Study of Bax Gene Expression Relationship for Two Different Periods with Follicles Size in Local Iraqi Sheep

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Abstract

This research was conducted at Al-Mussaib Technical College/Department of Animal production techniques /Physiology Laboratory in cooperation with Jisser Al-Mussaib Company for Biological research which located in Baghdad / Karrada Dakhla to study and evaluate Bax gene expression and measure the size of the follicles in the ovaries of the Iraqi local sheep. In this study, (50) female genital organs of sheep were collected from Karbala and Najaf butchery for two periods of time: the first period (August 2021) and the second period (January, 2022). Follicle sizes were measured and divided into three categories (<3 mm, 3-5 mm, and >5mm) and a biopsy of tissue was taken from the surface of the ovary for the purpose of Bax gene expression measurement. The obtained results showed a lower relative expression in the first period in comparison with the second period. While for the size of the follicles, the gene expression was the highest in the first category (<3 mm) and then It was followed by the second category (3-5mm) and finally the third category(>5mm).This study clearly indicate an increase in the gene expression of the programmed death gene (Bax gene) during the period of decline in reproductive activity represented by cold days (January) compared to a decrease in gene expression in August, which is a period of reproductive activity, and the reason behind that is the increasing in the activity of reproductive hormones which inversely act with the programmed death genes. As for the size of the follicles, it seems that the increased activity of programmed death genes in the developing small follicles may have come as a result of the increase in errors in the cell divisions of the developing cells as a way to get rid of the abnormal cells.

Keywords: Bax gene, Beta actin gene, follicles sizes

1. Introduction

Bax gene (B-lymphocyte associated protein 2) is a pro-apoptotic member of the Bcl-2 gene family; Encoding a 21 kDa protein called BAX-alpha. In general, researchers believe that Bcl-2 with Bcl-2 gene plays a vital role in the regulating endogenous apoptosis (Apte SS.etal, 1995). As well as considered as a potent promoter of the Bcl-2 family which present in the cytoplasm. Cells when received apoptotic signals, Bax protein will translocate to the mitochondrial membrane and then bind with other protein to form hybrid dimer (Bax-Bcl-2 heterodimer), this heterodimer will inhibit Bcl-2 protein and therefore prevent the activity of anti-apoptosis. BAX proteins can form a similar Bax-Bax homodimer in the mitochondrial membrane, thus increasing mitochondrial membrane permeability and release of cytochrome C as an apoptosis factor in the cytoplasm to activate the cascade response to apoptosis. Therefore, Bax gene plays a very important role in regulating the signaling pathway of mitochondrial apoptosis (Uhiliarova B and Hajtman A., 2018). Follicles development in connection with necrotic, apoptosis or development until its ovulation is determined by complex interactions and reactions between Bcl-2 family member's proteins. The survival of granulosa cells which promoted by gonadotropin hormones is associated with a decrease in the expression level of Bax, with no significant changes in Bcl-2 and Bcl-XL levels.
High levels of Bax gene are combined with granulosa cell death and follicular atresia. Gene expression encoding Bax was found in fetal germ cells and increased expression was found to correlate with the occurrence of fetal germ cell apoptosis, but without any change in Bcl-2 level (Kim and Tilly, 2004). It has been observed that in the absence of Bax protein, granulosa cells are unable to enter into apoptosis process, thus this will prolong mice reproductive life which were genetically engineered with this deficiency. The apoptosis mechanism induced by the heat shock response is still unknown. Several studies showed that the rate of apoptosis can increase depending on the ratio of Bcl-2/Bax proteins as well as Ca+2 and P53 are thought to be involved in the regulation of this protein (Yic, 1997; Ross, 1988; Fadhel, 2019; Villunger, 2003; perfettini, 2004). Regarding to above, this research aimed to study two periods of times, the first one representing the summer and the second is winter, to better understand the effect of heat on Bax gene expression.

2. Materials and Methods

Gene analysis

A biopsy of ovarian tissue was taken from the experimental sheep ovaries using special autopsy tools and taken to the laboratory. The process of extracting the RNA genetic material was carried out and a GENEzol TrRNA Pure Kit from Macrogen Company (Korea, Kit) was

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used. Next, rtPCR technique was applied to obtain the quantitative expression of Bax gene using the primers (F: CATGGAGCTGCAGAGGATGA)

(R: GTTGAAGTTGCCGTCGGAAA)

2.2 Removing of ovaries and measuring their dimensions.

The ovaries (100) were removed from their attachment to the ovarian ligament using Surgical Scissors, and the distant tissues and ligaments were cleaned using scalpel blade. Next, ovaries were washed with cooled physiological saline, and placed on filter papers to dry them. After that, the dimensions of the right and left ovaries (length, width and thickness) were measured using vernier clippers, as its length was measured from the side of the cut area with the ovarian ligaments, or the length of the space between the front and back end and its width from the widest area or line outside of the line Length, and thickness from the dorsal surface to the ventral surface using vernier clippers

2.3. Follicles measurement

Visible follicles on the surface of the ovary were measured starting with those with a diameter of 1 mm or above, and the outer diameter of the follicle was measured using vernier clippers and the follicles were classified according to these measurements into three categories: small with a diameter less than 3 mm (< 3 mm), and the second medium with a diameter (5 - 3 mm), and the third is large, with a diameter greater than 5 mm (>5 mm) (Nandi et al., 2007).

