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ORIGINAL ARTICLE

Depletion of serum-derived exosomes aggravates heat stress-induced damage of bovine mammary epithelial cells

Yue Wang[1] · Hui-Li Wang[1,3] · Zhi-Ping Lin[3] · Ji-Feng Zhong[1,3] · Kun-Lin Chen[1,3] · Xing Duan[2]

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Abstract

Background Exosomes are involved in intercellular communication, affecting many physiological and pathological process. The present study evaluated the effects of serum exosomes on the function of bovine mammary epithelial cells (BMECs) and milk synthesis under heat stress.

Methods and results We cultured the BMECs in fetal bovine serum (FBS) or exosome-free FBS medium and examined, their viability using CCK-8 kit. The results showed that culturing the cells in an exosome-free medium decreased viability and increased the levels of reactive oxygen species. The BMECs cultured in the exosome-free medium had reduced mitochondrial membrane potential, decreased manganese superoxide dismutase activity, and disrupted mitochondrial dynamics. They exhibited apoptosis due to upregulated Drp1, Fis1, Bax and HSP70. Lastly, we observed downregulation of milk fat and lactoprotein-related genes: mTOR, PPARγ, p-mTOR and ADD1 and SREBP1, ELF5, and CSN2, respectively, after culturing the cells in an exosome-free medium. These negative effects of the exosome-free medium on the BMECs could be further reinforced under heat stress.

Conclusion Our results demonstrated that exosomes from serum are critical for maintaining the normal function of BMECs.

Keywords Exosomes · Bovine mammary epithelial cells · Heat stress · Mitochondrial function · Milk fat · Milk protein

Abbreviations

BMECs	Bovine mammary epithelial cells
HS	Heat stress
Ctr	Contrast
qRT-PCR	Quantitative real-time PCR
PBS	Phosphate buffer solution
SDS	Sodium dodecyl sulfate
TBST	Tris-buffered saline containing 0.1% Tween 20
Exo	Exosomes
ELF5	E74-like Factor 5
CSN2	β-Casein
STAT5	Signal transducer and activator of transcription 5
SREBP1	Sterol regulatory element binding protein 1
ADD1	Adipocyte determination and differentiation factor-1
TG	Triacylglycerol
AMPK	Adenosine 5′-Monophosphate (AMP)-activated protein kinase
PI3K	Phosphoinositide3-kinase
AKT	Threonine-protein kinase
mTOR	Mammalian target of rapamycin
ROS	Reactive oxygen species
Mn-SOD	Manganese superoxide dismutase

✉ Kun-Lin Chen
chenkunlin@jaas.ac.cn

✉ Xing Duan
duanxing@zafu.edu.cn

[1] Institute of Animal Science/Key Laboratory for Crop and Animal Integrated Farming of Ministry of Agriculture and Rural Affairs, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

[2] Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A&F University, Hangzhou 311300, China

[3] Youyuan Research Institute of Dairy Industry Co., Ltd, Nanjing 211100, China

Introduction

Heat stress-induced by high temperature and humidity causes many health issues in dairy cows, including increased body temperature and respiratory rate, changed in nutrient metabolism and milk yield and quality. Moreover, heat stress reduced the number of mammary acinus and induced mastitis in lactating dairy cow. Therefore, maintaining the normal function of bovine mammary gland is critical for lactation performance in dairy cows [1]. Bovine mammary epithelial cells (BMECs) are essential for lactation in dairy cow mammary gland. Studies have shown that heat stress could induce the occurrence of oxidative stress and apoptosis in BMECs by disrupting mitochondrial function, which further affect the lactation performance of dairy cows [2–4]. The main nutrients in milk-milk fat and milk protein-are the most important traits in dairy cows, which are critical standards to evaluate milk quality [5]. It has been shown that heat stress can cause the decrease of milk protein content in milk [6, 7] and the effectiveness of amino acids required for milk protein synthesis [8] through changing mTORC1 signal pathway [9, 10]. Therefore, finding a strategy to prevent heat stress-induced injury and changes of milk synthesis-related genes in BMECs is necessary, which will attenuate heat stress-induced low milk performance in dairy cows.

