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CD44 GENE EXPRESSION IN MATURE, IMMATURE OOCYTES AND FETAL KERMANI, BALUCHI SHEEP AND RAYENI, TALI GOATS

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ABSTRACT

Family CD44 is belonging to a large group of binding proteins to hyaluronic acid. It has important role in oocyte maturation, fertilization and embryo development. We analyzed the CD44 in oocytes and embryos goat and different breeds of sheep. We used both of kermani and balouchi breeds of sheep, two rayeni and tali goat. They kept domestic animals for 3 months, and then gave embryos and oocyte. After this animals were cured by hormone progesterone for 14 days in vaginal after exiting of that from vagan injection GNRH hormone that animals were ready for sampling after 1 day After that for exiting mature oocyte from animals uterus enter to slaughters and that group used from animals that for product embryos by keeping male animal beside them this work did. After 6 days this animal entered slaughters. We gave embryos from them. another way ovary collected from slaughters and by exiting immature oocyte, laboratory ways produced immature and mature oocyte. So embryos by keeping sperm in environment condition producted culture embryos. Sampling production for exiting RNA they used by kit way after that for production cDNA used special protocol. The production produced by conventional PCR and Real time PCR was studied. The Result showed that gene expression didn't exist in immature oocyte sheep. In tali goat expression of this gene was more than Rayeni goat.

Keywords: CD44 Gene, Gene Expression, Real Time PCR, Goat, Sheep

INTRODUCTION

The communication between the oocyte and the granulosa cells surrounding is crucial for the acquisition of oocyte competence **(3)**. One way of oocyte and cumulus cells communication is characterized by the secretion of several growth factors such as glycosaminoglycans (GAGs), which play an important role in proliferation and differentiation of a variety of cell types **(25)**. Among the GAGs, hyalurunic acid (HA) is a high molecular weight polysaccharide found in the extracellular matrix of most animal tissues and is one of the most abundant GAGs in the uterine, oviductal and follicular fluids in woman **(23)**, mouse **(4)**, sow **(2)**, and cow **(18)**. During the process of ovulation, cumulus cells secrete HA **(22)** that actively participates in processes of cytoskeletal modification, gap junction losses that accompany cumulus expansion in cumulus-oocyte complexes (COCs) and oocyte meiotic progression **(1)**. The expansion of cumulus cells may be positively correlated to the ovulation, fertilization, and subsequent zygote development **(13)**. The degree of cumulus cells expansion is often cited as a major indicator oocyte selection for *in vitro* fertilization protocols **(32)**. It has also been demonstrated that HA delays death and prevents fragmentation of porcine oocytes **(29)** and plays a role in cell migration during the early embryonic development **(21)**. Moreover, HA added to the culture medium supports the development of 1- and 2-cell porcine embryos **(19)** as well as improving *in vitro* bovine embryo development to the blastocyst stage **(9)**. HA mainly binds to CD44, which is a glycoprotein widely expressed on the surface of many mammalian cells. CD44 exists as multiple isoforms expressed in a specific manner for different cell types. These isoforms result from splicing and post-translational modifications, where they can be differently glycosylated **(14)**. Cell surface glycoprotein CD44 is present in mature oocytes and preimplantation embryos in some species of mammals such as in humans **(21)**, bovine **(20)**, porcine **(28)**, and mouse (**30)**. Furthermore, CD44 was not detected in immature oocytes in porcine **(33)**, bovine **(10)**, mice **(8)**, and humans (**5)**. These data indicate that CD44 is expressed during the maturation process suggesting its importance in this phase. It is reasonable to assume that HA profile is directly proportional to the amount of CD44 in somatic cells surrounding the growing oocyte.

The purpose of this study is to investigate whether this gene in immature oocytes, embryos rhetoric and teach goats expression in which the expression occurs.

MATERIALS AND METHODS

Natural Ways to Collect Mature Oocytes

This procedure was performed as follows: First, plan synchronize To create an estrous cycle in the vagina with a sponge containing 60 mg medroxyprogesterone acetate for 14 days, Estrus 24 h after sponge removal using rams were identified, 90 hours after the slaughter of sheep and uterus extracted sheep were placed in the vagina. Another syringe was then vacuum the liquid into the lab, the fetus was confirmed under a microscope with a magnification of 10 to 50X. Only the morula stage embryos. For goats in estrus synchronization using CIDR and injections were carried out for 14 h before CIDR removal was performed in 5/2 mL of GnRH were given 48-24 hours after CIDR removal of goats of heat, that for every 10 female goats, a mail goat was used for mating other procedures such as sheep.

