# Study on the effect of *Asparagus officinalis* stem extract on the induction of molecular chaperone and cellular function of bovine granulosa cells

**(牛顆粒膜細胞への分子シャペロン誘導と細胞機能発現に及ぼすアスパラガス茎抽出物の作用)**

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# Contents

## Abbreviation

| | |
|---|---|
| 3β-HSD | 3β-hydroxysteroid dehydrogenase |
| ANOVA | Analysis of variance |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| CL | Corpus luteum |
| $CO_2$ | Carbon dioxide |
| CYP11A1 | Cytochrome P450, family 11, subfamily A, polypeptide 1 |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| EAS | A standardized extract of *Asparagus officinalis* stem |
| ELISA | Enzyme-linked immunosorbent assay |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| GC | Granulosa cells |
| GCL | Glutamate cysteine ligase |
| GS | Glutathione synthetase |
| GSH | Glutathione |
| H2AFZ | Histone H2A family member Z |
| HS | Heat stress |
| HSF | Heat shock factor |
| HSPs | Heat shock protein |
| KEAP1 | Kelch-like ECH-associated protein 1 |
| mRNA | Ribonucleic acid |
| NFE2L2 (NRF2) | Nuclear factor erythroid 2-related factor 2 |
| P4 | Progesterone |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| PES | Pifithrin-μ |
| PFA | Paraformaldehyde |
| PRDX | Peroxiredoxin |
| PVDF | Polyvinylidene Di-Fluoride |
| ROS | Reactive oxygen species |
| RT-qPCR | Reverse-transcription quantitative polymerase chain reaction |
| SEM | Standard error of the mean |
| SOD | Superoxide dismutase |
| STAR | Steroidogenic acute regulatory protein |
| TBS-T | Tris-buffered saline and Tween-20 |
| TX red | Texas Red |

Unit measures abbreviations

| | |
|---|---|
| % | Percentage |
| × *g* | Times gravity |
| °C | Degree Celsius |
| h | Hour |
| IU | International unit |
| mg | Microgram |
| Min | Minute |
| mL | Milliliter |
| ng | Nanogram |
| nM | Nanomolar |
| V/V | Volume by volume |
| W/V | Weight by volume |
| μM | Micromolar |
| μl | Microliter |

## Abstract

The defence response to stress from outside enviroment is important for maintaining the functions of cells, tissues, organs and whole animal bodies, and therefore lives have various defence mechanisms. Heat stress (HS) negatively affects various cell functions, including intracellular protein activity, gene expressions, and viability. In contrast, cells also have a defensive response mechanism to protect themselves. Heat shock protein (HSP) 70 is a well-known HS-induced protein and acts as an intracellular molecular chaperone to protect cells against stress conditions. Although HSP70 is induced by HS to confer stress resistance to cells, simultaneously HS causes cell toxicity by increasing reactive oxygen species (ROS). Recently, a standardized extract of *Asparagus officinalis* stem (EAS), produced from the by-product of asparagus, has been shown to induce HS independent induction of HSP70 in several somatic cells such as Hela cell, liver cell and pheochromocytoma cells in relation with regulating intracellular redox balance. However, the effects of EAS on reproductive cell function remain unknown.

In this chapter 2, I investigated the effect of EAS on HSP70 induction and oxidative redox balance in cultured bovine granulosa cells (GC). EAS treatment significantly increased *HSP70* expression level, whereas no significant effect was observed on *HSP27* and *HSP90* expression level under non-HS condition. EAS treatment decreased ROS generation and DNA damage with increase in glutathione (GSH) synthesis under both non-HS and HS conditions. Moreover,

EAS synergistically increased *HSP70* and *heat shock factor* (*HSF*)*1* and progesterone (P4) levels by HS treatment in GC. In order to know the effect of EAS under HS condition, the bovine GC were exposed to HS at 41 °C along with control groups (38.5 °C). Besides, treatment with an HSP70 inhibitor significantly increased ROS level, decreased GSH level, and decreased *HSF1*, *nuclear factor erythroid 2-related factor 2* (*NFE2L2*, also known as *NRF2*), and *Kelch-like ECH-associated protein 1* (*KEAP1*) in the presence of EAS. Furthermore, EAS treatment significantly increased P4 synthesis in CG. Thus, EAS improves HSP70-mediated redox balance and cell function in bovine CG.

P4 is a well-known steroid hormone and has role in ovarian function including oocyte growth and maintenance of pregnancy in mammals. HS by high ambient temperature in summer season is known to decrease P4 synthesis in the ovarian corpus luteum (CL) with collapsing a balance of intracellular redox status. P4 biosynthesis occurs in mitochondria. For utilizing, cholesterol from lipid droplet is transported into the mitochondrial inner membrane by steroidogenic acute regulatory protein (STAR). The enzyme Cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) conversed cholesterol to pregnenolone which is catalysed into P4 by 3β-hydroxysteroid dehydrogenase (3β-HSD).

In chapter 3, to clarify the steroidgenesis pathway including mitochondrial could be affected by HS and possibly be controled by HSP70, I investigated the effect of EAS on P4 level, expression of steroidogenesis genes, mitochondrial activity and lipid metabolism of GC under non-HS and HS conditions. P4 level was increased

by EAS treatment in association with the increase in *STAR, 3β-HSD*, mitochondrial membrane activity and lipid droplet both under non-HS and HS conditions. Notably, synergistic effect of EAS with HS co-treatment was observed to show a greater increase in P4 synthesis when comparison with EAS treatment under non-HS condition. Furthermore, inhibition of HSP70 significantly reduced EAS-induced P4 synthesis, mitochondrial activity and synthesis of lipid droplets. Overall results show that EAS-induced P4 synthesis is mediated by activation STAR and 3β-HSD pathway together with improvement mitochondrial and lipid metabolism through HSP70-mediated redox balance and chaperone function in bovine GC.

The main purpose of cell freezing is to maintain sufficient cell number to provide a stable cell supply and fewer passage number. Freezing of bovine GC is commonly applied as coculture cells with supporting the embryo growth for embryo transfer and donor cells for nuclear transfer. However, little is known about the effect of EAS pre-freezing treatment on post-freezing viability and redox status.

In chapter 4, I investigated the effect of EAS on the post-freezing viability of HS treated bovine GC in relation to the redox balance. HS pre-treatment significantly decreased the post-freezing viability, whereas EAS pre-treatment significantly increased the post-freezing viability of HS treated cells. ROS levels were increased by HS and decreased after EAS pre-treatment. GSH levels were increased by EAS pre-treatment. These results suggest that EAS affects the post-

freezing viability of HS bovine GC by improving intracellular molecular chaperones and the redox status.

In conclusion, EAS improved reproductive function and cellular protection in bovine GC. Moreover, synergistic effect of EAS and HS co-treatment on HSP70 production can contribute to improvement of P4 synthesis and cellular survival.

Chapter 1 **General Introduction**

By the end of the 21st century, the average global temperature is estimated to increase from 0.3°C to 4.8°C, and it may cause a negative impacts on animal production efficiency (Rojas-Downing et al., 2017). During rising of average global temperatures, dairy cattle are also at high risk of heat stress (HS) which leads to the reduction in milk yield, feed intake, average daily gain, and reproductive functions. Harmful effect of HS caused by the increase in body temperature, which inhibits the ovarian and uterine functions (Sartori et al., 2002; Collier et al., 2006; Nabenishi et al., 2011; Roth, 2015). The average body temperature of cattle under normal condition is 38.6 ± 0.5°C (Andersson B.E., 1993). However, maximum rectal temperature in cattle raised to 41.1 °C during summer (Lees et al., 2019). Therefore, increase in body temperature of cattle negatively affects various aspects of reproduction such as secretion of reproductive hormones, ovarian functions, including oocyte growth, fertilization, embryo development and implantation (Sartori et al., 2002; Roth, 2015).

Bovine oocyte–cumulus cell complexes, including an oocyte that main subject for ovulation, fertilization and formation of an embryo is surrounded by granulosa and cumulus cells which responsible for maturation signals and hormones secretion for growing and maturation of oocyte (Su et al., 2004; Voronina et al., 2007). Growth of the ovarian follicle can be affected by increase of environment temperature (de S Torres-Júnior et al., 2008). HS reduced about 60% fertility in cattle by hinder steroid production and follicular characteristics in follicles (Roth

et al., 2001b). In the cattle, reduction of steroid production has been led to compromising the quality/competence of oocyte and finaly reduces pregnancy rate (Rensis and Scaramuzzi, 2003). Nowadays, HS reduced oocyte maturation in bovine oocyte during *in vitro* maturation (Nabenishi et al., 2012; Maya-Soriano et al., 2013). HS bovine oocyte showed damages in intracellular protein synthesis of (Edwards et al., 1997), cortical granule translocation to the oolemma (Payton et al., 2004), and proper rearrangement of the cytoskeletal elements (Roth and Hansen, 2005). Moreover, the harmful effect of HS on GC are also reported to decrease the developmental competence of the oocytes through inhibiting glutathione synthesis (deMatos et al., 1997) which works as protecting against the oxidative damage during maturation (Tatemoto et al., 2000). *In vivo* study indicated that HS modified gene expression in GC that lead to decrease follicular growth in dairy cows (Vanselow et al., 2016). HS also reduced steroidogenesis in bovine GC and altered matrix metallopeptidase 9 of bovine cumulus cells under *in vitro* study (Rispoli et al., 2013; Li et al., 2016). Collectively, impaired function of GC may cause dysfunction in oocyte growth and ovarian functions which lead to disorder fertility of cow under HS condition.

The intracellular redox status contributes to important cellular functions, such as proliferation, differentiation, protein synthesis and cell death, and is defined as the balance between oxidants (or pro-oxidants) and antioxidants (Hiroi et al., 2005). Reactive oxygen species (ROS) such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide are free radicals that cause damage to cells by

breaking DNA strand, lipid peroxidation and inactivation of enzymes (Guerin et al., 2001). The increase of ROS production also activate cell death signaling pathway through the imbalance of the redox status of the cells (Murdoch, 1998). On the other hands, antioxidant response pathway, which produce antioxidants as role for removal of ROS by activating antioxidant genes including catalase, peroxiredoxin, superoxide dismutase and GSH have important roles to protect the cells against ROS (Amin et al., 2014; Espinosa-Diez et al., 2015). In addition, accumulated evidences indicate that HS induces ROS and reduces GSH in bovine oocyte (Roth, 2015). Taken together, HS impairs ovarian function through dysregulation of redox balance by inducting ROS and reducing GSH in mammals, including cattle.

Activation of defense system to HS is triggered in various ways including the induction of heat shock response proteins (Calderwood et al., 2012). Heat shock protein (HSP)s that work as molecular chaperones are known to fold, assemble and transport of proteins damaged under HS conditions (Hendrick and Hartl, 1993). HSPs are classified according to molecular weight: HSP of 27-kDa (HSP27) 60-kDa (HSP60), 70-kDa (HSP70), 90-kDa (HSP90) and 100-kDa (HSP100) (Jeng et al., 2015). The main HSPs induced by HS are HSP70 (J.Y. Li et al., 2016; Khan et al., 2020). In the cellular level, HSP70 works as a primary component of folding processes, as well as the folding and assembling newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organellar and secretory proteins, and manage of the activity of

regulatory proteins (Bukau et al., 2000; Hartl and Hayer-Hartl, 2002). The folding of non-native proteins by HSP70 contains three activities, 1) prevention of protein aggregation, 2) promotion of folding to the native state, and 3) solubilization and refolding of aggregated proteins (Mayer and Bukau, 2005). Under HS condition, HSP70 enhances tolerance of cell by binding and stabilizing protein against denaturation or aggregation (Mayer and Bukau, 2005). In addition, stressed cells induce expression of HSP90 which is responsible for cell cycle, meiosis and cytokinesis (McClellan et al., 2007). Similarly, HSP27, a smaller member of the HSP family, induced to protect cellular function in HS treated cell (Parida et al., 2020). Therefore, molecular chaperone is important for cellular survival under HS condition, HSP70 is one of key factors for improvement cellular survival and function.

However, it should be noted that HSP induction by HS simultaneously increase the generation of intracellular ROS. Therefore, for protecting cells against HS-induced stress, improving HSP as chaperone without oxidative stress is important to maintain the cell functions.

Generally, HSPs have role in cellular protection and function against environmental stress, especially by HS. Overexpression HSP70 was induced by whole body HS or gene transfer in heart and brain tissue (Massa et al., 1996; Carroll and Yellon, 1999). However, HS or gene transfer induced toxic effect to the cells and tissues, thus it would be extremely helpful to discover non-toxic or natural chaperone inducer for apply to prevention or treatment against injuries

causing by stress factors and disease relation with protein misfolding and protein aggregation.

A number of studies have revealed many chaperone inducer compounds, and they are classified as non-steroidal anti-inflammatory drugs, arachidonic acid and prostaglandins, anti-ulcer drugs and herbal medicines (Ohtsuka et al., 2005).

Herbal medicines are extraction from plants and have advantages as natural and safety. Notably, compounds as Paeoniflorin, Dihydrocelastrol and Celastrol induces HSP70 by itself without HS that are known as HSP70 inducers (Ohtsuka et al., 2005). In contrast, Bimoclomol only induced HSP70 in combination with HS that definition as HSP70 co-inducer (Hargitai et al., 2003). Interestingly, isolation from plants as Paeoniflorin and Celastrol have both HSP70 inducer and co-inducer function, so they induced cytoprotection under HS condition (Westerheide et al., 2004; Ohtsuka et al., 2005).

In the present thesis, HS decreased post-freezing viability while HSP70 inducer increased post-freezing viability of HS treated cells. Collectively, HSP70 inducers from plant sources have potential application to enhance HSP70 both *in vitro* and *in vivo* environment and potential synergistic effect of HSP70 inducer and HS may critical asset to improve additional cell survival and function under high temperature environment.

In this context, asparagus (*Asparagus officinalis* L.) is a potential candidate because of its role as herbal medicine for the treatment the disease (Kim et al., 2009). In addition, functional food ingredients are more valuable when they

promote the effective use of uneatable parts such as stem which is abandoned during the processing. A standardized extract of *Asparagus officinalis* stem (EAS) is produced from the unused bottom part of asparagus, and EAS ingestion induced HSP70 production in blood of adult men (Tomohiro Ito et al., 2014). In liver cells, *Asparagus officinalis* Extracts protect the cells against ROS-induced toxicity (Kim et al., 2009). In adult rat, aqueous crude extract from *Asparagus officinalis L.* roots enhanced ovarian follicles, CL and serum levels of estrogen, and progesterone (Jashni et al., 2016). Collectively, EAS as HSP70 inducer may improve cell survival and steroidogenesis by enhance redox balance or chaperone function.

In this study, I investigated the effect of EAS from *Asparagus officinalis* on HSP70 production and oxidative redox balance which may influence on cryoprotection and steroidogenesis in bovine GC.

Chapter 2 **Effect of a standardized extract of *Asparagus officinalis* stem on HSP70 induction, redox balance and cell functions in bovine granulosa cells under non-HS and HS conditions**

***Introduction***

In the ovarian follicles, GCs constitute complexes with oocyte and play a vital role in the oocyte growth, maturation and acquisition of developmental competence in mammalian oocytes as well as hormone synthesis (Fatehi, 2005). Cumulus cells and oocytes communicate metabolically via gap junctions, which provide important points of entry for nutrient transfer and signaling between both cells (Russell et al., 2016). The absence of GC has harmful effects on the maturation, fertilization, and embryo development in cattle (Leibfriedrutledge et al., 1989). Besides, GC enhance the nuclear and cytoplasmic maturation of oocytes during maturation (Suzuki et al., 2000) and fertilization rate (Nandi et al., 1998). In addition, GC protect oocytes against oxidative stress damage during maturation (Tatemoto et al., 2000). After ovulation, the remaining GC differentiate into large luteal cells to form corpus luteum (CL) (Abedel-Majed et al., 2019). Bovine luteal cells produce P4, an important steroid hormone, to maintain pregnancy (Rekawiecki et al.). In addition, the *in vitro* environment has a higher concentration of $O_2$, which is a source of ROS, than the *in vivo* environment (Luvoni et al., 1996). Cumulus cells have been suggested to play a critical role in defending bovine oocytes against cell damage due to ROS production (Fatehi, 2005). Moreover, during *in vitro* maturation, cumulus–oocyte complexes exhibit higher levels of

GSH than cumulus-denuded oocytes, and bovine GC contribute to GSH synthesis in cumulus–oocyte complexes (Tatemoto et al., 2000). Therefore, bovine GC play a major role in keeping the intracellular redox status by GSH synthesis, which can reduce ROS. As a result, the balance between ROS and GSH levels can prevent cells by maintaining the intracellular redox balance.

Previous studies have evaluated the effect of ROS on DNA damage, which causes toxicity and induces cell death (Kuo and Yang, 2008). In contrast, GSH, synthesized from the γ-glutamyl cycle, is one of the major antioxidants present in high concentration in mammalian cells, and provides a powerful antioxidant defense against oxidative stress (Meister, 1982). The appropriate balance between ROS production and GSH levels can reduce DNA damage by modulating the cellular redox status, thus improving cell survival.

HSP70 is known as a molecular chaperone that assists in the folding, unfolding, and homeostasis of cellular proteins (Saibil, 2013). The main function of HSP70 allows the cell to protect against several stresses, such as physical, chemical, and environmental stresses (Kregel, 2002). Experimental evidence has suggested that HSP70 expression regulates both GSH and ROS generation, indicating an inter-relationship between HSP70 and the redox status (Guo et al., 2007). Various plant sources have been studied to identify the HSP70 inducing activity (Hirakawa et al., 1996; Vigh et al., 1997; Yan et al., 2004; Hesselink JM, 2016).

Several products, such as isolation from *Paeonia lactiflora*, isoprenoid compound geranylgeranylacetone, and hydroxylamine compounds bimoclomol,

have been found to induce HSP70 (Hirakawa et al., 1996; Vigh et al., 1997; Yan et al., 2004; Hesselink JM, 2016). Bimoclomol induces HSP expression under HS conditions but does not influence HSP activity under non-stress conditions (Hargitai et al., 2003). Paeoniflorin and geranylgeranylacetone increased HSP70 expression under HS conditions in experiments on human neuroblastoma cells (Yan et al., 2004). These data suggested that the HSP70 inducer and HS have a potential synergistic effect on *HSP70* expression.

EAS has been discovered to increase expression of HSP70 gene and protein in human cervical adenocarcinoma cell lines (Tomohiro Ito et al., 2014). EAS contains active ingredients, such as asparaprolines, that enhance HSP70 expression (Inoue et al., 2020). In addition, asparaprolines has been found to increase *HSP70* mRNA levels in a human promyelocytic leukemia cell line (Inoue et al., 2020). In *in vivo* study, EAS increased HSP70 protein level in mouse organ (Tomohiro Ito et al., 2014). In addition, EAS also enhanced protection against damage caused by nitric oxide donor sodium nitroprusside or the hypoxia mimic reagent cobalt chloride in neuron cells (Sakurai et al., 2014). These evidence suggest that EAS is a potential inducer of HSP70, which may regulate the balance between GSH and ROS generation.

In this chapter, I investigated the effect of EAS on HSP70 induction and oxidative redox balance in cultured bovine GC.

***Materials and Methods***

EAS, which is produced from the sterm of asparagus (*A. officinalis L.*) grown in Hokkaido, was provided by Amino Up Co., Ltd. (Sapporo, Japan). EAS was manufactured according to a previously described method (Ito et al., 2013).

*Bovine GC cell culture*

Bovine ovaries were obtained 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), which is not showing obvious of atresia, using a disposable 18-gauge needle attached to a 10-mL syringe. Aspirated follicular fluids contaning cumulus-oocyte complexes follicular cells were transferred into a sterile plastic Petri dish, and cumulus-oocyte complexes were picked up. Then, the suspension of follicular fluid with floating GC were carefully transferred into 15 mL tubes. Follicular fluid with floating GC were then resuspended 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 overnight incubation, attached theca cell clusters were gently peeled from the culture dish surface. Bovine GC remaining in the culture dish were washed with calcium- and magnesium-free phosphate-buffered saline (PBS) (–) and cultured in 5% FBS in DMEM at 38.5°C under 5% $CO_2$ in air. After reaching

70% confluency, bovine GC were washed with PBS (–) and dissociated from the substratum 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 $1.0 \times 10^5$ cells/mL onto 4-well culture plates (Thermo Fisher Scientific Inc., Massachusetts, USA) or 8-well slides and chambers (SPL Life Sciences Co., Ltd, Pocheon, Korea) and cultured at 38.5°C under 5% $CO_2$ in air. After cells became 70% confluency, 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. The cells were then cultured at 38.5°C (Control; bovine normal body temperature) or at 41°C (HS condition; bovine body temperature in high temperature) under 5% $CO_2$ in air.

*Experimental design*

Cells were cultured for 6 h at 38.5°C with 0.5, 1, and 5 mg/mL of EAS to examine mRNA expression of *HSP27*, *HSP90* and, *HSP70*. For western blot analysis of HSP70, cells were cultured with 5 mg/mL of EAS for 6h at 38.5°C. To compare the effect of EAS treatment under non-HS and HS conditions, cells were cultured for 6 h at 38.5°C and 41°C with 5 mg/mL of EAS for western blotting, immunostaining, fluorescent staining and mRNA expression of *HSP27*, *HSP90*, *HSP70*, *HSF1 HSF2*, *glutamate cysteine ligase (GCL)*, *glutathione synthetase*

*(GS), NRF2, KEAP1, superoxide dismutase (SOD)1, SOD2, peroxiredoxin (PRDX)2 and PRDX6* listed in table 1.

To investigate the effect of induced HSP70, cells with 5 mg/mL of EAS supplementation were treated with or without pifithrin-μ (PES) (StressMarq Biosciences Inc., Victoria, Canada), inhibitor of HSP70, under non-HS conditions for 12 h.

To determine the optimal concentration of PES, cells were treated with 5 μM, 10 μM, or 20 μM PES under non-HS conditions for 12 h and cell viability was analyzed using the Live-Dead Cell Staining Kit (ALX-850-249, Enzo Life Sciences AG, Lausen, TX, USA) according to the manufacturer's instructions.

To determine the influence of EAS on P4 synthesis, GC were cultured for 12 h at 38.5°C with 5 mg/mL of EAS.

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

After 12h of culture, all media were removed, and the attached GC were washed with PBS (–). After removal of PBS (–), cells were lysed by ISOGEN II (Nippon Gene, Tokyo, Japan) for RNA extraction according to the manufacturer's instruction. All RNA samples were stored at −80°C until use. RNA concentration was measured using spectrophotometry (NanoDrop ND-2000; Thermo Fisher Scientific Inc.). Complementary DNA was synthesized by reverse transcription of 0.1 ug/uL of total RNA 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 at –30°C until qPCR analysis. Specific primers (Supplemental list, 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 THUNDERBIRD 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 seconds (denaturation), 45 cycles at 95°C for 10 seconds (denaturation), 55°C for 15 seconds (primer annealing), and 72°C for 30 seconds (extension). Relative mRNA abundance was calculated using the ΔΔCt method, with Histone H2A family member Z (*H2AFZ*) as a reference gene.

*Western blotting*

After culture with EAS, GC were lysed in lysis buffer consisting 1% SDS (Nacalai Tesque), 1% 2-mercaptoethanol (Nacalai Tesque), 20% glycerol (Nacalai Tesque)in 50 mM Tris–HCl (pH 6.8) and denatured at 95° C for 5 min. Sample solutions were separated by electrophoresis with 10–20% gradient SDS–polyacrylamide precast gels (Atto, Tokyo, Japan). At the same time, pre-stained marker proteins (Bluestar, 10-180kDa, Nippon Genetics, Tokyo, Japan) were used as molecular standards. The electrophoretically separated proteins in the

polyacrylamide gel were transferred onto Polyvinylidene Di-Fluoride (PVDF) membranes using an iBlot Gel Transfer System (Thermo Fisher Scientific). The PVDF membranes were then incubated in 4% skim milk (FUJIFILM Wako Pure Chemical, Osaka, Japan) for 10 min and then washed three times with Tris-buffered saline and Tween-20 (TBS-T) at room temperature. Membranes were then incubated with a rabbit anti-human HSP70 polyclonal antibody (1:1,000 dilution) (SPC-103; StressMarq Biosciences Inc.) and β-Actin monoclonal antibody (1:1,000 dilution; cat. no. 66009-1-Ig; Proteintech Group, Rosemont, USA) in Immunoreaction enhancer solution 1 (Can Get Signal™, Toyobo Co., Ltd, Osaka, Japan) at 4°C overnight. After 3 times of washing 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) for 1 h at room temperature. The primary and secondary antibodies were diluted with an immunoreaction enhancer, Can Get Signal (Toyobo). Membranes were washed with TBS-T before detection of bound antibodies using the WSE-7120EzWestLumi plus (Atto). Chemiluminescent signals were captured using LumiCube (Liponics, Tokyo, Japan). The intensity of the bands was analysed using ImageJ software v1.52A (National Institutes of Health; http://imagej.nih.gov/ij/).

*Immunostaining of HSP70 and γH2AX*

After treatment, bovine GC were fixed in 4% paraformaldehyde (PFA) diluted with PBS (–) for 15 min. After washing the cells 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 the cells with PBS (–), they were blocked with 2% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, MO, US) in PBS (–) for 1 h at room temperature. The samples were washed with PBS (–) and incubated with a rabbit anti-human HSP70 polyclonal antibody (SPC-103; Stress Marq Biosciences, British Columbia, Canada) diluted 1:500 with 0.1% (w/v) BSA (Sigma-Andrich) or rabbit anti γH2AX (ab11174; Abcam, Cambridge, MA, USA) polyclonal antibody for diluted 1: 1,000 in PBS (–) at 4°C overnight. After primary antibody treatment, the samples were washed three times with PBS (–) for 5 min 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 diluted with 0.1% (w/v) BSA (Sigma-Aldrich) in PBS (–) at room temperature. Cells were then washed with PBS (–) for 5 min, and then mounted with 10 μL of the mounting solution (Fluoro-KEEPER Anti Fade Reagent) (Non-Hardening Type with DAPI; Nacalai Tesque) and covered with a cover glass. Fluorescence images were acquired using an EVOS M5000 Imaging System (Thermo Fisher Scientific, Massachusetts, USA). Fluorescence intensity was quantified using ImageJ software.