Exosomes (Exo) are tiny cell-secreted vesicles with a diameter of 20–150 nm that contain proteins, lipids, or nucleic acids, which can be used as signaling molecules to regulate cellular functions [11]. Chen et al. demonstrated that exosomes derived from mesenchymal stem cells could protect β cells from hypoxia-induced apoptosis, reduce cellular stress, and inhibit activation of the P38-MAPK signaling pathway [12]. The bioactive substances in exosomes have important regulatory effects on low-density lipoprotein receptors and fatty acid synthase, and their disruption can lead to lipid metabolism disorders [13, 14]. Exosomes are mainly involved in intercellular communication and intercellular macromolecule transfer. They can shuttle from one cell to another and affect the protein expression of receptor cells [15]. Studies have shown that the distribution of miRNAs in goat milk exosomes changes at different lactation stages. miR-27a and miR-183 can increase the content of unsaturated fatty acids and medium chain fatty acids in milk [16, 17]. In addition, donor cell specific exosomes can change the lactation curve of cows and activate the pathways involved in lactation process [18]. Song et al. demonstrated that miR-29a/b/c induces glucose metabolism disorder in vivo by inhibiting cysteine-rich secreted protein (SPARC) in adipocytes [19]. Therefore, exosomes play a critical role in regulating milk fat and protein synthesis; however, their effects have not been well studied, especially under heat stress condition.

Given the potential function of exosomes in anti-oxidative function and milk synthesis, in the present study, we used high temperature-induced BMECs heat stress model to investigate whether exosomes involved heat stress-induced oxidative stress and the reduction of milk performance in dairy cows.

Materials and methods

Cell culture

BMECs were cultured with Dulbecco's modified Eagle's medium (DMEM) medium containing 10% normal fetal bovine serum (Gibco, CAT: A210808) or 10% exosome-free fetal bovine serum (SBI System Biosciences, CAT: EXO-FBS-50A-1) in a humidified incubator with 5% CO_2 at 37 ℃. The cells were transferred to a high temperature incubator (42 ℃) for 2 h to induce heat stress response until cells reached 80% confluence, after that the cells were collected for the following experiments.

Cell viability

Cell viability was determined by CCK-8 kit (APExBIO, CAT: K1018, US). Briefly, BMECs were seeded in 96-well culture plates; after experimental treatment, the cells were treated with CCK-8 (10 μL) for 2 h in 37 ℃. The light absorption value of each hole (OD450) was detected by a full-wavelength microplate analyzer at 450 nm. The cell viability was calculated based on the absorbance. Cell viability = (experimental group OD450/ control group OD450) × 100%.

Determination of reactive oxygen species and superoxide dismutase

For detecting reactive oxygen species (ROS), BMECs were incubated with DCFH-DA (10 μm/L) (Beyotime, CAT: S0033S, Shanghai) for 20 min at 37 ℃. After that the cells were washed with serum-free medium for three times; then examined by fluorescence microscopy (Olympus, Tokyo, Japan).

For detecting the activity of superoxide dismutase, the manganese superoxide dismutase (Mn-SOD) kit (Beyotime, CAT: S0103, Shanghai) was used to examine the activity of Mn-SOD after different experimental treatment according to the manufacturer's instructions Then the activity of Mn-SOD was calculated based on the absorbance.

Measurement of mitochondrial membrane potential (JC-1)

The mitochondrial membrane potential was detected by JC-1 kit according to the manufacturer's instructions. Briefly, the cells were incubated with JC-1 solution (1 mL cell medium + 1 mL JC-1 staining solution) at 37℃ for 20 min. After washing twice with JC-1 staining buffer (1×), the fluorescent images were taken by fluorescence microscope (Olympus, Tokyo, Japan).

Determination of triglyceride

The triglyceride was detected by TG detection kit (CAT: A110-1-1, Nanjing Jiancheng Biological Engineering Research Institute) according to the manufacturer's instructions. Briefly, the cells were collected in the centrifuge tube and crushed with ultrasonic wave, then incubated with working solution at 37 ℃ for 10 min. The light absorption value at 510 nm was measured with a microplate tester. The content of triglyceride was calculated based on the absorbance.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from BMECs with Trizol reagent kit (Bioteke Gorporayion, CAT: RP1202, Beijing). The total RNA was reverse transcribed to cDNA by reverse transcription kit (CAT: R212-01/02, Vazyme), then the relative genes expression levels were quantified by real-time PCR with ChamQ™ SYBR qPCR Master Mix (CAT: Q321-02/03, Vazyme). Expression levels of all genes were normalized to those of endogenous reference gene β-actin, according to an optimized comparative Ct($2^{-\Delta\Delta Ct}$) value methods, where $\Delta\Delta = \Delta Ct_{target} - \Delta Ct_{\beta-actin}$. The primers [20–22] that used in this study are listed in Table 1.

