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Assessment of Some Physiological, Immunological and Molecular Biomarkers in Sample of Iraqi Women Undergoing Caesarean Section and Normal Delivery

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Abstract

Pregnancy and delivery are physiological conditions that are marked by abrupt alterations to hormones, immunological and molecular characters. The current study aimed to evaluate oxytocin (OT), prolactin (PRL), cortisol and insulin growth factor-2 (IGF-2) levels as physiological biomarkers; programmed cell death protein-1 (PD-1), programmed cell death ligand-1 (PD-L1),interleukin-6 (IL-6) as immunological biomarkers, and single nucleotide polymorphisms (SNPs; rs53576 and rs2254298) of oxytocin receptor gene *OXTR* as molecular factors in samples of Iraqi women undergoing caesarean section (CS) and normal delivery (ND). Blood samples were collected from 96 pregnant women at term with ages ranging between 16-43 years. Regarding the physiological biomarkers, the study findings indicated that the change in OT, cortisol and IGF-2 between CS and ND groups was not significant, While the PRL level indicated a highly significant (P≤0.001) decrease in the CS group, especially in comparison with the ND group. Immunological biomarkers demonstrated a significant (P≤05) elevation for PD-1 as well as for PD-L1 in the CS group as compared to the ND group. However, PD-1/PD-L1 ratio revealed a high significant (P≤0.001) rise in CS compared with ND. The IL-6 results revealed a significant (P≤0.001) reduction in the CS groups compared to the ND group. And regarding the SNP of *OXTR* (rs53576), the findings revealed no notable association in genotypes CC, TC and TT between both groups. In addition, the mutant C allele and the wild T allele revealed no significant association between CS and ND groups [OR=1.26 (0.68-2.3%)]. The SNP results of *OXTR* (rs2254298) showed a high positive association (P≤0.001) in genotypes GG, AG and AA between CS and ND groups. Also, the mutant G allele and the wild A allele revealed high significance (P≤0.001) between both groups [OR=5.3 (2.8-9.76%)].

Keywords: Normal delivery, Caesarean section, Immune tolerance, Oxytocin receptor, Genetic polymorphism.

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تقييم بعض المعلمات الحيوية الفسيولوجية والمناعية والجزيئية في عينة من النساء العراقيات الخاضعات للولادة القيصرية والولادة الطبيعي

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الخلاصة

يعتبر الحمل والولادة من الحالات الفسيولوجية التي تتميز بتغيرات جذرية في الصورالهرمونية والمناعية والجزيئية. هدفت الدراسة الحالية الى تقييم مستويات الأوكسيتوسين والبرولاكتين والكورتيزول و IGF-2 كمؤشرات حيوية فسيولوجية، و PD-1 و DP-L1 و IL-6 كمؤشرات حيوية مناعية، و SNPs (rs2254298) (rs53576)لجين مستقبلات الأوكسيتوسين كعوامل جزيئية في النساء الخاضعات للولادة القيصرية والولادة الطبيعية. تم جمع عينات الدم من 96 من النساء الحوامل باعمار16-43 سنة.فيما يتعلق بالمؤشرات الحيوية الفسيولوجية، أظهرت نتائج الدراسة وجود فروق غير معنوية فيما يتعلق بـ OT ، الكورتيزول و IGF-2 بين مجموعتي الولادة القيصرية (CS) والولادة الطبيعية(ND) ،بينما أظهر مستوى PRLانخفاض معنوي($P\leq0.001$) عالي في مجموعة CS مقارنة بمجموعةND.أظهرت المؤشرات الحيوية المناعية ارتفاعاً معنوياً ($P<0.05$) في PD-1 و PD-L1 في مجموعة CS مقارنة بمجموعةND ، بينما أظهرت نسبة PD-L1 / PD-1ارتفاعاً معنوياً ($P\leq0.001$) في CS مقارنة معND.أظهرت نتيجةIL-6انخفاض معنوي ($P\leq0.001$)في مجموعة CS مقارنة بمجموعةND.فيما يتعلق بـ SNP لـ (rs53576) *OXTR* ، أظهرت النتائج عدم وجود ارتباط معنوي في التركيب الوراثي CC و TC و TT بين المجموعتين CS وND.بالإضافة إلى ذلك ، لم يكشف أليل Cالطافر وأليل T البري عن أي ارتباط معنوي بين الممجموعتين [OR=1.26 (0.68-2.3%)].أظهرت نتائج SNP لـ (rs2254298) *OXTR* ارتباطاً معنوياً عالياً ($P\leq0.001$)في التركيب الوراثي GG و AG و AA بين مجموعتي CS وND. أيضًا، أظهر أليل G الطافر وأليل A البري ارتباطاًمعنوياًعالياً ($P\leq0.001$)بين المجموعتين
[OR=5.3 (2.8-9.76%)].

