Chapter 3

Studies on Gene Expression and Developmental Competence of Bovine Embryos Produced Under Different Conditions of Heat Stress

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Additional information is available at the end of the chapter

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Abstract

Gene expression is required in all steps of embryonic development and therefore heat stress is known to reduce developmental competence after direct exposure of oocytes and embryos to different conditions of heat shock, by decreasing protein synthesis. Moreover, as in somatic cells, the heat stress befuddles the integration of RNA and posttranscriptional modification of RNA, the assumption was that during meiotic maturation heat shock may mutate RNA within oocytes, with the possibility of altering the surrounding cumulus cells, causing, thus, reductions in development. Heat shock proteins (HSP) are among the first proteins produced during embryonic development and are crucial to cell function. The HSP70 (*HSPA14* gene) is an important part of the cell's machinery for folding, unfolding, transport, localization of proteins and differentiation, regulation of the embryonic cell cycle and helping to protect cells from stress. Therefore, *HSPA14* is an apoptotic gene induced by heat shock is associated with embryonic loss, playing an important role of control mechanism of processes involved in growth, cellular differentiation, and embryonic development. In addition the connexin proteins (e.g. *Cx43*), related to gap junctions, are expressed in numerous tissues including gonads, act as a mediator of heat stress effect on cells. In the present review, the effect of heat stress on bovine embryonic development in a physiologic and genetic point of view is fully discussed.

Keywords: heat stress, oocyte, maturation, gene expression, *HSPA14*, *Cx43*, real-time PCR

1. Introduction

Climate changes influence the biogeography and phenology of animals, thus affecting their reproduction, physiological development, and metabolism [1]. The global warming refers to

the continued increase of the earth's temperature and ecosystems change, as when the sun heats the earth, the earth radiates only some of the sun's energy into space, while some energy is trapped by atmospheric gases, such as carbon dioxide and water vapor. This energy builds up in the earth's atmosphere, the earth gradually becomes hotter and leads to increase in the temperature, and hence heat stress can happen [2]. Depending on the proportion of CO_2 emission, the global surface temperature is predicted to rise by 1.1–6.4°C. Heat stress is the main factor responsible for lower productive and reproductive performance in cattle during the summer months. The extreme temperature events due to climate change coupled with high rate of relative humidity decrease the ability of reproduction in animals [3]. Heat shock impacts on the ovulatory oocyte, as well as on functions of follicular granulosa and theca cells, therefore delays and alters the efficiency of follicle selection and lengthening of the follicular wave, which adversely affects the quality of oocytes [4]. The first-wave dominant follicle is depressed by heat stress; hence, lactating cows were found to be smaller in diameter. Thus during the first 7 days of the cycle showed that follicular dynamics have been altered, as indicated by the decrease in the number of medium-sized follicles that associated with depressed summer fertility [5, 6]. During hot summer months, high temperature can affect endocrine responses that may increase foetal abortions, shorten the gestation length, lower calf birth weight, and reduce follicle and oocyte maturation associated with the postpartum reproductive cycle. Postpartum heat stress can significantly decrease the pregnancy rates with impacts lingering well into the fall months [7]. The summer period adversely affects endocrine system, follicular phase, metabolism, function of oocytes, and embryos. The towering temperature causes several cellular changes during the maturation period and germinal vesicle stage in the oocytes [8]. Therefore, the heat stress influences and impacts on *in vitro* oocytes maturation, their nuclear maturation, and further embryos development to the stage of blastocysts after *in vitro* fertilization [9]. The low fertility of cattle females has led to an increased interest in *in vitro* embryo production (IVEP) technologies for achieving rapid genetic improvement and providing an excellent source of embryos for carrying out basic research on developmental physiology [10]. Moreover, cytoplasmic and molecular maturation of oocytes are thought to be critically involved in the ability of the oocyte to support fertilization and early development stages. The major activation of the bovine embryonic genome occurs at the 8- to 16-cell stage. Before embryonic genome activation, mRNAs, ribosomes, and proteins synthesized during oocyte growth and maturation contribute to early development [11]. As known, heat stress induces apoptosis or expression of *HSPA14* gene of *in vitro* produced embryos. Stress proteins are assorted into families depending on their molecular weight and provide two main functions: primarily as a molecular chaperone having key roles in folding/unfolding of proteins and secondarily as a stabilizer factor from deteriorating proteins contributing in the protection of cells against stress/apoptosis, granting an opportunity for rehabilitation or degradation in the cells suffering from cellular stress [12]. Some activities of HSP70 include folding, unfolding, transport, and localization of proteins and differentiation and regulation of the embryonic cell cycle [13]. Hence, the piling up of HSPs has long been considered a sign of cellular damage. There is an evident overlap of the signals that induces a protective stress response and those that initiate apoptosis [14]; therefore, heat shock protein HSP70 plays a protective role in the embryos. During the early 2-cell of embryo to the blastocyst stage, the HSP70 is the prevailing gene. The induction of stress proteins (HSP) synthesis during 1- or 2-

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cell stage until blastocyst stage is intensely motivated by heat shock [15]. Thus, *HSPA14* gene is produced by embryonic cells, which protect the embryos from environmental stress. During early development, increased temperatures, free radicals, and oxygen stress have deleterious effects on embryonic viability and development. The *HSPA14* confers thermotolerance against variety of stressors. Thus, the preferential maintenance of *HSPA14* gene expression would allow this gene product to help maintain cellular function by acting as molecular chaperones to stabilize or refold proteins damaged by heat, and by blocking apoptosis [16]. There are many ways in which cells communicate with other cells, and gap junctional communication is one of these ways. Ovarian folliculogenesis and the production of fertilizable oocytes depend on gap junction channels which allow inorganic ions, second messengers, and small metabolites to pass from cell to cell, and this permeability is supposedly for underpinning the physiological roles played by gap junctions, for example, connexin 43 (*Cx43*) gene expressed within the oocyte–granulosa cell complex depending on the species [17]. The expression of *Cx43* gene in the ovary is regulated by gonadotropins like follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Increase in the *Cx43* expression in the large antral follicles was observed where FSH was relatively elevated. Moreover, germline and somatic cell during mammalian ovarian follicle have elements for cellular interactions, development, and function. Somatic cell-to-oocyte communication is essential for oocyte growth and the regulation of meiotic maturation via gap junctions [18]. *Cx43* was later detected in the cytoplasm of germ cells. Therefore, tight junction molecules were found and *Cx43* were distributed at the cell–cell contact of the adjacent cells. Such progressive reorganization of germ cells, *Cx43* gene, is probably coupling with conjugation role of other gap junctions and their specific connexins (Cxs), between the different cell types that structure the animal reproductive system [19]. Furthermore, germline and somatic cell during mammalian ovarian follicle have elements for cellular interactions, development, and function, essential for oocyte growth and the regulation of meiotic maturation via gap junctions [23]. On the other hand, during embryonic early development, increased temperatures lead to free radicals and oxygen stress, having also deleterious effects on embryonic viability and development. As far as *in vitro* embryo production is concerned, more and more motives are actually presented in the cattle breeding, such as the faster propagation of superior germplasm because of the low efficiency of superovulation in programs of embryo transfer. Moreover, embryo production is actually also a reproductive method used to improve the number of progeny with high genomic merit from bovines, both *in vivo* and *in vitro*. *In vitro* embryo production (IVEP) also including some of the applications serves to solve infertility problems, genetic recovery, and production of clones or even transgenic animals. In the present review, physiological and genetic factors affecting embryonic development under heat stress conditions are fully discussed.

2. Ovarian physiology: folliculogenesis, oocytes growth, and development: oocytes growth and development

The fertilization of mature oocyte occurs in the oviduct and there are three distinct phases that can be divided during this process. During the first phase (the oocyte growth phase),

the developmental competence of the oocyte and cell structure is generated, when oocyte growth accompanies follicular growth from the primordial to the small (2–3 mm) tertiary (antral) follicle. When follicles in a cohort reach a diameter of about 3–5 mm, one dominant follicle is selected during the antral phase. During the third phase, the oocyte undergoes to change (oocyte maturation) almost 24 hours between the peak ovulation and the rise of LH.