2.4. RNA Extraction

A biopsy of (1mm) tissue was taken from the surface of the ovary with a medical scissors, and it was frozen in (2ml) tubes then a GenezolTM TriRNA Pure kit was used and then the tissue samples were washed with phosphate buffer solution to get rid of (RNA Later). Then the tissue was cut into small pieces and preparation was made by the process of (Vortex mixer. Then the tissue was precipitated from the saline solution through Centrifuge. Furthermore, Phosphate buffer was added and the process was repeated. A 700 µl of Trizol was added and tissue was then crushed with a K Max into smaller pieces to facilitate RNA extraction. Then the process of (Vortex mixer) was repeated and left for a period of (10) minutes to precipitate the filtrate. The filtrate was withdrawn using micropipette to extract the RNA (filtrate has become carrying the RNA). Ethanol was added by the volume of the liquid (the filtrate) that was withdrawn (650) microliters. Then 1ml of washing buffer was added to wash the RNA. The DNA was digested by DNase enzyme and the RNA was extracted. It was left for 15 min, then (400) microliters of washing buffer (pre wash buffer) and added into Micro-Centrifuge device for centrifugation. A 600 µl of Wash buffer was added and the process was repeat twice, then dried completely by placing in Micro-Centrifuge device for Centrifugation without adding any filtrate. (100) microliters of RNAs-Free Water (which collects RNA) were added, then placed in a Micro-Centrifuge device. Then the purity and concentration of RNA was measured by adding 2 microliters of the sample with a NanoDrop device, where it was Determination of RNA concentrations by measuring the absorbance at 260 nm wavelength (A260) and wavelength 280 nm (A280). Optical spectroscopy was performed at these specific wavelengths. Whereas, the acceptable ratios are usually from 1.8 to 2.0 ng ul/ RNA.

3. Results and Discussion

3.1. Season

Table (1) showed that there were no significant differences in the critical cycles (Ct) of the beta-actin gene expression between the first and second period, and there were significant differences ($P \leq 0.01$) of the Bax gene with 21.26 ±0.45 in the first period and 19.35 ±0.26 for the second period and this represents the direct expression of both genes. As for the relative expression, it decreased in the first period from what it is in the second period, and this result appears to be expected, because the first period represented in the month of August which has a remarkable reproductive performance that is reflected in the hormonal activity, this result is consistent with the fact that the level of estrogen hormone is high in the first period compared to its level in the second period (Table 6), as there is an inverse relationship between these hormones and the occurrence of apoptosis as a result will inhibit or reduce the gene expression of programmed death genes including Bax. In general, the relative expression difference is slightly between the two seasons, and this may be due to the lack of clarity of the seasonality of breeding in Iraqi sheep, or to the small size of the sample available in the study.

Table 1. Critical cycles (CT) and the relative expression of beta-actin and Bax genes for the first and second periods.

periods	Mean ± SD		2Δct	Relative expression
	Beta actin Gene	Bax Gene		
The first period (August)	0.24±19.89	0.26±19.35	0.6877	0.9592
The second period(January)	0.54±20.78	0.45±21.26	0.7169	1
Morale level	NS	**		
(**P≤0.01)،NS :Not Significant.				

Follicles size

The results in Table (2) exhibited that there were no significant differences in the (Ct) value of the beta-actin gene expression (comparison gene) in regard

to the size of the follicles, while there were highly significant differences (P≤0.01) in the critical cycle (Ct) for the direct gene expression of Bax gene with the highest value for 3-5 mm category and then 5 mm (21.13 ± 0.57 and 20.88 ± 0.45) respectively. Also, the results indicated, that there were non-significant differences between (3-5 mm) and (> 5 mm) with 21.31 ± 0.57 and 20.88 ± 0.45 respectively. Then, it was followed by (<3mm) follicle size with (19.36 ± 0.38). This indicates that the direct gene expression was the highest in the category of sizes less than 3 mm compared to the other two categories, but the relative expression is the most important as it takes into account the expression of the target gene compared to the comparison gene (reference gene). Additionally, Table (2) showed that the relative expression of Bax gene was higher in the first group (<3mm) compared to the other two groups (3-5 mm) and (> 5 mm), and this indicates that the expression decreased with the increase in the size of the follicles in the ovary.

Table 2. Critical cycles (CT) and relative expression of beta-actin and Bax genes depending on the size of ovarian follicles.

Categories (follicle size)	Mean ± SD	2Δct	Relative expression	
	Beta actin Gene	Bax Gene		
First class (Less than 3mm)	0.45±19.82	0.38±19.36 b	1.3755	1
Second class (3-5mm)	0.49±21.30	0.57± 21.13a	1.1250	0.8178
Third class (Greater than 5mm)	0.56±19.83	0.45± 20.8 a	0.4829	0.3510
Morale level	NS	**		
(**P≤0.01)،NS :Not Significant.				

4. Conclusions

This study clearly indicate an increase in the gene expression of the programmed death gene (Bax gene) during the period of decline in reproductive activity represented by cold days (January) compared to a decrease in gene expression in August, which is a period of reproductive activity, and the reason behind that is the increasing in the activity of reproductive hormones which inversely act with the programmed death genes. As for the size of the follicles, it seems that the increased activity of programmed death genes in the developing small follicles may have come as a result of the increase in errors in the cell divisions of the developing cells as a way to get rid of the abnormal cells.

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