Dehydroepiandrosterone reduced lipid droplet accumulation *via* inhibiting cell proliferation and improving mitochondrial function in primary chicken hepatocytes

Long-Long LI, Dian WANG, Chong-Yang GE, Lei YU, Jin-Long ZHAO, Hai-Tian MA*

[1] *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, People's Republic of China*

[2] *Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture, Nanjing 210095, People's Republic of China*

Corresponding author

Haitian Ma, College of Veterinary Medicine, Nanjing Agricultural University, No. 1 Weigang, Nanjing, Jiangsu 210095, PR China. Email: mahaitian@njau.edu.cn; Tel: (+86)25-8439 6763; Fax: (+86)25-8439 8669

Short title: DHEA regulated lipid droplet accumulation

Summary

Dehydroepiandrosterone (DHEA) possesses fat-reducing effect, while little information is available on whether DHEA regulates cell proliferation and mitochondrial function, which would, in turn, affect lipid droplet accumulation in broiler. In this study, the lipid droplet accumulation, cell proliferation, cell cycle and mitochondrial membrane potential were analysis in primary chicken hepatocytes treated with DHEA. The results showed that total area and counts of lipid droplet were significantly decreased in hepatocytes after DHEA treated. DHEA treatment significantly increased the cell viability, while the cell proliferation was significantly inhibited in a dose-dependent manner in primary chicken hepatocytes treated with DHEA. DHEA treatment significantly increased the cell population of S phase and decreased the population of G2/M in primary chicken hepatocytes. Meanwhile, the *cyclin A* and *cyclin-dependent kinases 2* (CDK2) mRNA abundance were significantly decreased in hepatocytes after DHEA treated. No significant differences were observed on the number of mitochondria, while the mitochondrial membrane permeability and succinate dehydrogenase (SDH) activity were significantly increased in hepatocytes treated with DHEA. In conclusion, our results demonstrated that DHEA reduced lipid droplet accumulation by inhibiting cell proliferation and enhancing mitochondrial function in primary chicken hepatocytes.

Key words: Dehydroepiandrosterone · Lipid metabolism · Cell growth · Membrane permeability · Mitochondrial enzyme

Introduction

Dehydroepiandrosterone (DHEA), one of the most abundant steroids in human, is an intermediate in the biosynthesis of androgens and/or estrogens in peripheral tissues (Labrie 2003, Dharia *et al*. 2005). The conversion of DHEA to active steroids hormone depends upon the expression levels of various steroidogenic and metabolizing enzymes according to local needs of target tissues (Labrie 1991, Labrie *et al*. 2017). DHEA had been termed "the wonder hormone" due to it protects against mood depressed (Souza-Teodoro *et al*. 2016), obesity (Hansen *et al*. 1997, Sato *et al*. 2012), cancer (Arnold *et al*. 2008), cardiovascular (Boxer *et al*. 2010) and aging-induced changes to the brain (Kurita *et al*. 2013, Soma *et al*. 2015). Previous study certified that DHEA promoted a variety of illnesses associated with aging, and it is widely available as a dietary supplemental over all the United States (Vacheron-Trystram *et al*. 2002).

DHEA has anti-proliferative effect *in vivo* and *in vitro* (Yoshida *et al*. 2003, Jiang *et al*. 2005, Cheng *et al*. 2011, Kłobucki *et al*. 2017) due to its inhibitory effects on glucose -6- phosphate dehydrogenase (G6PD) activity, which subsequently block the formation of NADPH and ribose-5-phosphate, and both of which are essential for cell growth (Batetta *et al*. 1995, Di *et al*. 1997). These results indicated that G6PD might a target conduit by which DHEA regulates cell proliferation. Previous study reported that DHEA inhibits white preadipocyte (3T3-L1) cell proliferation by arresting in the G1 phase of cell cycle (Rice *et al.* 2010). In addition, DHEA inhibits the proliferation of HPV-positive and HPV-negative cervical cancer cells (Giron *et al*. 2009). Zapata *et al.* (2015) confirmed that DHEA inhibits the proliferation of mesodermal cell types. Although many studies had reported that DHEA regulates the cell proliferation in various cell types, while the exact mechanism about these physiological roles of DHEA is not fully understood.

Except for regulating metabolism, mitochondria are involved into many other functions that make them important to all cells. Swierczynski and Mayer (1996) found that *in vivo* administration of DHEA to rats induces lipid peroxidation in liver, kidney and heart mitochondria. It demonstrated that the peroxidation of mitochondrial membrane lipids increased the permeability of mitochondrial

membrane (Maciel *et al*. 2001). In addition, DHEA induced the fast release of accumulated matrix Ca^{2+} and inhibited the oxidation of malate–glutamate by blocking Site I electron transport in the respiratory chain in rats (Maciel *et al*. 2001). Correa *et al.* (2003) demonstrated that DHEA inhibits malate-glutamate oxidation by blocking Site I electron transport in the respiratory chain, and induces mitochondrial swelling and transmembrane electrical gradient collapse in isolated rat kidney mitochondria. The above results implied that DHEA could regulate the mitochondrial function, while further investigation is needed to validate this action of DHEA in cells more precisely.

Interestingly, previous studies about biological functions of DHEA were mainly focused on mammalian species, while little information is available on poultry, both *in vivo* and *in vitro*. Unlike mammalian species, the liver is the most important organ of lipids metabolism in poultry (Griffin *et al*. 1992). Our previous studies found that DHEA reduces fat deposition in broiler chicken or chicken embryos (Tang *et al*. 2007, Zhao *et al*. 2007, Huang *et al*. 2010). In addition, we found that DHEA accelerates lipid catabolism by direct regulation of hepatic gene expression, and this action was mainly achieved by activation of cAMP/PKA signaling pathway in primary chicken hepatocytes (Tang *et al*. 2009a, Tang *et al*. 2009b). Most of the mitochondria in DHEA-induced hepatocytes presented with a high electron density based on morphological observations, indicating that DHEA might influence mitochondrial function in primary chicken hepatocytes (Tang *et al*. 2009a). Various models have been used to study the biology of obesity in humans, including fetuses and children (Bluml *et al*. 2014), rat embryos or pups (Benitez-Diaz *et al*. 2003) and chicken embryos (Sato *et al*. 2009). Chicken embryonic development is enclosed in an eggshell that is hardly influenced by external factors and is independent from the mother; therefore, chickens also represent a model to study the mechanisms of adipocyte hyperplasia during development, a process that may exacerbate adult obesity (Bo *et al*. 2012). Importantly, chickens as a widely used model for studies of adipose tissue biology, metabolism and obesity was mainly due to its genetic makeup is ~70% homologous to that of humans (Shi *et al*. 2014). Chickens also represent a model to study

the mechanisms of adipocyte hyperplasia during development, a process that may exacerbate adult obesity (Shi *et al*. 2014). Furthermore, it had reported that although the chicken primary hepatocytes have just left the body, while the biological characteristics of primary hepatocytes are close to their physiological state *in vivo*.

In addition, chicken provides a widespread and relatively inexpensive source of dietary protein for humans. However, it is well known that rapidly accumulate excess fat as a result of intensive genetic selection for rapid growth in commercial broiler chickens, and which not only induced broiler ascites syndrome, sudden death and other metabolic diseases, but also lead to adverse effect in the consumer's health due to who are increase of diet fat intake. Therefore, the present study was conducted to investigate the effects of DHEA on proliferation, cell cycle and mitochondrial function in cultured primary chicken hepatocytes, which will provide useful information to understand the biochemical mechanisms of fat deposition control by DHEA in commercial broiler chickens. It is also provided valuable information for chickens as a model for further investigation of the mechanism of obesity and insulin resistance in humans.

Materials and methods

Materials

DHEA, dimethyl sulfoxide (DMSO), methyl thiazolyl tetrazolium (MTT), penicillin-streptomycin, transferring and trypsin were purchased from Sigma (St Louis, MO, USA); L-glutamine and HEPES were obtained from Amresco (Solon, OH, USA); Medium 199 and trypsin were purchased from Gibco (Grand Island, NY, USA); The TRIZOL Reagent Kit was purchased from Invitrogen (Carlsbad, CA, USA); The M-MLV reverse transcriptase, RNase inhibitor and dNTP mixture were obtained from Promega (Madison, WI, USA); Ex Taq DNA polymerase purchased from TaKaRa Bio Inc. (Shiga, Japan) and SYBR Green PCR Master Mix obtained from Roche (Basel, Switzerland). Anti-CDK2 rabbit polyclonal antibody and Anti-Cyclin A rabbit polyclonal antibody were purchased from Sangon Biotech Co. Ltd (Shanghai, China); β-actin was

purchased from Cell Signaling Technology (Boston, MA, USA); Goat anti-rabbit IgG and horseradish peroxidase conjugate were purchased from Boster (Wuhan, China); Protein assay kits were from the Beyotime Biotechnology Institute (Shanghai, China).

