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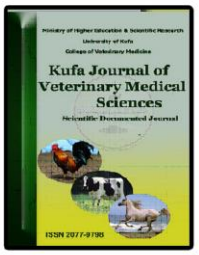

Effect Of Castration Methods On Gene Expression Of Androgen Receptor Gene In Skeletal Muscles Of Awassi Sheep

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Abstract

The effect of castration on skeletal muscle development in awassi sheep were studied at the gene expressed for androgen receptor. The result showed that live weigh for epididymectomy rams was heavier than control and orchidectomy rams, respectively. There was significant difference ($p < .05$). By using the (2^ΔCT) with reference gene method, the findings documented that there was higher up-regulated in mRNA expression for androgen receptor in splenius muscles cells for epididymectomy rams compare with interact rams. In contrast, there was down-regulated for mRNA expression for androgen receptor cells for orchidectomy rams. In conclusion the continuous producing of testes hormones lead to growth of skeletal muscles of wether's significant increase the growth of skeletal muscles of wethers by epididymectomy companied with other rams.

Key words: Awassi Sheep, Orchidectomy, Epididymectomy, Androgen gene expression, skeletal muscle

تأثير طريقة الاخصاء على التعبير الجيني لجين مستقبلات الاندروجين في العضلات الهيكلية لأغنام العواسي

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الخلاصة:

تم دراسة تأثير نوع الأخصاء على تطور العضلات الهيكلية في اغنام العواسي من خلال تعيين التعبير الجيني لجين مستقبلات الاندروجين, حيث أظهرت النتائج بان الوزن الحي للاكباش المخصية بطريقة ازالة جزء من البربخ كان وزنها اعلى من الاكباش في مجموعة السيطرة والاكباش المخصية بطريقة ازالة الخصية حيث انه كات هناك فرق معنوي ($p < .05$) كما واثبتت الدراسة بان هناك مستوى عالي للتعبير الجيني للحامض النووي الرايبوزي الناقل لمستقبلات الاندروجين في خلايا العضلة العنقية للاكباش المخصية بطريقة ازالة جزء من البربخ مقارنة مع الاكباش السليمة على العكس من ذلك فأن النتائج اظهرت أن التعبير الجيني لجين مستقبلات الاندروجين كان واطئ لنفس الخلايا في الاكباش المخصية بطريقة ازالة الخصية, من هنا نستنتج بأن استمرارية الخصية بانتاج الهرمونات يؤدي الى زيادة معنوية في نمو العضلات الهيكلية للاكباش المخصية بطريقة ازالة جزء من البربخ مقارنة مع بقية الاكباش الاخرى.

Abbreviations:

AR (Androgen Receptor), (mRNA) messenger RNA, cDNA (complementary DNA is double-stranded DNA synthesized from a mRNA template), SM (Splenius Muscle), RT-PCR (Reverse Transcription polymer change reaction),q (rt-PCR) quantitative (real time PCR) , GAPDH (glyceraldehyde 3-phosphate dehydrogenase), DEPC (Diethyl pyrocarbonate), Se (Stander error), oligo (dT)Primer is suitable for use in first-strand cDNA synthesis with reverse transcriptase.

Introduction

The awassi is the dominant fat-tail breed of sheep in Iraq, it's a utilized for meat, milk, and wool production, found in north of the Al-Amarah liwa and in the Al-Kūt and the lower Tigris marshes, up between the rivers through Al-Hayy, Ad-Daghgharah, As-Samāwah, Al-Hillah and Al-Jazirah (1, 2, 3).

The orchidectomy method is a surgical removal of testes to stopped the production of male hormones (4, 5), its essential that good technique using when applying the surgical method of castration (6), yet the weight and meat from wether high quality than that from interact ram (7). The epididymis is a single, long duct (160 foot) of very small diameter which is coiled into numerous loops, its divided into three areas; the head, body and tail, this function is store sperm cells which produced in the testicle and sperm transport from the testicle to the vas deferens (8), the epididymectomy is meaning of removing the tail of the epididymis is one of the simplest and most economical means of preparing a teaser bull (9).

The androgen is implicated in the increased neck muscle mass in sexually mature rams (10, 11), it have anabolic actions in some skeletal muscles, but the mechanism of androgen control of muscle size is poorly understood (12, 13), this actions of androgens can enhance muscle strength and increase muscle size clinically (12, 14, 15). The ram have relatively greater muscle in the neck and forequarter than wether, although the presence of testicular hormones is related to greater muscle growth capacity in intact males (11). The number of AR varies among skeletal muscles, which may explain regional differences in the physiological response to androgen administration (16, 17, 18).

