RESEARCH REPORT
ANIMAL SCIENCE

Title: Development of an in vitro model of heat stress during pig oocyte maturation and its impact on embryonic developmental competency - **NPB #09-249**

Investigator: Jason W. Ross, Assistant Professor of Swine Reproductive Physiology

Institution: Iowa State University

Date Submitted: August 3, 2011.

Industry Summary:

Heat stress has a significant negative impact on agriculture production. A significant portion of this loss is recognized through lost reproductive ability in swine herds during periods of elevated environmental temperatures. The objective of this project was to develop an in vitro model of heat stress during oocyte maturation and demonstrate the impact on subsequent development in thermal neutral conditions following in vitro fertilization. The completion of this research project has: 1) Established an in vitro model of heat stress during porcine oocyte maturation, 2) Demonstrated impaired developmental competency of porcine embryos produced through *in vitro* fertilization following heat stress to the maturing oocyte, and 3) characterized potential molecular markers associated with heat stress during oocyte maturation prior to fertilization. Utilization of the molecular markers of developmental competency following oocyte heat stress will be useful towards the development of strategies to mitigate the negative effects of heat stress during oocyte maturation in pigs.

Keywords:

embryo, development, heat stress, pig, gene expression

Scientific Abstract:

The objective of this project was to develop an *in vitro* model to evaluate the impact of heat stress during oocyte maturation and subsequent embryonic development competency in pigs. We characterized the impact of three different heat stress treatments during porcine oocyte maturation on subsequent embryonic developmental competency. To evaluate the molecular response to heat stress in oocytes that were exposed to heat stress treatments during maturation we measured several markers to determine oocyte viability and heat stress response. Three levels of heat stress were administered during in vitro maturation, 1) heat stress of oocytes at 41^{o}C for the first 21 hours, 2) heat stress for the last 21 hours of in vitro maturation, or 3) heat stress for the duration (42 hours) of in vitro maturation. Reduced maturation rates and developmental competency of embryos

produced following in vitro maturation was affected by the timing and duration of heat stress. Compromised developmental capacity from the 4-cell stage to the blastocyst stage was greatest in embryos produced from oocytes heat stressed for the duration of in vitro maturation and was correlated to MIR21, PDCD4 and HSP90a expression at the 4- to 8-cell stage of development. These data demonstrate a temporal relationship between MIR21 and its target mRNA, PDCD4, and suggest their utility as molecular markers of developmental competence following heat stress. Four to eight cell stage embryos produced form oocytes subjected to 42 hours of heat stress had greater MIR21 and HSP90a expression and significantly lower PDCD4 expression, while HSF1 gene expression was not affected by oocyte heat stress treatment. These gene expression data represent biological mechanisms in the 4- to 8-cell stage embryo that are impacted by heat stress during oocyte maturation and provide potentially novel molecular markers of embryonic developmental competence as a result of heat stress during female gamete production.

Introduction:

The swine industry in Iowa and the United States suffers considerably due to impaired reproductive performance during periods of seasonal infertility, particularly the months of July, August and into September. The impact is quite visible with day-28 pregnancy rates reaching their lowest levels in August into October and reduced farrowing rates in November and December. While seasonal infertility could arguably be related to factors such as photoperiod, seasonal infertility in pigs is associated with periods of excessive heat, which has been repeatedly demonstrated to negatively impact reproductive efficiency, particularly due to lost embryonic development (Omtvedt et al. 1971; Tompkins et al. 1967).

Germinal vesicle breakdown, the process involving the breakdown of the nuclear membrane and the resumption of meiosis signifies a critical stage in oocyte development prior to maturation, fertilization and early embryonic development. Prior to germinal vesicle breakdown, growing oocytes are capable of transcription and de novo synthesis of coding genes that result in the production of a translated protein. Following germinal vesicle breakdown the oocyte possesses a defined inventory of proteins and messenger RNA capable of being translated into proteins. This period, following germinal vesicle breakdown but prior to the 4-cell stage of embryonic development is particularly vulnerable to stressors such as elevated temperature due to their inability to initiate a transcriptional response to stress. The impact of heat stress during oocyte maturation and early embryonic development is evidenced in that sows exposed to heat stress for 5 days following breeding have significantly reduced number of viable embryos after day 27 of gestation, with control pigs possessing an

average of 11.0 (68.8% survival) viable embryos while heat stresses sows containing only 6.8 (39.1% survival) viable embryos (Tompkins et al. 1967). In this study, heat stress was administered following breeding, which generally *occurs prior to ovulation* and complete oocyte maturation, as pigs typically ovulate in the mid to latter half of estrus (Soede et al. 1992). Similar effects have been documented in gilts exposed to heat stress on days 0 to 8 or 8 to 16 of gestation (Omtvedt et al. 1971). Compared to control gilts, heat stress during either time period reduced the number of viable embryos per pregnant gilt on days 30 to 36 of gestation. Additionally, while all control gilts (n=14) remained pregnant at day 30 of gestation, only 8 of 14 (57%) of gilts exposed to heat stress on days 0 to 8 of gestation were still pregnant (Omtvedt et al. 1971).

