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Investigating The Effect Of Heat Treatment on The Expression Pattern of Different Genes on Cultured Primordial Germ Cells and Gonads in Chicken

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ABSTRACT

Heat stress due to high environmental temperature negatively influences animal performance. The aim of this study was to determine the effect of both early and late heat treatment on cultured primordial germ cells (PGCs) and gonads and the response of different gene expressions on the cultured PGCs and gonads. To better understand the biological impact of heat stress, dual purpose 2-day old chicks were subjected to heat treatment (38.5^{o}C) for the first 12 hours followed by heat stress (30^{o}C) at the age of 23 weeks for 12 weeks (treatment 1) and heat stress (30^{o}C) at the age of 23 weeks for 12 weeks (treatment 2). Circulating PGCs in the blood were isolated from the dorsal aorta of an embryo from Hamburger and Hamilton (HH) stage 14–16 (2.5 days old embryo) while the gonadal and chicken embryonic fibroblast samples were isolated from 10 and 12 days old embryos, respectively. The expression of all the genes was quantified by quantitative real-time PCR (qPCR). PGCs cultured for 23, 30 and 50 days regularly expressed Cvh, cPouV, cNanog and cHsp70 at an increasing level. ANOVA analysis demonstrated no significant (P > 0.05) treatment effect on cHsp70 expression for the 30 days cultured PGCs. The heat treated and heat stressed group had a significantly (P<0.05) higher cHsp70 expression than the control group but, no significant (P>0.05) difference was observed between the two treatment groups. It can be concluded that the expression of Cvh and cHsp70 was detectable in cultured PGCs and also 10 days old embryonic gonads.

1. INTRODUCTION

The key to the survival of a species is its ability to reproduce. In sexually reproducing organisms this requires the formation of gametes to generate the next generation. Germ cells are the only type of cells that can transmit all genomic information to the next generation. The gametes derive from PGCs. PGCs are the precursors of the germ cell lineage, differentiating to form oocytes in the ovary and spermatogonia in the testis. They arise early in embryonic development prior to sexual determination, in an extra-gonadal region. During embryonic development in the chicken, PGCs migrate into the genital ridge, where they differentiate into either spermatogonia in the testis or oogonia in the ovary (Nakamura et al. 2013). In chickens, segregation of the germ and somatic cell lineages occur in the epiblast of the stage X embryo (Eyal-Giladi & Kochav 1976) in contrast to mammals where PGCs are specified later in development at the start of gastrulation (Petitte et al. 1997).

High ambient temperature can generate a state of stress and evokes a combination of behavioral, biochemical and physiological changes. This generally results in a reduction in chicken performance. The major effects of heat stress are decreased feed intake, growth rate, feed efficiency, lower weight gain and increased fat deposition (Geraert et al. 1996; Yahav et al. 1996; Xie et al. 2014). Stressful experiences in the early stages of development could have a considerable impact on the overall growth and development of chickens. One of the practical approaches that have yielded promising results is altering the bird's abilities to survive with high ambient temperatures through stimulation early in life (Al-zhgoul et al. 2013). There is evidence that stressful experiences during the neonatal stage can have a considerable impact on various aspects of an animal's physiology and behavior. Exposure of 5-day-old broiler chicks to elevated temperature improved survivability in otherwise lethal heat treatment at 42 days of age (Yahav et al. 1996; De Basilio et al. 2001). This is based on the fact that full-blown homeothermic growth of chicks starts at approximately ten days post-hatch (Hemid et al. 2010). For increasing the thermotolerance capacity of the birds and also the inhibition of economic losses as a result of heat stress, two management models have been suggested. These are heat shock and adaptation of birds to desired environmental conditions (Horowitz 1998). These models have provided some suitable results for broiler industry. The fast response to heat shock can be achieved by application of this technique at early growth phase (Yahav et al. 1996; Collin, et al. 2004).

Extensive studies (Yu et al. 2008; Xie, J. et al., 2014) have been conducted to assess mechanisms of heat stress in chickens that have mostly focused on acute heat stress in broilers. However, laying broiler breeder chickens are also negatively affected by exposure to high temperature. For instance, chronic heat challenge produced prolonged decreases in progeny performance (Farnell et al. 2001; Bertin et al. 2013; Xie, J. et al., 2014) and reduced fertility in broiler breeders (Singh 1999; Sharifi et al. 2010).