Isolation of hepatocytes

Fertilized chicken eggs were purchased from a commercial hatching factory and incubated at 37 °C and at a relative humidity of 60%. Primary culture of embryonic hepatocytes was conducted based on the method described by Kennedy *et al.* (Kennedy *et al.* 1993). The embryos were killed on incubation day 9, and the livers were removed under sterile conditions and washed with cold PBS (phosphate-buffered saline). After washed in M199 medium, the livers were aseptically minced into small fragments (about 1 mm^3) and suspended in fresh medium for 1-2 min. The liver tissues were incubated in PBS (Ca^{2+} and Mg^{2+} free) trypsin solution (0.25 mg/mL) in a vibrating water-bath (90 cycles/min at 37 °C) for 10-15 min. Meanwhile, the solution was pipetted frequently by pipette to facilitate cell dissociation, until it consisted of 3-5 cells in each aggregate. The hepatocytes were collected by centrifugation (1000 rpm, 5 min) and filtrated through a 150 μm mesh. The material was washed three times with fresh M199 medium, and the cell number was determined using a hemacytometer. Cell survival was determined by the Trypan blue exclusion test and resulted in a survival rate always greater than 90% (Strober 2015).

Primary culture of chicken hepatocytes

Hepatocytes were seeded in monolayers in 6-well or 96-well plastic culture plates (Corning, USA) with a density of 2×10^6 cells per well in 2 mL or 1×10^5 cells per well in 100 μL serum-free M199 medium with 5 mg/mL transferrin, 2 mM glutamine and 1.75 mM HEPES. The culture medium also contained 100 IU/mL penicillin and 100 μg/mL streptomycin. Hepatocytes were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2.

Oil Red O staining

Hepatocytes were cultured in 6-well plates (2×10^6 cells per well) and treated with 0, 0.1, 1 or 10 μM DHEA for 24 h. The methods were previously reported of our study (Li *et al*. 2017). Briefly, the cells were fixed with 10% buffered formalin for at least 30 min, and incubated with 60% isopropanol for 15 min at room temperature, then stained with oil red O solution for another 15 min. Cells were washed 4 times with deionized water and then allowed to air dry. To normalize for the cell number, following Oil Red O staining the cell monolayers were stained with hematoxylin for 5 min. The slides were photographed with an optical microscope (Olympus BX53; Tokyo, Japan). Then, twenty photos were randomly selected from each treatment group and ten independent visual field in each photo were analyzed the counts and area of lipid droplets using Image-pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Cell viability assay

Hepatocytes were cultured in 96-well plates (1×10^5 cells per well) and treated with 0, 0.1, 1 or 10 μM DHEA for 24 h before addition of MTT solution, and then 20 μL of 5 mg/mL MTT were added to each well. Four hours later, the culture medium was removed and the formed blue formazan crystals were dissolved in 150 μL DMSO. The optical density of the formazan generated from MTT was measured at 490 nm using a model 550 Microplate reader (Bio-Rad, California, USA).

EdU-based cell proliferation assays

Cell proliferation assays were performed using a Click-iT EdU assay kit according to the manufacturer's instructions. Briefly, hepatocytes were cultured in 96-well plates (1×10^5 cells per well) and treated with 0, 0.1, 1 or 10 μM DHEA for 24 h, then 100 μL 5'-ethynyl-2'-deoxyuridine (EdU) solution was added at a 50 μM final concentration for 6 h. Cells were washed with PBS and fixed with 100 μl 4% formaldehyde for 15 min. Following formaldehyde fixation, cells were incubated with 100 μL saponin-based permeabilization buffer for 15 min and then incubated with 500 μl Click-iT reaction buffer for 1 h and washed with 3 mL permeabilization buffer. EdU-stained cells were mounted and imaged by fluorescence microscopy.

Cell cycle assessment by flow cytometry

Hepatocytes were plated in 6-well plates (2×10^6 cells per well) and treated with 0, 0.1, 1 or 10 μM DHEA for 24 h. After incubation, the cells were harvested and fixed in 1 mL 75% cold ethanol, and then incubated at -20 °C for 18 h. The cells were centrifuged at 1000 rpm for 5 min, and the cell pellets were re-suspended in 500 μL propidium iodine (50 μg/mL) containing 5 U RNase and incubated on ice for 30 min. Cell cycle distribution was calculated from 10,000 cells with ModFit LTTM software (Becton Dickinson, CA, USA) using FACScaliber (Becton Dickinson, San Jose, CA).

Real-time quantitative RT-PCR (qPCR)

Hepatocytes were cultured in 6-well plates (2×10^6 cells per well) and treated with 0, 0.1, 1 or 10 μM DHEA for 24 h. The cells were harvested and total RNA was extracted using the TRIZOL reagent kit (Invitrogen, USA) according to our previous laboratory reported (Li *et al*. 2017). Total RNA (2 μg) were reverse transcribed into cDNA using the Superscript II kit (Promega, USA) according to the manufacturer's recommendation. An aliquot of cDNA sample was mixed with 20 μL SYBR Green PCR Master Mix (Roche, Switzerland) in the presence of 10 pmol of each forward and reverse primers for *β-actin* (use as an internal control), *cyclin A, cyclin B* and *CDK2* (Table 1). All samples were analyzed in duplicate using the IQ5 Sequence Detection System (Bio-Rad, California, USA) and programmed to conduct one cycle (95 °C for 3min) and 40 cycles (95 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s). The $2^{-\Delta\Delta CT}$ method was used to calculate the fold change in mRNA levels. The primers were designed by Primes Premier 5 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Invitrogen Biological Company (Shanghai, China).

Western blotting analysis

Hepatocytes were cultured in 6-well plates (2×10^6 cells per well) and treated with 0, 0.1, 1 or 10 μM μM DHEA for 24 h, and scraped for the subsequent determination of protein level. The protein concentration was measured using a bicinchoninic acid (BCA) protein determination kit (Beyotime, Nantong, China). The extracted protein was separated on a 10% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 3 h with 5% BSA in TBST and then incubated with a rabbit polyclonal antibody against CDK2, Cyclin A (1:1000 dilution). After washing with TBST, goat anti-rabbit IgG with horseradish peroxidase-conjugated (1:5000 dilution) was added and incubated for 2 h at room temperature. The protein expression abundance was detected by ECL superSignalTM West Pico substrate (Pierce, Rock-ford, IL, USA). β-actin monoclonal antibody (dilution 1:1000) was used as the loading control, CDK2 and Cyclin A protein expression abundance were normalized to β-actin.

Quantitation of mitochondria

Hepatocytes were cultured in 6-well plates (2×10^6 cells per well) and treated with 0, 0.1, 1 or 10 μM DHEA for 24 h. The cells were fixed in 0.1 M sodium phosphate (pH 7.4) containing 2.5% glutaraldehyde, centrifuged at 3000 rpm for 4 min and rinsed in the same buffer and then post-fixed in 1% osmium tetroxide in Millonig's buffer. Cell samples were then processed by standard techniques for transmission electron microscopy (TEM). Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed in an H-7650 transmission electron microscope (Hitachi Company, Japan). Thirty photos were randomly selected from each treatment and fifteen independent cells in each photo were counted the number of mitochondria. The results were tabulated as the mean number of mitochondria in all treatment groups.

Evaluation of mitochondrial permeability by JC-1 staining assay

Mitochondrial membrane potential (ΔΨm) was detected using a JC-1 mitochondrial membrane potential detection kit following the manufacturer's instructions (Beyotime Institute of Biotechnology, China). Briefly, 2×10^6 cells were collected and re-suspended in 0.5 mL of medium. After adding 0.5 mL working solution of JC-1 dye, the cells were mixed thoroughly and incubated at 37 °C for 20 min in the dark, prior to analysis by flow cytometer (BD Bioscience, San Jose, CA). The JC-1 monomer has an excitation wavelength of 490 nm and an emission wavelength of 530 nm, and the JC-1 polymer has an excitation wavelength of 525 nm and an emission wavelength of 590

nm. The average fluorescence intensity of 10 randomly selected visual fields was calculated using the IPP software, and the fluorescence intensity ratio of 590/530 nm was used as a convenient index for the ΔΨm.

Succinate dehydrogenase activity assay

Hepatocytes were cultured in 6-well plates (2×10^6 cells per well) and treated with 0, 0.1, 1 or 10 μM DHEA for 24 h. The cells were harvested and succinate dehydrogenase (SDH) activity was measured using commercial kits according to the manufacturers' protocols (Jiancheng Biotechnology Institution, Nanjing, China). The data were normalized to the sample protein concentration as determined by a protein assay kit, and expressed as U/mg protein.

Data analysis and statistics

Data were analyzed with one-way ANOVA and expressed as the means ± standard error. Treatment differences were subjected to a Duncan's multiple comparison tests. Differences were considered significant at $P < 0.05$. All statistical analyses were performed with SPSS 20.0 for Windows (StatSoft, Inc., Tulsa, OK, USA).

