Dehydroepiandrosterone Reduced Lipid Droplet Accumulation via Inhibiting Cell Proliferation and Improving Mitochondrial Function in Primary Chicken Hepatocytes

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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 the broiler. In the present study, the lipid droplet accumulation, cell proliferation, cell cycle and mitochondrial membrane potential were analysis in primary chicken hepatocytes after DHEA treated. The results showed that total area and counts of lipid droplets were significantly decreased in hepatocytes treated with DHEA. The cell viability was significantly increased, while cell proliferation was significantly inhibited in a dose-dependent manner in primary chicken hepatocytes after DHEA treated. DHEA treatment significantly increased the cell population in S phase and decreased the population in 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 in the number of mitochondria, while the mitochondrial membrane permeability and succinate dehydrogenase (SDH) activity were significantly increased in hepatocytes after DHEA treated. In conclusion, our results demonstrated that DHEA reduced lipid droplet accumulation by inhibiting hepatocytes proliferation and enhancing mitochondrial function in primary chicken hepatocytes.

Key words

Dehydroepiandrosterone • Lipid metabolism • Cell growth • Membrane permeability • Mitochondrial enzyme

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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 biotransformation of DHEA into active steroids 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).

Many studies demonstrated that DHEA has anti-proliferative actions *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

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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 may be a target factor by which DHEA regulate cells proliferation. Rice *et al.* 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 and induces the death 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 there have many studies reported that DHEA could regulate the cell proliferation in various cell types, while the exact mechanism about these physiological actions of DHEA is not fully understood.

Except for regulating metabolism, mitochondria also involved into many other functions which make them important to all cells. Swierczynski *et al*. (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 lipids could promoted permeability of mitochondrial membrane (Maciel *et al*. 2001). Previous study showed that 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). In addition, 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 might regulate the mitochondrial function, while further investigation is needed to validate this action of DHEA in cells.

Interestingly, previous study about biological functions of DHEA were mainly focused on mammalian species, little information is available on poultry, both *in vivo* and *in vitro*. Unlike mammalian species, the liver is the most important organ of fat metabolism in poultry (Griffin *et al*. 1992). Our previous study found that DHEA treatment reduced 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 mainly achieved by activation of cAMP/PKA signaling pathway in primary chicken hepatocytes (Tang *et al*. 2009a, Tang *et al*. 2009b). Importantly, most of the mitochondria in DHEA-treated hepatocytes presented a high electron density based on morphological observations, indicating that DHEA might influence mitochondrial function in primary chicken hepatocytes (Tang *et al*. 2009a). As well known, the expansion of fat mass is due to increased adipocyte hypertrophy and hyperplasia in broiler chickens (Butterwith 1997). 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 metabolism regulation by DHEA in broiler chickens.

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.* (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 (1,000 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 twenty microliter 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 saponins-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 1,000 rpm for 5 min, and the cell pellets were resuspended 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, San Jose, CA, USA) using FACScaliber (Becton Dickinson, San Jose, CA, USA).

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 3 min) 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).

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	Forward	300
		TGCCAGGGTACATTGTGGTA	Reverse	
Cyclin A	NM_205244	ATGTCAGCGATATCCACACG	Forward	363
		GCTCCATCCTCAGAACTTGC	Reverse	
Cyclin B	NM_001004369	AGGGGTGGAGAATGCCGTGA	Forward	162
		TGCCAGGTCCTTTCGTAGCCTT	Reverse	
CDK2	NM_001199857.1	ATGGAGAACTTTCAAAAGGTGGAGA	Forward	185
		GGCTGTCCCCCACCTGCGCCTGTGA	Reverse	

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 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 (1:1,000 dilution). After washing with TBST, goat anti-rabbit IgG with horseradish peroxidase-conjugated (1:5,000 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 1:1,000) 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 3,000 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, USA). 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, hepatocytes viability was significantly increased in the 1 and 10 μM DHEA treated groups at 1-48 h than that of control group ($P<0.01$). Meanwhile, 0.1 μM DHEA treated group significantly increased hepatocytes viability at 3-48 h when compared with the control group ($P<0.01$).

