Effect of A Standardized Extract of *Asparagus Officinalis* Stem on HSP 70 Induction and Redox Balance in Bovine Cumulus-Granulosa Cells

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Research Article

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Abstract

Heat shock protein 70 (HSP70) is a well-known heat shock (HS)-induced protein that acts as an intracellular chaperone to protect cells against stress conditions. Although HS induces HSP70 expression to acquire stress-resistant ability to cells, HS causes toxicity to cells by increasing reactive oxygen species (ROS). Recently, a standardized extract of *Asparagus officinalis* stem (EAS), produced from the by-product of asparagus, was found to induce HSP70 expression without HS and regulate cellular redox balance in the cells. However, the effect of EAS on the function of reproductive cells remains unknown. In the present study, we investigated the effect of EAS on HSP70 induction and oxidative redox balance in cultured bovine cumulus-granulosa(CG) cells. EAS significantly increased *HSP70* expression, whereas no effect was observed in *HSP27* and – *90* under non-heat stress condition. EAS decreased ROS generation and DNA damage, and increased glutathione (GSH) synthesis both under non-HS and HS conditions. Moreover, EAS synergistically increased *HSP70* and *HSF1* expression. EAS also increased progesterone (P4) levels in CG cells. HSP70 inhibitor significantly decreased GSH and increased ROS, as well as decreased *HSF1*, *Nrf2*, and *Keap1* in the presence of EAS. These results suggest that EAS regulates redox balance through HSP70 in bovine CG cells.

Introduction

Cumulus-granulosa cells surround oocytes and play a vital role in the maturation and acquisition of developmental competence in mammalian oocytes [1]. Cumulus cells and oocytes communicate metabolically via gap junctions, which provide important points of entry for nutrient transfer and signaling between both cell types [2]. The absence of cumulus-granulosa cells has harmful effects on maturation, fertilization, and embryo development in cattle [3]. Cumulus-granulosa cells enhance the nuclear and cytoplasmic maturation of oocytes during maturation [4] and the fertilization rate during fertilization [5]. In addition to playing an important role in oocyte development, cumulus-granulosa cells protect oocytes against damage caused by oxidative stress during maturation [6]. After ovulation, the remaining cumulus-granulosa cells differentiate into corpus luteal cells to produce progesterone, an important steroid hormone, to maintain pregnancy [7]. In addition, the *in vitro* environment has a higher concentration of O_2, which is a source of ROS, than the *in vivo* environment [8]. Cumulus cells have been suggested to play a critical role in defending bovine oocytes against cell damage due to ROS production [1]. Moreover, during *in vitro* maturation, cumulus–oocyte complexes have higher levels of GSH than cumulus-denuded oocytes, and bovine cumulus-granulosa cells contribute to cumulus–oocyte complex GSH synthesis [6]. Therefore, bovine cumulus-granulosa cells play a major role in the generation of GSH, which can reduce ROS production. As a result, the balance between ROS and GSH can prevent cell death by maintaining the balance of the cellular redox status.

The cellular redox status contributes to important cellular functions, such as regulation of proliferation, differentiation, and cell death, and is defined as the balance between oxidants (or pro-oxidants) and antioxidants [9]. ROS are free radicals, such as superoxide anion radicals, hydroxyl radicals, and hydrogen

peroxide, that cause damage to cells by lipid peroxidation and enzyme inactivation [10]. The upregulation of ROS production can induce cell death signaling through an imbalance in the redox status of the cells [11]. In addition, previous studies have evaluated the effect of ROS on DNA damage, which induces toxicity in cells or the death of cells [12]. In contrast, GSH, which is synthesized from the γ-glutamyl cycle, is one of the major antioxidants present in mammalian cells and provides a powerful antioxidant defense against oxidative stress [13]. The balance between ROS and GSH can reduce DNA damage by altering the cellular redox status, thus improving cell survival.

HSP70, also called stress protein, is a molecular chaperone that assists in the folding, unfolding, and homeostasis of cellular proteins [14]. Therefore, the main functions of HSP70 allow the cell to survive during several stresses, such as physical, chemical, and environmental stress [15]. Experimental evidence has suggested that HSP70 expression regulates both GSH and ROS generation, indicating that an interrelationship between HSP70 and the redox status exists [16]. Various plant sources have been studied in an attempt to identify HSP70 induction activity. In this context, asparagus (*Asparagus officinalis* L.) is a potential candidate because of its antioxidant ability [17]. In addition, functional food ingredients are more valuable when they promote the effective use of unused parts. EAS is produced from the unused bottom part of asparagus, and this extract contains active ingredients that enhance HSP70 expression, such as asparagine [18]. In addition, asparagine has been found to increase the HSP70 mRNA levels in a human promyelocytic leukemia cell line [18]. This evidence suggests that EAS is a potential inducer of HSP70, which may regulate the balance between GSH and ROS generation.

Several products, such as paeoniflorin, geranylgeranylacetone, and bimoclomol, have been found to stimulate HSP70 [19 20]. Bimoclomol induced heat shock protein (HSP) under HS conditions, but did not influence HSP activity under non-stress conditions [19]. Paeoniflorin and geranylgeranylacetone increased HSP70 expression under HS conditions in experiments on cells and rats [20]. These data showed that the HSP70 inducer has a synergistic effect with HS on HSP70 expression.

I In the present study, we investigated the effect of EAS on HSP70 induction and oxidative redox balance in cultured bovine cumulus-granulosa(CG) cells.

Results

Effect of EAS on the induction of HSPs

To determine the concentration of EAS required to induce HSP genes (*HSP27*, *-70*, and *− 90*) in bovine cumulus-granulosa cells, we examined various concentrations of EAS (0.5, 1, and 5 mg/mL). A significant increase ($P < 0.05$) in *HSP70* expression was observed after treatment with 5 mg/mL EAS (Fig. 1a). However, the expression of *HSP90* and *HSP27* was not influenced by EAS (Fig. 1b and c). Similar to the induction of *HSP70*, the HSP70 protein levels were significantly increased ($P < 0.01$) by 5 mg/mL EAS (Fig. 3a, b). Since *HSP*s are induced by heat shock, we evaluated the effect of EAS on HSP expression

after 6 h of HS (41°C) treatment by comparing against non-HS conditions in bovine CG cells. Similar to Fig. 1, EAS specifically increased *HSP70* expression under non-HS control conditions (Fig. 2a) compared with *HSP90* expression (Fig. 2b) and − *27* (Fig. 2c).

HS induced an increase in *HSP70* and − *90* expression. However, interestingly, HSP70 was synergistically increased by HS and EAS (Fig. 2a). The immunodetection of HSP70 also showed a similar increase in *HSP70* gene expression (Fig. 3c, d).

HSF1 is a transcription factor that induces HSP70 by heat shock [24]. As shown in Fig. 4A, *HSF1* expression was significantly increased by HS and EAS, whereas no effect of HS and EAS was observed in *HSF2* expression (Fig. 4b).

Effect of EAS on ROS generation

After HSP70 expression was enhanced by EAS in bovine cumulus-granulosa cells, we investigated the effect of EAS on HSP70 and oxidative and redox balance [25]. As shown in Fig. 5a and c EAS significantly reduced the ROS levels under both non-HS and HS conditions.

Effect of EAS on DNA damage by γH2AX levels

ROS are known to cause apoptosis associated with mitochondrial dysfunction and single- and double-strand DNA breaks [26]. Therefore, we hypothesized that EAS could reduce DNA damage by heat shock-induced ROS generation. The levels of γH2AX were significantly increased ($P < 0.05$) by HS (Fig. 5b, d). The levels of γH2AX in HS were significantly decreased by EAS.

Effect of EAS on GSH synthesis

GSH was detected more strongly in the nuclei than in the cytoplasm (Fig. 6a). EAS significantly increased the GSH levels ($P < 0.05$) in non-HS cells (Fig. 6a, b).

Effect of EAS on expression of oxidative stress and redox-regulated genes

To determine whether EAS protects against DNA damage by maintaining the redox status, we focused on genes related to oxidative stress and redox balance. The expression of *GCL* and *GS*, which are involved in GSH synthesis in mammalian cells [27], was significantly increased by EAS under non-HS conditions (Fig. 7a, b). Since GSH synthetic pathways, such as GCL and GS activation, are dependent on Nrf2 regulation [28], we analyzed the expression of *Nrf2* and *Keap1*. As a result, the expression both *Nrf2* and *Keap1* was found to be significantly increased by HS treatment (Fig. 7c, d). The *Nrf2* levels in the HS and HS + EAS (5 mg/mL) groups were significantly increased compared to the control ($P < 0.01$). In contrast, the HS + EAS (5 mg/mL) group had a lower *Keap1* mRNA expression than the control, EAS (5 mg/mL), and HS groups ($P < 0.01$) (Fig. 7d).

EAS extracted from *Asparagus officinalis* L., which contains high levels of antioxidants, such as carotenoids, steroidal saponins, and flavonoids [29]. Antioxidant activity has been demonstrated in other

extracts of this vegetable [29]. Therefore, we analyzed antioxidant enzymes, including *SOD1, SOD2, PRDX2*, and *PRDX6*, to evaluate the antioxidant effect of EAS under non-HS conditions. The expression of *SOD1* and *SOD2* was significantly increased by HS treatment (Fig. 7e, f). Although no effect of EAS was observed on *SOD1* and *SOD2* expression under non-HS conditions, EAS significantly increased both expression under HS conditions. Similar to the expression patterns of *SOD1* and *SOD2*, EAS significantly increased the expression of *PRDX2* and *PRDX6* (Fig. 7g, h).