In Vivo Embryo Production

Four hair ewes aging 1 to 5 years were submitted to the same hormonal treatment as Previous described. Additionally, females were mated at the beginning of estrus and 24 h afterward, using rams of proven fertility. Embryo recovery was performed by laparotomy six Days the first mating. Shortly, after genital tract exposure, each uterine horn was retrogradely with 20-40 mL DMPBS. Embryo quality and development stage were evaluated under a stereomicroscope at 10 to 50X magniication. Only embryos at the morula stage were subsequently used for indirect immunoluorescence or frozen (-80°C) for nested PCR.

RNA

RNA was extracted by using RNA Purification Kit, Method CDNA In this protocol, the addition of materials and reactions to a system of numbered and color-coded labels were showed. Replication method using (PARSGENOME MiR-Amp kit) included a three-step protocol.

Real-time PCR method

Replication method by using (PARSGENOME MiR-Amp kit). It included a three-step protocol; the cDNA amplification was conducted with two rounds of Real-time using PCR primers to increase the specificity and yield of the PCR product. The total 20-µL PCR product contained 2 µL reverse-transcribed cDNA, 0.5 IU GoTaq DNA polymerase (Promega), 0.2 mM of each dNTP, 2 mM MgCl2 and 0.2 µM of each primer **(Table 1)**. For both PCR, the amplification parameters consisted of an initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 50 s, annealing at 60°C for 50 s, and extension at 72°C for 50 s, and a final extension at 72°C for 6 min. For the Real-time PCR, 2 µL primary products were added to 18 µL

freshly prepared mix, as above. The amplified product was subjected to 2% agarose gel electrophoresis using 100-bp DNA ladder (Invitrogen) as a reference for fragment size and stained with ethidium bromide. In order to produce visible amplicons, the second round of PCR was not necessary for b-actin gene.

The primer was designed with multiple alignments of Homo sapiens (NM_001001391), Bos Taurus (NM_001009784)

RESULT

Quality and quantity of extracted DNA& RNA extracted from highly and free desirable from any contamination and after the bands were observed on agarose gel, using Spectrophotometer quantity and quality of extracted DNA was calculated and recorded.

Using the device in the presence of (Epndorf original) mix temperature gradient Real time PCR was attempted. This test, respectively, for oocytes matured in Kermani sheep, Baluchi sheep, were lower than goats Rhine and baction and CD44 genes at a temperature range of 55 to 60 was conducted. CT criteria for selecting optimal binding temperature was high and fewer ΔRn. Based on temperature 59.5°C and 60.7 for CD44 gene to gene were selected.

The device of graphs for temperature gradient show Ct at different temperatures and ct is the cycle begins his Sigma growth chart. **Table (2)** CT findings of this study the thermal gradient. Melting point of the CD44 gene in all tissues of the animal, and 62°C and 62°C for the gene. No additional shoulder was observed for the gene.CD44 gene and the gene has been shown to control the temperature gradient **Table 2**.

By using Software line Reg PCR, the application cycle curve shows the fluorescence light and the data analysis based on the chart CD44 expression in all tissues and races the following **Figure 2** the CD44 gene in all tissues for the show.

CT results of this study shows the thermal gradient. Melting point of the CD44 gene in all tissues of animals and 62 degrees Celsius and 62 degrees Celsius for the gene. No additional shoulder was observed for the gene. Apart from CD44 gene in all tissues of sheep and goats were expressed in immature oocytes and this expression in other tissues were indistinguishable. Rates in PCR efficiency was different tissues of different animals. Expression levels in sheep and goat were different due to the significant different in sheep and goat breeds were studied in the kind of results **Table 3** is shown.

So we can conclude that CD44 expression is a significant test for comparison was done by examining a sample that will be explained below. In addition to the effect of

race on expression levels of CD44 gene expression comparison between sheep and goats were also compared in the **Table 4** is shown. F that was obtained from **Table 3** is higher than F in base table (P <0.05). So this test is more significant in the expression of these genes and also the obtained result was different differ between sheep and goat. **Table 5** in the CD44 gene in comparison to the races at (P <0.05) according to Duncan's method was studied. Base on the Table, it is clear that the greatest differences were between fetal goats and sheep oocytes matured and minimum difference were between mature oocytes Baluchi sheep and immature oocyte rayeni goats, the greatest differences were between oocytes matured oocytes of goats and sheep Kermani this table we can conclude, CD44 gene expression in fetal sheep and goats were higher than the other samples.