2.1. Oogenesis

The mature oocytes are differentiated, released, and established in mammalian ovaries to undergo oogenesis process for fertilization. The ovaries have individual follicles consisting of an innermost oocyte, surrounding granulosa cells, and outer layers of thecal cells, controlled by the endocrine system. Moreover, mammalian ovary produces steroids and peptide growth factors, which allow the development of female secondary sexual characteristics and support pregnancy [20]. The oocytes and surrounding granulosa cells have amiable connections together to support oocyte viability and growth mediated by the gap junctions, which are efficient conduits for low molecular weight substances. The granulosa cells have some metabolized molecules, which play a role in transporting the oocytes. Additionally, the KIT ligands and c-kit receptor are localized to oocytes and granulosa cells, respectively, to promote oocyte growth and follicular development. Moreover, some of the growth factors derived from oocytes, such as GDF-9 and BMP-15, contribute in follicular development by regulating the differentiation of surrounding somatic cells, as these communications are important for oocyte growth and follicular development [21]. The occurrence of events of oogenesis are concomitantly with folliculogenesis, as oogenesis can be explained as the process of formation and maturation of the egg by development and differentiation of the female gamete during the meiotic division, and this is the first phase of progress for the fetus. Thus, female ovaries have fixed number of oocytes, which decreases by time passing with several years, without potential to renew. During the ovarian start to deplete the auxiliary of oocytes, it evolves to a senescence/aging stage born with a fixed number of oocytes, which through several years reduce, without potential to renew. When the ovarian start to deplete the auxiliary of oocytes, it evolves to a senescence stage, leading the female to menopause [22]. During meiosis, mammalian oocytes undergo two consecutive asymmetric cell divisions, which are essential for the formation of a functional female gamete, without an intermediate replicative phase. Each division must ensure accurate segregation of the maternal genome and highly asymmetric partition of the cytoplasm, further that a tiny polar body and a large oocyte are generated. It leads to an asymmetric organization (or polarization) of the egg, which determines the geometry and the success of fertilization. Asymmetric divisions are tightly controlled by microtubule and microfilament cytoskeletons. During the beginning of the first meiotic division, this process allows the separation of the duplicated centrosomes and therefore to the gathering of a bipolar spindle formed by microtubules. Thus, it is referred to as the process that produces gametes with half of the number of chromosomes from the parent cells. For the position of spindle surrounding the oocytes and tossing the first polar body in parallel with separation of chromosome, the microfilaments are carried

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out in meiosis I. The microtubule spindle is positioned at the surrounding of the oocytes until fertilization, causing the emission of the second polar body during second meiotic division (meiosis II). Additionally, the loss of asymmetry is a mark of low-quality oocytes and a signature of pre- and postovulatory aging [23].

2.2. Folliculogenesis

Meiotic prophase consists of several temporary stages: preleptotene, leptotene, zygotene, pachytene, and the diplotene stage in which the first meiotic division of the ovarian follicles begin to develop as primordial structure that oocyte arrested. Primordial follicle activation is characterized by the possession of complete layer of 11–20 granulosa cells around the oocytes. Secondary follicle stage starts to show features of a second layer of granulosa cells. Zona pellucid is the initial deposition material around the oocyte, and at the same time, cortical granules are formed within the oocyte cytoplasm. It is at this point of development that follicles appear to become responsive to gonadotrophins [24]. The progress of ovarian follicle is a combination of many sides of a compound process that starts with the foundation of limited pool of primordial follicles and attains in either atretic degradation of the follicle or liberation of mature oocytes for fertilization. Through the primary, preantral and antral stages, the primordial follicles must be grown during these stages before reaching to the preovulatory stage where they are eligible to release oocytes for starting the fertilization stage. The corpus luteum structure (CL) is formed from the differentiation of the residual of granulosa and thecal cells after ovulation [25]. Large stock of oocytes is enclosed in primordial follicles in mammalian ovaries, and some of these follicles initiate growth toward a possible ovulation undergoing activity of the ovarian cycle. Additionally, most of these follicles end their growth at any moment and degenerate through atresia. During the growth of follicles, only a subset of oocytes is capable to support meiosis, fertilization, and early embryo development to the blastocyst stage. This proportion of eligible oocytes depends on the size of the follicular cell. Developing lines of evidence propose that the competent oocytes increase the storage of gene production leading to the determinant to support the precocious stages of developmental embryos, before the activation of embryonic genome. Thus, these transcripts may be stored during early folliculogenesis as the oocyte grows and displays high transcription activity [26]. Young and McNeilly [27] classified the system into five types: type I represents the primordial follicles, which are a resting stage before their activity begins. In this stage, follicles have only one layer of granulosa cells and this is the follicle transitioning through the primary stage, when the granulosa cells become cuboidal. The second type of follicles includes one layer of cuboidal granulosa cells. While antral follicles include two to four layers of granulosa cells going to the third type. At this stage, the large preantral follicles consists of four to six layers of granulosa cells, considering this stage as the fourth stage, increasing the number of layers, thus reaching the fifth type. Then, the *antum* arises and the thecal cells start to appear and protrude. Thus, begins the formation of the layer around the granulosa cells of the oocyte. Therefore, most follicles are observed at early stages of development. At the antral stage, follicles become gonadotropin dependent and form large antral follicles, most of which undergo atresia, and few are selected for ovulation. Estrogens

produced from the ovary of vertebrates, which have a fundamental endocrine function, leads the females to develop reproductive organs, while the corpus leteum produces progesterone, which is essential for the foundation of pregnancy. Thus, these functions are tightly coordinated during folliculogenesis, in which a dynamic process includes a continuous differentiation of three types of cells, theca of granulosa cells, and the oocytes themselves. The antrum formation from the granulosa cells proliferate, which is directly related to the follicular growth leading to a restricted number of follicles to complete their development to the stage of ovulation, while others undergo atresia. In the stage of antral follicle, theca cells are divided into two layers, internal and external, which can be distinguished by the surrounding selected follicles [28]. After ovulation, which is triggered by a peak of LH, theca cells and mural granulosa cells luteinize to produce progesterone (**Figure 1**). Once an ovarian follicle increase from the pool of resting follicles and initiates growth, it takes approximately around 100 days to arrive to the point where ovulation can execute and the oocyte included within the follicle is released [29].

2.3. Oocytes morphology and classification

In vitro maturation (IVM) is the starting point of a whole lot of biotechnological applications in animals like *in vitro* fertilization (IVF). These techniques combined with marker-assisted selection at embryonic stage will hasten acceleration for improving the production potency of cattle. The selection of oocytes depends only on the good quality cumulus-oocytes complexes (COCs), which were selected based on cumulus cells and ooplasm characteristics. The good oocytes are surrounded with at least three layers of cumulus cells and their ooplasm should be dark and homogeneous. Therefore, the oocytes were classified into four classes: (1) Grade A: oocytes were characterized with more than five

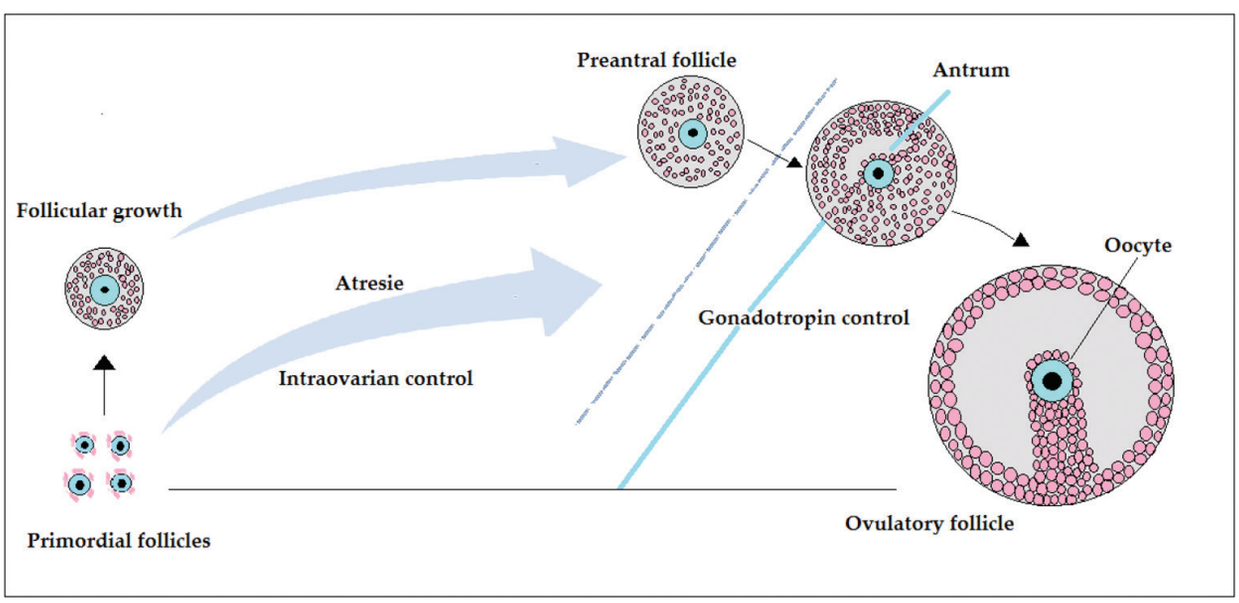

Figure 1. Outline of the main steps of folliculogenesis.

complete layers of cumulus cells, which are uniform granulation of ooplasm and healthy follicle. (2) Grade B: follicles with intact and well-organized granulose cells with three to five complete layers of cumulus cells and uniform granulation of ooplasm. (3) Grade C: follicles with one to two complete layers of cumulus cells and uniform granulation and less regular of ooplasm with some dark area. (4) Grade D: denuded oocytes with uniform granulation of ooplasm [30].