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**

Data are presented as means ± SE from three individual experiments (n=12). * $P<0.05$, ** $P<0.01$, DHEA treated group vs. control group.

Effect of DHEA on lipid droplet accumulation in primary chicken hepatocytes

To analyze the distribution of lipid droplets in primary chicken hepatocytes after DHEA treated, Oil Red O staining (Fig. 1A) was used to analyze the counts and total area of lipid droplets. 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 numbers of lipid droplet also significantly decreased in 0.1-10 μM DHEA treated groups than that of 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 different dose DHEA treatment significantly inhibited hepatocytes proliferation in a dose-dependent manner (Fig. 2).

Effect of DHEA on cell cycle in primary chicken hepatocytes

As shown in Figure 3, no significant differences were observed in the population of G0/G1, S and G2/M phase in hepatocytes treated with 0.1 μM DHEA ($P>0.05$). Compared to 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 ($P<0.01$).

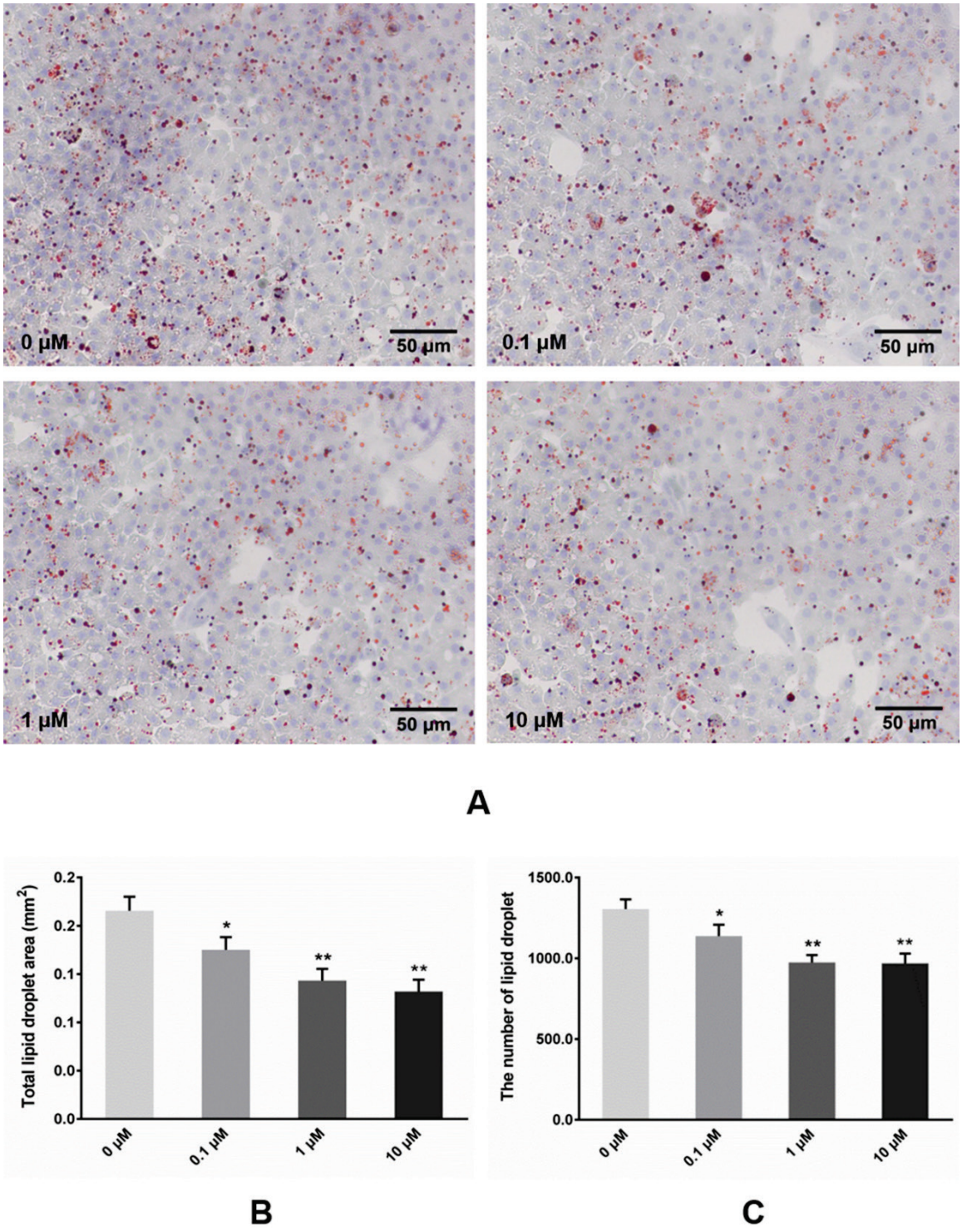

Fig. 1. Effect of DHEA on lipid droplets accumulation in primary chicken hepatocytes. (**A**) Representative photomicrographs of hepatocytes stained with Oil Red O, the red represents lipid droplet and blue represents the cell nucleus. (**B**) Total area of lipid droplets. (**C**) The number of lipid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

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

Compare with control group, the cyclin A mRNA level was significantly decreased in 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 hepatocytes when compared to the control group ($P<0.05$) (Fig. 4C). To further verified these results, we consequently detected the protein expression abundance of cyclin A and CDK2, and the results showed that

0.1-10 μM DHEA treatment significantly decreased the cyclin A and CDK2 protein expression abundance in hepatocytes (P<0.05) (Fig. 4E and 4F).

Morphological observations and quantization of mitochondria

Compared with control group, the histological organization was not obviously altered in hepatocytes treated with 0.1-10 μM DHEA (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 hepatocytes treated with different dose 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 hepatocytes treated with 0.1 or 1 μM DHEA (P>0.05), while 10 μM DHEA treatment significantly decreased the ΔΨm in hepatocytes when compared to the control group (P<0.05) (Fig. 6).