From all Heat Shock Protein (HSP) families that protect cells, tissues and whole organisms from severe thermal stress, heat shock protein 70 (*cHsp70*) is the most important and well-studied protein. Different studies reported that high level of *cHsp70* expression probably imposes costs on individuals exposed to thermal stress by influencing their fertility, growth or survival (Feder & Hofmann 1999; Hamdoun et al. 2003; Karl et al. 2009; Shatilina et al. 2011). These studies infer links between thermal adaptation and the high expression of *cHsp70* by comparing *cHsp70* and heat tolerance of animals from different thermal environments or from different seasons.

However, only Anand et al., (2016) has explored the transcriptional response of genes including *cHsp70* expression to early age heat treatment and later age heat stress in the PGCs and gonads of the developing embryo from the heat-treated parents of a dual-purpose chicken breed.

Therefore, this study was designed to provide basic information on the expression of *cHsp70* gene by comparing PGCs and gonads of the dual purpose Speckled Transylvanian Naked Neck chicken under stress and control conditions. Thus, the specific objectives of the study were (a) to determine the germline-specific marker (*Cvh*/Vasa) and stem cell-specific markers (c*PouV* and c*Nanog*) gene expression on cultured PGCs, (b) to determine

the effect of both early age heat treatment and later age heat stress on cultured PGCs and gonads and (c) to determine the response of c*Hsp70* gene expression on cultured PGCs and gonads of Speckled Transylvanian Naked Neck chicken.

2. Materials and Methods

2.1. Egg, PGCs and gonads sample collection

Fertilized eggs were collected from the Research Center for Farm Animals Gene Conservation in Gödöllő, Hungary and incubated in an incubator at 38°C at 60% humidity. The control group (EKTK) was raised under normal conditions without exposure to any heat treatment and stress. The second group heat treated and heat stressed (EK) was subjected to heat treatment (38.5ºC) at the age of 2 days for the first 12 hours followed by heat stress (30ºC) at the age between 23-34 weeks for about 12 weeks. The third group heat stressed (EKNK) was heat stressed (30ºC) at the age of 23-34 weeks for about 12 weeks. The males used for mating were also from the same control and treatment groups.

Circulating PGCs were isolated from the 2.5-day-old embryos. After staging the embryos to keep only those between 14 and 16 Hamburger and Hamilton (HH) stages, 3 to 4 µl of blood was taken by a glass micro-pipette from the dorsal aorta of an embryo under a stereomicroscope and kept in a petri dish. After 1–2 weeks, the red blood cells had died and the PGCs became visible. The PGCs were cultured for a total of 23, 30 and 50 days. After a short centrifugation of PGCs suspension, the supernatant was removed and 500µl Trizol was added. A 10 minutes incubation followed by suspending the cell lysate, all the samples were stored under -70ºC before RNA isolation.

Gonadal samples were collected from 10-day-old embryos by making a hole in the blunt end of the egg and removing the embryo from the yolk sac with curved forceps. The embryo was placed in a petri dish containing Phosphate Buffered Saline (PBS) and gonads were dissected from the embryo with the assistance of very fine straight forceps under a stereomicroscope.

2.2. Preparation of chicken embryonic fibroblasts (CEFs)

The method used for isolation of CEFs was based on a modified version of the protocol for isolation and handling of primary mouse embryonic fibroblasts (Jain, Verma, & Liu, 2014). The medium used for the culture of primary CEFs (CEF medium) consisted of DMEM/F12 (GIBCO) medium supplemented with 10% FBS (HY-Clone), containing penicillin and streptomycin (GIBCO). The CEF was used as a control sample for qPCR analysis.