There is some documented evidence of *in vitro* heat stress models during the transition between germinal vesicle breakdown and the 4-cell stage of development. A nine hour culture of pig embryos at 42^{o}C following porcine *in vitro* fertilization significantly reduced blastocyst formation rate from 20.6% to 8.8% (Isom et al. 2007) and heat shock of 41.5^{o}C for 4 hours following in vitro maturation also reduced the developmental ability of parthenogenetically activated oocytes (Tseng et al. 2006). So while it is clear that heat stress during oocyte maturation both *in vitro* and *in vivo* are detrimental to embryonic development in the pig, what is needed now is an appropriate model that can be used to comprehensively investigate the impact of heat stress during oocyte maturation on the abundance of specific RNA molecules during porcine embryonic development.

Objective:

Develop *in vitro* conditions that effectively demonstrate compromised development of zygotes produce from oocytes exposed to heat stress during maturation. Our working hypothesis is that heat stress during oocyte maturation will impair development of the zygote despite being cultured at optimum temperature following fertilization. Having an established model of thermal stress during meiotic progression and oocyte maturation will be beneficial for many future experiments.

Materials & Methods: This section should include experimental design, methods and procedures used, number of animals, etc.

Oocyte Collection and In Vitro Maturation

Sow ovaries were obtained from an abattoir and transported to the laboratory in a thermos maintained at 30-35˚C. Antral follicles (3-6 mm) were aspirated by using an 18-gauge needle attached to a 10 ml disposable syringe. The cumulus-oocyte complexes (COCs) with multiple layers of intact cumulus cells and uniform ooplasm were selected for GV oocyte collection or maturation. In vitro maturation was accomplished by culturing COC for 42 h in maturation media (TCM 199 medium (Gibco BRL, Grand Island, NY) supplemented with 0.1% polyvinylalcohol (PVA) (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 1 μg/ml gentamicin, 0.57 mM cysteine, 0.5 μg/ml luteinizing hormone, 0.5 μg/ml follicle-stimulating hormone, and 10 ng/ml epidermal growth factor) in 5% CO_2. During in vitro maturation, cumulus oocyte complexes were subjected to four environmental conditions: In vitro maturation at 39^{o}C for 42 h (control), in vitro maturation for 42 hours at 41.0^{o}C, (HS1); in vitro maturation for 21 hours at 39.0^{o}C followed by 21 hours at 41.0^{o}C, treatment 4 (HS2); in vitro maturation for 21 hours at 41.0^{o}C followed by 21 hours at 39.0^{o}C (HS3). Following in vitro maturation, matured COCs were separated from oocytes by vortexing the COCs in 0.1% hyaluronidase in Hepes-buffered Tyrode medium containing 0.01% PVA.

In vitro fertilization and Culture

In vitro fertilization and embryo culture was performed as previously described (Hao et al., 2006; Zhao et al., 2009). Briefly, oocytes with a polar body following *in vitro* maturation were rinsed and transferred into equilibrated 50-μl droplets of mTBM medium (modified Tris-buffered medium) plus 2mM caffeine and 2mg/ml BSA (bovine serum albumin) with around 35 oocytes per drop. Fresh Duroc boar semen was rinsed twice using Dulbecco phosphate buffered saline (DPBS) plus 1 mg/ml BSA and diluted to a concentration of 1×10^6 cells/ml using mTBM medium plus caffeine and BSA. Fifty microliters of the sperm sample was added to the droplets with oocytes to give a final sperm concentration of 0.5×10^6 cells/ml followed by incubation for

approximately 5 hours at 39°C in 5% CO_2. Following fertilization, oocytes were washed and cultured in 500 µl of PZM3 in four-well Nunclon dishes (Nunc) at 39.0°C in 5% CO_2.

Quantitative RT-PCR

Quantitative RT-PCR was used to analyze the expression of specific putative markers of oocyte and embryo quality and response to heat stress. MIR21 and programmed cell death 4 (PDCD4) were measured to determine effects on cell cycle regulation, viability and apoptosis. Heat shock factor 1 and heat shock protein 90α were measured to determine response to heat stress by the oocyte. Small pools of MII oocytes or embryos (n=25) were collected from each treatment and all markers were measured within the same group and time point. Statistical differences in gene expression between treatments in MII arrested oocytes and 4-cell stage embryos was determined using the MIXED procedure in SAS.

Results:

In vitro Maturation and Developmental Competency

A total of 667, 698, 697 and 716 cumulus oocyte complexes were in vitro matured for control, HS1, HS2 and HS3 treatment conditions, respectively (Figure 1). Control oocytes demonstrated the highest maturation rate (71.2 ± 3.7%). This was not different from HS2 (70.2 ± 0.7 %) but was significantly greater than HS1 (55.1 ± 6.3 %) and HS3 (54.0 ± 6.2 %) (Table 1, Figure 2) suggesting that oocytes experiencing HS during the early stages of oocyte maturation are more tolerant of heat stress than those exposed to heat stress during later stages of oocyte maturation or for the duration of in vitro maturation.

