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The expression of gonadotropin releasing hormone receptor gene in ovaries and uterus cells of Iraqi and Damascus goat breed

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Abstract

Iraqi goats have a major economic role in production of meat, milk and leather as well as it considered a financial source for owners as reproduce twice a year, yet the Damascus goats have great importance than Iraqi goats owing to the number of twin births. The gonadotropin releasing hormone (GnRH) and its receptors have great importance in the reproduction and eugenics. To make a comparison between the Iraqi and Damascus goats in terms of this receptor gene expression in the ovaries and uterus tissue cells, the study was performed, in which used the (ΔCt Using a Reference Gene method) by quintitive -real time PCR technique. Results were found a significant difference ($p<0.05$), as the gene expression of (GnRH-R) higher in the ovaries and uterus tissue cells in Damascus goats compared with the Iraqi goats. In conclusion; the multiple pregnancies of twins in Damascus goats may be due to an increase gene expression of (GnRH-R) in the ovaries and uterus tissue.

Key words: (GnRH-R) gene, Damascus goats, Iraqi goats, ovaries, uterus.

Introduction:

The Iraqi breed goats (capris hircus) consider one of small farm animals which can be produce meat and milk daily, its seasonally polyestrous and can breed one to two in year (1,2,3). The Damascus goats can be breeding every year and each time produces one or pair of kids it reach sexual maturity at the age of 10-12 months, its seasonally polyestrous comes in heat every 18-22 days in fall season when days are shortening (4, 5). The gonadotropin releasing hormone (GnRH) is main to the sexual development and performs an important role in mammalian reproduction by controlling synthesis and release of gonadotropins (6,7, 8, 9). The gonadotropin releasing hormone receptor (GnRH-R) stimulates the pituitary cells for the synthesis of FSH and LH, and helps the maturation of gonads and reproduction process (6,10). A GnRH functions as an important neuroendocrine regulator of the "hypothalamic-pituitary-gonadal axis" and their receptor have been recognized consist of reproductive tissue including the gonads, a GnRH-R is found in epithelium of ovarian surface, may it has important role in function of the reproductive system (11,12). The GnRH-R increasing the physiological actions in mammals (13, 14), as well as this receptors detected in other reproductive tissues such as the epithelium of normal ovaries (12), gonads, oviduct, epithelial cells in bovine uterus (15), this tissues and cells responsiveness to GnRH will depend on the number of GnRH-R on the cell surface (16). Reverse transcription polymerase chain reaction (RT-PCR) is widely uses for detection and quantification of mRNA because it has high sensitivity for detection of gene expression levels (17, 18). The study aim to investigate the differences between mRNA level of GnRH-R gene of Iraqi and Damascus goats by studying the distribution gene expression changes of GnRH-R gene in ovaries and uterus tissue cells using the "quintitive - real time polymerase chain reaction" (q-rt PCR) technique.

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Materials and Methods:

Samples collection and RNA extraction

The study was conducted on 16 female internal genitalia in follicular stage (presence of the follicles on the ovaries surface) of Iraqi goats (capris hircus) (n=8) and Damascus goats (n=8) at 2.5-3 years during the period from Oct. 2016 to Dec. 2016, collected from Al-Najaf abattoir and transported in normal saline in cool container to the veterinary hospital of Al-Najaf laboratory within 30 min. and rinsing these samples with PBS (phosphate buffer saline pH 7.4 (Bioland Scientific, LLC) and stored in deep freeze (Gesellschaft Fur Labortechnik, GFL, Germany) at -70°C until RNA extraction. Transport all freezing samples to research lab. of Biotechnology College, University of Al-Qadisiyah. The ovaries and uterus tissues of Iraqi and Damascus goats breed were cut by sterile scissors and freed from the surrounding tissues, each ovaries and uterus was cured to three washings in PBS (pH 7.4) and snap frizzing 100 mg from tissue by a liquid nitrogen (-196°C) and homogenization by mortar and pestle to isolated of the total RNA, followed by purification using TRIzol® Reagent-Life Technologies (Thermo Fisher Scientific, USA) in line with the company's recommendations. The concentration and purity of total RNA were determined using Nano- spectrophotometer (UV/Vis SPECTROPHOTO - METER, OPTIMA, Japan), then stored at -70°C until synthesize cDNA.