Table 1: Primer sequence, annealing temperature and fragment size of both hemi-nested PCRs used to detect the presence of CD44 receptor in immature, mature oocytes and embryos sheep and goat

Gene	Nested PCR step	Nucleotide sequence	GenBank accession No.	Product size (bp)
CD44	**1st PCR**	**5-CAACACCTCCCASTATGACAC-3 5-TTCTTCTGCCCACACCTTCT-3**	**NM_001001391 EE765662**	**570**
b-actin	**1st PCR**	**5-CAACTGGGACGACATGGA-3 5-TGGTGGTGAAGCTGTAGC-3**	**NM_001009784**	**377**

Table 2. Results of Thermal Gradients CT Internal Control Gene CD44

Temperature gradient	internal control gene CT	CD44 gene CT
57	**23.2**	29.1
58.2	**22.6**	28.62
61.9	**21.8**	24.31

Table 3. Variance Analysis of CD44 gene expression in sheep and goat

F	Mean-square	sum of squares	DF	Sources changes
7.47	**84.141**	**84.141**	**1**	**Treatment**
	11.25	**112.509**	**10**	**Error**
		196.65	11	**Total**

DF= degrees of freedom; Minimum significance level = P <0.05

Table 4: Sheep, Goat CD44 Gene Expression Data Analysis

F	Mean-square	sum of squares	DF	Sources changes
2616	65.4	196.4	3	**Treatment**
	0.025	111.26	8	**Error**
		196.65	11	**Total**

DF= degrees of freedom; Minimum significance level = P <0.05

Table 5: Comparison of Average					
Sample	**Goat Embryo**	**Sheep Embryo**	**mature oocyte tali goat**	**mature oocyte balouchi sheep**	**mature oocyte rayeni goat**
Sheep Embryo	2.97 *	-	-	-	-
mature oocyte tali goat	2.34 *	0.63	-	-	-
mature oocyte baloochi sheep	8.43 ***	5.45 **	6.99 * *	-	-
mature oocyte rayeni goat	10 ***	6.03 **	6.66 **	0.561	-
mature oocyte kermani sheep	14.4 ***	11.5 ***	12.06 ***	5.971 ***	5.98 **