Effect of HSP70 inhibition on redox status of bovine cumulus-granulosa cells treated by EAS

The high level of HSP70 reduced the levels of ROS and induced GSH levels during EAS treatment, suggesting that HSP70 plays a role in the balance of cellular redox status in bovine cumulus-granulosa cells. We hypothesized that HSP70 induction by EAS triggers the regulation of the balance of ROS and GSH, which is required for maintaining cellular redox status in bovine cumulus-granulosa cells. To test this hypothesis, cells were treated with EAS (5 mg/mL) and 10 μM PES. Cell viability of 10 μM PES was 77% (Supplemental Fig. 1)

Decreased levels of ROS by EAS treatment were significantly increased by PES treatment (Fig. 8a, c). In contrast, the levels of GSH that had been increased by EAS were significantly decreased (Fig. 8b, d). Moreover, the expression of *HSF1* and *Nrf2* increased by EAS was significantly decreased by the inhibition of HSP70 (Fig. 9a, b). In addition, *Keap1* expression was also significantly decreased by the inhibition of HSP70 (Fig. 9c).

EAS increased P4 synthesis

The role of bovine cumulus-granulosa cells not only supports oocyte development, but also fulfills the endocrine role of steroid hormone secretion [30]. To further investigate the effect of EAS on steroidogenesis, the P4 levels were measured, and were found to be significantly increased by EAS supplementation compared to the control group ($P < 0.05$) (Fig. 10).

Discussion

EAS has been shown to increase the expression of HSP70 mRNA and protein in HeLa cells, neuronal cell NG108-15, and hepatocyte cells [31, 32,33]. Moreover, HSP70 overexpression increased the GSH/GSH disulfide ratio while reducing the ROS levels under hypoxia and glucose deprivation conditions [16]. Therefore, the current study investigated whether EAS increases HSP70 expression. GSH is an antioxidant enzyme whose synthesis reduces heat shock-induced ROS generation with DNA damage to maintain the redox status in bovine cumulus-granulosa cells.

In the present study, EAS significantly increased the *HSP70* mRNA and protein levels in bovine cumulus-granulosa cells at 5 mg/mL during 6 h of incubation. This result is consistent with a previous study by [33], who studied the dose and incubation time of EAS treatment in cultured hepatocyte cells. In addition, HeLa

cells also increased *HSP70* mRNA and protein expression when treated with a lower dose of EAS (4 mg/mL), but had a longer incubation time (24 h) than in the current study (6 h) [31]. The variation in the dose of EAS in cells may be due to differences in cell type and culture conditions. HSP70 expression-enhancing activity by EAS may arise from asparagine, which has been found to elevate HSP70 in human monocyte HL-60 cells [18]. On the other hand, resistance to anti-cancer drugs due to overexpression of HSP27 and HSP90 has been reported in several studies, but the expression of HSP27 and HSP90 in bovine cumulus-granulosa cells was not affected by EAS [34]. Therefore, our results confirm the unique effect of EAS on HSP70 induction in bovine cumulus-granulosa cells under non-HS conditions.

In vivo and *in vitro* studies have shown a synergistic effect between HS and heat shock protein-inducing compounds on HSP70 expression [20], [35]. In this study, HSP70 induction by EAS occurred in both non-HS and HS conditions, and it was highest under HS conditions. Paeoniflorin, an HSP70-inducing compound derived from peony (Paeonia lactiflora), increased HSP70 expression under HS conditions in HeLa cells [20]. In addition, geranylgeranylacetone, a non-toxic HSP70 inducer, induced HSP70 levels in the brains of heatstroke rats [35]. These data support that the combination of EAS with HS synergistically affects HSP70 induction in bovine cumulus-granulosa cells. The induction of HSF1, which is one of the main transcription factors of HSP70, by EAS has been reported in neuronal cells NG108-15 [33]. The significant increase in *HSF1* gene expression by EAS in the present study supports the involvement of EAS in the HSP70 inducing pathway in bovine cumulus-granulosa cells. It is known that HSF1 is also available in non-stressed cells and binds to HSP70 as an inert monomer [36]. Under cellular stress, HSF1 is activated and releases HSP70 to prevent the formation of misfolded polypeptides [36]. In the present study, *HSF1* gene expression was induced by EAS supplementation in both non-HS and HS conditions, and it was highest with EAS supplementation under HS conditions. In addition, the activator of HSF1 can also be stimulated by endogenous metabolites, synthetic compounds, and phytochemicals [37]. Therefore, HSF1 enhancement by EAS was induced by the phytochemical compounds of asparagus, such as carotenoids, steroidal saponins, and flavonoids [29]. These data suggest that EAS and HS have synergistic effects on the induction of HSF1 in bovine cumulus-granulosa cells. HSF2 is a heat shock transcription factor co-expressed with HSF1 that is activated in response to distinct developmental cues or differentiation stimuli [36]. The interaction between HSF1 and HSF2 was discovered through their coiled-coil domains adjacent to their DNA-binding domains [37], however, *HSF2* was not affected by EAS in the current study. In the present study, supplementation with EAS led to increased HSP70 expression under non-HS conditions. However, *HSF1* expression was only induced under HS conditions with a synergistic effect by EAS. In addition, treatment with an HSP70 inhibitor reduced *HSF1* gene expression. The inconsistency with results from previous studies may be due to the involvement of several factors. EAS may regulate the expression of HSP70 not only through *HSF1* gene expression but also through other regulatory mechanisms. Additionally, research on different species and cell types has led to reports of different regulatory effects of EAS on *HSF1* gene expression [33]. Hence, it is conceivable that an increase in HSP70 expression by EAS is induced as a result of the asparagine content and enhancement of HSF1, with EAS exerting synergistic effects with HS on HSP70 induction.

Several studies have indicated that HSP70 has apoptosis-suppressive effects and anti-inflammatory activity, indicating that HSP70 exerts a cytoprotective function against various stresses [38]. In addition, the overexpression of HSP70 reduces ROS induced by hypoxia and glucose deprivation [16]. These results indicate that EAS with unique inducible HSP70 could protect cells against ROS under various stress conditions. Moreover, EAS was found to significantly reduce the amyloid beta peptide-induced production of ROS in differentiated PC12 cells [39]. In the present study, EAS treatment significantly inhibited ROS expression in both normal and HS conditions in bovine cumulus-granulosa cells by activating GSH generation and antioxidant enzymes, such as SOD and PRDX. SOD is the first line of defense against ROS, and is comprised of three classes: Cu/Zn SOD (SOD1), Mn SOD/Fe SOD (SOD2), and Ni SOD [40]. The reaction of the superoxide anion ($O_2^{\bullet -}$) to form hydrogen peroxide (H_2O_2) is catalyzed by SOD [40]. PRDX has six isoforms, PRDX1, 2, and 6 in the cytosol, PRDX3 in mitochondria, PRDX4 in the endoplasmic reticulum, and PRDX5 in both mitochondria and peroxisomes [41]. PRDX contributes to the reduction of H_2O_2 to H_2O [41]. In addition, GSH concentration is used by PRDX6, which contributes to the elimination of H_2O_2 production [42]. GSH has been shown to play an important role in antioxidant defense by interacting with ROS [43]. Moreover, it is known that ROS activate c-Jun N-terminal kinase protein, which also increases under HS conditions [44]. EAS clearly reduced the c-Jun N-terminal kinase protein induced by hydrogen peroxide in fibroblast cells [45]. Quercetin, a flavonoid in asparagus, reduced the ROS levels while enhancing the antioxidant activity of bovine embryos under hyperoxic conditions [46]. In conclusion, EAS reduced heat shock-related ROS generation in bovine cumulus-granulosa cells, which can be explained by the upregulation of GSH generation together with antioxidant enzymes and the downregulation of c-Jun N-terminal kinase protein.

ROS-induced damage can cause both single- and double-stranded DNA breaks [26]. In the present study, EAS reduced *γH2AX* expression under non-HS conditions, however, the difference was not significant. Moreover, EAS significantly reduced the *γH2AX* levels under HS conditions. ROS generation from normal cellular metabolism and HS induces DNA damage in cells [47]. In a previous study, EAS was shown to reduce cell damage induced by nitric oxide donor sodium nitroprusside or the hypoxia mimic reagent cobalt chloride of NG108-15 cells [32]. HSP70 has the ability to repair DNA damage caused by HS [26]. In the present study, HSP70 induction by the synergistic effect of EAS and HS contributed to reducing DNA damage in bovine cumulus-granulosa cells supplemented with EAS under HS conditions. Moreover, GSH also contributes to DNA repair activity, and the expression of GSH in the nucleus enhances protection against DNA damage [48]. In the present study, GSH generation was increased by EAS in bovine cumulus-granulosa cells under non-stress and HS conditions; however, it was only significantly higher under non-stress conditions. These results indicate that EAS reduced DNA damage under non-HS conditions and was within the acceptable range of non-toxic levels of ROS production due to the enhancement of HSP70 and GSH levels.

