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The efficacy of human recombinant luteinizing hormone for in vitro embryo production in sheep

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ABSTRACT

Objective: This study was carried out to assess the ability of recombinant human luteinizing hormone (r-LH) to be used instead of sheep hypophyseal luteinizing hormone (h-LH) in the maturation of sheep oocytes and its influence on embryonic development and quality.

Materials and Methods: The oocytes were obtained from slaughtered sheep ovaries. For oocyte maturation, grade 1 oocytes were incubated at 38.5°C with 5% CO_2 for 24 hours. The maturation medium for the hypophyseal LH (h-LH) group was supplemented with 0.1 IU/mL LH derived from sheep pituitary LH (Sigma®), while the medium for the recombinant LH (r-LH) group contained 0.1 IU/mL recombinant LH (Luveris® 75 IU, Serono). In vitro fertilization (20h) and embryo culture were performed at 5% CO_2, 5% O_2 and 38.5°C incubation conditions. The maturation rates were reported based on the MII stage chromosomal formation and the existence of first polar body by bisBenzimide (Hoechst 33342). Embryonic developments were controlled on the 3rd and 8th day of in vitro culture. For the embryonic cell count and determination of inner cell mass (ICM) and trophectoderm cell (TC), the differential staining technique was used with Hoechst 33342 + Propidium Iodide (PI).

Results: The proportion of cleavage (%), the rate of embryos developing the morula (%) and blastocyst stage (%), and the ICM, TC, and total cell numbers of the embryos were found to be statistically similar in the h-LH and r-LH groups ($p>0.05$).

Conclusion: It was concluded that r-LH could be used as an alternative LH source instead of hypophyseal LH.

Keywords: *Embryo development, Hypophyseal LH, In vitro maturation, Recombinant human LH, Sheep oocyte*

INTRODUCTION

Currently, in vitro embryo production procedures are increasingly used in sheep with high breeding values in veterinary practice and biotechnological research (Wani, 2002). So far, researchers have tried various culture systems, different hormone sources and media to increase the in vitro success of embryo production (Zhu et al., 2018). Nevertheless, the success rate of oocytes to mature and reach to blastocyst stage in vitro has been lower than those produced in vivo (Moor and Trounson, 1977; Rizos

et al., 2002; Birler et al., 2003; Paramio and Izquierda, 2016; Zhu et al., 2018).

In vivo cytoplasmic and nuclear maturation of oocytes are achieved by luteinizing hormone (LH) and follicle stimulating hormone (FSH) as a part of gonadal-pituitary axis and local cellular factors (Wani, 2001). Hormones, serum, and various growth factors added to maturation media increase the rates of mammalian oocytes reaching the blastocyst stage.

In vitro semi-defined culture systems use gonadotropins derived from pituitary or recombinant proteins for oocyte maturation. However, hypophyseal gonadotrophins that participate in the media may cause different contaminants which enter into the culture systems (Vanroose et al., 2001).

There are some problems such as; non-standard hormone activity in every production batch and transmission of undesired hormones (e.g., TSH) (De Koning et al., 1994; Kanitz et al., 2002; Baruselli et al., 2023) or diseases (Hesser et al., 2011), low efficiency and high cost of the method (Rosano and Ceccarelli, 2014; Baruselli et al., 2023).

Gonadotropic hormones obtained from the pituitary gland are widely used in the field of reproductive biotechnology (Demir et al., 2019; Baruselli et al., 2023). There are recent works obtained various results by using different types of recombinant gonadotropins (Törnell et al., 1995; Andresiez et al., 2000; Accardo et al., 2004; Sha et al., 2010; Tharasanit et al., 2014; Gifre et al. 2017; Arıcı et al., 2022).

This study aims to compare the effect of recombinant human luteinizing hormone (r-LH) and hypophyseal luteinizing hormone (h-LH) on ovine oocyte maturation, blastocyst ratios and total cell counts of blastocysts.

MATERIALS and METHODS

Unless otherwise noted, all of the chemicals used in the current research were purchased from Sigma (St. Louis, MO, USA).

Oocyte collection

A total of 80 ovaries from adult ewes were obtained from a local abattoir and conveyed to the laboratory within 3 hours in Dulbecco's phosphate buffered saline (DPBS) solution at approximately 35-37°C. After washing with fresh DPBS, ovaries were sliced and the follicle contents were flushed in TCM-199 (M5017) medium with 25 mM HEPES (H6147), 50 IU/ml penicillin (P4687), 50 µg/ml streptomycin (S1277), and 0.4% Bovine Serum Albumin (BSA) (A8806). A total of 370 cumulus-oocyte complexes (COCs) were retrieved. Out of these, 270 COCs with three to ten layers of cumulus cells and homogeneous cytoplasm were selected for in vitro maturation (Arıcı et al., 2022).