Results

Effect of DHEA on cell viability in primary chicken hepatocytes

As described in Table 2, cell viability was significantly increased in the 1 and 10 μM DHEA treated groups at 1-48 h when compared to the control group in primary chicken hepatocytes ($P < 0.01$). Meanwhile, 0.1 μM DHEA treated significantly increased cell viability at 3-48 h than that of the control group in primary chicken hepatocytes ($P < 0.01$).

Effect of DHEA on lipid droplet accumulation in primary chicken hepatocytes

To analyze the distribution of lipid droplet in primary chicken hepatocytes after DHEA treated, Oil Red O staining (Fig. 1A) was used to analyze the total area and counts of lipid droplet. The results showed that 0.1-10 μM DHEA treatment significantly decreased the total area of lipid

droplet when compared to the control group ($P < 0.05$) (Fig. 1B). Similarly, the counts of lipid droplet also significantly decreased in 0.1-10 μM DHEA treated groups than that of the control group ($P < 0.05$) (Fig. 1C).

Effect of DHEA on cell proliferation in primary chicken hepatocytes

EdU assay method was used to investigate the cell proliferation, and the results showed that the cell proliferation was significantly inhibited with a dose-dependent manner in primary chicken hepatocytes after DHEA treated (Fig. 2).

Effect of DHEA on cell cycle in primary chicken hepatocytes

As shown in Fig. 3, no significant differences were observed on the population of G0/G1, S and G2/M phase in primary chicken hepatocytes treated with 0.1 μM DHEA ($P > 0.05$). Compared with the control group, the population of S phase was significantly increased in hepatocytes treated with 1 or 10 μM DHEA ($P < 0.01$). Whereas, 1 or 10 μM DHEA treatment significantly decreased the population of G2/M in hepatocytes than that of the control group ($P < 0.01$).

Effect of DHEA on cell cycle related factors expression levels in primary chicken hepatocytes

Compare with the control group, the *cyclin A* mRNA level was significantly decreased in primary chicken hepatocytes after 1 or 10 μM DHEA treated ($P < 0.05$) (Fig. 4A). No significant changes were observed on the *cyclin B* mRNA level (Fig. 4B), whereas 0.1-10 μM DHEA treatment significantly decreased the *CDK2* mRNA level in primary chicken hepatocytes when compared to the control group ($P < 0.05$) (Fig. 4C). To further verified these results, we consequently detected the protein abundance of cyclinA and CDK2, and the results showed that 0.1-10 μM DHEA treatment significantly decreased the cyclin A and CDK2 protein expression level in primary chicken hepatocytes ($P < 0.05$) (Fig. 4E and 4F).

Morphological observations and quantization of mitochondria

Compared with the control group, the histological organization was not obviously altered in primary chicken hepatocytes after DHEA treated (Fig. 5A). Thirty photos were randomly selected

and fifteen independent cells of each photo were counted the number of mitochondria. No noticeable changes were observed on the numbers of mitochondrial in primary chicken hepatocytes treated with 0.1-10 μM DHEA ($P > 0.05$) (Fig. 5B).

Effect of DHEA on the mitochondrial permeability in primary chicken hepatocytes

No significant changes were observed on the mitochondrial membrane potential (ΔΨm) in primary chicken hepatocytes treated with 0.1 or 1 μM DHEA ($P > 0.05$), while 10 μM DHEA treatment significantly decreased the ΔΨm when compared to the control group in primary chicken hepatocytes ($P < 0.05$) (Fig. 6).

Effect of DHEA on succinate dehydrogenase activity in primary chicken hepatocytes

No significant changes were observed on the succinate dehydrogenase activity (SDH) in primary chicken hepatocytes treated with 0.1 μM DHEA ($P > 0.05$), while 1 or 10 μM DHEA treatment significantly increased the SDH activity when compared to the control group in primary chicken hepatocytes ($P < 0.05$) (Fig. 7).

Discussion

Dehydroepiandrosterone (DHEA) is commercially available as a non-prescription nutritional supplement to control body weight gain and inhibit fat accumulation (Legrain *et al*. 2003). Many studies found that DHEA treatment reduces fat accumulation in chickens (Tang *et al*. 2007, Huang *et al*. 2011), rodents (Sato *et al*. 2012) and humans (Hernandez-Morante *et al*. 2011). In this study, DHEA treatment decreased the total area and counts of lipid droplet, which indicated that DHEA reduced fat accumulation in primary chicken hepatocytes. DHEA possesses fat-reducing effect, and which may be accomplished through multiple mechanisms (Pergola 2000). The adipocyte hypertrophy and hyperplasia are the main forms of fat deposition in broiler chickens (Butterwith 1997). Therefore, we subsequently investigated the impact of DHEA on cell proliferation and mitochondrial function in primary chicken hepatocytes.

Although there reported that DHEA possesses anti-proliferate action in many cell types (Dashtaki *et al*. 1998, López-Marure *et al*. 2011), while little is known about its effect on chicken hepatocytes. In the present study, DHEA treatment decreased primary chicken hepatocytes proliferation in a dose-dependent manner, and this result was consistent with the previous reported that DHEA modulates growth factor-induced primary bovine chromaffin cell proliferation in an age-dependent manner (Sicard *et al*. 2007). Evidence suggested that the inhibitory effect of DHEA on cell proliferation is associated with the changes of cell cycle (Rice *et al*. 2010). Our results showed that DHEA increased the S phase cell population and decreased the G2/M population in primary chicken hepatocytes, which implied that DHEA inhibited hepatocytes proliferation and caused cell cycle arrest in S phase. In addition, DHEA treatment decreased the cyclin A and CDK2 protein abundance in primary chicken hepatocytes. In eukaryotes, the cell cycle is regulated by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDK1). It reported that cyclin A/CDK2 are mainly involved in regulating the progression of S phase and cyclin B/CDK1 are involved in regulating the progression of G2/M phase (Han *et al*. 2008). Thus, we speculated that DHEA inhibited primary chicken hepatocytes proliferation by decreasing the cyclin A and CDK2 protein expression level, which finally led the decreasing of fat accumulation in primary chicken hepatocytes.

DHEA has been characterized as a peroxisome proliferator, which directly affects the β-oxidation in rat hepatocytes (Yamada *et al*. 1992). Our previous study also found that DHEA caused a significant up-regulation of PPARα mRNA level in primary chicken hepatocytes (Tang *et al*. 2007). It reported that DHEA increase PPARα expression in hepatocytes, and which increases the rate of β-oxidation and decelerates the transport of acetyl-CoA from the mitochondria to the cytosol (Schoonjans *et al*. 1996). Mitochondria are associate with cellular energy metabolism and provide energy for all kinds of life activities. In addition, mitochondria are the main site for the β-oxidation of fatty acid in the body. Previous study reported that DHEA alters the number and configuration of mitochondria in liver of rats (Bellei *et al*. 1992). In this study, no noticeable

changes were found on the number of mitochondria in chicken hepatocytes after DHEA treated. The probable explanation for this discrepancy may be attributed to the different cell types and treated time in these studies. The MTT reduction assay detecting cell viability is measure the capacity of NAD(P)H-dependent cellular oxidoreductase enzymes to transform MTT into formazan in living cells (Ferrari *et al*. 1990). Thus, the mitochondrial membrane might play an important role in blocking the MTT entrance into the mitochondria. The mitochondrial membrane potential ($\Delta\Psi m$) was significantly decreased in this study, and which indicated that DHEA increased the mitochondrial membrane permeability in primary chicken hepatocytes. Previous study found that high concentration of DHEA decreases the $\Delta\Psi m$ of kidney cortex mitochondria, and this effect may be due to the opening of non-specific pores (Correa *et al.* 2003). In addition, Liu *et al.* (2016) reported that mitochondrial membrane permeability was significantly increased in Leydig cells treated with DHEA. It demonstrated that peroxidation of mitochondrial membrane lipids promotes the increasing of membrane permeability (Wang *et al*. 2005). These data may explain, at least partially, DHEA improved chicken hepatocytes viability by increasing mitochondrial membrane permeability. The mitochondrial membrane potential is prerequisite for maintaining mitochondrial oxidative phosphorylation and respiratory chain produces ATP by using the proton gradient energy across the membrane (Lin 1999). To further elucidate the effect of DHEA on mitochondrial functions, the succinate dehydrogenase (SDH) activity, which is the only enzyme that participates in both citric acid cycle and electron transport chain (Rutter *et al*. 2010), were evaluated in present study. Our results showed that DHEA significantly increased the SDH activity in primary chicken hepatocytes. Previous studies showed that DHEA inhibited complex I of the mitochondrial respiratory chain *in vitro* and *in vivo* (Safiulina *et al*. 2006), which play a crucial role in ATP production (Davies *et al*. 2011). But, DHEA does not inhibit succinate oxidation (Complex II) (Safiulina *et al*. 2006). It reported that DHEA primarily affects fatty acid mobilization, resulting in enhanced mitochondrial respiration in chicken (Tang *et al*. 2009a). In addition, our recently research showed that administration of DHEA accelerated glucose catabolism by enhancing some

mitochondrial enzyme activity in rats (Kang *et al*. 2016). Based on these reported, we speculated that DHEA enhanced the mitochondrial function may be associated with the increasing of fatty acid β-oxidation in chicken hepatocytes, and this supposition requires further investigation.

