rescued the heat treatment-induced downregulation of TJ proteins. By contrast, *AMPK* knockdown using small interfering RNA (siRNA) further decreased the expression levels of TJ proteins. In addition, claudin 11 was almost undetectable post heat treatment. Collectively, this study demonstrated that heat treatment could reversibly perturb the expression of TJ proteins in immature porcine Sertoli cells by inhibiting the AMPK signaling pathway.



## 1. Introduction

Mammals have a certain capacity to regulate their body temperature, and scrotal hypothermia is essential for normal spermatogenesis [1–4]. Sertoli cells play the role of “nurse cells” for developing germ cells during spermatogenesis [5]. Although Sertoli cells have a stronger tolerance to heat stress compared with germ cells [6], heat stress damages their structure, reduces their physiological functions, and renders them unable to aid germ cells in their development [7,8]. The structures and physiological functions of boar testes and human testes are similar; therefore, exploring the mechanism of heat stress-induced dysfunction of boar Sertoli cells might help us understand the mechanism of heat-induced infertility in men.

Tight junctions (TJs) between adjacent Sertoli cells partially form the blood-testis barrier, which compartmentalizes meiotic spermatocytes and post-meiotic spermatids away from the vasculature; therefore, preventing autoantibody production against these immunogenic germ cells [9–13]. In the testis, TJs comprise claudins, occludins, junction adhesion molecules (JAMs), and zonula occludens 1 (ZO1) [14–16]. Claudins play critical roles in tight junction assembly in the testis [17–19]. Claudin 11 deficiency in mice resulted in the loss of Sertoli cell epithelial phenotypes in testes [20]. In monkeys and mice, heat stress can transiently downregulate the expression of TJ proteins in mature Sertoli cells,

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destroy the structure of TJs, and cause failure of spermatogenesis [6,7,21]. However, little is known about whether heat stress could affect the expression of TJ proteins in immature porcine Sertoli cells.

Adenosine 5′-monophosphate-activated protein kinase (AMPK) is a regulator of cellular energy metabolism [22–24]. The phosphorylation of AMPK is regulated by upstream kinases (i.e., liver kinase B1[LKB1], $Ca^{2+}$/calmodulin-dependent protein kinase kinase B [CaMKKB]) and the cellular AMP/ATP ratio [25–27]. Heat stress activates AMPK in muscles [28]. Activated AMPK phosphorylates downstream effectors to regulate the biosynthesis and catabolism of ATP [29], and enhances TJ assembly [30–33]. Moreover, specific deletion of *AMPKA1* (AMPK alpha 1) resulted in downregulation of TJ proteins and disorganization of the blood-testis barrier [34]. By contrast, heat stress inhibits the phosphorylation of AMPK in cell lines (e.g., HepG2, 293T, Hepa1-6, and bEnd3) and tumor cells without affecting the ATP level [35,36]. However, how AMPK responds to heat treatment in Sertoli cells, and whether AMPK plays a regulatory role in the response of immature Sertoli cells to heat stress, remains to be determined.

In the present study, we showed that heat stress decreased the viability and increased the early apoptosis rate of immature porcine Sertoli cells, and decreased the expression of TJ proteins. In this process, heat stress inhibited the AMPK signaling pathway via CaMKKB, but not LKB1. These findings partially reveal the molecular mechanism whereby heat stress affects the expression of TJ proteins in immature porcine Sertoli cells through the AMPK signaling pathway.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Lipofectamine® RNAi MAX reagent, Lipofectamine 2000, Opti-MEM, and Dulbecco's modified eagle's medium/F12 (DMEM/F12) were purchased from Invitrogen Technologies (Carlsbad, CA, USA). STO609, collagenase IV, trypsin, fetal bovine serum, newborn bovine serum, penicillin, and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit 8 (CCK8) came from Dojindo Molecular Technologies Inc (Kumamoto, Japan). The mitochondrial membrane potential assay kit with JC1, phenylmethanesulfonyl fluoride (PMSF), phosphatase inhibitor cocktail A, enhanced bicinchoninic acid (BCA) protein assay kit, enhanced ATP assay kit, and the AMPK activator 5-aminoimidazole-4-carboxamide-1-B-d-ribofuranoside (AICAR), were obtained from Beyotime Biotechnology (Shanghai, China).