Following in vitro fertilization and development, the percentage of MII arrested oocytes producing embryos capable of development to the four cell stage within 60 hrs was not different between treatments despite being numerically lower in oocytes from the HS1 (Control, 51.3 ± 6.2%; HS1, 38.3 ± 4.4%; HS2, 48.2 ± 6.3%; HS3, 52.0 ± 8.6%) (Table 1, Figure 3). However, of the embryos capable of developing to the 4-cell stage, those capable of continued development to the blastocyst stage was significantly affected by exposure to heat stress during in vitro maturation (Table 1, Figure 4). Control embryos produced the greatest number of

blastocysts on Day 6 as a percentage of 4-cell embryos at 60 hours post fertilization (29.4 ± 4.5%) compared to other treatments (HS1, 1.6 ± 1.1%, HS2, 13.3 ± 1.0%; HS3, 21.6 ± 3.8%). Heat stress during the entire 42 hour period of in vitro maturation significantly impaired the ability of the embryo to develop to the blastocyst stage. However, the negative impact of the heat stress appears to primarily take effect during the first 21 hours of in vitro maturation as developmental ability, as measured by blastocyst formation rate, is significantly lower in HS2 compared to HS3 ($P < 0.001$).

Characterization of gene expression in MII arrested oocytes and 4- to 8-cell stage embryos following *in vitro* maturation

MicroRNA-21 (MIR21) and mRNA target programmed cell death 4 (PDCD4)

Mature MIR21 expression was increased in MII-arrested oocytes in HS2 and HS3 treatments compared to HS4 ($P< 0.05$) but not significantly different from HS1 ($P > 0.05$) (Figure 5A). In 4-8 cell stage embryos produced from oocytes for each treatement, mature MIR21 expression was similar between control, HS2 and HS3 ($P < 0.01$) and significantly increased embryos from HS1 oocytes ($P = 0.02$) (Figure 5B).

PDCD4 mRNA expression was not significantly different in MII-arrested oocytes between the heat stress treatments ($P = 0.35$) (Figure 6A). However, PDCD4 mRNA expression in 4-8 cell embryos was lowest in those embryos produced from HS1 oocytes compared to embryos produced from control, HS2 and HS3 oocytes ($P = 0.03$) (Figure 6B).

Heat Shock Proteins

To measure a direct effect of heat stress on the oocytes during in vitro maturation, Heat Shock Factor 1 (HSF1) and Heat Shock Protein 90α (HSP90a) mRNA were measured in all treatments. Heat shock factor 1 was not significantly different ($P = 0.18$) in MII stage oocytes for all heat stress treatments (Figure 7A). Heat shock factor 1 was also not different ($P = 0.50$) between treatments among embryos at the 4-8 cell stage (Figure 7B). Similarly, Heat shock protein 90α was not different ($P = 0.41$) in MII oocytes for all heat stress treatments

(Figure 8A), however, in 4-8 cell stage embryos, heat shock protein 90α mRNA expression was affected by treatment ($P < 0.05$) with lowest expression in control embryos and greatest in embryos produced from HS1 oocytes (Figure 8B).

Tables and Figures

Table 1. Summary of maturation rates for heat stress oocytes at MII, 60 hr and blastocyst stage.

Treatment[1]	% of oocytes arrested at MII[2]	Cleavage Rate within 60 hr post fertilization[3]	Percentage of oocytes developing to blastocyst stage[4]	Total cell number of day six blastocyst[5]
Control	71.2[a]	51.3	29.4[a]	46.0 ± 8.47
HS1	54.0[b]	38.2	1.6[b]	N/A
HS2	70.2[a]	48.2	13.3[c]	44.2 ± 6.8
HS3	55.1[b]	52.0	21.6[d]	46.6 ± 8.2

Numbers with a different superscript within a column represent statistical difference ($P < 0.05$).
[1]Oocyte maturation treatment conditions as described in Material and Methods.
[2]Percentage of oocytes arrested at Metaphase II of meiosis following in vitro maturation.
[3]Percentage of oocytes achieving ≥ 4-cell of development within 60 hours following *in vitro* fertilization.
[4]Percentage of embryos at 60 hrs achieving blastocyst stage within six days following in vitro fertilization.
[5]Total number of cells in day six blastocysts from each treatment.

Figure 1.

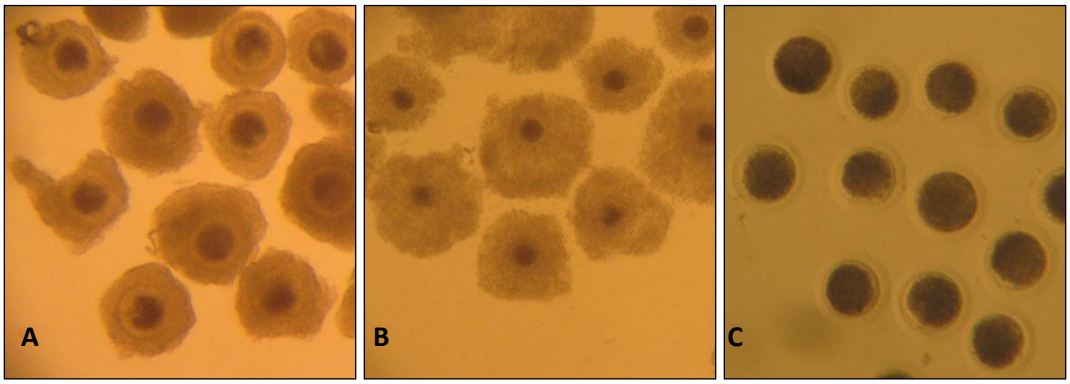


Figure 1. Representative images of porcine oocyte in vitro maturation. A. Germinal vesicle stage cumulus ooctye complexes (COCs) freshly aspirated from 3-5 mm antral follicles from sow ovaries. **B**. COCs after culture for 42 hours in maturation media **C**. MII oocytes denuded of cumulus cells just prior to fertilization.