In vitro maturation (IVM)

COCs obtained from ovaries were divided into two separate maturation groups. Basic maturation media consisting of bicarbonate-buffered TCM199 (M5017) with 0.4% BSA (A8806), 2 mM L-glutamine (G7513), 0.3 mM sodium pyruvate (P4562), 1 µg/ml estradiol-17β (E2758), 100 IU/ml penicillin (P4687), 100 µg/ml streptomycin (S1277), 0.1 IU/ml FSH (Folltropin-V®) addition (Birler et al., 2002). The maturation media for the hypophyseal LH (h-LH) group was supplemented with 0.1 IU/ml LH (from sheep Piturity LH, Sigma, L5269) and the Recombinant LH (r-LH) group was supplemented with 0.1 IU/ml LH (Luveris® 75 IU, Serono).

Oocytes were incubated in four-well plates (500 µL per well), with 35-40 oocytes per well, at 38.5°C, 5% CO2 for 24 h. Subsequent to maturation, the cumulus cells surrounding the oocytes were denuded by vortexing for 2-3 min. After a complete denudation, oocytes were incubated in 5µg/ml Hoechst 33342 added DPBS for 15 minutes.

Following incubation, a total of 40 oocytes were examined for each group under an epifluorescence microscope (IX 70, Olympus, Japan) with a 365 nm excitation filter and a 410 nm barrier filter. Each oocyte was evaluated in conformity with chromatin structure. Evaluation of maturation was made according to Metaphase II (MII) or the other stages of meiosis respectively.

In vitro fertilization (IVF)

Mature COCs were then washed three times in ovine fertilization medium (bSOF) [3 mg/mL BSA-fraction V (A3311), 0.72 mg/mL D-glucose (G7021), 0.06 mg/mL sodium pyruvate (P4562), 0.25 mg/mL L-glutamine (G7513), 0.12 mg/mL kanamycin monosulfate (K1377), 0.075 mg/mL penicillin (P4687), and 0.05 mg/mL streptomycin (S1277)] at 38.5°C with 2% estrous sheep serum. After maturation, oocytes of the study groups were transferred to a four-well plate (Nunc, Thermo Scientific, USA) containing 500 µl fertilization medium (Birler et al., 2002).

Fresh sperm from a Kivircik ram was prepared for IVF using a modified swim-up technique based on the protocol described by Shirazi et al. (2010). A

volume of 100 µL of semen was placed under 1 mL bSOF in a 15 mL conical tube and incubated at 37°C for up to 1 h. The supernatant of each tube (one third of the volume) was aspirated and mixed with h-SOF then it was centrifuged at 1700 rpm for 5 minutes.

After centrifugation, the spermatozoa concentration was calculated by haemocytometer. The spermatozoa concentration was adjusted to 0.8×10^6 spermatozoa/ml, washed and centrifuged (200×g for 5 min) twice in h-SOF, and added to the fertilization medium.

Maturated oocytes (35-40 per well) and spermatozoa were carried out co-incubated at 38.5°C, 5% CO_2 for 24 h.

In vitro culture (IVC)

At the 20th hour post-fertilization, the pipetting technique was used to remove the cumulus cells and spermatozoa residues. Then, cumulus-free cells of all groups were cultured by placing 12-15 in a 50 µl droplet of synthetic oviduct fluid (SOF) supplemented with 1% (v/v) essential amino acids, 1% (v/v) MEM-nonessential amino acids (M7145), 1mM glutamine and 10% fetal bovine serum (FBS) (F2442). They were incubated with 5% O_2, 5% CO_2 and 90% N_2 at 38.5°C.

Embryos were transferred into 15 mM glucose (G7021) including SOF medium on day 4 post-fertilization and culturing continued until the 8th day.

Embryonic developmental stages were evaluated by inverted microscope. The quality of blastocysts from h-LH and r-LH maturation media were determined with a differential staining of blastocysts depicted by Thouas et al. (2001) with modifications. Embryos were held in PBS with 1% (v/v) Triton X-100 and 100 µg/ml propidium iodide (PI) for approximately 15-20 seconds. During this treatment trophectoderm's colour turning to red were transferred to 500 µl fixative solution consisted of 25 µg/ml bisBenzimide (Hoechst 33342) and absolute alcohol and incubated overnight at 4°C in dark. Then, the blastocysts were put in a glycerol droplet on a glass slide to be covered with a coverslip.