Complement deoxyribonucleic acid (cDNA) Synthesis

The cDNA were synthesized from total RNA by RT-PCR with reference housekeeping (GAPDH) gene (19, 20) use thermal cycle device (MULTIGENE OPTIMAX /THERMAL CYCLE, LABNET, USA), by RT-PCR method of the first strand cDNA, using oligo deoxy thymidine (dT) (Invitrogen Oligo (dT) Primer, Fisher Scientific) according to manufactory protocol of (Omni script™ Reverse Transcriptase, USA) kit. The RT-PCR (cDNA) products samples were equalized at the same concentrations (100 ng/µl) by add the diethyl pyro carbonate (DEPC) water use dilution forma (V1.C1=V2.C2), then electro-phoresed by (MULTI SUB ELECTRO-PHORESIS, CLEAVER, KOREA), 0.9% agarose gel mixed with the electrophoresis buffers 1X Tris-borate-EDTA (TBE) (21), the voltages were 90 volts for 30 minutes according to (22), and were visualized under UV system used (DESKTO GEL IMAGER SCOPE, OPTIMA INC., JAPAN).

Design and optimize primers

The GAPDH-R gene primer as a housekeeping (references) gene, so the GnRH-R gene primer as a target gene. These primers sequences were designed by using NCBI- GENE BANK data base and PRIMER 3 DESIGN ONLINE, and used for GABDH-R and GnRH-R amplified a 557,556 base pair (bp) respectively (Table 1).

Dissolve primers

According manufactory recommended, we making the stock solution 100 pmols/µl for each primers**,** the GABDH-R Primer concentration (forward 34.2, reveres 30.0 nmols) dissolving by add (342) µl, (300) µl respectively by sterile DW**,** so dissolving the GnRH-R Primer concentration (forward 44.5, reveres 43.8) nmols by add the sterile DW (445, 438) µl respectively, yet were making the work solution (10 pmols/ µl) for each primers, we take 10 µl from each stock solution and equal of this volume to 100 µl by sterile DW.

Relative quantification and gene expression analysis

The q-rtPCR were carried out by an (QuantiTect® SYBR Green PCR, USA) Kit and applicator according to manufactory recommended by (Exicycler™ 96 Real-Time Quantitative Thermal Block instrument, Bioneer, Korea) system in veterinary hospital laboratory of Al-Najaf. Each 25 µL reaction

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contained 12.5 µL of SYBR Green super mix, 1 µl from working solution of each primers, cDNA 100 ng / 3 µl and 7.5 µl RNase-free water, under the following PCR conditions: the initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 sec and annealing-extension at 52°C for 1 min, extension at 72°C for 1 min and then a final extension at 72°C for 5 min. Amplification of GnRH-R cDNA was performed in separate tubes, the relative gene expression analysis by ΔCT using a Reference Gene method with specific formula (Fold change of relative gene expression = $2^{-((CT_{target} - CT_{reference})}$) (23).

Statistical analysis

The means ± SE values of expression fold change data to each GnRH-R gene in ovaries and uterus cells of the Iraqi and Damascus goats breed were analyzed using student t-test to estimate the significant differences between this groups, P value less than 0.05 ($p \leq 0.05$) was considered significant a suggested by (24), which were done using SPSS (version 23).

Table (1): Primers sequences with melting temperature (Tm) and product size bp (base pair) of GABDH-R gene and GnRH-R gene.

Primers	Sequences	Tm	Product size (bp)
GABDH-R-forward GABDH-R- reveres	5'- AGCCCCGTTGTCTCTTTAGC-3' 5'-CCACTGCACTTACCCTCAGG -3'	56.3ºC 58.3ºC	557 bp
GnRH-r forward GnRH-r reveres	5'-AGTTCTCGCGAGACTTTGCA -3' 5'-CCAGCATCACCCCACTTGAT -3'	54.2ºC 56.3ºC	556 bp

Results:

The relative expression of target genes in the ovaries and uterus cells in Iraqi and Damascus goats breed calculated by used (ΔCt Using a Reference Gene method) with (QuantiTect® SYBR Green PCR ,US) Kit which described by (20), this samples were analyzed using Microsoft Excel.