Overall, our results demonstrated that DHEA reduced lipid droplet accumulation, and this action mainly achieved *via* inhibiting hepatocytes proliferation and enhancing mitochondrial function in primary chicken hepatocytes. This information is necessary to understand the mechanisms by which DHEA exerts its fat-reducing actions in broiler chicken, it also provide valuable information for chickens as a model for further investigation of the mechanism of obesity and insulin resistance in humans. Certainly, further deeply investigation is required to validate this mechanism more precisely in primary chicken hepatocytes.

Acknowledgments

This work was supported by the National Natural Science Foundation of Jiangsu (NO. BK20151434) and Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Competing interests

The authors declare that they have no competing interests.

References

ARNOLD JT, GRAY NE, JACOBOWITZ K, VISWANATHAN L, CHEUNG PW, MCFANN KK, LE H, BLACKMAN MR: Human prostate stromal cells stimulate increased PSA production in DHEA-treated prostate cancer epithelial cells. *J Steroid Biochem Mol Biol* **111**: 240-246, 2008.

BATETTA B, BONATESTA R, SANNA F, PUTZOLU M, PIRAS S, SPANO O, PANI P, DESSI S: Effect of DHEA on cell proliferation, cholesterol metabolism and G6PD gene expression in human G6PD normal and deficient lymphocytes. *Aging Clin and Exp Res* **7**: 483-484, 1995.

BELLEI M, BATTELLI D, FORNIERI C, MORI G, MUSCATELLO U, LARDY H, BOBYLEVA V: Changes in liver structure and function after short-term and long-term treatment of rats with dehydroepiandrosterone. *J Nutr* **122**: 967-976, 1992.

BENITEZ-DIAZ P, MIRANDA-CONTRERAS L, MENDOZA-BRICENO RV, PENA-CONTRERAS Z, PALACIOS-PRU E: Prenatal and postnatal contents of amino acid neurotransmitters in mouse parietal cortex. *Dev Neurosci* **25**: 366-374, 2003.

BLUML, WISNOWSKI JL, NELSON MD, PAQUETTE L, PANIGRAHY A: Metabolic Maturation of White Matter Is Altered in Preterm Infants. *Plos One* **9**: e85829, 2014.

BO J, ERNEST B, GOODING JR, DAS S, SAXTON AM, SIMON J, DUPONT J, MéTAYERCOUSTARD S, CAMPAGNA SR, VOY BH: Transcriptomic and metabolomic profiling of chicken adipose tissue in response to insulin neutralization and fasting. *BMC Genomics* **13**: 441, 2012.

BOXER RS, KLEPPINGER A, BRINDISI J, FEINN R, BURLESON JA, KENNY AM: Effects of dehydroepiandrosterone (DHEA) on cardiovascular risk factors in older women with frailty characteristics. *Age Ageing* **39**: 451-458, 2010.

BUTTERWITH SC: Regulators of adipocyte precursor cells. *Poult Sci* **76**: 118-123, 1997.

CHENG ML, SHIAO MS,CHIU DT, WENG SF, TANG HY, HO HY: Biochemical disorders associated with antiproliferative effect of dehydroepiandrosterone in hepatoma cells as revealed by LC-based metabolomics. *Biochem Pharmacol* **82**: 1549-1561, 2011.

CORREA F, GARCÍA N, GARCÍA G, CHÁVEZ E: Dehydroepiandrosterone as an inducer of mitochondrial permeability transition. *J Steroid Biochem Mol Biol* **87**: 279-284, 2003.

DASHTAKI R, WHORTON AR, MURPHY TM, CHITANO P, REED W, KENNEDY TP: Dehydroepiandrosterone and analogs inhibit DNA binding of AP-1 and airway smooth muscle proliferation. *J Pharmacol Exp Ther* **285**: 876-883, 1998.

DAVIES KM, STRAUSS M, DAUM B, KIEF JH, OSIEWACZ HD, RYCOVSKA A, ZICKERMANN V, KUÜHLBRANDT W: Macromolecular organization of ATP synthase and complex I in whole mitochondria. *Proc Natl Acad Sci U S A* **108**: 14121-14126, 2011.

DHARIA SP, FALCONE T: Robotics in reproductive medicine. *Fertil Steril* **84**: 1-11, 2005.

DI MM, PIZZINI A, GATTO V, LEONARDI L, GALLO M, BRIGNARDELLO E, BOCCUZZI G: Role of glucose-6-phosphate dehydrogenase inhibition in the antiproliferative effects of dehydroepiandrosterone on human breast cancer cells. *Br J Cancer* **75**: 589-592, 1997.

FERRARI M, FORNASIERO MC, ISETTA AM: MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. *J Immunol Methods* **131**: 165-172, 1990.

GIRON RA, MONTANO LF, ESCOBAR ML, LOPEZ-MARURE R: Dehydroepiandrosterone inhibits the proliferation and induces the death of HPV-positive and HPV-negative cervical cancer cells through an androgen- and estrogen-receptor independent mechanism. *Febs J* **276**: 5598-5609, 2009.

GRIFFIN HD, GUO K, WINDSOR D, BUTTERWITH SC: Adipose tissue lipogenesis and fat deposition in leaner broiler chickens. *J Nutr* **122**: 363-368, 1992.

HAN YH, KIM SZ, KIM SH, PARK WH: Arsenic trioxide inhibits the growth of Calu-6 cells via inducing a G2 arrest of the cell cycle and apoptosis accompanied with the depletion of GSH. *Cancer Lett* **270**: 40-55, 2008.

HANSEN PA, HAN DH, NOLTE LA, CHEN M, HOLLOSZY JO: DHEA protects against visceral obesity and muscle insulin resistance in rats fed a high-fat diet. *Am J Physiol* **273**: R1704-R1708, 1997.

HERNANDEZ-MORANTE JJ, CEREZO D, CRUZ RM, LARQUE E, ZAMORA S, GARAULET M: Dehydroepiandrosterone-sulfate modifies human fatty acid composition of different adipose tissue depots. *Obes Surg* **21**: 102-111, 2011.

HUANG J, RUAN J, TANG X, ZHANG W, MA H, ZOU S: Comparative proteomics and phosphoproteomics analyses of DHEA-induced on hepatic lipid metabolism in broiler chickens. *Steroids* **76**: 1566-1574, 2011.

HUANG J, TANG X, RUAN J, ZHANG W, MA H, ZOU S: Use of comparative proteomics to identify key proteins related to hepatic lipid metabolism in broiler chickens: evidence accounting for differential fat deposition between strains. *Lipids* **45**: 81-89, 2010.

JIANG Y, MIYAZAKI T, HONDA A, HIRAYAMA T, YOSHIDA S, TANAKA N, MATSUZAKI Y: Apoptosis and inhibition of the phosphatidylinositol 3-kinase/Akt signaling pathway in the anti-proliferative actions of dehydroepiandrosterone. *J Gastroenterol* **40**: 490-497, 2005.

KANG J, Long-Term Administrationof Dehydroepiandrosterone Accelerates Glucose Catabolism via Activation of PI3K/Akt-PFK-2 Signaling Pathway in Rats Fed a High-Fat Diet. *Plos One* **11**: e0159077, 2016.

KENNEDY SW, LORENZEN A, JAMES CA, COLLINS BT: Ethoxyresorufin-O-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. *Anal Biochem* **211**: 102-112, 1993.

KŁOBUCKI M, GRUDNIEWSKA A, SMUGA DA, SMUGA M, JAROSZ J, WIETRZYK J, MACIEJEWSKA G, WAWRZENCZYK C: Syntheses and antiproliferative activities of novel phosphatidylcholines containing dehydroepiandrosterone moieties. *Steroids* **118**: 109-118, 2017.

KURITA H, MAESHIMA H, KIDA S, MATSUZAKA H, SHIMANO T, NAKANO Y, BATA H, SUZUKI T, ARAI H: Serum dehydroepiandrosterone (DHEA) and DHEA-sulfate (S) levels in medicated patients with major depressive disorder compared with controls. *J Affect Disord* **146**: 205-212, 2013.

LÓPEZ-MARURE R, CONTRERAS PG, DILLON JS: Effects of dehydroepiandrosterone on proliferation, migration, and death of breast cancer cells. *Eur J Pharmacol* **660**: 268-274, 2011.

LABRIE F: Intracrinology. *Mol Cell Endocrinol* **78**: C113-118, 1991.

LABRIE F: Extragonadal synthesis of sex steroids: intracrinology. *Ann Endocrinol* **64**: 95-107, 2003.

LABRIE F, MARTEL C, BEéLANGER A, PELLETIER G: Androgens in women are essentially made from DHEA in each peripheral tissue according to intracrinology. *J Steroid Biochem Mol Biol* **168**: 9-18, 2017.