(19, 20) reported that the identifying genes that are differentially expressed in response to testosterone and growth state could lead to the identification of molecular markers contributing variation to muscle anabolism. The intact male have relatively greater muscle in the neck and forequarter than females or castrates, although the presence of testicular hormones is related to greater muscle growth capacity in intact males (11), these results suggest that the regulation of AR expression are important for sexually dimorphic muscle growth patterns (20 , 21)

The sexual dimorphism in muscle growth relates to the protein anabolic effect of testicular hormones, the SM in rams and wethers implanted with testosterone was heavier and had a biphasic growth pattern (11). The effects of castration and androgen replacement differentially affect

skeletal muscle mass and AR levels (22), the castration in adult animals and guinea pig produces atrophy of several muscles (12, 13). The biological action of androgen is mediated through the AR by binding to specific DNA sequences that influence transcription of androgen-responsive genes (23, 24).

The aim of this study was to investigate variation of the AR gene expression in the skeletal muscles of awassi breed rams after castrated by orchidectomy and epididymectomy methods to construction of the molecular mechanisms in which castration type on skeletal muscle development, and relation this defect in live weight difference in awassi breed wethers.

Materials and Methods

The animals were managed under normal husbandry conditions, twenty-four awassi breeding pubertal rams (n=24) at (13-15) month in al-Diwaniyah, was distribution in to two groups, control group (A) (n=8) and sixteen animals of treated group which divided in to two subgroups, group B1 (n=8) castrated by orchidectomy, and group B2 (n=8) castrated by epididymectomy method, this animals was weighted before and after 60 days from castration, as well as weighted of the group A in same two period.

The biopsies samples taken from SM of group A, yet group B1 & group B2 after 60 days from castration during the summer, the muscle biopsies were performed using a biopsy drill, and transported to laboratory of biotechnology college of Al-Qadisiyah university, then snap-frozen in liquid nitrogen and stored at −70°C until RNA extraction in veterinary hospital laboratory of Al-najaf.

The orchidectomy operated by sedation use xylazine hydrochloride 0.2 mg/kg IM, in dorsal recumbence, the scrotum is pulled distally and excised to exposed the testes then applying traction to each testis, the subcutaneous tissues are closed by absorbable suture material, the skin are closed by an absorbable suture, the wound is allowed to drain. (9, 25).

The epididymectomy method deal with removing the tail of the epididymis, the surgical sites are on the ventral aspect of the scrotum over the epididymal tails, in lateral recumbence, performed local anesthesia (injected 2 ml of 2% lidocaine according to (26)), then incisions above each cauda of epididymis, and separated easily from testes and ligated proximally and distally, so removal of a two cm. segment of the epididymal tails with ligation (9, 27).

The set of primers were designed by using web-based program for primer design is Primer3 http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 _www.cgi. (28), and using the oligo primer Analysis, the GAPDH gene as a housekeeping gene and AR gene primer (target gene), this primers used in quantification of gene expression using quantitative rt-PCR techniques based SYBER Green DNA binding dye, and supported from (BIONEER, KOREA) company. table (1)

