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A CLINICAL AND ULTRASONOGRAPHICAL STUDY TO INVESTIGATE THE LHR RECEPTOR GENE AND EMBRYONIC DEATH OUT SEASON IN SHEEP

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Abstract

In the present study, the embryonic death of Iraq sheep was investigated by LHR gene and Ultrasonographical after estrus synchronization by using 40 mg impregnated sponges with MAP for 12 days with 400 IU PMSG on the day before sponges withdrawal. All 20 Iraqi sheep showed (100%) estrus after sponge withdrawal after the natural mating. They were diagnosed by ultrasonography weekly, 14, 21, 28 and 35 days of pregnancy. Embryonic mortality was diagnosed in ewes by Transrectal examination with liner probe 5 MHz. The ultrasounds have well accurate to an estimation of the embryo a vitality. Our results showed that twining pregnancy in sheep high rates of early embryonic mortality than a single pregnancy. Blood samples were collected for measurement of progesterone concentration before and during pregnancy. the study aimed to identification of sheep neonate death using ultrasonography and study the effect of progesterone hormone on neonate death of sheep and study the effect of LH Receptor) on the neonate death.

Key words: LH Receptor embryonic death Iraq sheep

Introduction

The implantation process and trophoblast .invasion is currently considered as the most limiting factor for the establishment of pregnancy (Herrler *et al.,* 2002**)**. The main role of LH in the myometrium of females is stimulation .of growth and hyperplasia and relaxation of uterine motility through LHR, with the rise in estrogen hormone, LHRs also expressed on the maturing follicle that produces an increasing amount of estradiol (Regan *et al.,* 2017). To maintain pregnancy we requires to reciprocal interactions between the conceptus and endometrium of uterus, also there are available evidence supports the idea that hormones from the placenta act directly on the uterine endometrium to regulate cell differentiation and function (Spencer And Bazer, 2004). Embryo existence could be also affected by the ambient environmental indications such as nutrition and temperature. Regimes containing high protein increase rate of early embryonic loss by increasing blood plasma urea that alters uterine environment through decreasing PH (Nancarrow, 1994). Decreases Progesterone synthesis and damages oocyte quality The study aimed on identification of sheep neonate death using ultrasonography to investigation, also study the effect of progesterone hormone on neonate death of sheep and study the effect of (LH Receptor) on the neonate death.

Materials and Methods

Animals of study: The animals of the study were (20) healthy mature sheep with (4) sexually mature males, housed in Agricultural Researches, Ruminant Researches Station – Ministry of Agriculture. All animals kept under observation along a period of study. The clinical examination was done to determine that they are healthy and free from disease. The animals were submitted to veterinary preventive health measures such as the vaccination against enterotoxaemiaat dose of 1ml/per animal s/c (Ultrachoice, U.S.A) Estrus synchronization all sheep treated with progesterone hormone (Vaginal sponges rich with progesterone hormone) for about 12 days, at the pre last day we used of pregnancy Mare Serum gonadotrophin (PMSG) Intervet International co

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European Union, Holland. to induce ovulation. All ewe were mated (hand mating) naturally with fertile rams at the standing position when ewe appears signs of heat a after sponge removal. The mating was begun at 24 h after sponge removal and injection of PMSG 500 IU. The day of mating was considered as day 0 for calculating the gestational period. After (24 h) the females mixed with males to make a pregnancy. Tittering of serum progesterone hormone: blood collection samples drawing from all females to isolate serum and testing the titter of progesterone hormone alt the day of vaginal sponges withdrawal. The serum samples sent to a private laboratory for identifying the results. Early pregnancy diagnosis via Ultrasonography. Pregnancy diagnosis of all inseminated sheep to detect pregnancy in (14, 28, 30 and 35 days of pregnancy). Testing of (LH receptors): All samples which send to the laboratory to identifying RNA using Kit contents of RNA extraction (Direct-zol™ RNA MiniPrep, R2051, ZYMO RESEARCH/USA.

The procedure of RNA isolation done by conventional phase separation to selectively enrich for some miRNA species. The method of Direct-zol™ assures unbiased recovery of small RNAs including miRNA. Prepared samples in TRI Reagent® directly to the Zymo-Spin™ IIC Column and then spin, wash and elute the RNA. The RNA eluted is high quality and suitable for subsequent molecular manipulation and analysis (including RT-PCR).