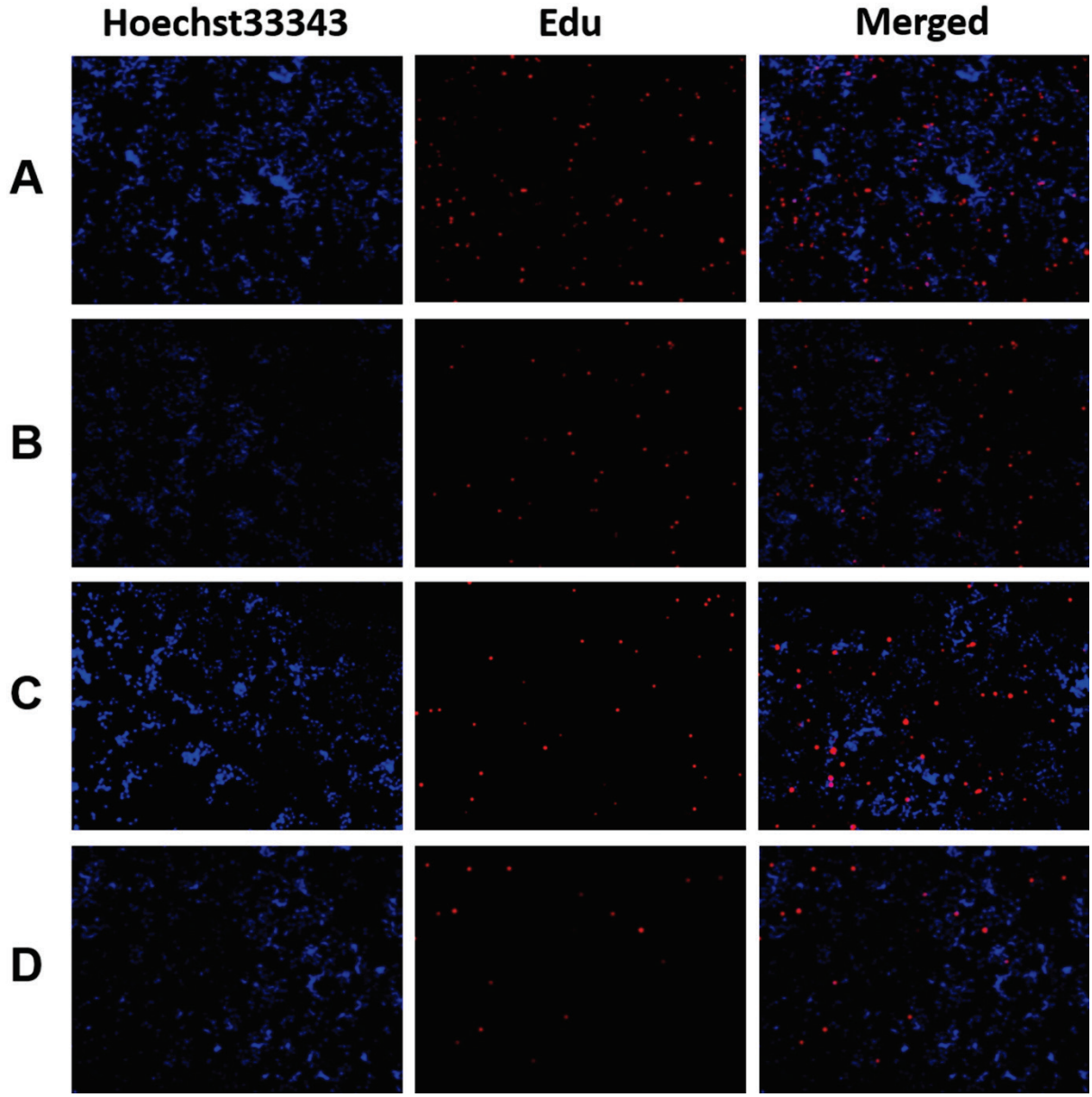

Fig. 2. EdU (5-ethynyl-2′-deoxyuridine) labels cells proliferating in primary chicken hepatocytes. (**A**) Control group. (**B**) Hepatocytes treated with 0.1 μM DHEA. (**C**) Hepatocytes treated with 1 μM DHEA. (**D**) Hepatocytes treated with 10 μM DHEA. The cells in each group were stained with DNA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