LEGRAIN DS, GIRARD L: Pharmacology And Therapeutic Effects of Dehydroepiandrosterone In Older Subjects. *Drugs Aging* **20**: 949-967, 2003.

LI L, CAO Y, XIE Z, ZHANG Y: A High-Concentrate Diet Induced Milk Fat Decline via Glucagon-Mediated Activation of AMP-Activated Protein Kinase in Dairy Cows. *Sci Rep* **7**: 44217, 2017.

LI L, PENG M, GE C, YU L, MA H: (-)-Hydroxycitric Acid Reduced Lipid Droplets Accumulation Via Decreasing Acetyl-Coa Supply and Accelerating Energy Metabolism in Cultured Primary Chicken Hepatocytes. *Cell Physiol Biochem* **43**: 812-831, 2017.

LIN Q: Mitochondria and apoptosis. *Science* **31**: 116-118, 1999.

LIU L, WANG D, LI L, DING X, MA H: Dehydroepiandrosterone inhibits cell proliferation and improves viability by regulating S phase and mitochondrial permeability in primary rat Leydig cells. *Mol Med Rep* **14**: 705-714, 2016.

MACIEL EN, VERCESI AE, CASTILHO RF: Oxidative stress in Ca(2+)-induced membrane permeability transition in brain mitochondria. *J Neurochem* **79**: 1237-1245, 2001.

PERGOLA GD: The adipose tissue metabolism: role of testosterone and dehydroepiandrosterone. *Int J Obes Relat Metab Disord* **24**: S59-63, 2000.

RICE SPL, ZHANG L, GRENNAN-JONES F, AGARWALL N, LEWIS MD, REES DA, LUDGATE M: Dehydroepiandrosterone (DHEA) treatment in vitro inhibits adipogenesis in human omental but not subcutaneous adipose tissue. *Mol Cell Endocrinol* **320**: 51-57, 2010.

RUTTER J, WINGE DR, SCHIFFMAN JD: Succinate dehydrogenase - Assembly, regulation and role in human disease. *Mitochondrion* **10**: 393-401, 2010.

SAFIULINA D,PEET N, SEPPET E, ZHARKOVSKY A, KAASIK A: Dehydroepiandrosterone Inhibits Complex

I of the Mitochondrial Respiratory Chain and is Neurotoxic and at High Concentrations. *Toxicol Sci*, **93**: 348-356, 2006.

SATO M, TOMONAGA S, DENBOW DM, FURUSE M: Changes in free amino acids in the brain during embryonic development in layer and broiler chickens. *Amino Acids* **36**: 303-308, 2009.

SATO K, IEMITSU M, AIZAWA K, MESAKI N, AJISAKA R, FUJITA S: DHEA administration and exercise training improves insulin resistance in obese rats. *Nutr Metab* **9**: 47-53, 2012.

SCHOONJANS K, STAELS B, AUWERX J: The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. *Biochim Biophys Acta* **1302**: 93-109, 1996.

SHI L, KO ML, HUANG CC, PARK SY, HONG MP, WU C, KO GY: Chicken Embryos as a Potential New Model for Early Onset Type I Diabetes. *J Diabetes Res* **2014**: 354094-354103, 2014.

SICARD F, EHRHARTBORNSTEIN M, CORBEIL D, SPERBER S, KRUG AW, ZIEGLER CG, RETTORI V, MCCANN SM, BORNSTEIN SR: Age-dependent regulation of chromaffin cell proliferation by growth factors, dehydroepiandrosterone (DHEA), and DHEA sulfate. *Proc Natl Acad Sci U S A* **104**: 2007-2012, 2007.

SOMA KK, RENDON NM, BOONSTRA R, ALBERS HE, DEMAS GE: DHEA effects on brain and behavior: insights from comparative studies of aggression. *J Steroid Biochem Mol Biol* **145**: 261-272, 2015.

SOUZA-TEODORO LH, DE OLIVEIRA C, WALTERS K, CARVALHO LA: Higher serum dehydroepiandrosterone sulfate protects against the onset of depression in the elderly: Findings from the English Longitudinal Study of Aging (ELSA). *Psychoneuroendocrinology* **64**: 40-46, 2016.

STROBER W: Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* **111**: A3.B.1-3, 2015.

SWIERCZYNSKI J, MAYER D: Dehydroepiandrosterone-induced lipid peroxidation in rat liver mitochondria. *J Steroid Biochem Mol Biol* **58**: 599-603, 1996.

TANG X, MA H, ZOU S, CHEN W: Effects of Dehydroepiandrosterone (DHEA) on Hepatic Lipid Metabolism Parameters and Lipogenic Gene mRNA Expression in Broiler Chickens. *Lipids* **42**: 1025-1033, 2007.

TANG X, MA H, HUANG G, MIAO J, ZOU S: The effect of dehydroepiandrosterone on lipogenic gene mRNA expression in cultured primary chicken hepatocytes. *Eur J Lipid Sci Tech* **111**: 432-441, 2009a.

TANG X, MA H, SHEN Z, XU X, LIN C: Dehydroepiandrosterone activates cyclic adenosine 3',5'-monophosphate/protein kinase A signalling and suppresses sterol regulatory element-binding protein-1 expression in cultured primary chicken hepatocytes. *Br J Nutr* **102**: 680-686, 2009b.

VACHERON-TRYSTRAM MN,CHEREF S, GAUILLARD J,PLAS J: A case report of mania precipitated by use of DHEA. *Encephale* **28**: 563-566, 2002.

WANG XJ, WANG ZB, XU JX: Effect of salvianic acid A on lipid peroxidation and membrane permeability in mitochondria. *J Ethnopharmacol* **97**: 441-445, 2005.

YAMADA J, SAKUMA M, SUGA T: Induction of peroxisomal beta-oxidation enzymes by dehydroepiandrosterone and its sulfate in primary cultures of rat hepatocytes. *Biochim Biophys Acta* **1137**: 231-236, 1992.

YOSHIDA S, HONDA A, MATSUZAKI Y, FUKUSHIMA S, TANAKA N,TAKAGIWA A, FUJIMOTO Y, MIYAZAKI H, SALEN G: Anti-proliferative action of endogenous dehydroepiandrosterone metabolites on human cancer cell lines. *Steroids* **68**: 73-83, 2003.

ZAPATA E, VENTURA JL, DE LA CRUZ K, RODRIGUEZ E, DAMIAN P, MASSO F, MONTANO LF, LOPEZ-MARURE R: Dehydroepiandrosterone inhibits the proliferation of human umbilical vein endothelial cells by enhancing the expression of p53 and p21, restricting the phosphorylation of retinoblastoma protein, and is androgen- and estrogen-receptor independent. *Febs J* **272**: 1343-1353, 2005.

ZHAO S, MA H, ZOU S, CHEN W: Effects of In Ovo Administration of DHEA on Lipid Metabolism and Hepatic Lipogenetic Genes Expression in Broiler Chickens During Embryonic Development. *Lipids* **42**: 749-757, 2007.

Figure Legends

Fig. 1. Effect of DHEA on lipid droplets accumulation in primary chicken hepatocytes. A: Representative photomicrographs of hepatocytes stained with oil red O, the red represents lipid droplet and blue represents the cell nucleus. **B:** Total area of lipid droplets. **C:** The number of lipid droplets. Data are presented as means ± SE. *P < 0.05, **P < 0.01, DHEA treated group vs. control group.

Fig. 2. EdU (5-ethynyl-2'-deoxyuridine) labels cells proliferating in primary chicken hepatocytes. A: Control group. **B:** Hepatocytes treated with 0.1 μM DHEA. **C:** Hepatocytes treated with 1 μM DHEA. **D:** Hepatocytes treated with 10 μM DHEA. The cells in each group were stained with DNA marker (Hoechst33342) and EdU, the merged images in the right column and the pink color in the merged image shows the proliferating cells.

Fig. 3. Effect of DHEA on cell cycle in primary chicken hepatocytes. **A:** Control group. **B:** Hepatocytes treated with 0.1 μM DHEA. **C:** Hepatocytes treated with 1 μM DHEA. **D:** Hepatocytes treated with 10 μM DHEA. The cell cycle was evaluated using flow cytometric analysis, and cell cycle distribution was calculated from 10,000 cells with ModFit LTTM software using FACSCaliber. Data are presented as means ± SE from three individual experiments (n= 12). ** P < 0.01, DHEA treated group vs. control group.

Fig. 4. Effect of DHEA on cell cycle related factors expression level in primary chicken hepatocytes. A: Cyclin A mRNA expression level. **B:** Cyclin B mRNA expression level. **C:** CDK2 mRNA expression level. **D:** Chicken hepatocytes were treated with 0, 0.1, 1 or 10 μM DHEA, and the protein were immunoblotted against Cyclin A and CDK2. **E:** Cyclin A protein expression abundance. F: CDK2 protein expression abundance. Data are presented as means ± SE from three individual experiments (n= 12). *P < 0.05, **P < 0.01, DHEA treated group vs. control group.