Introduction

Pregnancy and delivery are physiological conditions that are indicated by extreme variations in the hormonal, immunological and molecular characteristics. Through these periods, the placenta synthesizes and then secretes a variety of hormones and cytokines which are essential for the management of certain pregnancies and delivering phases, for instance decidualization, implantation and labor, and also for the mother's metabolic adaptation and arranging for breast feeding [1].

Oxytocin is a potent uterotonic agent that is used clinically for the induction and augmentation of labor and initiates uterine contractions by activating the OXTR [2]. During pregnancy and delivery, stressful situations are correlated with an elevation in the PRL and cortisol secretion. Exposure to stress in the early prenatal period may impact the individual phases of neonatal development [3]. On the other hand, IGF-2 is an essential growth factor regulating growth, especially during the normal physiological fetal development [4].

Likewise, gestation is seen as a distinct immunologic paradigm; while it necessitates mother tolerance towards the semi-allogeneic embryo, this also necessitates the conservation of an extremely active immune function to defend both the maternal and the embryo from infections [5]. Failure to build immunological resistance in order to respond to paternal antigens can lead to pregnancy difficulties, including recurrent miscarriage, pre-eclampsia, fetal growth restriction and CS delivery, all of which are connected with an excessive inflammatory reaction [6]. The immune PD-1 and its ligand PD-L1 are important modulators of T cell immunity reactions and the induction of peripheral tolerance in this field [7]. Furthermore, various cytokines have been functionally related to different biological procedures that sustain gestation and induce labor and delivery. In this regard, IL-6, often assumed as a pro-inflammatory cytokine, is considered to be one of the most searched cytokines during pregnancy and delivery as well as in adverse pregnancy conditions [8]. Thus, any disruptions in these physiological and immunological biomarkers contribute to adverse pregnancy consequences such as preterm, labor dystocia and CS delivery [9]. On the other hand, genetic variations that occur in some loci on the OXTR may be related to some problems of pregnancy and delivery. A previous study has suggested that recurrent miscarriage is linked to a genetic variation in the OXTR gene which has the mutation on the site of rs53576 [10]. Moreover, another research on OXTR SNPs has linked a genetic variation in the rs53576, rs2254298 locations to preterm birth [11]. The present study aimed to evaluate OT, PRL, cortisol and IGF-2 levels as physiological biomarkers; PD-1, PD-L1, and IL-6 as immunological biomarkers, and single nucleotide polymorphisms (SNPs; rs53576 and rs2254298) of OXTR as molecular factors in sample of Iraqi women undergoing CS and ND.

Materials and Methods

Studied subjects:

A total of 96 women at term whose ages ranged between 16-43 years and were attending the Hospital of Al-Immamain Al-Kadimain in Baghdad, Iraq through the duration from December 2020 till June 2021, were enrolled in this study. These women were separated into two groups; the first group composed of 48 women with CS, while the second group had 48 ND females as control samples. All women in this study gave consents before participating in this study. Furthermore, this study was approved by ethical committees of Department of Biology, College of Science, University of Baghdad (Reference No. CSEC/0322/0040).

Collection of Blood Samples:

Five ml of blood was obtained from each participant by vein puncture using disposable syringes. The blood samples were divided into two aliquots (4 and 1 ml). The first aliquot was dispensed in a gel tube and was then left to coagulate at room temperature and later centrifuged at 5000 rpm for 10 minutes to collect the blood serum that was then transferred to Eppendorf tubes and kept at-20º C until used for the physiological and immunological examination. The second aliquot was dispensed into vacuum tubes containing EDTA to be used for the molecular examination.

Assessment of the Physiological Biomarkers:

The physiological biomarkers (OT, PRL, cortisol and IGF-2) were estimated by using an ELISA technique(Biotek / USA).

Assessment of the Immunological Biomarkers:

The immunological biomarkers (PD-1, PD-L1 and IL-6) were estimated by using an ELISA technique(Biotek / USA).

Determination of the Molecular Biomarkers:

The molecular biomarkers [*OXTR* SNPs (rs53576, rs2254298)] were determined via allele specific-polymerase chain reaction (AP-PCR) for *OXTR* (rs 53576) SNP and restriction fragment length polymorphisms (RFLP-PCR) for OXTR (rs 2254298) SNP.

Statistical Analysis:

Statistical analysis was carried out using the statistical package for social sciences (SPSS), version 21.0 and Microsoft Excel 2013. The mean ± standard error was used to characterize as numerical data (SE). Independent sample t-test was performed to compare both groups (CS and ND). The genotype and allele frequencies for SNPs were estimated using a straight count approach. Hardy-Weinberg equilibrium (HWE) for each SNP was investigated via the use of the online calculator of Michael H. Court's (2005- 2008). Where *p*-value ≥ 0.05 indicates population consistency with HWE. Odds ratio and its 95% confidence interval were used to evaluate the possible risk of mutation with study groups. The lower level of accepted statistically significance acceptable was ≤ 0.05. aq1

Levels of the Physiological Biomarkers:

The statistical analysis found no significant (P>0.05) difference in means of OT, cortisol, and IGF-2 in the CS group (380.52±29.61 pg/ml), (39.88±2.55 µg/dl) and (11.91±1.72 ng/ml) respectively, compared with the ND group (383.64±31.26 pg/ml), (40.45±1.98 µg/dl) and (14.68±2.30 ng/ml) respectively (Table 1). While PRL level highly significantly (P≤0.001) decreased in the CS group (120.33± 7.36 ng/ml) when compared with the ND group (169.39± 6.94 ng/ml).