Western blot

The total protein was extracted by protease lysate, then the protein concentration was determined by BCA kit (CWBIO, CAT: CW0014S, Jiangsu). The protein samples were denatured at 100 ℃ for 10 min. Then proteins were separated on 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis mini gels (GenScript) and blotted to polyvinylidene fluoride membranes (Millipore). The blots were incubated with 5% non-fat milk in Tris-buffered saline, 0.05% Tween-20 (TBST) for 1 h and incubated with primary antibody overnight. Then the bolts were washed three times with TBST; after incubation with secondary antibody for 1 h, the blots were washed with TBST for three times and developed with ECL chemiluminescence reagent, analyzed with Image J the grayscale value of target bands. The primary antibodies and all secondary antibodies GAPDH (CAT: 60004-1-Ig), ADD1 (CAT: 10791-1-AP), SREBP1 (CAT: 14088-1-AP), Bax (CAT: 50599-2-Ig), HSP70 (CAT: 10995-1-AP), Drp1 (CAT: 12957-1-AP), Fis1 (CAT: 10956-1-AP), PPARγ (CAT: 16643-1-AP), ELF5 (CAT: Ag14861) were purchased from Proteintech. CSN2 (CAT: PAJ332Bo01) antibody was purchased from Clous-Clone Corp. mTOR (CAT: AF6308) and p-mTOR (CAT: AF3308) antibodies were purchased from Affinity.

Data analysis

All statistic results were given as mean ± SEM. All results were analyzed by One-Way analysis of variance (ANOVA) multiple comparisons, using the software GraphPad Prism 8.0.1 (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant. * means $P < 0.05$, ** means $P < 0.01$, *** indicates $P < 0.001$.

Table 1 Primer sequences of mRNA

Gene name	Accession no	Primer pairs sequence (5′→3′)
HSP70	NM_203322.3	CTGAACCCGCAGAACACG/CCTTGGTCTCCCCTTTGT
Bax	NM_173894.1	TGGACATTGGACTTCCTTCG/CCAGCCACAAAGATGGTCAC
Drp1	NM_001273017.1	CAGAGAGCTCATCCTTCGGT/TGCGACCATCTGGATCTACC
Fis1	NM_001034784.2	CACAGAACAACCAGGCCAAA/TGCCGTCTCCTTCAGGATTTT
SREBP1	NM_001113302.1	TGGACCAGGCAAGAGAAGAG/TCTTCCTCCAGCTTGACAGG
ELF5	NM_001024569.1	ATGTCGTGGACTGACCTGTT/GCTTGTACTGGTCACAGCAG
CSN2	XM_010806178.2	GAGGCTATGGCTCCTAAGCA/GACAGTTGGAGGAAGAGGCT
mTOR	XM_002694043.6	TGAACTGGAGGCTGATGGACAC/TGACTGGCCAGCAGAGTAGGAA
ADD1	XM_005208232.4	ATAGAACTGGCTACCCTTACCG/AGCAAGTGCCCGAATCA
PPARγ	NM_181024.2	CTCCAATGTTCTCAAACTTAC/GATGAGTCATGTAAGTTGACC
β-Actin	NM_173979.3	TCACCAACTGGGACGACA/GCATACAGGGACAGCACA

Results

Exosomes-free FBS accelerated heat stress-induced apoptosis

We determined the cell viability using the CCK-8 assay examine the effect of exosomes on BMECs. The cell viability significantly decreased in the exosome-free (N-Exo) groups (Fig. 1A). Furthermore, cell activity was lower in N-Exo groups under heat stress compared to that in the controls, indicating that serum exosomes play critical roles in BMEC growth. We used RT-qPCR and western blotting to access whether exosomes are involved in heat stress-induced BMEC apoptosis. The results showed upregulation of HSP70 and Bax in the 37 ℃ + N-Exo and 42 ℃ + N-Exo groups compared with the controls 37 ℃ and 42 ℃, respectively (Fig. 1B–F).

Exosomes-free FBS facilitates heat stress-induced oxidative stress

To study the association between exosomes, heat stress-induced BMEC apoptosis, and oxidative stress, we measured the ROS levels using DCFH-DA kit. The intracellular ROS level in the N-Exo group was significantly higher than that in control group at 37 ℃ and 42 ℃ (Fig. 2A, B). Moreover, SOD2 was significantly more active in the N-Exo groups than in the control cultured in normal FBS medium at 37 ℃ and 42 ℃ (Fig. 2C).

