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Alpha lipoic acid improves heat stress-induced reduction of serum oestradiol and progesterone levels by affecting oxidative and endoplasmic reticulum stress in hens

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Abstract: Alpha lipoic acid (ALA), a natural lipophilic compound, plays an important role in regulating several metabolic pathways due to its antioxidant properties. This study aims to investigate whether ALA could be used as a feed additive to enhance the antioxidant capacity of the ovary tissue in hens exposed to heat stress (HS). One hundred and sixty 128-days-old female chickens were randomly assigned into four groups: the control group (Con), ALA treatment group (ALA), ALA and HS treatment group (ALA + HS), and HS treatment group (HS). The ALA and ALA + HS groups were fed a basal diet with 0.25% ALA, whereas the Con and HS groups were fed a basal diet only. Serum oestradiol, progesterone levels, biomarkers of antioxidant capacity, and endoplasmic reticulum (ER) stress markers were detected in the ovaries of heat-stressed chickens. HS decreased serum oestradiol and progesterone concentrations compared with the control group, whereas dietary ALA (0.25%) increased oestradiol and progesterone levels in the serum of heat-stressed hens. Malondialdehyde concentration in the ovary was higher in the HS group than that of the ALA + HS group. Compared with the HS group, ALA increased the enzymatic activities of superoxide dismutase, glutathione peroxidase, and catalase in the ovaries of ALA + HS group. Simultaneously, ALA enhanced the total antioxidative capacity of the ovaries of heat-stressed hens. Moreover, ALA also significantly inhibited the increased expression of glucose-regulated protein 78 and CCAAT/enhancer-binding protein homologous protein, which are two markers of ER stress, and heat shock protein 70, a key biomarker of heat stress, in the ovaries of the ALA + HS group as compared to those of the HS group. This work implied that dietary ALA supplementation improved the antioxidant capacity and attenuated the HS-induced reduction of serum oestradiol and progesterone levels and modulated the oxidative and ER stress, which are involved in the protective effect of ALA in hens exposed to hyperthermia.

Keywords: antioxidant; hyperthermia; steroid hormone; oxidative damage; ovarian function

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The occurrence of heat stress (HS) is usually affected by many factors, including environmental temperature, humidity, radiant heat from animals, and ventilation. Chickens are more sensitive to HS than other animals (Goo et al. 2019). Once the ambient temperature is above the hens' tolerance, a series of abnormal changes is observed in egg production, egg quality traits, many blood constituents, and the activities of enzymes related to the antioxidant capacity of the ovary (Saeed et al. 2019). HS, as an important environmental stressor, can impair the physiological function of the ovary to decrease the reproductive efficiency of hens. Because of global warming and rising cooling costs, more and more studies have focused on therapeutic strategies for preventing a detrimental effect of HS on chickens (Mirzaie et al. 2018).

Oxidative stress, as a result of disturbing the homeostasis of the oxidant and antioxidant systems, has been proved to be responsible for cell and tissue damage caused by various pathological factors, such as HS (Houston et al. 2018). At the same time, previous research has indicated that oxidative stress often induces the activation of the endoplasmic reticulum (ER) stress signalling pathway to attempt to re-establish the balance between oxidant and antioxidant systems, repairing the pathological lesions (Luo et al. 2019). In eukaryotes, the ER is indispensable for controlling the process of synthesis, folding, and assembly of various proteins, including secreted and membrane proteins before they carry out their physiological functions. A variety of pathological perturbations can disrupt the process of protein folding in the ER, triggering a protective response called ER stress to attempt to improve the folding capability of ER. Nevertheless, sustained or severe ER stress is not helpful for re-establishing ER homeostasis. Sustained ER stress will eventually induce the cell death (Hu et al. 2019).