The study design

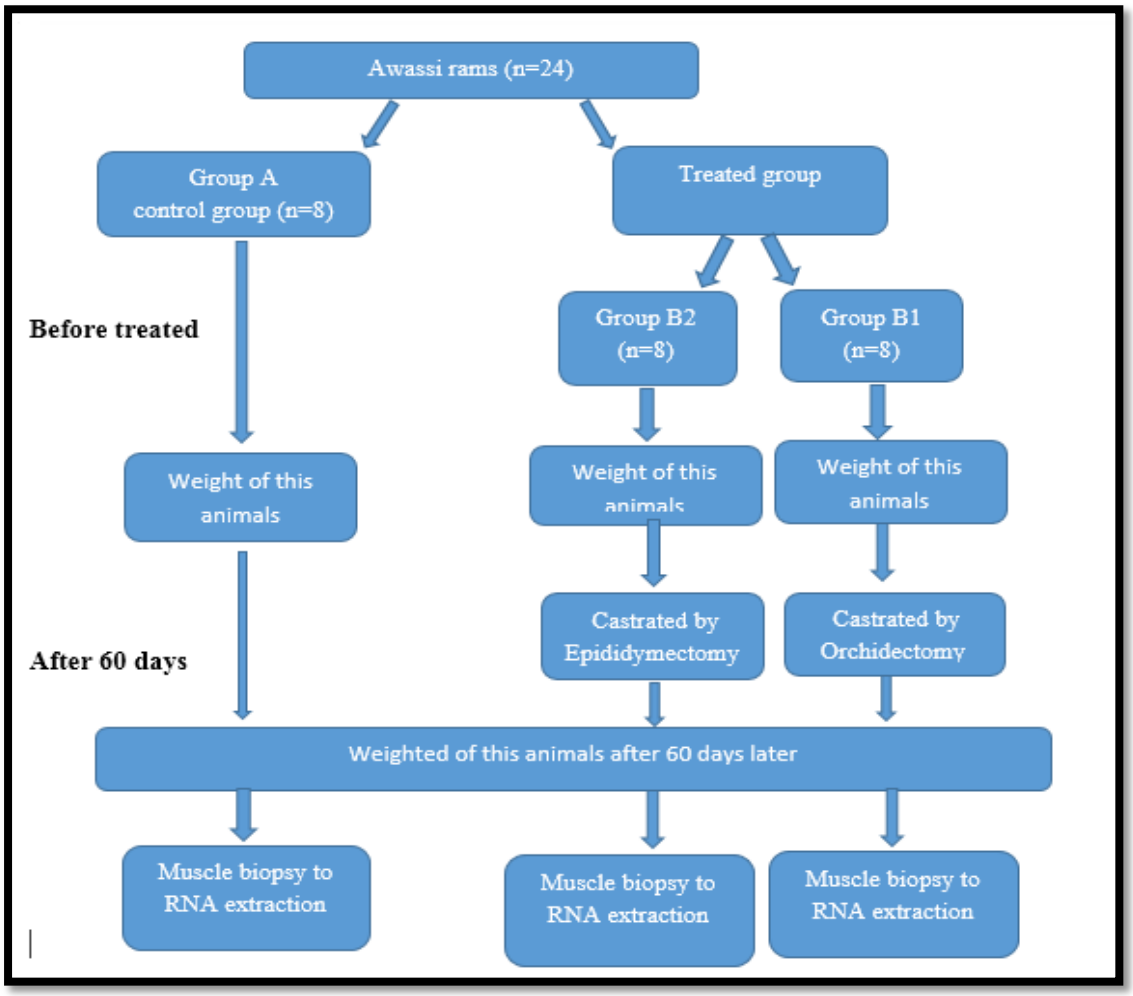

Table (1): The AR and GABDH gene primers with their sequences, product size and PCR conditions.

Primer	Sequences	Product size (bp)	PCR conditions
GABDH- forward GABDH- reveres	5'- AGAGATGCTCTGCTCTGGGA -3' 5'-TCCAAACTGCCTATGCCTGG -3'	bp 567	2 min. 95 °C ,30 sec. 95 °C , 30 sec. 59.3 °C , 60 sec. 72 °C , 5 min. 72 °C
AR-r forward AR-r reveres	5'- AGTTCTCGCGAGACTTTGCA -3' 5'- CCAGCATCACCCCACTTGAT -3'	bp 556	2 min. 95 °C ,30 sec. 95 °C , 30 sec. 58.3 °C , 60 sec. 72 °C , 5 min. 72 °C

Total RNA Extraction

The total RNA was extracted from the SM cells using (Accuzol® Usere manual, BIONEER-Korea) according to the manufacturer's protocol, firstly we adjusted concentrations and purity of the total RNA samples performing by nanodrop spectrophotometer machine (OPTIZEN POP. MECASYS,KOREA) to equals 1.0 for the 40 µg/ µl solution of RNA, and this purity was verified by measuring of the OD_{260}/OD_{280} absorption ratio were applied to all RNA samples in this study was 1.8 -1.9 OD, as well as the RNA integrity by agarose gel electrophoresis were seen two bands of (18S rRNA and 28S rRNA) by U.V light in figure (1), then we treated the extracted total RNA by DNase enzyme to remove the trace amounts of genomic DNA by using (DNase I enzyme, BIOBASIC, USA), according to company instructions. The PCR is a powerful and sensitive tool to quantify a small amounts of mRNA but physiologically relevant changes in gene expression (20), therefore the reverse transcription reaction (RT-PCR) was performed, by used the 1500 ng of RNA to synthesized the first-strand cDNA by oligo (dT) (Oligo-dT (18) Primer, 100ul, BIO PIONEER), according to recommended of the AccuPower® RocktScript RT PreMix kit (BIONEER, KOREA) company, by using real time -PCR system (Excecycler 96) ®, in PCR condition recorded in table (1), the cDNA bands were seen by U.V light, as figure (1).