GSH is a pivotal intracellular antioxidant that exerts potential cytoprotective ability by protecting cells against oxidants and electrophiles [49]. In our study, EAS increased GSH in both non-HS and HS conditions,

but only cells supplemented with EAS under non-HS conditions were statistically significantly different when compared with the control group. In agreement with this observation, previous reports have suggested that the overexpression of HSP70 enhances GSH expression [16]. However, the GSH levels in the control group were similar to those in the HS group, which were exposed for 6 h at 41°C. In HeLa cells, HS treatment for 1, 2, or 3 h at 42°C resulted in the highest increase in GSH at 1 h and the lowest at 3 h [28]. GSH biosynthesis is required by the action of two ATP-dependent enzymes: GCL, which assembles the formation of c-glutamyl-cysteine from glutamate-cysteine, and GS, which is involved in the ligation of c-glutamyl-cysteine to glycine in another ATP-dependent reaction to yield GSH [27]. Our results showed that EAS increased the expression of GCL and *GS* under non-HS conditions. The transcription factor Nrf2, which has the potential to induce GCL and GS, is activated by ROS production [50]. The transcription factor Nrf2 binds to Keap1 under normal conditions, known as the Nrf2/Keap1 signaling pathway, and is translocated to the nucleus in the presence of HS [51]. In the nucleus, Nrf2 binds to antioxidant response elements (ARE) via the DNA-binding domain of small Mafs, thereby activating the transcription and translation of GCL and GS [51]. Moreover, HSF1 may induce Nrf2 by activating p62, which can separate Nrf2 from Keap1 [52]. In this study, the GSH levels were not decreased by HS because of the compensatory effect of HSF1 with Nrf2, which can influence the GSH levels after 6 h of HS treatment. Therefore, there is a need to study the effect of HS treatment on GSH synthesis and the induction of genes. A previous study showed that reduced Keap1 expression is logically related to the induction of Nrf2 [53]. Together with the induction of *Nrf2*, EAS supplementation reduced *Keap1* and induced *HSF1* expression in the present study. In addition, supplementation of quercetin, a plant-derived flavonoid, comprised mainly of polyphenolic compounds from fruits and vegetables, induced Nrf2 expression in bovine embryos [46]. EAS treatment increased the Nrf2 protein levels in NG108-15 neuronal cells [32]. In conclusion, EAS increased GSH expression and γ-glutamyl cycle mRNA expression due to Nrf2, which was induced by the compensation effect of HSF1 with Nrf2 and the antioxidant content of EAS.

The higher expression of antioxidant enzyme genes in the HS group in the present study was similar to that reported in a previous study, in which HS induced SOD and PRDX expression in pig skeletal muscle [54] and HeLa cells [28], respectively. In another study, HS was reported to upregulate HSP70 and SOD2 in Chinese hamster lung fibroblast V79 cells [44]. Interestingly, the heat shock protein family and antioxidant system reduce the harmful effects of ROS [55]. In the present study, EAS increased *HSP70* expression, antioxidant enzymes, and reduced ROS levels. Nrf2 expression has been shown to induce PRDX activity in HeLa cells [27] and SOD activity in mesenchymal stem cells [56]. The binding of Nrf2 to ARE activates PRDX and SOD activity [27 56]. In this study, *Nrf2* expression was highest in bovine cumulus-granulosa cells treated with EAS under HS conditions, indicating that the induction of *PRDX* and *SOD* was due to *Nrf2* expression. EAS extracted from asparagus (*Asparagus officinalis* L.) contains high levels of antioxidant, including carotenoids, steroidal saponins, and flavonoids [29]. In addition, quercetin, which is one of the six subclasses of flavonoid compounds, increased the expression of Nrf2 and antioxidant genes, such as PRDX1 and SOD1 [46]. Therefore, the enhanced antioxidant activity of EAS treatment results from the induction of Nrf2 expression and the antioxidant content of asparagus (*Asparagus officinalis* L.).

PES has been shown to inhibit the function of HSP70 [57]. In the present study, PES reversed the effect of EAS on the GSH and ROS levels, indicating that HSP70 induction by EAS regulated the levels of GSH and ROS in bovine cumulus-granulosa cells. A previous study reported a correlation between the transcription factors Nrf2 and HSF1 for the protection of cells [58]. There is evidence that Nrf2 and HSF1 compensate each other; the induction of HSP70 by methionine deprivation is dependent on Nrf2 but independent of HSF1 [59]. Both Nrf2 and HSF1 play important roles in cellular redox processes due to their ability to influence the levels of HSP70 and GSH. Therefore, distinct cell survival pathways, such as the HS response and Keap1/Nrf2/ARE pathway, are regulated by Nrf2 and HSF1 [51]. In the present study, PES also reversed the induction effect of both *Nrf2* and *HSF1*. PES reduced the nuclear translocation of the nuclear factor-κB (NF-κB) pathway, which regulates the transcription of various gene families, including stress response, apoptosis, and receptor genes, and influences cell survival, differentiation, and proliferation [60]. The nuclear translocation of NF-κB p65 enhances the ability of Nrf2 and plays a role in the antioxidant response in human kidney-2 cells [61]. In addition, HSF1 activation in intestinal epithelial cells during HS is regulated by the NF-κB pathway [62]. Moreover, PES reduced the levels of Keap1, which combined with Nrf2 to regulate the antioxidative protection system [51]. These results indicate that HSP70 induction by EAS improved the redox balance by regulating the ROS and GSH levels via regulating the heat shock response through HSF1 and the antioxidant response through the Nrf2/Keap1 pathway in bovine cumulus-granulosa cells.

The beneficial effect of EAS on P4 synthesis demonstrated in the present study was similar to that reported in a previous study, in which the oral administration of asparagus root extract enhanced P4 levels in rats [63]. P4 levels were enhanced by EAS was due to the presence of steroid saponins, such as sarsaponin, protodioscin, and diosgenin, in asparagus extract, which act as precursors of progesterone [63]. This indicated that bovine cumulus-granulosa cells were important for reproductive function, especially steroidogenesis. As such, these findings indicate that EAS contributed to the improvement of P4 synthesis in bovine cumulus-granulosa cells.

In conclusion, EAS was shown to induce HSP70 under non-HS conditions, exerting a synergistic effect with HS on HSP70 induction in bovine cumulus-granulosa cells. Furthermore, EAS had beneficial effects, reducing the DNA damage induced by ROS, as well as increasing GSH synthesis and antioxidant enzyme levels to maintain the redox status, in addition to the P4 levels, in bovine cumulus-granulosa cells. HSP70 induced by EAS regulated the Nrf2/Keap1 pathway and HSF1 transcription factor levels, which contributed to the ROS and GSH levels in bovine cumulus-granulosa cells.

Taken together, the findings presented in this study demonstrate that EAS has potential uses in the regulation of reproductive functions by reducing physical stress and improving the properties of reproductive cells.

Methods

EAS was provided by Amino Up Co., Ltd. (Sapporo, Japan), produced from asparagus (*A. officinalis* L.) grown in Hokkaido. EAS was manufactured according to a previously described method [21].

Culture of bovine cumulus-granulosa cells

Bovine ovaries were collected from a local abattoir and transported to the laboratory at 20°C. The ovaries were washed several times with sterile saline solution. Cumulus—oocyte complexes were aspirated from follicles (2–8 mm in diameter) using a disposable 18-gauge needle attached to a 10-ml syringe. After picking up the COCs, the bovine cumulus-granulosa cells remaining in the follicular fluid were used for the experiment. These cells were cultured in 5% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (high glucose) (DMEM) (Wako, Osaka, Japan) supplemented with 0.06 g/l penicillin G potassium (Nacalai Tesque, Kyoto, Japan) and 0.1 g/L streptomycin sulfate (Nacalai Tesque) at 38.5°C under 5% CO_2 in air. After reaching confluency, bovine cumulus-granulosa cells were washed with calcium- and magnesium-free phosphate-buffered saline (PBS) (–) and separated with PBS (–) containing 0.05% trypsin and 0.53 mM EDTA for 2 min at 38.5°C in a CO_2 incubator. After supplementation with 5% FBS in DMEM to inhibit trypsin activity, the cell suspension was centrifuged at 1,200 × *g* for 3 min.

Viable cells were plated at a density of 1.0×10^5 cells/mL onto 4-well culture plates (Thermo Fisher Scientific) for analysis of gene expression and western blot or 8-well slides and chambers (SPL Life Sciences Co., Ltd, Pocheon, Korea) for ROS, GSH, HSP70, and γH2AX. The cells were then cultured at 38.5°C under 5% CO_2 in air. After cells became 70% confluent, the medium was replaced with 0.9 ml of 5% FBS in DMEM together with 0.1 ml of EAS stock solution in PBS (–), and 0.1 ml of PBS (–) was added in control group. After that, the cells were cultured at 38.5°C under 5% CO_2 in air for non-HS conditions or 41°C under 5% CO_2 in air under HS conditions. A previous study showed that the maximum rectal temperature in cows was 41.1°C during summer (heat load index > 86) [22]

Experiment design

Cells were cultured at 38.5°C for 6 h with 0.5, 1, and 5 mg/mL of EAS for mRNA expression of *HSP27*, *HSP90*, *HSP70*, and 5 mg/mL of EAS for western blot analysis of HSP70 at 38.5°C for 6 h. To compare the effect of EAS under non-HS and HS conditions, cells were cultured at 38.5°C and 41°C for 6 h with 5 mg/mL of EAS for immunostaining, fluorescent staining, mRNA expression of *HSP27*, *HSP90*, *HSP70*, *heat shock factor* (*HSF*) *1*, *HSF2*, *glutathione synthetase* (*GS*), *glutamate cysteine ligase* (*GCL*), *nuclear factor erythroid 2-related factor2 (Nrf2), Kelch-like ECH-associated protein 1* (*Keap1*) *Keap1, Superoxide dismutase* (*SOD*) *1*, *SOD2*, *peroxiredoxin* (*PRDX*)*-2*, and *PRDX-6*.

To inhibit HSP70 activity, cells with 5 mg/mL of EAS supplementation were treated with 10 μM pifithrin-μ (PES) or without PES under non-HS conditions for 12 h.

To determined suitable concentration of PES, cells were treated with 5 μM, 10 μM, 20 μM PES at non HS condition for 12 h and cell viability was analysed by Live-Dead Cell Staining Kit (ALX-850-249, Enzo Life

Sciences AG, Lausen, TX, USA) according to the instructions by the manufacturer.