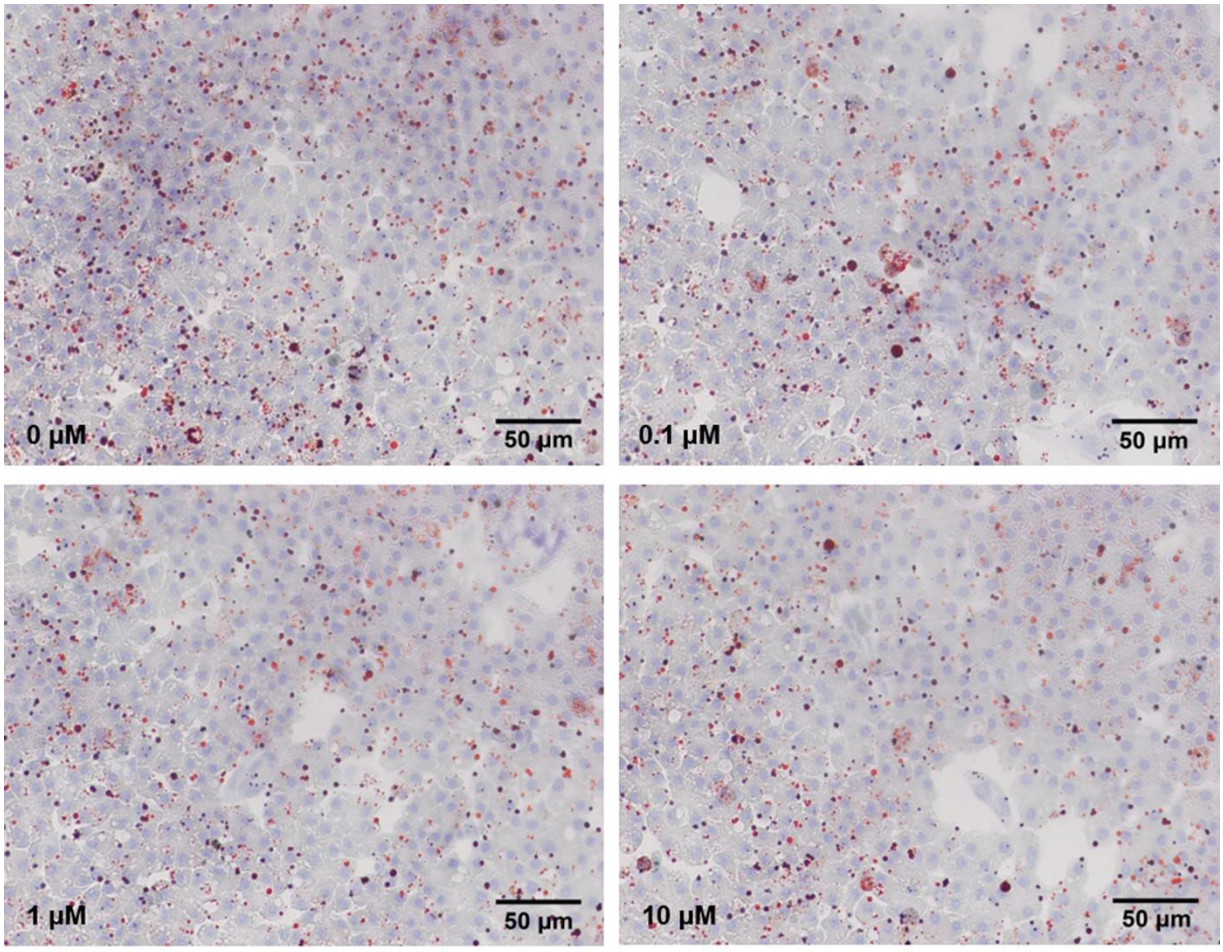
Fig. 5. Electron micrographs and the number of mitochondria in primary chicken hepatocytes treated with DHEA. A: Electron micrographs. B: The number of mitochondria. After incubation, cell samples were processed by standard techniques for transmission electron microscopy, and ultra-thin sections were observed with magnification×2500. Thirty photos were randomly selected from each treatment group, and fifteen independent cells in each photo were counted the number of mitochondria. The results are displayed as the average number of mitochondria per cell in all treatment groups and

presented as means ± SE.

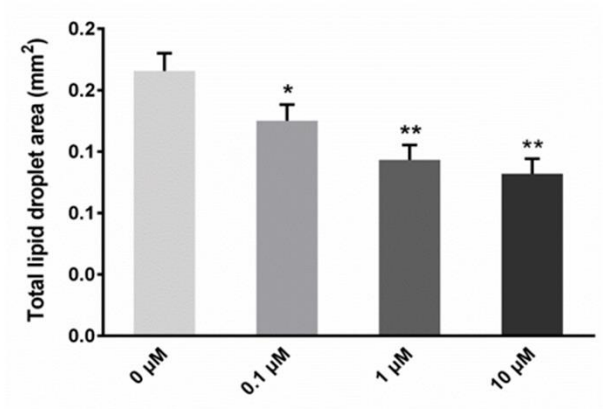
Fig. 6. Effect of DHEA on mitochondrial permeability in primary chicken hepatocytes. A: Typical mitochondrial permeability images from chicken hepatocytes treated with DHEA. **B:** The mitochondrial membrane potential as indicated by the 590/530 nm fluorescence intensity ratio. Data are presented as means ± SE. *P < 0.05, DHEA treated group vs. control group.

Fig. 7. Effect of DHEA on succinate dehydrogenase activity in primary chicken hepatocytes. The activity of succinate dehydrogenase (SDH) was normalized to the sample protein concentration as determined by a protein assay kit, and expressed as U/mg protein. Data are presented as means ± SE from three individual experiments (n= 12). *P < 0.05, **P < 0.01, DHEA treated group vs. control group.