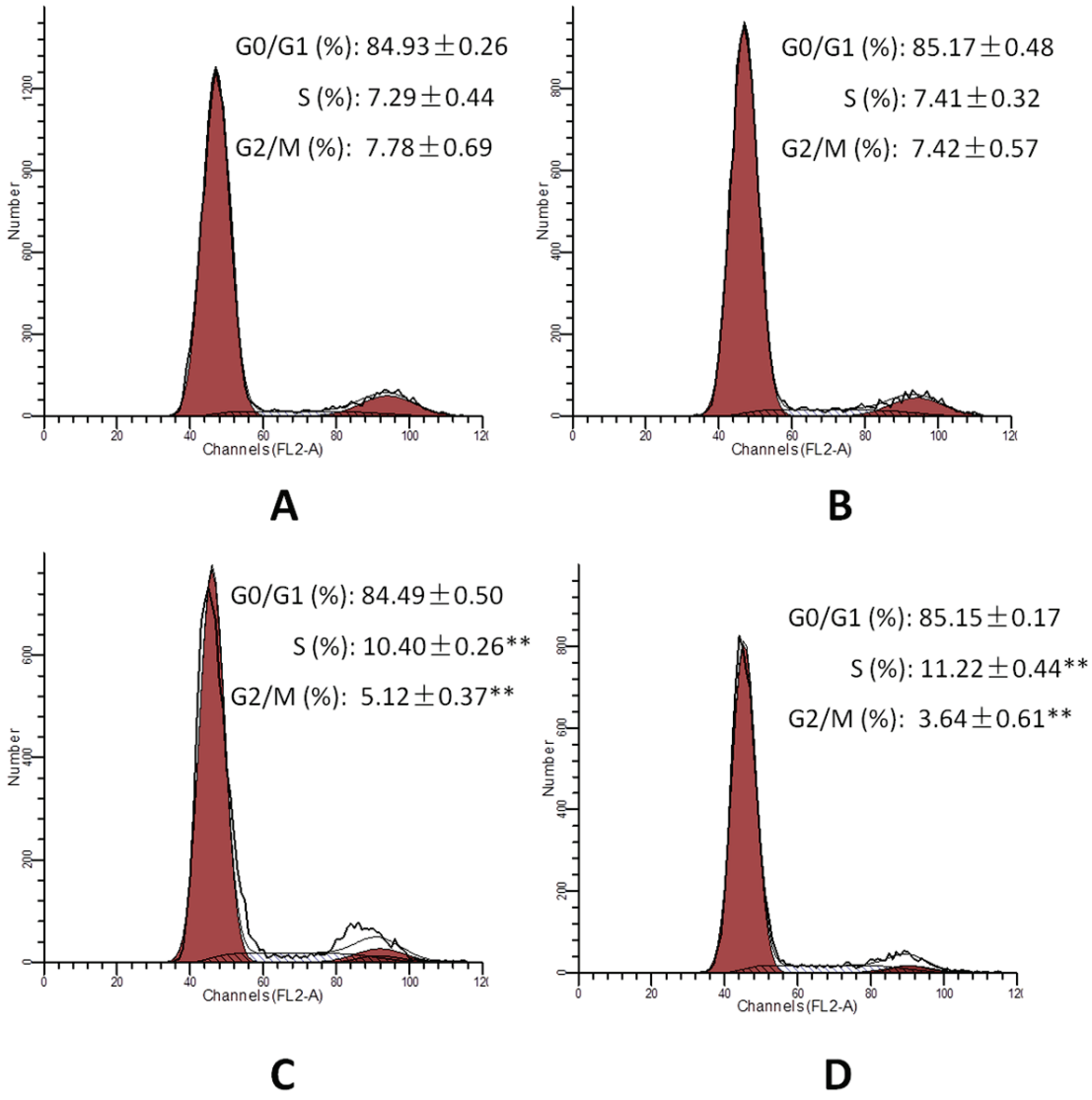

Fig. 3. Effect of DHEA on cell cycle in primary chicken hepatocytes. (**A**) Control group. (**B**) Hepatocytes treated with 0.1 μM DHEA. (**C**) Hepatocytes treated with 1 μM DHEA. (**D**) Hepatocytes treated with 10 μM DHEA. The cell cycle was evaluated using flow cytometric analysis, and cell cycle 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.

Effect of DHEA on succinate dehydrogenase activity in primary chicken hepatocytes

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

Discussion

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 had found that DHEA administration reduced 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 numbers and total area of lipid droplet, which indicated that DHEA reduced fat accumulation in primary chicken hepatocytes. DHEA possesses fat-reducing effect, which may be accomplished through multiple mechanisms (De Pergola 2000). The adipocyte hypertrophy and hyperplasia are the main forms of fat deposition in broiler chickens (Butterwith 1997). Therefore, we subsequently investigated the effect of DHEA on cell proliferation and mitochondrial function in primary chicken hepatocytes.