Figure 2.

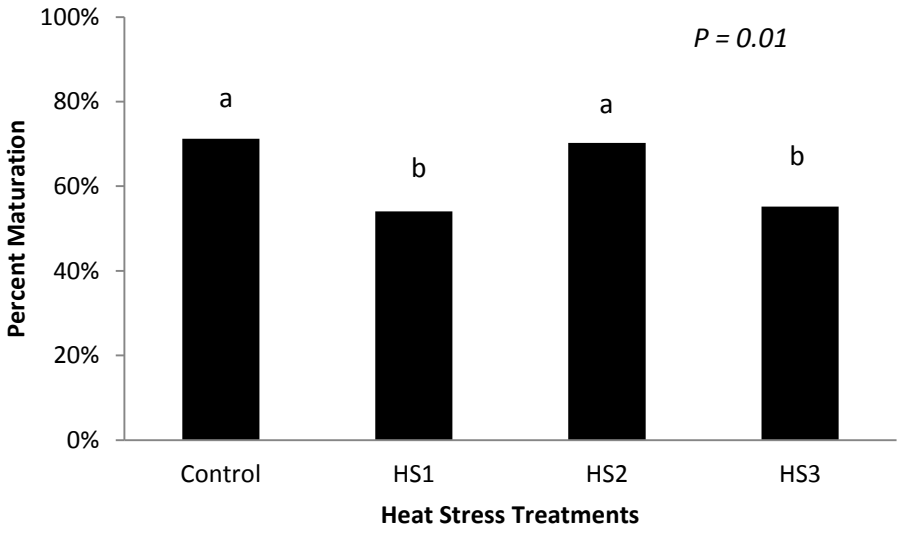


Figure 2. Maturation rates for MII development in heat stress samples. Control and HS2 had similar maturation rates (approximately 70%) while HS1 and HS3 were significantly lower (*P = 0.01*).

Figure 3.

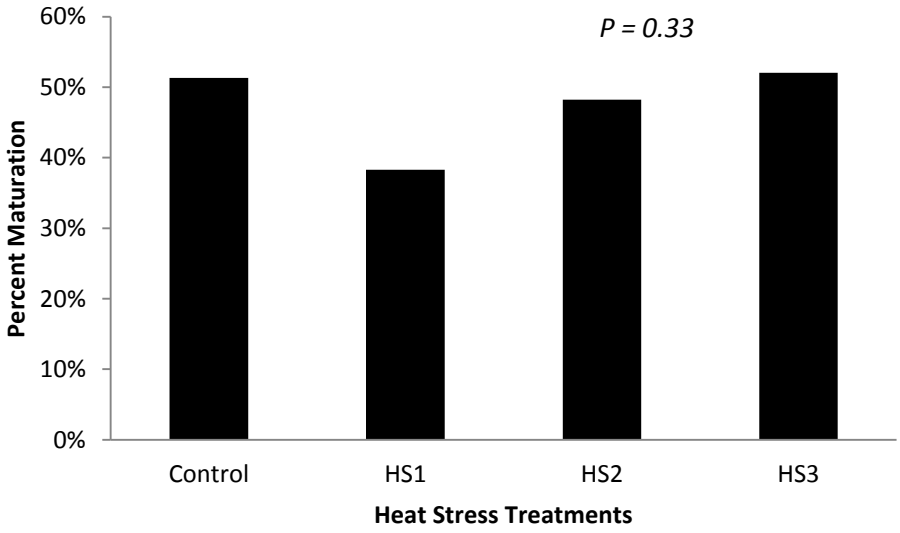


Figure 3. Percentage of IVF embryos which developed to >4 cell stage at 60 hr post fertilization. The 4-8 cell embryo development rate was similar for all heat stress treatments (*P= 0.33*).

Figure 4.