Samples were examined by using a fluorescent microscope (IX70, Olympus, Japan) equipped with an UV filter. By Hoechst 33342 and PI, the inner cell mass (ICM) was observed blue, and trophoblastic cells (TC) nuclei as pink to red. The total cell count, ICM, and TC numbers were assessed in 17 blastocysts from the h-LH group and 23 blastocysts from the r-LH group, respectively.

Statistical analysis

The chi-square test was used to compare the effect of LH type added to the culture in the growth of cultured oocytes up to MII, blastocyst stage and total cell of blastocysts. All analyses were performed with the SPSS Version 20. Each experiment was repeated at least five times. Differences were considered significant at $p<0.05$.

Ethical approval

This study was approved by the Experimental Animals Local Ethics Committee at the Istanbul University-Cerrahpaşa with decision number 2017-295820.

RESULTS

In vitro maturation

No statistically significant difference was determined between the groups in the maturation medium when h-FSH and h-LH (67.50%) or h-FSH and r-LH (65.00%) were compared to the MII stage ($p>0.05$) (Table 1).

Table 1. Effect of different LH sources on oocyte maturation.

Group	No. of oocytes examined (n)	Metaphase II (%)
h-LH	40	27 (67.50)[a]
r-LH	40	26 (65.00)[a]

Different letters indicate statistical difference within each column ($p<0.05$).

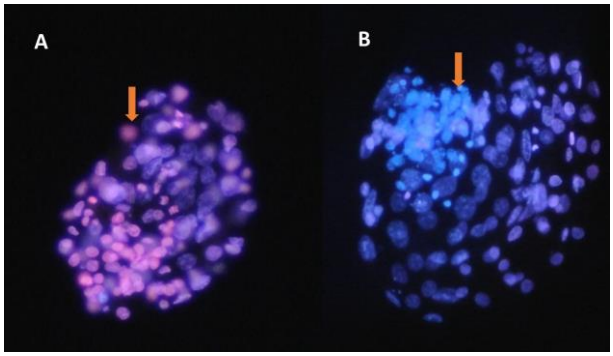

Figure 1. Image of inner cell mass (ICM) and trophectoderm cells (TC) of blastocysts. Since the membranes of TC are lysed, their nuclei appear dark pink in colour (A). The cytoplasm is blue / pink with the effect of flurochrome dyes of propidium iodine and bisbenzimide. However, as the membranes of ICM cells are intact, only the bisbenzimide dye can pass through and stain the nucleus making the cells to be seen blue (B).

Embryo development

Oocytes were maturated for 24 hours in maturation medium containing different types of LH, fertilized and transferred to culture. For the evaluation of embryonic development after IVC, 90 oocytes were used in the h-LH group and 108 oocytes in the r-LH group. Cleavage, morula, and blastocyst rates were determined as 76.66%, 45.55%, and 18.88% in the h-LH group, and 84.25%, 50.92%, and 21.29% in the r-LH group, respectively (Table 2). There was no statistically significant difference in the cleavage, morulae, and blastocyst rates of cultured oocytes ($p>0.05$).

Embryonic cell evaluation

Although, ICM, trophectoderm and total cell numbers of embryos reaching the blastocyst stage in culture medium containing h-LH were superior to another group, there was no statistical difference between the two groups ($p>0.05$) (Table 3). Images of ICM and TC of blastocysts belonging to LH groups are given in Figure 1.

Table 2. Embryonic development rates of recombinant and pituitary LH groups.

Group	Oocytes (n)	Cleavage (%)	Morulae* (%)	Blastocyst*(%)
h-LH	90	69 (76.66)[a]	41 (45.55)[a]	17 (18.88)[a]
r-LH	108	91 (84.25)[a]	55 (50.92)[a]	23 (21.29)[a]

Different letters indicate statistical difference within each column ($p<0.05$). * Morulae and blastocyst rates / oocytes.

Table 3. Means of inner cell mass (ICM), trophectoderm (TC) and total cell numbers of blastocysts.

Group	Blastocyst (n)	Cell number (mean±SEM)			ICM:TC ratio
		Total	ICM	TC	
h-LH	17	210.33±71.90[a]	75.33±26.02[a]	135.00±47.96[a]	1:1.79
r-LH	23	171.60±27.43[a]	58.80±10.42[a]	112.80±17.39[a]	1:1.90

Different letters indicate statistical difference within each column ($p<0.05$).

DISCUSSION

Adding gonadotropins to the in vitro maturation media, provides nuclear and cytoplasmic maturation of oocytes and increases the number of embryos developing from in vitro fertilized oocytes (Moor and Trounson, 1977; Sha et al., 2010; Demir et al., 2019).