*Detection of ROS and GSH*

After treament, cells were gently washed with PBS (–) and incubated at 38.5°C for 30 min with 5 μM CellROX Oxidative Stress Reagents (cat. no. C10444; Life Technologies, Carlsbad, CA, USA) for ROS staining. For GSH staining, treated cells were gently washed with PBS (–) and incubated with 20 μM ThiolTracker Violet (cat. no. T10095, Molecular Probes, Eugene, OR, USA) for 30 min at 38.5°C.. Nuclei were stained with 25 μg/mL Hoechst 33342 (Sigma-Aldrich) and incubated for 30 min at 38.5°C..

Fluorescence images were acquired using an EVOS™ M5000 imaging system (Thermo Fisher Scientific). Fluorescence intensity was quantified using ImageJ software v1.52A (National Institutes of Health; http://imagej.nih.gov/ij/). For corrected total cell fluorescence (CTCF), we used the following formulas (Gavet and Pines, 2010):

1. CTCF = integrated density (total area of selected cells × mean fluorescence of background readings)
2. CTCF per cell = CTCF/ Ncells

where “Integrated Density” 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 that was measured by counting fluorescently labelled nuclei from images.

*P4 measurement*

After treatment, culture medium was collected and centrifuged. The supernatant of the culture medium was used to measure P4 with enzyme-linked immunosorbent assay using commercial kit by following manufacturer's instructions (ADI- 900– 011, Enzo Life Sciences, PA, USA).

*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 $P < 0.05$.

***Results***

*Effect of EAS on the induction of HSPs*

To determine the HSP70-specific expression and concentration of EAS in bovine GC, expressions of *HSP27*, *HSP70*, and *HSP90* were examined with various concentrations of EAS (0.5, 1, and 5 mg/mL). A significant increase ($P < 0.05$) in *HSP70* expression was observed after treatment of 5 mg/mL EAS (Figure 1a). In contrast, *HSP90* and *HSP27* were not significantly induced by EAS (Figure 1b and c) at non-HS culture condition (38.5°C). Since HSPs are induced by HS, I investigated the effect of EAS on expressions of *HSP27*, *HSP70*, and *HSP90* after 6 h of HS (41°C) treatment in bovine GC in comparison with that under non-HS conditions. Similarly, EAS treatment specifically increased *HSP70* (Figure 2a), whereas the expressions of *HSP90* (Figure 2b) and *HSP27* (Figure 2c) were not induced under control conditions.

HS treatment significantly increased *HSP70* (Figure 2a) and *HSP90* (Figure 2b). Interestingly, expression of *HSP70* was synergistically increased by HS and EAS (Figure 2a). Similar to the increase of *HSP70* mRNA level, HSP70 protein level was also significantly increased ($P < 0.05$) by 5 mg/mL EAS treatment both under control and HS conditions (Figure 3a, b). Significant increase of HSP70 ($P < 0.01$) was also observed by HS and/or EAS (Figure 3). The immunolocalization of HSP70 protein also showed an increase by EAS treatment and/or HS condition in GC (Figure 4).

HSF1 is a transcription factor that induces *HSP70* under HS condition (Lindquist, 1986). In addition, HSF1 and HSF2 were co-expressed in most tissue

and cell (Pirkkala et al., 2001), and HSF2 enhance *HSP* expression through interact with HSF1 (Östling et al., 2007). Therefore, I investigated whether *HSF1* and *HSF2* were induced by EAS and/or HS as a HSP70 inducer in bovine GC.

As shown in Figure 5a, *HSF1* expression was significantly increased by HS and HS + EAS, however, significant difference was not observed in expression of *HSF1* by single treatment of EAS. Besides, no effect of HS and HS + EAS on *HSF2* expression was also observed (Figure 5b).

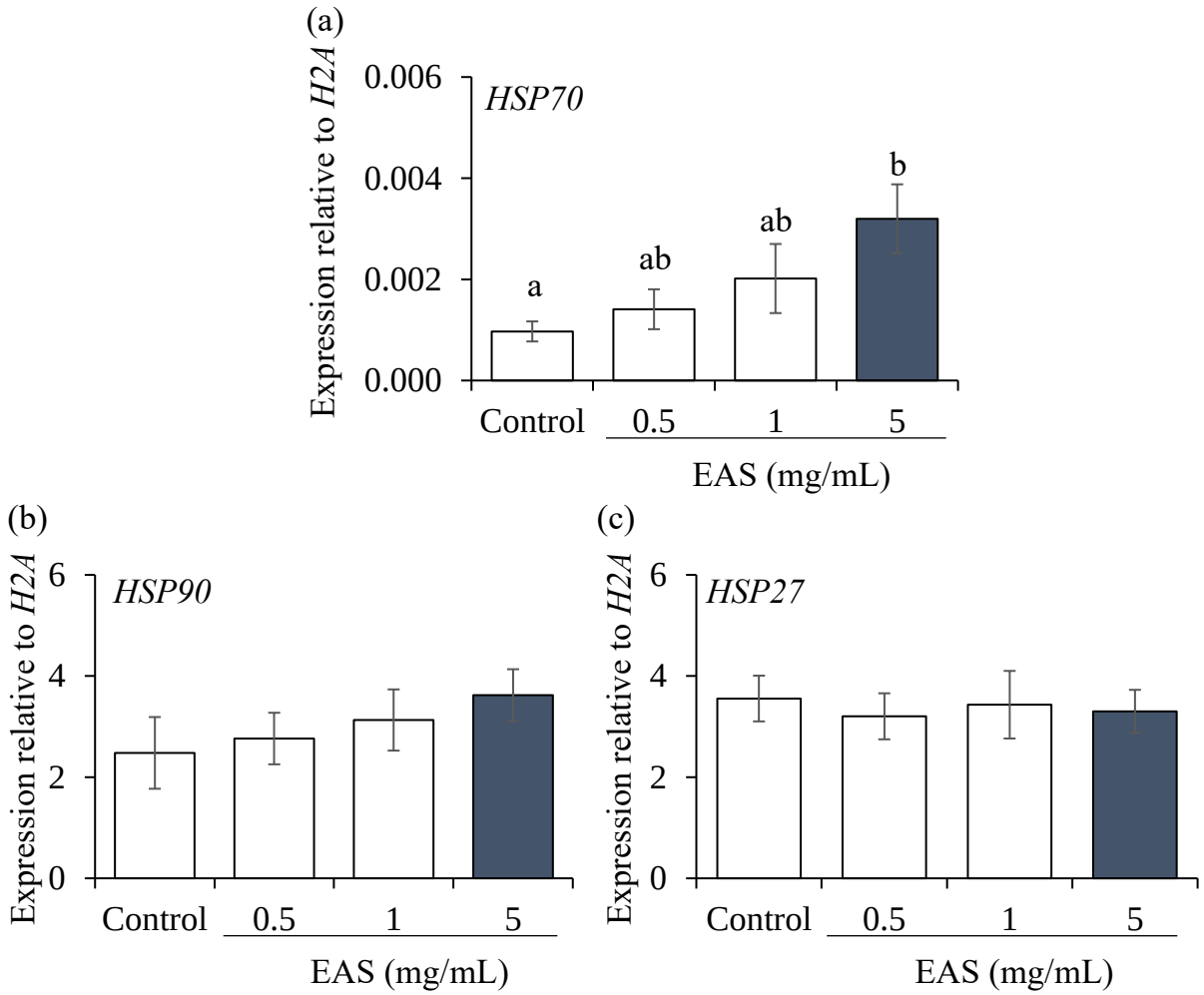


**Figure 1.** Effect of EAS on the expression of HSP70, HSP90, and HSP27 in bovine GC

Cells were treated with various concentrations of EAS (0.5, 1, and 5 mg/mL) for 6 h at 38.5° C and analysed for gene expression. The expression levels of HSP70, HSP90, and HSP27 were examined using real-time quantitative PCR by normalizing to H2AFZ as a reference gene. **(a)** HSP70, **(b)** HSP90, and **(c)** HSP27. Data are shown as the mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.05$).

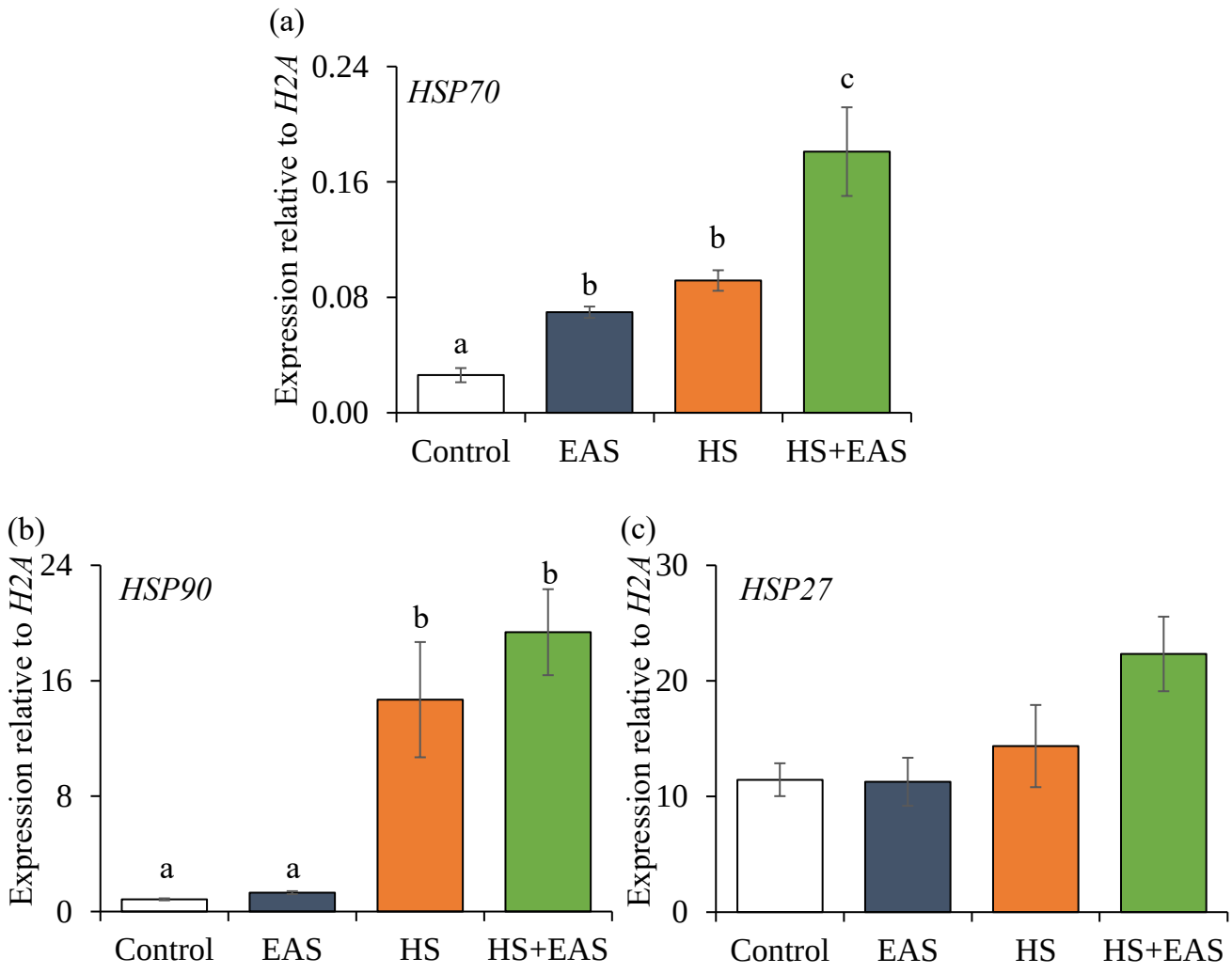


**Figure 2**. Effect of EAS on the expression of HSP70, HSP90, and HSP27 in bovine GC under non-HS and HS conditions

Cells were treated for 6 h with or without EAS (5 mg/mL) under non-HS condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). **(a)** HSP70, **(b)** HSP90, and **(c)** HSP27. Data are shown as the mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.05$), a vs. c ($P < 0.01$), and b vs. c ($P < 0.01$).

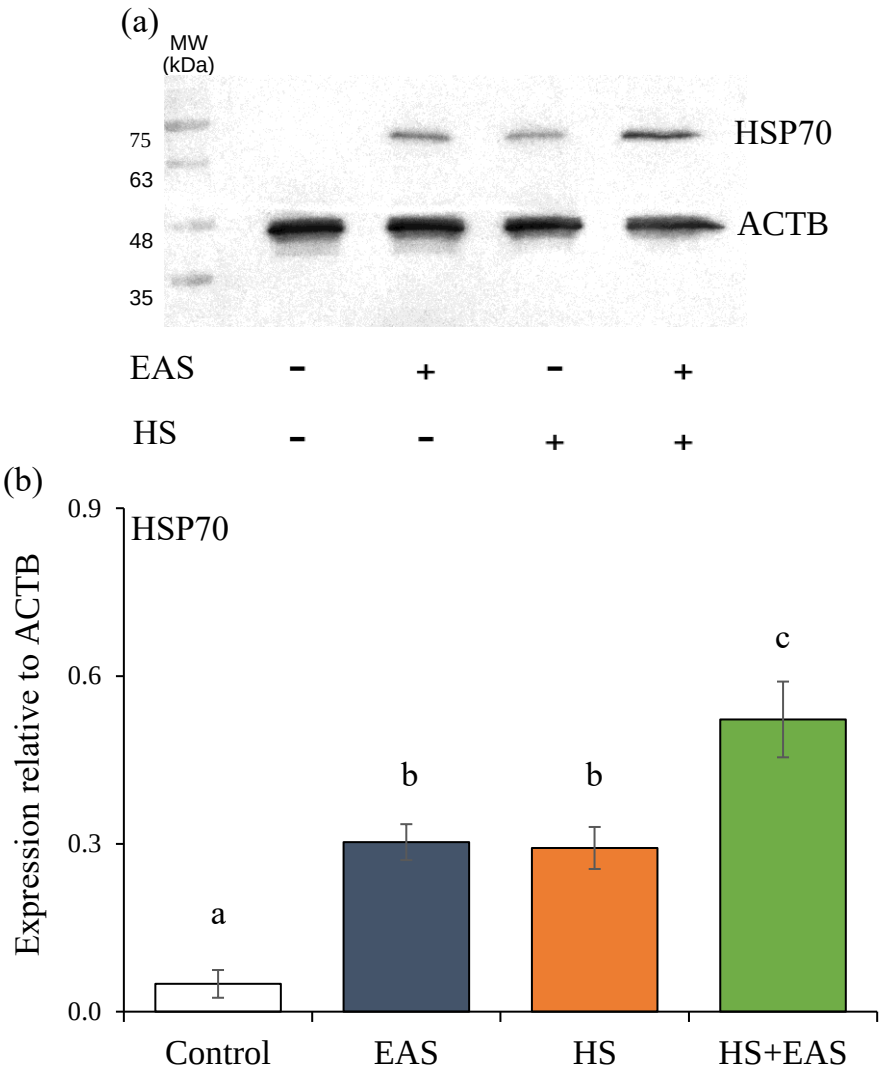


**Figure 3.** Effect of EAS on the expression of HSP70 protein in bovine GC

Cells were treated for 6 h with or without EAS (5 mg/mL) under non-HS conditions at 38.5°C (Control, EAS group) and HS conditions at 41°C (HS, HS + EAS group). **(a)** Expression of HSP70 (upper bands) and ACTB (lower bands) with or without HS and EAS. Left lane shows the molecular marker from 35 to 75 kDa. **(b)** Quantitative analysis of HSP70. The expression level of HSP70 was normalized to that of ACTB. Data are shown as the mean ± SEM. Experiment were repeated 3 times. a vs. b ($P < 0.05$), a vs. c ($P < 0.01$), and b vs. c ($P < 0.05$).

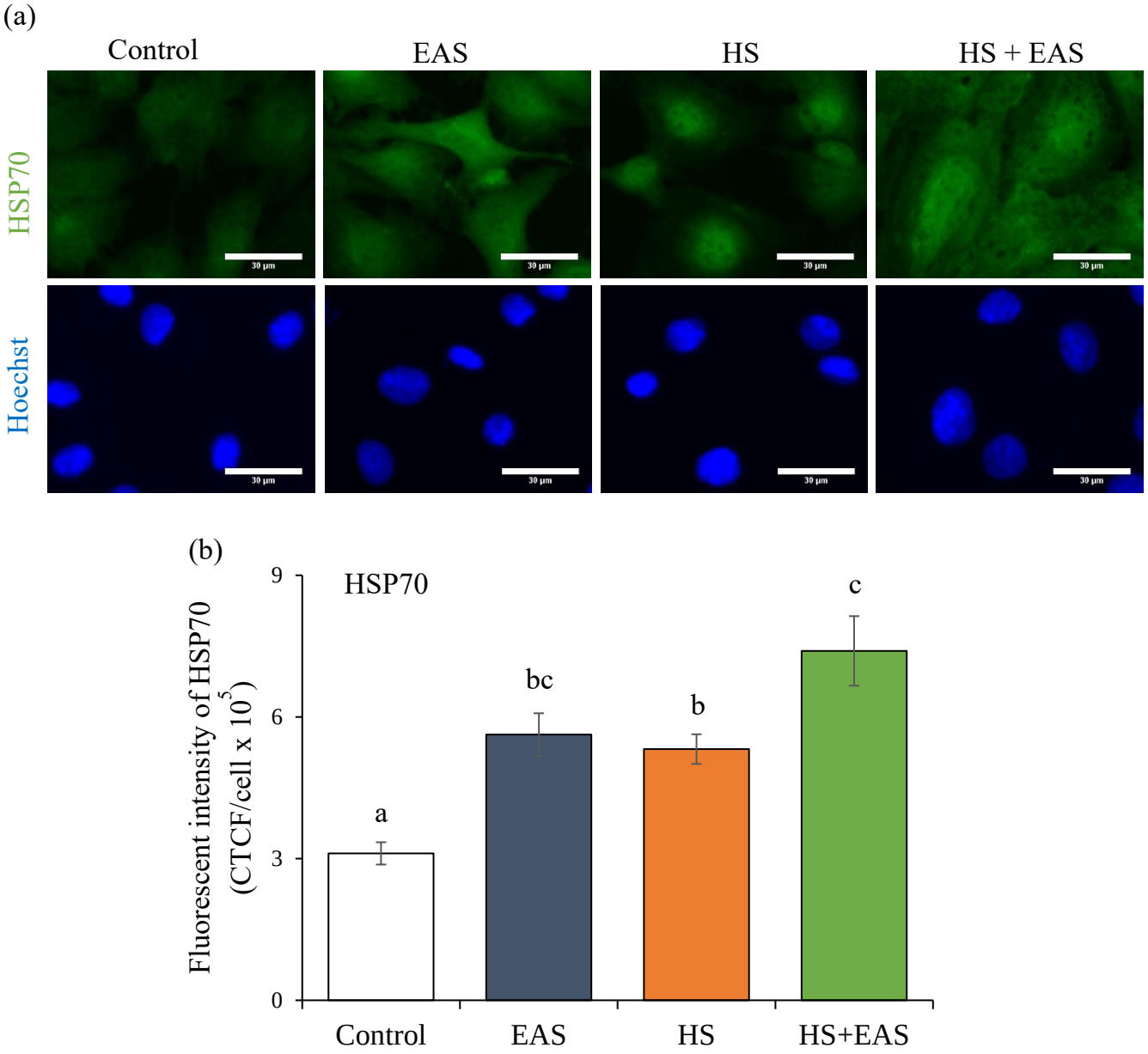


**Figure 4**. Immunodetection of HSP70 in bovine GC treated with EAS and HS

Cells were treated for 6 h with or without EAS (5 mg/mL) under non-HS condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). **(a)** Immunostaining for HSP70 and nuclear staining by Hoechst. Scale bar shows 30 µm. **(b)** Expression of HSP70 by CTCF analysis of fluorescence intensity. Data are shown as the mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.05$), a vs. c ($P < 0.01$), and b vs. c ($P < 0.05$).

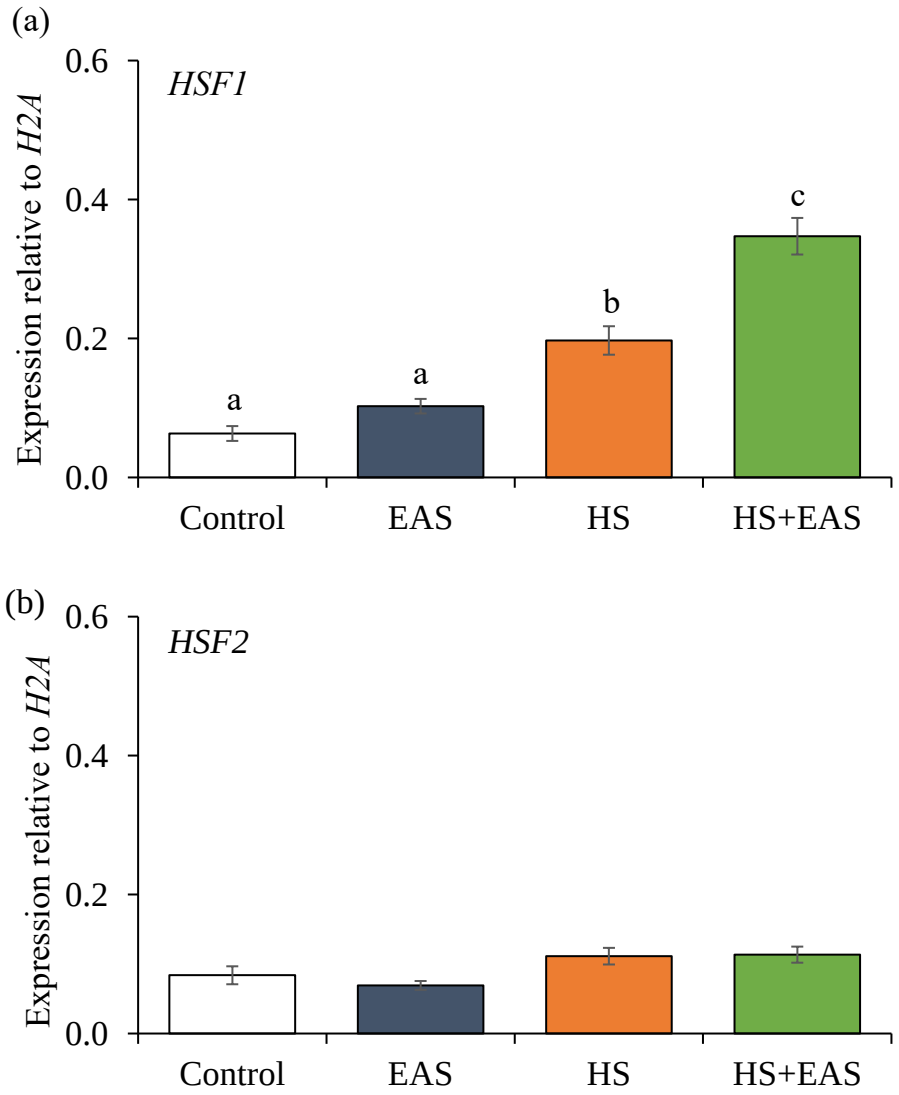


**Figure 5.** Effect of EAS on the expression of HSF1 and HSF2 in bovine GC under non-HS and HS conditions

Cells were treated for 6 h with or without EAS (5 mg/mL) under non-HS condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). The expression levels of **(a)** HSF1 and **(b)** HSF2 were analyzed by real-time quantitative PCR normalized to H2AFZ as a reference gene. Data are shown as the mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.05$), a vs. c ($P < 0.01$), and b vs. c ($P < 0.01$).

*Effect of EAS on ROS generation*

Since expressions of HSP70 gene and protein were induced by EAS treatment in bovine GC, I investigated the effect of EAS on the role of HSP70 and oxidative and redox balance.

As shown in figure 6a, ROS production was mainly detected in the nucleus of bovine GC. EAS treatment significantly reduced ROS levels compared with control under non-HS ($P < 0.05$, Figure 6b). HS groups showed highest ROS levels compared with other treatment groups. Interestingly, EAS strongly reduced ROS levels of HS treated cells ($P < 0.05$, Figure 6b), and ROS levels of HS + EAS co-treated cells was similar with single treatment of EAS and control cells (Figure 6b).

*Effect of EAS on DNA damage based on γH2AX levels*

ROS are well known to cause apoptosis associated with mitochondrial dysfunction and single and double-stranded DNA breaks (Sottile and Nadin, 2018). HS increase the ROS generation, which triggers DNA damage (Shahat et al., 2020). Therefore, I hypothesized that EAS could reduce DNA damage caused by HS-induced ROS. Similar with ROS localization, γH2AX protein, a marker for DNA damage, expressed mainly in nucleus of bovine GC (Figure 7a). γH2AX level of HS treated cells were highest when compared with other treatment groups ($P < 0.05$, Figure 7b). However, γH2AX level was significantly reduced by combining treatment of HS and EAS ($P < 0.05$, Figure 7). On the other hand, no

significant difference was found between γH2AX level of single treatment of EAS and control bovine GC (Figure 7b).