### 2.2. Isolation, in vitro culture, and heat stress induction of primary porcine Sertoli cells

All animal procedures were approved by the Southwest University Institutional Animal Care and Use Committee. Testes were obtained from 3-week-old piglets under sterile conditions at a local livestock farm. Sertoli cells were isolated as previously described, with modifications [37,38]. Briefly, decapsulated testes were cut into small pieces and digested with 0.03 g/mL collagenase IV for 40 min at 32 °C (70 vibrations/min). After centrifugation at 1000 × g for 5 min, the sediment was digested with 0.0025 g/mL of trypsin for 20 min at 32 °C (70 vibrations/min). The reaction was terminated by adding newborn bovine serum, and the suspension was filtered through stainless steel sieves with 0.2-mm and 0.038-mm apertures in turn. Sertoli cells were suspended in DMEM/F12 (containing 10% [v/v] fetal bovine serum) at $1 \times 10^6$ cells/mL. Medium with cells were added to flasks (25 $cm^2$, containing 4 mL of medium, which included about $4 \times 10^6$ cells), 24-well plates (500 μL of medium per well, which included about $5 \times 10^5$ cells), or 96-well plates (100 μL of medium per well, which included about $1 \times 10^5$ cells), and cultured at 32 °C in incubators containing 5% $CO_2$. After 24 h, the medium was replaced to remove unattached germ cells. Attached cells were treated with hypotonic solution (20 mM Tris-HCl, pH 7.0) for 3 min at room temperature to eliminate residual germ cells [37,38]. The purity of the Sertoli cells was determined by measuring the immunofluorescence of the cell-specific molecular marker GATA binding protein 4 (GATA4) [39]. The calculated fluorescence ratio of GATA4 showed that the purity of the Sertoli cells was 91.95 ± 0.17%.

According to previous studies [21,40–44], when the cells in culture became confluent (approximately 48 h post attachment), we subjected Sertoli cells to transient heat treatment using two methods: An air bath and a water bath. Briefly, flasks containing Sertoli cells were placed in a 43 °C sterile water bath for 30 min (water bath) or in a 43 °C incubator (5% $CO_2$) for 30 min (air bath). After heat treatment, the flasks were transferred back to the 32 °C incubator (5% $CO_2$) for recovery. The control cells were at 32 °C in an incubator (5% $CO_2$) throughout. Culture was terminated at the indicated time points for further analysis [44]. As the culture time did not affect Sertoli cells viability at the pre-experiment stage, we ignored the effect of culture time in the following experiments.

### 2.3. Cell viability assay

Cell viability was measured using the CCK8 kit. Briefly, $1 \times 10^5$ cells were cultured in 96-well plates containing 100 μL of DMEM/F12 per well for 48 h at 32 °C (5% $CO_2$). After heat treatment, 10 μL of CCK8 solution was added to each well, and the plates were incubated for 1–4 h. The absorbance at 450 nm of each well was then measured using a microplate reader (Bio-Rad, Hercules, CA, USA). According to the manufacturer's instructions, cell viability was calculated using the following formula: viability = (absorbance of the experimental group − absorbance of the blank group)/(absorbance of the control group − absorbance of the blank group) [45].

### 2.4. ATP level assays

The ATP concentration was measured using an enhanced ATP assay kit (Beyotime Biotechnology) according to the manufacturer's instructions [39]. In brief, Sertoli cells were lysed using ATP lysate buffer and then centrifuged at 12000 × *g* for 5 min at 4 °C. The supernatant was then used to assay the ATP concentrations using a standard curve of known ATP concentrations (1–1000 nmol/L). Relative ATP concentrations were determined using a luminometer (Glomaxmultidetection system; Promega).

### 2.5. Assessment of mitochondrial membrane potential ($\Delta\Psi m$ or MMP)

Sertoli cells were stained with 5,5′,6,6′-tetraethylbenzimidazolylcarbocyanine iodide (JC1) dye (Beyotime Biotechnology) for 20 min at 32 °C in a $CO_2$ incubator. At the end of the incubation, the cells were washed with staining buffer and centrifuged at 600 × *g* for 3 min. Then, the mitochondrial membrane potential (MMP) was measured using fluorescence activated cell sorting in a FACScan machine (Becton-Dickinson, Franklin Lakes, NJ, USA). Green fluorescence was analyzed in the fluorescein isothiocyanate (FITC) channel (monomeric JC1) and red fluorescence (aggregated JC1) was analyzed in the phycoerythrin (PE)-A channel. The ratio of aggregated JC1 to monomeric JC1 represented the MMP of the Sertoli cells. Reduced MMP levels can be considered as an index of the early apoptosis rate [46]. Carbonylcyanide-3-chlorophenyl