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Effect of exogenous hormones in the expression level of *OXTRs* gene in cows using Rt PCR

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Abstract

We aimed in the current study to investigate the effect of oxytocin and prostaglandin on the expression level of the oxytocin receptor gene (*OXTRs*) in local Iraqi cows at postpartum period. A total of 30 local Iraqi cows were divided randomly into three groups; the first group was considered a control group. The second group was injected with oxytocin 100 IU/IM twice weekly for four weeks postpartum. The third group was injected with PGF2α at a dose of 500 µg/I.M. twice weekly for four weeks postpartum. The blood was collected twice weekly for four weeks from the jugular vein for DNA extraction and to measure the *OXTRs* receptor gene by real-time PCR. The current study showed that the *OXTRs* gene expression level was insignificant in the first week between the three groups. In the second, third, and fourth weeks, the oxytocin group showed the highest significant *OXTRs* expression level, followed by the progesterone group compared to the control group. In conclusion, this study provides evidence that *OXTRs* expression in bovine blood plasma regulates by oxytocin and prostaglandin hormones during the postpartum period.

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Introduction

Oxytocin is an essential hormone released from the pituitary gland from the posterior part under the influence of the hypothalamus. It is a polypeptide that acts mainly as a neuropeptide hormone (1). and is released directly into the blood, and has a significant role in the uterine contraction to reduce labor and increase milk production. It is released and affected by the sexual activity of animals and during the birth process (2). This initial release and further uterine contraction will induce positive feedback to the pituitary and hypothalamus to increase the level of oxytocin in the bloodstream; a similar pathway and positive feedback also can be observed during lactation in animals (3). Recent studies showed that the *OXTRs* have great importance in reproduction (4) and the lactation process, especially after revealing the fog about *OXTRs* expression sites (5), in which the *OXTRs* are overexpressed in sexual activities and postpartum periods; even more, it is known as love receptor (6). The *OXTRs* can observe in different types of tissues, including liver cells, kidney tissues, testis tubules, pituitary gland, heart myofibers, endothelial cells of the vascular system, osteoclast, and myofibers in the uterus, and even more in the cancerous cells (7). The *OXTRs* are activated as a response to the activation of the G-protein receptor on the cell membrane to induce their desired effect. It has seven transmembrane domain receptors, a class I family of G-protein-coupled receptors (GPCRs) (8). In addition, the vasopressin receptor on the cell membrane act for a substantial increase in uterine sensitivity toward *OXTRs* (9). At postpartum, *OXTRs* in myometrium suffer from down expression, while *OXTRs* in mammary glands overexpress to promote the lactation period (10). This regulation in *OXTR* receptor over and down expression allows the circulating *OXTRs* to change their target tissue and exert their effects during estrus, ovarian rebound, parturition, and lactation (11).

The current study investigates the effect of exogenous injection of oxytocin and prostaglandins in the expression of oxytocin receptor gene in local Iraq cows during the postpartum period using the R- PCR technique.

Materials and methods

Animals

Thirty local Iraqi breed cows were randomly divided into three groups (10 cows in each group). The first group was considered a control group. The second group was injected with oxytocin 100 IU/IM (Interchemie Werken, Holland) twice weekly for four weeks postpartum. The third group was injected with PGF2α at a dose of 500 µg/I.M. (Interchemie Werken, Holland) twice weekly for four weeks, starting from the 3rd day after parturition.

Samples collecting

The blood collected from the jugular vein in a vacutainer tube without anticoagulant is used for DNA extraction in real-time PCR to measure the *OXTRs* receptor gene. The collected samples were centrifugated at 3600 rpm for 10 minutes, and the serum was kept at -20ºC.

Conventional PCR primers

The primers used to sequence *OXTRs* describe in table 1, and the EasyTaq® PCR SuperMix kit (Transgenbiotech Beijing, China) use to conduct the real-time PCR protocol.

Conventional PCR

This procedure was carried out in a reaction volume of 25 µl according to EasyTaq® PCR SuperMix Catalog Nos. As111-01 manufacturer's instruction (Tables 2 and 3).