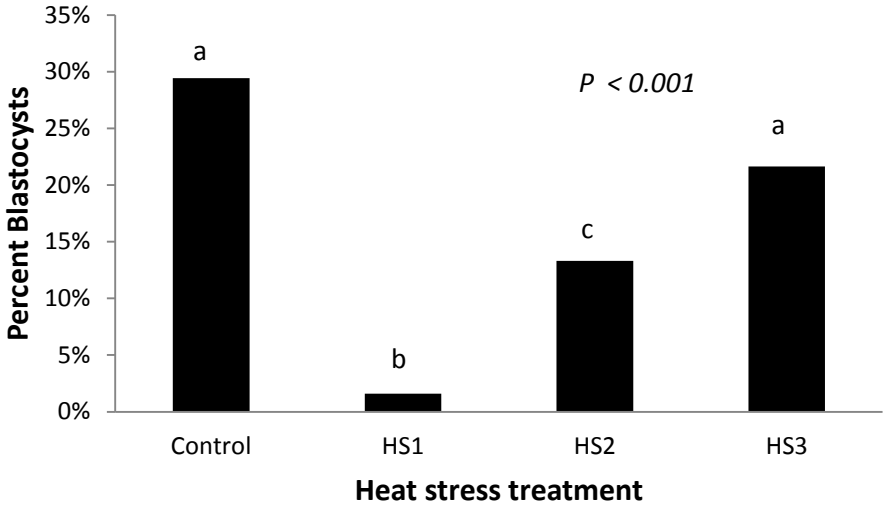
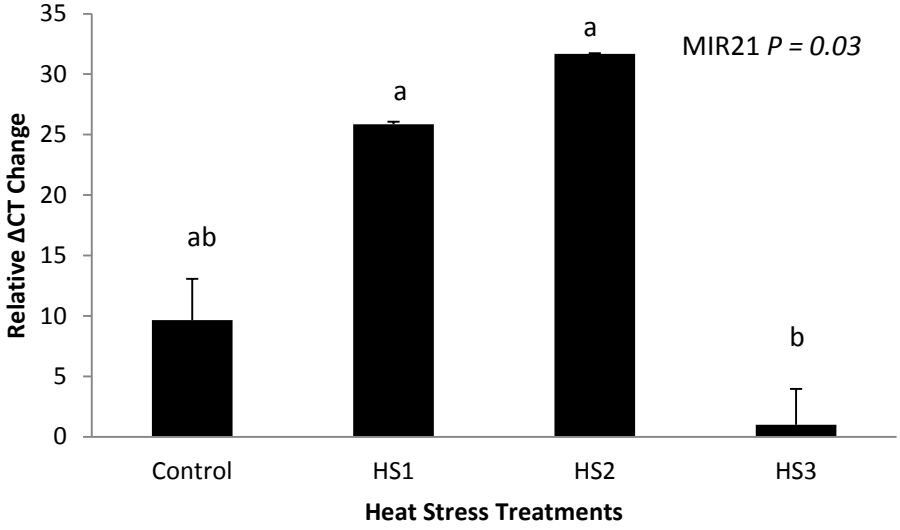


Figure 4. Percent blastocyst development for heat stressed oocytes. Heat stress during the first half or during the duration of in vitro oocyte maturation, significantly reduced the blastocyst development rate following in vitro fertilization.

Figure 5.

(A)



(B)

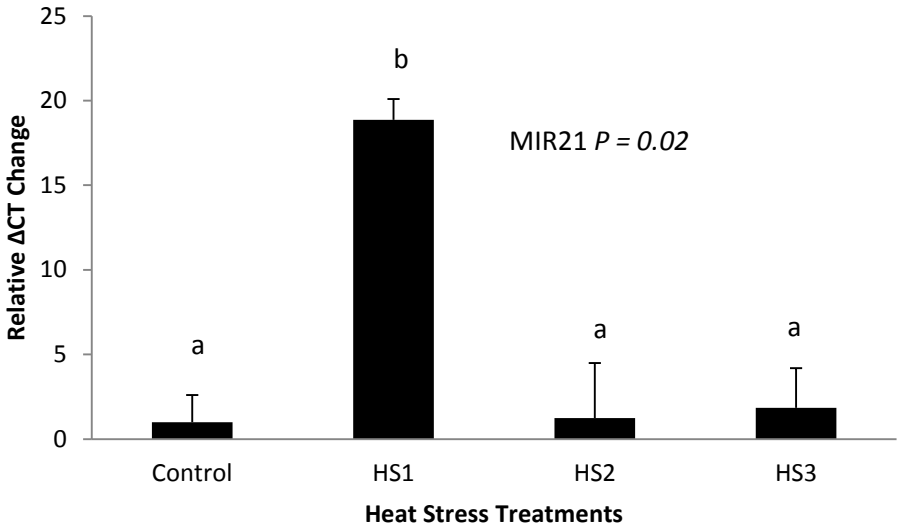
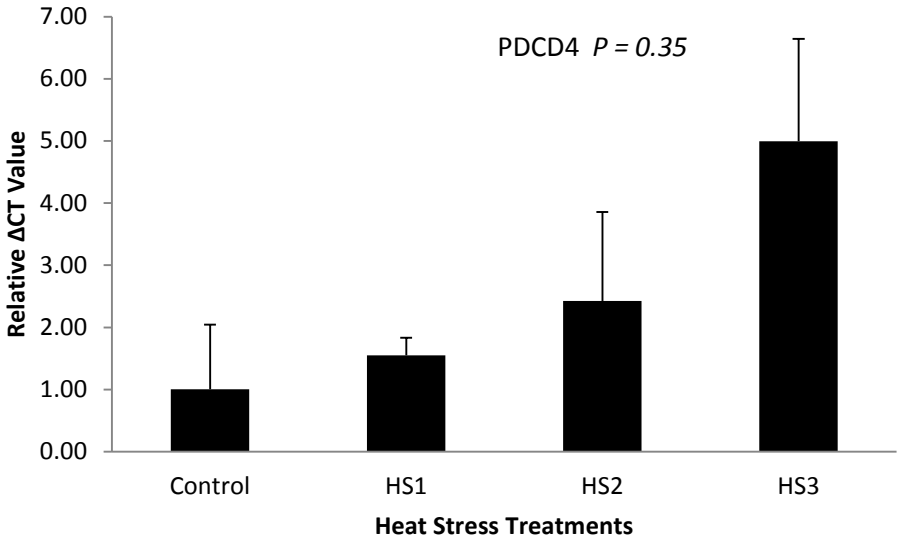


Figure 5. Mature MIR21 expression in heat stressed MII oocytes and subsequently produced emrbyos. (A) Mature MIR21 expression in MII arrested oocytes was greater in HS1 and HS2 compared to HS3 ($P < 0.05$) but not significantly different from control ($P > 0.05$). **(B)** Mature MIR21 expression in 4-8 cell stage IVF embryos. Expression was similar for control, HS2 and HS3 ($P > 0.1$) although was significantly greater in 4-8 cell embryos derived from HS1 treated oocytes ($P = 0.02$).