Exosome-free serum increased heat stress-induced mitochondrial damage

Mitochondrial dysfunction can induce oxidative stress and apoptosis. Therefore, we evaluated the effect of exosomes on mitochondrial function. We found a significant reduction in mitochondrial membrane potential in the N-Exo group than in control group under heat stress (Fig. 3A, B). Meanwhile, the western blot results showed the increased Drp1 and Fis1 phosphorylation in the BMECs cultured with the exosome-free medium. This result indicate that exosomes might be involve in heat stress-induced apoptosis and oxidative stress as they help maintain mitochondrial function and dynamics (Fig. 3C–E).

Exosome-free serum suppressed milk protein synthesis of BMECs

As BMECs are critical for milk protein and fat syntheses, we accessed whether serum exosomes have a positive effect on them. The mRNA levels of *SREBP1*, *ELF5* and *CSN2* in the

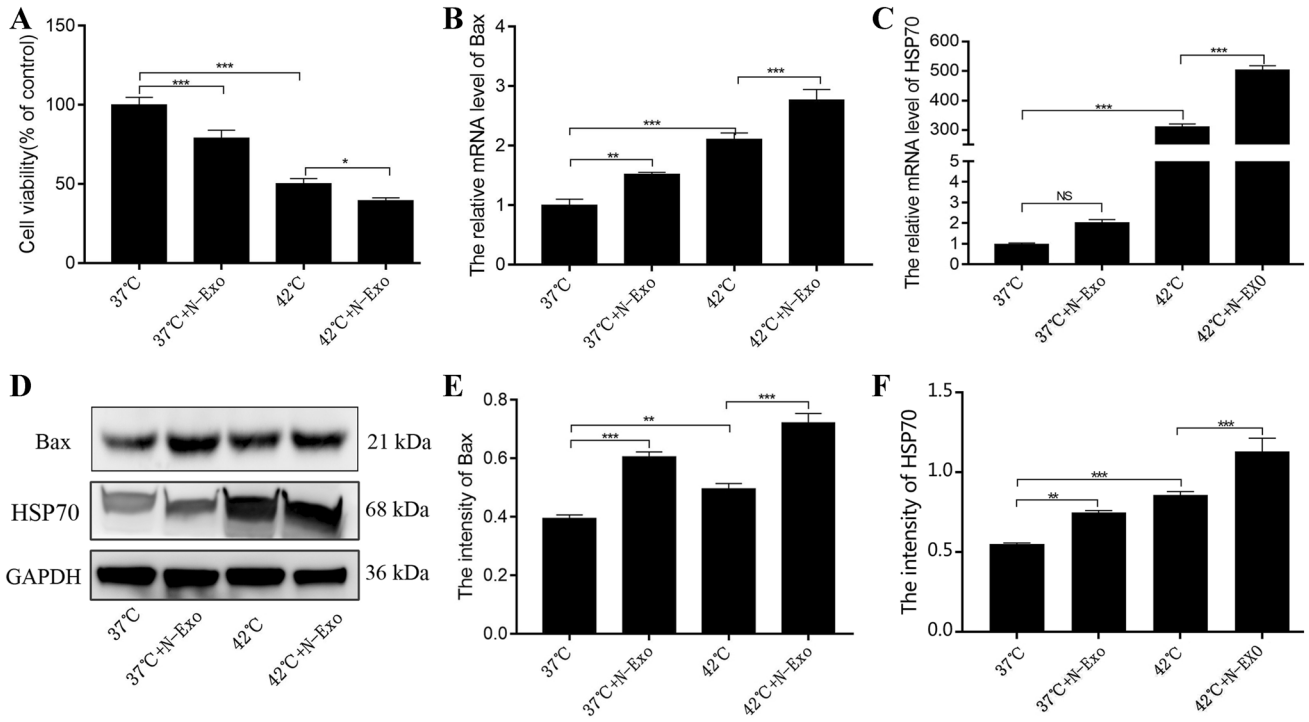

Fig. 1 Exosome-free FBS accelerates heat stress-induced apoptosis in BMECs. **A** BMECs activity detected using the CCK-8 kit under heat stress. **B**, **C** mRNA levels of Bax and HSP70 in BMECs detected using RT-qPCR. **D**–**F** Protein expression levels of Bax and HSP70 in BMECs examined using western blot