*Effect of EAS on GSH synthesis*

GSH was detected more strongly in the nuclei than in the cytoplasm of bovine GC (Figure 8a). EAS treatment significantly increased GSH levels ($P < 0.05$) in non-HS treated cells (Figure 8b). Results from determined fluorescence intensity, GSH levels were not changed by HS treatment (Figure 8b). However, combination of HS and EAS treatment tended to be higher in GSH synthesis level than control and HS treatment groups ($P = 0.1$, Figure 8b).

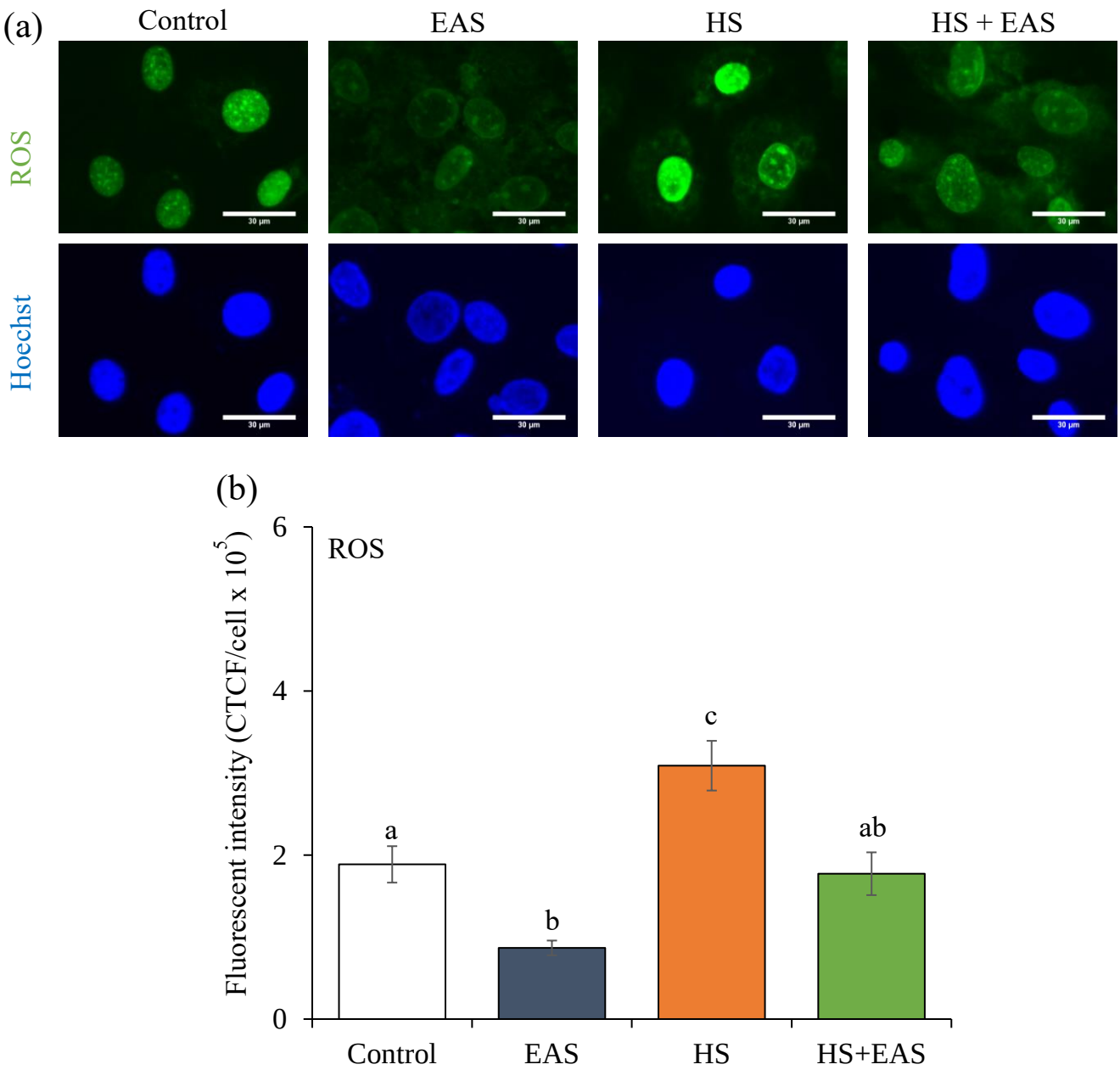


**Figure 6.** Effect of EAS on induction ROS in the bovine GC under non-HS and HS conditions

Bovine GC were treated for 6 h with or without EAS (5 mg/mL) under non-HS condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). **(a)** Fluorescence of ROS (upper) and nuclei stained with Hoechst (lower panel). **(b)** Fluorescence intensity of ROS (CTCF). Bar shows 30 µm. Data are shown as the mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), b vs. c ($P < 0.05$), and ab vs. c ($P < 0.01$).

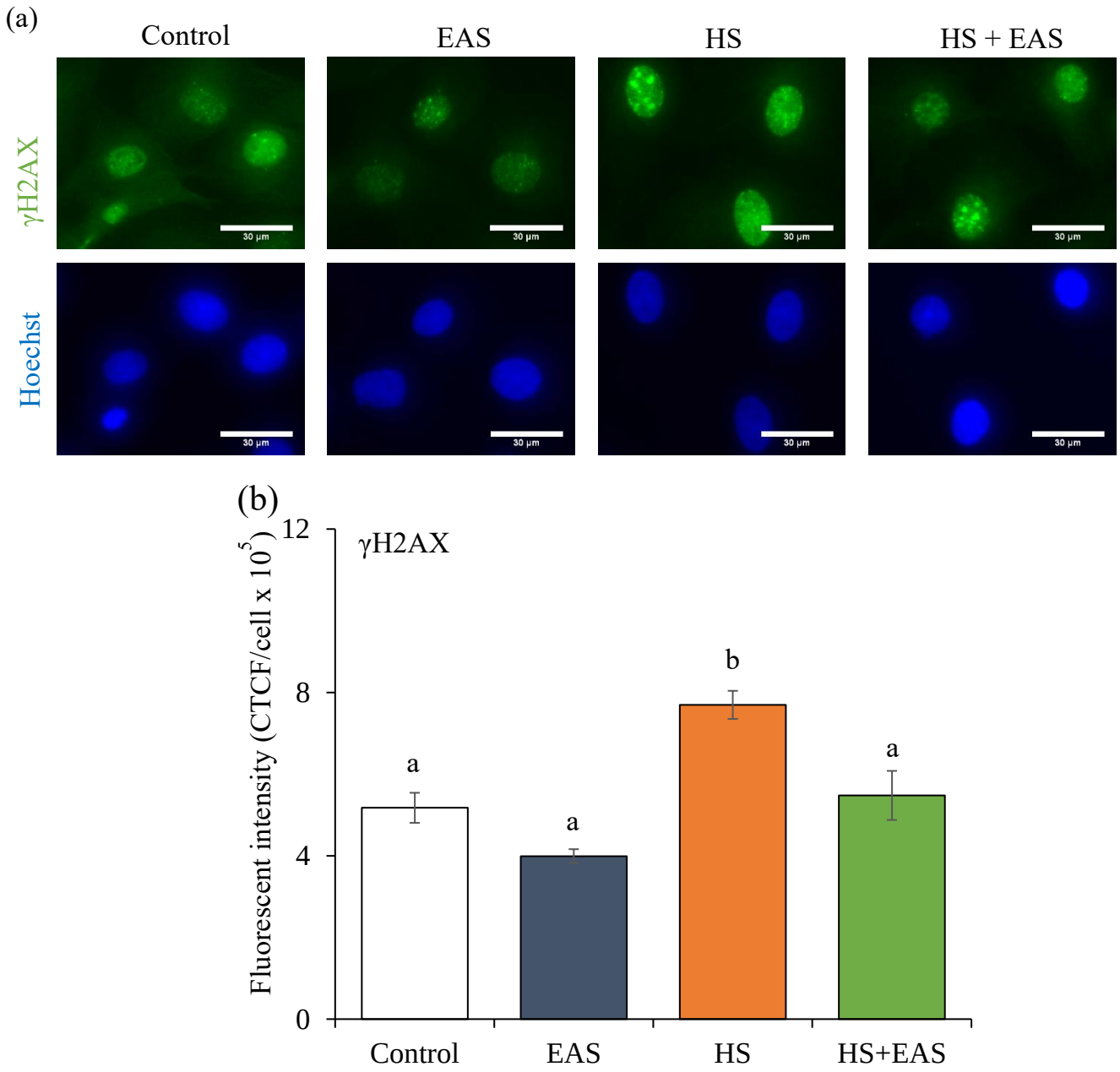


**Figure 7.** Effect of EAS on DNA damage stained γH2AX immunostaining in the bovine GC under non-HS and HS conditions

Bovine GC were treated for 6 h with or without EAS (5 mg/mL) under non-HS condition at 38.5°C (Control, EAS group) and HS condition at 41°C (HS, HS + EAS group). **(a)** Immunostaining for γH2AX (upper panel) and nuclei stained with Hoechst (lower panel). Bar shows 30 μm. **(b)** Fluorescence intensity of γH2AX (CTCF). Data are shown as the means ± SEM. Experiment were repeated 3 times. a vs b ($P < 0.05$).

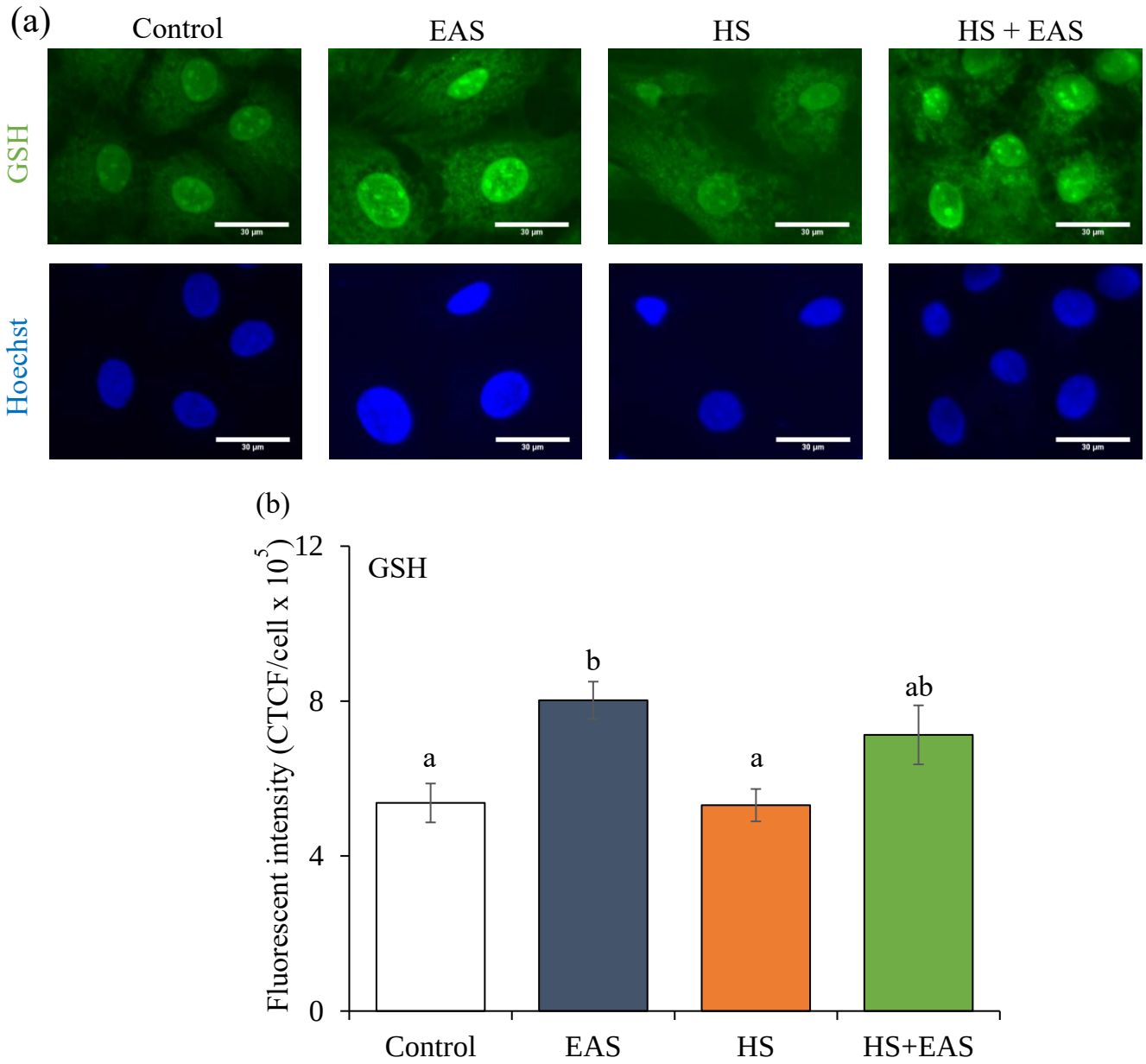


**Figure 8.** Effect of EAS on GSH synthesis in the bovine GC under non-HS and HS conditions

Cells were treated for 6 h with or without EAS (5 mg/mL) under non-HS 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 with Hoechst (lower). Scale bar shows 30 µm. **(b)** Fluorescence intensity of GSH (CTCF). Data are shown as the mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.05$).

*Effect of EAS treatment on expression of genes related to oxidative stress and redox balance*

To determine whether EAS protects against DNA damage by maintaining the redox status, I focused on the expressions of genes related to oxidative stress and redox balance. The expression of *GCL* and *GS*, which are involved in GSH synthesis in mammalian cells (Bachhawat and Yadav, 2018), was significantly increased by EAS treatment under non-HS conditions (Figure 9a, b). Since GSH synthetic pathways, including GCL and GS activation, are dependent on NRF2 regulation (Tchouague et al., 2019), I analysed the expression levels of *NRF2* and *KEAP1*. The expression of both *NRF2* and *KEAP1* was significantly increased by HS treatment (Figure 9c, d). The *NRF2* levels in the HS + EAS (5 mg/mL) groups were significantly higher than the HS group ($P < 0.01$) (Figure 9c). In contrast, the HS + EAS (5 mg/mL) group had a lower *KEAP1* mRNA expression than the HS group ($P < 0.01$) (Figure 9d).

Extract of *Asparagus officinalis L.*is known to contain high levels of antioxidants, such as carotenoids, steroidal saponins, and flavonoids, and antioxidant activity has been demonstrated in other extracts of this vegetable (Makris and Rossiter, 2001). Thus, I investigated whether EAS has similar antioxidative abilities to induce the expression of antioxidant enzymes, including SOD1, SOD2, PRDX2 and PRDX6 under non-HS condition. The expression of *SOD1* and *SOD2* was significantly increased by HS treatment ($P < 0.05$) (Figure 10 a, b). Although EAS treatment had no effect on *SOD1* and *SOD2* expression under non-HS condition, significantly increased both *SOD1* and *SOD2* expressions under HS conditions. Also, EAS treatment

significantly increased the expression of *PRDX2* and *PRDX6* ($P < 0.05$) (Figure 10 c, d).

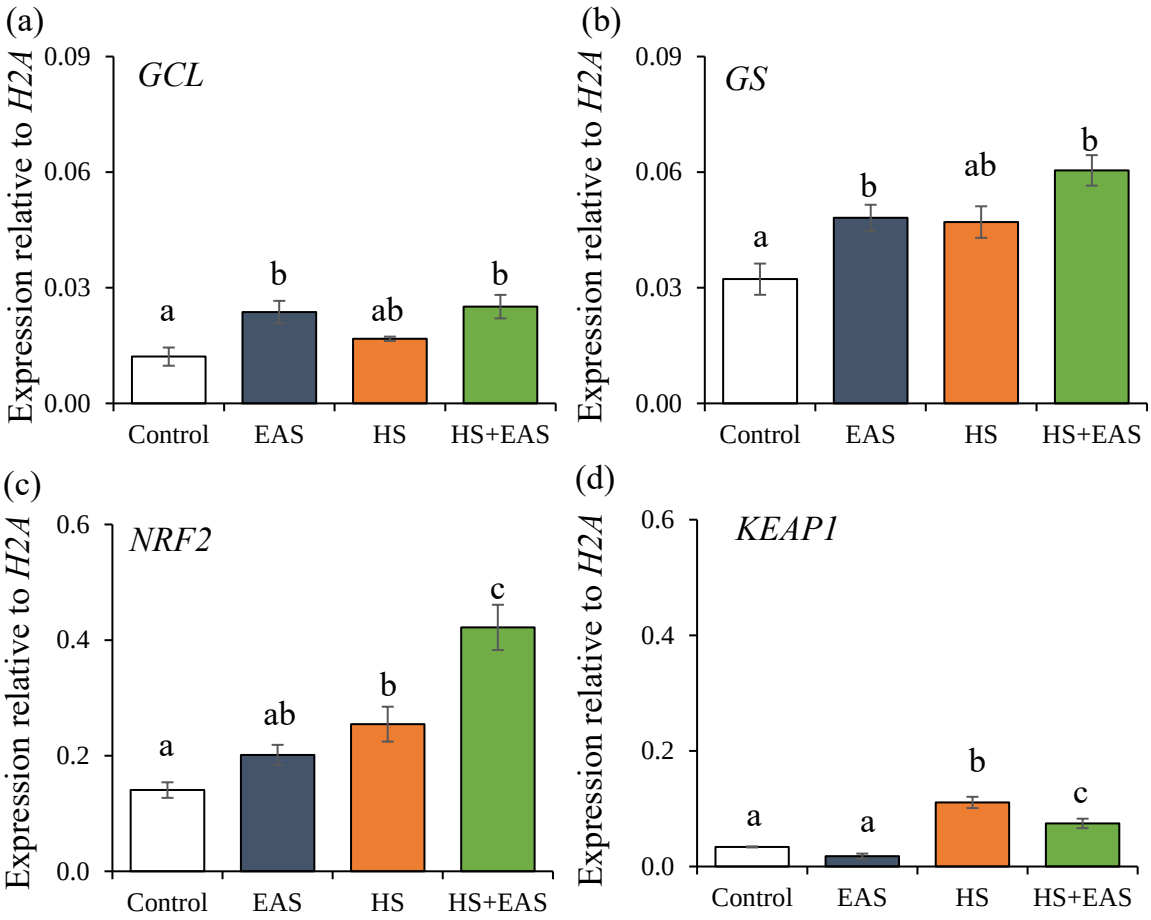


**Figure 9**. Effect of EAS on expressions of GCL, GS, NRF2, and KEAP1 under non-HS and HS conditions

The expression levels of mRNA were examined using real-time quantitative PCR normalized to H2AFZ as a reference gene. **(a)** GCL, **(b)** GS, (c) NRF2, **(d)** KEAP1. Data are shown as the mean ± SEM. Experiment were repeated 5 times. **(a)** a vs. b ($P < 0.05$), **(b)** a vs. b ($P < 0.05$), **(c)** a vs. b ($P < 0.05$), a vs. c ($P < 0.01$), b vs. c ($P < 0.01$) **(d)** a vs. b ($P < 0.01$), a vs. c ($P < 0.01$), b vs. c ($P < 0.01$).

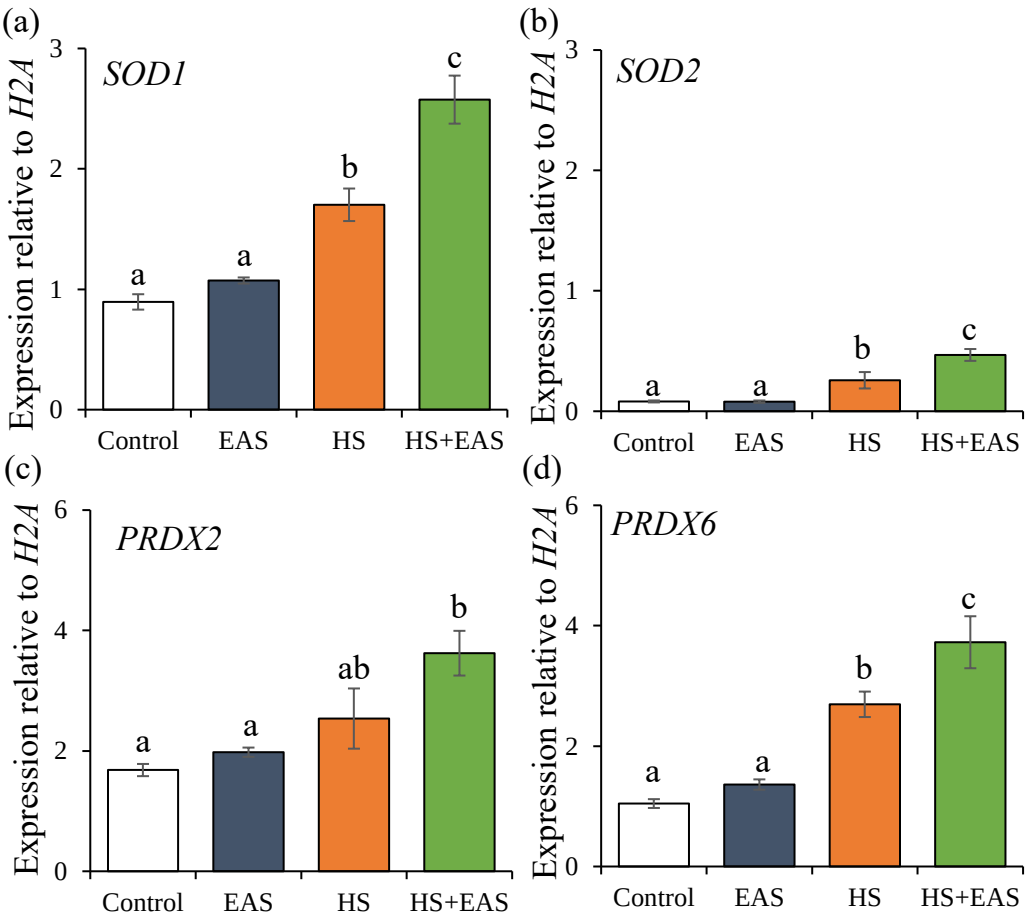


**Figure 10.** Effect of EAS on expressions of SOD1, SOD2, PRDX2 and PRDX6 under non-HS and HS conditions

The expression levels of mRNA were examined using real-time quantitative PCR normalized to H2AFZ as a reference gene. **(a)** SOD1, **(b)** SOD2, **(c)** PRDX2, **(d)** PRDX6. Data are shown as the mean ± SEM. Experiment were repeated 5 times. **(a)** a vs. b ($P < 0.01$), a vs. c ($P < 0.01$), b vs. c ($P < 0.01$), **(b)** a vs. b ($P < 0.01$), a vs. c ($P < 0.01$), b vs. c ($P < 0.01$), **(c)** a vs. b ($P < 0.05$), **(d)** a vs. b ($P < 0.01$), a vs. c ($P < 0.01$), b vs. c ($P < 0.05$).

*Effect of HSP70 inhibition on the redox status of bovine GC treated with EAS*

EAS-induced high level of HSP70 was asociated with reduced ROS levels and increased GSH levels suggests that HSP70 plays a role in the modulation of the cellular redox balance in bovine GC. I hypothesized that HSP70 induction by EAS triggers the regulation of the balance between ROS and GSH levels, which is required for maintaining the cellular redox balance in bovine GC. To test this hypothesis, cells were treated with EAS (5 mg/mL) and 10 μM PES. PES has been reported to inhibit HSP70 by interacting with the C-terminal peptide substrate-binding domain of HSP70 (Leu et al., 2009). The viability of cells treated with 10 μM PES was 77% (Figure 11). Similar with my study, 10 μM PES was widely applied in several difference cell line as human non-small cell lung carcinoma cell line (Balaburski et al., 2013), *C.elegans* intestinal cells (Fang et al., 2019) and ovarian granulosa tumour cells (Manousakidi et al., 2018). In addition, 10 μM was less toxic than 20 μM when PES was added in human fibroblasts cell line (Leu et al., 2009). HSP70 interacts with the nuclear transcription factor NF-κB which is one of the main regulator of cellular stress response (Salminen et al., 2008). Notably, 10 μM PES inhibited NF-κB pathway activity in human non-small cell lung carcinoma cell (Leu et al., 2009). Collectively, these studies and my data indicated that 10 μM PES has sufficient HSP70 inhibition less toxic to bovine GC.

As shown in Figures 6 and 12, ROS levels were significantly decreased ($P < 0.05$) by EAS treatment. In contrast, PES treatment with EAS significantly increased ROS levels in the nuclei of GC cells (Figure 12). The levels of GSH that had been increased by EAS treatment were significantly decreased ($P < 0.01$) by

PES (Figure 13). Moreover, the expression of *HSF1* and *NRF2* increased by EAS treatment was significantly decreased by inhibition of HSP70 (Figure 14a, b). In addition, *KEAP1* expression was significantly decreased by inhibition of HSP70 (Figure 14c).

