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GENE EXPRESSION STUDIES ON BOVINE OOCYTES AND EMBRYOS SUBJECTED TO VARIOUS HEAT STRESS AND HEAT SHOCK CONDITIONS

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By

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To my family

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GENERAL ABSTRACT

Thesis Title: Gene expression studies on bovine oocytes and embryos subjected to various heat stress and heat shock conditions

Increasing temperature mainly by global warming has been showing rapid environmental temperature chances, unpredictable climatic changes. Increasing temperature has been showing a greater effect on reproductive performance of lactating cows, ultimately affecting dairy economy. Ambient temperatures in subtropical zones during summers affecting cows, as the temperature level were reaching more the upper critical temperature or else above theromonetual zone. Terceira-Azores being considered a dry summer tropical climate, it is important to study the seasonal changes impact on Holstein cows in the islands. Besides this, they still lot unknown factors effecting the heat stress oocytes and embryos, as fertility is a multifactorial problem that affects physiological and cellular functions in several tissues.

To improve and study the reproductive performance of the cows, the following tasks were performed :

Chapter 2: to evaluate how environmental factors in a dry-summer subtropical climate in Terceira-Azores (situated in the North Atlantic Ocean: 38° 43' N 27° 12' W) can affect dairy cow (Holstein) fertility, as well as seasonal influence on in vitro oocytes maturation and embryos development. Impact of heat shock (H.S) effects on in vitro oocyte's maturation and further embryo development after in vitro fertilization (IVF) was also evaluated.

Chapter 3 :A standardized reagent protocol for total RNA extraction was designed for bovine oocytes and embryos, which is considered specific and less expensive. This protocol is mandatory for the gene expression in further experiments.

Chapter 4: Three assays were performed. In assay 1, oocytes harvested during winter months were subjected to kinetic heat shock by stressing the oocytes at 39.5 °C (HS1) and at 40.5 °C (HS2) for either 6 h, 12 h, 18 h or 24 h and then matured at control temperature (38.5 ºC). The nuclear maturation rates (NMR) of all oocytes were recorded after 24 h. In assay 2, oocytes collected year-round maturated, were implanted via in vitro fertilization (IVF) and developed for nine days. Gene expression analysis was performed on target genes (Cx43, CDH1, DNMT1, HSPA14) with reference to the two housekeeping genes (GAPDH and SDHA) in embryos. Similarly, in assay 3, genetic analysis was performed on the embryos produced from heat-stressed oocytes (from HS1 and HS2).

Chapter 5: In Assay 1, oocytes from winter months (December-March) (n = 100) and summer months (June-September) (n = 100) were collected and matured to analyze their heat shock tolerance. Total RNA was extracted from the matured oocytes, based on the seasons in which they were obtained, and further cDNA synthesis was performed, followed by qPCR for selected genes (Cx43, CDH1, DNMT1, HSPA14) and was compared to the two reference genes (GAPDH and SDHA). In Assay 2, oocytes collected during winter months were subjected to kinetic heat shock by stressing the oocytes at 39.5 °C (HS) for periods of 6, 12, 18 or 24 hours and then matured at control temperature (38.5 ºC). Matured oocytes were subjected to the previously described gene analysis procedure.

To meet all these experiments following procedure were followed:

Chapter 2: The result of the first artificial insemination (AI) performed 60-90 days after calving of 6300 cows were recorded for one year. In parallel, climatic data was obtained at different elevation points (n = 5) from 0 to 1000 m and grazing points (GP) from 0 to 500 m, in Terceira island, and the temperature humidity index (THI) was calculated. For in vitro experiments, oocytes (n = 706) were collected weekly during all year, for meiotic maturation and IVF. Further, to evaluate H.S effect, 891 oocytes were

collected in the cold moths (December, January, February and March) and divided in three groups treated to H.S for 24 h during in vitro maturation (IVM) at: C (Control = 38.5ºC), H.S1 (39.5ºC) and H.S2 (40.5ºC). Oocytes from each group were used for meiotic assessment and IVF. Cleavage, morula and blastocyst development were evaluated respectively on day 2, 6 and 9 after IVF.