To determine the influence of EAS on progesterone synthesis, CG cells were cultured at 38.5°C for 12 h with 5 mg/mL of EAS.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

The cells were collected with a cell scraper and subjected to RNA extraction using ISOGEN II. All RNA samples were stored in a freezer at − 80°C until use. RNA concentration was measured by spectrophotometry (NanoDrop ND-2000; Thermo Fisher Scientific). Complementary DNA was synthesized by reverse transcription using the ReverTra Ace® qPCR RT Master Mix with gDNA remover (Toyobo Life Science, Osaka, Japan) according to the manufacturer's instructions using a thermal cycler (Astec GeneAtlas Type G Thermal Cycler; ASTEC, Fukuoka, Japan). All cDNA samples were stored in a freezer at − 30°C until further use. Specific primers (Supplementary Table 1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The relative expression levels were assessed via qRT-PCR using a LightCycler® Nano (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRDTM SYBR® qPCR Mix (Toyobo Life Science) at a final primer concentration of 0.5 μM for each primer. The thermal cycling conditions were as follows: 1 cycle at 95°C for 30 s (denaturation), followed by 45 cycles at 95°C for 10 s (denaturation), 55°C for 15 s (primer annealing), and 72°C for 30 s (extension). Relative mRNA abundance was calculated via the ΔΔCt method using H2AFZ as a reference gene.

Western blot analysis

Bovine cumulus-granulosa cells were lysed in 1% SDS (Nacalai Tesque), 1% 2-mercaptoethanol (Nacalai Tesque), 20% glycerol (Nacalai Tesque), and 50 mM Tris–HCl (pH 6.8) and denatured at 95°C for 5 min. Sample solutions were separated by electrophoresis on 10–20% gradient SDS–polyacrylamide precast gels (Atto Corporation, Tokyo, Japan). Pre-stained marker proteins with known BlueStar (range, 10–180 kDa) (cat. no. MWP03; Nippon Genetics Co., Ltd., Tokyo, Japan) were run simultaneously as standards. The electrophoretically separated proteins were transferred onto PVDF membranes using an iBlot Gel Transfer System (Invitrogen, Thermo Fisher Scientific Inc., Massachusetts, USA). The membranes were incubated in 4% skim milk (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 10 min and then washed three times with Tris-buffered saline and Tween-20 (TBS-T) at room temperature. Membranes were incubated with rabbit anti-human HSP70 polyclonal (1:1,000 dilution) (SPC-103; StressMarq Biosciences Inc.) and β-Actin monoclonal (1:1,000 dilution) (cat. no. 66009-1-Ig; Proteintech Group, Rosemont, USA) at 4°C overnight. After three washes with TBS-T, the membranes were incubated with HRP-labeled anti-rabbit IgG for HSP70 (1:25,000 dilution) (cat. no. NA934; GE Healthcare, Buckinghamshire, UK) or anti-mouse IgG secondary antibody for β-actin (1:25,000 dilution) (cat. no. NA931; GE Healthcare, Buckinghamshire, UK) or at room temperature for 1 h. Primary and secondary antibodies were diluted with an immunoreaction enhancer, Can Get Signal (Toyobo). Membranes were washed extensively with TBS-T before detection of bound antibodies using the WSE-7120EzWestLumi

plus (Atto Corporation, Tokyo, Japan) according to the manufacturer's instructions. Chemiluminescent signals were captured using the ChemiDoc System (Bio-Rad) and analyzed using ImageJ software.

Immunostaining for HSP70 and γH2AX

Bovine cumulus-granulosa cells were washed with PBS (−) and fixed in 4% paraformaldehyde diluted with PBS (−) for 15 min. After washing three times with PBS (−) for 5 min, the samples were permeabilized with PBS (−) containing 0.2% (v/v) Triton X-100 for 10 min. After washing with PBS (−), the cells were blocked with 2% (w/v) BSA (Sigma-Aldrich) in PBS (−) for 1 h at room temperature. The samples were washed with PBS (−), incubation with a Rabbit Anti-Human HSP70 Polyclonal (SPC-103; Stress Marq Biosciences Inc.) diluted 1:500 with 0.1% (w/v) BSA (Sigma-Andrich) or primary rabbit polyclonal antibody for γH2AX (ab11174; Abcam, Cambridge, MA, USA) diluted 1:1,000 in PBS (−) at 4°C overnight, was performed for immunoreaction. The samples were washed three times with PBS (−) for 5 min each and incubated for 1 h with a fluorescein-conjugated secondary antibody (Alexa Fluor® 488 donkey anti-rabbit IgG) (A21206; Thermo Fisher Scientific) diluted 1:500 for HSP70 or 1:1,000 for γH2AX with 0.1% (w/v) BSA (Sigma-Aldrich) in PBS (−) at room temperature. Cells were then washed with PBS (−) for 5 min, and 10 μL of the mounting solution (Fluoro-KEEPER Anti fade Reagent) (Non-Hardening Type with DAPI; Nacalai Tesque) was added to the samples, which were then covered with a cover glass. Then, γH2AX and HSP70 staining was examined under a fluorescent microscope with an EVOS™ M5000 Imaging System (Invitrogen, Thermo Fisher Scientific Inc., Massachusetts, USA). Fluorescence intensity was quantitated using ImageJ software.

ROS and GSH staining and detection

Cells were washed with PBS (−) and treated with 5 μM CellROX® Oxidative Stress Reagents (cat. no. C10444; Life Technologies, Carlsbad, CA, USA) for ROS staining, or 20 μM ThiolTracker™ Violet (cat. no. T10095, Molecular Probes, Eugene, OR, USA) for staining the reduced form of GSH. After each staining, the cells were incubated for 30 min at 37°C.

Fluorescence images were acquired using an EVOS™ M5000 imaging system (Thermo Fisher Scientific Inc.). Fluorescence intensity was quantitated using ImageJ software. For corrected total cell fluorescence (CTCF), we used the following formulas [23]:

CTCF = integrated density − (total area of selected cell × mean fluorescence of background readings)

CTCF per cell = CTCF/ Ncells

where "Integrated Density" is the total cell area is the integrated intensity of the pixels for all cells in the image, total cell area is the number of pixels of all of the cells, background fluorescence is the average mean gray value of nearby regions containing no cells, and Ncells is the number of cells in the image.

P4 measurement

After 12 h of incubation, the culture medium was collected and spun down. The supernatant of the culture medium was used for P4 measurement by ELISA.

Statistical analysis

All data are shown as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA), Tukey's test, and Student's t-test were performed using R (version 3.5.3; https://www.r-project.org/). Statistical significance was set at $P < 0.05$.

Declarations

Acknowledgements

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Author contributions statement

M.T. conceived the experiments, H.T.K mainly conducted the experiments, H.B. and M.K. supported bovine ovarian cell collection, K.H and J. T. conducted the EAS purification, providing and data analysis, H.T.K , T.K.K. and M.T. analysed the results. All authors reviewed the manuscript.

Additional information

There are no conflicts of interest to declare.