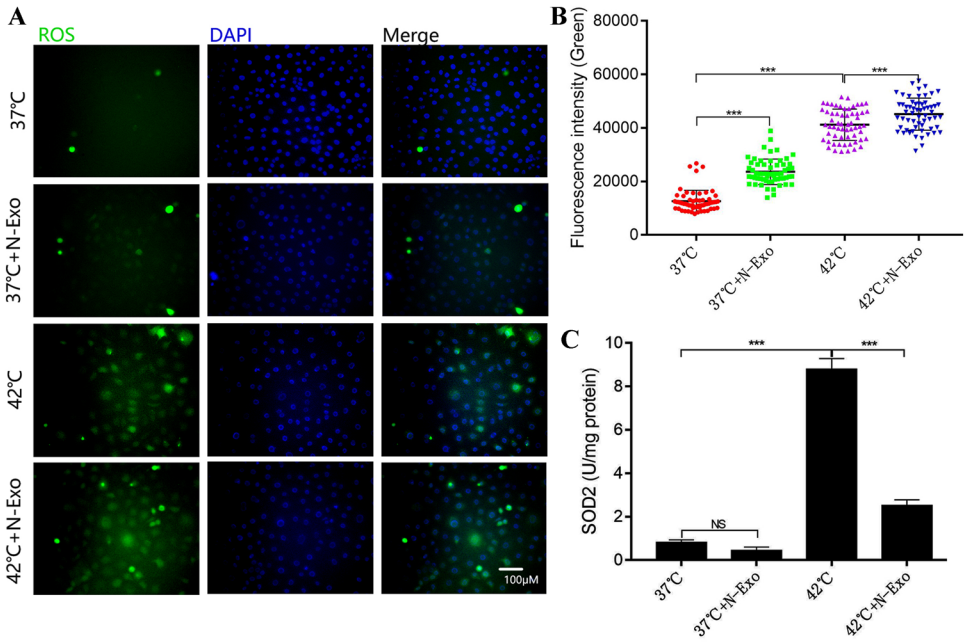

Fig. 2 Exosome-free FBS facilitates heat stress-induced oxidative stress in BMECs. **A**, **B** Reactive oxygen species (ROS) level was determined using DCFH-DA kit cultured after treatment of cells with exosome-free serum under heat stress. **C** The SOD2 content was determined using a manganese superoxide dismutase assay kit. Data are presented as the mean ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

37 ℃ + N-Exo and 42 ℃ + N-Exo groups were significantly decreased compared with those in the control groups at 37 ℃ and 42 ℃ (Fig. 4A–C). Western Blotting also showed that culture with exosome-free serum had reduced levels of SREBP1, CSN2, and ELF5 proteins in BMECs (Fig. 4D–G), indicating that exosomes play important roles in milk protein synthesis.

Exosome-free serum inhibited milk fat synthesis of BMECs

We determined the TG content in BMECs to assess the effects of exosomes on the synthesis of milk fat. The cells in the exosome-free serum had lower TG content compared with the controls at 37 ℃ and 42 ℃ groups (Fig. 5A). In addition, the expression levels of mTOR and ADD1 in BMECs cultured in exosome-free serum were significantly decreased (Fig. 5B, C). The PPARγ expression in the N-Exo group was significantly decreased, while the phosphorylation of mTOR was increased (Fig. 5D–G).

Discussion

With the global warming, heat stress has been considered as one of serious factors that affected dairy production and milk quality. The ability of mammary glands to synthesize and store milk is associated with normal function of bovine mammary epithelial cells during lactation [2]. Studies have demonstrated that heat stress could induce the generation of reactive oxygen species (ROS) in mammary glands and cause oxidative stress, resulting in the occurrence of apoptosis and autophagy [21, 23]. Exosomes, an important carrier of intercellular communication signals, play critical roles in various diseases. Exosomes carry various proteins, RNA, DNA and other components that involved in cell proliferation, immune regulation and apoptosis [24]. In this study, we cultured BMECs in vitro and investigated the effects of serum-derived exosome on BMECs proliferation under heat stress condition. The results showed that exosome-free serum induced apoptosis and oxidative stress, inhibited the synthesis of milk fat and protein in BMECs.

Mammary epithelial cells are responsible for lactation in dairy cows, usually suffering from environment-induced oxidative stress and injury [22]. Studies showed that the genes expression involved in cell structure, biosynthesis and transportation were down-regulated in cow mammary epithelial cells after heat stress treatment [25]. In addition, under high temperature environment, oxidative stress of bovine mammary epithelial cells induces cell mitochondrial damage, leading to programmed cell death [26]. It has been shown that exosomes can reduce oxidative stress by activating survival-promoting signals and restoring cell bioenergy [27]. Meanwhile, human mesenchymal stem cell-derived exosomes could promote chondrocyte proliferation and inhibit apoptosis [28]. Study has showed that pretreating with milk-derived exosomes for 24 h significantly enhanced cell viability and anti-oxidative capacity in IEC-6 cells [29]. Consistently, we found that culture with exosome free medium accelerated heat stress-caused oxidative stress and apoptosis in BMECs. In addition, our results displayed that depletion of exosomes from serum could increase the activity of SOD2, indicating that serum exosomes played critical roles in protecting the BMECs from heat stress-induced