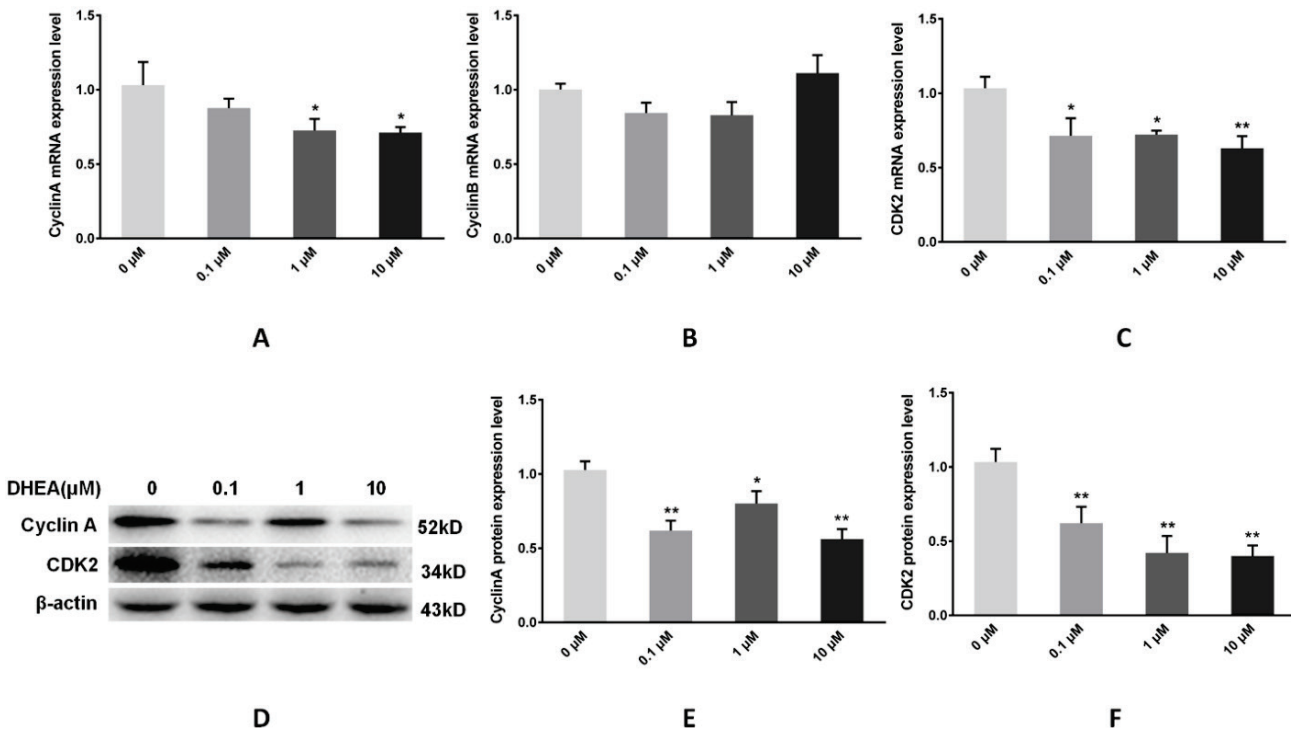

Fig. 4. Effect of DHEA on cell cycle related factors expression level in primary chicken hepatocytes. (**A**) Cyclin A mRNA expression level. (**B**) Cyclin B mRNA expression level. (**C**) CDK2 mRNA expression level. (**D**) Chicken hepatocytes were treated with 0, 0.1, 1 or 10 μM DHEA, and the protein 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.