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Figures

Fig 1

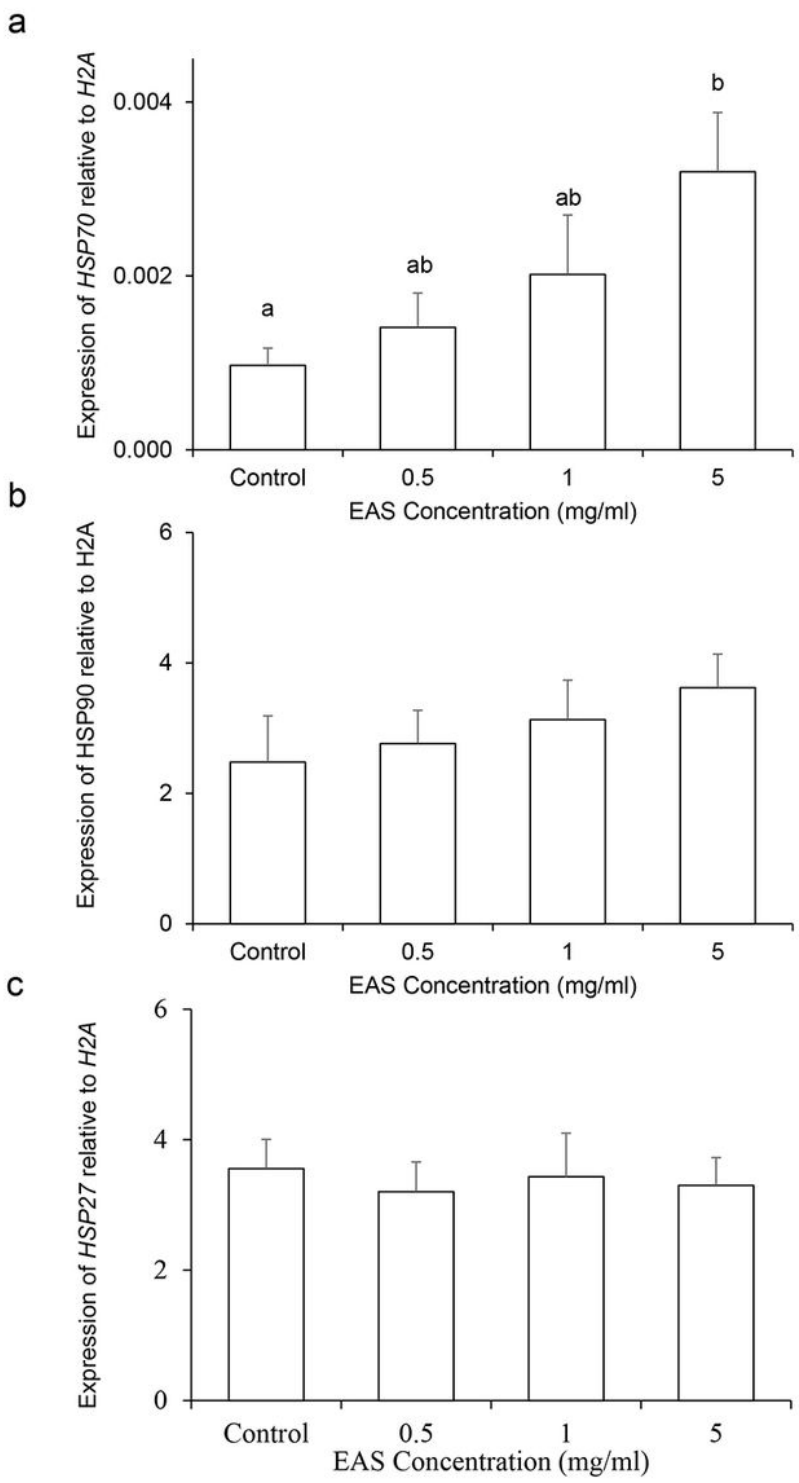

Figure 1

Effect of EAS concentration on the expression of HSP70, HSP90 and HSP27 in bovine cumulus-granulosa cells Cells were treated with various concentration of EAS (0.5, 1 and 5 mg/ml) for 6 h at 38.5°C and analysed for gene expression. The expression level of HSP70, HSP90 and HSP27 was examined using real time quantitative PCR normalized to H2AFZ as a reference gene. (a) HSP70, (b) HSP90, (c) HSP27. Data are shown as the means ± SEM, n=5, a vs b (P<0.05).

Fig 2

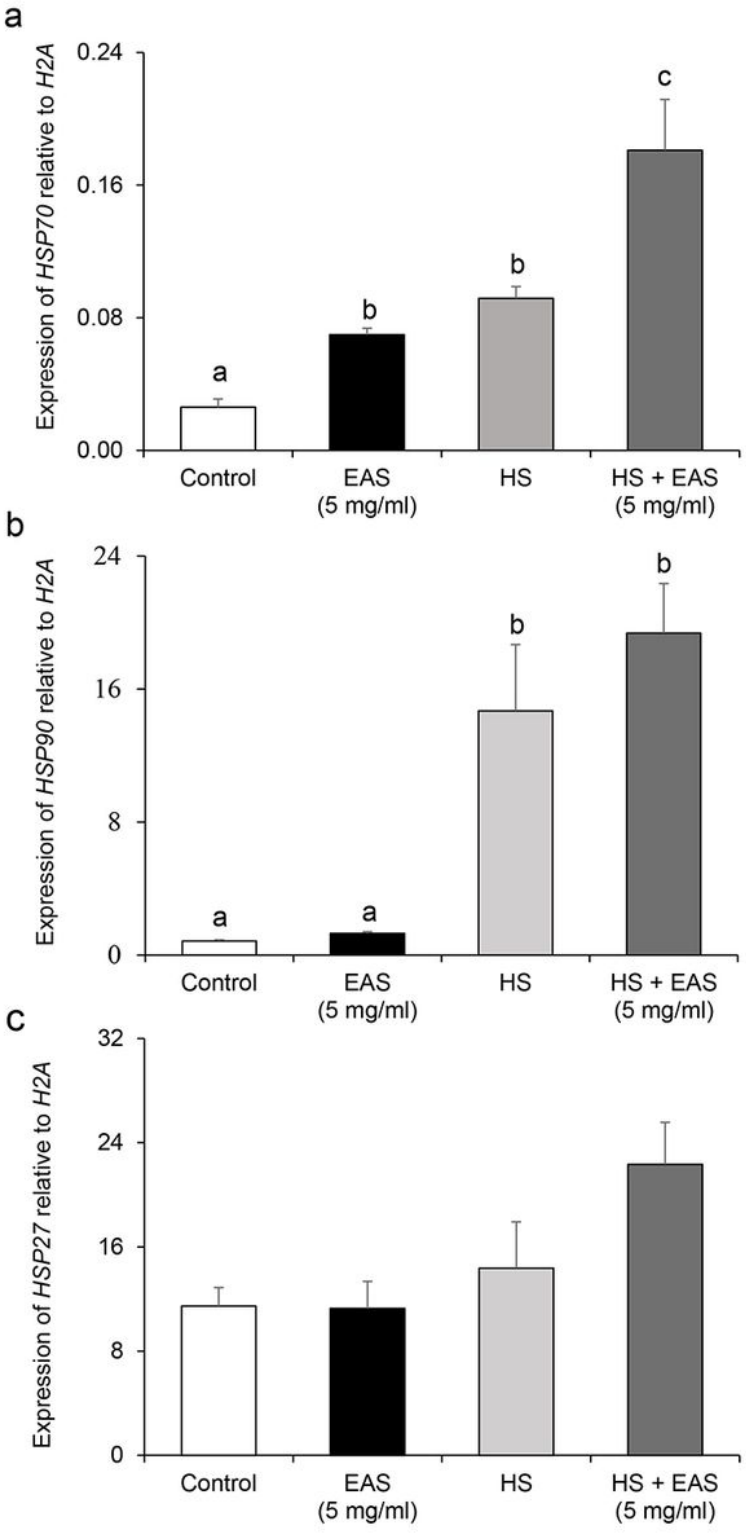

Figure 2

Effect of EAS on the expression of HSP70, HSP90 and HSP27 in bovine cumulus-granulosa cells under non and HS condition Cells were treated for 6 h with or without EAS (5 mg/ml) under normal condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). (a) HSP70, (b) HSP90, (c) HSP27. Data are shown as the means ± SEM, n=5, a vs b ($P<0.05$), a vs c ($P<0.01$), b vs c ($P<0.01$).

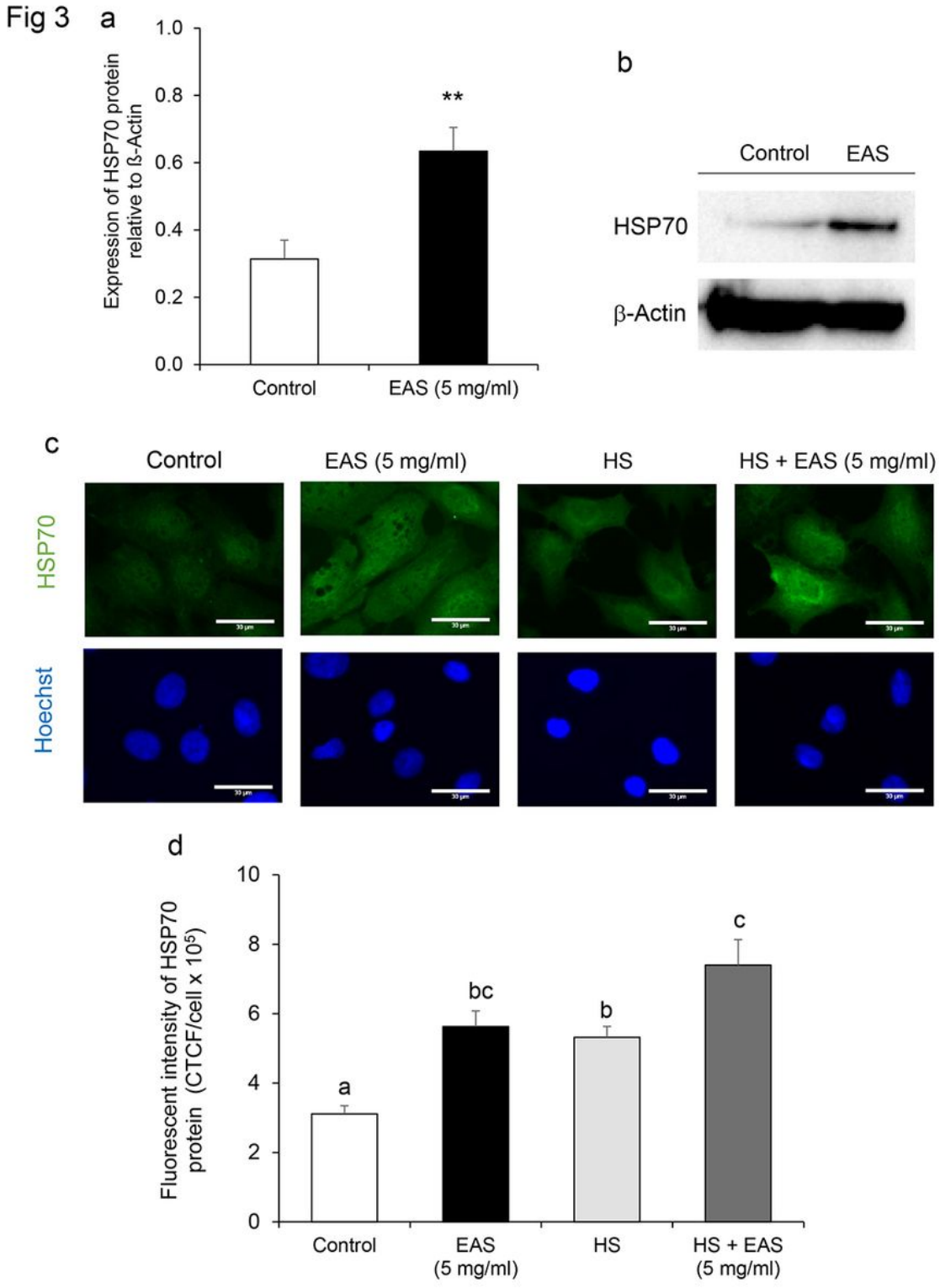

Figure 3

Effect of EAS on the expression of HSP70 protein in bovine cumulus-granulosa cells Cells were treated with 5 mg/ml of EAS for 6 h at 38.5°C. (a) The expression level of HSP70 protein was normalized to that of the β-actin. (b) Western blotting elucidates the temporal expression profile of HSP70. (c) Immunostaining for HSP70, image of Hoechst and HSP70. Immunostaining images are shown at magnification × 40 and the scale bar shows 30 μm. (d) Bars showing CTCF analysis of fluorescence

intensity. (a) Data are shown as the means ± SEM, n=5, *P<0.05 vs. Control, **P<0.01 vs. Control. (d) Data are shown as the means ± SEM, n=5, a vs b (P<0.05), a vs c (P<0.01), b vs c (P<0.05).