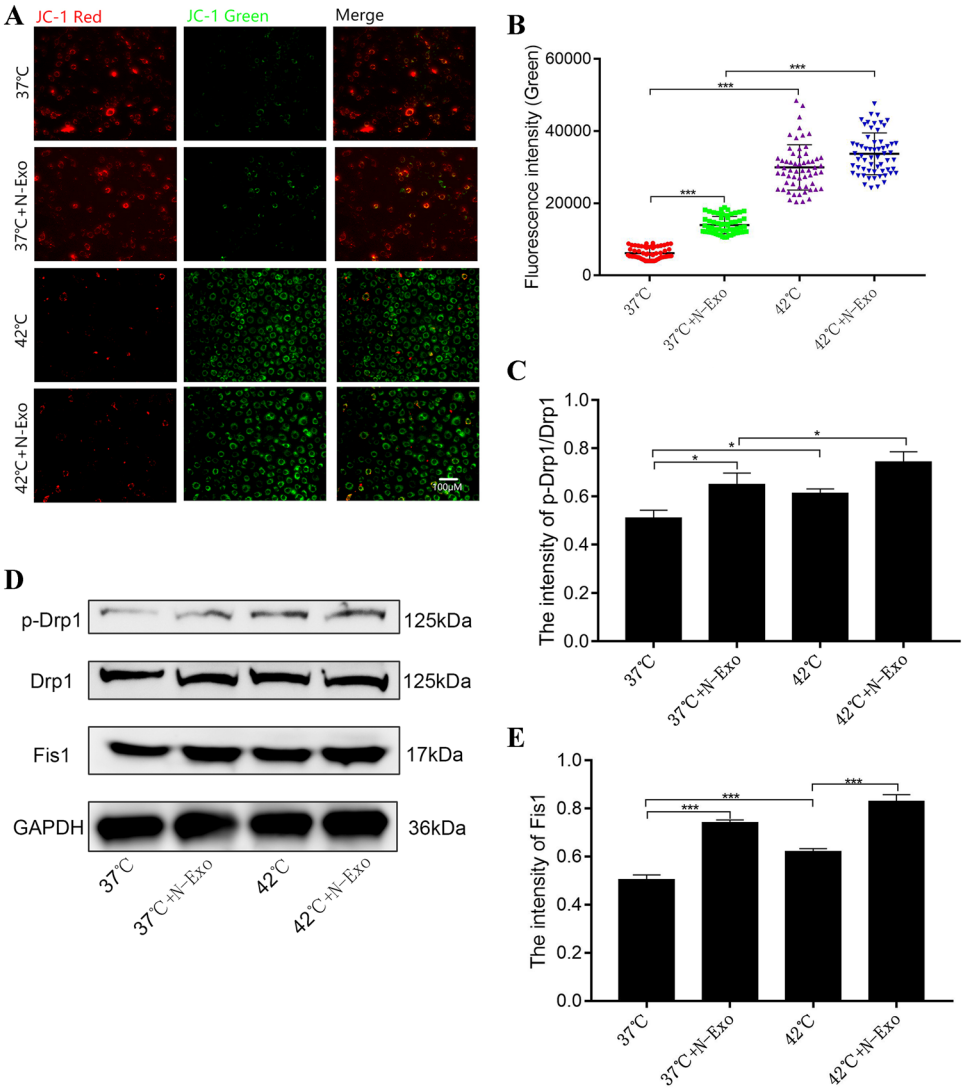

Fig. 3 Exosome-free serum increases heat stress-induced mitochondrial damage in BMECs. **A**, **B** Mitochondrial membrane potential examined using JC-1 kit. **C**–**E** The protein expression levels of Drp1, p-Drp1, and Fis1 determined using western blot. Data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

damage. However, the detail regulatory mechanism of serum exosomes in anti-oxidative function need to be further explored.

