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The Effect of High Temperature on Viability, Proliferation, Apoptosis and Anti-oxidant Status of Chicken Embryonic Fibroblast Cells

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ABSTRACT

The effects of oxidative stress induced by high temperature on the cell viability, proliferation, apoptosis and oxidative status of chicken embryonic fibroblasts (CEF) were analyzed. The viability, proliferation, apoptotic and anti-oxidative status were measured after incubating CEF at the temperatures of 37ºC (control) and 40-44ºC (experimental groups) for 6,12 and 24 hours. The results showed that at high temperature (42-43ºC), the viability of CEF cells decreased after 6, 12 and 24 h of incubation, but the difference was significant only at 43ºC. Cell proliferation was significantly reduced at 44ºC/6h. The apoptotic rate of CEF cells was increased following heat treatments in a time-dependent manner. ROS formation increased with increasing temperature, but the difference was only significant at 44ºC/6,12h. Heat stress did not significantly affect the superoxide dismutase (SOD) activity. CAT activity was significantly decreased at 43ºC/24h and 44ºC/12 and 24h. Malondialdehyde (MDA) formation was significantly increased at 43ºC/12h and 44ºC/12 and 24h. In conclusion, heat stress induced the oxidative stress, decreasing the viability, proliferation and anti-oxidative response of CEF cells.

INTRODUCTION

Livestock are exposed to numerous types of stressors which disrupts their reproduction, production, and health. The continuously increasing ambient temperature is one of the most damaging stressors for livestock because of its detrimental effects, especially in poultry sector. In arid and tropical regions heat stress is considered as key because it directly and indirectly affects animal production and health.

In the poultry sector, high temperatures may significantly damage meat and egg production. Temperature has been suggested to be the most important factor controlling the embryonic and post-hatch development of chicks. Moreover, the negative effects of heat stress on animal growth performance also have been reported in the tropical and subtropical regions of the world. Reduced feed intake, daily gain, body weight, and growth rate were reported in broilers (Sohail *et al.*, 2010). The reduction of feed intake of animals under heat stress is usually believed to be the primary reason for low production performance. However, it has lately been shown that high temperate change the steady state concentrations of free radicals, causing both mitochondrial and cellular oxidative damage.

High environmental temperature (physical stressor) challenges the homeostatic system and stimulates the excessive production of reactive oxygen species (ROS), which is a chemical stressor (Ibtisham *et al.*, 2016). ROS are chemically-reactive molecules containing oxygen and are natural byproducts of the normal oxygen metabolism (Abdel-

Moneim *et al.*, 2015). At normal physiological levels, ROS play an important role in hemostasis and cell signaling (Liu *et al.*, 2015), but at high levels, ROS may promote apoptosis (Sinha *et al.*, 2013). ROS cause irreversible damage to molecules as they enhance lipid peroxidation and damage protein and DNA, increasing the production of malondialdehyde (MDA) and thiobarbituric acid-reactive species (TBARS). MDA and TBARS can significantly damage cell structures(Di Meo *et al.*, 2016). High environmental temperatures were shown to increase both of MDA and TBARS levels in broilers (Mujahid *et al.*, 2005). Superoxide is the main ROS produced by mitochondria as an outcome of the reaction with oxygen molecules presenting unpaired electrons (Zolkipli-Cunningham & Falk, 2017). In general, cells maintain a balance between ROS formation and elimination, whereas imbalances lead to oxidative stress. Under oxidative stress, the body protects itself against damages caused by ROS through enzymes, including catalases, lactoperoxidases, superoxide dismutases (SODs), glutathione peroxidases and peroxiredoxins (Michiels *et al.*, 1994). Antioxidant enzyme activities are enhanced as a result of increasing ROS levels in order to maintain the steady state concentrations of generated free radicals. Furthermore, studies showed that concentrations of endogenous antioxidants, such as catalase and glutathione, decrease when environmental temperature is high (Kumar *et al.*, 2011). This is due to the mobilization of cellular antioxidants to detoxify free radicals produced by high temperature. SOD transfer the superoxide produced as the key ROS into hydrogen peroxide and subsequently, glutathione peroxidase and catalase detoxify it. Avian species have comparatively lower ROS loads then mammals (Lu & Finkel, 2008), which explains the longer life span potential of most bird species compared with mammals with similar body weight (Barja, 1998).