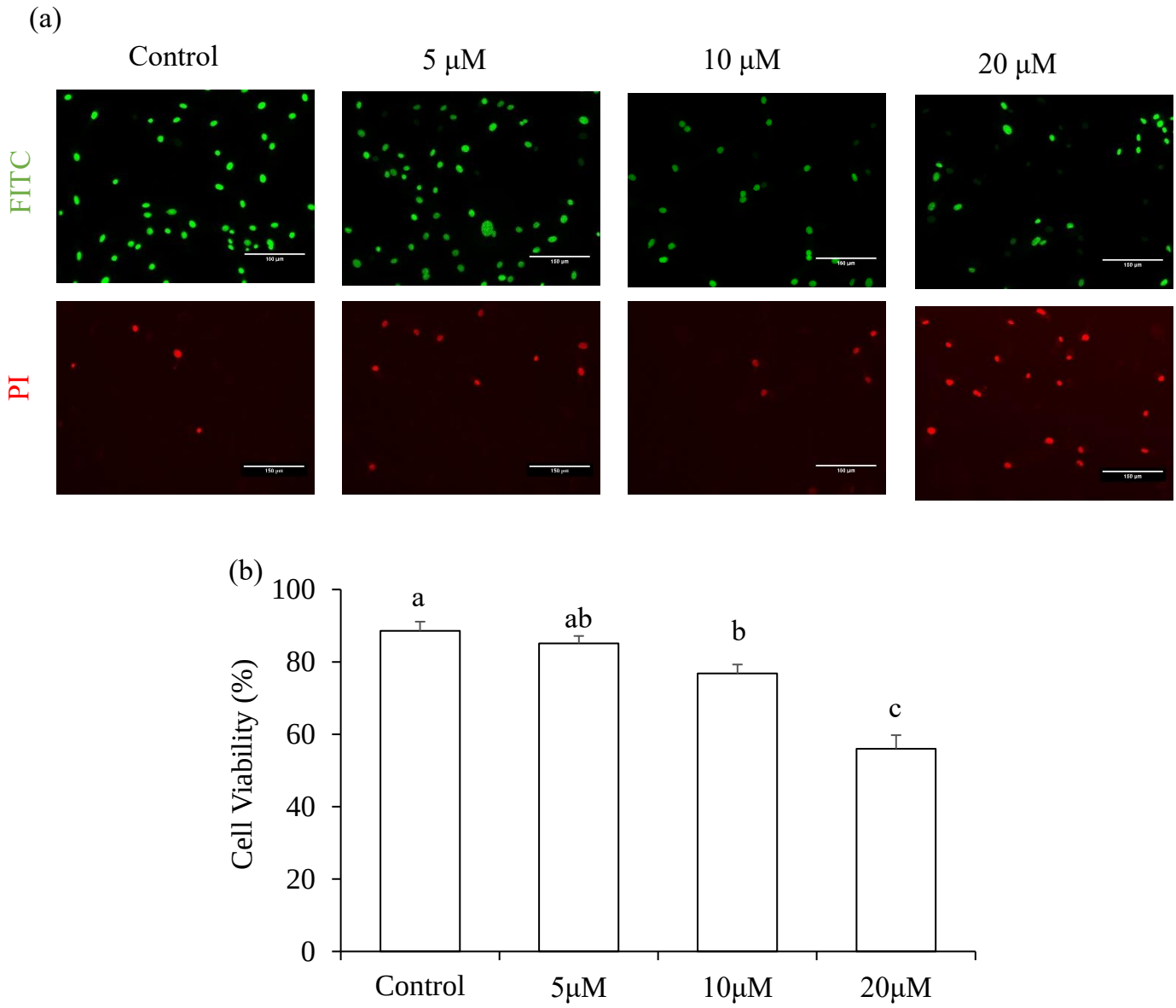


**Figure 11.** Effect of PES on the viability of bovine GC

Bovine GC were treated for 12 h with 0, 5, 10 and 20 μM PES under 5% $CO_2$ condition at 38.5°C. cell viability was analysed by Live-Dead Cell Staining Kit **(a)** Fluorescence of FITC indicates for live cells (Upper) and PI indicates for dead or damaged cells (Lower). Bar shows 150 μm. **(b)** Cell viability (%). Data are shown as the means ± SEM. Experiment were repeated 4 times. a vs b ($P < 0.05$), a vs c ($P < 0.01$).

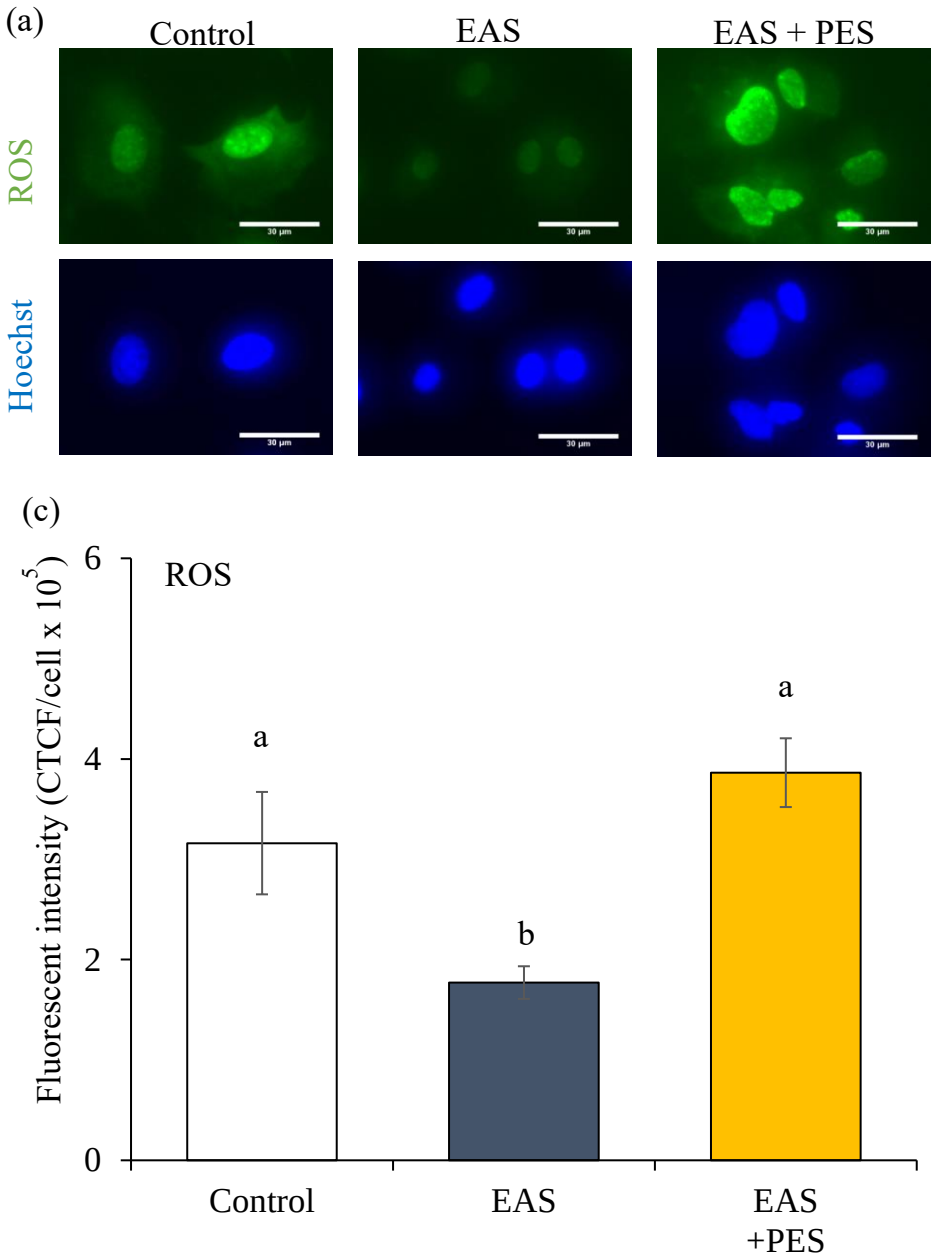


**Figure 12.** Effect of EAS-induced HSP70 inhibition on ROS generation in bovine GC

Cells were treated for 6 h with or without EAS (5 mg/mL) under non-HS 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 with Hoechst (lower). **(b)** Fluorescence intensity of ROS (CTCF). Data are shown as mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.05$).

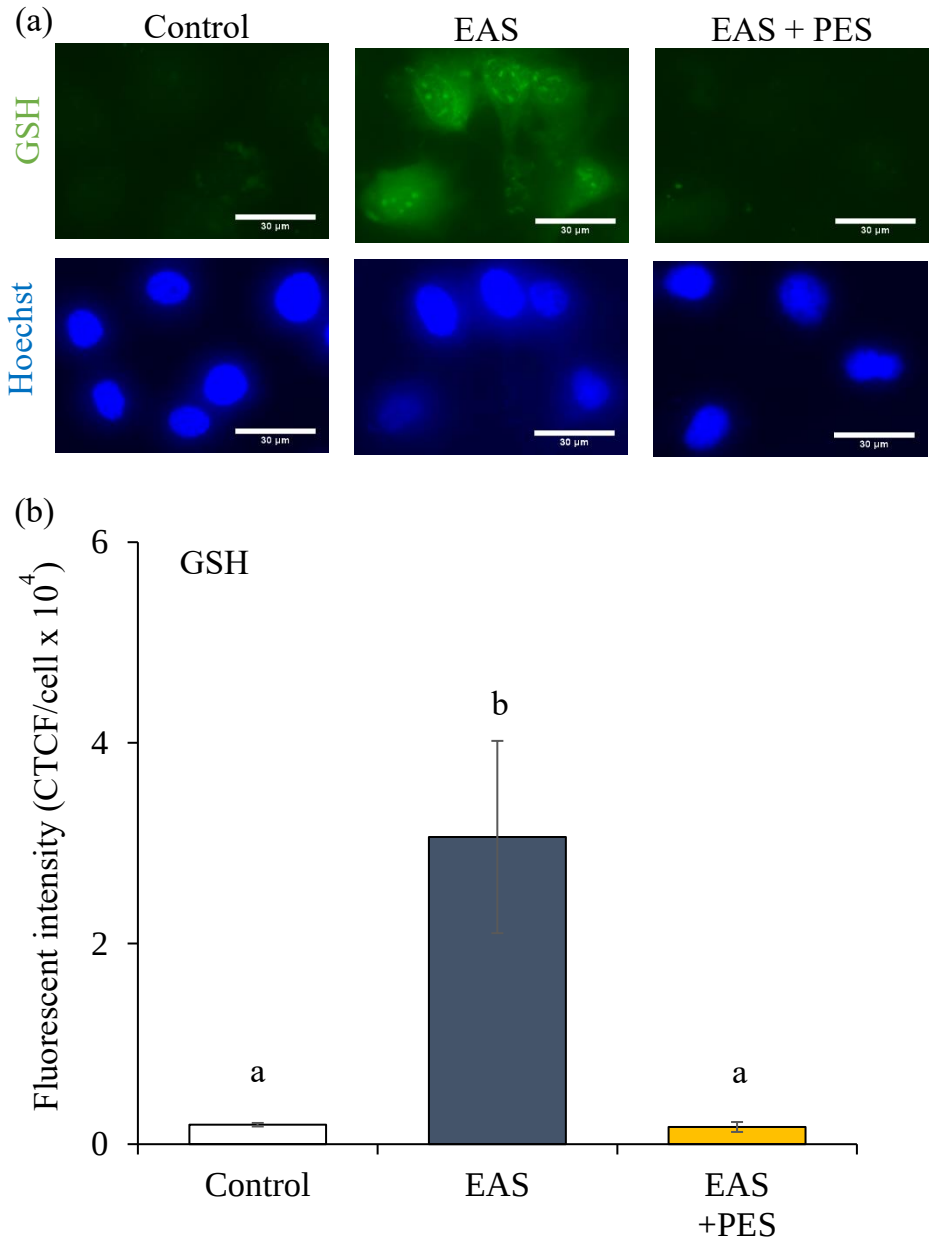


**Figure 13.** Effect of EAS-induced HSP70 inhibition on GSH synthesis in bovine GC

Cells were treated for 6 h with or without EAS (5 mg/mL) under non-HS conditions at 38.5°C (control, EAS group) and 10 µM PES together with 5 mg/mL EAS (EAS + PES). **(a)** Fluorescence of GSH (upper panel) and nuclei stained with Hoechst (lower panel). Scale bar shows 30 µm. **(b)** Fluorescence intensity of GSH (CTCF). Data are shown as the means ± SEM. Experiment were repeated 5 times, a vs b ($P < 0.01$).

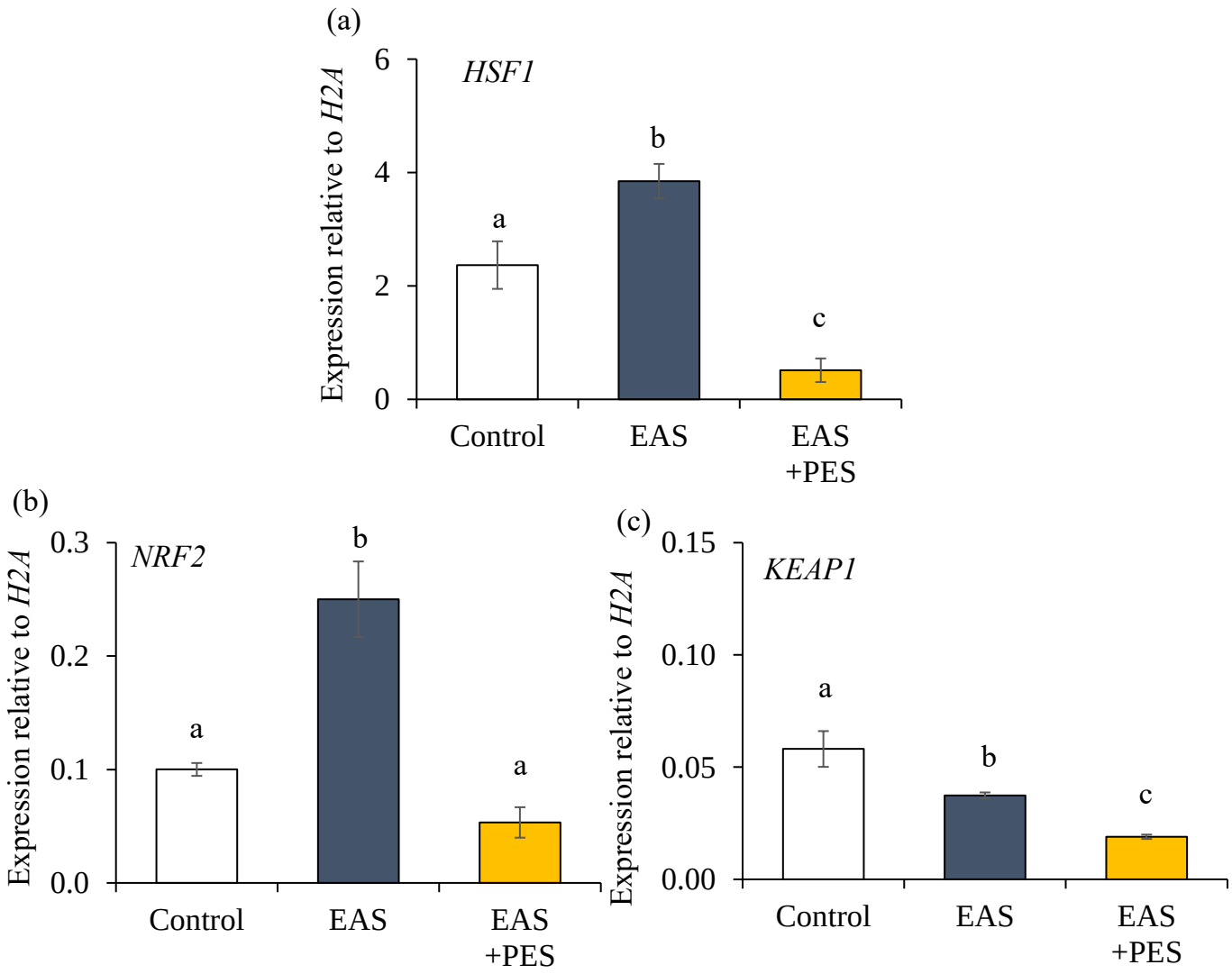


**Figure 14.** Effect of EAS-induced HSP70 inhibition on expression of HSF1, NRF2 and KEAP1

Cells were treated for 12 h with or without EAS (5 mg/mL) under non-HS condition at 38.5°C (Control, EAS group) and 10 µM PES together with 5 mg/mL EAS (EAS + PES). The expression levels of HSF1, NRF2, and KEAP1 were examined using real-time quantitative PCR, normalized to H2AFZ as a reference gene. **(a)** HSF1, **(b)** NRF2, **(c)** KEAP1. Data are shown as the mean ± SEM. Experiment were repeated 5 times. **(a and b)** a vs. b ($P < 0.01$), a vs. c ($P < 0.05$), b vs. c ($P < 0.01$). **(c)** a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.05$).

*Effect of EAS on P4 synthesis under normal culture condition*

Bovine GC not only play a role in supporting the follicular and oocyte development, but also perform an endocrine role in the secretion of steroid hormone (Su et al., 2009). P4 plays a major role in maintaining pregnancy and conceptus growth in cattle (Lonergan et al., 2016). I measured P4 levels to further investigate the effect of EAS treatment on steroidogenesis and found that P4 levels were significantly increased by EAS supplementation compared with that in the control group ($P < 0.05$) culture ordinary condition at 38.5$^{o}$C (Figure 15).

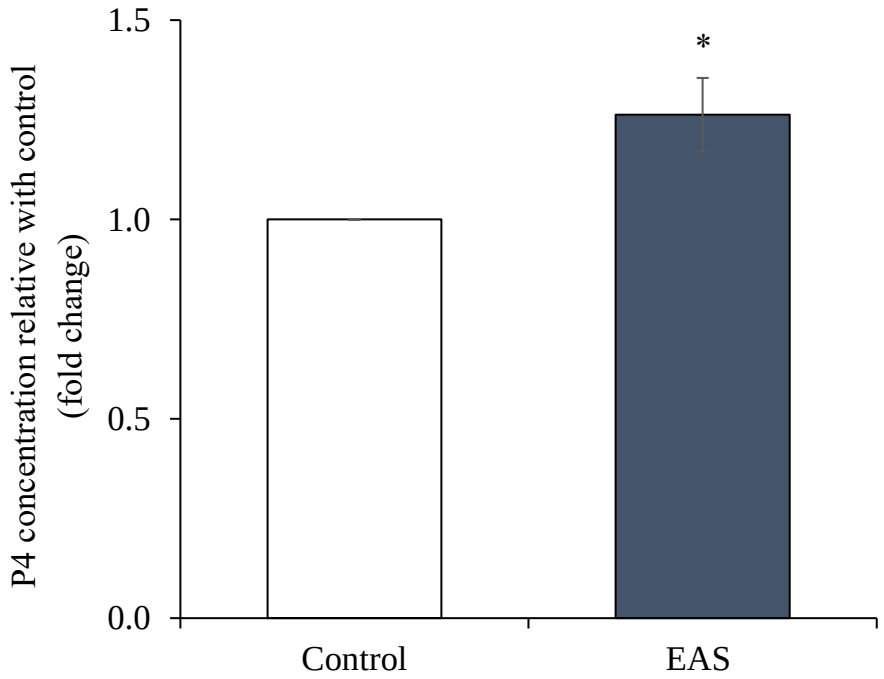


**Figure 15.** Effect of EAS treatment on P4 synthesis in bovine GC

Cells were treated for 12 h with or without EAS (5 mg/mL) under non-HS condition at 38.5°C (Control, EAS group). Data are shown as the mean ± SEM. Experiment were repeated 8 times. *P < 0.05, vs. Control.

***Discussion***

In the present study, I found that EAS increases HSP70 expression, associated with redox balance through decrease is ROS generation and increase in GSH synthesis in the bovine GC both under non-HS and HS conditions.

Five mg/mL of EAS treatment significantly increased both HSP70 mRNA and protein levels in bovine GC following 6 h of incubation under non-HS condition. This result is consistent with that of a previous study, in which hepatocytes were treated with 4 mg/mL EAS during incubation for 4 h (Nishizawa et al., 2016). In addition, mRNA and protein expression of HSP70 in human cervical epithelioid carcinoma cells were increased at a lower dose (2 mg/mL) of EAS after a longer incubation time (24 h) (Ito et al., 2014), than in the current study (6 h). The variation in the effect of EAS dose in cells may be a cause of differences in cell type and culture conditions. HSP70 expression-enhancing activity by EAS may arise from asparaprolines, which has been found to elevate HSP70 expression in human monocyte HL-60 cells (Inoue et al., 2020). On the other hand, several studies have reported that overexpression of other HSP such as *HSP27* and *HSP90*, resulted in the resistance to anticancer drugs. (Lu et al., 2012). However, *HSP27* and *HSP90* in of bovine GC was not affected by EAS in present study. Therefore, my results indicate that the effect of EAS on bovine GC under non-HS condition is to preferentially induce HSP70, which is expected to improve GC without adverse effects.

*In vitro* studies have shown a synergistic effect between HS and HSP-inducing compounds on HSP70 expression (Westerheide et al., 2004; Yan et al., 2004). In

the present study, HSP70 induction by EAS occurred both under non-HS and HS conditions, and it was highest under HS conditions. These data suggest that the combination of EAS and HS synergistically induces HSP70 induction in bovine GC. The induction of *HSF1*, the main transcription factors of HSP70, by EAS has been reported in mouse Neuroblastoma × rat glioma hybrid NG108-15 cells (Sakurai et al., 2014). The significant increase in *HSF1* by EAS in the present study supports the previous findings (Lees et al., 2019) that EAS is involved in the HSP70 induction pathway in bovine GC. Under stress conditions, HSF1 is activated and releases HSP70 to prevent the formation of misfolded polypeptides (Santoro, 2000). In current study, *HSF1* gene expression was induced by EAS both under non-HS and HS conditions, and it was highest with EAS supplementation under HS conditions. These data suggest that EAS and HS have synergistic effects on the induction of *HSF1* in bovine GC. HSF2 is a HS related transcription factor co-expressed with HSF1, which is activated in response to distinct developmental cues or differentiation stimuli (Santoro, 2000). However, *HSF2* was not affected by EAS treatment in the present study in spite of increased HSP70 expression under non-HS conditions. However, *HSF1* expression was only induced under HS conditions, with a synergistic effect of EAS. In addition, PES reduced *HSF1* expression. The inconsistency with results from previous studies may be due to several factors. EAS may regulate the expression of HSP70 not only through *HSF1* gene expression but also through other regulatory mechanisms as signal-transducer and activator of transcription protein and nuclear factor of IL6 gene/ CCAAT/enhancer binding protein beta (Zorzi and Bonvini, 2011). Additionally,

research on different species and cell types has led to reports of different regulatory effects of EAS on *HSF1* gene expression (Nishizawa et al., 2016). Hence, it is conceivable that an increase in HSP70 expression by EAS treatment is induced as a result of enhancement of HSF1, with EAS exerting synergistic effects with HS on HSP70 induction.

Several studies have indicated that HSP70 has anti-apoptotic effects and anti-inflammatory activity, indicating that HSP70 exerts a cytoprotective function against various stresses (Wu et al., 2013). In addition, the overexpression of HSP70 reduced ROS induced by hypoxia and glucose deprivation (Guo et al., 2007). These results indicate that induced HSP70 by EAS treatment 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 rat pheochromocytoma PC12 cells (Ogasawara et al., 2014). In this study, EAS treatment significantly inhibited ROS generation both in normal and HS conditions in bovine GC with increase in GSH synthesis. Moreover, ROS activates c-Jun N-terminal kinase protein, which is increased under HS conditions (Dasgupta et al., 2010). EAS clearly reduced the c-Jun N-terminal kinase protein induced by hydrogen peroxide in murine skin fibroblast cells (Shirato et al., 2016).

In conclusion, EAS reduced HS-induced ROS generation in bovine GC, which can be explained by the upregulation of GSH generation and the downregulation of c-Jun N-terminal kinase protein.

ROS-induced damage can cause both single- and double-stranded DNA breaks (Sottile and Nadin, 2018). In this study, EAS significantly reduced γH2AX levels

under HS conditions. ROS generation from normal cellular metabolism and HS induces DNA damage in cells (Tubbs and Nussenzweig, 2017). In the previous study, EAS reduced cell damage induced by nitric oxide donor sodium nitroprusside or the hypoxia mimic reagent cobalt chloride in mouse Neuroblastoma× rat glioma hybrid NG108-15 cells (Sakurai et al., 2014). HSP70 has the ability to repair DNA damage caused by HS (Sottile and Nadin, 2018). In this study, HSP70 induction highly contributed to the reduction of DNA damage in bovine GC 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 (Cotgreave, 2003). In the present study, GSH levels were increased by EAS in bovine GC both under non-stress and HS 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.

EAS increased GSH both under non-HS and HS conditions. In agreement with this observation, previous reports have suggested that overexpression of HSP70 enhanced GSH levels in Madin-Darby canine kidney cell under *in vitro* condition (Guo et al., 2007). 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, 3h of HS treatment increased GSH level than that in control cells (Tchouague et al., 2019). GSH biosynthesis is required by the action of two ATP-dependent enzymes: GCL, which assembles the formation of gamma-glutamyl-cysteine from glutamate-cysteine, and GS, which is involved in the ligation of gamma-glutamyl-

cysteine to glycine in another ATP-dependent reaction to yield GSH (Bachhawat and Yadav, 2018). My results showed that EAS increased the expression of *GCL* and *GS* under non-HS conditions.

The transcription factor NRF2, which has the potential inducer of GCL and GS, is activated by ROS generation (Steele et al., 2013). The transcription factor NRF2 binds to KEAP1 under normal conditions, known as the NRF2/KEAP1 signalling pathway, and is translocated to the nucleus under HS condition (Glory and Averill-Bates, 2016). 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 (Glory and Averill-Bates, 2016). Moreover, HSF1 may induce NRF2 by activating p62, which can release NRF2 from KEAP1 (Ichimura et al., 2013). In this study, GSH levels were not decreased after 6h of HS treatment, because the compensatory effect NRF2 maintained GSH levels. Therefore, there is a need to study the effect of HS treatment on GSH synthesis and the induction of GSH synthesis-related genes. A previous study has shown that reduced KEAP1 expression is related to the induction of NRF2 (Bae et al., 2013). In this study, together with the induction of *NRF2*, EAS reduced *KEAP1* and induced *HSF1* expression. EAS treatment increased NRF2 protein levels in NG108-15 neuronal cells (Sakurai et al., 2014). In summary, EAS increases GSH biosynthesis by enhancing expression of γ-glutamyl cycle related gene due to NRF2 induction, which might be caused by the compensation effect of HSF1 with NRF2 and the antioxidant content of EAS.