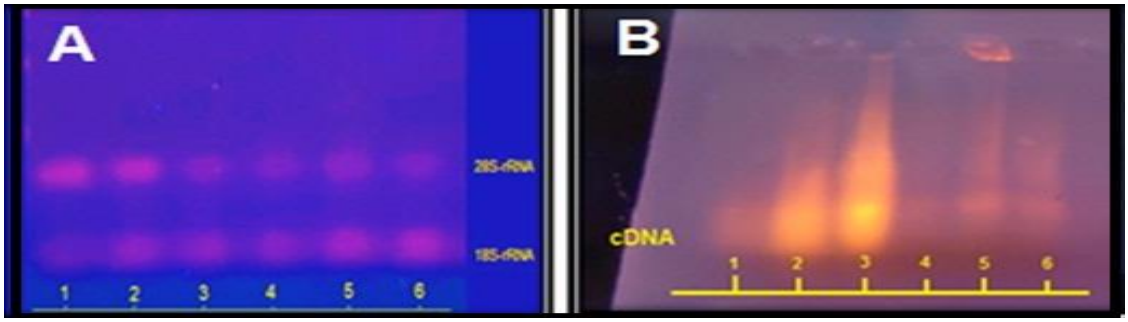

Figure (1): Agarose gel electrophoresis, (A) rRNA(28S-rRNA & 18S-rRNA), (B) cDNA of AR gene in SM cells for (1,2) samples in group A, (3,4) samples in group B1 (after orchidectomy method) and (5,6) samples in group B2 (after epididymectomy).

Data analysis of SYBR green I based rt-PCR assay were divided into primers efficiency estimation and relative quantification of target genes expression level which normalized by housekeeping gene expression (GAPDH). According to method described by (29) calculated the relative expression by q (rt-PCR) for target gene AR in splenius muscle cells of group A in comparison with group B1 and group B2, the ΔCT reference gene method can be used by normalizing gene expression of target genes with gene expression of housekeeping gene (GABDH) as a reference gene. This method used the difference between reference and target Ct values for each sample, the expression level of the reference genes are taken into account using following

formula: Expression value **(fold yield) = $2^{\wedge CT\ (reference) - CT\ (target)}$.**

The q(rt-PCR) was performed by using the SYBR Green PCR Master Mix (AccuPower® Greenstar™ qPCR PreMix reagent kit (BIONEER, KOREA) with cDNA (10 µL) and gene-specific primers (2 µL each forward & revers) completed with DEPC water to 20 µL ml (final volume) reaction mixture on an Exicycler™ 96 Real-Time Quantitative Thermal Block (BIONEER, KOREA) System, the primer sequences were shown in table (1). An RT-minus control was included to detect any contaminating genomic DNA, according to method described by (29).

The regulation of AR mRNA expression was investigated earlier in a variety of cell types and was shown to be both complex and tissue-specific (30, 31), therefore the quantification of AR gene expression in SM cells was perform with internal control gene as a housekeeping gene (GAPDH) was used for normalization of gene expression levels, and Performance of the loaded the specific Exicycler™ 96 Program to relative quantification, according to kit instruction. All the cDNA samples were randomly used for PCR program which consists from initial step at 95℃ for five minutes for one cycle, to activate the Taq DNA polymerase, followed by different five cycles of denaturation at 95℃ for 20 seconds and a combined primer annealing/extension at the 64℃ and 67℃ annealing temperature for 45 seconds for 35 and GABDH-r successively.

Statistical analysis

All the values are expressed as mean ± SE. data of results were analyzed using student t-test and appropriate p-values of less than 0.05 ($P<0.05$) were considered as statistically significant. (32)

Results

The live weight before treated was approximated (20.5375 ± 0.382, 20.6673 ± 0.554 and 20.9443 ± 0.554) kg in groups A, B1 and B2 successively. But the weights were differenced in wethers of group B1 (26.8125± 0.687). kg and in group B2 (31.1875 ± 0.49) kg heavier than rams weight of control group (A) (24.5832 ± 0.382) kg at same age and the difference was significant ($p < .05$), yet that the live weight of wethers in group B2 higher compartment (high Significant differences) with weights data of group B1, table (2). The value of total RNA concentration was highly significant different ($p < .05$), in SM cells of group A, B1 and B2 were 85.89906 ± 5.253, 88.37 ± 4.073, 91.7555 ± 3.583 (ng/µl) successively. table (2)

Table (2): The live weight and total RNA conc. (ng/µl) in the group A, group B1 (after orchidectomy) and group B2 (after Epididymectomy).