Hyperthermia is cytotoxic, because it modifies metabolic reactions and biological molecules, causing oxidative cell damage and triggering both apoptosis and necrosis pathways. Hyperthermia upregulates ROS production, which subsequently triggers the fundamental pathway of the apoptosis progression, which is a mitochondrial pathway based on cytochrome c liberation from the outer mitochondrial membrane (Du *et al.*, 2008). Indeed, several researchers have demonstrated that heat stress triggers cell necrosis (Shimizu *et al.*, 1996).

Both chronic and acute heat stress cause different effects on metabolism and production. Researchers involved in studies on hyperthermia are trying to produce special animal breeds either via genomic selection (Ibtisham *et al.*, 2017) or by determining the molecular and cellular responses of animals under heat stress condition, and are also trying to explore how animals can be managed in hot environments. This means that being able to better characterize the cell response by different heat stress in terms of duration or intensity is of great interest. The study of these fundamental molecular and cellular routes may provide methods to select high temperature-tolerant animals with great production potential. Among the various biological tools that have been used to study the stress response, avian cells grown in tissue culture have shown to be very valuable. Chicken embryo fibroblast (CEF) cells are commonly used in embryology and differentiation studies, as well as in vaccine production (Jeon *et al.*, 2016). The objective of the current study was to determine the effects of high temperature on the proliferation, apoptosis and antioxidant status of CEF.

MATERIALS AND METHODS

Materials

HEPES and dimethyl sulfoxide (DMSO), MTT and Cell Counting Kit (CCK-8) were purchased from Sigma Aldrich Chemicals (St. Louis, MO, USA). Phosphate-Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin were purchased from Gibco, Invitrogen (Carlsbad, CA, USA). Catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA) and reactive-oxygen species (ROS) kits were purchased from Nanjing Institute of Bioengineering, Nanjing, China. All other chemicals used were of high purity biochemistry grade.

Cell Culture

Primary CEF cells were prepared using nine-day old embryos isolated from Lohmann chicken embryos. The cells were trypsinized and collected by centrifugation. Primary CEF cells and land continuous CEF cell line were cultured in DMEM with 10%FBS at 37°C in a humidified incubator under 5% CO_2 and 95%air. Upon confluence, the cells were purified via serial passages. Experimental cells were used in exponential phase and from passages 3–6.

Cultured CEF cells plates were divided into six groups (four replicates each), including a control group (NC) and five experimental groups (H1, H2, H3, H4, and H5). Experimental plates were moved to a

separate incubator (95% air/5% CO_2) at the desired experimental temperature (40°, 41°, 42°, 43° and 44°, respectively), whereas NC group plates remained at 37°C.

Cell Viability and Proliferation Assay

For the measurement of cell viability and proliferation, CEF cells were cultured in 96-well culture plates at a density of 10^4 cells/cm^2, with the heat stress experimental plates being transferred to the variable temperature cell culture incubator following 24 h of attachment. After 6, 12 and 24 h of heat stress treatment, cells were separately incubated in medium containing 1.25mg/mL of the 10ul MTT salt, and 10μL CCK-8, for 4hoursand 2 hours, respectively, at 37°C. After an incubation period of 4 hours at 37°C, formazan was solubilized by adding DMSO. The optical densities were measured at 490 nm (Wei *et al.*, 2012).

Analysis of Apoptosis by Annexin V-FITC/ Propidium Iodide Staining

Samples were labeled with annexin V-fluorescein isothiocyanate (FITC; BD Biosciences, San Diego, CA) and propidium iodide (PI) double staining (Zhang *et al.*, 1997) according to the manufacturer's instructions. Briefly, CEF cells were cultured in 96-well culture plates at a density of 10^4 cells /cm^2, with the temperature experimental plates being transferred to the variable temperature cell culture incubator following 24 h of attachment. After 6, 12 and 24 h of heat stress treatment, cells were incubated in 500 μL of binding buffer with AnnexinV-FITC/PI in dark for 20 min, and following samples were analyzed by fluorescence microscope.

Antioxidant Activity Measurement

After the heat stress treatment, CEF cells were washed twice with PBS. Cells were then incubated with 0.2% tritonx-100, at 4° for 30min. Cells were ruptured with ultrasonic cell oscillators and SOD activity, CAT activity, MAD and ROS content were measured using respective assay kits obtained from Nanjing Jiancheng Bioengineering Institute (strictly according to kit manufacturer protocol).

Statistical Analysis

Data were analyzed using the software SPSS (version 20.0) (Chen *et al.*, 2017) and Duncan's multiple range test was applied to test the difference among groups. Differences were considered significant when $p<0.05$. All the results are expressed as means ± standard error.