2.3. Isolation of total RNA, synthesis of cDNA and quantitative real-time PCR (qPCR)

Total RNA from cultured PGCs, gonads and CEF was extracted using TRIzol (Rio et al. 2010) following the manufacturer's protocol. Finally, the concentration of RNA in each sample was determined by NanoDrop Spectrophotometer. The isolated RNA samples were reverse transcribed into cDNA using high capacity cDNA reverse transcription Kit following the manufacturer's protocol (Life Technologies, Carlsbad, USA). Synthesized cDNA was subjected to qPCR using SYBR Green PCR master mix as a double-stranded DNA-fluorescent specific dye according to the manufacturer's instructions for all genes (Table 1). Eppendorf Master Cycler Real plex equipment was used to perform the qPCR. For each gene examined, triplicate from each cDNA was analyzed, fluorescence emission was detected and relative quantification was calculated automatically by using GenEx program of MultiD company.

Table 1. List of sequences of primers used in the amplification process

Gene	NCBI number	Primers		Length of the product (bp)
cGapdh	NM_204305.1	FW	GACGTGCAGCAGGAACACTA	112
		RV	CTTGGACTTTGCCAGAGAGG	
cNanog	NM_001146142.1	FW	ATACCCCAGACTCTGCCACT	100
		RV	GCCTTCCTTGTCCCACTCTC	
cPouV	NM_001110178.1	FW	GAGGCAGAGAACACGGACAA	109
		RV	TTCCCTTCACGTTGGTCTCG	

Cvh	NM_204708.1	FW	GAACCTACCATCCACCAGCA	113
		RV	ATGCTACCGAAGTTGCCACA	
cHsp70	NM_001006685.1	FW	GACAACCGCATGGTAAACCG	108
		RV	CCTCTCACAAGCTGTACGCA	
P2/P8-Z	NC_006127.3	FW	TCTGCATCGCTAAATCCTTT	345
P2/P8-W	NW_003766117.1	RV	CTCCCAAGGATGAGAAATTG	362

2.4. Immunostaining of PGCs

Isolated PGCs and PGC colonies were fixed with 4% PFA for 10 minutes. After washing with PBS (three times, five minutes each), cells were permeabilized with 0.5% Triton X-100 (Merck-Millipore, USA) for 5 minutes. After washing with PBS, to minimize nonspecific binding of antibodies, the fixed cells were blocked for 45 minutes with a blocking buffer containing PBS with 5% (v/v) BSA. Then, cells were washed three times with PBS and were incubated with each of the primary antibodies including mouse anti-stage-specific embryonic antigen-1 (SSEA-1) (Hybridoma Bank, 1:10), rabbit anti-VASA (1:1000; kindly provided by Bertrand Pain, Lyon, France). After incubation for a night in the primary antibody solution in a humid chamber at 4ºC, the cells were washed three times with PBS. Then, cells were incubated with the secondary antibodies (donkey anti-rabbit IgG FITC (Jackson Immuno Research, USA), donkey anti-rabbit IgG conjugated to Daylight 549 (Jackson Immuno Research, USA)) in a dark humid chamber for 1 hour at room temperature. After washing with PBS, the nucleus was stained with TO-PRO®-3 stain, which is a far red-fluorescent (642/661) nuclear and chromosome counterstain. Coverslips were mounted on the slide with the application of 40µl antifade plus DAPI (Vectashield) and analyzed by confocal microscope (Leica TCS SP8). Negative controls, without the use of a primary antibody, were only stained with the secondary antibody.

2.5. Sex determination from chicken embryonic tissue samples

Following dissection, soft tissue was removed and placed into the lysis buffer which contains 0.1 mM Tris with PH of 8.5 (5 ml), 5 mM EDTA (0.5 ml), 0.2 M NaCl (2 ml), 0.2 % SDS (0.5 ml), and sterile water (42 ml). 10 µl Proteinase K 1 ml of lysis buffer was added according to the number of samples to 100 µl lysis buffer mix. The samples were vortexed and allowed to stand for 3 hours at 55ºC. After short vortex, all the samples were centrifuged by 13,000 g centrifugation for 1 minute at 4ºC. The samples were allowed to stand for 10 minutes at 100ºC followed by 13,000 g centrifugation at 4ºC for 5 minutes.