Animals	Live weight before treated (mean ± SE)Kg	Live weight after 60 days (mean ± SE)Kg	Total RNA con. before treated (mean ± SE) ng/µl
Group A (n=8)	20.5375 ± 0.382	24.5832 ± 0.382	85.8990 ± 5.253
Group B1 (n=8)	20.6673 ± 0.554	26.8125±0.687 *	88.37 ± 4.073
Group B2 (n=8)	20.9443 ± 0.554	31.1875 ± 0.49**	91.7555 ± 3.583

(*) Significant differences.
(**) High significant difference.
LSD= 4.34

In the our study, calculated the relative expression of target genes in the SM cells used method described by (33) ($2^{-\Delta Ct}$ with a Reference Gene (GAPDH) in this study), and normalized the target genes expression of AR genes in group A was significant ($p < .05$) up-regulated (2.3556),compartment with down-regulated genes expression of AR genes in group B1 (0.3454), as well as this gene expression vary high expression in group B2 significant ($p < .05$) (3.9789). table (3) & figures (2,3,4,5,6)

Table (3): The mean of Ct values of reverence gene (GABDH) and expression value of the AR gene in cells of the SM for group A with group B1 (after orchidectomy method) and group A with group B2 (after Epididymectomy).

samples	Mean of Ct values		ΔCT	(2^ΔCT) Expression value
	GABDH	Target gene (AR)		
Group A (n=16)	26.4435	25.2074	1.2361	2.3556*
Group B1 (n=8)	26.8674	28.4009	-1.5335	0.3454
Group B2 (n=8)	26.6083	24.6160	1.9923	3.9789*

(*)Significant differences.
t-cal.=1.343 ($p < .05$) of group A with group B1
t-cal.=3.0302 ($p < .05$) of group A with group B2

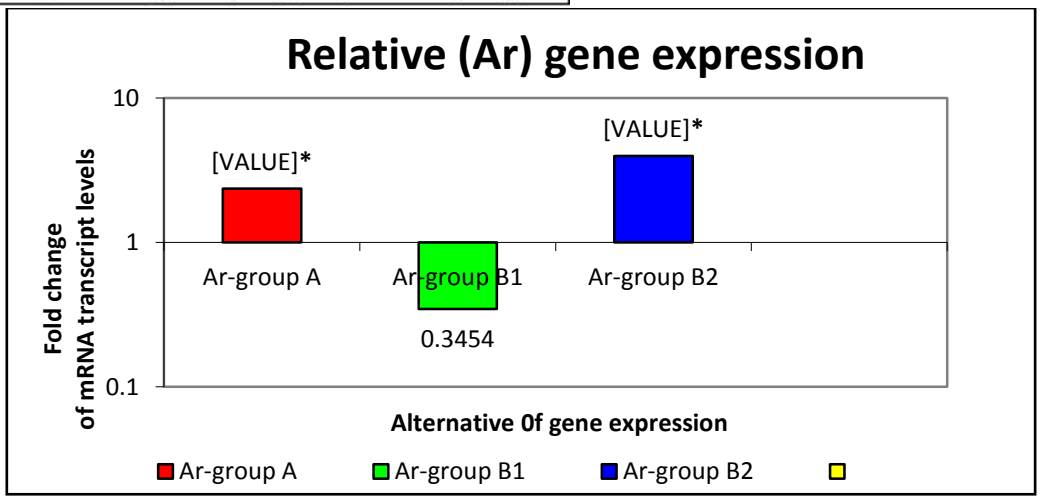

Figure (2): Fold change of mRNA transcript levels of the AR gene in cells of the SM for group A, group B1 (after orchidectomy method) and group B2 (after epididymectomy).
(*)Significant differences.