Alpha lipoic acid (ALA), a natural lipophilic compound, acts as an important modulator of several metabolic pathways because of its antioxidant actions (Rochette et al. 2015). A previous study documented that the antioxidative effect of ALA was observed in the breast meat of broiler chickens exposed to a hyperthermic environment. And it can induce fatty acid metabolism and antioxidative effect persisting during the heat stress, even though a sexual difference in the responsiveness was seen in broiler chickens (Hamano 2014). Simultaneously, ALA has a protective effect against ovary-related diseases, such as polycystic ovary syndrome in women (Di Tucci et al. 2018), methotrexate-induced ovarian oxidative injury in rats (Soylu et al. 2017), and ovarian ischaemia-reperfusion injury in a rat model (Dokuyucu et al. 2014). However, it remains unclear whether ALA has protective effects against HS-induced reproductive hormone secretion dysfunction and a decrease in the antioxidant ability in the ovaries of chickens.

Therefore, the aim of this study was to determine whether ALA can ameliorate the HS-induced secretion disorder of steroid hormones, including oestradiol and progesterone, and to improve the antioxidative stress ability of the ovary by affecting the activities of antioxidant enzymes and ER stress in chickens exposed to hyperthermia.

MATERIAL AND METHODS

Experimental design, animal care, and treatment

All experimental animal protocols complied with the rules and procedures for the welfare and care of experimental animals of the Ministry of Science and Technology of China and approved by the Animal Care and Use Committee of Anhui Science and Technology University (approval No. 006-398). One hundred and sixty 128-days-old female chickens (Arbor Acres, Chuzhou, China) were randomly assigned into four groups (n = 40 each group): the control group (Con), ALA treatment group (ALA), ALA and HS treatment group (ALA + HS), and HS treatment group (HS).

The chickens in the ALA + HS and HS groups were housed in an artificially high temperature environment at 33 ± 1 °C (relative humidity: 55–60%) for 6 h/day from 11:00 to 17:00, while the Con and ALA groups were kept at 25 ± 1 °C (relative humidity: 55–60%). In addition, the ALA and ALA + HS groups were fed a basal diet with 0.25% ALA (Shengxuan Biotech, Guangzhou, China), whereas the Con and HS groups were fed a basal diet only. The nutritional ingredient of the basal diet used in this study was the same as in a previous study (He et al. 2019), which meets the National Research Council recommended requirements for all nutrients of the grower diet. All treatments in this experiment lasted for 14 days. Finally, 20 chickens/group (five replicates; four chickens/replicate) were randomly selected and sacrificed to collect the blood and ovary tissue

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samples for further analysis including the analysis of serum oestradiol (E_2), progesterone (P_4), and the malondialdehyde (MDA) levels, antioxidant enzyme assays of the ovaries, and western blotting.

Analysis of serum E_2 and P_4 levels

After blood samples were collected from all hens, serum E_2 and P_4 levels were analyzed using a commercial E_2 or P_4 ELISA Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The absorbance (λ = 450 nm) was recorded using a spectrophotometer (1510; Thermo Fisher Scientific, Waltham, MA, USA).

Analysis of redox state in ovary tissues

First, the ovary samples were homogenized to collect the supernatants under cold conditions at 4 °C. Then, the MDA levels and the superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and total antioxidative capacity (T-AOC) levels were analyzed using colorimetric testing kits according to the manufacturer's instructions (Jiancheng Bioengineering, Nanjing, China).