Figure 6.

(A)



(B)

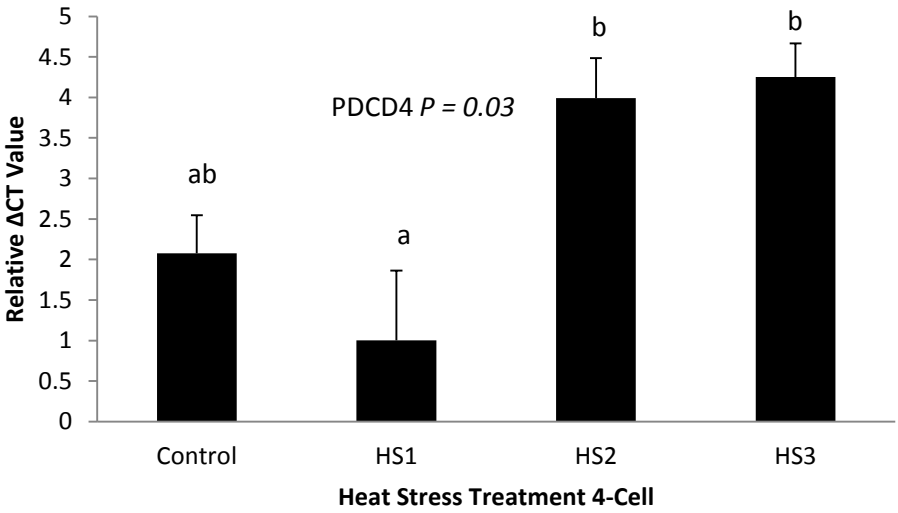
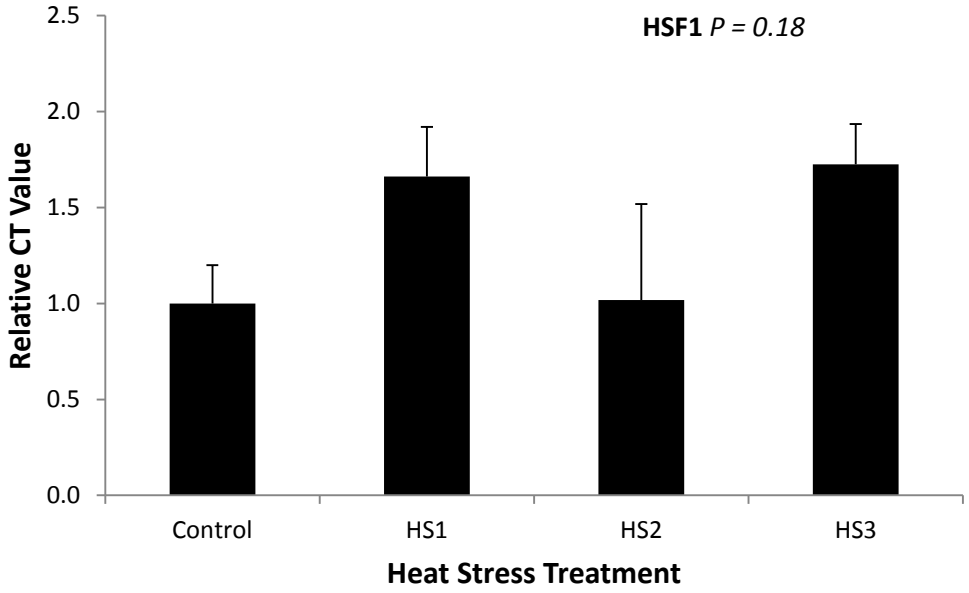


Figure 6. PDCD4 mRNA expression in heat stressed oocytes and 4-8 cell embryos. (A) PDCD4 mRNA expression was not significantly different among the heat stress treatments (*P = 0.35*). **(B)** HS1 4-8 cell embryos had the lowest relative PDCD4 mRNA expression compared with Control, HS2 and HS3 (*P = 0.03*).

Figure 7.