RESULTS

Effect of Different Temperatures on CEF Cell Viability

The viability of CEF cells was measured at 6, 12, and 24 h after heat stress (40, 41, 42, 43 or 44°C) and control (37°C) treatments. Table1 lists the effect of heat stress on the viability of CEF cells. After 6 h of heat stress, cell viability of H1, H4 and H5 treatments were reduced compared with the NC, but the difference was not significant. After 6 h of heat stress treatment, H3 presented significantly lower number of viable cells compared with NC. Comparing cell groups at 12 h, the viability of H3 and H5 cells were significantly lower compared with NC. At 24 h of treatment, only H5 group had significantly lower number of viable cells compared to NC group. H2 cells presented significantly higher viability compared with NC at 6,12 and 24 h of treatment.

Table1 – Effect of different temperatures on the viability of chicken embryonic fibroblasts.

Parameter	Duration (h)	NC	H1	H2	H3	H4	H5
MTT (OD)	6	0.210±0.018	0.201±0.009	0.272±0.007*	0.179±0.009*	0.182±0.012	0.191±0.009
	12	0.205±0.018	0.195±0.030	0.255±0.041*	0.181±0.017*	0.197±0.020	0.176±0.012*
	24	0.187±0.058	0.195±0.011	0.215±0.015*	0.152±0.027	0.167±0.027	0.119±0.013*

*Indicates significant differences among treatments ($p<0.05$)

Effect of Different Temperatures on CEF Cell Proliferation

The effects of heat stress on the proliferation of CEF cells are shown in Table 2. Cell proliferation was examined using Cell Counting Kit-8 at the time points of 6, 12and 24h after heat stress. After 6 h of heat stress, the proliferation ofH2 group cells was significantly higher and of H5 cells was significantly lower compared with NC cells. By 12 h of treatment, no statistical differences in proliferation were detected among treatments. The H5 group exhibited reduced proliferation compared to NC, but the difference was not significant. At 24 h, the proliferation of H1, H3, H4, and H5 cells was not statistically different from NC cells. The H2 group showed significantly higher proliferation compared with NC at 24 h.

Table2 – Effect of different temperatures on the proliferation of chicken embryonic fibroblasts.

Parameter	Duration (h)	NC	H1	H2	H3	H4	H5
CCK8 (OD)	6	0.177±0.010	0.215±0.57	0.238±0.66*	0.214±0.031	0.187±0.031	0.128±0.031*
	12	0.225±0.032	0.226±0.40	0.259±0.067	0.235±0.023	0.233±0.017	0.214±0.018
	24	0.185±0.023	0.178±0.39	0.215±0.36*	0.177±0.014	0.171±0.012	0.163±0.012

*Indicates significant differences among treatments ($p<0.05$)

Effect of Different Temperatures on ROS Production

In order to evaluate heat stress-induced cell toxicity, intracellular ROS generation using a ROS kit (Nanjing Jiancheng Bioengineering Institute, China) was analyzed. Table 3 lists the effect of heat stress on ROS production. At 6 h, H1 and H2 group showed lower ROS content compared to NC, but the difference was not significant. H3, H4, and H5 groups presented higher ROS content compared with NC, but the difference was not significant. By 12 h of heat treatment, the H2 group showed lower ROS level compared to NC. H3, H4, and H5 had a higher level of ROS content compared to NC, but only H5 showed a significant difference. At 24h, H2, H3, H4, and H5 exhibited a higher level of ROS compared to NC, but the difference was only significant for the H4 and H5 groups.

Table 3 – Effect of different temperatures on ROS content of chicken embryonic fibroblasts.

Parameter	Duration (h)	NC	H1	H2	H3	H4	H5
ROS	6	63.91±8.64	60.48±7.36	62.91±4.50	67.00±4.05	70.91±8.50	73.48±20.36
	12	95.22±5.28	95.59±7.40	90.84±6.54	105.54±10.92	112.84±6.54	120.59±7.40*
	24	130.91±4.22	129.48±7.36	134.99±6.64	156.48±10.36	167.01±8.64*	189.18±9.06*

*Indicates significant differences among treatments ($p<0.05$)

Effect of Different Temperatures on CEF Cell Apoptosis

In order to examine the effect of heat stress on CEF cells apoptosis in vitro, Annexin V-FITC (green)/ Propidium Iodide (red) staining was performed on CEF cells exposed to the different temperature (37, 40-45ºC) at 6, 12 and 24h. The apoptosis of CEF cells submitted to heat stress increased in a time-dependent manner (Figure 1A, 1B and 1C). H1 and H2 groups showed lower numbers of apoptotic cells compared to H3, H4, and H5.