Western blot analysis

Using the whole protein extraction kit (Keygen, Nanjing, China), ovary tissues were mixed with the pre-cooled lysis buffer and homogenized on ice. These lysates were centrifuged at 800 × *g* for 0.5 h to separate the supernatants. A BCA assay kit (Keygen, Nanjing, China) was used to quantitate the protein content of the supernatants according to the manufacturer's protocol. Using 10% SDS-PAGE, 40 μg of protein sample was separated with an output voltage of 115 V, and then the sample gels were electrically transferred onto the methanol-pretreated polyvinylidene difluoride (PVDF) membranes (output voltage: 60 V). Following fully immersing in QuickBlock™ Blocking Buffer (Beyotime, Shanghai, China) for blocking for 0.5 h at 25 ± 1 °C, the membranes were soaked in different antibodies including cytochrome P450 family 19 subfamily A member 1 (CYP19A1; 1 : 1 000; Beyotime, Shanghai, China), cytochrome P450 family 1 subfamily B member 1 (CYP1B1; 1 : 1 000; Beyotime, Shanghai, China), steroidogenic acute regulatory protein (StAR; 1 : 1 000; Santa Cruz, TX, USA), glucose-regulated protein 78 (GRP78; 1 : 2 000; Proteintech, Wuhan, China), CCAAT/enhancer-binding protein homologous protein (CHOP; 1 : 2 000; Proteintech, Wuhan, China), heat shock protein 70 (HSP70; 1 : 1 500; Proteintech, Wuhan, China), and β-actin (1 : 3 000; Beyotime, Shanghai, China) for 12 h at 4 °C. Subsequently, the PVDF membranes were washed with TBST (Tris-buffered saline containing 0.1% Tween-20) and were fully soaked in Biotin-labelled secondary antibodies for 1.5 h at 37 ± 1 °C. Following washes in TBST buffer, membranes were incubated with Western ECL Substrate (Bio-Rad, Hercules, CA, USA) to detect the blot signals using the FlourChem HD2 Western Blot Gel Imager (ProteinSimple, San Jose, CA, USA). Quantitative analysis of densitometry of the immunoreactive signals was performed using Quantity One v4.6.2 (Bio-Rad, Hercules, CA, USA).

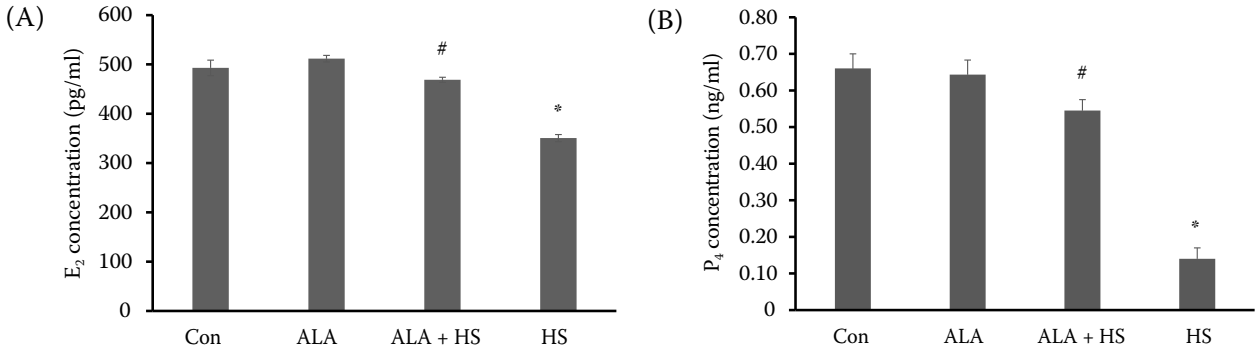

Figure 1. Effect of alpha lipoic acid (ALA) on oestradiol (E_2; A) and progesterone (P_4; B) serum levels of heat-stressed hens
ALA = the basal diet with ALA groups were housed at 25 ± 1 °C; ALA + HS = the basal diet with ALA groups were subjected to HS treatment; Con = the basal diet groups were housed at 25 ± 1 °C; HS = the basal diet groups were subjected to HS treatment
*P < 0.05 vs Con group; #P < 0.05 vs HS group