The isolated DNA was diluted to 25ng/µl concentration for PCR reaction and gel electrophoresis. A 13 µl PCR mix was prepared for each sample (4.25 µl nucleus-free water, 0.5µl forward primer/P2, 0.5µl reverse primer/P8 (Table 1), 6.75 µl MayTaq Ready Mix, and 1 µl DNA sample). For positive control, two sex controls (male and female) and for negative control, nucleus-free water was used instead of DNA. After gentle shaking and short centrifugation, the PCR program was run by using "*cSEX P2 P8 MyTaq"* PCR program of the PCR machine according to the following program. Cycling parameters were, 95ºC for 60s, 95ºC for 15s, 48ºC for 45s, 72ºC for 10s, 72ºC for 2.5 minutes and 4ºC forever.

The optimal primer concentration of the two primers was 20 µM. The PCR products (345 bp for males and 362bp for females) were then separated by electrophoresis, using 3% agarose gel stained with ethidium bromide at 100 V for 1.5-2.0 hours. The DNA bands were then visualized under UV illumination and photographed.

2.6. Validation of the housekeeping gene

To confirm the housekeeping gene for this study, expression of *cGapdh* was determined by quantitative real-time PCR using SYBR green dye for the three treatment groups before the actual research work (Figure 3 A and 4 A). Housekeeping genes are expected to be expressed in all cells of an organism under normal conditions, irrespective of tissue type, developmental stage, cell cycle state, or external signal (Eisenberg & Levanon 2013).

2.7. Data analysis

Individual embryo served as the experimental unit for all statistical analyses. Statistical differences between each group were assessed by a one-way analysis of variance (ANOVA) using the GenEx 6.0 software. Comparisons between the mean values of the control group and those of each experimental group were performed using Tukey's post hoc test for multiple comparisons. The data are presented as mean ± SD and P values of less than 0.05 were regarded as statistically significant. The expression of the target gene relative to the internal control gene in each sample was calculated in GenEx 6.0 using the following formula (Wang et al. 2015):

$2^{-\Delta\Delta CT}$ where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal control}}$

and $\Delta\Delta Ct = \Delta Ct_{\text{test sample}} - \Delta Ct_{\text{control sample}}$

3. Results and Discussion

3.1. Characterization of cultured PGCs

The isolated PGCs from Hamburger and Hamilton stages 14-16 contained a large nucleus and many prominent vacuoles in the cytoplasm after 30 days of culture (Figure 1 A, B, and C). The PGCs grew as round, granular single cells with off-center nuclei or in small clusters and remained unattached. Many antibodies are used for showing the cell epitopes of primordial germ cells. For example, in humans, the stage-specific embryonic antigen-3 (SSEA-3), SSEA-4 and TRA-1 antibodies can be referred (Henderson et al. 2002), in mouse, the SSEA-1 is recognized as the pluripotency marker (Resnick et al. 1992). In chicken, different markers like SSEA-1, SSEA-3, SSEA-4 and EMA-1 have been reported (Jung et al. 2005). In the present study, immunostaining with the pluripotency marker, SSEA-1 demonstrated that PGCs were strongly stained for this marker (Figure 1 D). In addition, to determine whether isolated PGCs also express the germ cell-specific protein, immunofluorescence was used to detect chicken vasa homologue (VASA /*Cvh*), RNA processing protein important for germ cell survival and specification (Kuramochi-Miyagawa et al. 2010). Immunostaining with an antibody to VASA illustrated that *Cvh* was localized throughout the cytoplasm in PGCs (Figure 1 E). This is consistent with the reported cytoplasmic localization of *Cvh* in avian germ cells (Tsunekawa et al. 2000).