Relative quantification normalized of Iraqi and Damascus goats

1- The GnRH-R gene in ovaries cells Iraqi goats and Damascus goats by the ΔCT using a Reference Gene method:

Ratio (reference/target) = $2^{Ct\ (reference) - Ct\ (target)}$

The cDNA (100 ng/µl) representing of total RNA which isolated from both ovaries cells was assayed in triplicate for (target) GnRH-R gene and (reference) GAPDH-R gene. The results of ovaries samples are shown below:

Ovaries sample	Ct /GnRH-R	Ct /GABDH-R
Iraqi goats	17.0484	18.2643
Damascus goats	16.0971	18.665

To calculate relative expression, simply normalize GnRH-R expression for each sample use formula:

$2^{CT((GAPDH\text{-}R) - CT(GnRH\text{-}R))}$ = Expression

For ovaries of Iraqi goats cells, this yields **$2^{(18.2643 - 17.0484)}$ = 2.339**

For ovaries of Damascus goats cells, this yields **$2^{(18.665 - 16.0971)}$=6.014.** The evaluation of the ratio between the two samples will reveal that these give the results:

Ovaries of Iraqi goats expression = Normal/Normal = 2.339 /2.339 = 1

Ovaries of Damascus goats expression = Damascus /Normal = 6.014/2.339 = 2.572

Therefore, the ovaries cells of Damascus goats are expressing GnRH-R gene at a 2.572 fold higher level than ovaries cells of Iraqi goats.

2- The GnRH-R gene in uterus cells in Iraqi goats and Damascus goats by the ΔCT using a Reference Gene method:

Uterus samples	Ct GnRH-R	Ct GABDH-R
Iraqi goat	20.8413	18.2643
Damascus goats	19.207	18.665

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For uterus of Iraqi goat cells, this yields $2^{\wedge(18.2643 - 20.8413)} = 0.1733$

For uterus of Damascus goats cells, this yields $2^{\wedge(18.665 - 19.207)} = 0.7054$

Uterus of Iraqi goat expression = Normal/Normal = 0.1733 /0.1733 = 1

Uterus of Damascus goats expression = Damascus /Normal = 0.1733 /0.7054= 0.245

Therefore, the uterus cells of Damascus goats are expressing GnRH-R gene at a 0.245 fold higher level than uterus cells of Iraqi goat.

Expression of GnRH-R gene in ovaries and uterus cells

The target gene expression normalization was very high -regulated significant (P≤0.05) of GnRH-R genes in the ovaries cells ($6.0614^{**} \pm 0.25$) and in uterus cells (0.7054 ±0.059) of the Damascus goats, compared with low-regulated genes expression significant (P≤0.05) of this receptors genes in the ovaries cells (2.339±0.104) and uterus cells (0.1733 ± 0.017) of Iraqi goats (Table 2) & Fig. (1,2, 3).

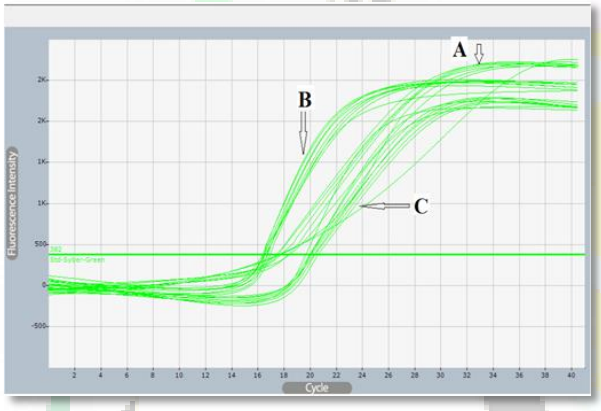

Figure (1): Real time-PCR amplification curve of iraqi goats. X-axis represented cycle number (Ct), Y-axis refered to the amplified production; A. GABDH-R (Ct mean = 18.2643) , B. GnRH-R (Ct mean =17.0484) in ovaries cells,C. GnRH-R (Ct mean =20.8413) in uterus cells.