Mitochondria are the major source of ROS, mitochondrial dysfunction often causes excessive production of reactive oxygen species (ROS), resulting in oxidative stress and apoptosis [23, 30]. Inhibition of exosomal secretion deteriorated 1,4-benzoquinone-mediated mitochondrial fission by regulating Drp1 function [31]. In line with this, our result showed that depletion of exosomes from medium aggravated heat stress-induced mitochondria dysfunction by increasing Drp1 and Fis1 expression, indicating that exosome could serve as a self-protective mechanism to against heat stress-caused mitochondrial damage and mitochondrial dynamics. It has been showed that the daily milk yield was highly associated with climate change. High temperature and humidity usually resulted in a significant decrease in milk production and quality. Study showed that synthesis of milk proteins was regulated by mTOR-SREBP1 signaling pathway [32]. Previous study showed that miR-143 promoted milk fat synthesis by increasing SREBP1 expression in BMECs through the TGF-β/ Smad3-axis [33]. Consistently, our result showed that the mRNA and protein levels of SREBP1a were declined under heat stress condition, which accompanied by the decreased ELF5 and CSN2. Because exosomes miRNAs are biomarkers of mammalian milk quality, and exosomes-derived miR-142-3p can inhibit the expression of mTOR, CSN2 and SREBP1 in BMECs, indicating that exosomes are critical for milk synthesis and quality [34]. In the present study, we found that culture with exosomes-free medium aggravated the negative effect of heat stress on milk protein synthesis-related genes expression, indicating the serum-derived exosomes involved in the regulation of milk synthesis in BMECs. PPARγ is the most important transcriptional regulator of adipogenesis and adipocyte differentiation, and has been shown to regulate milk synthesis by mediating

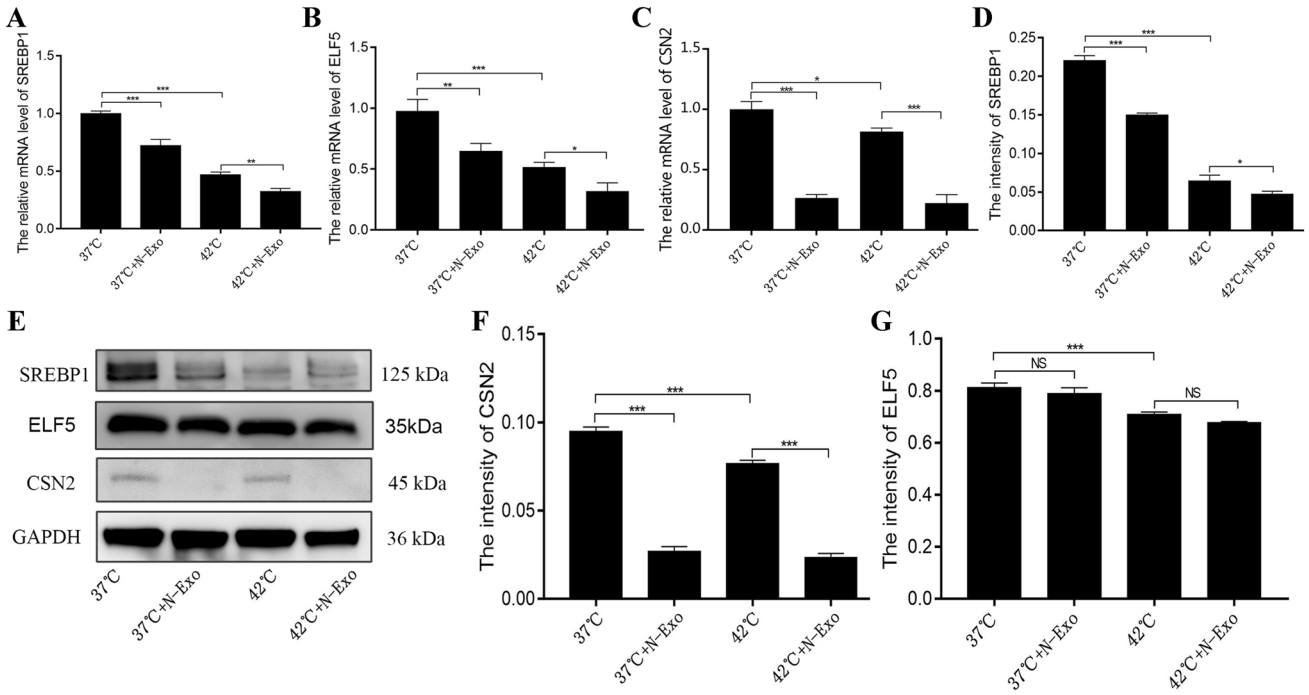

Fig. 4 Exosome-free serum suppresses milk protein synthesis in BMECs. **A-C** The mRNA levels of SREBP1, ELF5, and CSN2 detected using RT-qPCR. **D-G** Western Blot used to detect the protein expression levels of SREBP1, ELF5 and CSN2 in BMECs. Data are presented as the mean ± SEM (n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