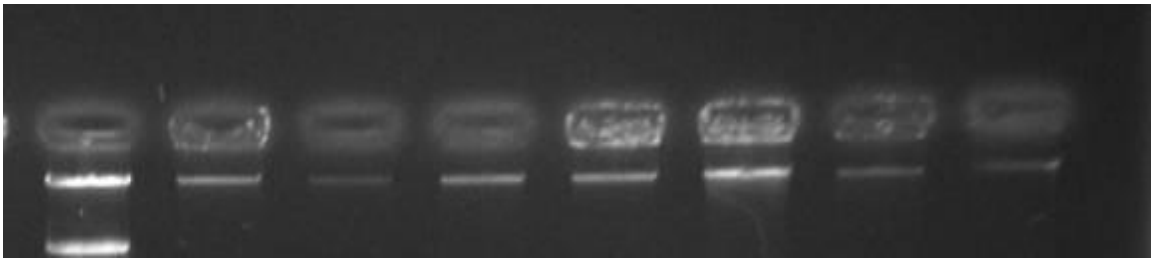

Figure 1: RNA was Extracted From the Agarose Gel Shows

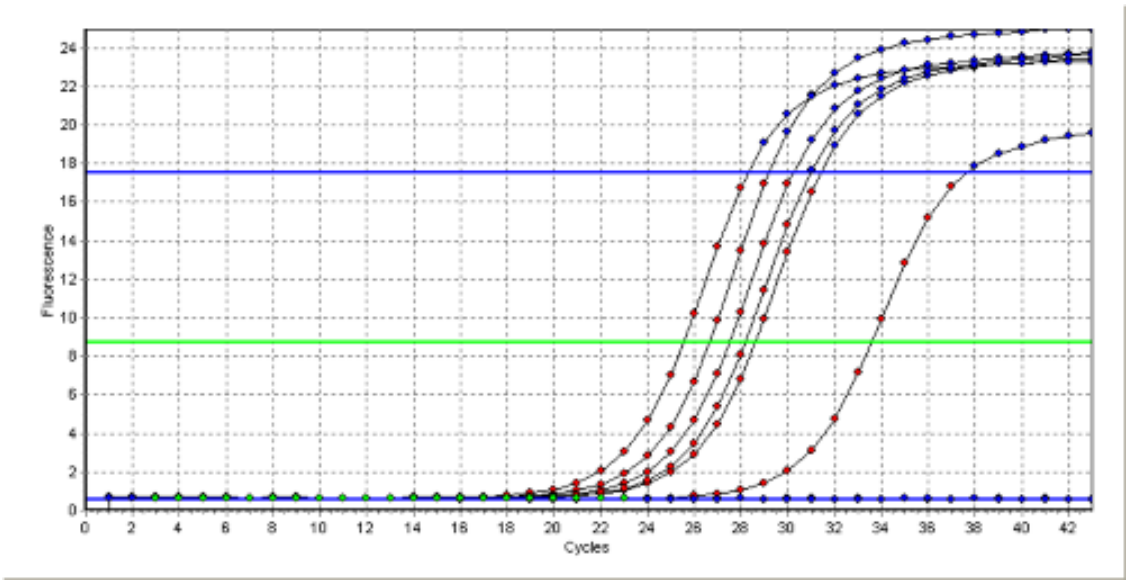

Figure 2: Curve of CD44 Gene Replication in Immature Oocytes, Mature and Fetal

DISCUSSION

In the present study, for the first time to our knowledge, we demonstrated that CD44 is expressed on goat mature oocytes and embryos. Using hemi-nested PCR and indirect immunofluorescence, it was

detected the expression at transcript and mature protein levels respectively. The same findings were already reported in matured oocytes in other mammalian species such as human **(5)**, rat **(8)**, bovine **(26)**, and porcine **(16)** and in different preimplantation stages of embryos in bovine **(10)**, porcine **(28)** and human **(6)**. On the other hand, we found that neither CD44 mRNA nor transmembrane protein was detected in immature oocytes. This is coherent since immature oocytes should be in contact with surrounding granulose cells to allow nutrient passage and reach its proper development. This agrees with data previously reported for other mammalian species such as porcine **(14)**, bovine **(8)**, mice **(15)**, and human **(9)**, who did not detect CD44 in immature oocytes.

No studies have established the role of the HA-CD44 system in oocyte maturation. However, one study **(27)** demonstrated that the degradation product of HA (3 to 10 disaccharides) induces the phosphorylation of the CD44 receptor, leading to the activation of kinase proteins, which are subsequently translocated to nucleus. This cascade is important for mitogenic signal transduction and sufficient for the induction of cell proliferation through the stimulation of proto-oncogenic transcription factors **(7)** furthermore, it plays a crucial role in activation and stabilization of the M phase promoting factor (MPF) during oocyte maturation **(12)**. Since the predominant component in the expanded cumulus is HA **(23)**, this probably explains the presence of the CD44 receptor in mature oocytes.

It is already known that CD44 has an influence on the expansion of the cumulus cells during the oocyte maturation **(31)**, on fertility and quality of oocytes **(11)**. Since the hyaluronan-CD44 interaction is involved in the induction of meiotic resumption it was presumed that this receptor was expressed in the oocytes. However, it was demonstrated that this receptor is present only in cumulus cells, not in the oocyte. Recent studies have shown that the meiotic maturation of oocytes is also subject to regulation by the somatic compartment of the ovarian follicle. MPF activation at the onset of meiotic resumption is inhibited by intra-oocyte cAMP, which is transferred from cumulus cells via gap junctional communication within COCs. Interruption of gap junctions in the COCs, which occurs in response to the pre-ovulatory surge of gonadotropins **(3)**, leads to a drop in the intra-oocyte concentration of cAMP, followed by MPF activation and meiotic resumptions. The reduction of the intra-oocyte cAMP concentration was suppressed by the inhibition of the interaction between hyaluronan and CD44. This result supports the concept that hyaluronan-CD44 interaction is involved in the regulation of

gap junctional communication and the termination of the cAMP flux from cumulus cells to oocytes **(33)**.

One study **(17)** reported that early production of HA occurs approximately 18 h after the onset of maturation, stimulated by the growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), which induce hyaluron synthase enzyme expression, responsible for synthesis of HA. Optimal expansion of cultured COCs requires the presence of substrates of HA synthesis and an expanded cumulus mass may positively influence oocyte viability **(6)**. HA produced naturally by granulosa cells also prevent fragmentation or segmentation of oocytes *in vitro* **(24)**.

CD44 also plays a role on embryo development up to blastocyst stage **(15)**. One experiment **(9)** supplemented l mg/mL HA to the culture medium and found the rate of bovine embryos that developed to the blastocyst stage higher than in medium alone. These authors reported that the incorporation of HA in a chemically defined medium clearly demonstrated the effect of HA in the improvement of blastocyst formation. This is in agreement with **(19)**, who verified that the proportion of degenerated porcine embryos was lower in the presence than in the absence of HA. It has been suggested that HA might benefit embryo development *per se* or by regulating the action of factors synthesized by the embryo, acting in an autocrine way **(30)**.

The presence of CD44 in mature oocytes and preimplantational embryos suggests the expression profile of the HA during maturation and preimplantational development. The findings of this study could be useful in the definition, investigation and also understanding of the physiological role of CD44 in the reproductive processes involved in the ovine species. Further studies are necessary to clarify the events in which CD44 and HA are involved during maturation and preimplantational embryo development in goat.

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