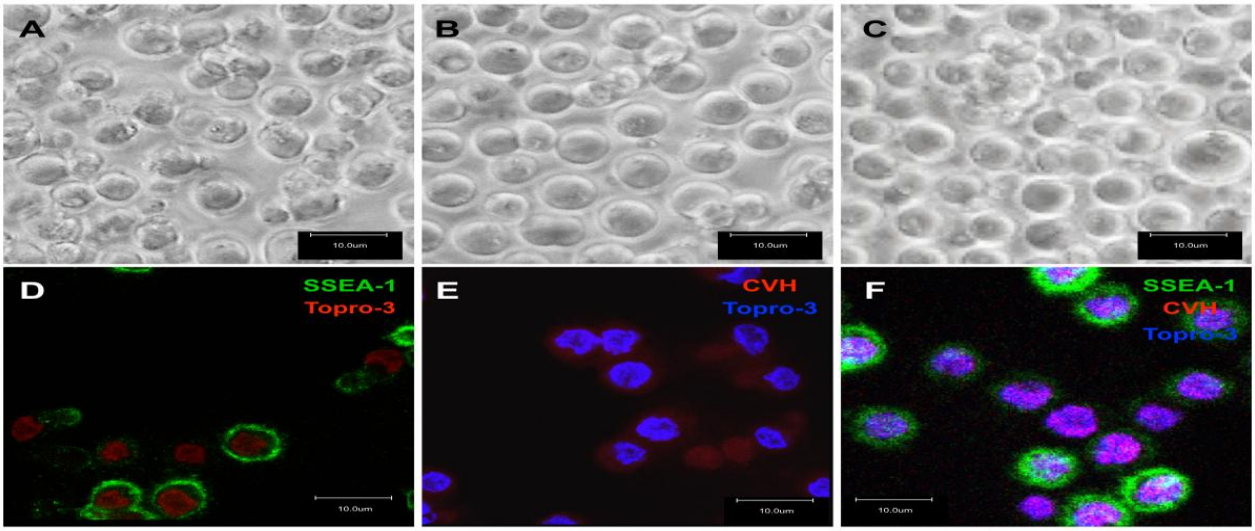

Figure 1 Characterization of 30 days cultured PGCs

A) Heat treated and heat stressed (EK) PGCs under a light microscope, **B**) Heat stressed (EKNK) PGCs under a light microscope, **C**) Control (EKTK) PGCs under a light microscope, **D**) PGCs immunostained for SSEA-1, **E**) PGCs immunostained for *Cvh*, **F**) PGCs immunostained for both SSEA-1 and *Cvh*.

In previous studies, it has been shown that freshly isolated PGCs express pluripotency genes, including the triad *PouV* (*Oct4*), *Sox2* and *Nanog* (Cañón et al. 2006; Motono et al. 2008; Macdonald et al. 2010; Naeemipour et

al. 2013). In addition, a study on White Leghorn, Isa Brown, Dekalb White, Herve and Ardennaise PGCs cell lines were characterized by RT-PCR for the expression of germline-specific markers (*Cvh*), chicken dead-end homologue (*Cdh*) and deleted in azoospermialike (*Dazl*), pluripotency markers (*Sox2*, *Oct4*, *Nanog*), telomerase reverse transcriptase (*Tert*) and chemokine (*C-X-C motif*) receptor 4 (*Cxcr4*) receptor and the expression of the markers was maintained after a very long-term culture (151–540 days) and cryopreservation (Tonus et al. 2016).

In this study, the PGCs cultured for 23, 30 and 50 days regularly expressed germline-specific marker (*Cvh*), stem cell-specific markers (c*PouV* and c*Nanog*) and the heat shock protein (c*Hsp70*) at an increasing level during long-term cultivation (Figure 2). The c*Hsp70* mRNA expression was the highest at 50th day and lowest on the 23rd day of the culture period. Similarly, the expression level of pluripotency markers c*PouV* and c*Nanog* was maximum at day 50th and minimum in the 23rd-day old cultures. In comparison, the expression of *Cvh* was highest in 23rd-day old culture. This result is in agreement with the result of Tonus et al. (2016) which reported that the expression of all the markers was maintained after a very long-term culture (151–540 days) and cryopreservation.