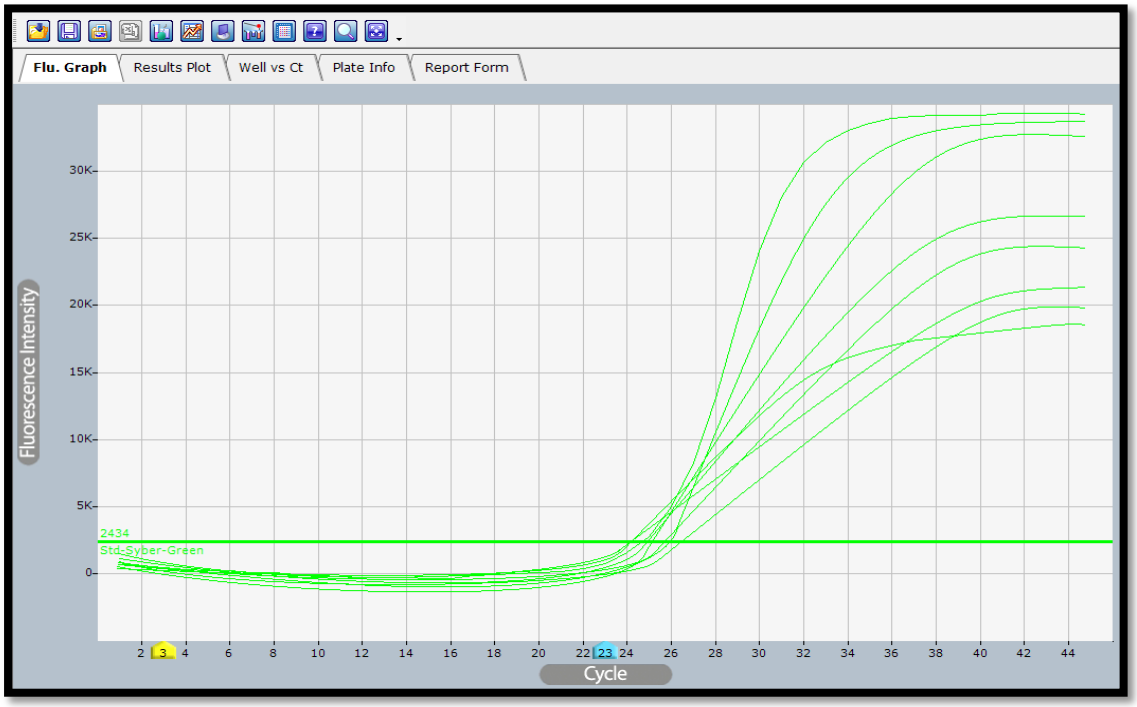

Figure (3): Plot Amplification of rt-PCR of control group A (after 60 days) for AR gene in SM cells (CT=25.2074), by (Excecycler 96) ® system.

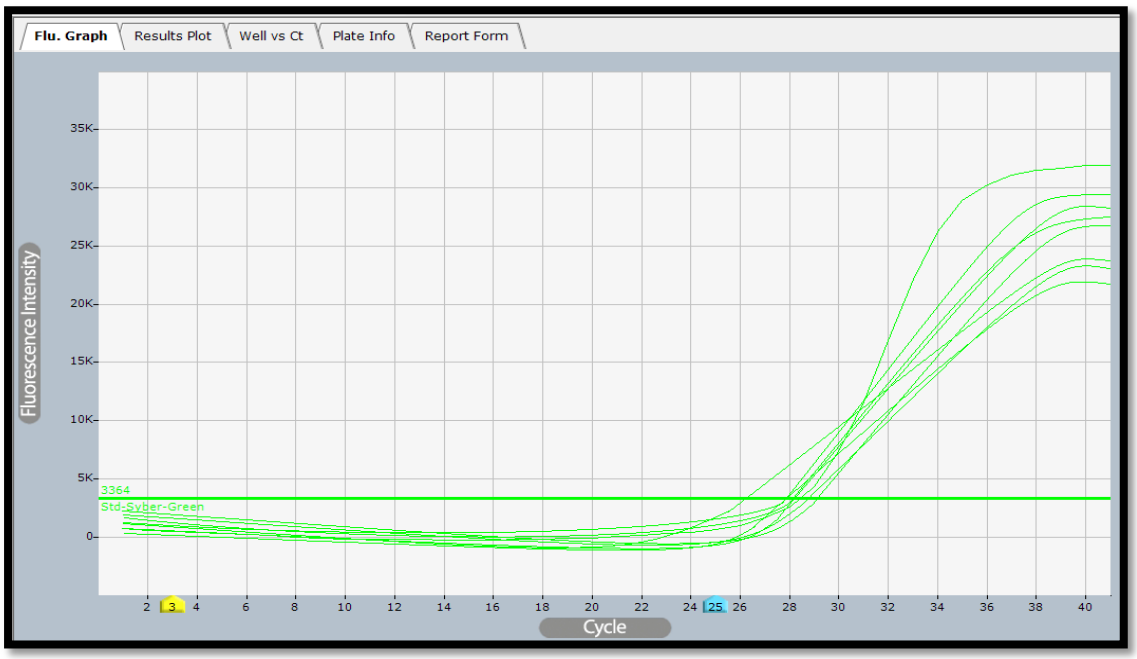

Figure (4): Plot Amplification of rt-PCR of group B1 (after orchidectomy method) for AR gene in SM cells (CT=28.4009), by (Excecycler 96) ® system.