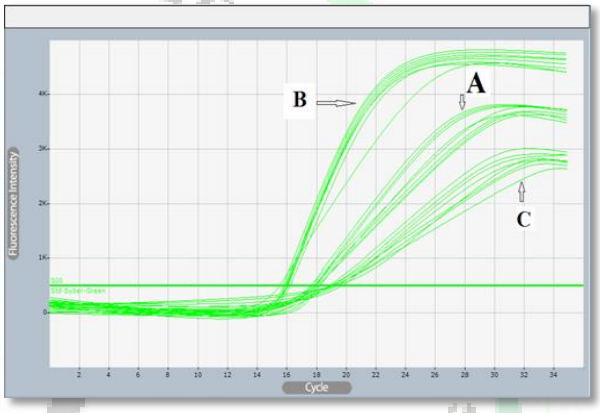

Figure (2): Real time-PCR amplification curve of Damascus goats. X-axis represen-ted cycle number (Ct), Y-axis refered to the amplified production: A. GABDH-R (Ct mean = 18.665) , B. GnRH-R (Ct mean =16.0971) in ovaries cells, C. GnRH-R (Ct mean =19.207) in uterus cells.

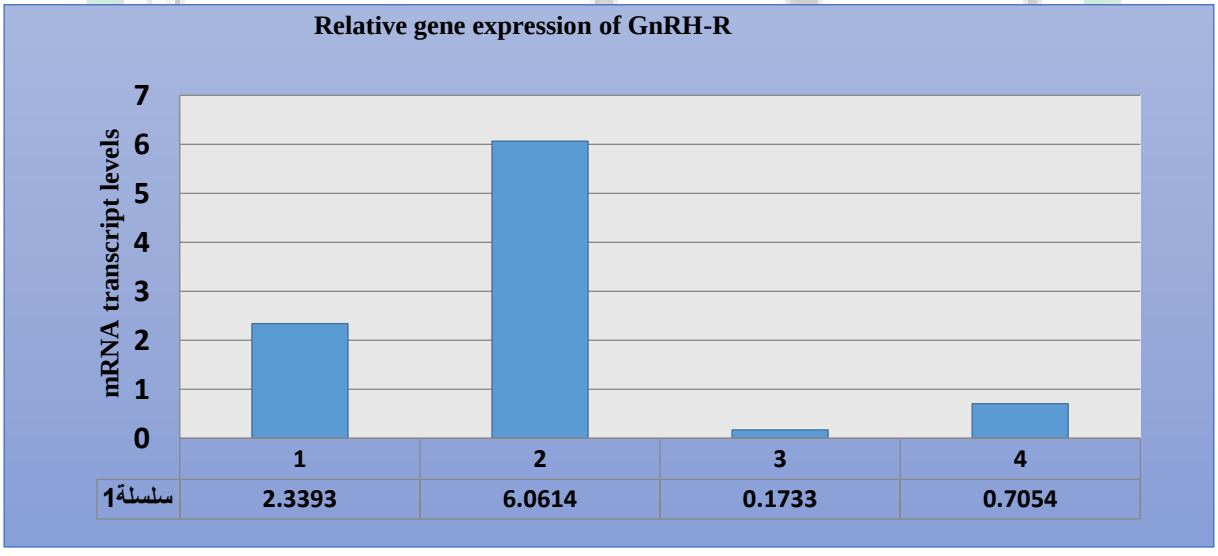

Figure (3): Fold change of mRNA transcript levels of the GnRH-R (1) ovaries of Iraqi goat, (2) ovaries of Damascus goat, (3) uterus of Iraqi goat (4) uterus of Damascus goat.

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Table (2): Data analysis of threshold cycle (Ct) and expression values of GnRH-R genes (Mean ± SE) and Coefficient of variation (C.V %) in the ovaries and uterus cells of Iraqi and Damascus goat