Table 1: Oligonucleotide primer used in PCR for detection of the target gene

Gene	Sequence (5'-3')	Amplified fragment (bp)	Gene ID
OXTRs	F: GCATGTTCGCGTCCACCTACCT R: CCCGTGAAGAGCATGTAGATCC	634	28137

Table 2: Reaction components and volume for PCR

Component	Volume
2×EasyTaq® PCR SuperMix	12.5
Forward Primer (10 picomols)	1
Reverse Primer (10 picomols)	1
Template DNA	3
Nuclease-free Water	7.5

Table 3: Stages and temperature of PCR

Stage	Interval	Temperature	Time	cycle
Stage 1	Denaturation	94ºC	30 sec	1
Stage 2	Denaturation	94ºC	5 sec	35
	Annealing	65ºC	30 sec	
	Extension	72ºC	30 sec	
Stage 3	Extension	72ºC	60 sec	1

DNA extraction

The DNA extraction was applied from 100 µl serum. The Quick-gDNA™ Blood MiniPrep (Biosciences, U.K.) Catalog Nos. D3072 and D3073 kit is used for DNA extraction (Table 4).

RNA extraction

Storage temperature - all kit components are stored at room temperature. Before use: 1 Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml RNA Wash Buffer concentrate (R1054) or 192 ml 100% ethanol (208 ml 95% ethanol) to the 48 ml RNA Wash Buffer concentrate (R1055). 2 Reconstitute lyophilized DNase I with DNase/RNase-Free Water, mix by gentle inversion and store frozen aliquots (#E1009-A, 250 U), add 275 µl water (#E1009-A-S, 50 U), add 55 µl water.

Strand cDNA synthesis

The necessary components for cDNA synthesis from total RNA or mRNA are mentioned in table 5. EasyScript® First-Strand cDNA Synthesis SuperMix efficiently synthesizes the cDNA (Cat. No. AE301).

Table 4: Kit composition for Quick-gDNA™ Blood MiniPrep for both D3072 and D3073

Quick-gDNA™ Blood MiniPrep (Kit Size)	D3072 (50 Preps.)	D3073 (200 Preps.)	Storage
Genomic Lysis Buffer	50 ml	2*100 ml	23-25 ºC
DNA Pre-Wash Buffer	15 ml	50 ml	23-25 ºC
gDNA wash Buffer	50 ml	100 ml	23-25 ºC
DNA Elution Buffer	10 ml	2*10 ml	23-25 ºC
Zymo-Spin™ IIC Columns	50	200	23-25 ºC
Collection Tubes	100	400	23-25 ºC
Instruction Manual	1	1	-

Table 5: Kit composition for EasyScript® First-Strand cDNA Synthesis SuperMix

Component	Volume
Random Primer(N9)	1 ul
2×ES Reaction Mix	10 ul
EasvScript®RT/R.I. Enzyme Mix	1 ul
RNase-free Water	to 20 ul
Eluted RNA	5 ul

Real-time PCR

Real MOD TM Green W2 2x qPCR mix is an optimized ready-to-use solution for real-time quantitative PCR assays, incorporating SYBR Green I dye. It comprises Taq DNA Polymerase, ultrapure dNTPs, $MgCl_2$, and SYBR Green I dye. was activated the DNA Polymerase at 95°C (Table 6). This prevents the extension of nonspecifically annealed primers and primer dimers formed at low temperatures during q PCR setup (Table 7).

Table 6: Materials and kits used in real-time PCR

Reagent	Volume
Real MOD™ Green W2 2x qPCR mix	10 µl
Forward Primer (10µM)	2.0 µl
Reverse Primer (10 µM)	2.0 µl
Template DNA	4 µl
DNase/RNase-free Water	Up to 20 µl

Table 7: The PCR Programs Conditions for each Primer understudy

qPCR Steps	Temp.	Time	Cycle
Initial activation	95°C	10 minutes	1
Denaturation	95°C	30 seconds	
Annealing	60°C	30 seconds	40
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	1

Results

The result of conventional PCR techniques for detection of *OXTRs* mRNA expression in experimental cows revealed a band of the nucleic acid of *OXTRs* 634 bp from study animal (n=30) cows in postpartum to period at four weeks animals disrupted for three groups, control group, treated group with oxytocin, lastly treated group of $PGF_2\alpha$. The effect of oxytocin treatment on *OXTRs* mRNA expression in cows was examined, there was an increase in *OXTRs* mRNA band in the first, second and third week after treatment by injection with oxytocin twice doses per week, and $PGF_2\alpha$ treatment this increase in *OXTRs* mRNA expression reach a significant level in the third and fourth week from experimental. At the same time, the result showed that the *OXTRs* mRNA expression was low identified in the control group in fourth-weeks postpartum from experimental (Figures 1-3).