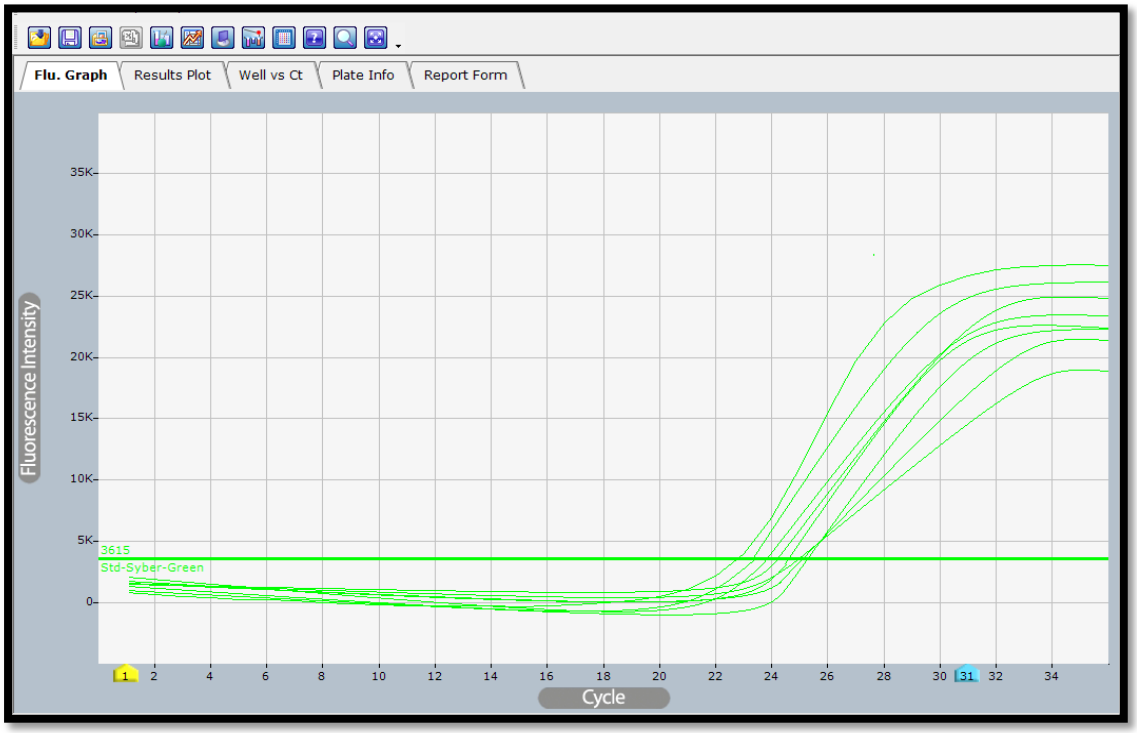

Figure (5): Plot Amplification of rt-PCR of group B2 (after orchidectomy method) for AR gene in SM cells (CT=24.6160), by (Excecycler 96) ® system.