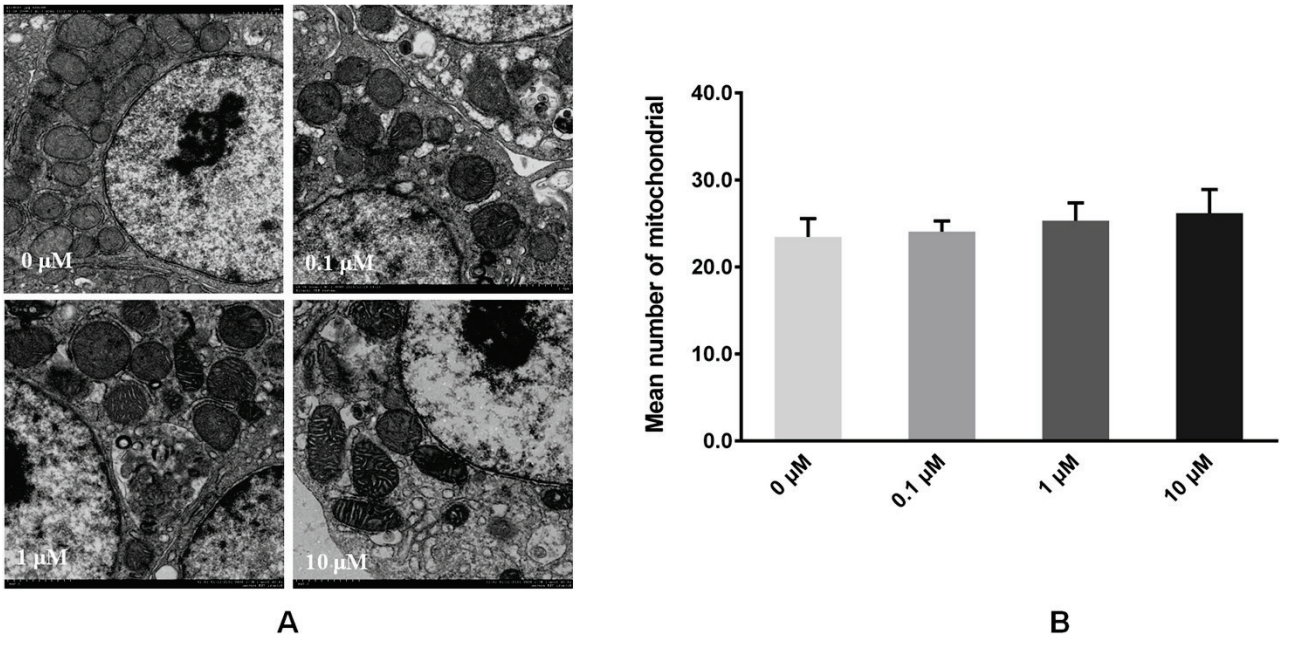

Fig. 5. Electron micrographs and the number of mitochondria in primary chicken hepatocytes treated with DHEA. (**A**) Electron micrographs. (**B**) The number of mitochondria. After incubation, cell samples were processed by standard techniques for transmission electron microscopy, and ultra-thin sections were observed with magnification ×2,500. 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.