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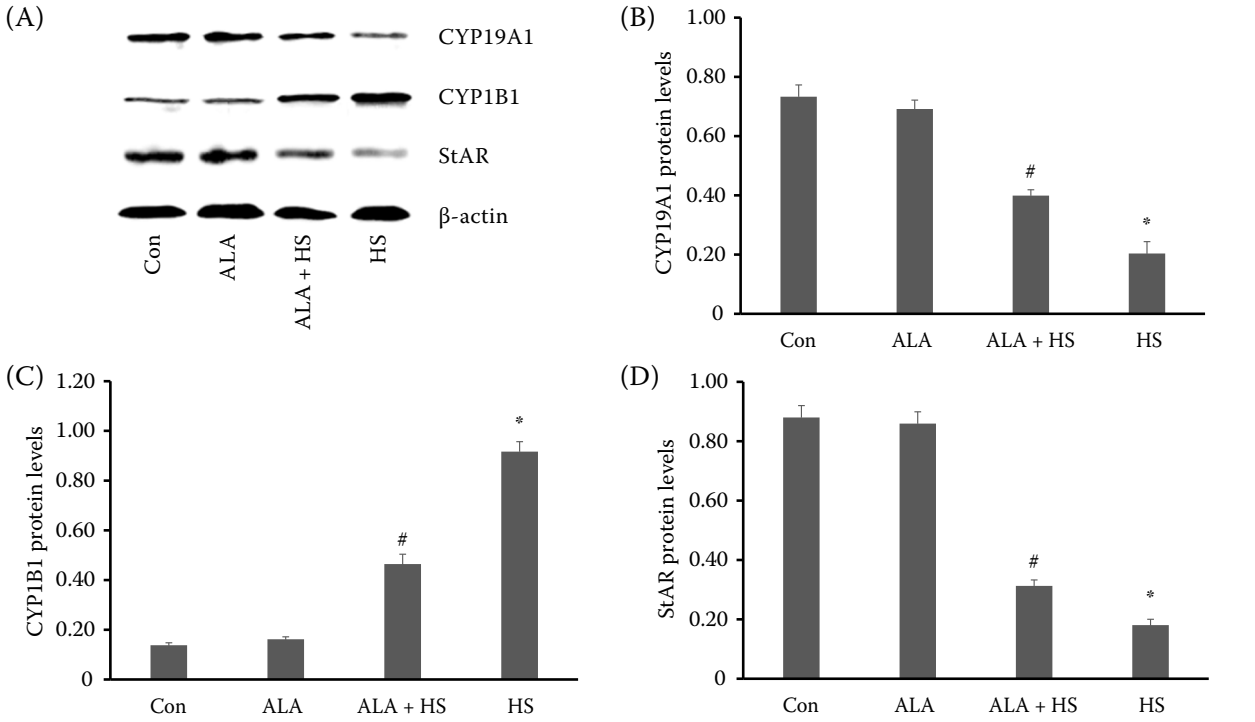

Figure 2. Effect of alpha lipoic acid (ALA) on protein expressions of key steroidogenic-related modulators in the ovary of heat-stressed hens (A), Western blotting was performed to analyze the protein expression of CYP19A1 (B), CYP1B1 (C), and StAR (D)

ALA = the basal diet with ALA groups were housed at 25 ± 1 °C; ALA + HS = the basal diet with ALA groups were subjected to HS treatment; Con = the basal diet groups were housed at 25 ± 1 °C; HS = the basal diet groups were subjected to HS treatment

*$P < 0.05$ vs Con group; #$P < 0.05$ vs HS group

Statistical analysis

The results are expressed as mean ± SEM, and SPSS software (v17.0; SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis by one-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

RESULTS

The effect of ALA on serum E_2 and P_4 levels in hens exposed to HS

Figure 1 shows the serum E_2 and P_4 levels in hens exposed to HS. Compared with the Con group, the serum E_2 and P_4 concentrations were decreased in the HS group ($P < 0.05$). Although there was no significant difference between the Con group and ALA-supplemented groups, the E_2 and P_4 levels in serum from the ALA + HS groups showed a significant increase under the HS conditions ($P < 0.05$).

Effect of ALA on key steroidogenic-related modulators in the ovaries of hens exposed to heat stress

As shown in Figure 2, compared with the Con group, the protein expression of CYP19A1 and StAR was significantly decreased, while CYP1B1 was increased in the ovaries of HS groups ($P < 0.05$). Moreover, the protein levels of these steroidogenic-related modulators showed no significant difference between the ALA-supplemented groups and the Con group ($P > 0.05$). However, the CYP19A1 and StAR protein levels in the ALA + HS groups were significantly higher than those in the HS group, while CYP1B1 expression was decreased in the ALA + HS group ($P < 0.05$).