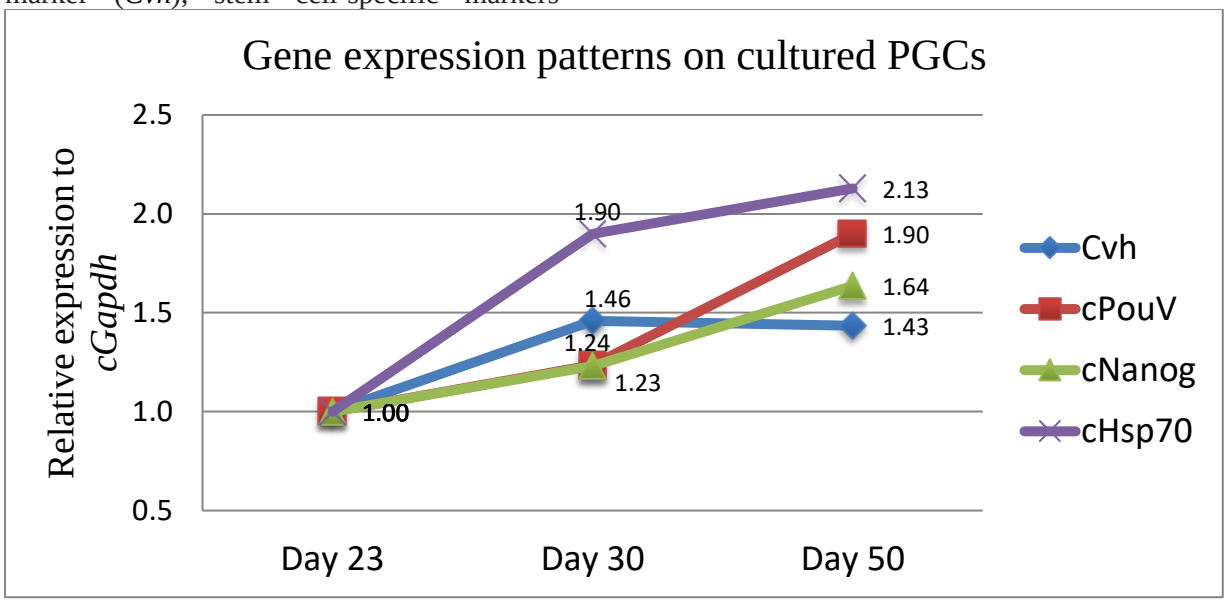

Figure 2 *Cvh, cPouV, cNanog and cHsp70* gene expression patterns of 23, 30 and 50 days cultured PGCs relative to housekeeping gene *cGapdh*.

3.2. Expression of cHsp70 in 30 days cultured PGCs

The results of mRNA expression levels of the *cHsp70* gene in 30 days cultured PGCs from the two treatments and control groups are shown in Figure 3 B. Housekeeping genes are genes that are required for the maintenance of basal cellular functions that are essential for the existence of a cell, regardless of its specific role in the tissue or organism. Thus, they are expected to be expressed in all cells of an organism under normal conditions, irrespective of tissue type, developmental stage, cell cycle state, or external signal (Eisenberg & Levanon 2013). To confirm the reference gene for this study, expression of *cGapdh* was determined by quantitative real-time PCR using SYBR green dye for the 3 treatment groups before the actual research work. The results of mRNA expression levels of *cGapdh* based on cycle threshold (CT) value in 30 days old cultured PGCs are shown in Figure 3 A. ANOVA analysis demonstrated that no significant ($P > 0.05$) difference between treatments and control group were observed. Because of this, *cGapdh* was used as housekeeping gene for both treatments and control group which were derived from the same cell type of the three treatment groups.