The current study showed that the OXTRs gene concentration was insignificant in the first week between the control group 1.0689±0.1243, oxytocin group 1.0645±0.230, and prostaglandin group 1.0599±0.1299 (Table 3). The current study showed that the OXTRs gene concentration in the second week was the lowest significant concentration in the control group, 0.9011±0.0980, increased in the prostaglandin group at 0.9048±0.0450, and the highest significant concentration in the oxytocin group 2.0957±0.2603 (Table 3).

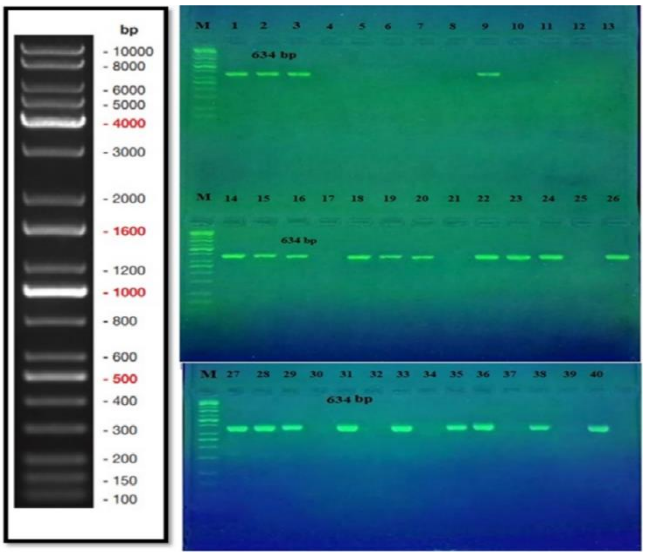

Figure 1: Agarose gel electrophoresis of PCR products for the control group. The positive result for *the OXTRs* gene at 634 bp. The product was electrophoresis on 2% agarose at 5 volt/cm^2, 1x TBE buffer for 1:30 hours, N: DNA ladder (100), *and OXTRs* gene for 1-4 weeks.

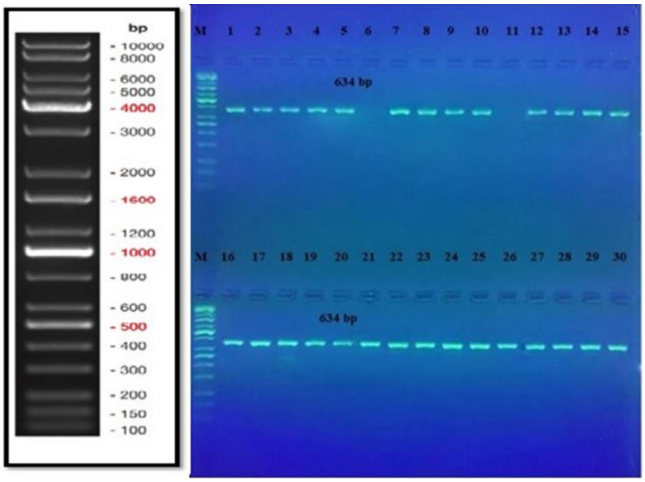

Figure 2: Agarose gel electrophoresis of PCR products for oxytocin group. The positive result for *the OXTRs* gene at 634 bp. The product was electrophoresis on 2% agarose at 5 volt/cm^2, 1x TBE buffer for 1:30 hours, N: DNA ladder (100), *and OXTRs* gene for 1-4 weeks.

In the third week, the OXTRs gene concentration was in the lowest significant concentration in the control group at 0.5321±0.3641, increased significantly in the oxytocin group at 4.5415±0.6785, and in the high significant concentration in the prostaglandin group 2.1318±0.1711 (Table 3). While

in the fourth week, the OXTRs gene concentration was at the lowest considerable concentration in the control group at 0.2447±0.0694, increased significantly in the prostaglandin group at 4.7898±0.3275, and in highest significant concentration in the oxytocin group 6.8704±0.2674 (Table 8, Figures 4 and 5).