2.4. Oocyte maturation

Immature oocytes begin to develop in the ovaries, with each oocyte possessing a large nucleus that is referred to as the germinal vesicle (GV). Therefore, immature oocytes start to give response and undergo maturation process, which starts the nucleus of oocyte to have disassembly during a sequence called germinal vesicle breakdown (GVBD). After this stage, the immature oocyte turns into mature being eligible for developing until fertilization. Moreover, regardless of the timing of GVBD relative to fertilization and at the end, all oocytes must be matured enough to be able to consequently develop to continue to proceed normally [31]. During dictyate stage of prophase I, the oocytes are arrested, which can be identified by the presence of a germinal vesicle (GV). Additionally, meiosis I is marked by germinal vesicle breakdown (GVBD) following which bivalents are brought to alignment at the spindle equator by metaphase I. Anaphase I then ensues when chromosomes segregate between the secondary oocyte and the polar body. Following first polar body extrusion (PBE), oocytes progress without a hiatus into meiosis II where they are arrested for a second time at metaphase II [32]. During the maturation process, mammalian oocytes accompany a comprehensive, extensive rearrangement of the cytoskeleton and associated proteins. In oocytes, during the MI takes nearby 6-11 hours and the spindle migration toward the egg cortex occurs at this time. When the chromosome-spindle complex moves to the egg cortex, it involves a spindle pole close to the cortex [33]. Spindle movement induces a cortical differentiation performed by the accumulation of actin filaments and a scarcity of microvilli. After polar body extrusion, chromosomes realign progressing to metaphase II.

2.4.1. Nuclear and cytoplasmic maturation

The oocytes for bovine with an inside zona diameter smaller than 95 μm are unable to start meiosis *in vitro*. A high percentage of bovine oocytes are able to start meiosis to the MI stage once the oocyte diameter is at least 100 μm. However, the oocyte must measure 110 μm or more to reach the MII stage. The ability to develop to the blastocyst stage *in vitro* increases with oocyte growth. Cleavage and blastocyst rates increase in parallel with meiotic competence and significantly higher developmental rates have been obtained when the diameter of fertilized oocytes is greater than 120 μm. The developmental potential is apparently similar in oocytes originating from nonatretic and early atretic follicles [34], including spindle transfer, chromosome condensation, germinal vesicle breakdown, the progression to MI, and separation of the homologous chromosomes with polar body extrusion, where the nuclear

membrane starts to fold, the nuclear pores disappear, and then the nuclear membrane undergoes fragmentation and rapidly disappears. It appears that nuclear maturation follows the same pattern *in vivo* and *in vitro*. Nuclear maturation involves changes in protein synthesis patterns. Bovine oocytes undergo marked changes in the patterns of protein synthesis after GVBD *in vitro* and *in vivo*, whereas oocytes that remain at GV stage have consistent protein synthesis patterns [35]. Meiotic competence is known as the ability of the oocyte to complete meiosis. Additionally, during follicular growth, the meiotic competence is obtained and is acquired progressively. Oocytes first acquire the capacity to undergo condensation of chromosome, germinal vesicle breakdown, and follicular growth and are desired to acquire the capacity to progress to MI and eventually acquire the capacity to reach MII. The ability to complete the MI to MII transition coincides with the achievement of full size and with the process of nucleolar compaction [36].

The diameter of the follicle increases depending on the number of Golgi apparatus present in the oocyte. The change in location of cortical granules constitutes the most obvious ultrastructure sign of cytoplasmic maturation. According to the oocytes in the GV stage, cortical granules are distributed in clusters throughout the cytoplasm. However, as the oocytes progress to metaphase I stage, the cortical granules translocate to the periphery of the oocyte and become attached to the plasma membrane. At the end of the maturation period, when these oocytes reach the MII stage, the granules are distributed through the inner surface close to the plasma membrane [37]. The mitochondria make a homogeneous distribution throughout the cytoplasmic and are more common at the germinal vesicle (GV) stage, while heterogeneous distribution is more commonly observed in the oocyte of metaphase I or II. During oocyte maturation, the mitochondria disperse distribution throughout the cytoplasm, until reaching metaphase II (MII), when the central position in the cell operates in the mitochondria because high-energy supply around the nucleus is very important during embryonic development. It is also observed that morphologically poor quality embryos are only characterized by the homogenous distribution of the mitochondria [38]. Additionally, the cytoplasmic maturation describes both the ultrastructural changes that take place in the oocyte from the germinal vesicle (GV) to the metaphase II (MII) stage and the possession of developmental competence of the oocyte. Mammalian oocyte's cytoplasmic maturation can be described as the ability of a mature egg to undergo regular fertilization stage, all stages of cleavage, and further development of the blastocyst. Other indirect morphological parameters considered to evaluate cytoplasmic maturation include cumulus cell expansion, polar body expulsion, and increased perivitelline space (PVS) of Ref. [39].

3. RNA synthesis and molecular maturation

The molecular maturation coincides with the maturation and growth of oocyte corresponding with transcription and mRNAs expression by genes of oocytes. In mammalians ovaries, meiosis occurs mainly in two steps: during foetal life (step 1) and through the period of preovulatory life (step 2). The genetic information must be stored in advance because of nuclear instructions, when the chromosomes are the subjects of transformation. Thus, in the

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form of mRNA, the chromosomes arrive and are silenced at the resumption of meiosis. After DNA synthesis at double helix, the chromosomes are partially condensed and rearranged by the process of crossing over. The chromatin then reaches a special conformation that is an intermediate between chromatin condensation and interphase, the dictyate phase. Additionally, in some species during that special prophase period, the oocyte remains static and the chromosome appearance changes little. When an oocyte begins to grow in the primordial follicle, the uncondensed loops of chromatin in the dictyate state ensure the transcription of required elements. Thus, the mRNA produced is either translated immediately [40]. Through the *in vivo* and *in vitro* maturation, oocytes can have the ability to elucidate the signaling pathways by gene expression. These signaling pathways are involved in the intricate mutual among the oocyte and its somatic compartment through morphogenetic and differentiation processes, and the origin of disturbances in oocyte maturation is theoretically involved in the decrease of the fertility; therefore, molecular maturation of oocytes is characterized by enhanced nucleolar activity and ribosome synthesis, and when the oocyte reaches the meiotic phase, the nucleolus becomes inactivated [41]. During the meiotic phase, for oocyte maturation, large numbers of macromolecules are accumulated. The mRNA and riRNAs produced in these cells are far in excess of those necessary to support protein synthesis. Thus, concomitantly the onset of germinal vesicle breakdown occurs after 6–10 h of IVM. In the course of IVM, *de novo* transcription strongly declines as determined by measuring the incorporation of [3H] uridine into RNA. In contrast to this finding, the incorporation of [3H] adenosine increased and showed a peak during the time interval of 6–10 h of IVM, parallel with the onset of germinal vesicle breakdown (GVBD) and translation. In the further course of maturation, only a moderate decrease of [3H] adenosine incorporation was observed. These findings indicate and betoken that during the time of germinal vesicle breakdown (GVBD) the translation is increased. Additionally, these operations were accompanied by polyadenylation of the mRNA, despite the decline of the transcription and accumulation of polyadenylated mRNA until MII [42]. As known, oocytes' growth normally undergoes cytoplasmic and nuclear maturation, and the bovine embryo has the ability to develop to the blastocyst stage, and it is important to clarify the contribution of the oocyte to the embryo quality. Currently, the most popular hypothesis is that specific mRNA and possibly some proteins are produced and added to the oocyte's stockpile in the last few days before ovulation, it is believed that molecular maturation represents the closest association with the intrinsic capacity of an oocyte to reach the blastocyst stage and probably beyond [43]. Gene expression patterns are responsible for the development of the early embryogenesis. Fertilization and the first zygotic cleavage involve major changes to paternal and maternal genome activation. Additionally, the function of genomes has regulatory mechanisms, including differential promoter activation, alternative RNA splicing, RNA modification, RNA editing, localization, translation and stability of RNA, expression of noncoding RNA, antisense RNA, and microRNAs. All of these mechanisms work together to produce the level of RNA for transcriptome functioning of an organism. Developmental embryonic stage is the first to rely on the stored maternal transcript, which is progressively exhausted untill the production of the embryo on its own transcripts, after shifting to the program for embryonic expression. Moreover, through gene expression patterns and RNA stability, oocytes and early embryos prior to the expression of embryonic activation are dramatically