Chapter 3: Oocytes (n=795) recovered from about 80 ovaries were divided in three groups in order to apply the different protocols: Group 1 modified trizol® (MTP n=355); Group 2 Guanidinium thiocyanate protocol (GNTC n= 140) and Group 3 Commercial Kit protocol (CKP n=60). Oocytes belonging to group 1 (n=100) and 3 (n=20) were subjected to vitrification using two cryoprotectants 1,2 propandiol (PROH) or Dimethylsulfoxide (DMSO). The 240 remaining oocytes were divided into 3 groups in which 100 were used, in fresh, for in vitro fertilization, and 140 oocytes were vitrified using PROH (n=70) and DMSO (n=70) as cryoprotectants, being then fertilized in vitro after thawing. Embryos were used nine days after fertilization. Gene amplification (SDHA, GAPDH and DNMT1) was performed in oocytes, and gene quantification (DNMT1) in in vitro produced embryos at the stage of blastocyst (n≈10). Regarding Chapter 4 and Chapter 5 it already explained in afore in the introduction.

By overall analysis each part of the experiment in presented thesis has added a new insight to the animal reproductive field. From chapter 2, *in vivo* studies have demonstrated that, up to a THI of 59, a decrease in CR occurs when AI is performed 60-90 days after calving. This falling in fertility was confirmed by the in vitro experiments, in which oocytes nuclear maturation and further in vitro development decreases significantly in warmer periods. Heat-shocked oocyte's maturation also confirmed this low ability of oocytes to maturate and develop after IVF. As THI values in hot months are lower in highest elevations, one could propose to locate animals in high elevation points during the

warmest season to reduce the impact of heat stress in cow conception rate. From chapter 3, a standardized protocol is designed which can be considered more effective than GNTC and CKP after analyzing the spectrophotometric, PCR amplification and gene quantification results. From chapter 4, results has provided a concrete evidence for the chapter 2 results that low embryo development rate in summer months when compared with winter is due to the altered expression of DNMT1, HSPA14 and Cx43 in these conditions. The results of the chapter 2 had also shown a low developmental rate in embryo samples where the embryos were developed from different heat shocked oocyte samples. This phenomenon is explained in chapter 4 as it is to the constant, high expression of the DNMT1 gene and variations in the expression of HSPA14, Cx43 and CDH1. From chapter 5, gene expression results had shown very good evidence for supporting the results of chapter 2 and chapter 4, where the gene expression analysis of in vitro matured oocytes during the summer months showed that its lower maturation rate compared to those collected in winter may be due to altered expression of HSPA14. The gene quantification of in vitro matured oocytes subjected to heat shock at different temperatures has provided additional evidence that oocytes exhibit a low maturation rate after 12 h of heat shock because there is a constant up-regulation of CDH1 and an up-regulation of DNMT1 in samples exposed to 24 h of heat shock. Overall, the gene regulation analysis of CDH1 and DNMT1 supports that, after prolonged heat shock, in vitro matured oocytes likely experience apoptosis. To concluded the presented thesis from overall results, as it shows a better and new insight in terms of how to increase the conception rate of cows in summer seasons, theromotelorance level of bovine oocyte. It also provided better evidence for in vitro and in vivo heat stress on cows through gene expression analysis.

Keywords: Environmental stress, Artificial insemination, Heat shock, Maternal heat stress, Kinetic Heat Shock,, Nuclear Maturation, Gene Quantification.

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CHAPTER ***1***

General Introduction

Global warming is one of the major factors responsible for the reduced fertility or conception rate (Stephenson *et al.,* 2010). As global warming directly affect fertility in two ways, firstly hot weather distresses sexual behavior, secondly elevated ambient temperatures had negative influence on the reproductive health factors such as sperm motility and menstruation (Hansen, 2009). Climatic change is the major problem for sustainability of livestock production systems (Koluman and Silanikove, 2014) across the world. Especially in the subtropical and temperate countries (Badinga *et al*., 1985), but surprisingly even countries located within the non-temperate zone are affected by changes in global warming. These changes are associated with unprecedented events of extreme ambient temperatures (above 40°C) and seasonal changes. It has been observed a noticeably increase in temperature humidity index (THI) above specific comfort zone threshold (>68) in European countries (Silanikove and Koluman, 2015).