PES has been shown to inhibit HSP70 (Leu et al., 2009). In this study, PES neutralized the effect of EAS on GSH and ROS levels, indicating that HSP70 induction by EAS regulates the intracellular redox balance by GSH and ROS in bovine GC. Previous study has revealed the correlation between the NRF2 and HSF1 for the protection of cells (Naidu et al., 2015). NRF2 and HSF1 compensates each other; the induction of HSP70 by methionine deprivation is dependent on NRF2 (Hensen et al., 2013). Both NRF2 and HSF1 play important roles in cellular redox processes because of 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 (Glory and Averill-Bates, 2016). In the present study, PES also inhibited 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 (Hayden and Ghosh, 2008). Nuclear translocation of NF-κB p65 enhances the ability of NRF2 and plays a role in the antioxidant response in human kidney-2 cells (George et al., 2012). In addition, HSF1 activation in intestinal epithelial cells during HS is regulated by the NF-κB pathway (Li et al., 2018).

Moreover, PES reduced *KEAP1*, which combined with NRF2 to regulate the antioxidative protection system (Glory and Averill-Bates, 2016). These results indicate that HSP70 induction by EAS improved the redox balance by regulating

the ROS and GSH levels through HSF1, and NRF2/KEAP1 pathway in bovine GC (Figure 16).

The beneficial effect of EAS on P4 synthesis demonstrated in the study was similar to that reported in a previous study, in which the oral administration of asparagus root extract enhanced P4 levels in rats (Jashni et al., 2016). This indicates that bovine GC are important for reproductive functions due to differentiation into luteal cell which responsibility for P4 synthesis (Montaño et al., 2009). From overall studies in this chapter, these findings indicate that EAS highly contributes to the improvement of P4 synthesis in luteinized bovine GC through HSP70 regulating intracellular conditions.

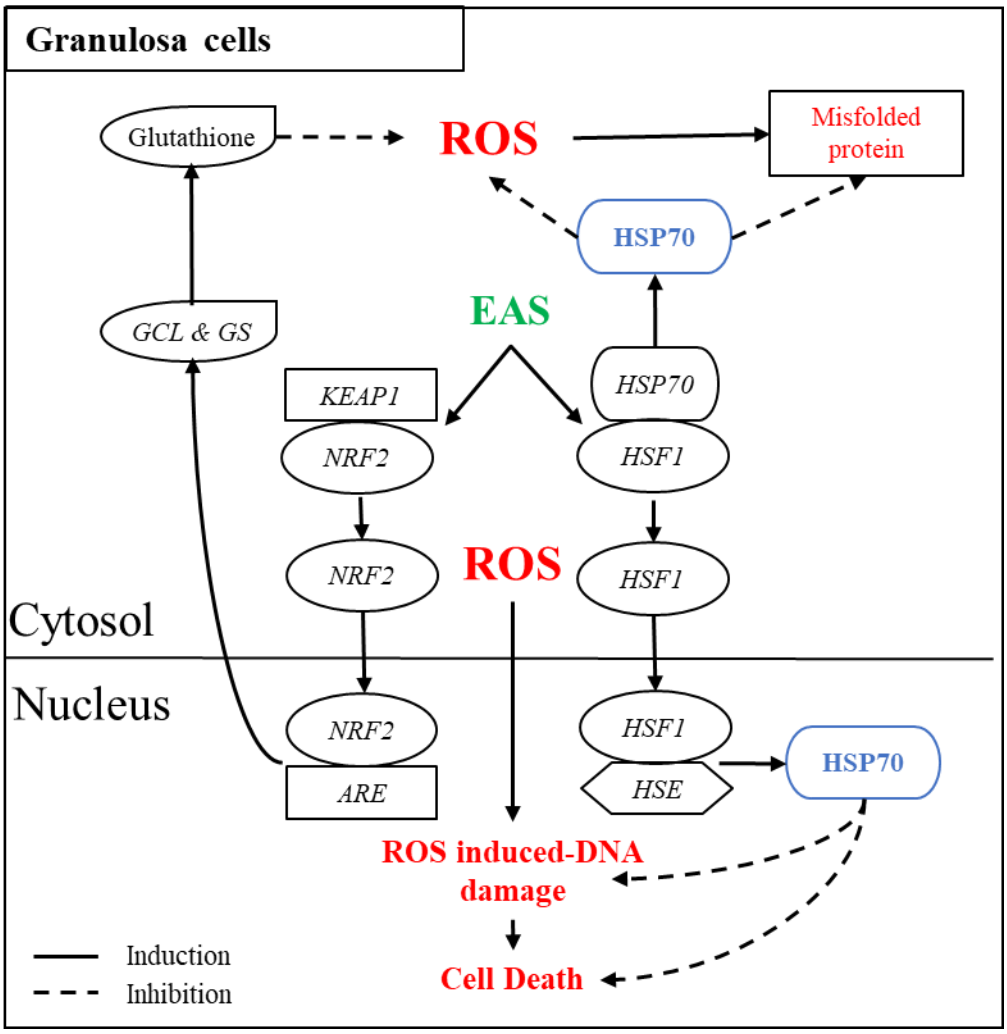


**Figure 16.** Schematic diagram of effect EAS on HSP70 production and redox status of bovine GC

Chapter 3 **Role of a standardized extract of *Asparagus officinalis* stem on the steroidogenesis in bovine granulosa cells under non-HS and HS conditions**

***Introduction***

P4 is an important steroid hormone, and it has pivotal role in establishing uterine receptivity, oocyte maturation and maintaining pregnancy (Bazer et al., 2011; Fair and Lonergan, 2012). Granulosa cells has fundamental role in the maturation and acquisition of developmental competence in bovine oocytes and it differentiate into large luteal cells which responsible for P4 production (Fatehi, 2005; Abedel-Majed et al., 2019). Therefore, GC are suitable model for the study of steroidogenesis in the ovary.

P4 synthesis in GC is carried out by cholesterol transport into mitochondria by steroidogenesis-related genes such as STAR, CYP11A1and 3β-HSD (Chapman et al., 2005; Bassi et al., 2021). Importantly, mitochondria has a central role for steroidogenesis (Rone et al., 2009; Bassi et al., 2021). Moreover, P4 synthesis in bovine GC related with lipid metabolism (Elis et al., 2015). In addition, cholesterol from lipid droplet is one of available source for P4 biosynthesis (Rone et al., 2009). Thus, P4 synthesis in bovine GC is regulated by steroidogenesis enzyme activity, mitochondria function and lipid metabolism.

One of the critical issues that affects the dairy farming is a reduction of reproductive performance in high-yielding dairy cows (Lucy, 2001). Besides, high body temperature in summer season decreases the reproductive functions in ovary such as oocyte growth, steroidogenesis, and developmental competence of oocytes (Wilson et al., 1998; Wakayo et al., 2015). Furthermore, negative impact of ROS

on P4 level is reported in the follicular fluid of human ovary (Pizarro et al., 2020). Follicular fluid harbours hormones and molecules in relation with redox homeostasis from bovine GC (Rodgers and Irving-Rodgers, 2010; de los Santos et al., 2012). P4 level in bovine GC was reduced by HS-induced ROS and hydrogen peroxide (Khan et al., 2020; Wang et al., 2021). ROS broadly causes imbalance of redox status with causing damage, alteration the cellular function and physiological processes in the ovaries (Murdoch, 1998; Shi et al., 2020).

Under stressed condition, cellular damage was rescued by HSP70 that regulate folding, unfolding, and homeostasis of cellular proteins (Saibil, 2013). In the chapter 2, I demonstrated that EAS-induced HSP70 improved the redox balance through reduction of ROS in bovine GC both under non-HS and HS conditions. EAS-induced HSP70 with keeping redox balance in both conditions may contribute to P4 synthesis in bovine GC. However, the effect of EAS on P4 biosynthesis in bovine GC has not been fully elucidated.

Therefore, in this chapter, I investigated the effect of EAS on P4 production, expression of steroidogenesis enzyme, mitochondria function and lipid metabolism in bovine GC under non-HS and HS conditions. Moreover, to confirm wheather EAS-induced P4 synthesis is dependent on HSP70, I investigated the effect of HSP70 inhibition on the P4 synthesis in EAS- treated bovine GC under non-HS and HS conditions.

***Materials and Methods***

*Bovine GC culture*

GC cell collection and culture was performed as described in chapter 2

*Experimental design*

To investigate the suitable concentration of EAS on P4 production, the GC were cultured for 12 h at 38.5°C with 0.5, 1, 5 and 10 mg/mL of EAS. Beside, to evaluate effect of EAS on P4 synthesis under non-HS and HS conditions, cells were cultured for 12 h at 38.5°C and 41°C with or without 5 mg/mL EAS. To confirm the role of EAS-induced HSP70 on P4 synthesis, EAS-treated cells were also cultured with or without 10 µM PES, HSP70 inhibitor both under non-HS and HS conditions.

After culture, cells and culture medium were collected and used for P4 measurement, mRNA expression analysis, immunostaining, detection of lipid droplet and mitochondrial activities.

*P4 measurement*

The concentration of P4 was performed as described in chapter 2.

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

RNA extraction, cDNA synthesis and qPCR were performed as described in chapter 2. Specific primers (Supplementary Table 1) used for qPCR were designed

using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized commercially.

*Immunodetection of HSP70*

Immunodetection of HSP70 was performed as described in chapter 2.

*Detection of Lipid droplet and mitochondrial activity*

For detection of lipid droplet, cells were washed with PBS (–) after treatment and fixed 4% PFA in PBS (–) for 10 min at room temperature. After washing three times with PBS (–) for 5 min, cells were treated with 1 µg/mL Nile Red (Wako, Osaka, Japan) for 30 min at 38.5°C in a 5% $CO_2$ in air.

For detection of mitochondrial activity, cells were washed with PBS (–) and added with 200 nM MitoTracker™ Green FM (Thermo Fischer Scientific) for 30 min at 38.5°C in a 5% $CO_2$ in air.

Cells from both detections were then washed with PBS (–), and 10 µl of the mounting solution (NacalaiTesque) was dropped on the cells and covered with a cover glass. Then, fluorescence images were observed using a fluorescent microscope (Leica). The fluorescence intensity was quantified by using ImageJ software v1.52A (National Institutes of Health; http://imagej.nih.gov/ij/). Corrected total cell fluorescence (CTCF) was carried out a formula as described in Chapter 2.

*Statistical analysis*

Statistical analysis was conducted as described in chapter 2.

***Results***

*Effect of EAS on P4 production*

I investigated the effect of various dose of EAS on P4 secretion in bovine GC. As shown in Figure 17, EAS significantly ($P < 0.001$) increased P4 level at 5mg/mL as a peak. Collectively, EAS increased P4 production in a dose dependent manner from 0.5 to 5 mg/mL and showed highest level at 5 mg/mL.

*Effect of EAS on P4 level in bovine GC under non-HS and HS conditions*

As described in chapter 2, EAS induced HSP70 and improved redox balance in both non-HS and HS conditions. In the present chapter, I investigated whether EAS affects P4 synthesis in both environments together with expression of steroidogenesis-related genes in EAS-treated bovine CG under non-HS and HS conditions. As shown in Figure 18a, P4 level was significantly ($P < 0.001$) increased by EAS treatment than untreated control. However, P4 level of HS treated cells tend to be lower than control cells ($P < 0.1$, Figure 18a). Interestingly, P4 level was significantly ($P < 0.001$) increased by EAS treatment under HS condition (Figure 18a).

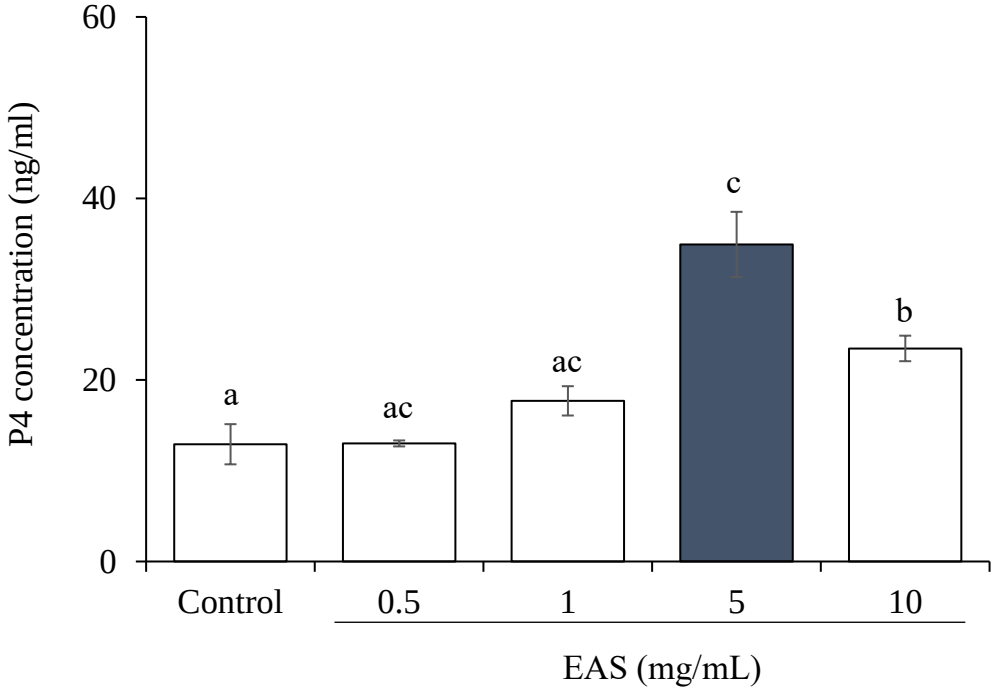


**Figure 17.** Effect of various concentration of EAS on P4 production in bovine GC

Cells were subjected with various concentrations of EAS (0.5, 1, 5 and 10 mg/mL) for 12 h at 38.5°C. Detection of P4 secretion by ELISA in cell supernatants. Data are shown as the mean ± SEM. Experiment were repeated 5 times. a vs. b ($P < 0.001$), a vs. c ($P < 0.05$), and b vs. c ($P < 0.05$).

*Effect of EAS on expression of steroidogenesis-related genes in bovine GC under non-HS and HS conditions*

Expression of steroidogenesis enzymes, *STAR* and *3β-HSD* were significantly increased in both EAS and EAS+HS groups similar with the P4 level ($P < 0.001$) (Figure 18b, d). Whereas *CYP11A1* was not changed by EAS treatment and/or HS conditions (Figure 18c).

These results confirmed that EAS-induced P4 production is affected through enhancement *STAR* and *3β-HSD* and it has greatest effect on P4 synthesis in bovine GC under HS condition. It should be noted that HS treatment had synergistic effect on the increase in *3β-HSD*.

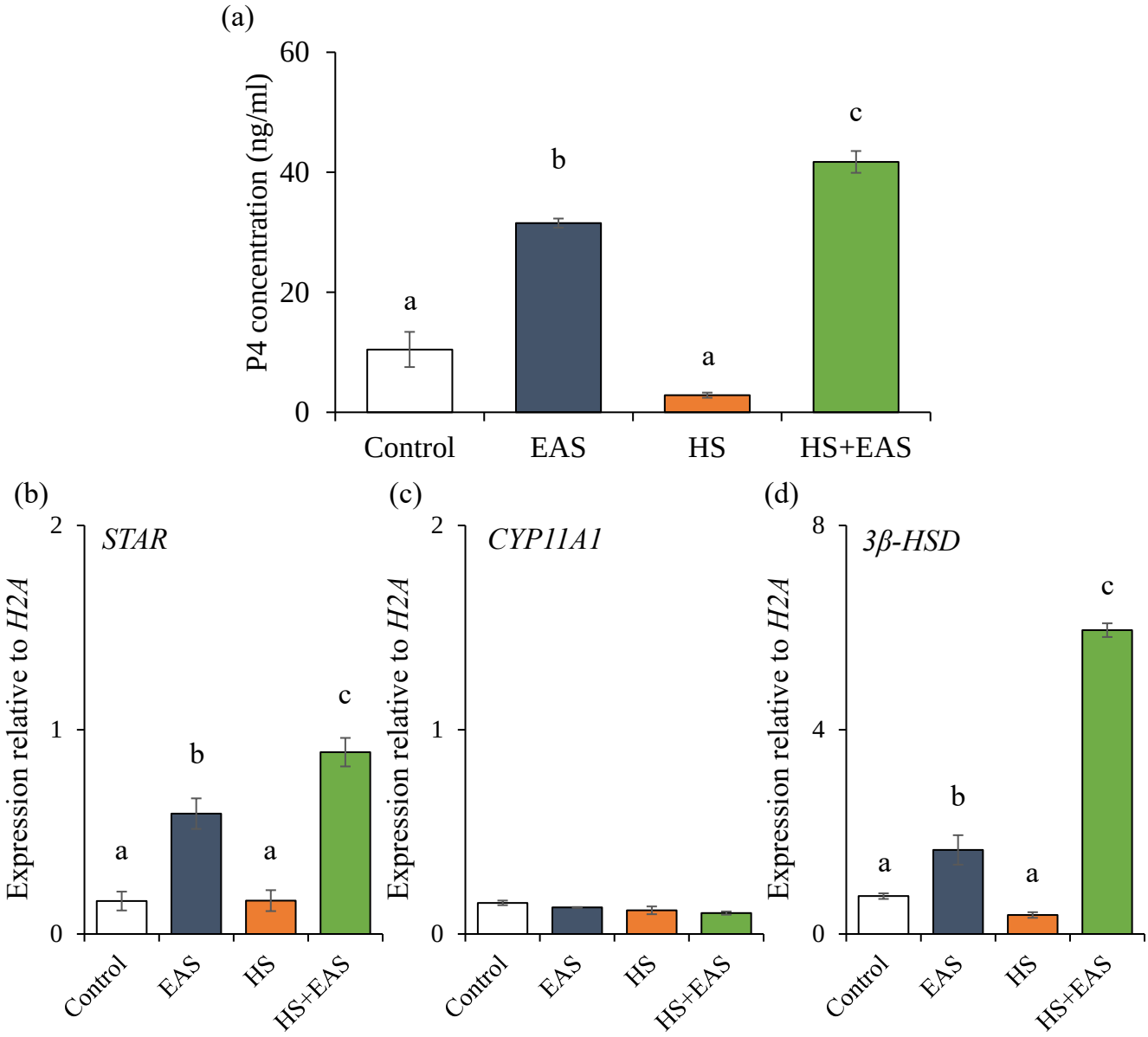


**Figure 18.** Effect of EAS on P4 synthesis and steroidogenesis-related genes in bovine GC under non-HS and HS conditions

Cells were subjected with or without 5 mg/mL of EAS at 38.5°C (Control, EAS) or at 41°C (HS, HS + EAS) for 12 h. Detection of P4 secretion by ELISA in cell supernatants. The mRNA expression levels of STAR, CYP11A1 and 3β-HSD are presented relative to H2AFZ as a reference gene. Data are shown as the mean ± SEM. **(a)** experiment were repeated 3 times, **(b to d)** experiment were repeated 5 times. **(a)** a vs. b ($P < 0.001$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.05$), **(b)** a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.05$), **(d)** a vs. b ($P < 0.01$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.001$).

*Effect of EAS on lipid metabolism and mitochondrial activity in bovine GC under non-HS and HS conditions*

To clarify the effect of EAS on P4 production and expression of steroidogenesis gene, I conducted the detection of lipid droplet and mitochondrial activity of EAS-treated cells under non-HS and HS conditions. As shown in Figure 19a, EAS treatment significantly increased the fluorescence of stained lipid droplet and active mitochondria in bovine GC both under non-HS and HS conditions. Remarkably, EAS-induced HSP70 protein expressed both in nucleus and cytoplasm, but HS-induced HSP70 localized mainly in the nucleus (Figure 19b). Notably, the increased number and size of lipid droplets and actively stained mitochondria were clearly detected in the cytosol around nucleus (Figure 19b). Similar to the results of P4 and expression of steroidogenesis-related genes, fluorescent intensity of lipid droplet and mitochondria activity of EAS + HS group were significantly ($P < 0.001$) higher than other groups (Figure 20). HS significantly ($P < 0.05$) increased fluorescent intensity of lipid droplet, but significantly ($P < 0.01$) reduced the number of active mitochondria (Figure 20). EAS treatment with HS enhanced lipid droplet and mitochondrial activity, and these results may be caused by EAS-induced HSP70.

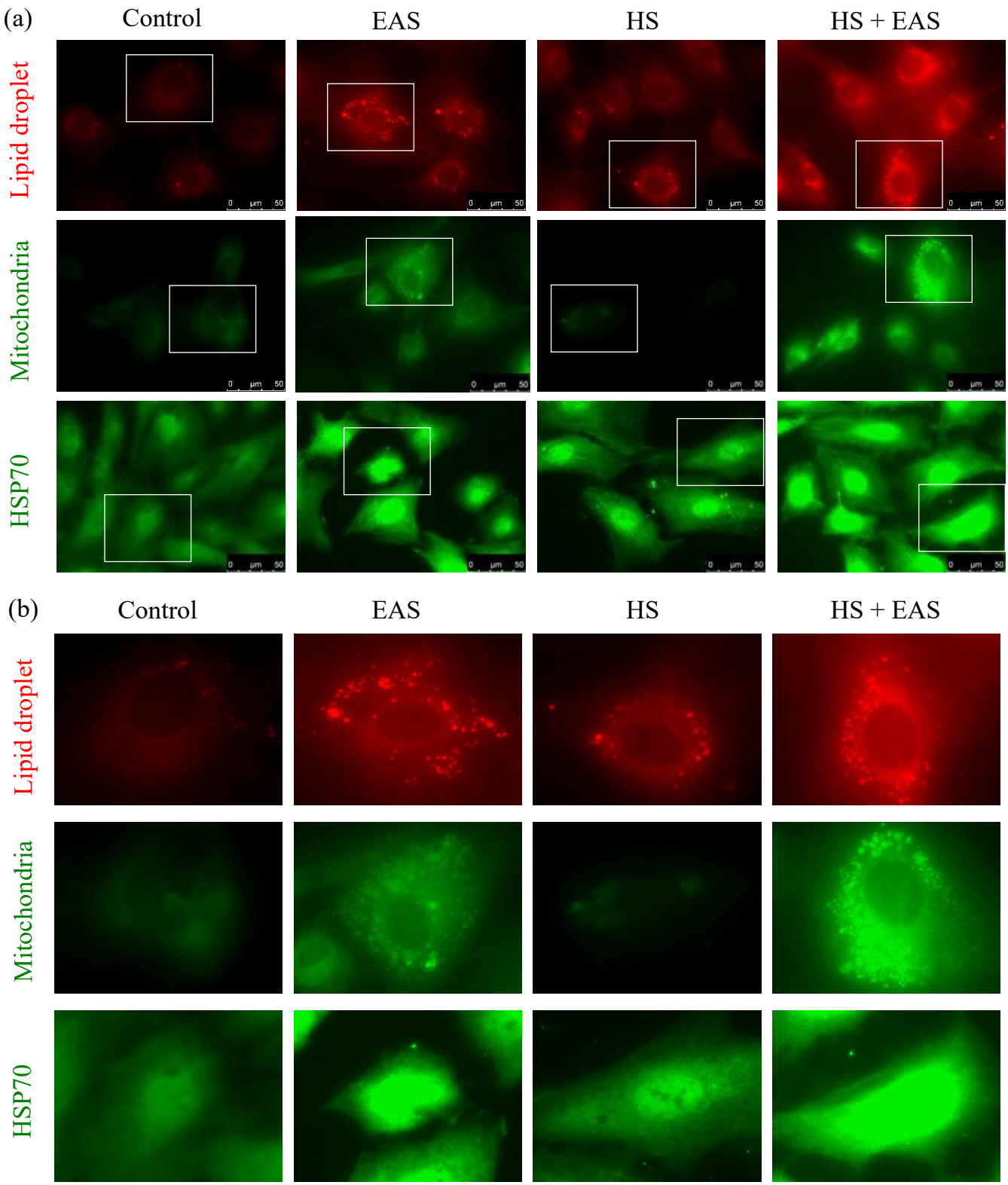


**Figure 19.** Fluorescent expression of mitochondrial activity and lipid droplet by EAS associated with HSP70 localization change in bovine GC under non-HS and HS conditions

Cells were subjected with or without 5 mg/mL of EAS at 38.5°C (Control, EAS) or at 41°C (HS, HS + EAS) for 12 h. Fluorescent staining and enlarged image (surrounded by white line) of mitochondria, lipid droplet and HSP70 **(a and b)**. Scale bar = 50 µm.