different and it leads to significantly different from what is observed after the main outset of embryonic transcription. The start for transcription of embryonic genes execute at a specific species at a specific time point. In bovine embryos, it occurs at 8- to 16-cell stage [44]. When the immature oocytes are removed from follicle, they spontaneously will convert to mature oocytes. Oocyte maturation can have deficiencies and the incompetence of cellular machinery, frequently causing a failure in embryonic development following fertilization and lower implantation rates. Additionally, the cytoplasmic and molecular maturation of oocyte might fail to promote male pronuclear formation. Hence, it might increase the chromosomal abnormalities after fertilization, and these abnormalities may result in an incompetent embryo in cleavage [45].

4. Production of embryos *in vitro*

There are many reasons for interest in *in vitro* generation embryos that can be produced in the laboratory. Among these reasons is the faster propagation of superior germplasm in cattle because of the low efficiency of superovulation (SO) and embryo transfer (ET) programs. *In vitro* embryo production (IVEP) consists of four steps: (1) aspiration of the ovaries, (2) *in vitro* maturation (IVM) of the recovered oocytes, (3) *in vitro* fertilization (IVF) of the IVM oocytes, and (4) *in vitro* culture of the IVM/IVF zygotes for development to the desired stages [46]. To evaluate the effect of oocyte source (live animals and abattoir ovaries) on subsequent embryo development in cattle, it is essential to perform IVEP from cow ovaries. The beginning of harvest of the oocytes from bovine ovaries and the cumulus-enclosed oocytes (COCs) suitable for IVEP were *in vitro* matured (IVM), fertilized (IVF), and cultured (IVC) to the tight morula (Tm) and blastocyst (Bl) stage. A higher overall IVEP efficiency is mainly related to the higher cleavage rate. Moreover, the production of embryos *in vitro* is a technique largely used as a method for increasing the production of progeny in the bovine with high genetic meritocracy, overcoming several of infertility problems in cattle, genetic recovery, and production of clones and transgenic animals. Despite several studies in this area, the *in vitro* development of cattle embryos to the blastocyst stage rarely surpasses the rate of 40%. Extrinsic and intrinsic oocyte factors can interfere with oocyte fertilization and development to the blastocyst stage. Among the intrinsic factors is the presence of the dominant follicle that, mainly through inhibin and estradiol secretion, has an inhibitory effect on the development of other follicles. Furthermore, they reported that oocytes derived from subordinate follicles have less capacity to become healthy embryos than oocytes derived from growing follicles [47].

In **Figure 2**, it can be observed different stages of embryonic development from day 0 to day 7, monitored by our team. Following fertilization, embryos undergo a series of mitotic cell divisions. Hence, the embryo compacts to form a morula that comprises of cells in a compact cluster including the pellucid zone (i.e., comprising of glycoproteins envelope of mammalian oocytes, which beset the embryo). Then, the blastocyst is formed and finally "hatches" from the zona pellucid. Besides, in humans, all this process takes about 1 week [48], and in cows, it can take up to 10 days.

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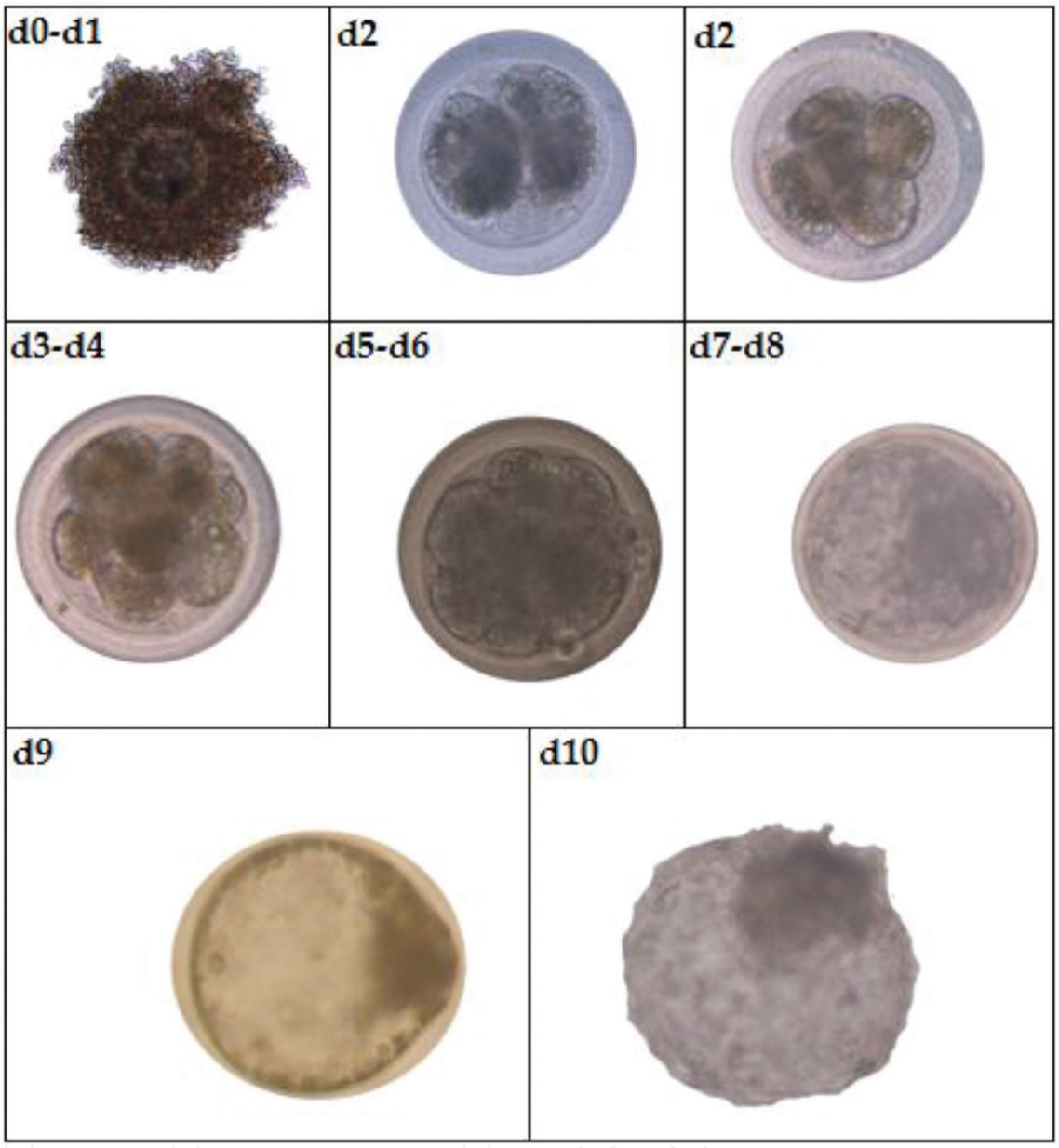

Figure 2. Stages of embryo development (photos made by the authors).