Effect of ALA on MDA concentration in the ovaries of chickens exposed to heat stress

As shown in Figure 3, compared with the Con group, the MDA concentration in the ovaries was in-

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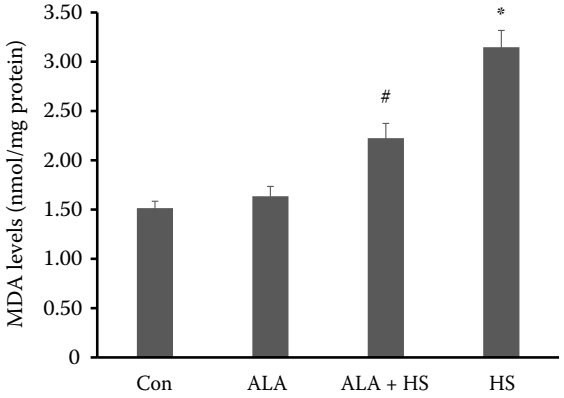

Figure 3. Effect of alpha lipoic acid (ALA) on malondialdehyde (MDA) content in the ovaries of heat-stressed hens
ALA = the basal diet with ALA groups were housed at 25 ± 1 °C; ALA + HS = the basal diet with ALA groups were subjected to HS treatment; Con = the basal diet groups were housed at 25 ± 1 °C; HS = the basal diet groups were subjected to HS treatment
*P < 0.05 vs Con group; #P < 0.05 vs HS group

creased in the HS groups (P < 0.05). Furthermore, the MDA content was not significantly different between the Con group and ALA-supplemented groups. However, the MDA content in the ALA + HS group was lower compared to that in the HS group (P < 0.05).

Effect of ALA on SOD, GPx, CAT, and T-AOC levels in the ovaries of chickens exposed to heat stress

Figure 4 shows the activities of key biomarkers of the redox status of the ovaries in hens exposed to heat stress. The enzyme activities of GPx, SOD, CAT, and the level of T-AOC were significantly lower in the ovary of the HS group as compared to those in the Con group (P < 0.05). However, the activities of these enzymes (GPx, SOD, CAT) and the T-AOC level were higher in the ALA +