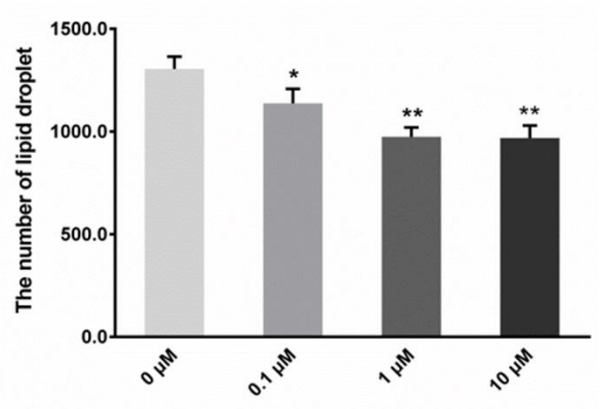
533 **Fig. 1**



A



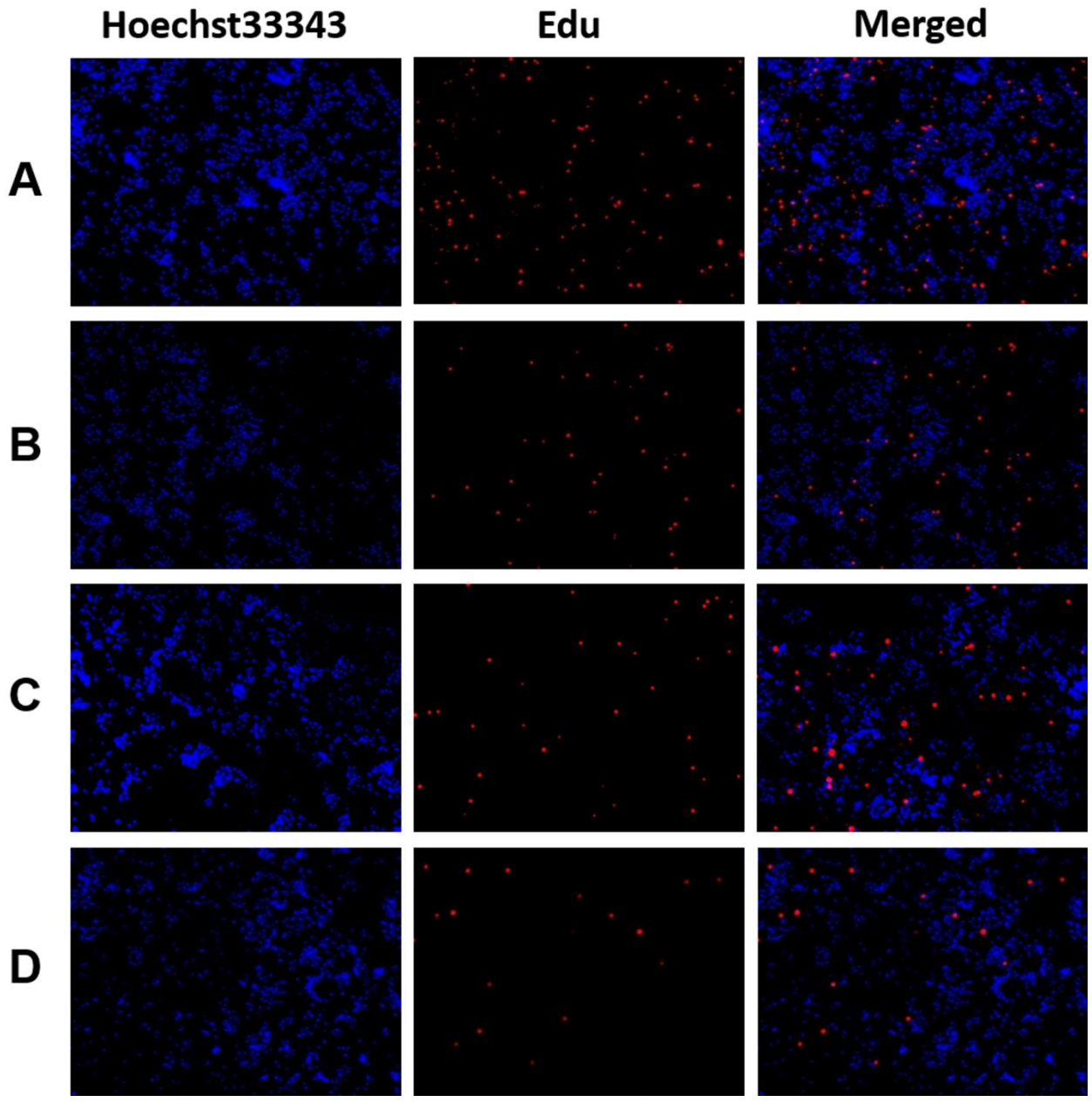
B



C

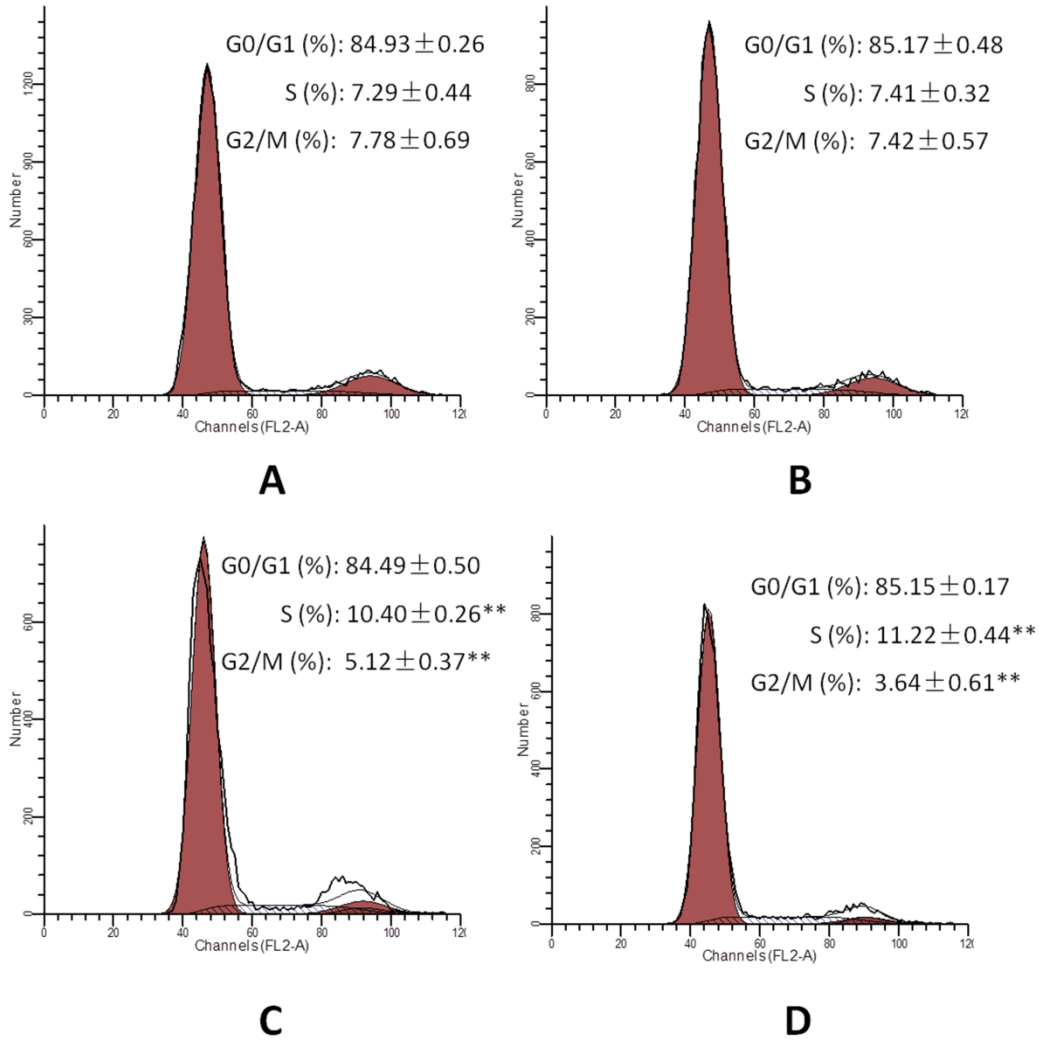
534

535 **Fig. 2**



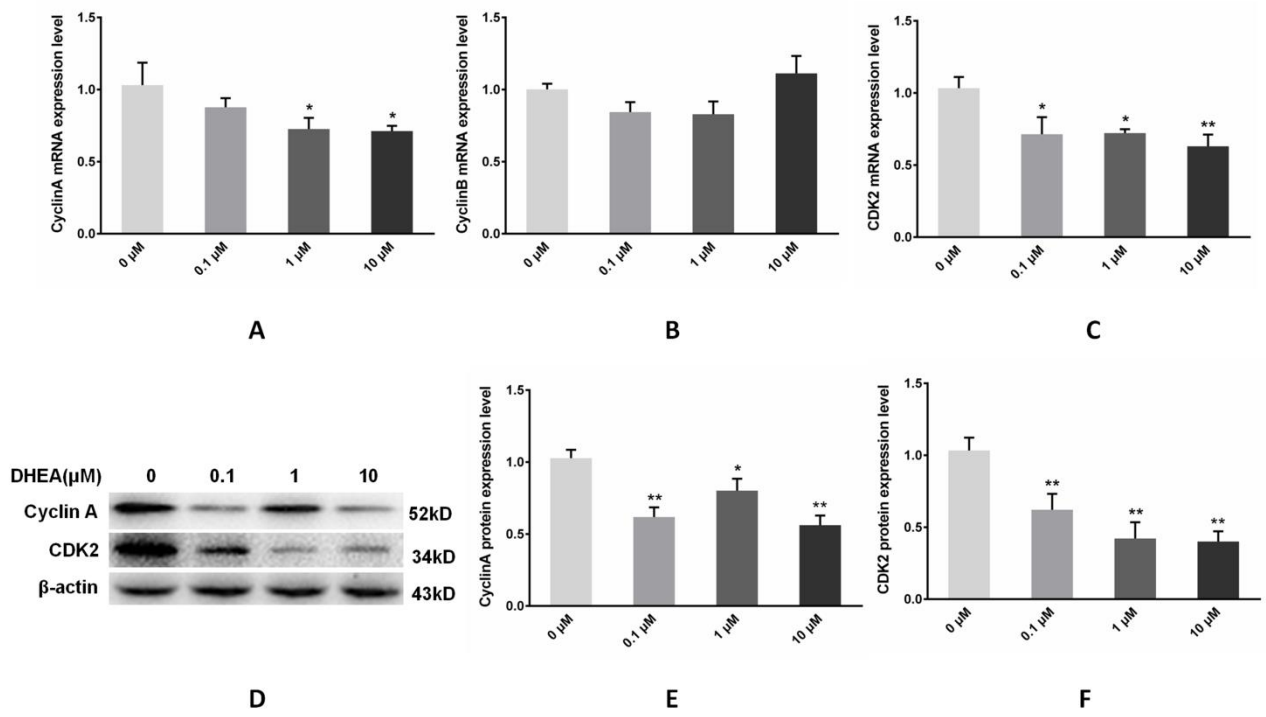
536

537 **Fig. 3**


G0/G1 (%): 84.93±0.26
S (%): 7.29±0.44
G2/M (%): 7.78±0.69
Number
Channels (FL2-A)
A
G0/G1 (%): 85.17±0.48
S (%): 7.41±0.32
G2/M (%): 7.42±0.57
Number
Channels (FL2-A)
B
G0/G1 (%): 84.49±0.50
S (%): 10.40±0.26**
G2/M (%): 5.12±0.37**
Number
Channels (FL2-A)
C
G0/G1 (%): 85.15±0.17
S (%): 11.22±0.44**
G2/M (%): 3.64±0.61**
Number
Channels (FL2-A)
D