Thermonetural zone/ Comfort zone is the range of environmental temperatures where normal body temperature is maintained and heat production is at basal level. The ranges of thermoneutral zone are from lower critical temperature (LCT) to upper critical temperature (UCT). LCT is the environmental temperature at which an animal needs to increase metabolic heat production to maintain body temperature, UCT is the environmental temperature at which the animal increase heat production as a consequence of a rise in body temperature resulting for in adequate evaporative heat loss (Yousef, 1985). Thermoneutral zone depends on the age, breed, feed intake, diet composition, previous state of temperature acclimatization, production, housing and stall conditions, tissue (fat, skin) insulation and external (coat) insulation, and the behavior of the animal.

The thermoneutal range of UCT is 25-26°C, LCT is from -16 to -37°C for dairy cows (Hamadal, 1971; Berman *et al*., 1985), where their physiological body temperature is maintained from 38.4 to 39.1°C (Yousef, 1985). Variation in thermoneutal/comfort zone

affects the dairy cattle to experience either heat stress or cold stress. Which is related to the seasonal effect on reproduction in dairy cows. Heat stress (HS) is a major contributing factors to the low fertility of dairy cows during summer months (Ray *et al*., 1992; Thompson *et al*., 1996; Al-katanani *et al*., 1999). Decrease in conception rate during the hot seasons can range between 20 to 30% compared to the winter seasons (Cavestany *et al*., 1985; Badinga *et al*., 1985; De rensis *et al*., 2002). There are clear seasonal patterns of estrous detection ,day to first service and conception rate in dairy cows (Badinga *et al*., 1985; Cavestany *et al*., 1985) and low conception rate are consistently observed in summer months compared to winter months. The reduced fertility associated with summer heat stress is multifactorial problem, which mainly includes hyperthermia leading to the following signs in cows : Restlessness, Crowding under shade or at water tanks, Panting (open-mouthed breathing), Increased salivation, Increased respiration rate, Rise of rectal temperature (40-41°C), Declined feed intake, Reduced heart rate, Declined feed intake, Increased water intake, Drop in daily milk production (McDowell *et al*., 1976; Silanikove, 1992; Shalit *et al*., 1991; Kadzere *et al*., 2002).

Hyperthermia can affect cellular function in various tissues of the female reproductive tract (Wolfenson *et al*., 2000; Hansen *et al*., 2001). Heat stress compromised ovarian follicular dynamics (Badinga *et al*., 1993) and their ability of dominant ovarian follicle to exert dominance (Wolfenson *et al*., 1995; Wilson *et al*., 1998). This loss of follicular dominance could be related to reduced plasma concentrations of Estradiol 17β and inhibin (Badinga *et al*., 1998) and increased plasma concentration of Follicular Stimulating Hormone (FSH) (Roth *et al*., 2000). Heat stress induced codominance (Sartori *et al*., 2004) which may compromise oocyte viability; indeed oocytes may be compromised by heat stress (Roch *et al*., 1998 Al-katanani *et al*., 2002). Oocytes harvested from follicles of Holstein cows during summer had reduced ability to develop to blastocyst stage after in vitro fertilization than

oocytes harvested during winter (Roch *et al*.,1998 Al-katanani *et al*., 2002). Besides , expuse of Holistein heifer to HS between the onset of estrus and developmentally retarded embryos as compared to heifers maintained at thermaneutrality (Putney *et al*., 1989). If the pool of ovarian oocytes was damaged by summer stress, it takes two or three estrous to get back to the normal competent oocytes (Roth *et al*., 2001). Hence follicles and oocytes can be damaged by heat stress during early stage of folliculogenesis, with a delayed deleterious effect on ovarian function.