5. Effects of heat stress on animal fertility

During summer, heat shock reduces pregnancy, conception rates, and leads to low fertility in lactating dairy cow. Progesterone secretion by luteal cells is decreased and this is reflected in plasma progesterone concentration, the endometrial function, and alters its secretory activity by high temperature, which leads to the termination of pregnancy. Moreover, heat shock impedes oocytes quality and embryo development and increases the mortality rate of embryos [49]. Therefore, there are different factors that lead to the decrease of fertility in cattle. The most important are the increased humidity and temperature that result in a decreased expression of overt estrus and a reduction in appetite and dry matter intake.

During the postnatal period "puerperium," the exposure to extreme heat stress prolonged, leading to negative energy balance and increase in the calving pregnancy interval. Heat stress influences by decreasing the dominance of the selected follicle and this decreases the steroidogenic ability of its granulose cells and theca cells, which declines the estradiol concentrations in blood. The elevation or reduction of the plasma progesterone levels can be relied on whether it is severe or chronic, and on the cattle metabolism state. The reduction of follicular activity and the alteration of ovulatory pattern execute endocrine changes, leading to the reduction of oocytes and the quality of embryos [50]. Moreover, oocyte's maturation from the meiotic stage of germinal vesicle (GV) to MII is affected by heat stress and it has been shown that high temperatures affect also the cattle cumulus-oocyte complexes (COCs), zona pellucida hardening, fertilization, and further cleavage of putative zygotes after fertilization [51]. In cattle, genes exist for regulation of body temperature and for cellular resistance to elevated temperature. These genes offer possibility for their incorporation into dairy cow through cross breeding or on an individual-gene basis and the physiological and genetic manipulation of the cow to improve embryonic resistance to high environmental temperature, which reduces fertility in lactating dairy cows, and as a result, pregnancy, oocyte, and early embryo are affected by heat stress [52]. Therefore, heat stress reduces gonadotropin secretion and the ovarian pool of oocytes and impairs fertility. Additionally, heat stress has immediate effects on follicle and its enclosed oocyte, follicular function, and follicular growth and disrupts steroidogenesis [53]. Heat stress has an impact on the reproductive function, that is, it reduces the intensity of the behavioral estrus and leads to low fertility in female and compromised sperm output and increased sperm abnormalities in male. The pregnancy rate and the embryos at earlier stages of development during heat stress are affected [54]. Furthermore, oocyte susceptibility to heat shock can be detected during the germinal vesicle (GV) and oocyte maturation periods. The bovine oocytes' exposure to high temperature *in vivo* and *in vitro* affects oocyte maturation, fertilization, and preimplantation of embryonic development. This heat-induced reduction in oocyte function occurs due to a series of cellular alterations that affect nuclear and cytoplasmic compartments of the bovine oocyte. Before ovulation, there are several physiological factors that can disrupt establishment of pregnancy changes by elevated thermal heat. Heat shock effects endometrial prostaglandin secretion, oocytes during maturation period, and decreases low fertility of dairy cows in hot seasons [9]. Moreover, heat stress was suggested to be similar to oxidative stress, because of correspondences in the genes expressed after heat exposure, such as genes encoding heat shock proteins (*HSPA14* gene), demonstrated heat-induced increase of reactive oxygen species (ROS) production, especially the superoxide anion. Additionally, heat stress disturbs protein synthesis, increasing the production of heat shock proteins (HSPs). Due to their chaperone function, HSPs ensure the folding, unfolding, and refolding of nascent or stress-denatured proteins. The HSP70 and HSP90 were correlated with the development of thermotolerance of embryos. Thus, heat stress was shown to increase HSP70 and HSP90 levels and that probably due as a mechanism of cellular defense and/or repair [55]. Dairy cows respond to heat stress in several ways, including reduced feed intake, lower milk yield and quality, and compromised fertility [56]. Thus, fertility traits in dairy animals show a very low heritability value, and this indicates that most of the variations in the fertility are determined by nongenetic factors or environmental effects. Therefore,

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summer season has been associated with reduced fertility in dairy cattle through its deleterious impact on oocyte maturation and early embryo development. Moreover, the highest pregnancy rate of cow was observed in September–November as 32% while the lowest pregnancy rate of 24% in March–May. The lower pregnancy rate is due to the delay of rebreeding cows in the summer hot months with a high level of heat stress. Additionally, there are some other genes such *ATP1A1* allowing resistance and adaptation to thermal stress in bovine. Whereas *ATP1A1* gene is known as Na+/K+ -ATPase subunit alpha-1, this gene is fully recognized as a nominee to responsd for heat shock because of its assembly to oxidative stress in bovine which have Na+/K+ -ATPase protein complex consisting of α and β subunits. The *ATP1A1* gene encodes the α1 isoform, for α subunit of Na+-K+ ATPase pump is considered as a major isoform. *ATP1A1* gene has been mapped on *Bos taurus* chromosome number 3 and is comprised of 22 introns and 23 exons. *ATP1A1* gene is responsible for the fundamental establishment of the electrochemical gradient of Na+ and K+ across the plasma membrane, which is fundamental for maintaining body fluid and cellular homeostasis. During the elevation of body temperature "hyperthermia," heat stress activates heat shock transcription factor-1 and promotes the expression of HSPs coupled with reduced expression and synthesis of other proteins having a fundamental role to activate the immune and endocrine system. For understanding of genes in the regulation of heat shock response in animals would be helpful to improve their thermal tolerance via gene manipulation. Therefore, the *HSPA14* genes were found highly expressed in summer months in cattle, which enhance their thermotolerance and the ability to adapt to the thermal environment [57]. Genetic adaptation to heat stress for cellular resistance to elevated temperature reduced the levels of gonadotropin receptors, aromatase activity of granulosa cells, the follicular fluid concentrations of oestradiol, and follicular function involving changes at the level of the follicle or the secretion of the pituitary hormones that control development of the follicle. Additionally, oxidative stress is a major cause for thermal damage of spermatogenic cells and leads to apoptosis and DNA strand breaks. Moreover, apoptosis plays a critical role in effects of thermal stress on the maturing of oocyte in cattle. Therefore, the inhibition of apoptosis in bovine embryos with a caspase inhibitor increased the magnitude of the reduction in development caused by the elevated temperature. Thus, apoptosis is limited to the most damaged cells of the embryo. Heat shock caused damage to the oocyte during preovulation, which seems to involve the generation of reactive oxygen species, as both are effects of heat stress *in vivo* and heat shock *in vitro*. Furthermore, there are indications that developmental competence of the resulting embryo can be reduced if fertilization is by a spermatozoon exposed to heat shock. *In vitro* fertilization with sperm recovered from male in which the scrotum was heated to 42°C resulted in embryos with reduced ability to complete development [58]. In cows, heat shock has negative effects on competence of oocytes from antral follicles, granulosa, or theca cells, which are responsible for the production of steroids. Moreover, heat stress applied on bovine oocytes during *in vitro* maturation as well as on embryos during *in vitro* culture reduces both oocyte maturation and embryo development rates. The expression of HSP70 was not affected in the oocyte, but increased in cumulus cells. Therefore, the negative effects in oocytes because of heat shock could be mediated through the surrounding cumulus cells. Interestingly, higher expression of HSP70 in immature bovine oocytes was observed. This mRNA could be translated throughout the IVM and consequently decreases its expression

in mature oocytes [59]. Heat stress accelerates cellular metabolism, resulting in the inability of the mitochondria to properly reduce oxygen that remains in its radical state [5, 6]. Thus, the poor developmental competence might be due to the lack of cytoplasmic maturation in oocytes maturing *in vitro*, even though they undergo normal nuclear maturation. In addition to the presence of heat shock proteins, there are several antioxidants, for example glutathione (GSH) that partially imparts the protection against oxidative stress. GSH appears to be the main antioxidant defense system against reactive oxygen species (ROS) in oocytes/embryos [60].