Fig 4

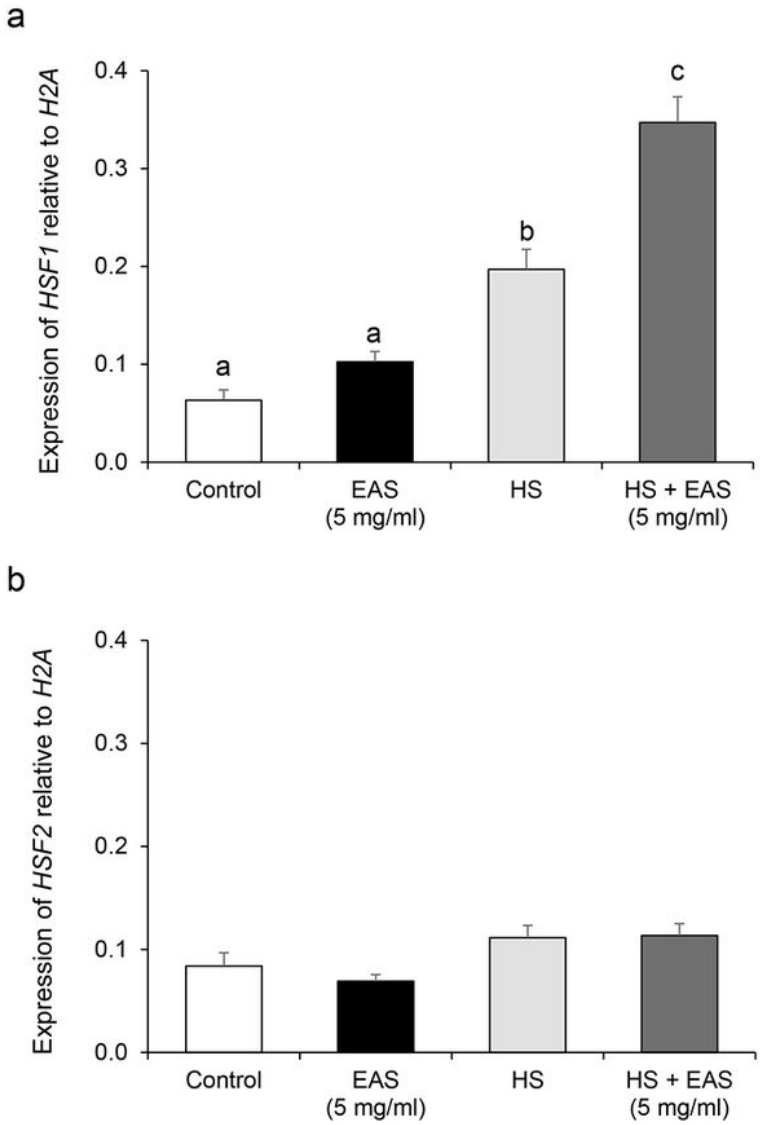

Figure 4

Effect of EAS on the expression of HSF1 and HSF2 in bovine cumulus-granulosa cells under non and HS condition Cells were treated for 6 h with or without EAS (5 mg/ml) under normal condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). The expression level of (a) HSF1

and (b) HSF2 was examined using real time quantitative PCR normalized to H2AFZ as a reference gene. Data are shown as the means ± SEM, n=5, a vs b (P<0.05), a vs c (P<0.01), b vs c (P<0.01).

Fig 5

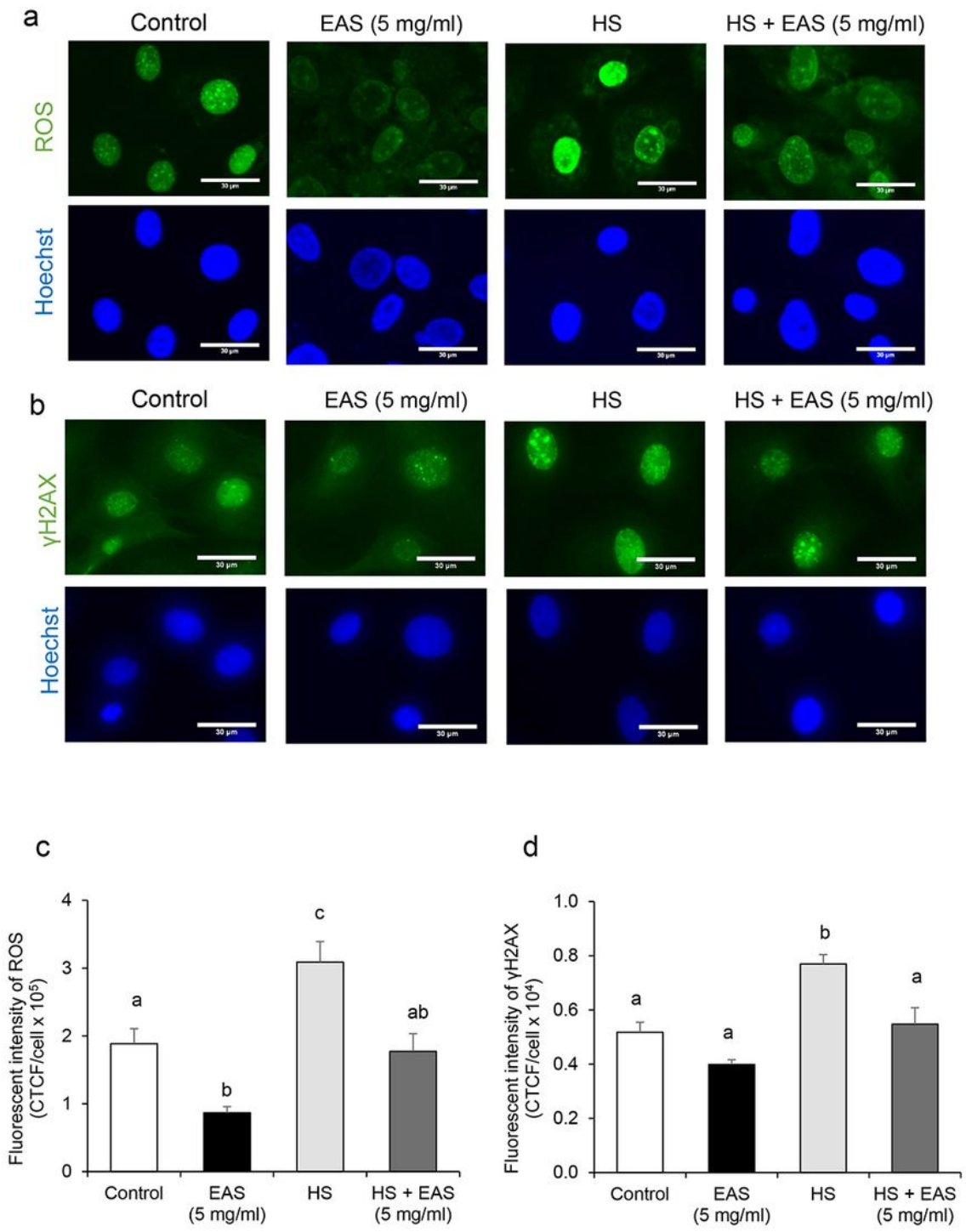

Figure 5

Effect of EAS on induction ROS and DNA damage stained γH2AX immunostaining in the bovine cumulus-granulosa cells under non and HS condition Bovine cumulus-granulosa cells were treated for 6 h with or without EAS (5 mg/ml) under normal conditions at 38.5°C (Control, EAS group) and HS conditions at

41°C (HS, HS + EAS group). (a) Fluorescence of ROS (Upper) and nuclei stained with Hoechst (Lower). (b) immunostaining for γH2AX (Upper) and nuclei stained with Hoechst (Lower). Bar shows 30 μm. (c) Fluorescence intensity of ROS (CTCF). Bar shows 30 μm. (d) Fluorescence intensity of γH2AX (CTCF). (c) Data are shown as the means ± SEM, n=5, a vs b (P<0.05), a vs c (P<0.001), b vs c (P<0.05) and ab vs c (P<0.01). (d) Data are shown as the means ± SEM, n=3, a vs b (P<0.05).