Table 1: The Direct-zol™ RNA MiniPrep provides a streamlined method for the purification of up to 50 ìg (per prep) of high-quality RNA directly from samples in TRI Reagent. Total RNA, including small RNAs (17-200 nt), is effectively isolated from a variety of sample sources (blood).

Direct-zol™ RNA MiniPrep Kit Size (Preps)	**R2051(50)**
TRI Reagent®	50 ml
Direct-zol™ RNA PreWash1 (concentrate)	40 ml
RNA Wash Buffer2 (concentrate)	12 ml
DNase I3 (lyophilized)	1
DNA Digestion Buffer	4 ml
DNase/RNase-Free Water	6 ml
Zymo-Spin™ IIC Columns	50
Collection Tubes	100

Table 2: Component of the qRT-PCR reaction.

Component	**20 µL (Final volume)**	**Final concentration**
SYBR Fast qPCR master mix	10 µL	1 X
Forward primer (10µM)	0.5 µL	0.2 µM
Reverse primer (10µM)	0.5 µL	0.2 µM
Dntp	0.5 µL	200 nM
KAPA RT mix (Reverse transcriptase)	1 µL	lX
Tamplet RNA	6 µL	100 ng/µL
dd-water	1.5 µL	N/A

1- RNA Purification Add an equal volume ethanol (95-100%) to a sample lysed in TRI Reagent and mix thoroughly. And Transfer the mixture into a Zymo-Spin™ IIC Column 2 in a Collection Tube and centrifuge should be performed at 10,000-16,000 × g for 30 seconds. Transfer the column into a new collection tube and discard the flow-through.

2- Real-Time PCR (one-step RT-qPCR):

Step 1: Preparation of qPCR master mix

The KAPA RT mix was kept on ice during use and assembled reactions on ice to avoid premature cDNA synthesis.

1. PCR master mix containing the appropriate volume of all reaction components common to all or a subset of reactions to be performed.
2. Included a no template control (NTC) and no RT control (NRT) when necessary. The NTC would enable detection of contamination in the reaction components, while the NRT would enable detection of contaminating genomic DNA.

The required volume of each component was calculated based on table 2.

Step 2: Setting individual reactions

1. The appropriate volumes of qPCR master mix, template and primers were transferred to each well of a PCR tube/plate.
2. The reaction tube/plate was caped and centrifuged briefly.

Step 3: Performing One-Step qRT-PCR.

Table 3: Performed conventional qRT-PCR with cycling protocol.

Step	**Temperature**	**Duration**	**Cycles**
Reverse transcription	42℃	5 minutes	Hold
Enzyme inactivation	95℃	3 minutes	Hold
Denaturation	95℃	3 seconds	40
Annealing	59℃	20 seconds	
Extension	72℃	20 seconds	

Step 4: Calculations

$\Delta Ct\ (patients) = Ct\ (patients)_{mean} - Ct\ (reference)_{mean}$

$\Delta Ct\ (Controls) = Ct\ (controls)_{mean} - Ct\ (reference)_{mean}$

$\Delta\Delta Ct = \Delta\ Ct\ (patients) - Ct\ (controls)$

Normalized target gene expression level $= 2^{(-\Delta\Delta Ct)}$

FAM Ct housekeep But CY5 Ct of gene:

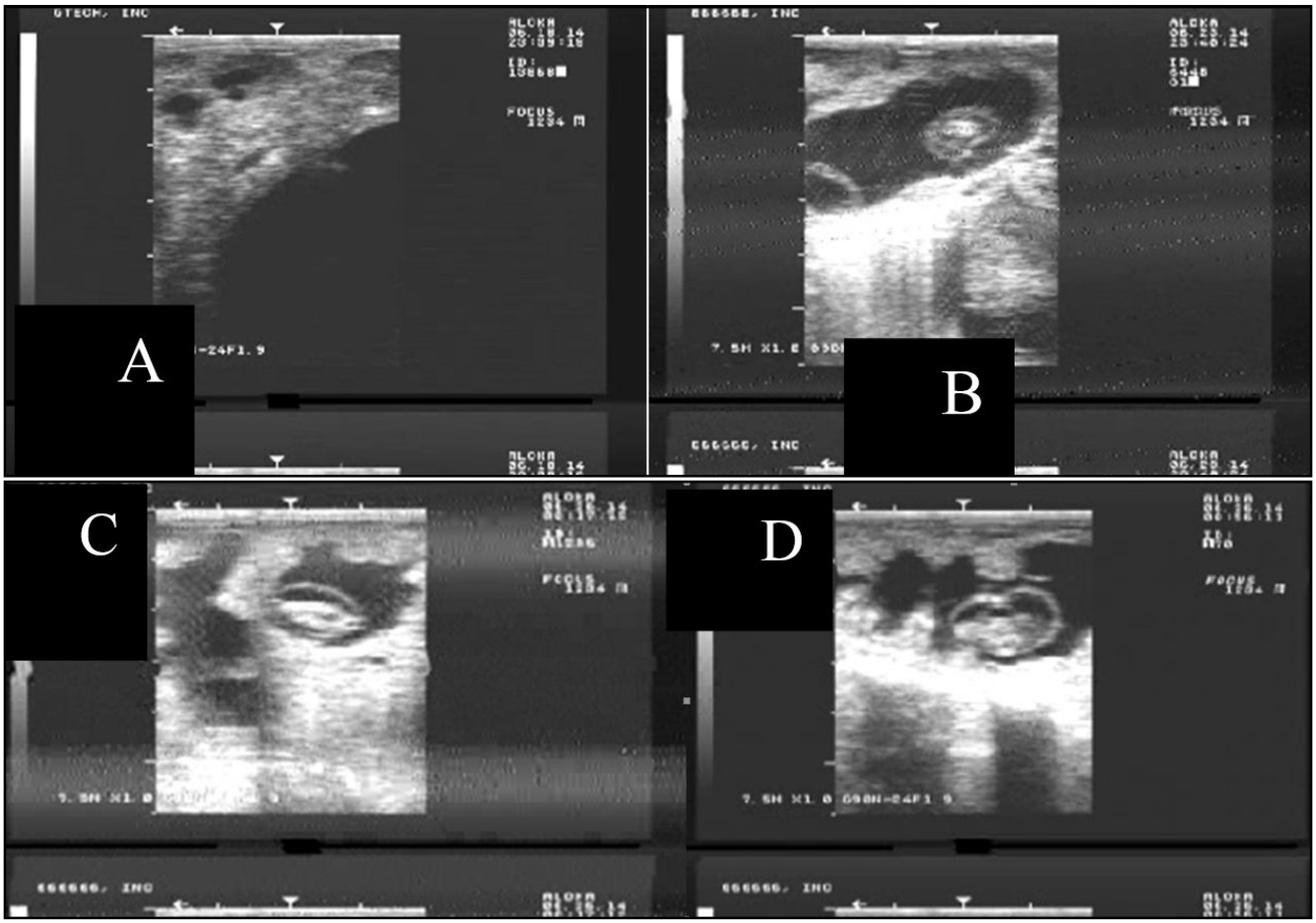

Fig. 1: Ultrasonographic image of pregnant sheep using trans-rectal examination on day 14 post mating, 3 amniotic Embryonic Vesicle. U.B: Urinary bladder (A), (B) Ultrasonographic image twin pregnancy at day 21 (C) pregnancy at day 28 and (D) pregnancy at day 35.

Table 4: Mean ± SD serum progesterone concentrations in sheep with single and twins pregnancy during different day of pregnancy.

Time of blood collectionpost mating	Progesterone concentration (ng/ml)Post different day of twining pregnancy	Progesterone concentration (ng/ml)Post different day of single pregnancy
First day	9.33 ± 0.85 a	10.36 ± 1.30 a
14 day	12.3 ± 0.7 a	10.26 ± 1.27 a
21 day	18.06 ± 0.3 a	14.33 ± 0.9 b
28day	22.1 ± 0.6 a	16.6 ± 0.8 b
35 day	24.53 ± 2.41 a	17.23 ± 1 b
42 day	28.53 ± 1.49 a	19.6 ± 0.79 b
----	(P < 0.05).	(P < 0.05).