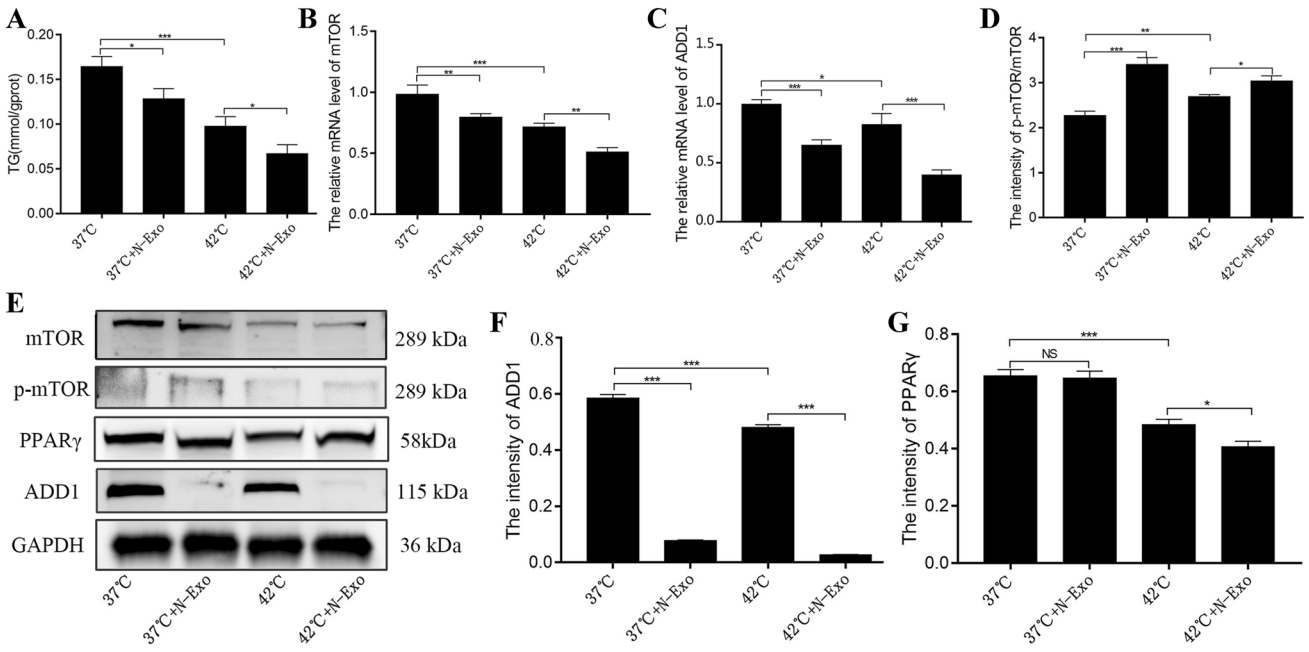

Fig. 5 Exosome-free serum inhibits milk fat synthesis in BMECs. **A** Triglyceride (TG) content in BMECs determined using the triglyceride detection kit. **B**, **C** mRNA levels of mTOR and ADD1 detected using RT-qPCR. **D**–**G** Western blot used to detect the protein expression levels of mTOR, PPARγ, p-mTOR and ADD1 in BMECs. Data are presented as the mean ± SEM (n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

triacylglycerol (TG) content in BMECs [35]. More interesting, our results revealed that exosomes also played critical roles in milk fat synthesis. Depletion of exosomes from culture medium reduced the expression level of PPARγ and TG content in BMECs under heat stress condition, indicating that exosomes not only involved in milk protein synthesis of BMECs, but also essential for milk fat synthesis.

Taken together, we were the first time to demonstrate that serum-derived exosomes can alleviate the negative effect of heat stress on BMECs. The protective effect of exosomes against heat stress in BMECs was associated with inhibition of oxidative stress and apoptosis, as well as increasing of milk protein and fat synthesis-related gene expression. Although multiple methods have been used to attenuate the adverse effect of heat stress dairy cows, there is no clinical trial that directly uses exosomes as a treatment for alleviating heat stress-induced damage. The data obtained from this study provided reliable information for preventing heat stress-induced decrease in milk production and quality in dairy cows in summer.

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Author contributions YW conceptualized the study and analyzed data. HLW, ZPL and JFZ carried out the molecular studies and sample collection. KLC and XD designed the research and drafted and revised the manuscript. All authors read and approved the final manuscript for publication.

Declarations

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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