Effect of Different Temperatures on Anti-oxidant Status

The effects of heat stress on CAT, SOD activity, and MDA content are shown in Table 4. At 6 h, H2 and H3 group showed improved CAT activity, while H4 and H5, reduced activity. At 12 h, H5 showed significantly decreased CAT activity compared with NC. At 24 h, H1 and H2 presented higher CAT activity, while H4 and H5 showed significantly lower activity compared with NC. Heat stress did not significantly affect SOD activity. At 6 h, MDA content was lower in H1 and H2, and significantly higher in H5 group compared with NC. By 12 h, H4 and H5 had significantly increased MDA content compared to NC.

DISCUSSION

Hyperthermia is considered as key threat to animal health and it has been shown to have a significant economic impact on the poultry industry,

Table 4 – Effect of different temperatures on antioxidant activity of chicken embryonic fibroblasts.

Parameter	Duration (h)	NC	H1	H2	H3	H4	H5
CAT (U/mL)	6	18.91±1.64	18.48±2.36	20.91±2.50	19.00±1.05	16.91±2.50	13.48±4.36
	12	35.22±3.24	38.59±4.40	37.84±3.54	35.54±4.92	32.84±2.54	19.59±6.40*
	24	53.91±4.22	60.48±7.36	58.99±6.64	50.48±10.36	44.01±8.64*	20.18±8.26*
SOD (U/mL)	6	63.91±8.64	60.48±7.36	62.91±4.50	67.00±4.05	60.91±8.50	56.48±20.36
	12	75.22±5.28	75.59±7.40	80.84±6.54	72.54±10.92	74.84±6.54	70.59±7.40
	24	80.91±4.22	79.48±7.36	84.99±6.64	76.48±10.36	67.01±8.64	69.18±9.06
MDA (nmol/mL)	6	0.35±0.11	0.33±0.10	0.30±0.09	0.36±0.10	0.49±0.16	0.57±0.15*
	12	0.75±0.11	0.68±0.14	0.70 ±0.19	0.78±0.15	0.90 ±0.19*	0.92 ±0.12*
	24	1.35±0.11	1.36±0.14	1.28 ±0.19	1.38±0.15	1.40 ±0.19	1.34 ±0.12

*Indicates significant differences among treatments ($p<0.05$)

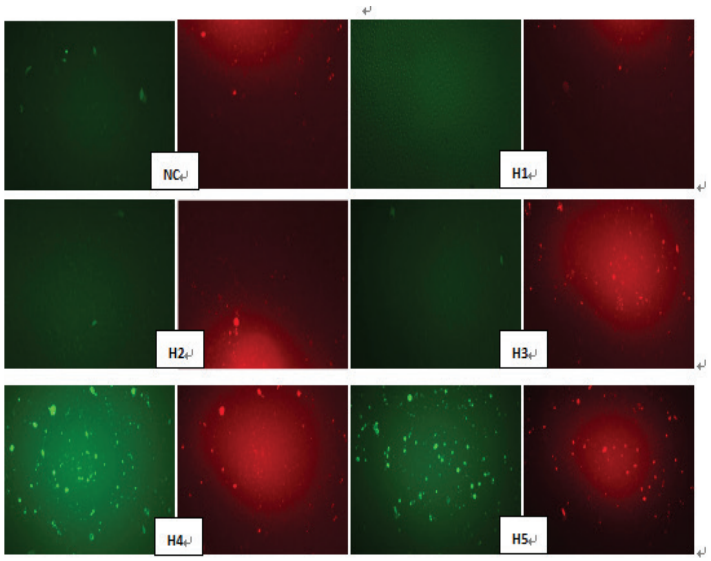

Figure 1 A – Effects of different temperature treatments on the apoptosis of chicken embryonic fibroblasts measure at 6 h(Annexin V-FITC -green/Propidium Iodide-red)