Serum progesterone (ng/ml) was determined by ELISA kit (Monobind Inc., Certified Company, Lake Forest, USA) and colometric microplate enzyme immunoassay with analytical sensitivity of 0.105 ng/ml with cross reactivity specificity of 100% read at 450nm *(Automatic ELISA Reader, Italy)*.

Table 4: Example Statistical analysis of gene expression in ooooooooooooooo and control group.

Groups	Group control	Group patient
Mean (Ct)	17.911	16.260
SD	1.111	0.470
SE	0.162	0.181
95% C.I. for Mean	1.161-2.142	
Min.	16.161	14.333
Max.	20.502	19.060
ΔΔCt(Cycle)	-1.931	
Fold change$2^{-\Delta\Delta Ct}$	3.813	
P-value	0.001 *	

The expression of gene was detected successfully by using new molecular technique which is Real time PCR (qRT-PCR) with used specific primer as described up. The amplification accuracy of gene product was noticed by the value of cycle threshold (Ct) for the triplicate reactions. The data obtained from real time experiments were detected according to the Ct values which calculated from cycles and was proportional to the starting target copy number (logarithmic scale) used

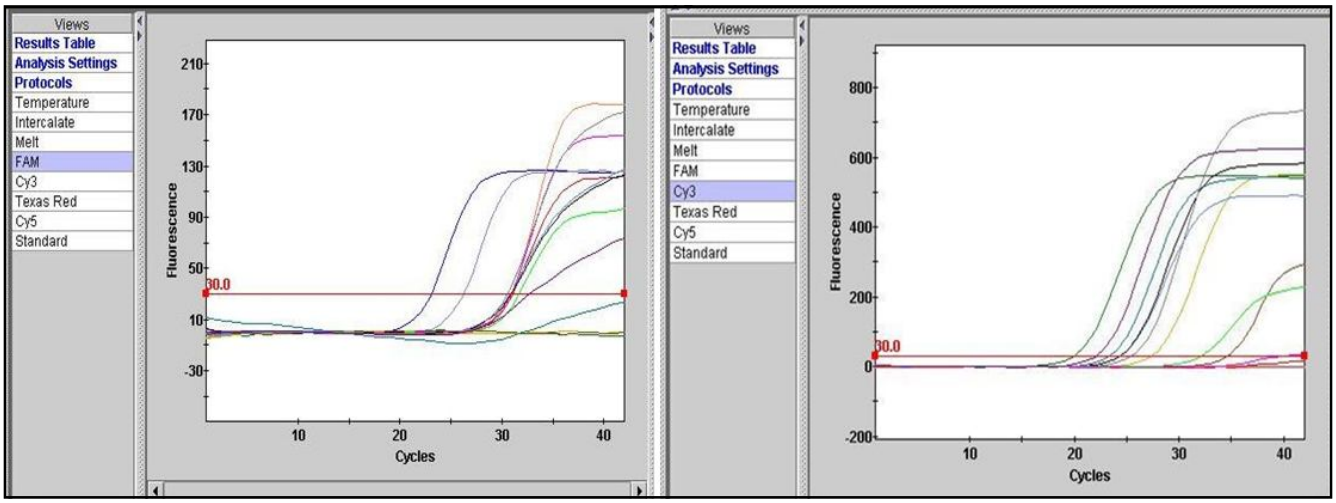

Fig. 2: Amplification plot for gene (the threshold fluorescence level as green line).