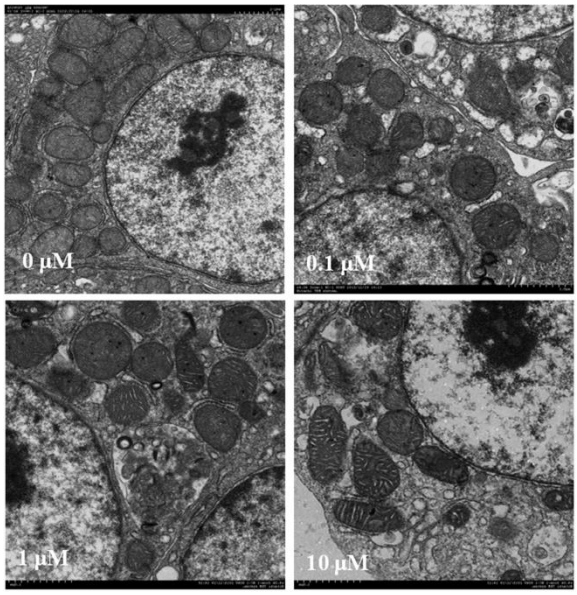
538

539 **Fig. 4**

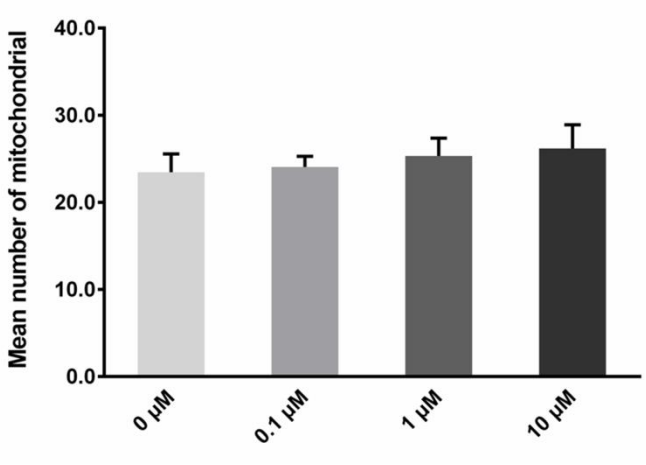

CyclinA mRNA expression level
CyclinB mRNA expression level
CDK2 mRNA expression level
0 μM
0.1 μM
1 μM
10 μM
A
B
C
DHEA(μM) 0 0.1 1 10
Cyclin A
CDK2
β-actin
52kD
34kD
43kD
CyclinA protein expression level
CDK2 protein expression level
D
E
F

540

541 **Fig. 5**



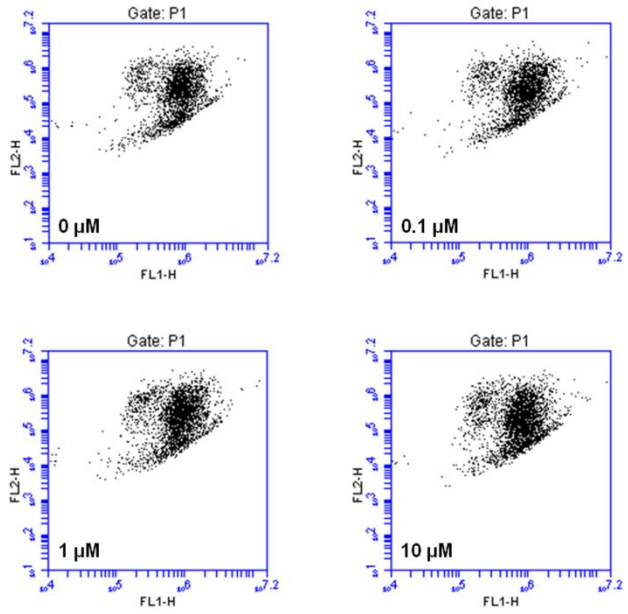
A



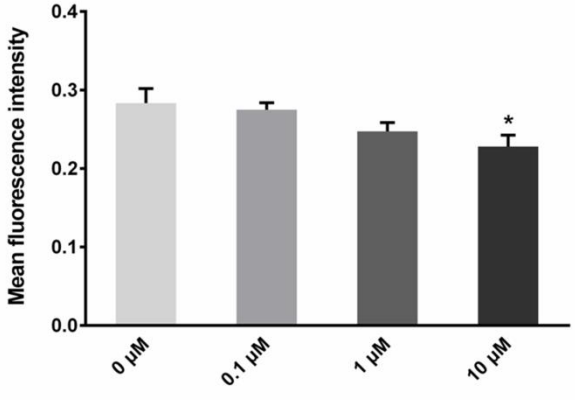
B

542

543 **Fig. 6**



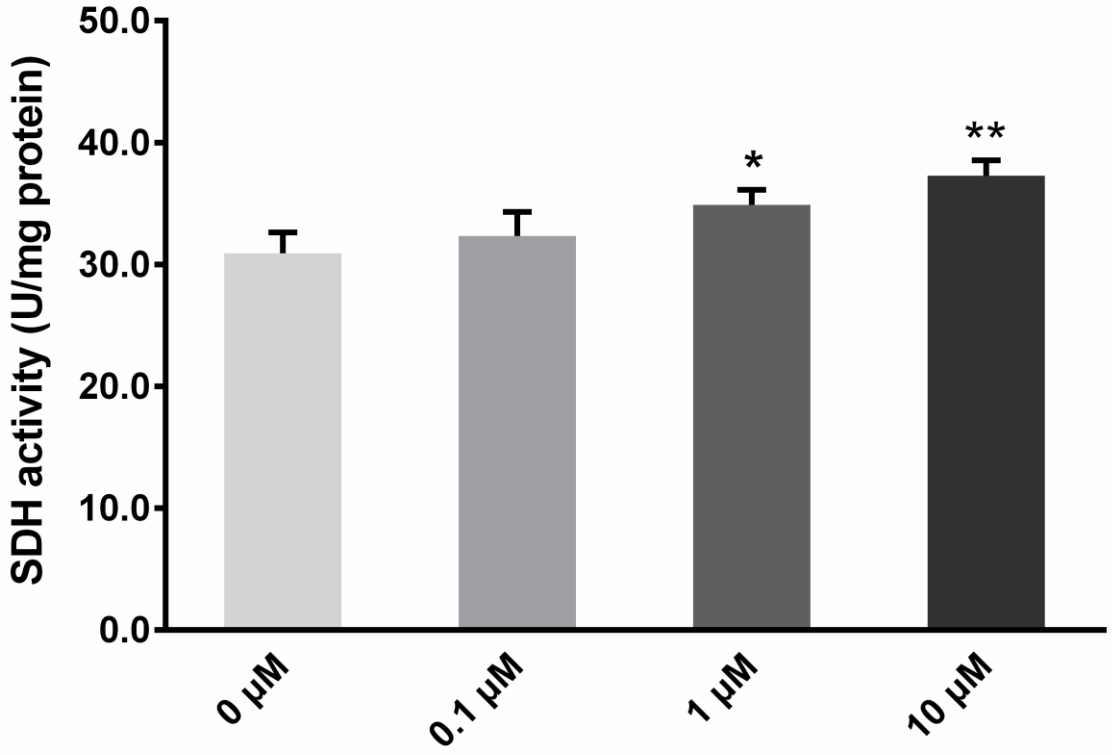
A



B

544

545 **Fig. 7**


SDH activity (U/mg protein)
50.0
40.0
30.0
20.0
10.0
0.0
*
**
0 μM
0.1 μM
1 μM
10 μM

546

547 **Table 1.** Prime sequence of targeted genes and β-actin

Gene	GenBank accession number	Primer sequences (5'–3')	Orientation	Product size (bp)
β-actin	L08165	TGCGTGACATCAAGGAGAAG TGCCAGGGTACATTGTGGTA	Forward Reverse	300
Cyclin A	NM_205244	ATGTCAGCGATATCCACACG GCTCCATCCTCAGAACTTGC	Forward Reverse	363
Cyclin B	NM_001004369	AGGGGTGGAGAATGCCGTGA TGCCAGGTCCTTTCGTAGCCTT	Forward Reverse	162
CDK2	NM_001199857.1	ATGGAGAACTTTCAAAAGGTGGAGA GGCTGTCCCCCACCTGCGCCTGTGA	Forward Reverse	185

548

549 **Table 2.** Effect of DHEA on the cell viability in primary chicken hepatocytes (OD_{490})

Treatment (μM)	Incubation time (h)					
	1	3	6	12	24	48
0	0.401±0.018	0.412±0.018	0.496±0.017	0.525±0.017	0.574±0.015	0.358±0.027
0.1	0.468±0.017	0.553±0.020**	0.566±0.009**	0.616±0.011**	0.655±0.009**	0.558±0.024**
1.0	0.513±0.014**	0.586±0.017**	0.604±0.014**	0.620±0.014**	0.679±0.014**	0.606±0.019**
10.0	0.549±0.012**	0.646±0.011**	0.695±0.020**	0.718±0.017**	0.742±0.013**	0.662±0.014**

550 Data are presented as means ± SE from three individual experiments (n= 12). *P < 0.05, **P < 0.01,

551 DHEA treated group vs. control group.