In vitro nuclear maturation of oocytes is the sequence of events that oocyte proceeds to meiotic divisions and progressing to the metaphase stage of the second meiosis and can easily be detected by observing discard of the first polar body.

When the polar body extrusion of matured oocytes in maturation media containing h-LH and r-LH were compared, a similar rate of MII development were observed ($p>0.05$). This indicates that LH coming from both sources can support nuclear maturation.

Pituitary-derived FSH and LH play an important role in the cytoplasmic and nuclear development of oocytes in vivo. Hormones used in the in vitro development stage are usually extracted from the pituitary glands of slaughtered animals. During this extraction, hormones are contaminated with various contaminants. This affects the purity of the products obtained and probably the fate of in vitro studies. In in vivo development process, meiosis takes place with the effect of LH before ovulation. It has been observed that r-LH and h-LH added to the maturation medium support oocytes to continue meiotic divisions at a similar rate with in vitro conditions. These results are consistent with the results obtained by Accardo et al. (2004).

In farm animals, embryos are produced using IVM, IVF, and IVC techniques, followed by embryo transfer. However, the success rates for embryo development and pregnancies achieved through these methods are notably lower compared to in vivo conditions. This discrepancy is primarily due to the inability to replicate the favorable conditions of the in vivo environment in vitro. Despite some progress in enhancing oocyte quality, achieving significant, large-scale improvements in in vitro oocyte maturation remains a considerable challenge. As a result, the application of assisted reproductive technologies to improve reproductive efficiency in both agricultural and biomedical fields

has faced limitations. To overcome these challenges, extensive research has focused on the influence of the in vitro environment on oocyte quality. Key components that impact oocyte developmental potential include proteins, energy substrates, hormones, growth factors, pH, oocyte-secreted factors, and antioxidants. Each of these elements plays a critical role in regulating the developmental competence of oocytes, emphasizing the complexity and importance of optimizing in vitro conditions to enhance reproductive outcomes (Lonergan et al., 2006; Krisher, 2013).

Oocyte meiotic maturation is an essential process for oocyte development, facilitating the progression from meiotic arrest to full maturation. The luteinizing hormone surge plays a pivotal role in this process by releasing oocytes from meiotic prophase arrest and triggering the resumption of meiosis, culminating in the completion of the first meiotic division (Mehlmann, 2005). This maturation process is initiated by the generation of an LH signal within the ovarian follicle, which activates key molecular pathways necessary for oocyte development. Luteinizing hormone binds to luteinizing hormone receptors (LHR) located on mural granulosa cells, initiating a cascade of intracellular events through G protein activation and subsequent cyclic adenosine monophosphate (cAMP) signaling. This LH-mediated signaling affects both the follicular compartment and the oocyte, orchestrating the regulation of oocyte meiotic maturation. The primary molecular targets of LH signaling within the ovarian follicle include the C-type natriuretic peptide (CNP)/natriuretic peptide receptor 2 (NPR2) system, the epidermal growth factor (EGF) network, and intercellular communication through gap junctions (Conti et al., 2012; Jaffe and Egbert, 2017). In the oocyte, the primary target of the LH signal is the maturation-promoting factor (MPF) (Adhikari and Liu, 2014). Upon activation, MPF phosphorylates several critical systems, including the spindle assembly checkpoint (SAC) and the anaphase-promoting complex/cyclosome (APC/C), as well as other downstream proteins. This cascade drives the progression of meiosis by inducing key cellular events such as germinal vesicle breakdown (GVBD), chromosome condensation, and chromosome segregation, thereby facilitating oocyte maturation (Arroyo et al., 2020).

Although the chromosomal development in the maturation of oocytes is demonstrated by detection of the first polar body, cytoplasmic maturation is not adequately demonstrated. Therefore, it is important to examine the effects of r-LH and h-LH used in maturation media on embryo development.

No statistically significant difference was found between groups in terms of cleavage, morulae and blastocyst after in vitro fertilization with h-LH and r-LH ($p>0.05$). These results are similar to previous studies (Andresiez et al., 2000; Accardo et al., 2004) suggesting the use of recombinant LH in sheep oocyte maturation.

The number of ICM and TC in blastocysts that develop in vivo or in vitro is an important indicator of embryo quality (Leppens et al., 1996; De la Fuente and King, 1997). When the cell numbers of blastocysts developed in Recombinant and Pituitary LH groups were compared, no statistically significant difference was observed ($p>0.05$).

CONCLUSION

Based on the results, it has been demonstrated that human recombinant luteinizing hormone is a suitable option for use in sheep in vitro embryo production and transfer programs.

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