Samples	Ct_1 GnRH-R	Ct_2 GABDH-R	(ΔCt) Ct_1 – Ct_2	$2^{(-\Delta Ct)}$ Express values	Mean ± SE.	C.V%
Ovary of Iraqi goat	16.8837	18.2643	-1.3806	2.6037	2.339 ± 0.104	0.126%
	16.998	18.2643	-1.2659	2.4048		
	17.111	18.2643	-1.1530	2.2238		
	17.324	18.2643	-0.9398	1.9183		
	17.276	18.2643	-0.9878	1.9831		
	16.775	18.2643	-1.4888	2.8065		
	17.008	18.2643	-1.2553	2.3873		
	17.008	18.2643	-1.2553	2.3872		
Ovary of Damascus goat	16.1112	18.665	-2.5764	5.9645	6.0614** ± 0.25	0.116%
	15.9973	18.665	-2.6903	6.4547		
	15.9883	18.665	-2.6993	6.4950		
	16.2156	18.665	-2.4720	5.5483		
	16.0077	18.665	-2.6802	6.4098		
	15.8793	18.665	-2.8078	7.0022		
	16.1222	18.665	-2.5653	5.9189		
	16.4556	18.665	-2.2320	4.6981		
Uterus of Iraq goat	20.983	18.2643	2.71909	0.1519	0.1733 ± 0.017	0.28%
	21.333	18.2643	3.06899	0.1191		
	21.227	18.2643	2.9633	0.1282		
	20.857	18.2643	2.59329	0.1657		
	20.321	18.2643	2.05669	0.2403		
	21.001	18.2643	2.73689	0.1500		
	20.782	18.2643	2.51769	0.1746		
	20.227	18.2643	1.96356	0.2565		
Uterus of Damascus goat	19.223	18.665	0.53565	0.6792	0.7054 ± 0.059	0.24%
	19.332	18.665	0.64522	0.6298		
	18.773	18.665	0.08585	0.9278		
	18.788	18.665	0.10035	0.9182		
	19.378	18.665	0.69035	0.6100		
	18.987	18.665	0.29965	0.7999		
	19.288	18.665	0.60111	0.6493		
	19.887	18.665	1.222	0.4286		

***The significant difference at (P≤ 0.05) compared of two breeds, The student t-test of the ovaries samples =8.09, The student t-test of the uterus samples = 6.115, The moderate t-test to this groups =3.326.**

Discussion:

The first time has determined the mRNA level of GnRH-R gene expression using GAPDH as an internal control in ovaries and uterus of the Iraqi goats and Damascus goats by used q-rt PCR, figures (1, 2, and 3). The genes expression of GnRH-R gene in ovaries of Damascus goats is vary up-regulated (6.0614**), compared with low-regulated of quantities expression in Iraqi goat (2.3393), or the ovaries cells of Damascus goats are expressing GnRH-R gene at a 2.572 fold higher level than ovaries cells of Iraqi goat, table (2) and fig. (3). This results in agreement with (9, 25) which revered to the pulsation of GnRH incitement is fundamental for proper GnRH-R gene expression levels, in the maintaining a strategic distance from down-regulation because of ceaseless

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hormonal incitement, because the fact by (26) which revered to that GnRH-R mRNA are accounted for to express in the ovaries tissues. The expressions in Damascus goats breed is (0.7054), but in uterus of Iraqi goat breed (0.1733) or the uterus cells of Damascus goats are expressing GnRH-R gene at a 0.245 fold higher level than uterus cells of Iraqi goat, table (2), fig. (3). The GnRH initiation of GnRH-R gene is subsequently a strong boost for expression of various qualities including the quality encoding the GnRH-R gene itself, as indicated by (10,27), this finding which affirm nearness the GnRH-R in ovaries and uterus tissue agreement with (28) which showed the GnRH and GnRH-R mRNA are expressed in myometrial smooth muscle cells and the Iraqi expression of GnRH and receptor alongside the immediate activity of GnRH analogs on the smooth muscle cell DNA synthesis creation recommend an autocrine/paracrine part for GnRH in these tissues. The present study is an endeavor to clarify the impacts of the GnRH-R gene in the ovaries and uterus of Damascus breed goats relative with Iraqi breed, which indicates that the expanded discharge of GnRH assumes a focal part in the start of sexual development gonadotropins, as study of (29,30) which their outcomes demonstrated that GnRH-R quality significantly affects reproduction.

Conclusion

The multiple pregnancy of twins in the damascene goats may be due to the high regulated of GnRH-R genes expression in the ovaries of this breeds, In addition to the high difference in gene expression of this gene in uterus cells when compared between Iraqi and Damascus goats. Investigations the GnRH-R gene have role of ovulation and twins pregnancy, could further add to the knowledge in the reproductive biology and result in improvements in goat fertility.

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