Fig 6

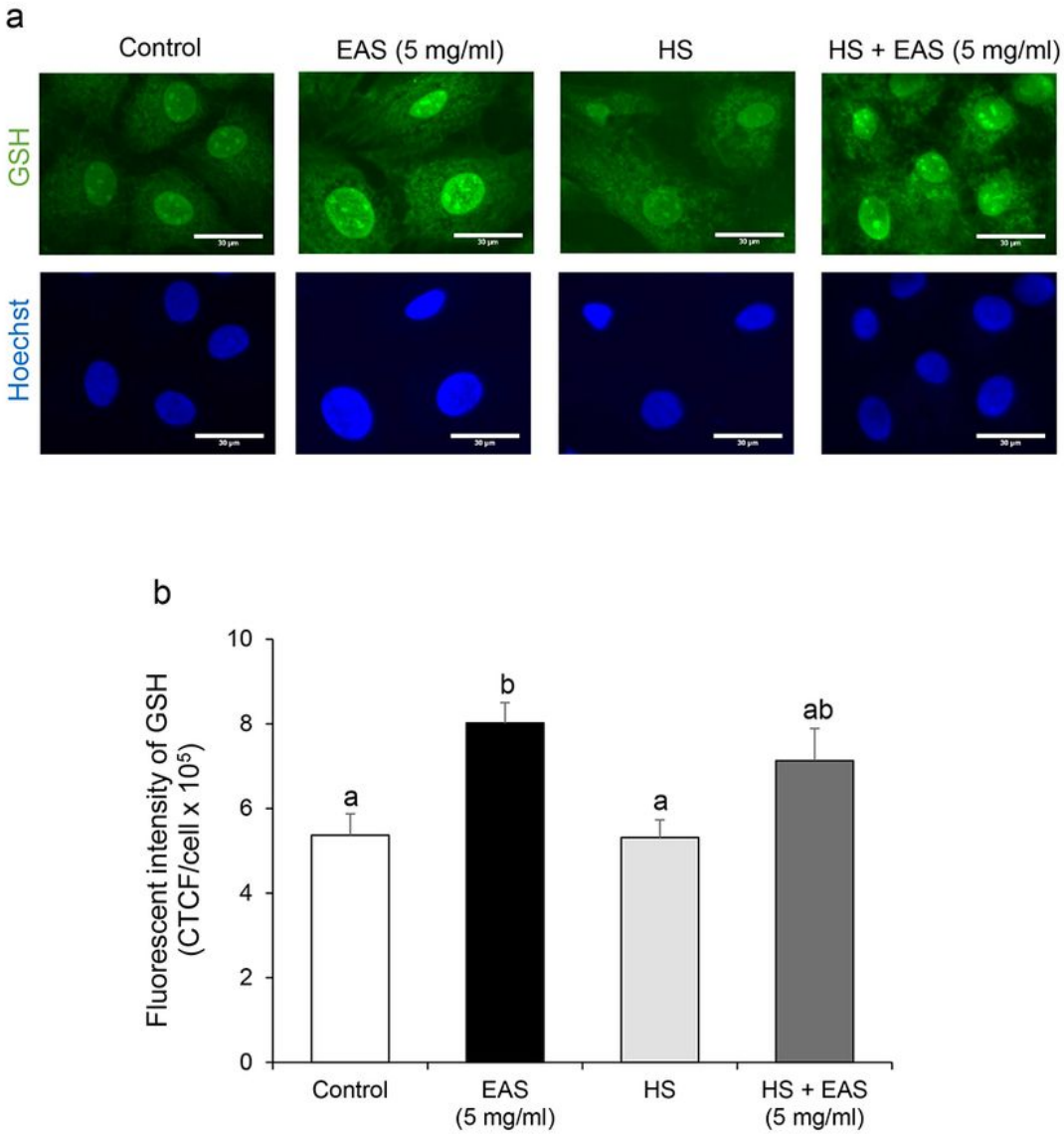

Figure 6

Effect of EAS on GSH synthesis in the bovine cumulus-granulosa cells under non and HS condition Cells were treated for 6 h with or without EAS (5 mg/ml) under normal condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). (a) Fluorescence of GSH (Upper) and nuclei stained by Hoechst (Lower). Scale bar shows 30 μm. (b) Fluorescence intensity of GSH (CTCF). Data are shown as the means ± SEM, n=5, a vs b (P<0.05).

Fig 7

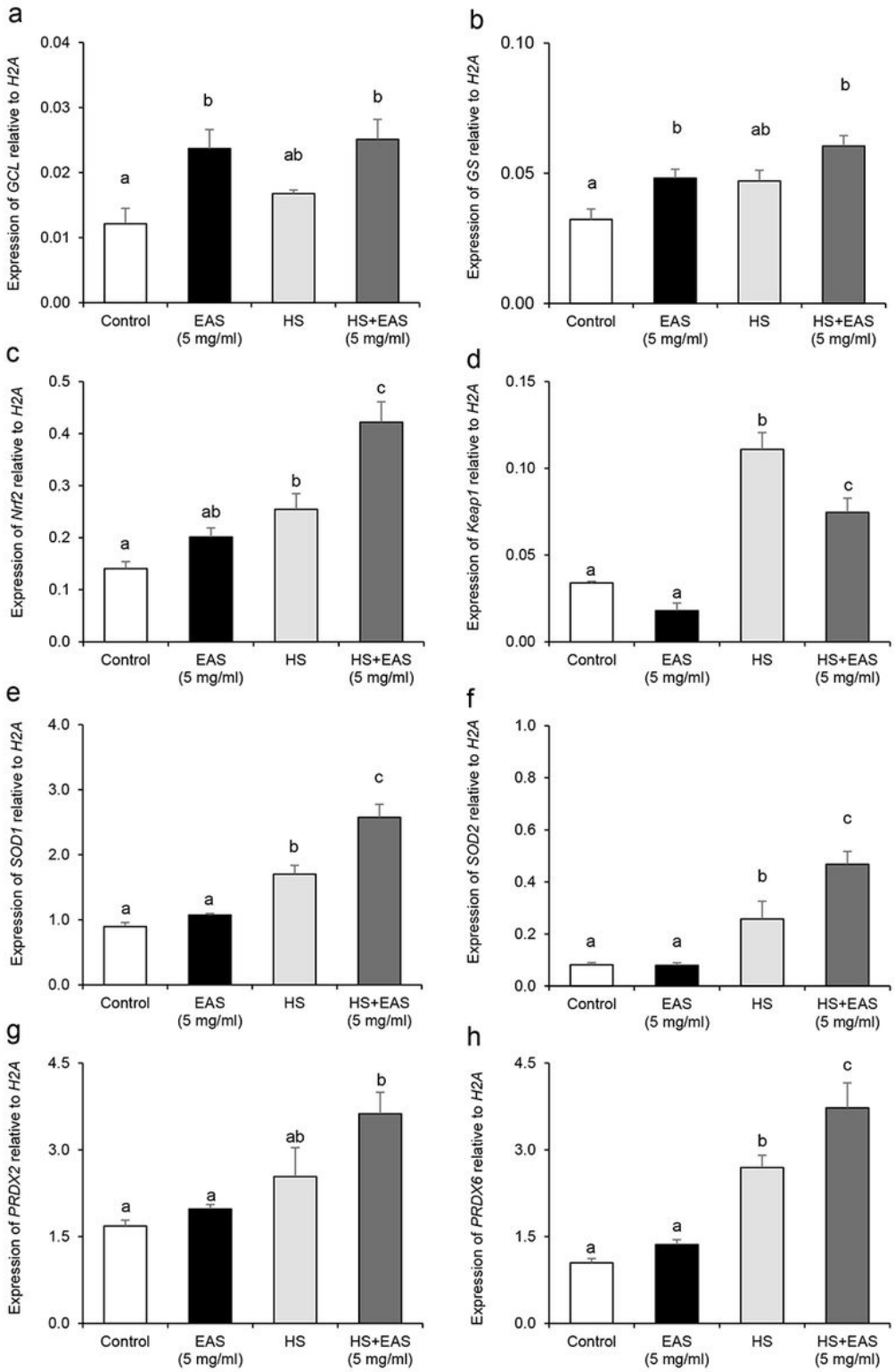

Figure 7

Effect of EAS on expressions of GS, GCL, Nrf2, Keap1, SOD1, SOD2, PRDX2 and PRDX6 under non- and HS condition Cells were treated for 6 h with or without EAS (5 mg/ml) under normal condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). The expression level of GS, GCL, Nrf2, Keap1, SOD1, SOD2, PRDX2 and PRDX6 was examined using real time quantitative PCR normalized to H2AFZ as a reference gene. (a) GCL, (b) GS, (c) Nrf2, (d) Keap1, (e) SOD1, (f) SOD2, (g) PRDX2, (h) PRDX6. Data are shown as the means ± SEM, n=5, Means with different letters (a~c) at each mRNA are significantly different ($p<0.05$).