6. Regulation of the cell cycle and oocyte maturation

Several mechanisms are involved in the activation of translationally inactive mRNA. These mechanisms involve the phosphorylation of many factors that initiate translation. Therefore, according to this model, polyadenylation (the addition of adenine) of the 3′ terminal portion of the cytoplasmic mRNA would stimulate the release of repressor molecules linked to the 5′ portion, thus beginning translation. The transport of this mRNA to the cytoplasm occurs through a characteristic splicing of the poly-(A) tail, which, after reaching the cytoplasmic compartment, becomes smaller and heterogeneous in size. The cytoplasmic elongation of the poly-(A) tail has been associated to the translation activation, meaning that during addition of adenine to mRNA in the cytoplasmic of oocyte through maturation leads to deadenylations, leading to the degradation of the particular mRNA. For this reason, protein synthesis starts when the two ribosomal subunits are linked onto the mRNA. This stimulates the degradation of particular mRNA which, when oocytes are acquiring developmental competence, the fundamental transcripts produced encode regulators of the cell cycle such as "maturation promoting factor" (MPF), the protein of the c-mos pro-oncogene (MOS), and mitogen-activated protein kinase (MAPK) [37]. In mitotic cells, S-phase always precedes M-phase in order to maintain euploidy. Additionally, the maturation (M-phase) promoting factor (MPF) plays a pivotal role in oocytes during their maturation. During the mitotic cell cycle, MPF activity shows different stages of vacillation and steadiness, i.e., MPF activity wobbles positively and negatively in time with the beginning and ending of M-phase in succession, while being precisely regulated during the two cell divisions of meiosis. MPF is a heterodimer protein kinase complex consisting of two subunits: the catalytic subunit CDK1 and the regulatory subunit cyclin B1. As phosphorylation is required by CDK1, cyclin B is the main determinant for CDK1 and this is a necessary factor for certain processes during cell cycle as (1) initiating germinal vesicle breakdown (GVBD) through phosphorylation of nucleoporin—a components of nuclear pore complex—among many protein components of nuclear envelope and (2) with a large protein complex termed as condensing that helps in supercoiling the DNA during mitosis. The kinase activity requires more than binding of CDK1 to cyclin B1. MPF, the heterodimer protein, is held in an inactive state termed as PRE-MPF by wee1 kinase that causes inhibitory phosphorylation of CDK1 ($p34^{cdc2}$). However, CDC25 activates CDK1 by dephosphorylation at the same sites. Although binding

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of CDK1 to cyclin B1 is necessary, it is not sufficient for kinase activity. Switching on MPF in all cells is further governed by the balance in the regulatory activity of Wee1/Myt1 kinases, which cause an inhibitory phosphorylation of CDK1 ($p34^{cdc2}$) (and hold the heterodimer in an inactive state called pre-MPF), and the CDC25 phosphatases, which cause activation of dephosphorylation of CDK1 at the same sites. Thus, the high CDC25 and low Wee1/Myt1 activity are needed for switching on the CDK1 component of MPF. In addition, before entry into mitosis, cyclin B1 (and so MPF) is spatially restricted to the cytoplasm, through a cytoplasmic retention sequence, containing a nuclear export signal. When cells commit to mitosis, cyclin B1 has to become phosphorylated within its cytoplasmic retention sequence, leading to rapid accumulation of cyclin B1 and MPF within the nucleus, and ensuing GVBD [61]. In the mammalian oocytes during the meiotic maturation, two consecutive divisions execute without an intermediate phase of DNA replication where even haploid gametes are produced. In addition, these two divisions are asymmetrical, maintaining and ensuring the maximum number of oocytes possessed for maternal stores. In addition, immature oocyte contains only a small amount of cyclin B, just enough to induce entry into the first meiotic M-phase. Furthermore, MPF activity is regulated by a translation-dependent mechanism that determines the level of cyclin B1 synthesis. Among the different mechanisms that control the expression of maternal mRNAs, polyadenylation has been implicated in cyclin B1 translation in Xenopus and mouse oocytes. The immature oocyte contains only a small amount of cyclin B1, just enough to induce entry into the first meiotic M-phase increases progressively, reaching its maximum at the end of the first meiotic M phase, and the newly synthesized protein becomes associated immediately with the $p34^{cdk1}$ kinase to form an active complex. Cyclin B degradation is required for polar body extrusion. Changes in cyclin B1 levels, through changes in MPF activity, regulate not only the timing of the cell-cycle phases during meiosis but also the orderly events leading to the formation of functional meiotic spindles and asymmetric divisions, MPF activity controls the formation of a functional spindle in the oocyte. The MPF activity required for GVBD (sufficient for entry into M-phase) only allows the formation of a single aster of microtubules around the condensed chromosomes. A first threshold in MPF activity is then required to organize the microtubules into a bipolar structure. In contrast, the further migration of the chromosomes toward the vicinity of the spindle equator does not depend on changes in the MPF level [62]. The meiotic maturation is an essential process for the development of an immature oocyte into fertilization egg. In vertebrates, it is compatible with the transition from the prophase arrest of the first meiotic division to the metaphase arrest of the second meiotic division. Thus, MPF is activated in response to the hormonal signal. Therefore, steroids bring about meiotic maturation through functionally redundant pathways involving synthesis of Mos or of cyclin proteins. The c-mos pro-oncogene (MOS) protein is a Serine/Threonine kinase, which is specifically expressed in germ cells where it functions only during the short period of meiotic maturation from maternal mRNA in vertebrates before being proteolyzed at fertilization. Although it is not necessary for GVBD, Mos remains a powerful inducer of meiotic maturation when microinjected. Two roles could be envisioned for Mos in the process of GVBD induction: (1) The mRNA coding for Mos could, intrinsically or via associated proteins, affect the translation of other mRNA. (2) Synthesized Mos kinase provokes a

strong pathway that most certainly participates in the amplification of MPF, as well as being necessary for normal GVBD events (for the repression of DNA synthesis) [63]. Mammalian oocytes reach the prophase diplotene stage of meiosis I before or after birth, and they remain arrested at this stage until resumption of meiosis, characterized by germinal vesicle breakdown (GVBD) that follows preovulatory gonadotropin stimulation after puberty. Thus, family of serine and threonine kinases is activated in the somatic compartment of the follicle. Intra oocyte MAPK cascade activation is more closely related to post-GVBD events such as meiotic spindle organization. Stimulation of meiosis resumption by activation of MAPK can be accomplished by prompting synthesis of downstream meiosis resumption inducing factors. Additionally, the phosphorylation of gap junctional proteins blocks communication between oocyte and around somatic cells, and subsequently prevents the meiosis from inhibiting signals from entering into oocytes from the vicinity of somatic cells. Lutropin causes activation MAPK in follicular somatic cell, which have fundamental role, which in turn phosphorylates connexins, leading to a reduction in gap junction permeability between the somatic cells prior to germinal vesicle breakdown. The suppression of gap junction communication is enough and essential for reinitiating meiosis. Mos kinase is a universal mediator of oocyte meiotic maturation and is produced during oogenesis and destroyed after fertilization. Thus, mos limits the number of meiotic division to just two rounds (meiosis I and II) and to avoid from ingress into meiosis III. Strikingly, the maintaining of Mos/MAPK pathway activities after fertilization close to physiological levels prompts additional rounds of meiosis, and the spindle is positioned symmetrically resulting in further rounds of asymmetric cell division [64]. Additionally, in the mammalian oocytes the meiotic maturation is described by special asymmetric cytokinesis through chromosomes moving from the center toward the cortex zone of an oocyte. After GVBD, a meiotic spindle congregates surrounding centrally positioned metaphase chromosomes and then moves in the vicinity of the cortex in an actin filament relying on the process. Furthermore, cortical reorganization executes an ectopic actin-rich actin cap and a cortical granule-free domain (CGFD) formed in the vicinity of the cortex devoid of microvilli in a MOS-dependent manner. Establishing cortical polarity, including spindle migration, positioning and cortical reorganization is critical for oocyte asymmetric divisions. Then cytokinesis occurs and the polar body extrudes, forming a high-polarized MII oocyte [65]. Additionally, in humans, oocyte growth unfolds over 110–120 days, during which cell mass undergoes an astounding, more than 100-fold, increase and cell diameter shifts from <40 to ~120 mm. At the same time, the macromolecules and organelles are produced and stored in very large amounts. In such a way, the oocyte meets a fundamental for preimplantation embryo developmental growth, storing enough for cytoplasmic mass desired for fertilized egg to accomplish multicellularity without the presence of net growth. Remarkably, the oocyte growth is much more than a quantitative increase in cell mass finalized to the housekeeping needs of the early embryo. For example, maternal effect genes have been characterized as sequences whose transcriptional and translational products are generated during oocyte growth and whose regulatory function is required only after fertilization. In particular, many maternal effect gene products are transcriptional regulators whose loss of function causes major developmental failures, such as arrest at cleavage or blastocyst stages, or inability to activate the zygote genome [66].