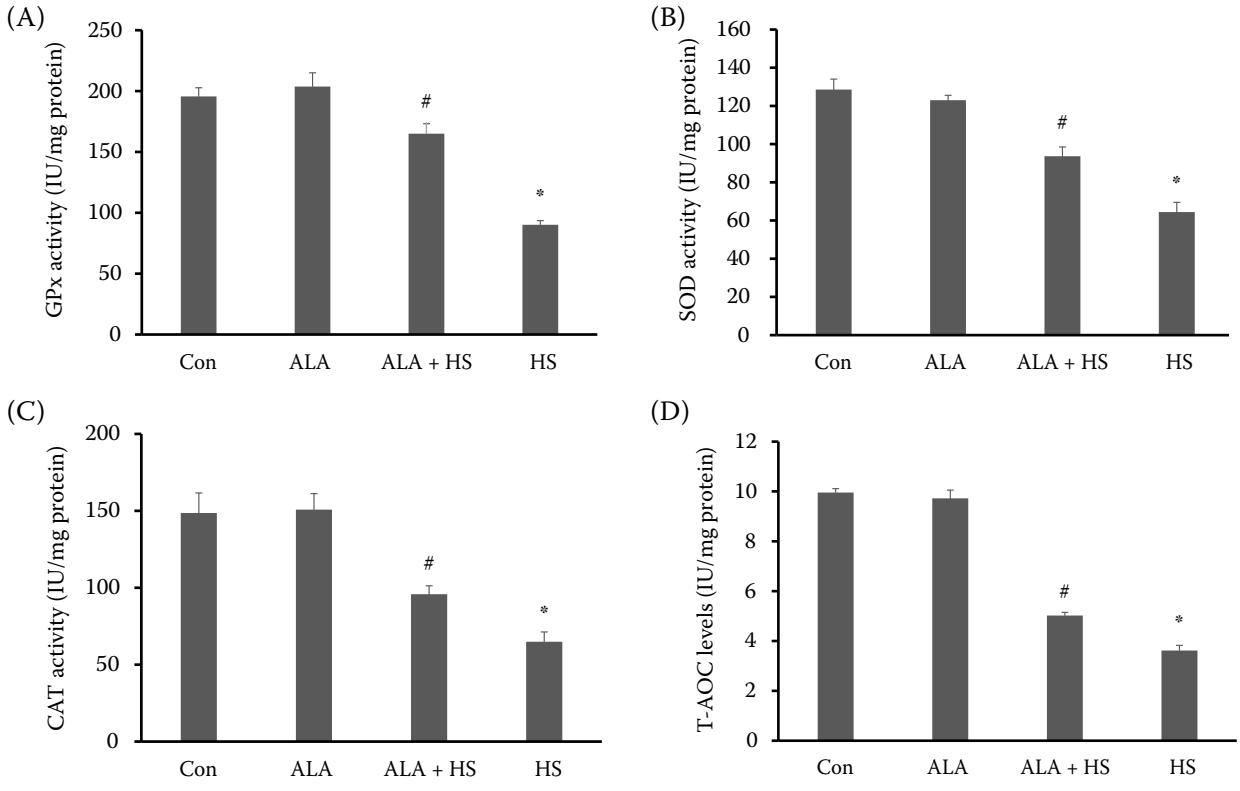

Figure 4. Effect of alpha lipoic acid (ALA) on antioxidant capacity of the ovaries of heat-stressed hens
The activities of antioxidant enzymes including GPx (A), SOD (B), and CAT (C) were measured using the colorimetric method, respectively. (D) T-AOC levels were analyzed using the colorimetric method
ALA = the basal diet with ALA groups were housed at 25 ± 1 °C; ALA + HS = the basal diet with ALA groups were subjected to HS treatment; Con = the basal diet groups were housed at 25 ± 1 °C; HS = the basal diet groups were subjected to HS treatment
*P < 0.05 vs Con group; #P < 0.05 vs HS group

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HS group than those in the HS group ($P < 0.05$). In addition, neither GPx activity nor SOD, CAT, or T-AOC levels in the ovary of the ALA group were significantly different from those in the Con group.

Effect of ALA on ER stress and HSP70 protein expression in the ovaries of hens exposed to heat stress

As shown in Figure 5, compared with the Con group, the protein expression levels of GRP78, CHOP, and HSP70 were upregulated in the HS group ($P < 0.05$), while none of them showed a significant difference in the ALA group. However, the expression of GRP78, CHOP, and HSP70 proteins was decreased in the ALA + HS group compared with the HS group ($P < 0.05$).

DISCUSSION

For female chickens, reproductive performance is closely related to the physiological function of the ovary. An important function of the ovary in female animals is to produce steroid hormones which are involved in folliculogenesis, follicle maturity, ovulation, and follicular atresia. It is well-known that E_2 and P_4 are two key steroid hormones secreted in the ovary and play an indispensable role in maintaining the normal physiological function of the ovary. Serum E_2 and P_4 levels are considered as a standard indicator for assessing the ovarian function. The existing evidence indicates that HS could cause secretion disorders of E_2 and P_4 in female animals (Dickson et al. 2018). In the present study, it was found that HS induced decreases in both E_2 and P_4 concentrations in the serum, whereas dietary ALA significantly increased serum E_2 and P_4