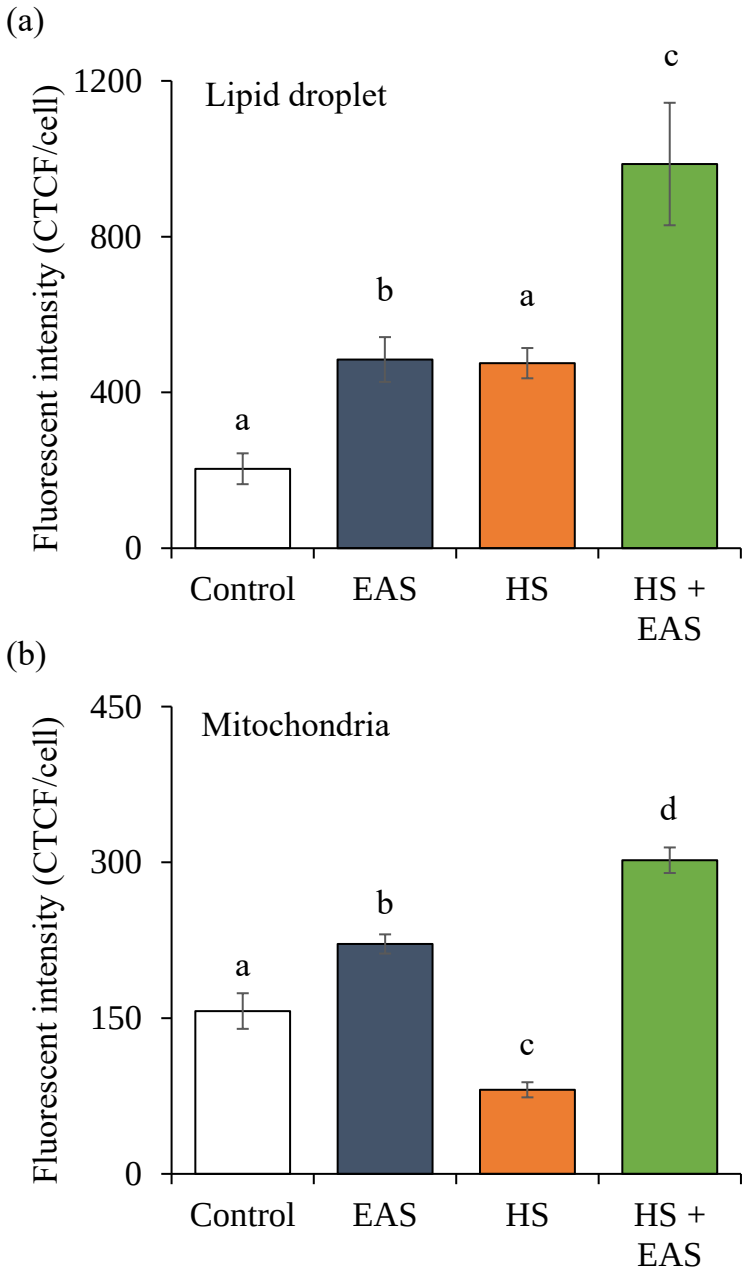


**Figure 20.** Fluorescence intensity of mitochondria and lipid droplet by EAS in bovine GC under non-HS and HS conditions

Cells were subjected with or without 5 mg/mL of EAS at 38.5°C (Control, EAS) or at 41°C (HS, HS + EAS) for 12 h. Fluorescence intensity was analyzed by CTCF (**a and b**). Data are shown as the mean ± SEM. Experiment were repeated 5 times. **(a)** a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.001$), **(b)** a vs. b ($P < 0.01$), a vs. c ($P < 0.01$), a vs. d ($P < 0.001$), b vs. c ($P < 0.001$), b vs. d ($P < 0.001$), c vs. d ($P < 0.001$).

*Inhibition of HSP70 attenuates P4 synthesis, lipid droplet and mitochondrial activity in the EAS-treated bovine GC under non-HS and HS conditions*

I hypothesized that EAS-induced HSP70 might be involved in effect of EAS on P4 synthesis, lipid droplet and mitochondrial activity in bovine GC under non-HS and HS conditions. To test this hypothesis, I evaluated the effect of HSP70 inhibitor PES on P4 synthesis, gene expression of steroidogenesis enzymes, lipid droplet and mitochondrial activity in EAS-treated cells under both non-HS and HS conditions. As expected, PES significantly inhibited EAS-induced P4 synthesis related with reduced P4 level, *STAR* and *3β-HSD* in EAS-treated cells both under non-HS and HS conditions ($P < 0.05$) (Figure 21, Figure 22 a,c). However, PES did not affect *CYP11A1* (Figure 22b). In addition, PES inhibited the EAS-induced mitochondrial activity and lipid droplet, while HSP70-induction was not affected (Figure 23). Consistently, PES suppressed lipid droplet synthesis and mitochondrial activity in EAS treated bovine GC under non-HS and HS condition ($P < 0.05$) (Figure 24). Taken together, these data suggest that EAS-induced P4 synthesis is regulated by EAS-induced HSP70.

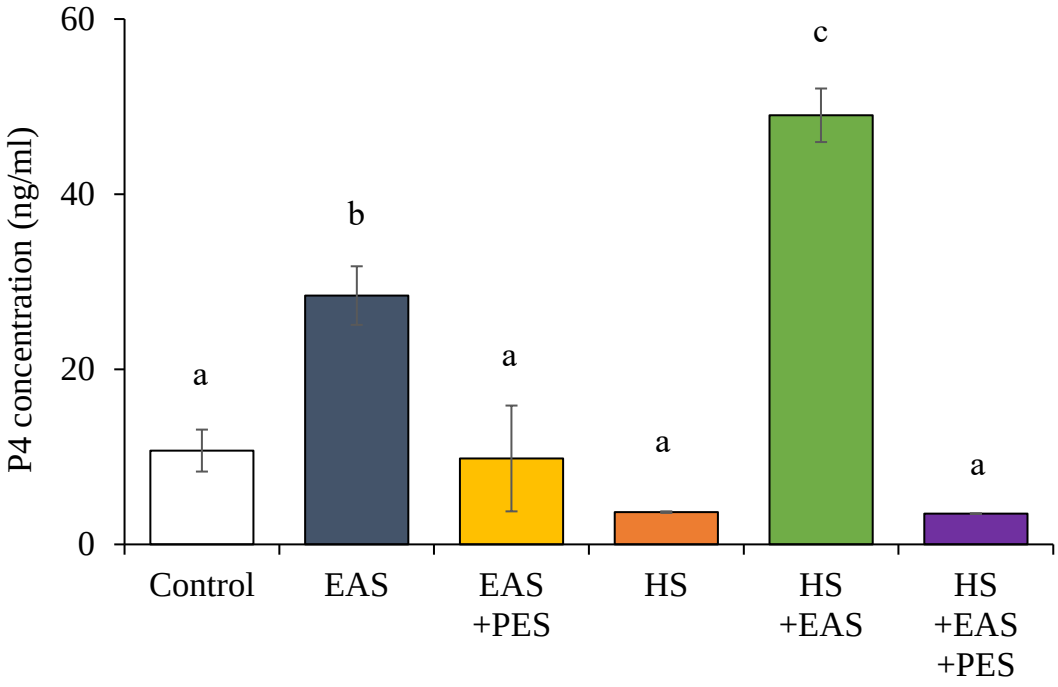


**Figure 21.** Effect of HSP70 inhibitor on P4 production in bovine GC under non-HS and HS conditions

EAS and non-EAS treated cells were subjected with or without 10 µM of HSP70 inhibitor, PES at 38.5°C (Control, EAS, EAS + PES) or at 41°C (HS, HS + EAS, HS + EAS + PES) for 12 h. Detection of P4 secretion by ELISA in cell supernatants. Data are shown as the mean ± S.E.M. Experiment were repeated 3 times. a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.01$).

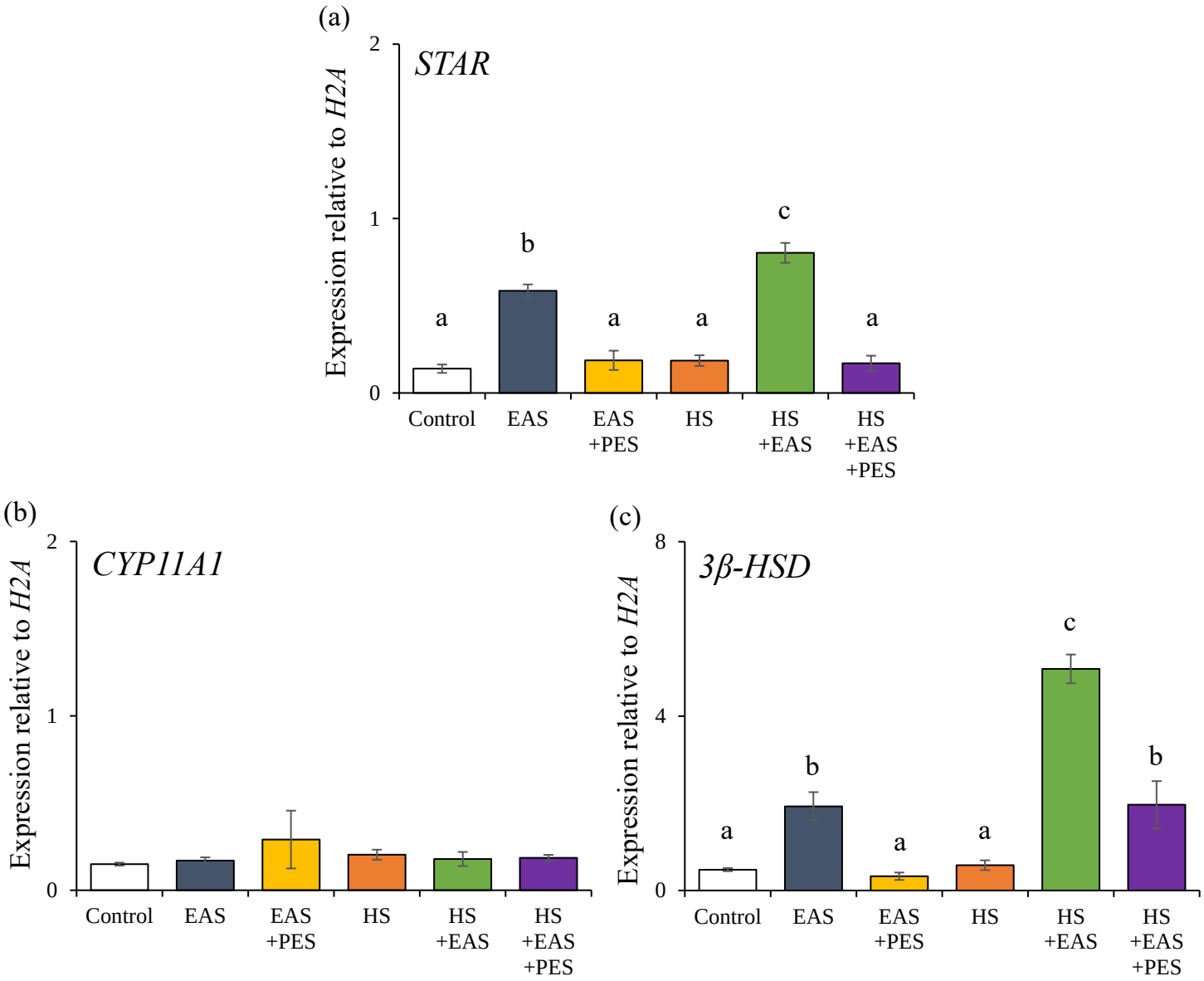


**Figure 22.** Effect of HSP70 inhibitor on steroidogenesis-related genes in bovine GC under non-HS and HS conditions

EAS and non-EAS treated cells were subjected with or without 10 µM of PES at 38.5°C (Control, EAS, EAS + PES) or at 41°C (HS, HS + EAS, HS + EAS + PES) for 12 h. The mRNA expression level of STAR, CYP11A1 and 3β-HSD are presented relative to H2AFZ as a reference gene. Data are shown as the mean ± SEM. Experiment was repeated 5 times. **(a)** a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.01$), **(c)** a vs. b ($P < 0.001$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.05$), (d) a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.001$).

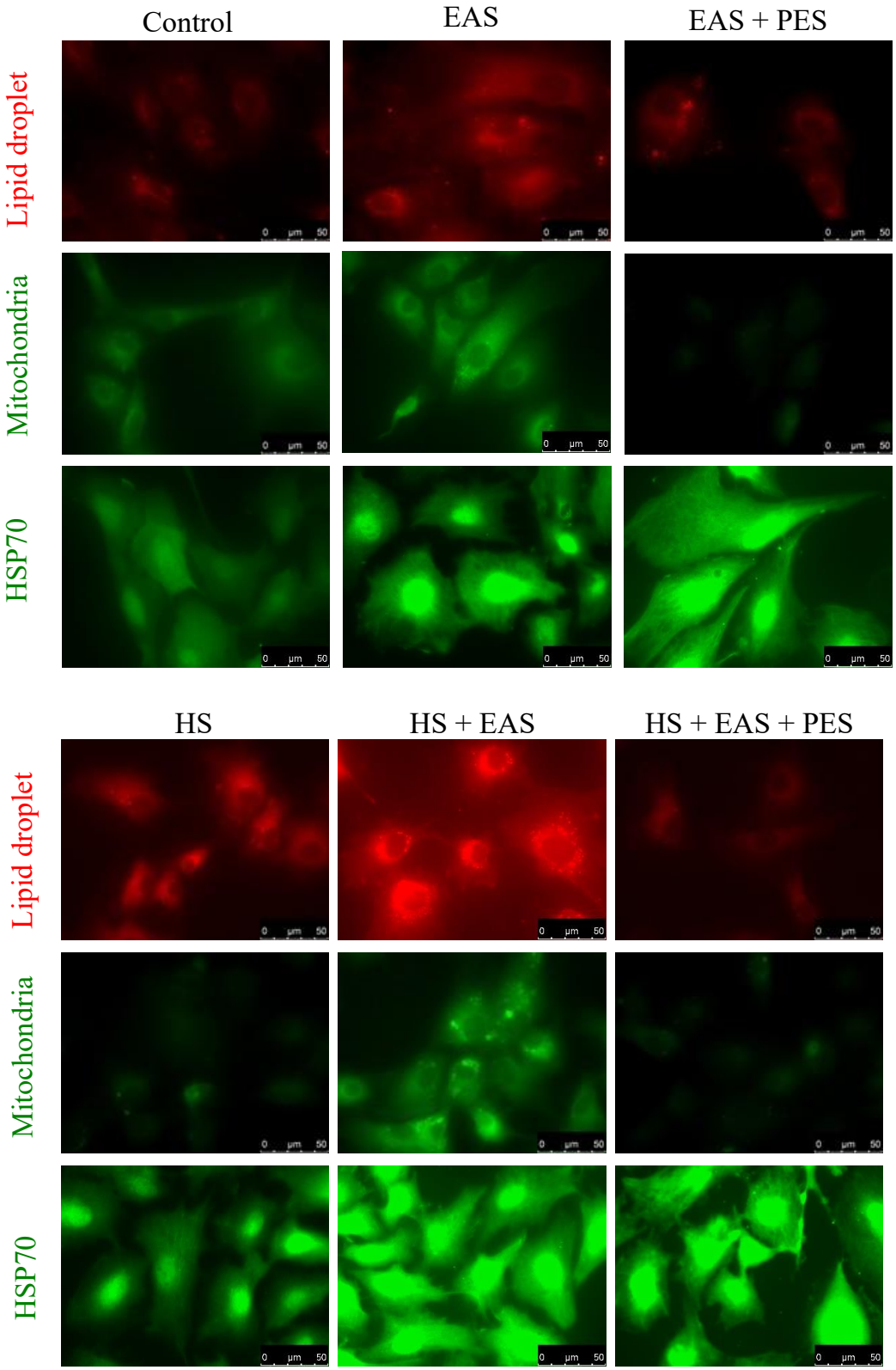


**Figure 23.** Effect of HSP70 inhibitor on mitochondria activity and lipid droplet in bovine GC under non-HS and HS conditions

EAS and non-EAS treated cells were subjected with or without 10 µM of PES at 38.5°C (Control, EAS, EAS + PES) or at 41°C (HS, HS + EAS, HS + EAS + PES) for 12 h. Fluorescent staining image of mitochondria, lipid droplet and HSP70. Scale bar = 50 µm.

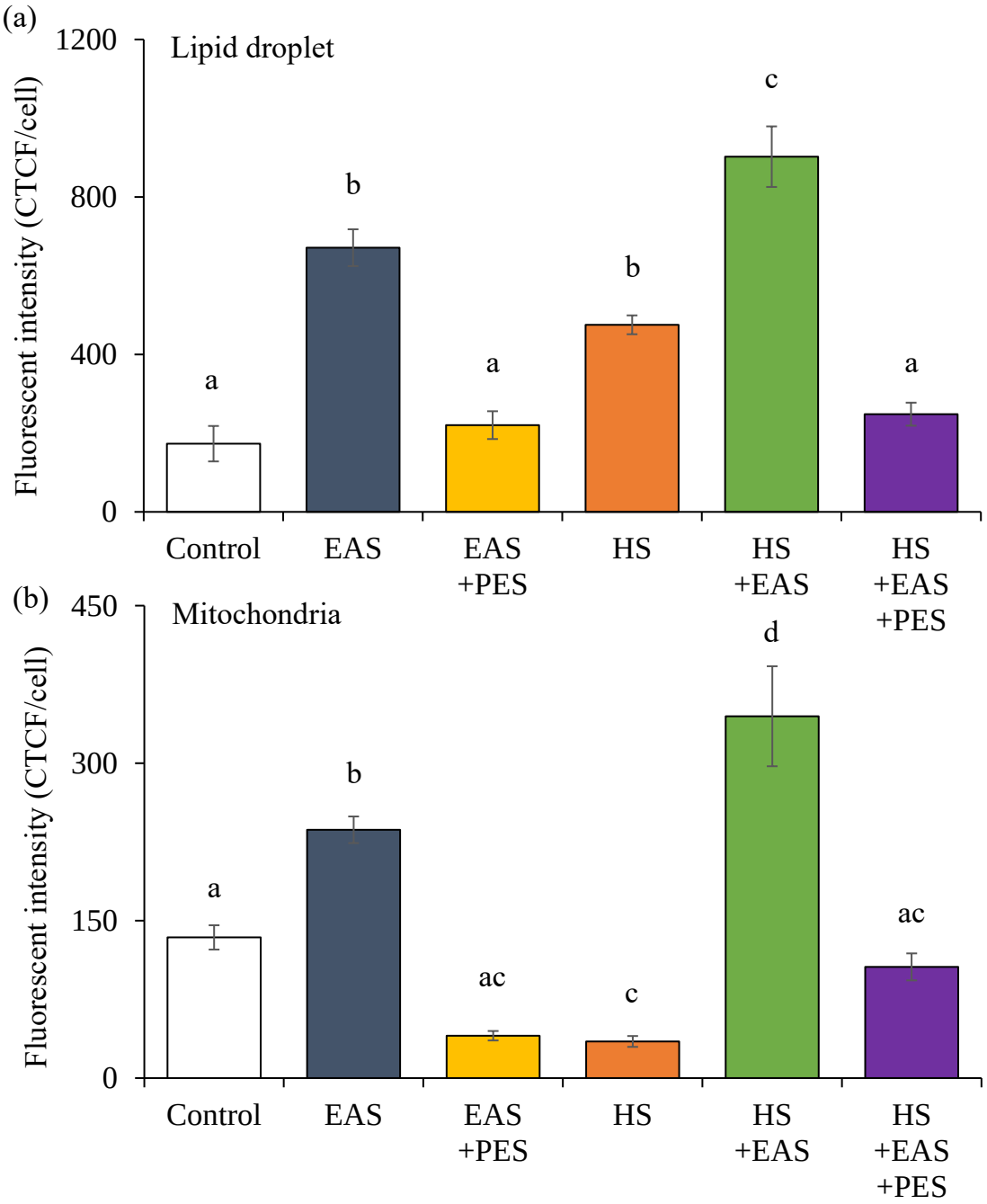


**Figure 24.** Effect of HSP70 inhibitor on fluorescence intensity of mitochondria activity and lipid droplet in bovine GC under non-HS and HS conditions

EAS and non-EAS treated cells were subjected with or without 10 µM of PES at 38.5°C (Control, EAS, EAS + PES) or at 41°C (HS, HS + EAS, HS + EAS + PES) for 12 h. Fluorescence intensity was analysis by CTCF. Data are shown as the mean ± SEM. Experiment was repeated 5 times. **(a)** a vs. b ($P < 0.05$), a vs. c ($P < 0.001$), and b vs. c ($P < 0.05$), **(b)** a vs. b ($P < 0.05$), a vs. c ($P < 0.05$), a vs. d ($P < 0.001$), b vs. c ($P < 0.001$), b vs. d ($P < 0.05$), c vs. d ($P < 0.001$).

***Discussion***

In the present study, a new finding was confirmed that EAS induces P4 synthesis through steroidogenic genes associated with induction of lipid metabolism and mitochondrial activity in bovine GC both under non-HS and HS conditions. Notably, EAS treatment under HS treatment strongly increased P4 production, expression of *STAR* and *3β-HSD* mRNA, lipid metabolism and mitochondrial activity.

The present results showed that P4 level of bovine GC was highest when treatment with 5mg/mL of EAS. EAS has been found to provide beneficial effect on anti-stress and improve sleep quality in human (Ito et al., 2014). Other several studies have revealed the beneficial effect of EAS on brain cell function (Takahashi et al., 2016). Recently, an evidence has suggested that central nervous system has the ability to synthesis steroid hormone from cholesterol (Bates et al., 2005). P4 synthesis is started by cholesterol import into mitochondria by STAR, after that, CYP11A1 transforms cholesterol to pregnenolone which is converted into P4 by 3β-HSD (Conley and Bird, 1997). In this study, EAS increased P4 level and expression of *STAR* and *3β-HSD* but did not affect the expression of *CYP11A1*. These results suggest that EAS regulates P4 synthesis through STAR and 3β-HSD pathway in mitochondria of bovine GC. Furthermore, HS can synergistically promote this pathway by EAS treatment, resulting in promote P4 synthesis. Importantly, mitochondria has a central role for steroidogenesis enzymic activity (Rone et al., 2009; Bassi et al., 2021). Surprisingly, in the present study, EAS induced mitochondrial activity was synergistically improved with HS. In contrast,

mitochondrial activity was decreased by single HS. Generally, HS in summer season causes the decrease in ovarian function with reduced of P4 level and CL size (Howell et al., 1994; Burke et al., 2001; Wolfenson et al., 2002). In mammalian cells, cholesterol preservation for steroid hormone is lipid droplet (Zweytick et al., 2000). My present result showed that lipid synthesis was induced both by EAS and HS at the same level, and it showed greatest expression by co-treatment of EAS and HS. Collectively, EAS induced P4 synthesis through enhancement STAR, 3β-HSD enzyme activity, mitochondrial activity and lipid droplet in bovine GC. In addition, significant effect of EAS and HS co-treatment on P4 synthesis was also found in the present study.

In chapter 2, EAS was revealed to enhance redox balance through reduction of ROS levels under ordinary non-HS condition, and surprisingly EAS had significant effect for HSP70 induction and ROS reduction in bovine GC even under HS condition compared with the fact of increased ROS and decreased GSH under HS without EAS.

In the previous study, STAR, a key enzyme of P4 synthesis, was inhibited by ROS in rat Leydig cells (Tsai et al., 2003). ROS also blocked P4 synthesis, CYP11A1 and 3β-HSD in human granulosa luteal cells (Endo et al., 1993). In bovine GC, HS-induced ROS reduced *STAR* and *CYP11A1* gene expression (Khan et al., 2020). Hydrogen peroxide, most popular ROS, decreased P4 level, *STAR* and *3β-HSD* gene expression in bovine GC (Wang et al., 2021). In addition, high concentration of ROS caused negative impact to mitochondria in mouse GC cell (Hoque et al., 2021). Therefore, a redox balance might also be important to regulate

steroidogenesis-related genes and mitochondrial activity in bovine GC. Besides, lipid droplet is a marker of cellular stress, and it has important role for maintaining redox status under stressed condition (Jarc and Petan, 2019). Under stressed conditions, lipid droplet is sequestrated toxic lipid and delayed release of lipid, and this lipid droplet biosynthesis maintains redox balance (Jarc and Petan, 2019). Thus, EAS-treated GC in this study with keeping redox balance may caused the stabilization of lipid droplet as a source of cholesterol than non EAS treated cells. Collectively, enhancement of P4 synthesis by EAS treatment with keeping redox status depend on ROS level in bovine GC.

In the present study, HS induced lipid droplet, while reduced mitochondrial activity in bovine GC. Overall results obtained in chapter 2 suggest that ROS and ROS-induced DNA damage were detected mainly in nucleus of bovine GC under HS condition. In bovine somatic cell nuclear transfer embryos, mitochondrial and DNA damage were induced by increase of ROS (Hwang et al., 2013). Thus, impairment of DNA damage in nucleus reduced mitochondria function in HS bovine GC as well as affecting gene expressions. Previous studies have proved that lipid droplet has dynamic function in cellular metabolism (Olzmann and Carvalho, 2019). In mice, mitochondrial damage by hypoxia-induced formation of lipid droplet (Lee et al., 2013). Thus, high expression of lipid droplet in bovine GC by HS can be explained due to mitochondrial dysfunction. Collectively, HS-induced ROS damage in nucleus impaired mitochondria function which accumulation of lipid droplet in bovine GC. EAS and EAS plus HS treatment also induced lipid

droplet, these results were related with expression of HSP70 which will discuss in more details in next paragraph.

In chapter 2, overall data confirmed the beneficial effect of EAS and HSP70 production via regulating redox balance by HS treatment in bovine GC. HSP70 inhibitor, PES did not affect expression of HSP70 protein, however HSP70 inhibition clearly inhibited mitochondrial activity and synthesis of lipid droplet. Previous study showed that PES disrupted co chaperone and substrate protein of HSP70 without affecting HSP70 expression, thus PES could inhibit function of HSP70 protein in multiple cell signaling pathways (Leu et al., 2009). PES inhibited P4 synthesis, expressions of *STAR*, *3β-HSD*, mitochondrial activity and lipid droplet synthesis in bovine GC even in the presence of EAS both under non-HS and HS conditions. Therefore, these data support the idea that the increase of EAS-induced HSP70 promote P4 synthesis. In addition, localization of HSP70 was clearly detected both in nucleus and ctytoplasm of EAS-treated cells, particularly under HS condition. In mammalian cells, HSP70 have chaperone function in both nucleus and cytoplasm (Michels et al., 1997; Imamoto, 2018).