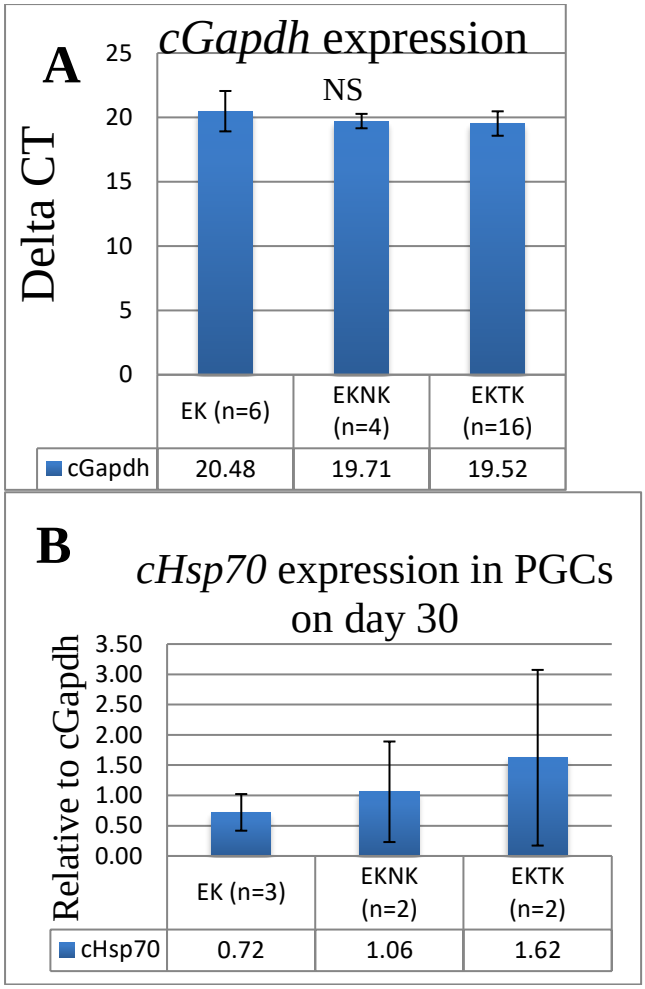

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Figure 3 ***cGapdh and cHsp70 gene expression in 30 days cultured PGCs***

A. Gene expression of *cGapdh* in the 3 treatment groups based on delta CT value **B**. *cHsp70* gene expression pattern of 30 days cultured PGCs relative to *cGapdh* of the 3 treatments. EK: heat treated and heat stressed; EKNK: heat stressed; EKTK: control.

In this study, the ANOVA analysis demonstrated that there was no significant ($P > 0.05$) treatment effect on *cHsp70* gene expression relative to the housekeeping gene c*Gapdh*. The reason for this result might be due to the use of fertilized eggs collected from heat-treated and non-treated parents. A study by Anand et al. (2016) reported an increased level of expression of *cHsp70* on 23, 30 and 50 days cultured PGCs of chicken collected from the embryo of non-treated parents. In previous studies, *cHsp70* expression exhibit spatial and temporal variations at basal and heat-treated levels (Tanguay et al. 1993; Vamvakopoulos 1993) during thermal manipulation and either acute or chronic heat exposure. In addition, the duration and severity of heat stress could also influence the expression pattern of *HSPs* (Yahav, Collin, et al. 2004; Yahav, R.Sasson, et al. 2004; Piestun et al. 2008).

Expression of cHsp70 in 10 days old embryonic gonads

The results of mRNA expression levels of *cGapdh* based on CT value in 10-days old embryo gonadal PGCs is shown in Figure 4A. ANOVA analysis demonstrated that no significant ($P > 0.05$) difference between treatments and control group were observed. Because of this result, *cGapdh* was used as housekeeping gene for both treatments and control group which were derived from 10-days old embryonic gonads.

In the present study, the expression of c*Hsp70* mRNA in the gonad of 10 days old embryo under normal, heat treated and heat stressed conditions were investigated. Figure 5B shows the results of c*Hsp70* expression of the gonad

in A 10 days old embryo of the three treatment groups from Speckled Transylvanian Naked Neck fertilized eggs. c*Hsp70* was detected in all embryonic gonads despite the sex, side of the gonad, heat treated or stressed condition. The heat treated and heat stressed (EK) had a significantly (P<0.05) higher c*Hsp70* expression than the control group (EKTK), but no significant (P>0.05) difference was observed between the two heat treatment groups (EK versus EKNK) and between heat stressed and control group (EKNK versus EKTK).