7. Regulation of mammalian oocyte gene expression at transcription level

There are six Obox (oocyte-specific homeobox) family transcripts and Obox-1, 2, 3, and 5 mRNA they have been detected in oocytes from growing primary follicles [67], playing then an important role in early embryogenesis [68, 69] by orchestrating gene transcription, either ubiquitously or in a tissue-specific manner. Mice lacking the Obox6 gene grow without morphological abnormalities and with normal fertility, indicating a functional redundancy among the Obox family members [70]. Moreover, several genes play key roles in oogenesis, folliculogenesis, or early embryonic development. In particular, GDF-9 and BMP-15 are necessary for folliculogenesis beyond primary follicles in mouse and sheep, respectively. Gene expression in oocyte is quite different from those in somatic cells. The messenger RNAs produced by these cells are not only required to support germ cell development but, in the case of oocytes, they are also used for maturation, fertilization, and early embryogenesis. It is very important to understand the oocyte mechanisms and transcription factors that play a role in the regulation of the transcriptional activity of the oocyte dictating its ultimate acquisition of developmental competence. The oocyte genome has evolved specialized transcription machinery to ensure proper activation of gene that is required for oocyte growth and early embryonic development [71].

8. Gene expression and the role of the *Cx43* and *HSPA14* genes during the embryonic development

8.1. The *HSPA14* gene

Experiments developed by our team show that exposing cumulus oocyte complexes (COCs) to 41°C did not alter the number of embryos that cleaved but reduced significantly the percentage of development in the blastocyst stage [9]. Additionally, the exposure of bovine embryos to heat shock during oocyte maturation leads to embryos with reduced development and induced alterations in protein synthesis and possibly gene expression as early as the two-cell embryos. In addition, cumulus cells are removed before maturation, which affects and reduces the protein synthesis at 42°C exposure oocytes and COCS. On the other hand, the developed oocytes at 39°C created heat shock protein 70 kDa but oocytes exposure to 42°C did not increase synthesis of any of these proteins, which was shown after examining the methionine- and cysteine-labeled proteins—by two-dimensional SDS-PAGE and fluorography. It has also been noticed that the reduction of protein synthesis caused a prominent decrease in the percentage of protein synthesis in oocytes with intact cumulus compared to those bared oocytes. Therefore, the heat shock increases the steady-state amounts of mRNA for the inducible form of heat shock protein 70 (HSP70) in embryos. The *HSPA14* is the most abundant, highly sensitive to culture environment and it is the major inducible heat shock gene important for protecting embryonic cells from cellular stress in bovine. Thus, HSP70 mRNA in 2- and 4-cell embryos was increased by exposure to 42°C. As findings indicate that the experience for embryos may undergo the transcription of heat shock exposure as early in

2-cell stage, the rates of cleavage and early development are reduced by transcription inhibitors, which show the significance of transcription on the earliest period of fetal life development [72]. The expression of the major heat-inducible protein HSP70 protects cells from a self-destruction known as apoptosis. Cells can also respond to stress by adaptive changes that increase their ability to tolerate normally lethal conditions. The expression of *HSPA14* gene would allow to produce and help maintain cellular function by acting as molecular chaperones to stabilize or refold proteins damaged by heat, and by blocking apoptosis by preventing cytochrome c release from mitochondria during the early stages of apoptosis [73]. Moreover, these authors postulated that the *HSPA14* during embryo development with the presence of antibodies to HSP70 significantly decreases progression to the hatched blastocyst stage in murine embryos. Correspondingly, antisense oligonucleotides complementary to HSP70 mRNA had a similar effect on embryo development, which was amplified ninefold by arsenic exposure at a subtoxic dose. Hence, the embryos that did reach the blastocyst stage despite the inhibition of HSP70 expression, with and without the presence of arsenic, were characterized as degenerate with cell death accompanied by membrane blebbing. Thus, suggested that the requirement for HSP70 during embryo development is amplified by exposure to adverse environmental conditions [12]. *In vitro* matured bovine oocytes, 2-cell and 8-cell embryos, and day 9 hatched blastocysts subjected to control and elevated temperature conditions were analyzed by semiquantitative reverse transcription polymerase chain reaction methods for HSP70 mRNA expression. In the cytoplasm of 8-cell embryos that were inferred under control conditions, HSP70 was evenly disseminated in the cytoplasm but appeared as aggregates in some embryos exposed to elevated temperature. The hatched blastocysts show a competence to react to elevated temperature that is shown in the increased distributions noted after heat stress [74]. As described before, several authors pointed out that heat shock protein can be induced by bovine embryos exposed to heat shock. Those bovine embryos, presented to heat shock, also produce other cellular changes to make cell more impervious to a subsequent heat shock. The 2-cell bovine embryos can deliver HSP70 in response to raised temperature although it is sensitive to heat shock. Therefore, to test for induced thermotolerance, 2-cell bovine embryos were first exposed to a mild heat shock 40°C, allowed to recover at 38.5°C and 5% CO_2. Subsequently, the way in which HSP70 take part in embryonic development was examined at two different temperature, 38.5 and 40°C, culturing embryos with a monoclonal immunoglobulin to the inducible form of HSP70 experiment, a reduction of the proportion of 2-cell embryos ($p < 0.05$) by adding anti-HSP70 to the culture medium. Thus, bovine 2-cell embryos appear incapable of thermotolerance owing to the increased sensitivity of 2-cell embryos to heat shock as compared to embryos at later stages of development. The results also implicated a role for HSP70 in normal development of bovine embryos. Studies developed by Mayer and Bukau [75] showed that bovine 2-cell embryos appear incapable of thermotolerance owing to the increased sensitivity of 2-cell embryos to heat shock as compared to embryos at later stages of development. The results also implicated a role for HSP70 in normal development of bovine embryos. The same authors [75] pointed out that one of the central components of the cell network of molecular chaperones are HSP70 proteins. An abundant array of protein folding processes are assisted by HSP70 proteins in the cell through association transiently of segments of short hydrophobic peptide—within substrate proteins—with their substrate binding area. When substrate binding, it releases cycle which is

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driven by exchanging of HSP70 between the high-affinity ADP bound state and low-affinity ATP bound state. Hence, the chaperone activity of HSP70 proteins need hydrolysis and binding if ATP *in vivo* and *in vitro*. Cochaperones of the J-domain proteins family control this ATPase, which make their substrates targeted by nucleotide exchange factor and by HSP70s, decide the lifetime of the HSP70 substrate complex. This chaperone cycle is tweaked by additional cochaperones. The HSP90 and HSP100 among other chaperones couple with the HSP70 cycle for specific tasks. As a different understanding, Kampinga stated that several assortments of cellular internal and external cases of the stress can be distributed as proteotoxic stresses, which can be described as stresses that raise the main fraction of proteins that are in an unfolded state, thereby promoting the possibility of the formation of intracellular aggregates. These accumulations of assemble, whether not disposed, may lead to the apoptosis. In response to the appearance of denaturation and damaged proteins, cells promote the expression of heat shock proteins. These can have fundamental function as molecular chaperones to avoid protein from the assembling and to maintain the proteins in a state competent for either refolding or degradation. As *Bos indicus* cows usually have better reproductive performance in tropical and subtropical regions than *Bos taurus* cows, presumably due to their better adaptation to tropical environments, Camargo et al. [76] evaluated the developmental competence and expression of the HSP70 gene in immature oocytes from *Bos taurus* (Holstein) and *Bos indicus* (Gyr) dairy cows raised in a tropical region. The total RNA extracted from Holstein and Gyr oocytes and the HSP70 transcripts was conducted, which was formed by real-time PCR after reverse transcription. Cleavage and blastocyst rates were ultimately greater ($p < 0.05$) for Gyr breed ($n = 390$ oocytes) than for the Holstein ($n = 505$) breed (66.7% vs. 53.1% of cleavage and 19.6% vs. 10.8% of blastocysts, respectively), showing an adaptation of these two breeds to the environmental conditions. Studies developed by Virenque et al. [77] hypothesized that the high structural and functional conservation of HSP during evolution suggests crucial roles in fertilization, embryo development, and thus, fertility in cattle. Also, some HSP are considered housekeeping genes that are essential for many cell functions, such the HSP40 combined with HSP70 acting as chaperones to protect cells from apoptosis [78]. All HSP isoforms are ATP-dependent molecular chaperones as they protect nascent or denatured proteins from aggregation and assist their folding or refolding into native conformation and regulation of heat shock response.