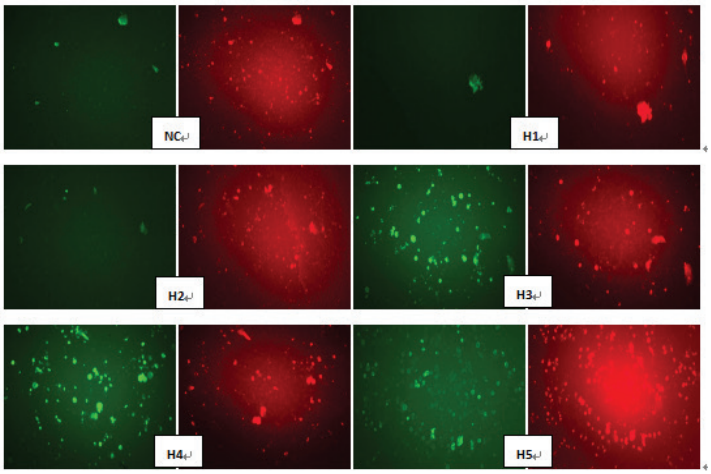

Figure 1 B – Effects of different temperature treatments on apoptosis of chicken embryonic fibroblasts cells at 12 h (Annexin V-FITC -green/Propidium Iodide-red)

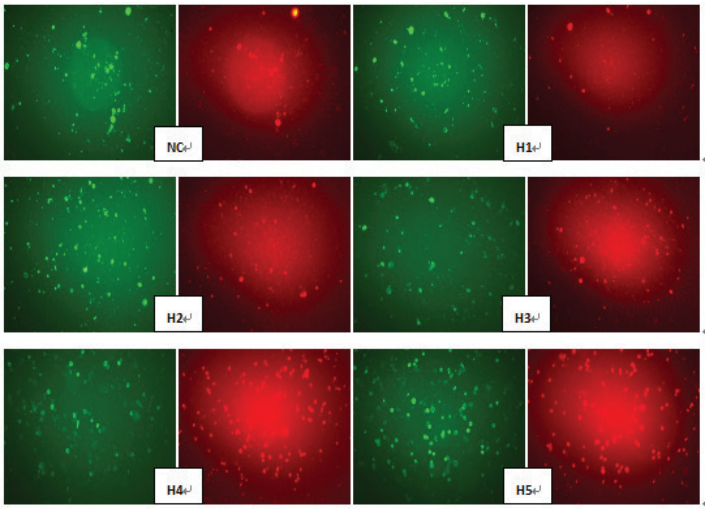

Figure 1 C – Effects of different temperature treatments on apoptosis of chicken embryonic fibroblasts cells at 24 h (Annexin V-FITC -green/Propidium Iodide-red)

and the losses are estimated to increase due to the rising environmental temperature around the globe. However, little is known about physiological response of chickens under heat stress. Therefore, the purpose of this study was to advance our understanding of cellular responses that may compromise the performance of chickens during heat stress. Collectively, the obtained results show that high temperatures induce oxidative stress, resulting in lower cell viability and proliferation and reduced anti-oxidative response of CEF cells.

Heat stress is physical stressor that may induce structural changes in proteins, leading to cell death. In the current study, heat stress-induced cytotoxicity was assessed using MTT, which confirmed that cell viability was greatly decreased under heat stress. The damage caused by the different temperatures was time-dependent. Although at 6 h of heat stress the viability of the CEF cells was not severely affected, the H2 group presented significantly higher cell viability compared with NC. At 12 and 24 h, H2 had significantly higher cell viability, while H5 showed significant lower cell viability compared with NC. This reduced cell viability as temperature increased temperate is in agreement with a previous study on the effect of heat stress on avian cells (Harding *et al.*, 2016), human mesenchymal stromal cells (Reissis *et al.*, 2013), mammalian epithelial cells (Du *et al.*, 2008), and fish hepatic cells (Cui *et al.*, 2013). Our results showed that like viability, the proliferation of CEF was also decreased when cells were maintained at high temperature for 24 h. Higher CEF proliferation was observed in the experimental groups H1, H2, H3, and H4 at 6 and 12 h. The results presented in this study provide the evidence of the beneficial effects of mild heat shock for a short duration on the viability and proliferation of CEF. This type of beneficial effects were also previously reported in human mesenchymal stromal cells (Choudhery *et al.*, 2015), where mild heat shock enhanced proliferation. Such results are useful for the development of safe strategies for the maintenance of cell potential when expansion is required for cell-based therapies.