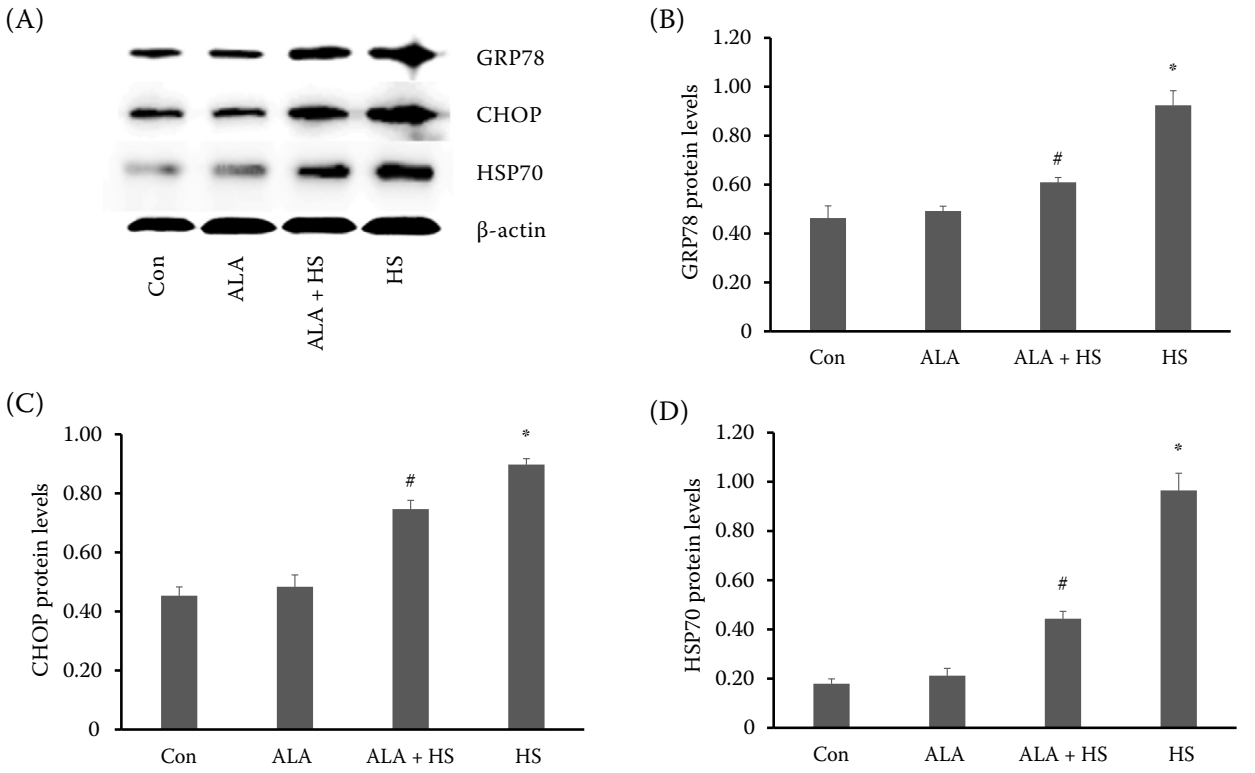

Figure 5. Effect of alpha lipoic acid (ALA) on protein expressions of endoplasmic reticulum (ER) stress markers and HSP70 in the ovaries of heat-stressed hens

(A) Western blot was performed to analyze the protein level of ER stress indicators GRP78 (B) and CHOP (C), and the HS marker HSP70 (D)

ALA = the basal diet with ALA groups were housed at 25 ± 1 °C; ALA + HS = the basal diet with ALA groups were subjected to HS treatment; Con = the basal diet groups were housed at 25 ± 1 °C; HS = the basal diet groups were subjected to HS treatment

*$P < 0.05$ vs Con group; #$P < 0.05$ vs HS group

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levels in hens under HS conditions. Furthermore, it was observed that the protein levels, including CYP19A1 and StAR, were significantly upregulated, while the CYP1B1 protein levels were downregulated in the ovaries of heat-treated hens after ALA treatment. Thus, we inferred that the ALA improved E_2 secretion levels might be due to increased protein level of CYP19A1, a key rate-limiting enzyme involved in E_2 synthesis (Belani et al. 2014), and lower expression of CYP1B1, which is a key rate-limiting enzyme in E_2 metabolism (Nishida et al. 2013). Likewise, the higher expression of StAR, which is responsible for transporting cholesterol for P_4 synthesis in chicken secretory cells (Sechman et al. 2011), might result in the increased P_4 concentration in the serum of hens suffering from heat stress. Moreover, it is worth noting that previous studies reported that ALA treatment is helpful for restoring the degenerated physiological function of the ovary by normalizing the abnormal E_2 production by the ovary exposed to several pathological factors, such as in 4-vinylcyclohexene diepoxide-induced primary ovarian failure in rats (Ozel et al. 2020) and cyclophosphamide-induced ovarian toxicity in rats (Nair et al. 2020). Based on these findings, we inferred that the addition of ALA may be beneficial for sustaining the normal physiological function of the ovary in the face of the adverse effects of heat stress by inhibiting the HS-induced decrease in E_2 and P_4 levels in chickens.