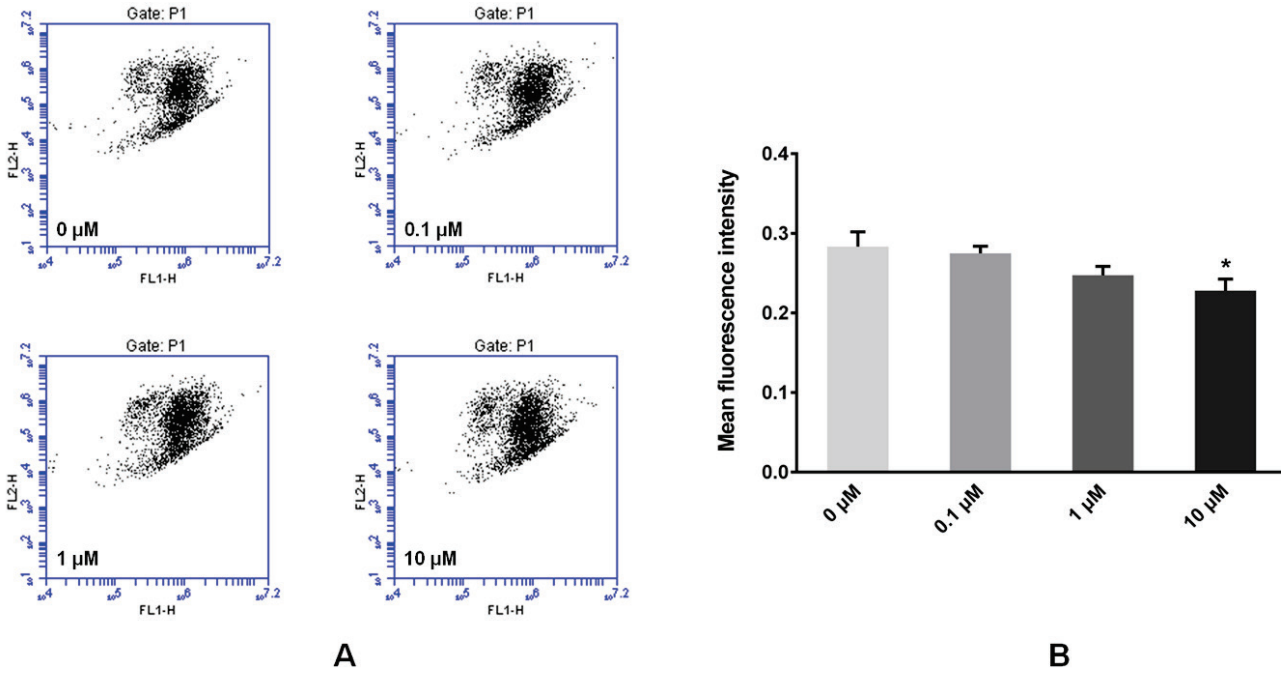

Fig. 6. Effect of DHEA on mitochondrial permeability in primary chicken hepatocytes. (**A**) Typical mitochondrial permeability images from chicken hepatocytes treated with DHEA. (**B**) The mitochondrial membrane potential as indicated by the 590/530 nm fluorescence intensity ratio. Data are presented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