(A)



(B)

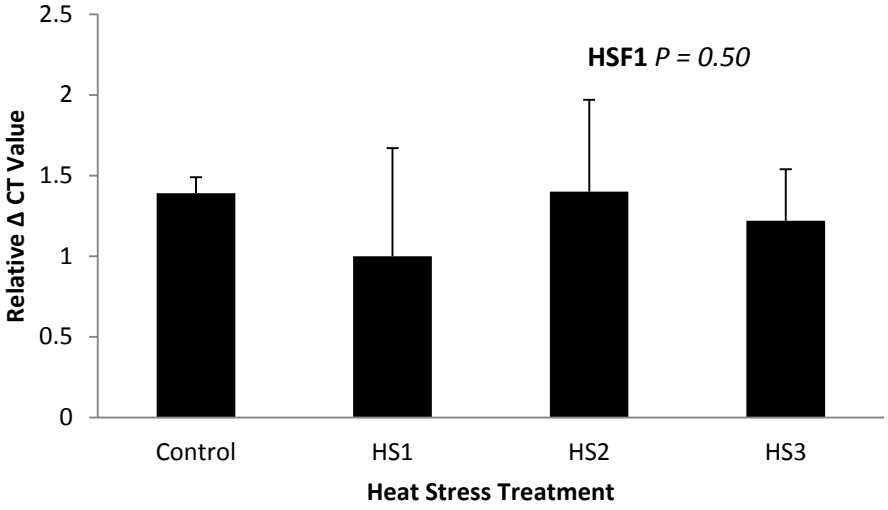
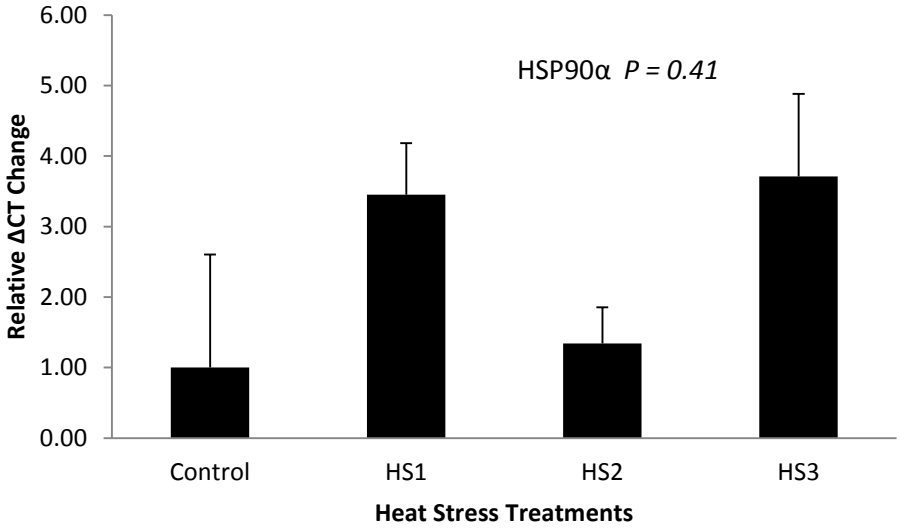


Figure 7. Heat Shock Factor 1 mRNA expression in heat stressed oocytes and IVF embryos. (A) Heat shock factor 1 was not significantly different in MII stage oocytes for all heat stress treatments (*P = 0.18*) however variation within the samples was high compared to all other genes we looked. **(B)** Heat shock factor 1 was also similar between all treatments among embryos at the 4 to 8 cell stage (*P = 0.50*).

Figure 8.

(A)



(B)

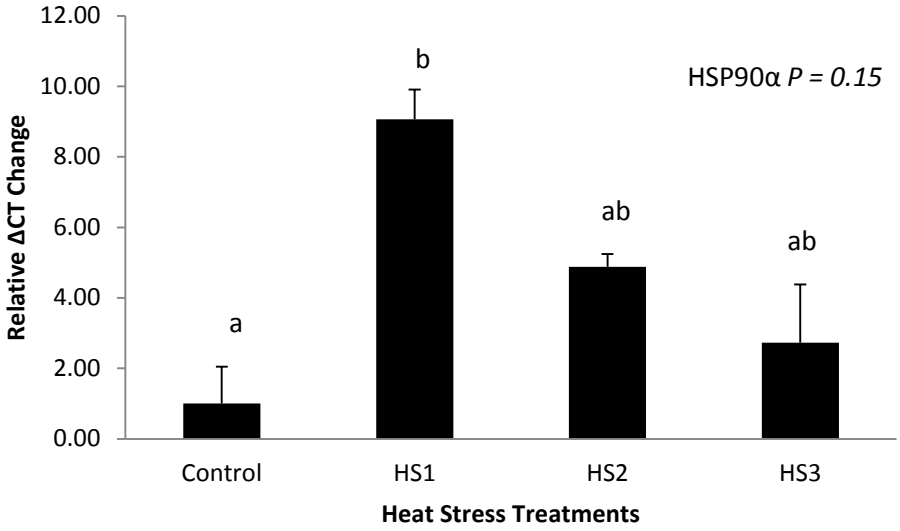


Figure 7. Heat Shock Protein 90α mRNA expression in oocytes and 4 cell embryos. (A) Heat shock protein 90α was not significantly different in MII oocytes for all heat stress treatments (*P = 0.41*). **(B)** Heat shock protein 90α mRNA expression was lowest in the control sample and highest in HS1(*P < 0.05*). HS2 and HS3 were intermediate (*P=0.15*).