A number of stress factors, including ischaemia and HS, can trigger oxidative stress (Sinning et al. 2017). The process of HS is usually accompanied by elevated levels of reactive oxygen species (ROS). The accumulation of ROS destroys cellular homeostasis by inducing lipid peroxidation, resulting in oxidative damage in cells and tissues in SD rats (Yang et al. 2019). MDA, a stable end product of lipid oxidation, is a key biomarker for lipid peroxidation. It has been demonstrated that the degree of lipid peroxidation in cells and tissues is positively correlated with MDA production (Zhang et al. 2019). Moreover, it was reported that ALA addition significantly inhibited the lipid peroxidation of ammonia-exposed broilers by suppressing MDA production (Lu et al. 2017). The MDA content was significantly increased in the rat ovary which had suffered oxidative damage (Kulhan et al. 2019). In the present study, we found that dietary ALA could effectively inhibit the HS-induced upregulation of MDA levels in the ovary tissues, suggesting that lipid peroxidation in the ovary was attenuated in heat-stressed hens, which corroborates the findings of previous studies. Additionally, substantial evidence has shown that the accumulation of ROS and free radicals was induced by a high temperature environment, which could suppress the activities of antioxidant enzymes, resulting in oxidative damage to the body (Shadmehr et al. 2018). GPx, SOD, and CAT are known to be key antioxidant enzymes, and the T-AOC level is a standard indicator of antioxidant capacity in the body. Changes in the activities or levels of these markers are used to evaluate the ability of cells and tissues to scavenge ROS and/or free radicals (Bai et al. 2016). Previous reports showed that supplementation with exogenous ALA is beneficial for improving the antioxidant capacity of the body by potentiating the activities of main antioxidant enzymes in broilers (Li et al. 2019). Similar to a previous report, our results showed that not only the antioxidative activities of GPx, SOD, and CAT, but also the T-AOC levels in the ovary were significantly upregulated in chickens treated with high environmental temperature after ALA supplementation, further verifying the antioxidant effects of ALA in the ovary of heat-stressed hens.

Previous studies have revealed that the ER stress signalling pathway is activated in the process of oxidative lesions in various cells and tissues (Dandekar et al. 2015). It has been previously documented that ER stress is implicated in diseases of the reproductive system caused by oxidative damage (Alemu et al. 2018). Furthermore, it was reported that ALA has a protective effect on pathological injuries in different species via inhibition of ER stress (Yuan et al. 2019). Thus, we analyzed the changes in the protein expressions of GRP78 and CHOP, two indicators of ER stress activation, in the ovary of hens housed in a high temperature environment. Our results showed that HS significantly induced GRP78 and CHOP protein expressions in the ovary tissue of heat-stressed hens, but the increased protein expressions of these ER stress markers were effectively inhibited by the addition of ALA, which is consistent with the results of previous studies by Yuan et al. (2019). At the same time, our data revealed that although elevated protein expression levels of HSP70, a well-established HS-induced chaperon and marker of stress (Galal et al. 2019), were observed in the ovaries of heat-stressed hens,

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ALA effectively suppressed the increase in HSP70 protein expression, further verifying that ALA not only inhibited the HS-induced activation of ER stress but also diminished the severity of HS. Based on these findings, we speculated that ALA could attenuate the detrimental effect of HS by inhibiting ER stress in the ovary and thus restoring the physiological function of the ovary, which further rescued the decreased E_2 and P_4 production caused by HS.

CONCLUSION

The present study indicated that dietary ALA supplementation attenuated the HS-induced reduction of serum E_2 and P_4 levels and improved antioxidant capacity in the ovary of heat-stressed hens. Furthermore, oxidative stress and ER stress were involved in these protective actions of ALA under HS conditions. Dietary supplementation with alpha lipoic acid may be a potential strategy to improve the physiological function of the ovary in hens under heat stress.

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Conflict of interest

The authors declare no conflict of interest.

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