In chapter 2, EAS-induced HSP70 clearly reduced DNA damage with inducting by ROS which is mainly generated in the nucleus in bovine GC. Modulation of redox balance-regulated mitochondrial activity and lipid droplet with reducing ROS damage in the nucleus of bovine GC. In addition, transcription of steroidogenesis enzyme mainly occurs in nucleus (Mizutani et al., 2015). Therefore, EAS-induced HSP70 balanced redox status to improve steroidogenesis enzyme activity, mitochondria function and lipid synthesis in bovine GC. On the

other hand, synthetic chemical chaperone increased 3β-HSD metabolic activity in inner mitochondrial membrane by promoting the folding speed of 3β-HSD from native into active state (Thomas and Bose, 2015). In rat adipocytes lipid droplets, HSP70 stimulation involved to stabilizing the droplet monolayer, protein folding and mobilization nascent proteins to the lipid droplets (Jiang et al., 2007). Therefore, EAS-induced HSP70 enhanced accumulation of lipid droplet and 3β-HSD activity in mitochondria in cytoplasm with chaperone function of bovine GC.

In summary, overall data support the idea about direct and indirect regulation of HSP70 by EAS on steroidogenesis-related genes, mitochondria activity and lipid synthesis both in nucleus and cytoplasm of bovine GC.

In the conclusion of this chapter, my present study showed enhancement effect of EAS on P4 level through steroidogenesis enzyme activity, mitochondrial activity and lipid droplet in bovine GC under both non-HS and HS conditions (Figure 25). Moreover, I also discovered the significant effect of EAS and HS co-treatment on P4 production bovine GC. Furthermore, P4 synthesis induction by EAS treatment was regulated directly and indirectly by HSP70 in bovine GC. These findings might be useful for application EAS for improvement the reproductive functions in mammals including bovine with combination of HS treatment.

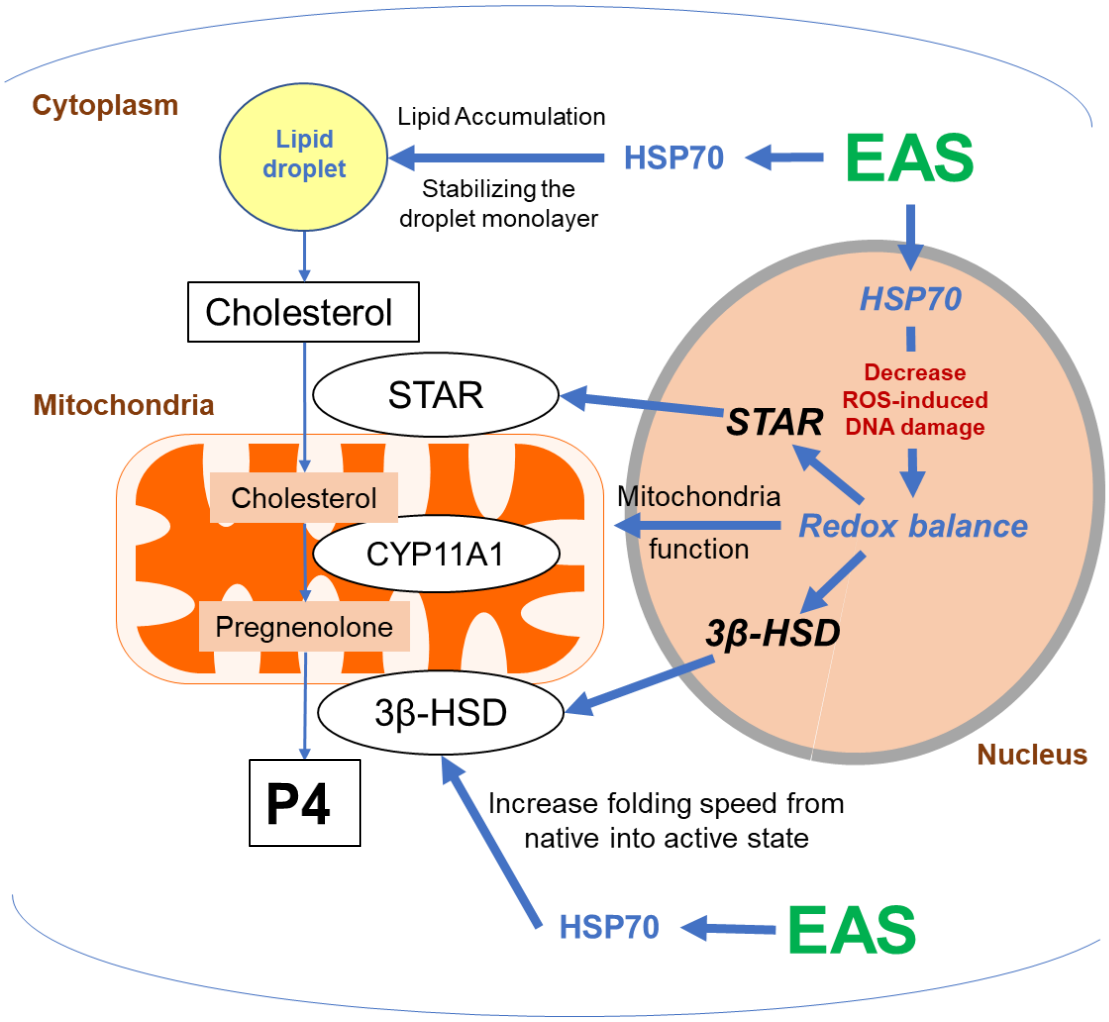


**Figure 25.** Schematic diagram of effect EAS on P4 synthesis of bovine GC

## Chapter 4 **Effect of a standardized extract of *Asparagus officinalis* stem on the cell viability after cryopreservation**

### ***Introduction***

GC are known to support oocyte growth and maturation in ovaries (Fatehi, 2005). Furthermore, the GC differentiate into corpus luteal cells after ovulation and which secrete progesterone to support the maintenance of pregnancy after successful fertilization *in vivo* (Abedel-Majed et al., 2019).

GC are also used for the *in vitro* research of cell function and co-culture to support oocyte maturation and embryo development by using freshly collected or frozen-thawed cells (Broussard et al., 1994). Cryopreservation is an important technique for long-term storage and transportation of many types of cells, including bovine and ovine GC (Broussard et al., 1994; Loi et al., 2008). However, freezing and thawing processes induce harmful effects on cell function, including damage to the membranes and nuclei, metabolic perturbations, and impaired mitochondrial activity and gene expression (Mazur et al., 1972; McGann et al., 1988; Linfor and Meyers, 2002) .

In humans, cryopreserved GC are used for coculture to support embryo development (McAllister et al., 1990). In addition, human GC have beneficial effects on the development of embryos under *in vitro* conditions (Plachot et al., 1993). Therefore, GC co-culture systems offer relatively secure and appropriate

methods enhancing embryo development. In case of co-culture of frozen-thawed human embryos, co-culture cells should be prepared with thawing and start culture when needed. Therefore, the use of frozen GC from oocytes as donors to supply additional cells for co-culture is a possible solution. In bovine oocytes, frozen GC also enhance *in vitro* maturation and fertilization (Broussard et al., 1994). In addition, GC are used as *in vitro* models for steroidogenesis research since GC differentiate into CL cells. The viability and function of these cells after thawing are important for steroidogenesis, and the pre-freezing status of cells is also important for maintaining higher viability and cellular functions. Therefore, the state of the cells before cryopreservation is likely to have a significant influence on the cell function and viability after thawing.

Several positive and negative factors influence cell quality and viability. HS is one of the negative stress factors that affects cell functions. In cattle, HS has harmful effects on reproductive performance, including follicular development, ovarian function, steroidogenesis, and fertilization (Wilson et al., 1998). Dysfunction of GC induce functional disorders in ovaries (Tatone et al., 2008). In addition, HS reduces hormone production in bovine GC, and induces apoptosis in swine GC (Roth et al., 2001a; Sirotkin, 2010).

In addition to the harmful effects of HS on cellular functions, cryopreservation processes also increase ROS generation through damage to the mitochondria

(Mazur et al., 1972; McGann et al., 1988; Linfor and Meyers, 2002; Dalcin et al., 2013). In general, high levels of ROS induce damage to the cells by inducing death signals to disturb the redox status (Murdoch, 1998; Halliwell, 2000). As mentioned in chapter 2, GSH is a major antioxidant that reduces ROS generation and maintains the cellular redox status (Murdoch, 1998). Redox balance and cellular defense against oxidative stress are essential for maintaining of cell functions including DNA replication, transcription, translation and functional protein function. GSH synthesis pathways including glutathione reductase, glutathione peroxidase, and glutathione *S*-transferase are regulated by NRF2 that a master regulator of antioxidant response (Krajka-Kuzniak et al., 2017). NRF2 and KEAP1 is known as components of the NRF2/KEAP1 signaling pathway and plays an important role in the antioxidant defense system via GSH synthesis (Steele et al., 2013). In addition, HS increases ROS levels in bovine GC (Khan et al., 2020). Therefore, the improvement of the cellular redox status represents a potential strategy for improving the post-freezing viability of cells under HS conditions.

Induction of HSP70 expression has been reported to enhance the quality of post-freezing goat semen and the viability of spermatozoa in bull (Elliott et al., 2009) and boar (Reddy et al., 2018). HSP70 also plays a role in regulating redox status with decrease in ROS generation and increase in kidney cells (Guo et al., 2007). These findings strongly suggest that HSP70 plays a role in the improvement of

post-freezing viability by maintaining cellular redox status. In chapter 2, I found that supplementation of EAS induced HSP70 production without affecting HSP90 and HSP27 in bovine GC. Furthermore, EAS significantly improvement redox status by decreasing ROS and reduced DNA damage even under HS condition. Therefore, I hypothesized that pretreatment of EAS improves the post-freezing viability of bovine GC under HS condition by improving the redox status and HSP70.

To prove the hypothesis, I investigated the effect of EAS treatment on post-freezing viability of bovine GC.

***Materials and Methods***

*Bovine GC culture*

Collection and culture of GC were achieved by the methods described in the section of materials and methods in Chapter 2.

*Experimental design*

GC were cultured with or without 5 mg/mL EAS for 12 h at 38.5°C and 41°C. Then, the cells were immediately processed for the following experiments including gene expression, HSP70 immunostaining, ROS, and GSH detection. For cryopreservation, cells were collected by washing with PBS (–) and followed by PBS (–) containing 0.05% trypsin and 0.53 mM EDTA for 2 min at 38.5°C in a 5% $CO_2$ to obtain a cell suspension. Subsequently, 5% FBS in DMEM was added to inhibit trypsin activity, and the cell suspension was centrifuged at 1,200 × *g* for 3 min, and then cryopreserved and stored at -80°C for 48 h. After cryopreservation, the cells were thawed and the percentage viability, ROS, and GSH levels analyzed immediately.

*Cryopreservation and thawing*

After treatment with EAS and HS, bovine GC were detached as washed as outlined above. Viable cells were counted by using a hemocytometer after the suspension with 0.4% trypan blue solution (Wako, Osaka, Japan). Cells were then adjusted to the final density of $1.0 \times 10^6$ cells/mL in cryovial (SPL Life Sciences, Pocheon, Korea) by adding of cryoprotectant solution (BAMBANKER™, Wako).

The vial are frozen at -80°C for 48 h. After 48 h of storage, the vials were then thawed rapidly (< 1 minute) with water bath at 37°C and promptly mixed with 10 mL culture medium containing 5% FBS in DMEM in 15 ml tube. After centrifugation at 200 × *g* for 5 min, the supernatants were removed, and cell pellets were collected for further experiments.

*Valuation of viability*

After thawing (< 1 minute), the cell viability was evaluated with a live-dead cell staining kit (ALX-850-249; Enzo Life Sciences AG, Lausen, TX, USA) according to the manufacturer's instruction. The stained cells were observed using hemocytometer under a fluorescent microscope with an EVOS™ M5000 Imaging System (Thermo Fisher Scientific) for the green fluorescent protein and Texas red dyes. Cell viability was evaluated with green fluorescence as live and propidium iodide (PI)-stained red fluorescence as damaged or dead.

*Detection of ROS and GSH*

For ROS detection, the cells were incubated with 250 μM CellROX® oxidative stress reagent (cat. no. C10444; Life Technologies, Carlsbad, CA, USA) for 30 min at 38.5°C. For GSH staining, cells were incubated with 10 μM ThiolTracker™ Violet (cat. no. T10095; Molecular Probes, Eugene, OR, USA) for 30 min at 38.5 °C. Nuclei were stained with 25 μg/mL Hoechst 33342 (Sigma-Aldrich) and incubated at 38.5°C for 30 min.

Fluorescence images were acquired and analysed similar as described previously (Chapter 2), materials and methods section.

*Statistical analysis*

Statistical analysis was conducted as described in chapter 2

***Results***

*Effect of EAS and HS on post-freezing viability of bovine GC*

To determine whether the post-freezing viability of bovine GC was enhanced by EAS pretreatment, I measured the cell viability of the cells in each treatment group using a live/dead cell staining kit. Live and dead cells were clearly detected (Figure 26a). The average cell viability of the control cells was 88.7 ± 1.7% (Figure 26b). Although post-freezing viability was not affected by EAS treatment in the control cells, c HS treatment significantly decreased the viability ($P < 0.05$) (Figure 26b). In contrast, EAS pretreatment significantly increased cell viability of HS-treated cells, which was similar level as the control ($P < 0.05$) (Figure 26b).

*Effect of EAS and HS on post-freezing redox status of bovine GC*

To investigate whether the post-freezing redox status of bovine GC was improved by EAS pretreatment, I detected ROS generation and GSH synthesis in post-thawed bovine GC. As shown in figure 27a, strong fluorescence of ROS was detected in the nuclei of HS treated cells whereas EAS-treated cells showed a weak fluorescence of ROS both in control and HS treated cells. Besides, EAS pretreatment significantly decreased ROS levels in the control cells ($P < 0.05$) after thawing (Figure 27). In addition, EAS pretreatment significantly decreased the HS-induced ROS levels in the nuclei after thawing ($P < 0.01$, Figure 27).

Fluorescence of GSH was detected clearly in the nuclei of the cells (Figure 28a). After image analysis, GSH levels were significantly higher by EAS pretreatment

both in the control and HS treated cells ($P < 0.05$, Figure 28b). HS treatment did not affect the GSH level after thawing (Figure 28b)

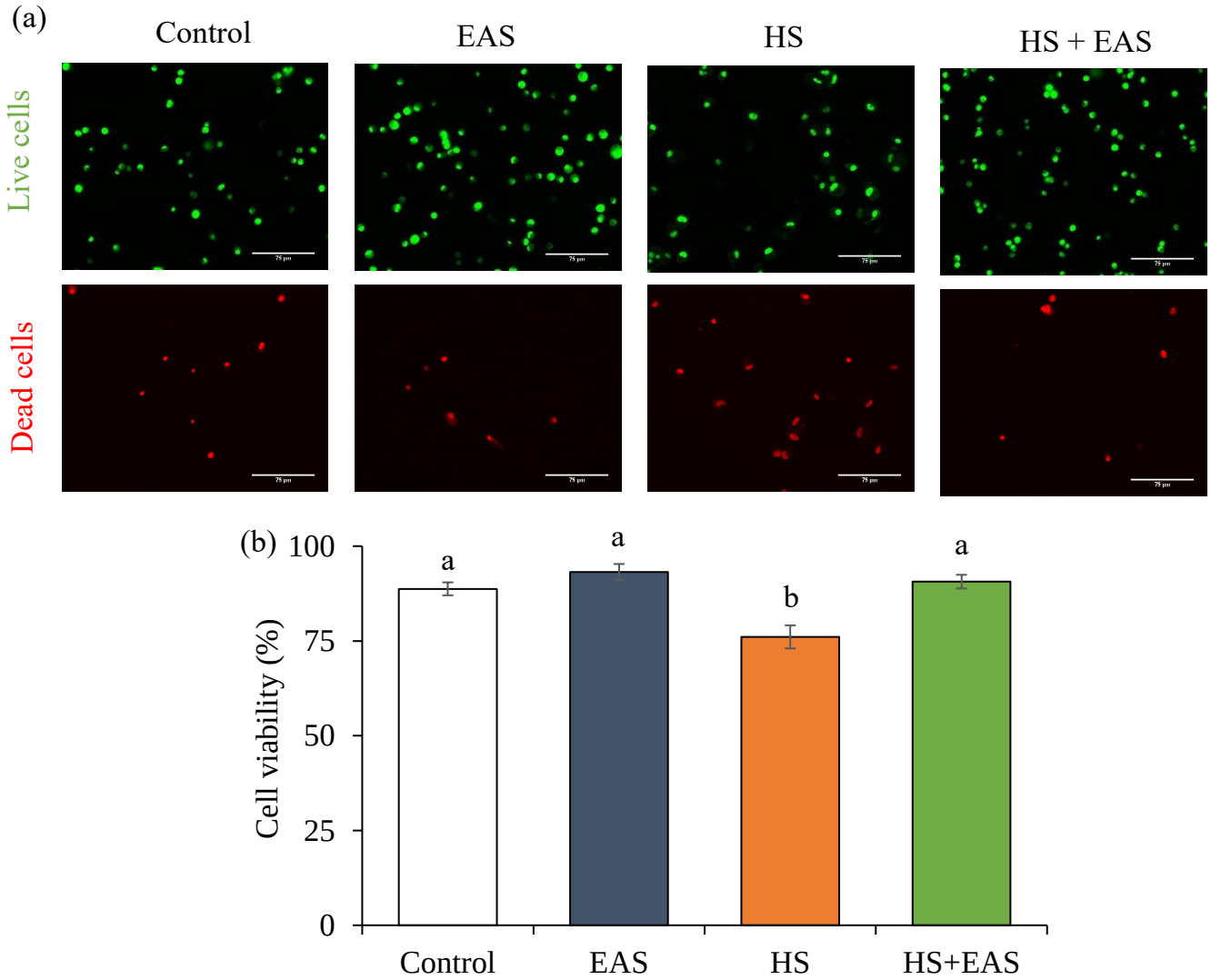


**Figure 26.** Effect of EAS pretreatment on post-freezing viability in bovine GC

Cells were cultured with or without 5 mg/mL of EAS at 38.5°C (Control, EAS) or at 41°C (HS, HS + EAS) for 12 h. **(a)** A cell-permeable green fluorescent dye (FITC), to stain live (upper) and dead cells can be easily stained by propidium iodide, a cell non-permeable red fluorescent dye (TXR) (lower). Scale bar, 75 µm. **(b)** Post-freezing cell viability (%). Data are shown as the mean ± standard error of the mean (SEM). Experiment were repeated 5 times. a vs. b ($P < 0.05$).

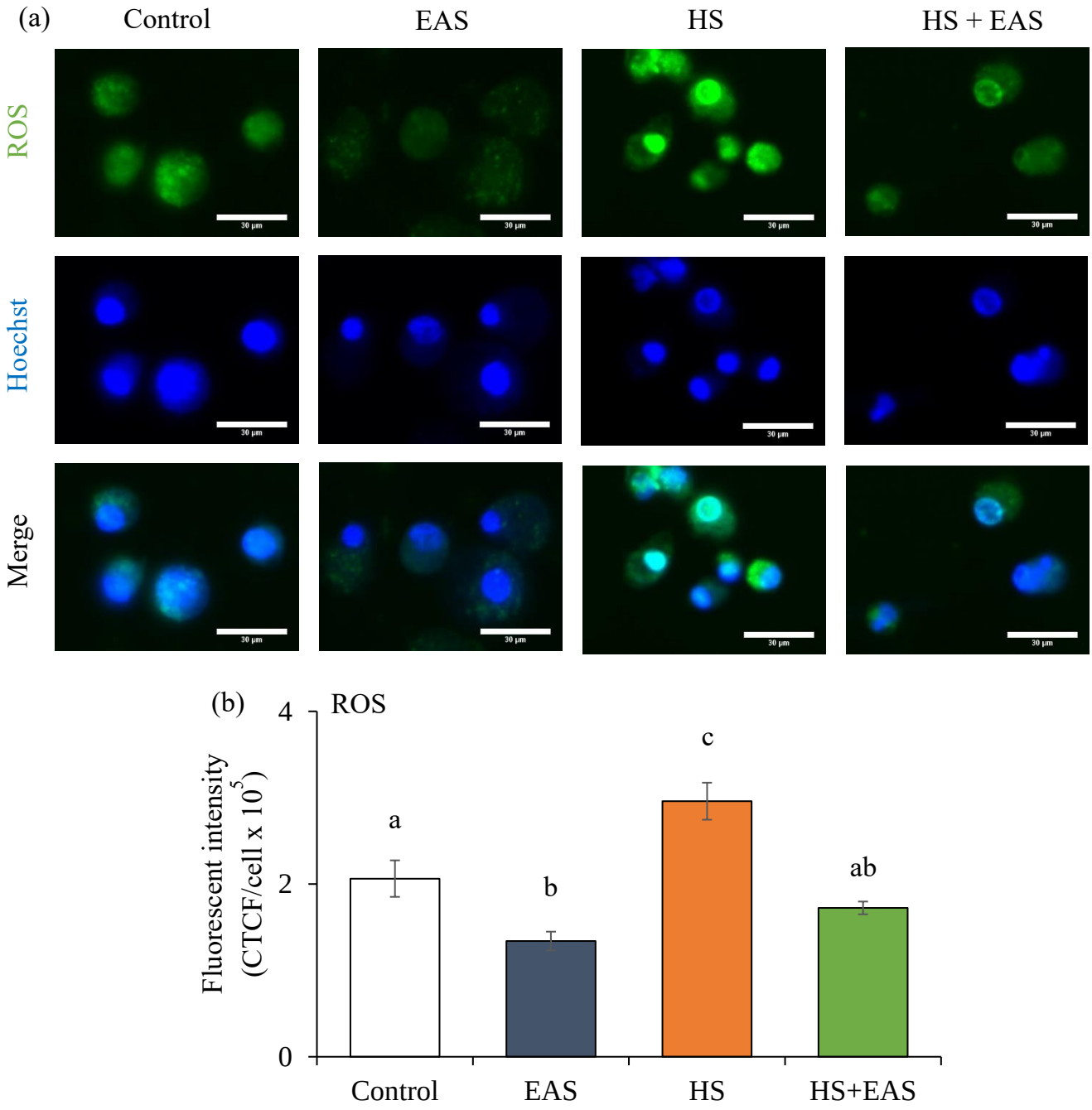


**Figure 27.** Effect of EAS pretreatment on ROS levels in post-freezing bovine GC

Cells were cultured with or without 5 mg/mL of EAS at 38.5°C (Control, EAS) or at 41°C (HS, HS + EAS) for 12 h. **(a)** Fluorescence of ROS (upper), nuclei stained with Hoechst (lower), and merged images. Scale bar, 30 µm. **(b)** Fluorescence intensity of ROS (CTCF). Data are mean ± standard error of the mean (SEM). Experiment were repeated 5 times. a vs. b ($P < 0.05$), a vs. c ($P < 0.05$), b vs. c ($P < 0.01$).

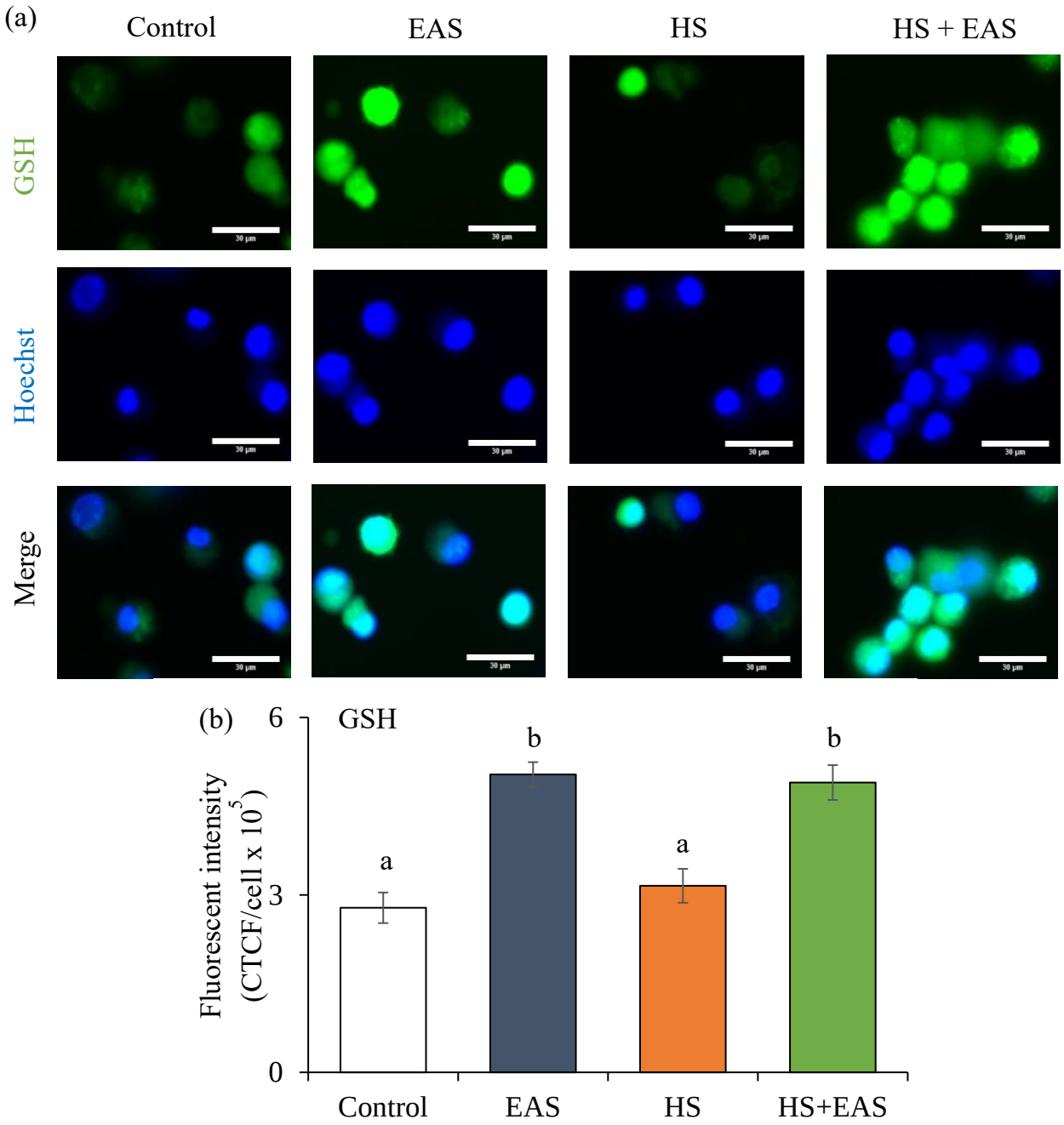


**Figure 28.** Effect of EAS pretreatment on GSH levels in post-freezing bovine GC

Cells were cultured with or without 5 mg/mL of EAS at 38.5°C (Control, EAS) or at 41°C (HS, HS + EAS) for 12 h. **(a)** Fluorescence of GSH (upper) and nuclei stained by Hoechst (lower) and merged images. Scale bar, 30 µm. **(b)** Fluorescence intensity of GSH (CTCF). Data are shown as mean ± standard error of the mean (SEM). Experiment were repeated 5 times. a vs. b ($P < 0.01$).