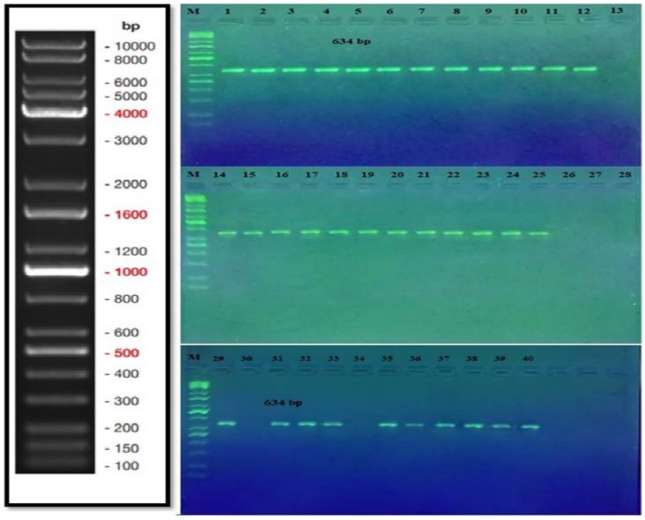

Figure 3: Agarose gel electrophoresis of PCR products for prostaglandins group. The positive result for *the OXTRs* gene at 634 bp. The product was electrophoresis on 2% agarose at 5 volt/cm^2, 1x TBE buffer for 1:30 hours, N: DNA ladder (100), and *OXTRs* gene for 1-4 weeks.

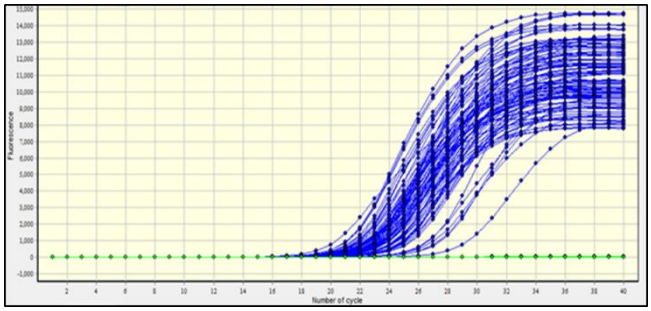

Figure 4: Stander housekeeper gene expression level of oxytocin receptor gene in blood plasma.

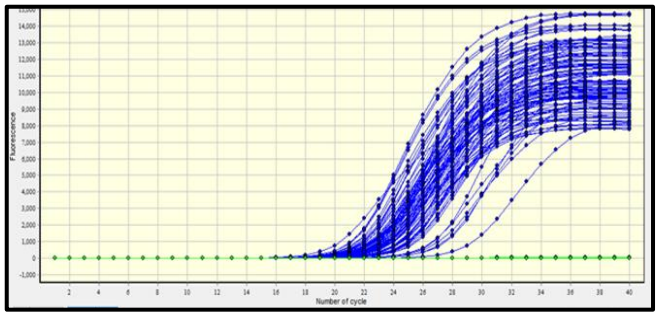

Figure 5: Real-time PCR of specific amplification curve of oxytocin receptor gene.

Table 8: *OXTRs* gene concentration in cows' postpartum

Postpartum time	OXTR-gen concentration means concentration (ng/ml) ± SE		
	Control group	Oxytocin group	Prostaglandin group
1st week	^{A} 1.0689±0.1243 ^{a}	^{D} 1.0645±0.1242 ^{a}	^{C} 1.0599±0.1299 ^{a}
2nd week	^{A} 0.9011±0.0980 ^{a}	^{C} 2.0957±0.2603 ^{b}	^{C} 0.9048±0.0450 ^{a}
3rd week	^{B} 0.5321±0.3641	^{B} 4.5415±0.6785 ^{a}	^{B} 2.1318±0.1711 ^{b}
4th week	^{C} 0.2447±0.0694 ^{c}	^{A} 6.8704±0.2674 ^{a}	^{A} 4.7898±0.3275 ^{b}

Different vertical capital letters mean significant differences between weeks within the same group. Different horizontal small letters mean significant differences between groups within the same week.