8.2. The *Cx43* gene

Connexins (CXs) are a family of transmembrane proteins with molecular masses varying from 26 to 60 kD; *Cx43* has a molecular mass of 43 kD. In vertebrates, CXs are the building blocks of gap junction channels, intercellular channels that connect the cytoplasm of two neighboring cells. Wrenzycki et al. [79] examined the presence of the mRNA encoding connexin 43 (*Cx43*) in bovine embryos derived *in vivo* and *in vitro* and in the bovine embryos through morula and blastocysts grown *in vivo*, the transcription of the *Cx43* were disclosed. Conversely, when the early *in vitro* stages from cumulus oocyte complexes (COCs) to morula expressed *Cx43*, blastocysts and hatched blastocysts cannot have disclosed concentrations of mRNA from this gene as because it is not activated or if embryonic gene expression had been active, it terminated prematurely. The differences in transcription between bovine embryos derived *in vivo*

or *in vitro* indicate that culture conditions affect gene expression. Melton et al. [80] reported that the gap junction is a major form of cell–cell communication and aggregations of intercellular channels composed of connexins (CXs), which are responsible for exchange of low molecular weight (<1200 Da) cytosolic materials. In ovarian tissue of mammals, investigators have established that the presence of *Cx43* is to determine the ontogeny of early stages of follicular development of embryos. Additionally, it has been found that the preovulatory LH surge caused a decrease in *Cx43* mRNA in granulosa cells of rat ovarian follicles. Therefore, the results reported indicated that increased expression of *Cx43* temporally correlates with the activation of follicular development and early differentiation of granulosa cells in follicles of prepubertal pig ovaries. Vozzi et al. [81] indicated that in ovarian follicles, oocyte is provided by cumulus cells with small molecules that license control maturation and growth. The germinal cell gets these nutrients through gap junction channels, present between the oocyte and cumulus cells. Goldberg et al. [82] reported that the gap junctions, composed of proteins from the connexin family, are the only channels that directly connect the cytoplasm of the adjacent cells to allow for the intercellular transfer of small hydrophilic molecules, essential for proper development and health in animals and humans. Kidder and Mhawi [83] showed that the gap junctions are a group of intercellular membrane channels which leads to adjacent cells to participate in small molecules (<1 kDa). Gap junction channels are composed of connexins, a homologous family of more than 20 proteins. For developing of follicles, the gap junction is coupled with the developing oocyte and its besetment of the follicle cells into an essential functional syncytium. Among the gap junctions and cumulus cells includes the majority connexin 43, and this connexin has also been detected utilizing immunoelectron microscopy in a small minority of gap junctions at the surface of the oocyte. The significance of connexin 43 for granulosa cell having fundamental function is demonstrated by the fact that follicles lacking this connexin arrest in early prenatal stages and produce incompetent oocytes. Connexin 37 is shown to be the only connexin participate by oocytes to the gap junctions coupling them with granulosa cells, and loss of this connexin interferes with the development of the secondary follicle. The expression of multiple connexins in growth follicles probably indicate the multiple functions served by gap junctional correspondence in folliculogenesis. In the absence of *Cx43*, granulosa cells block growing in an early preantral stage. To illustrate the fundamental role function of *Cx43*, dye injection experiments detected that granulosa cells from *Cx43* knockout follicles are not coupled, and this was confirmed by ionic current injections. However, electron microscopy detected that gap junctions are quite rare in mutant granulosa cells. Conversely, mutant granulosa cells were eligible to form gap junctions with wild-type granulosa cells in a dye preloading assay. It was accomplished that mutant granulosa cells comprise a population of connexins, composed of nonrecognized connexins that cannot contribute regularly to participate in gap junctions. Additionally, despite *Cx43* being the only gap junction protein existing in granulosa cells of early preantral follicles, it is the only one that makes a significant contribution to intercellular coupling [84]. Research conducted by Veitch et al. [85] uncovered that studies of mammalian animals with targeted disruption of specific connexin genes have revealed that at least two connexins, connexin 37 (*Cx37*) and connexin 43 (*Cx43*), play essential roles in ovarian follicle development. Immunofluorescence microscopy located *Cx37* within gap junction plaques between granulosa cells and the oocyte, and *Cx43* between surrounding granulosa cells. Gittens et al. [86] reviewed that the intercellular

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communication is required for ovarian folliculogenesis. Yogo et al. [87] reported that the connexin 43 (*Cx43*)-intermediate gap junctional communication in granulosa cells is crucial for germ line development and postnatal folliculogenesis. These authors showed that follicle-stimulating hormone (FSH) induced the phosphorylation of *Cx43* in primary granulosa cells and then further specified to identify Ser365, Ser368, Ser369, and Ser373 in the carboxy terminal tail as the major sites of phosphorylation by FSH, and found that the phosphorylation of these remains was substantial for channel activity. Furthermore, Borowczyk et al. [88] evaluated the role of gap junctions in the regulation of progesterone secretion. The changes in *Cx43* mRNA expression were positively correlated with changes in progesterone concentration. They demonstrated a relationship between gap junctions and progesterone secretion that was supported by (1) the positive correlations between progesterone secretion and *Cx43* mRNA expression and gap junctional interacelluer communication (GJIC) of luteal cells and (2) the inhibition of *Cx43* mRNA expression resulted in decreased production of progesterone by luteal cells. This suggested that gap junctions may be involved in the regulation of steroidogenesis in the corpus luteum. Gershon et al. [89] reported that the gap junctions that allow the direct communication between cytoplasmic compartments of neighboring cells are present in a variety of tissues and organs, allowing thus the rapid exchange of ions and metabolites. Besides in the bovine, follicles express *Cx43,* which is localized to granulosa cells from the primary follicle and increases upon antrum formation being necessary for follicle development and oocyte growth. The expression specificities of *Cx43* in ovary are still in discussion by several groups. During folliculogenesis, the granulosa cells give physical support and mediate signal between different types of follicular cells. Rhett et al. [90] reviewed that the *Cx43* is the most ubiquitous connexin, with expression in at least 46 different cell types being a gap junction protein widely expressed in mammalian tissues that mediates cell-to-cell coupling. Intercellular channels comprising GJ aggregates form from docking of paired connexons, with one each contributed by opposing cells. The regulation of gap junction coupling is a necessary component of cellular function and response to physiological and pathological stimuli. The cells can modify the expression, phosphorylation state, and protein interactions of *Cx43* throughout the cell cycle. Because of the short half-life of *Cx43*, which is as little as 1–2 h, regulation appears to exist on both short and long time scales through phosphorylation and protein interaction, and gene expression, respectively. Currently, there is a great deal of data on phospho- and transcriptional regulation of *Cx43* but less is known about the mechanistic basis and function of *Cx43* protein–protein interactions. Furthermore, concerning the *Cx43*, normally low expression of *Cx43* in different development stages of embryos has been associated with low quality and reduced survival capacity of embryos [91, 92].

9. Methods for studying differential gene expression

Several techniques have been developed for the screening of genomic alterations at the mRNA level, including subtractive hybridization, differential display-PCR, expressed sequence tag (EST), serial analysis of gene expression (SAGE), and microarray hybridization. Some of these techniques have been used to investigate changes in gene expression at oocyte cell

development and differentiation [93]. In addition, the effective and simple methods for identifying and isolating those genes that are differentially expressed in various cells or under altered conditions. Advantages of the technique include the ability to isolate genes with no prior knowledge of their sequence or identity and the use of common molecular biology techniques that do not require specialized equipment or analyses, its abilities to compare multiple experimental samples for any number of treatment conditions, phenotypes, or genotypes simultaneously, and to identify genes that are either up- or downregulated in one sample relative to another [94]. The differential display include as first step is reverse transcription of mRNA to cDNA using one of anchor primers (which is usually poly T oligonucleotide with one or two additional bases, e.g., T_{12} AC) designed to anneal to the 3′ poly-A tail of messenger RNA (mRNA). The resulting of cDNA species is subsequently used as templates in a PCR, utilizing the same anchor primer from reverse transcription (RT) reaction in combination with an arbitrary primer. The PCR products may be labeled by incorporation of a radiolabeled nucleotide fluorescently labeled primer. Electrophoresis pattern can be then evaluated by comparing the relative intensities of bands produced from different treatment samples. Bands that are present in one sample and absent in another or bands that are present at different relative intensities, across different treatment, represent potentially differentially expressed mRNA transcripts. The final phase of differential display is excising and purification of the band of interest from the polyacrylamide gel and reamplification by PCR, and eventually subjected to sequencing.

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