Homeostasis is continually challenged by intrinsic and extrinsic adverse forces or stressors. A stressful condition can stimulate ROS production and induce oxidative stress in cells, triggering protein and DNA oxidative damage as well as lipid peroxidation (Lin, Decuypere and Buyse, 2006).ROS has been associated with the number of pathological conditions, including infertility, bone disorders, ischemic/reperfusion injury, rheumatoid arthritis, cataract, diabetic nephropathy, neurological disorders and

aging by means of lipid peroxidation (Jin *et al.*, 2011). In the present study, ROS level increased with high temperature (42-44°C) and the CEF group exposed to 44°C presented significantly higher ROS level at 24 h. Previous studies reported that high temperature induces ROS production in fibroblasts (Grasso *et al.*, 2003), platelets (Wang *et al.*, 2013), leukemia cells (Katschinski *et al.*, 2000), skeletal muscle cells (Mujahid *et al.*, 2005) and diaphragm muscle cells (Zuo *et al.*, 2000). Mitochondrial injury is considered as an attractive source for the excessive production of ROS, which can promote apoptotic signaling (Orrenius, Gogvadze and Zhivotovsky, 2007). As temperature increases the frequency of biological and numerous chemical reactions increase, resulting in elevated body temperature, which in turn increases ROS production by enhancing metabolic reactions in cells. Hence, it is proposed that the upregulated production of ROS and the subsequent oxidative stress are partially responsible for heat-induced cellular damage. In the present study, we also found, as temperature increased, more ROS was produced, leading to higher apoptosis of CEF cells. The apoptotic cell ratio of CEF cells increased with heat treatments in a time-dependent manner. The heat treatment at 43°C for 6h did not increase apoptosis compared with the control group. Annexin V-FITC/Propidium Iodide staining showed that the number of apoptotic cells increased as temperature and the duration of heat stress increase. Several conditions, molecules, and organelles may be involved in apoptosis and ROS may play a key role in apoptosis (Wang *et al.*, 2008). Our finding are in agreement with previous studies reporting that heat stress induced apoptosis in chicken myocardial cells (Xu *et al.*, 2017), human umbilical vein endothelial cells (Li *et al.*, 2015)hepatocytes and hepatocellular carcinoma cells (Thompson *et al.*, 2014), and rat germ cells (Lizama *et al.*, 2009).

Under normal physiological states, the balance between ROS production and destruction is well regulated in cell metabolism. ROS is promptly removed by antioxidant enzymes, such as CAT, SOD, and non-enzymatic defenses, such as tocopherol, amino acids, and vitamins E, K, and C (Cheng *et al.*, 2015). When ROS is excessively produced under stress conditions, antioxidant defense systems play an impotent role to protect cells from oxidative damage. Under oxidative stress, SOD works as the primary defense line, modifying highly reactive superoxide radicals into less toxic hydrogen peroxides, which are subsequently decomposed by CAT.

It is believed that heat stress not only elevates ROS production, but also disturbs antioxidant systems in Japanese quails (Sahin *et al.*, 2003) laying hens (Xiao-Lan *et al.*, 2012), broiler chickens (Lin, Du & Zhang, 2000). This is why oxidative stress has been linked with both ROS production and changes in the scavenging ability of antioxidant systems (Lin *et al.*, 2008). In present study, the activities of SOD and CAT were reduced at 6 h of heat stress in the H2 and H3 groups, and at 24 h of heat stress, CAT and SOD activities were increased in the H1 and H2 groups. Under mild oxidative stress, SOD and CAT activities increase, but are reduced when oxidative stress is extended (Igor M *et al.*, 2016), which explains why H1, H2, and H3 groups presented better activity of these enzymes comparatively to NC. On the other hand, the groups submitted to higher temperatures (H4 and H5) showed reduced SOD and CAT activities, possibly due to the consumption or inhibition of antioxidant synthesis by ROS.

The content of MDA of H5 cells at 6h of heat stress and of H4 and H5 at 12 and 24 h of heat stress was significantly higher compares with NC. The increase in MDA content also demonstrates that heat stress induced oxidative stress in CEF cells as a result of lipid peroxidation and protein and DNA damage (Di Meo *et al.*, 2016). Together, these results showed that heat stress reduced the activity of the enzymatic scavenging systems.

In conclusion, the results of present study indicate that heat stress stimulates ROS production, and its excessive accumulation reduces cell viability and proliferation and induces apoptosis. Furthermore, excessive ROS production caused by heat stress also reduces the activity of antioxidant defense systems, leading to increased oxidative damage of chicken embryonic fibroblast cells. The information presented in this study may aid the elucidation of the mechanism apoptosis and oxidative stress induced by heat stress in avian cells.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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