Discussion

OXTRs mRNA gene was highly expressed in the oxytocin-treated twice-weekly for the four-week group because a sudden increase in oxytocin response coincided with a sharp rise in endometrial *OXTRs* density, suggesting that physiology regulation of oxytocin may be determined at the receptor level and involve *OXTRs*. (12), the *OXTRs* receptor up or down-regulation by the pattern of sex steroids hormones, especially estrogen and progesterone (4). This is why oxytocin stimulates the lining of the uterus to release $PGF_2\alpha$ to establish a positive feed loop with endometrial to regulate and recreative corpus luteum (13), myometrial concentration via oxytocin increases in intracellular calcium, possible by blocking Ca^{+2}-Mg^{+2} ATPase mediated calcium extrusion that stimulation of myometrial receptor (14). This positive feedback loop caused stimulation hypothalamus and pituitary gland to produce the gonadotropin hormones especially ovarian steroids have direct effect on *OXTRs* mRNA expression was indicated that estrogen induce a substantial rise in uterine *OXTRs* mRNA, this is in keeping with the impact on uterine oxytocin binding (9), that the estrogen - induced *OXTRs* gene up-regulation occurs by increased receptor biosynthesis (15) on *OXTRs* mRNA expression was indicated that estrogen cause a substantial rise in uterine *OXTRs* mRNA, this is in keeping with the

effects of estrogen on uterine oxytocin binding (16), that an increased production of *OXTRs* that lead to, rather, this affects the appearance and increase of oxytocin receptors in the blood plasma of treated cows, and this in turn will lead to an increase *OXTRs* receptor gene promotor (17), but in average condition this procedure occur typically and consuming more time depended on normal physiology and stander level of oxytocin secretion from pituitary gland to stimulate the endo thecal cell of uterus to produce $PGF_2\alpha$ may extend 15 to 17 days to complete and the corpus luteum decomposition (18), while in $PGF_2\alpha$ treated group the result showed in fourth week high expression of *OXTRs* mRNA which interacts with exogenous $PGF_2\alpha$ injection because the $PGF_2\alpha$ act as potent luteolytic agent specially during pregnancy and postpartum period (19), this fact lead to decline in progesterone in peripheral circulation (20). Moreover, progesterone has an inhibitory regulation *of OXTRs* receptor gene expression (21,22). Progesterone and estrogen withdrawal could affect the level of *OXTRs* mRNA (23).

Conclusions

In conclusion, *OXTRs* mRNA expression in bovine blood plasma regulates oxytocin and prostaglandin hormones during postpartum.

Acknowledgments

We thank the College of Veterinary Medicine, University of Mosul, for supporting this study.

Conflicts of interest

No conflicts.

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تأثير حقن الهرمونات على مستوى إظهار جين مستقبلات الاوكستوسين في الأبقار باستخدام تفاعل السلسلة المتبلمرة في الزمن الحقيقي

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الخلاصة

كان الهدف من الدراسة الحالية التحري عن تأثير الاوكستوسين والبروستوكلاندين في مستوى إظهار مستقبلات جين الاوكستوسين في الأبقار المحلية العراقية خلال الفترة بعد الولادة. تم استخدام ٣٠ بقرة محلية في الفترة بعد الولادة قسمت عشوائيا الى ثلاثة مجاميع، المجموعة الأولى اعتبرت مجموعة سيطرة. المجموعة الثانية تم حقنها بالاوكستوسين بجرعة ١٠٠ وحدة دولية/بقرة في العضلة مرتين أسبوعيا ولمدة أربعة أسابيع. المجموعة الثالثة تم حقنها بالبروستوكلاندين بجرعة ٥٠٠ مايكرو غرام/بقرة في العضلة مرتين أسبوعيا ولمدة أربعة أسابيع. تم جمع عينات من الدم مرتين أسبوعيا ولمدة أربعة أسابيع من الوريد الوداجي من اجل استخلاص الدنا لغرض قياس مستقبلات جين الاوكستوسين باستخدام تفاعل السلسلة المتبلمرة في الزمن الحقيقي. أظهرت نتائج الدراسة الحالية أن مستوى إظهار مستقبلات جين الاوكستوسين بين المجاميع المختلفة في الأسبوع الأول لم تسجل أية فروقات معنوية مقارنة فيما بينها. أما في الأسبوع الثاني والثالث والرابع سجلت المجموعة المعاملة بالاوكستوسين اعلى إظهار معنوي لمستقبلات جين الاوكستوسين، ثم المجموعة المعاملة بالبروستوكلاندين مقارنة بمجموعة السيطرة. في الخلاصة، زودت هذه الدراسة دليل على أن إظهار جين مستقبلات الاوكستوسين في بلازما دم الأبقار يتم التحكم به من خلال هرموني الاوكستوسين والبروستوكلاندين في الفترة بعد الولادة.