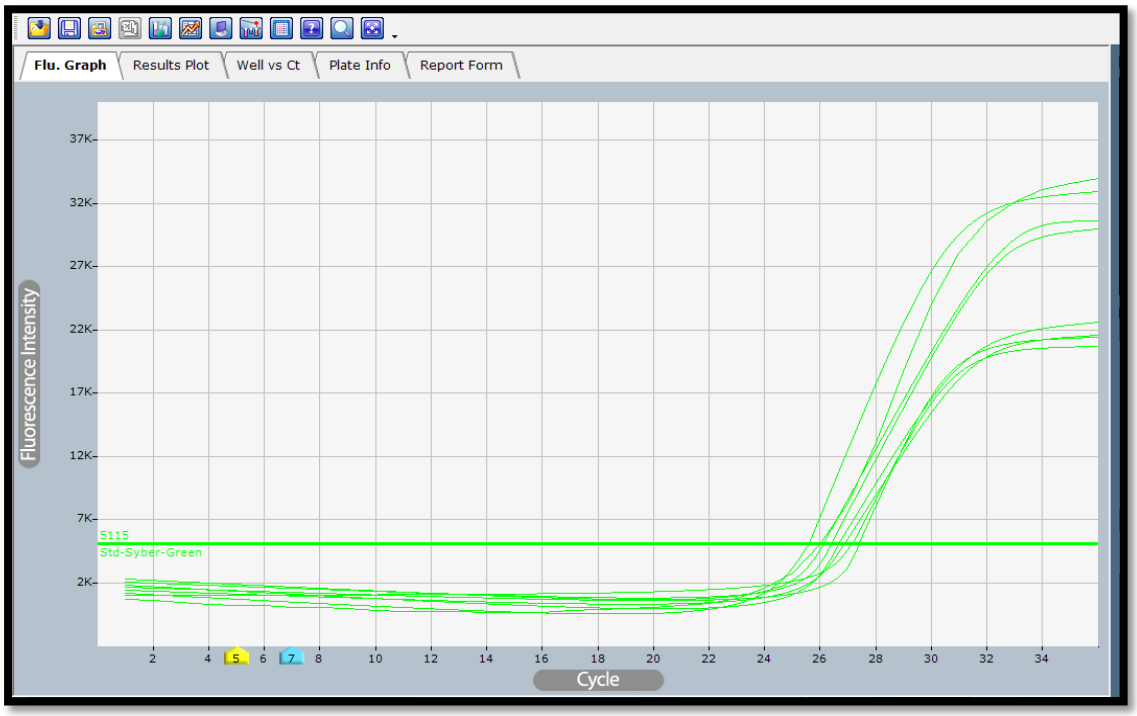

Figure (6): Plot Amplification of rt-PCR of control group A for GABDH gene in SM cells (CT=26.4435), by (Excecycler 96) ® system.

Discussion

The our results showed that the means and SE. of the live weight the interact ram (group A,B1 and B2) at three month age were (20.5375 ± 0.382, 20.6673 ± 0.554 and 20.9443 ±

0.554) Kg successively , but when we classified the treated group in to two group, castrated ram by orchidectomy method (group B1) this means and Se.(26.812 ± 0.687), whereas in castrated ram by epididymectomy method are significant ($p < .05$) (31.1875 ± 0.49) Kg (table 1), therefore in this study the results showed that the weight group B1 and group B2 more than group A at same age, yet the weight of group B2 higher than group B1, this finding refer to the actually that the male animals are generally have more muscle, especially in the neck and the forequarters. Different growth rates for individual muscles for meat production as improving muscle growth (34) and the atrophy of these muscles after castration (13).

The increase live weight of group B2 were supposed the study of (11) and (35) which hypothesis that the whether muscles of the neck (SM) are more responsive specifically to testosterone. Because the mechanisms of testosterone effects on the relationship of SM weight to combined muscle weight, and the testosterone-induced muscle synthesis in relation to muscle weight for these cellular aspects of muscle growth (11, 20).

The results of this study confirm the relative mRNA expression for the AR gene was high regulated in SM cells of group A (entire rams) alternative for mRNA expression of this genes in group B1 (castrated rams by orchidectomy method), table (3) & figures (2,3). This a high regulated result are helped to explain the pronounced muscle growth in the neck of maturing rams (11) and agree with previous studies like (36) and (37), that the down-regulation of AR gene expression following castration (table,3 & figures 2,3), agree with study of (19) which explain the negative effect of castration on skeletal muscle development in male Pigs, and in the orchidectomy of hamster has been same this result (Drengler et al., 1998), in bovine skeletal muscle (36), maybe that's alternative because reported of the (11) and (38) which are referred to the SM in rams with testosterone administration was heavier growth compared of the same muscle in wethers, yet the higher sensitivity to testosterone in the neck muscles regulate by specific genes (18, 10)

Conclusion

The our study concluded that the epididymectomy method of the rams (awassi breed) produces significant positive effects on skeletal muscle growth (SM), yet the orchiectomy method had been negative effected on growth of this muscles. In addition, we examined how the expression of AR mRNA is regulated in the skeletal muscles (SM) of this rams before and after castration. We showed that locally expressed AR gene mRNA levels are down-regulated following orchiectomy method, this is perhaps one of the reasons for the negative effects on skeletal muscle development in wethers, as well as the up-regulation of expression for AR gene mRNA levels after epididymectomy, that may be continues the testes hormonal produce lead to heavily growth of skeletal muscles in wethers.

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