Direct exposure of the cumulus oocytes complexes (COCs) to 41°C during the first 12 h of in vitro maturation (IVM) disrupted cytoskeletion architecture reduced oocyte maturation (Roth *et al*., 2005) oocyte death through apoptosis (Roth *et al*., 2004).These deleterious effects of heat-shock decreased the proportion of oocytes that became blastocysts following in vitro fertilization (Roth *et al*., 2004; Edwards *et al*., 2005). Studies on molecular events occurring during oocyte maturation under stress are few, only a few studies which are available it appeared that proteins synthesis was affected in heat stressed oocytes, both in cattle and mice (Hahnel *et al*. 1986; Curci *et al*. 1987; Edwards and Hansen 1996). So far, no experiments have been performed on the effect of kinetic heat stress on bovine oocytes, apart from these studies on the effects of heat shock on oocytes and embryos and a limited number of seasonal studies on the effects of maternal heat stress (Roth *et al*., 2001; Roth *et al*., 2008; Gendelman *et al*., 2010; Gendelman *et al*., 2012) as maternal hypothermia requires two to three estrus cycles to normalize competent oocytes. Research conducted by Payton *et al*. (2011) shows that heat stress may induce alterations in the transcriptional levels of genes involved in cell growth, cell cycle and programmed cell death. Also, Gendelman *et al*. (2012) has shown that specific developmental genes have less mRNA expression patterns in the summer than in the winter. So far few developmental genes were studied on heat stock

oocytes and embryos, it is important to analysis more genes. It has been proven that the *Cx43* gene is involved in embryonic development and maternal zygotic transition (Houghton, 2005), while the *CDH1* gene controls the embryonic compaction process (Vestweber *et al*., 1984; Riethmacher *et al*., 1995). Furthermore, the *DNMT1* gene is known to affect mammalian pre-implantation development, which represents a critical stage for the establishment of the epigenome (Golding *et al*., 2003). Studying DNMT1 in oocytes and embryos may provide a better understanding of the epigenetic rearrangements occurring in early stage embryos where first cell divisions impact chromatin configuration during cell differentiation (Giraldo *et al*., 2013a). Gene expression changes are an integral part of cellular response to heat shock. From the literature review conducted by Sonna *et al*. (2002), genes encoding heat shock proteins (HSPs) affect a substantial number of genes which are not directly associated with HSPs. So far, the *HSPA14* gene is not well understood, so analyzing the *HSPA14* gene may provide more functional details about HSPs.

1.1. Aims and Objectives

Increasing environmental temperatures have been showing a greater effect on the fertility of cattle, which eventually affecting global economy of dairy industry. As describe above there is an increasing global warming effect in temperate zone, and subtropical regions , Terceira-Azores (situated in the North Atlantic Ocean: 38° 43' N 27° 12' W) being a dry-summer subtropical climate presumed having similar effect. To determine this climatic / Heat stress effect and to study the molecular mechanism involved in reproductive performance of cows following objectives were performed.

Objectives

Chapter 2

The major objective of this study to evaluate reproductive performances of all day grazing Holstein cows in a warm temperature region of Azores, in relation to environmental stress, but also to determine the *in vitro* development of oocytes and embryos during cold and warmer months.

Apart from this effect of heat shock under different temperatures during *in vitro* maturation (IVM) of bovine oocytes and further embryonic development after IVF was also evaluated.

Chapter 3

To study molecular mechanism/gene expression analysis it is important to stabilize a standardized protocol for the extraction of total RNA from a minimum number bovine oocytes and embryos samples. As so far no proper standardize protocol was descried in specific to Bovine cells. Hence the major aim of this work is to design a standardize protocol which is specific for bovine oocytes and embryos and reliable for the downstream process (Gene amplification and Gene quantification).

Chapter 4

To understand the molecular mechanism involved in low fertility rate of cows under heat stress (*in vivo* and *in vitro*) the following objective has to be performed. Gene expression studies of developmental genes (*Cx43, CDH1, DNMT1* and *HSPA14*) in different developmental stages (2-cell, 4-cell, morula and blastocyst) of embryos developed from oocytes under prolonged heat shock, as well as oocytes collected during hot and cold seasons has to be studied.

Chapter 5

As it is important to understand maternal heat stress factors and to analyze the heat stock condition based on time and exposure, following objectives were performed. Gene expression analysis of kinetic heat shocked oocytes and oocytes matured in the summer and the winter.

Chapter 6

An overview and discussion of the results of these studies and their possible implications for the practice and for future research are given