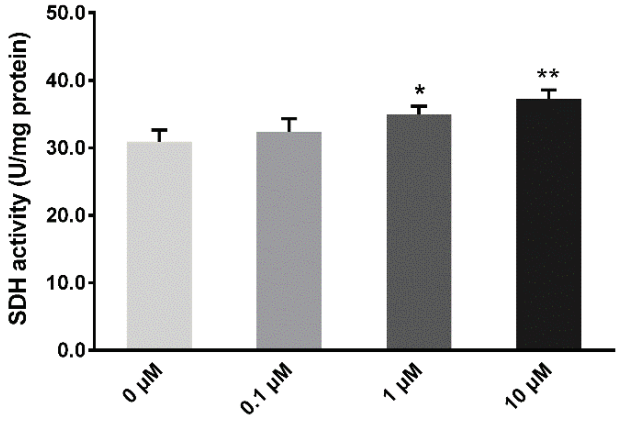

Fig. 7. Effect of DHEA on succinate dehydrogenase activity in primary chicken hepatocytes. The activity of succinate dehydrogenase (SDH) was normalized to the sample protein concentration as determined by a protein assay kit, and expressed as U/mg protein. Data are presented as means ± SE from three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

Although there had 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 hepatocytes proliferation in a dose-dependent manner, and this result was consistent with the previous report that DHEA modulates growth factor-induced proliferation in an age-dependent manner in primary bovine chromaffin cells (Sicard *et al*. 2007). Evidence suggests that the inhibitory effect of DHEA on cell proliferation is associated with the changes in the phases of the cell cycle (Rice *et al*. 2010). Our results showed that DHEA increased the S phase cell population and decreased the G2/M population in hepatocytes, which implied that DHEA inhibits hepatocytes proliferation and causes cell cycle arrest in S phase. In addition, DHEA treatment decreased the cyclin A and CDK2 protein expression abundance in hepatocytes. In eukaryotes, the cell cycle is regulated by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDK1). It had 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 hepatocytes proliferation by decreasing the cyclin A and CDK2 protein expression abundance, 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 found that DHEA caused a significant up-regulation of PPARα mRNA expression in primary chicken hepatocytes (Tang *et al*. 2007). It had reported that DHEA

increased PPARα expression in hepatocytes, and which increased the rate of β-oxidation and decelerated the transport of acetyl-CoA from the mitochondria to the cytosol (Schoonjans *et al*. 1996). Mitochondria are associated with cellular energy metabolism and provided 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 altered 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 was measured the capacity of NAD(P)H-dependent cellular oxidoreductase enzymes to transform MTT into formazan in living cells (Ferrari *et al*. 1990). The mitochondrial membrane may play an important role in blocking the MTT entrance into the mitochondria. The mitochondrial membrane potential (ΔΨm) was significantly decreased, which indicated that DHEA increased the mitochondrial membrane permeability in chicken hepatocytes. Previous study found that high concentration of DHEA decreased the ΔΨm 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 had 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 this study. Our results showed that DHEA significantly increased the SDH activity in chicken hepatocytes. Previous study showed that DHEA inhibited complex I of the mitochondrial respiratory chain *in vitro* and *in vivo* (Safiulina *et al*. 2000, 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 had reported that DHEA primarily affects fatty acid mobilization, resulting in enhanced mitochondrial respiration in chicken (Tang *et al*. 2009a). In addition, our recent research shows 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. Certainly, further deeply investigation is required to validate this mechanism more precisely in primary chicken hepatocytes.

Conflict of Interest

There is no conflict of interest.

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