Fig 8

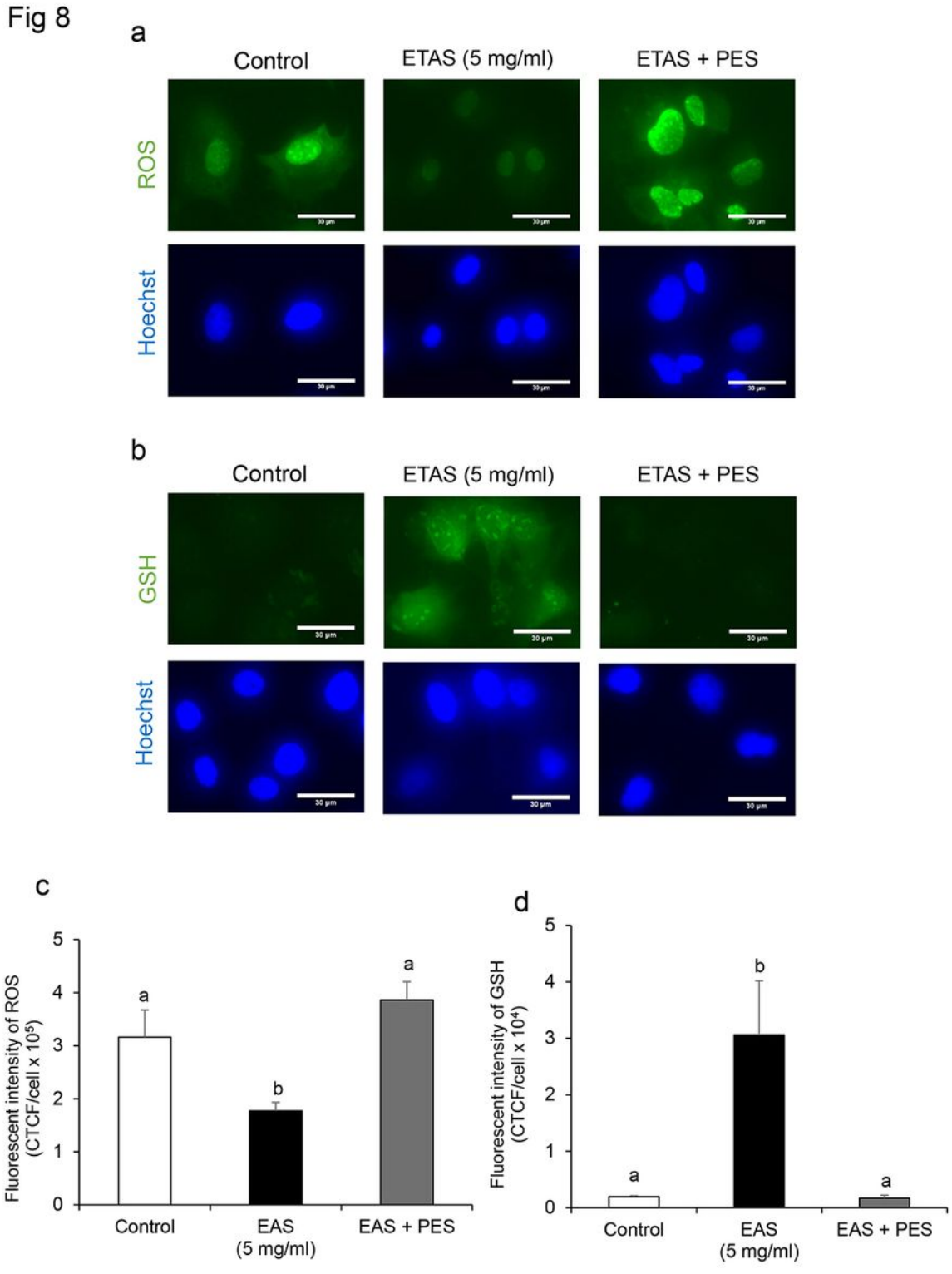

Figure 8

Effect of EAS-induced HSP70 inhibition on GSH synthesis and ROS generation in bovine cumulus-granulosa cells Cells were treated for 6 h with or without EAS (5 mg/ml) under normal conditions at 38.5°C (Control, EAS group) and 10 μM PES together with 5 mg/ml EAS (EAS + PES). (a) Fluorescence of ROS (Upper) and nuclei stained by Hoechst (Lower). (b) Fluorescence of GSH (Upper) and nuclei stained with Hoechst (Lower). Scale bar shows 30 μm. (c) Fluorescence intensity of ROS (CTCF). Bar shows 30 μm. (d) Fluorescence intensity of GSH (CTCF). (c) Data are shown as the means ± SEM, n=5, a vs b ($P<0.05$). (d) Data are shown as the means ± SEM, n=5, a vs b ($P<0.01$).

Fig 9

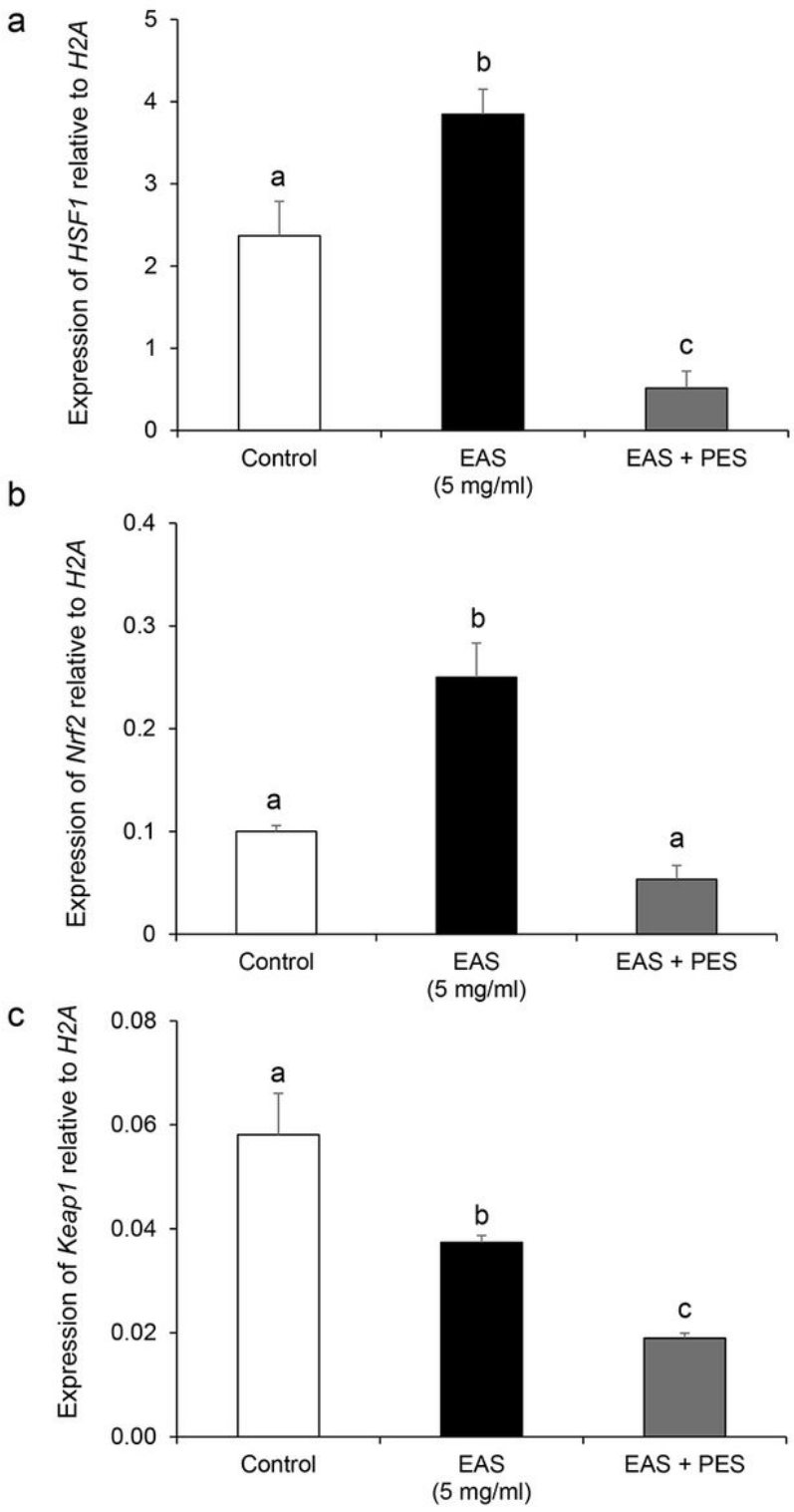

Figure 9

Effect of EAS-induced HSP70 inhibition on expression of HSF1, Nrf2 and Keap1 Cells were treated for 12 h with or without EAS (5 mg/ml) under normal condition at 38.5°C (Control, EAS group) and 10 μM PES together with 5 mg/ml EAS (EAS + PES). The expression level of HSF1, Nrf2 and Keap1 was examined using real time quantitative PCR normalized to H2AFZ as a reference gene. (a) HSF1, (b) Nrf2. Data are

shown as the means ± SEM, n=5, a vs b (P<0.01), a vs c (P<0.05), b vs c (P<0.01). (c) Keap1. Data are shown as the means ± SEM, n=5, a vs b (P<0.05), a vs c (P<0.001), b vs c (P<0.05).

Fig 10

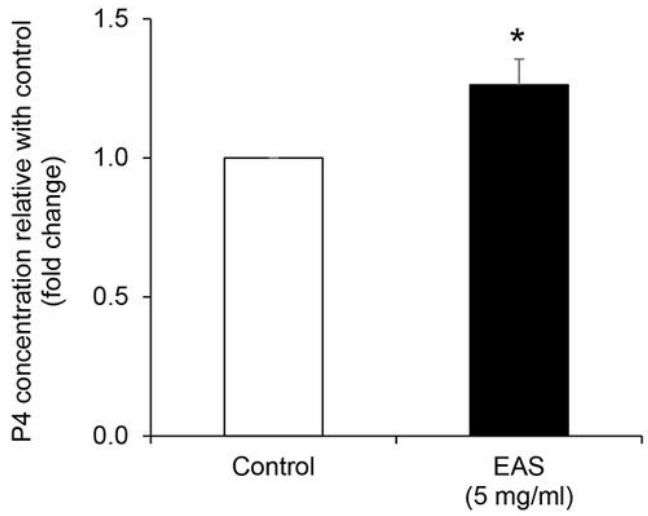

Figure 10

Effect of EAS on P4 synthesis in bovine cumulus-granulosa cells Cells were treated for 12h with or without EAS (5 mg/ml) under normal conditions at 38.5°C h (Control, EAS group). Data are shown as the means ± SEM, n=8, *P<0.05 vs. Control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CTSKoisupplTable1.docx
- CTSKhoiSupplFig.pdf