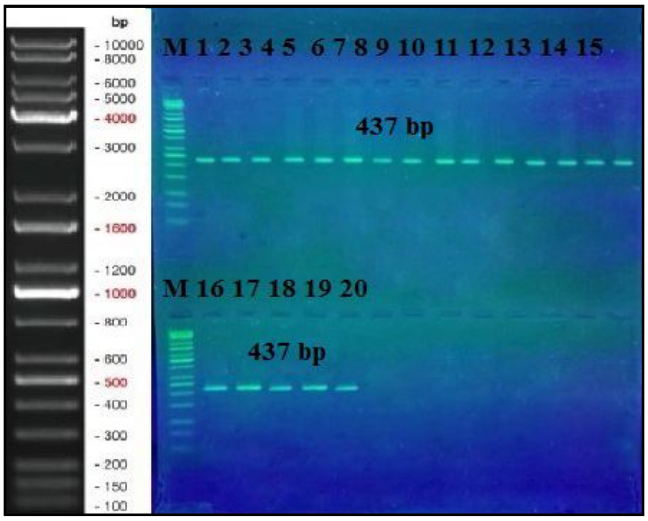

Fig. 3: The product was electrophoresis on 2% agarose gel at 5 volt/cm2, 1x TBE buffer for 2 hours. M: DNA ladder (100-10000bp), Lane 1-10 product for LHR gene of sheep, PCR product of band size 437 bp. Visualized under U.V light after staining with red stain safe.

for amplification (the point that the fluorescence signal increased above baseline is the threshold cycle) which are inversely related to the amount of starting template that mean the high value of Ct refers to the low levels of gene expression or amplification gene, while low Ct value indicate high level of gene expression or high copy of gene amplification. Amplification plots appeared when the fluorescent signal from sample is plotted against cycle number; however amplification plots include the accumulation of product through the period of qPCR experiment Fig. 2.

The total DNA produced by the standard protocol by intron kit (Korea) procedure. Two conserved primers, (437 bp) forward: 5-TCCCTGGAAAAGATAGAAGCT AATGCC-3 and reverse: 5'-AAACCTGCCAA-CAAAAGAG-3. Thermal cycling included : Denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 35s with final incubation at 72°C for 7 min. Polymerase chain reaction products were extracted using electrophoresis at 2% agarose gel with the visualized by contact with ultra violet light (302nm).

The Statistical Analysis System (SAS 2012) program was used to determine differences between treatments and means ($P<0.05$).

Results and discussion

The result of pregnancy diagnosis in post naturally inseminated sheep of the study using pre-rectum ultrasonography is shown in the Fig. 1, a-b. These pregnant sheep show one or more fetuses in the uterus early embryonic mortality investigated by ultrasonography with pregnancy diagnosis weakly from day 14 post mating to day 35 of pregnancy. Early embryonic mortality was exhibit in twin pregnancy. Ultrasonic scanning has been used in different features of fertility management in sheep including early pregnancy diagnosis, multi-pregnancy diagnosis and determination of embryonic mortality (Al-Rawi, 2005 (In sheep, inadequate luteal function was the major cause of pre-implantation losses (Nancarrow, 1994). Progesterone maintains pregnancy by inhibiting myometrial contractions, enhances maternal recognition (interferon-tau). (Nephew *et al.,* 1994, Spencer *et al.,* 2004a) and also affects implantation (Johnson *et al.,* 2001; Lee and DeMayo, 2004; Spencer *et al.,* 2004b).

The expression of gene was detected successfully by using new molecular technique which is Real time PCR (qRT-PCR) with used specific primer as described in chapter two. The amplification accuracy of gene product was noticed by the value of cycle threshold (Ct) for the triplicate reactions. The data obtained from real time experiments were detected according to the Ct

values which calculated from cycles and was proportional to the starting target copy number (logarithmic scale) used for amplification (the point that the fluorescence signal increased above baseline is the threshold cycle) which are inversely related to the amount of starting template that mean the high value of Ct refers to the low levels of gene expression or amplification gene, while low Ct value indicate high level of gene expression or high copy of gene amplification. Amplification plots appeared when the fluorescent signal from sample is plotted against cycle number; however amplification plots include the accumulation of product through the period of qPCR experiment.

The analysis of *LHR* gene polymorphism was carried out using PCR method. Genomic DNA of goat was successfully amplified by pair of primer that covers entire coding sequence of *LHR* gene. Genomic DNA of white blood cells was also used for amplification of *LHR* gene using PCR specific primers. The amplified fragment which is yielded of single band of the desired product of LHR gene with a molecular weight of 437 base pair appeared sharp in agarose gel through Gel electrophoreses technique and loaded with (100-1000bp) DNA ladder Fig. 3.

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