Discussion

Heat stress has a negative effect on reproductive efficiency, by determining the response of the female gamete to heat stress during maturation and its effect on early embryo development management and mitigation strategies can be developed to improve seasonal reproductive losses related to heat stress in the pig. Our in vitro model of heat stress during oocyte maturation was able to measure responses to heat stress in the oocyte and evaluate the impact on early embryo development to the blastocyst stage. Consistent with previous studies we also observed a decrease in blastocyst rates when oocytes were exposed to 41° for 22-44 hrs. Our in vitro model also found that oocyte maturation is significantly decreased ($P = 0.01$) in oocytes xposed to 41°C for the first 22 hr of maturation but not the second 22 hrs of maturation. HS1 oocytes which were exposued to 41° C for the full 44 hr of maturation also demonstrate decreased ($P = 0.01$) oocyte maturation to a similar rate as HS3 indicating the first 22 hr of maturation may be a critical period for oocyte maturation representing a stage of follicular development that susceptible to heat stress.

HS1 oocytes were able to achieve cleavage at a similar rate ($P = 0.33$) as the other treatments however almost no blastocyst development was observed for this group. Additionally gene expression was most often significantly different in HS1 groups of oocytes. Increased heat stress protein 90α mRNA abundance from MII to 4 cell stage indicates a possible endogenous mechanism in the oocyte to diminish the effects of a heat stress. This could potentially lead to strategies for managing and reducing the harmful effects of heat stress in the pig.

Our objective for this study was to define in vitro culture conditions to model the effects of heat stress during oocyte maturation on maturation rate and subsequent developmental potential. We determined that all heat stress groups were able to develop to MII although the heat stress significantly reduced oocyte maturation for HS1 and HS2 groups.

Importantly, this model demonstrates that oocytes that appear normal phenotypically, as determined by their ability to mature to metaphase II of meiosis, can still demonstrate significantly reduced developmental competency as a result of prior exposure to heat stress. This model allows the investigation of specific

molecular markers of competency at a time period (i.e. 4- to 8-cell stage of development) when developing embryos with different levels of developmental competency appear similar, phenotypically.

We evaluated this model and investigated HSF1 and HSP90a mRNA expression differences in MII oocytes and 4- to 8-cell stage embryos, in addition to PDCD4 and a non-coding regulatory miRNA, MIR21. While HSF1 was not different in oocytes or embryos produced from the different heat stress treatments, HSP90a was greater in 4- to 8-cell stage embryos created from oocytes exposed to heat stress during in vitro maturation suggesting its utility as a potential molecular marker of competency in swine embryos as affected by heat stress. Similar to HSP90a, MIR21 and PDCD4 also represent markers of developmental competency. In 4-to 8-cell stage embryos produced from heat stress treatment conditions, MIR21 was increased in abundance compared to control oocytes, with the greatest expression being in those produced from oocytes exposed to heat stress conditions for the entire duration of in vitro maturation. Interestingly, PDCD4 mRNA expression was significantly lower in the embryos produced from HS1. Taken together, we hypothesize that increased MIR21 expression results in reduced PDCD4 mRNA abundance at the 4- to 8-cell stage in developmentally compromised embryos, as this biological interaction has been observed in numerous cell types (Fujita et al. 2008; Talotta et al. 2009) including the pig oocyte during normal in vitro maturation from the GV stage to MII arrest (Wright et al. 2010).

Development of this in vitro model of heat stress during porcine oocyte maturation will be valuable for the development and characterization of potential mitigation strategies to reduce the seasonal reproductive losses related to the impact of heat stress on oocyte production and early embryo development. Molecular markers, such as MIR21, PDCD4 and HSP90a represent targets that can be utilized to develop mitigation strategies to improve oocyte quality during heat stress. Further understanding of the biological mechanisms by which MIR21, PDCD4 and HSP90a affect embryo competence is necessary.

References

Fujita S, Ito T, Mizutani T, Minoguchi S, Yamamichi N, Sakurai K, Iba H. 2008. miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. J Mol Biol 378(3):492-504.

Isom SC, Prather RS, Rucker EB, 3rd. 2007. Heat stress-induced apoptosis in porcine in vitro fertilized and parthenogenetic preimplantation-stage embryos. Mol Reprod Dev 74(5):574-581.

Omtvedt IT, Nelson RE, Edwards RL, Stephens DF, Turman EJ. 1971. Influence of heat stress during early, mid and late pregnancy of gilts. J Anim Sci 32(2):312-317.

Soede NM, Noordhuizen JP, Kemp B. 1992. The duration of ovulation in pigs, studied by transrectal ultrasonography, is not related to early embryonic diversity. Theriogenology 38(4):653-666.

Talotta F, Cimmino A, Matarazzo MR, Casalino L, De Vita G, D'Esposito M, Di Lauro R, Verde P. 2009. An autoregulatory loop mediated by miR-21 and PDCD4 controls the AP-1 activity in RAS transformation. Oncogene 28(1):73-84.

Tompkins EC, Heidenreich CJ, Stob M. 1967. Effects of post-breeding thermal stress on embryonic mortality in swine. J Anim Sci 26:4.

Tseng JK, Tang PC, Ju JC. 2006. In vitro thermal stress induces apoptosis and reduces development of porcine parthenotes. Theriogenology 66(5):1073-1082.

Wright EC, Yang C, Scanlon R, Selman B, Ross JW. Temporal Relationship of miRNA-21 and PDCD4 Expression during In Vitro Maturation of Porcine Oocytes

2010; Milwaukee, WI.