***Discussion***

HSPs are well-known molecular chaperones that support the folding of polypeptides (Hartl et al., 2011). Protein impairment by HS can interact with HSPs and either refold to the normal state or are degraded (Mosser and Morimoto, 2004). HSPs are classified based on their molecular weight, and HSP70 is one of the most extensively studied protein among the HSP family (Kim et al., 2018). HSP70 overexpression increased the viability of frozen normal human fibroblasts (Shaik et al., 2017). In chapter 2, I showed that EAS induced HSP70 synthesis in bovine GC, especially with significant effect of EAS and HS co-treatment with decrease in ROS generation and DNA damage and increase of GSH. These results show that EAS modulates the intracellular redox condition with increase in HSP70. Based on these results, I postulated that the induction of HSP70 in pre-freezing cells would prevent cell death following cryopreservation under HS condition.

Alternative HSP70 inducers from plant sources, such as bimoclomol, paeoniflorin, and geranylgeranylacetone, provide the synergistic effect on HSP70 induction (Vigh et al., 1997; Yan et al., 2004). This could prove useful in future studies aiming to strengthen the stress-induced intracellular environment by controlling molecular chaperones, including HSP70. In the present study, I showed that HSP70 inhibition may cause the increased ROS, reduced GSH, and DNA

damage, HSF1 and NRF2 gene expressions, and proved that HSP70 induced by EAS improve redox balance in bovine GC.

After thawing, the redox status of pre-frozen bovine GC was highly maintained by EAS pretreatment, but was highly impaired by HS. In the present data described in chapter 2 and previous report (Ogasawara et al., 2014) shows that the ROS levels in cells were decreased by EAS supplementation. High levels of ROS cause an imbalance in redox status, which induces cellular damage to DNA, proteins, and lipids (Orrenius et al., 2007). In addition, HSP70 overexpression influences the cellular redox status by regulating GSH synthesis enzymes (Guo et al., 2007). In this chapter, EAS-treated bovine GC enhanced redox status by decreasing ROS and increasing GSH levels. Therefore, EAS could maintain the cellular redox status of pre-frozen bovine GC by decreasing ROS generation and increasing GSH synthesis.

In the present chapter, the post-freezing viability of bovine GC decreased after exposure to HS, but was recovered by EAS supplementation. My results are consistent with previous study, HS induced cell death of normal human fibroblasts and frozen-thawed bovine blastocysts (Park et al., 2013; Mori et al., 2015). There are evidences that the cytoprotective effects of HSP70 aggravate cryopreservation damage in cells (Wang et al., 2005; Shaik et al., 2017). Exposure of cells to stress conditions, such as HS, is a popular method of inducing HSP70 (Lindquist, 1986).

However, HS not only induces HSP70 but also causes cell death by inducing apoptosis (L. Li et al., 2016).

In the present study, HS significantly increased the expression of both HSP70 gene and protein in bovine GC, and EAS led to similar levels of *HSP70* in non-HS control cells associated with *HSF1* expression. These results suggest that *HSP70* and *HSF1* expression is regulated by EAS treatments. However, HSP70 immunolocalizations were not similar, that is, HS treated-cells showed highly condensed HSP70 in the nucleus, whereas EAS-treated cells showed HSP70 localization in both the nuclei and cytoplasm. Notably, HS plus EAS-treated cells showed high expression and localization of HSP70 both in the nucleus and cytoplasm, similar to that in EAS-treated control cells. HSP70 activity has been observed to induce protection in hamster fibroblasts cells both in the nucleus and cytoplasm (Michels et al., 1997). Protein synthesis and folding activities occur in the cytoplasm; therefore, the molecular chaperone function of HSP70 is mostly reported in the cytoplasm (Imamoto, 2018). HSP70 is localized in the cytoplasm under conditions of hypoxemia or oxidative stress (Imamoto, 2018). However, HSP70 translocases to the nucleus under HS conditions, and translocation of HSP70 in the nucleus contributes to protecting cells from heat-induced DNA breaks (Kotoglou et al., 2009). In the results of chapter 2, EAS treatment clearly protected HS treated bovine GC against ROS-induced DNA damage by HS-

induced ROS levels in the nuclei significantly. Therefore, the localization of HSP70 in the nuclei of HS treated cells might be due to the translocation of HSP70 to protect cells against ROS-induced DNA damage.

In the present study, the reduction in post-freezing viability by HS treated cells was due to an increase in ROS generation. On the other hands, GSH levels were not decreased by HS treatment. The possible reason is that duration of HS treatment was not sufficient to reduce the GSH pool in the cells, whereas ROS was rapidly generated and accumulated. In contrast, EAS pre-treatment rescued HS-damaged cells by reducing ROS generation and inducing GSH levels in bovine GC. In frozen oocytes and embryos, ROS induce mitochondrial damage, adenosine triphosphate depletion, and impair development (Santoro, 2000; Steele et al., 2013). Besides, in frozen thawed sperm, generated ROS induce DNA and protein damage, thereby reducing sperm viability (Thomson et al., 2009). Moreover, ROS impair the cellular redox status, which enhances cell proliferation, differentiation, and death (Hiroi et al., 2005). GSH neutralizes ROS generation within cells rapidly (Wei and Lee, 2002) with other antioxidant enzymic activities like SOD and GPX. When the balance between ROS and GSH production was recovered, the balance of redox status rescued the post-freezing viability of bovine GC. Moreover, HSP70 enhances cell protection by repairing misfolded proteins and maintaining redox status (Guo et al., 2007; Saibil, 2013). In the present study, EAS and HS

significantly increased HSP70 expression both in the nuclei and cytoplasm. Overall, HS-induced ROS causes an imbalance in redox status, resulting in impaired post-freezing viability of bovine GC. EAS-induced HSP70 rescued post-freezing viability in bovine GC by maintaining redox status via a reduced in ROS levels and increased GSH levels.

NRF2 and KEAP1 bind together under non-stressed condition, and KEAP1 releases NRF2 under stressed conditions (Glory and Averill-Bates, 2016). NRF2 is responsible for antioxidants, redox regulators, and GSH when cells are exposed to oxidative stress (Hayes and Dinkova-Kostova, 2014). KEAP1 has been found to be an oxidative stress sensor that regulates NRF2 (Bryan et al., 2013). In the present study, HS increased both *NRF2* and *KEAP1* expression compared to that in the control cells. The induction of KEAP1 by HS might be due to an increase in ROS generation. HS-induced NRF2 indicated that the oxidative stress-defense pathway was activated, but it was not enough to overcome high levels of ROS, leading to reduced cell viability. In contrast, EAS treatment increased *NRF2* expression and decreased *KEAP1* expression. The results suggest that EAS induced antioxidant defense and reduced oxidative stress, leading to the rescue of HS treated cell viability.

In addition, the decrease in the post-freezing viability of HS-treated cells, indicating the incorporation of PI-stained nuclei by membrane damage, was

recovered by EAS. PI is a red-fluorescent cell viability dye which is excluded from live cells with intact membranes but penetrates dead or damaged cells and binds to DNA. The present result suggested that an increase in HSP70 in both the nuclei and cytoplasm protected the cell structure, including protection against membrane damage.

In summary, EAS induced HSP70 production without HS and had a significant effect with HS on HSP70 induction in bovine GC. EAS also maintained the cellular redox balance in pre- and post-freeze bovine GC. Furthermore, EAS was shown to rescue the post-freezing viability of HS treated bovine GC by maintaining the cellular redox status and reducing oxidative stress.

Further application of EAS to reduce the stress-caused cell damage will contribute to improve cell functions in other cells.

## Chapter 5 **General Discussion**

In my thesis, I confirmed that EAS induced HSP70 production through HSF1 pathway under non-HS condition in bovine GC. In addition, EAS-induced HSP70 reduced ROS production while induced GSH which leads to maintain redox balance in bovine GC in both non-HS and HS conditions. Notably, I figured out the effect of EAS and HS on HSP70 induction. EAS, which contain asparaprolines, an active ingredient of HSP70 at the cellular level (Ito et al., 2014; Nishizawa et al., 2016; Inoue et al., 2020). Proline-containing diketopiperazines is mainly compound of Asparaprolines and it increased HSP70 in brain cell (Faden et al., 2005; Inoue et al., 2020). These informations showed that EAS was purify extraction HSP70 inducer compound. HSF1 is a main regulation factor for HSP70 synthesis. Previous studies have shown that EAS induced HSP70 through HSF1 pathway (Sakurai et al., 2014; Nishizawa et al., 2016). My present study has revealed that EAS and HS significantly induced *HSF1* mRNA expression in bovine GC. Celastrol, HSP70 inducer, induced HSP70 by activation of HSF1, and it also synergized with HS on the induction of HSP70 (Westerheide et al., 2004). Therefore, the synergistic effect of EAS and HS on HSP70 induction may strongly related with HSF1 pathway. The synergistic effect of bimoclomol and HS on HSP70 was explained due to induction translocation of HSF1 into nucleus, binding to HSF1, modulation HSF1 DNA-binding and prolongation HSF1 binding with heat shock element (Hargitai et al., 2003). In addition, celastrol effected on DNA biding and enhanced phosphorylation of HSF1 (Westerheide et al., 2004). Collectively, EAS is identified as HSP70 inducer, and it is capable in both non-HS

and HS conditions, effect of EAS and HS co-treatment may explain by effect of EAS on the activity of HSF1 at multiple level (Figure 29). My result clearly confirmed the significant effect of EAS and HS on HSP70 by activation HSF1. However, further study is still needed to elucidate the mechanism.

Moreover, EAS also improved cell function due to induction P4 level in bovine GC. EAS-induced HSP70 increased P4 synthesis through enhancement steroidogenesis-related genes, lipid metabolism and mitochondrial activity. In addition, HSP70 increased lipid droplet size and fat accumulation due to enhancement of lipogenic gene as sterol regulatory element binding protein 1c, fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase in mice liver cells (Zhang et al., 2018). Therefore, the effect of EAS treatment on P4 synthesis may explain by maintenance of redox status and chaperone function of EAS-induced HSP70. Notably, significant effect of EAS and HS co-treatment also increased P4 synthesis in bovine GC (Figure 30). Besides, EAS-induced HSP70 strongly protected bovine reproductive cells after cryopreservation by maintain redox status.

In summary, EAS treatment and, EAS and HS co-treatment induced HSP70 production in bovine GC. EAS-induced HSP70 contributed to function and protection by maintain redox status and chaperone activity in bovine GC. Furthermore, EAS may have potential effect on the improvement reproduction function in bovine oocyte growth, so application of EAS should be a useful way to improve oocyte maturation and embryo development even under HS condition.

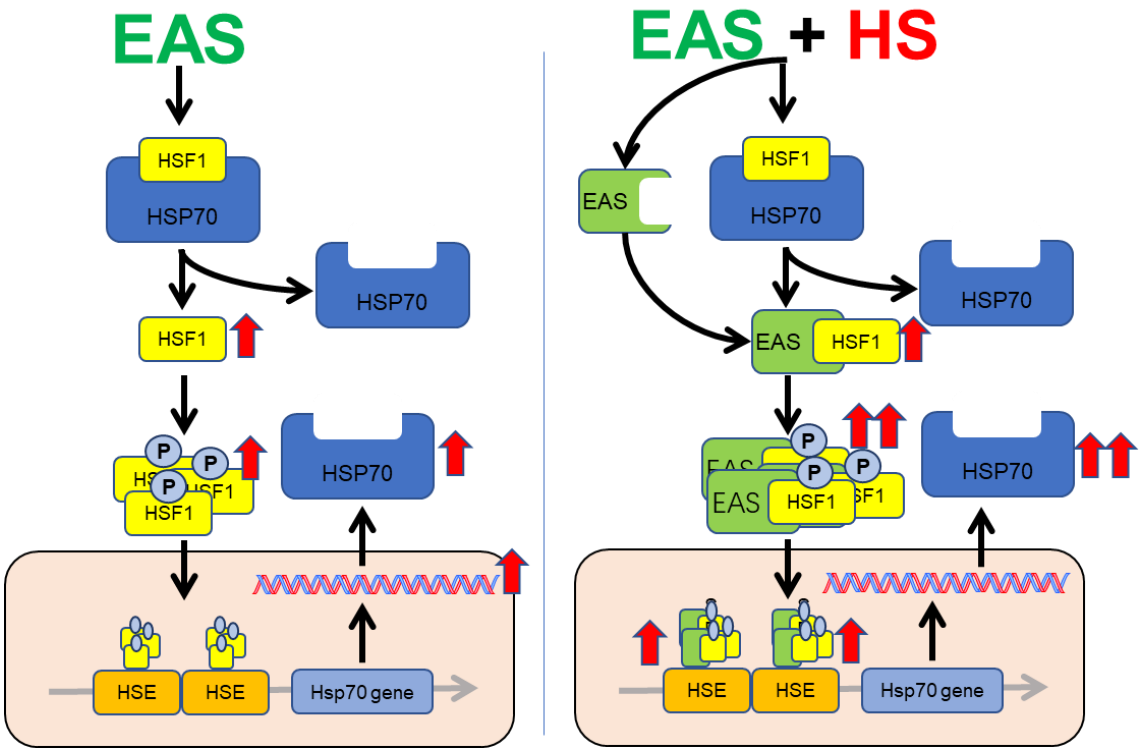


**Figure 29.** Schematic diagram of synergistic effect of EAS and HS on HSP70 production of bovine GC

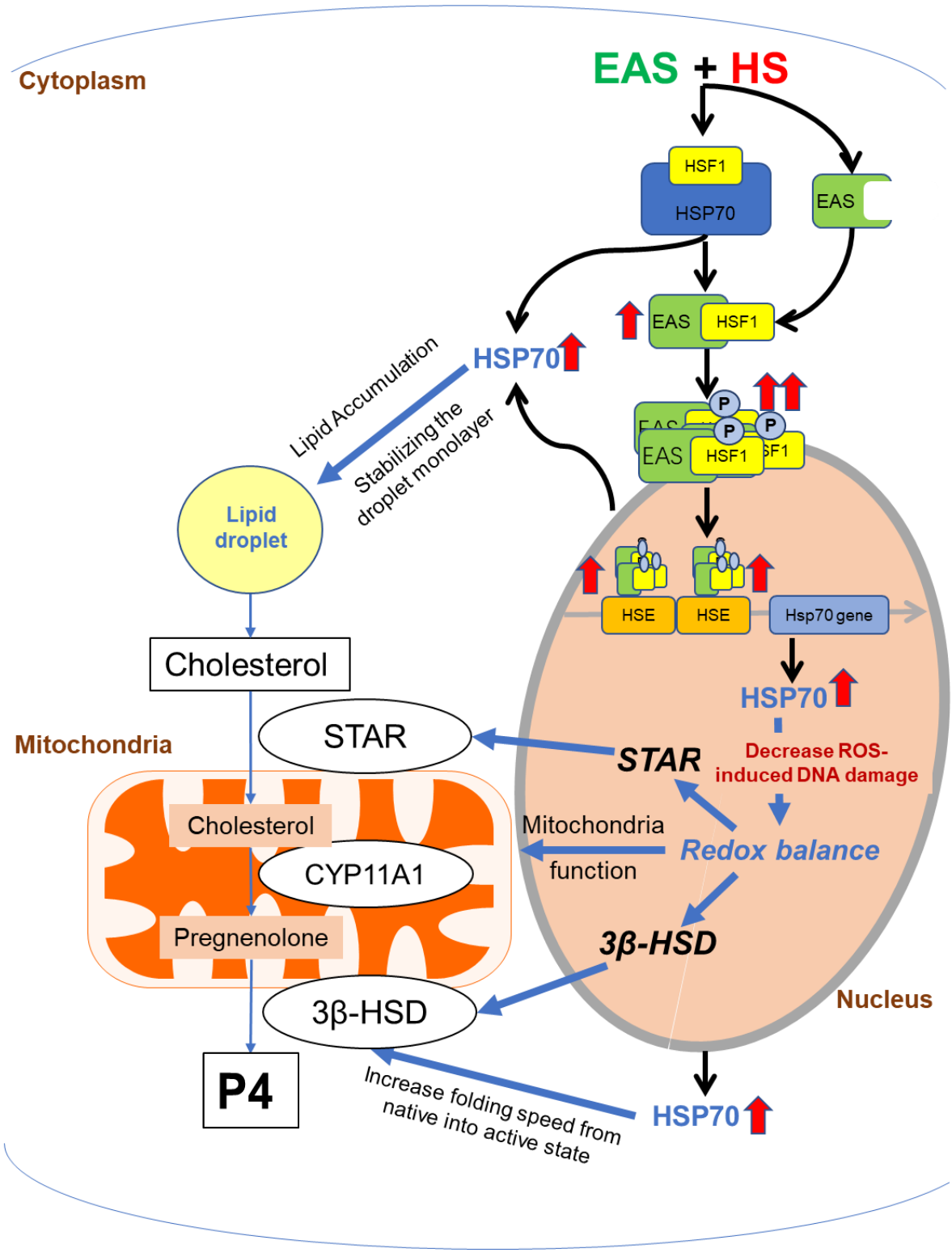


**Figure 30.** Schematic diagram of synergistic effect of EAS and HS on P4 synthesis of bovine GC

## Chapter 6 **Conclusion**

EAS induced HSP70 under non-HS conditions, exerting a synergistic effect with HS on HSP70 induction in bovine GC. In addition, EAS-induced HSP70 induced cellular function by enhancement of P4 synthesis and cellular protection against physical stress in bovine GC. Notably, synergistic effect of EAS and HS showed highest influence on HSP70 production, redox balance, steroidogenesis, cytoprotection of bovine GC. Collectively, these results indicate that EAS has potential uses in the regulation of reproductive function and cellular protection through HSP70 which has influence on redox balance and chaperon activity of bovine GC.

In chapter 2, EAS-induced HSP70 had beneficial effects as reducing DNA damage induced by ROS and increasing GSH synthesis and gene expression of antioxidant enzyme levels to maintain the redox status in bovine GC. In addition, EAS-induced HSP70 regulated the NRF2/KEAP1 pathway and HSF1 transcription factor which contributed to the ROS and GSH levels in bovine GC. Moreover, the synergistic effect of EAS and HS on HSP70 production was confirmed by increasing the expression of HSP70 mRNA and protein through enhancement of HSF1. Besides, the result of chapter 2 showed the beneficial effect of EAS on P4 production, thus EAS has potential effects on cellular reproductive function of bovine GC. Similarly, in chapter 3, EAS increased P4 synthesis by enhancement of specific steroidogenesis gene, lipid metabolism and mitochondria function in bovine GC. Especially, synergistic effect of EAS and HS has shown the most effective power on P4 synthesis. HSP70 inhibition experiment confirmed

a key contribution of HSP70 on P4 synthesis. Thus, synergistic effect of EAS and HS on HSP70 production was strongly strengthened cellular reproductive function in bovine GC. Finally, EAS was also applied for cryopreservation of bovine GC described in chapter 4. Synergistic effect of EAS and HS has also revealed the increase of cell viability with maintaining redox status in freezing bovine GC. Therefore, EAS has potential applications for cell treatments before cryopreservation based on its capacity to reduce physical stress and restore the redox function of the cells.

In conclusion, EAS can become a novel candidate for strengthening GC functions by increased HSP70, regulating an intracellular redox status to protect the cells against an oxidative stress with increasing the progesterone secretion as well as increasing the post-freezing viability. Furthermore, synergistic effect of EAS with mild heat stress has a strong increase of cellular functions with increasing hormone production which has never been reported.

These novels findings will contribute to improve the reproductive functions not only in mammals but also livestock and poultry under heat stressed conditions.

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## Supplementary list

**Supplementary Table 1-1**: Primer sequences used for qRT-PCR

| Gene | NCBI Accession No. | Primer sequence | Forward (5'-3')<br>Reverse (5'-3') | Product length (bp) |
|---|---|---|---|---|
| *HSP 70* | NM_203322.3 | GCAGTCGGACATGAAGGAGT<br>GATCTCCTCCGGGTAGAACG | | 109 |
| *HSP 90* | NM_001012670.2 | GATCTCCTCCGGGTAGAAC<br>CCGTTTGTTGTAAGGTGTGTATGTA | | 277 |
| *HSP27* | NM_001046570.2 | AAGACTGCAGGCTGGATCAC<br>CAGGACTTGGAAGCGGGATT | | 143 |
| *HSF1* | NM_001076809.1 | TTCAAGCACAGCAACATGGC<br>CAGAGTGGACACACTGGTCA | | 195 |
| *HSF2* | NM_001083405.1 | GTGCAGATGAATCCCACAGA<br>AGGGTTCCCATCGAGGAATG | | 180 |
| *GCL* | NM_001083674.1 | CATTTGCAAAGGTGGCAACG<br>GTAGGAGTTCAGGACGGGGA | | 157 |
| *GS* | NM_001015630.1 | GAGGCCAGAGTAAGGAACGC<br>GAGGTCCTCAGCAACACACC | | 150 |
| *NRF2* | NM_001011678.2 | ATGATGGACTTGGAGCTGCCG<br>TGCTCCTTCTGTCGTTGACTG | | 137 |
| *KEAP1* | NM_001101142.1 | AGGCTAGAGTGGGAACTCGT<br>CCAGGCCTAGTCTTGGGGTA | | 139 |
| *SOD1* | NM_174615.2 | ACACAAGGCTGTACCAGTGC<br>TGTCACATTGCCCAGGTCTC | | 105 |

| Gene | NCBI Accession No. | Primer sequence | Forward (5'-3')<br>Reverse (5'-3') | |
|---|---|---|---|---|
| *SOD2* | NM_201527.2 | GGATCCCCTGCAAGGAACAA | | 110 |
| | | TGGCCTTCAGATAATCGGGC | | |
| *PRDX2* | NM_174763.2 | ATGGTGCCTTCAAGGAGGTG | | 186 |
| | | GGTGGGTGAACTGAGAGTCG | | |
| *PRDX6* | NM_174643.1 | CTCCTCTTACTTCCCGCGTC | | 138 |
| | | GAATGCCCCATGAGTCTCCC | | |
| *STAR* | NM_174189.3 | TGGAAGTCCCTCAAGGACCA<br>GCAAGTTGGCCTTCAACACC | | 178 |
| *CYP11A1* | NM_176644.2 | AGGCAGAGGGAGACATAAGCA<br>GTGTCTTGGCAGGAATCAGGT | | 156 |
| *3β-HSD* | NM_174343.3 | GTGAGCGTTTCTCAGTGCTC<br>CTAGCACCCGGATTTCCTGC | | 136 |
| *H2AFZ* | NM_174809 | AGAGCCGGTTTGCAGTTCCCG | | 116 |
| | | TACTCCAGGATGGCTGCGCTGT | | |

| **1 ×PBS** | **(g/500mL)** |
|---|---|
| **NaCl (Wako)** | 4 g |
| **KCl (Wako)** | 0.1 g |
| **$Na_2HPO_4$・ $12H_2O$ (Wako)** | 1.4405 g |
| **$KH_2PO_4$ (Wako)** | 0.1 g |

Store at Room Temperature

| **TBS-T** | **(g/1000mL)** |
|---|---|
| **NaCl (Wako)** | 8 g |
| **KCl (Wako)** | 0.2 g |
| **Tris** | 3 g |
| **0.1% Tweeen 20** | 1 mL |

Store at Room Temperature

| **0.05% Trypsin/0.53mM EDTA** | **(g/500mL)** |
|---|---|
| **Trypsin** | 0.25 g |
| **EDTA** | 0.0986 g |
| **1x PBS (-)** | 500 ml |

Store at 4˚C

| **4% PFA** | **10 mL** |
|---|---|
| **Paraformaldehyde** | 0.4 g |
| **1x PBS (-)** | 10 ml |

Store at 4˚C

| **5% FBS DMEM (Wako)** | **10 mL** |
|---|---|
| **Streptomycin sulfate salt (Nacalai tesque)** | 0.03 g |
| **Penicillin G Potassium Salt (Nacalai tesque)** | 0.05 g |
| **FBS** | 25 mL |

Store at 4˚C

| **0.2 % TritonX-100** | **10 mL** |
|---|---|
| **TritonX-100** | 20 µl |
| **1x PBS (-)** | 10 mL |

Store at 4˚C

| **0.1% Tween 20 in PBS** | **10 mL** |
|---|---|
| **Tween 20 (Wako)** | 10 µl |
| **1x PBS (-)** | 10 mL |

Store at 4˚C