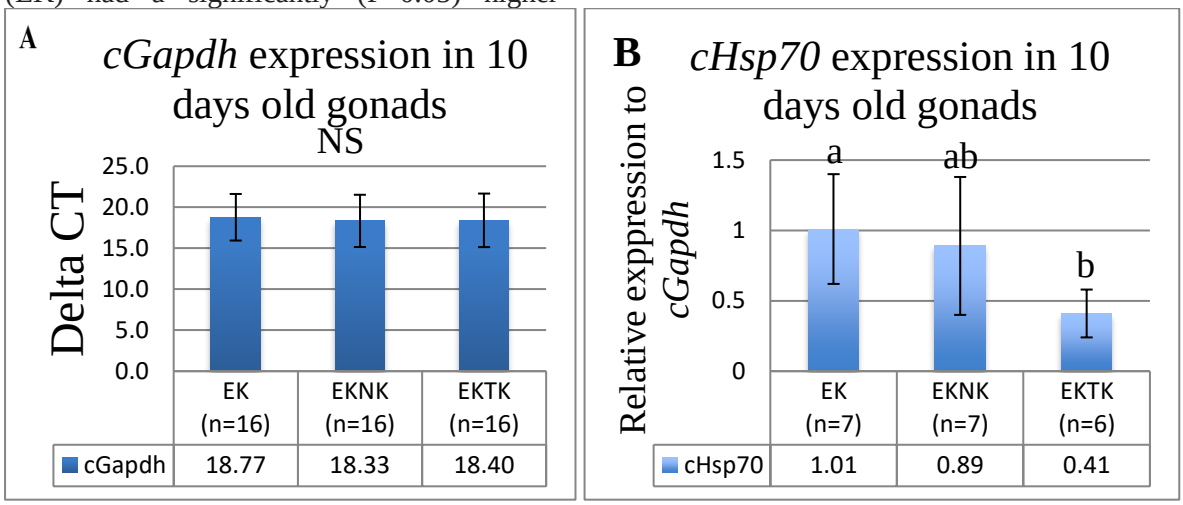

Figure 4 ***cGapdh and cHsp70 gene expression in 10 days old gonads***

A. Gene expression of *cGapdh* in the 3 treatment groups based on delta CT value **B**. c*Hsp70* expression in the gonad of the 10day old embryo. EK: heat treated and stressed; EKNK: heat stressed; EKTK: control.

Anand et al. (2016) also reported the higher expression of *cHsp70* in heat-treated groups than the control group which is collected from a 10-day old embryonic gonad. The concentration of induced *cHsp70* mRNA was higher than that of control *cHsp70* mRNA in the same gonad. It may be a reflection of the self-protection mechanism that animals under heat stress would bring self-regulation into full play to adapt to the environment.

Several studies have shown that Heat Shock Factor (HSF) genes can be induced by certain types of heat stress. In human epidermoid cells, heat treatment elevated the gene expression of HSF1 and HSP70 (Ding et al. 1996). In plants, gene expression of HSF was altered by heat stress, oxidative stress, and osmotic stress and the HSF expression changes exhibited a stress-specific and tissue-specific pattern (Swindell et al. 2007; Hu et al. 2009; Mittal et al. 2009). The quick response of HSFs, especially during heat stress, might be essential for the rapid transcription of HSPs. To date, mechanisms regulating transcription of HSF genes are poorly understood. Xue et al. (2012) reported that the C/EBPa -dependent pathway could be involved in glutamine-induced HSF 1 transcription.

The activation of HSP70 gene during heat challenge has been extensively studied in mammals and birds. In the present study, the patterns of *cHsp70* gene expression in response to different heat treatment and stress varied in the gonad of different treatment groups. The abundance of *cHsp70* mRNA was affected by the heat treatment and stress in the gonad. In broiler chickens, acute heat stress induces gene expression of *cHsp70* in the liver, lung, heart, kidney, blood vessels (Pei-Ming et al. 2007; Yu et al. 2008).

4. CONCLUSIONS

PGCs from HH stages 14-16 contained a large nucleus and many prominent vacuoles in the cytoplasm after 30 days of culture and grew as round, granular single cells with off-center nuclei or in small clusters and remained unattached. Immunostaining of these cells with the pluripotency marker, SSEA-1 (stage-specific embryonic antigen 1) demonstrated that PGCs were strongly stained for this marker. In addition, immunostaining with an antibody to VASA illustrated that *Cvh* was localized throughout the cytoplasm in PGCs. The expression of *cHsp70* in chicken is affected by heat treatment, and this treatment

altered expression of *cHsp70* gene in the 10 days old gonads, which results in thermotolerance. On the other hand, there was no treatment effect on the expression of *cHsp70* in 30 days cultured PGCs. Based on the above-mentioned results it can be concluded that the expression of *Cvh* and *cHsp70* was detectable in cultured PGCs and also 10 days old embryonic gonads.

The results of this study require further validation and a follow-up study is now under investigation. Furthermore, additional work is needed to evaluate the productive and reproductive performance and characterization of the specific roles of *cHsp70* in both heat treated and heat stressed chickens. In the future, the results of the comparison of indigenous breeds and intensive lines allow elaborating new lines, which can adapt easily to the extreme weather conditions.

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