Table 1: Levels of some hormones and IGF-2 in the studied groups

Biomarkers(Mean ± SE)	Study Groups		*P -value*
	CS	ND	
OT (pg/ml)	380.52± 29.61	383.64± 31.26	0.942 ^{NS}
PRL (ng/ml)	120.33± 7.36	169.39± 6.94	<0.001**
Cortisol (µg/dl)	39.88± 2.55	40.45± 1.98	0.859 ^{NS}
IGF-2 (ng/ml)	11.91± 1.72	14.68± 2.30	0.337 ^{NS}

NS: No-Significant (P>0.05), ** High Significant (P≤0.001)

The present findings, regarding non-effects of OT hormone on the delivery mode, disagree with a study by Malik *et al.* [12] who mentioned that OT is generally used over delivery to begin and enhance contractions of uterine and hemorrhage reduction at postpartum. Therefore, delivery begins when uterine contractions are more powerful and frequent as the cervix dilates, causing the fetus to gradually descend. Gradually or limited delivery advancement is among the most prevalent issues in intrapartum care, particularly for nulliparous pregnant women. Lack to progress is a big worry since it is one of the main reasons of CS delivery [13]. Previous research found that the OT system has an important part in delivery, although the effects may be indirect since OT is engaged in the activity of numerous transmitter systems, such as the HPA axis and GC receptor function which may be related to OXTR activity [14]. On the other hand, synthetic OT over exposure causes desensitization of OXTR, contributing to reduced uterus contractions, dystocia, instrumental delivery and postpartum hemorrhage [15].

A highly substantial decrease was detected in PRL level in the CS group compared with the ND group which agrees with a study by Kebede *et al.* [16] who found a noticeable reduction in PRL level pre- and postpartum in females with dystocia than in vaginal ND. They explained that dystocia was the cause of the result. There are numerous concurrent reasons of labor dystocia, where mother factors are being much more prevalent than child factors; the most frequent woman cause is primary uterine inertia that can be either complete or partial [17]. However, pregnancy-related levels of PRL rise at highest point in the third trimester, but high levels of PRL during pregnancy do not often result in copious milk production [18]. This is due to the inhibitory influence of progesterone which is produced by the placenta. Progesterone antagonizes the influence of PRL on casein transcription [19].

Regarding the finding of cortisol, no significant changes were found among the two studied groups. Also, a previous study reported no changes in cortisol levels between CS and ND groups [20]. In contrast, another study found a higher level of cortisol at the 3rd phase of delivery between ND females compared to CS delivery [21]. Cortisol plays an essential role as a response to tension and stress that influence the mind. Hence, high levels of stress and anxiety through gestation are related to contrary outcomes for a mother's health, like an elevated cortisol level, pro-inflammatory cytokines, obstetrical complications, CS delivery, postpartum depression and negative impact in the offspring in the postnatal development [22]. According to the present findings, IGF-2 indicated a non- noticeable variance among the 2 studied groups. A previous study [23] was conducted on the peripheral blood and umbilical cord blood at delivery where the results showed no significant difference in IGF-2 levels between the study groups. In contrast, [24] indicated that reduction of the IGF-2, IL-6 and TNF expression in the term is linked with the long duration of labor at the normal delivery

Levels of the Immunological Biomarkers:

Results of the immunological biomarkers showed a significant ($p \leq 0.05$) rise in the immune checkpoints PD-1(0.71±0.05 ng/ml) and PD-L1(0.47±0.02 ng/ml) for the CS group compared with their values in ND group (0.56±0.04 ng/ml) and (0.40±0.01 ng/ml) respectively (Table 2). The PD-1/PD-L1 ratio indicated a highly significant ($p \leq 0.001$) rise in the CS group (1.65±0.06 ng/ml) where compared to the ND group (1.33±0.05 ng/ml); while IL-6 level revealed a highly significant ($p \leq 0.001$) decline in the CS group (6.81±0.42 pg/ml) compared to the ND group (12.18±1.56 pg/ml).

Table 2: Levels of the immunological biomarkers in the studies groups

Biomarkers(Mean ± SE)	Study Groups		*P-value*
	CS	ND	
PD-1 (ng/ml)	0.71± 0.05	0.56± 0.04	<0.05*
PD-L1 (ng/ml)	0.47± 0.02	0.40± 0.01	<0.05*
PD-1/P (ng/ml)	1.65± 0.06	1.33± 0.05	<0.001**
IL-6 (pg/ml)	6.81± 0.42	12.18± 1.56	<0.001**

* Significant ($P \leq 0.05$), ** High Significant ($p \leq 0.001$)

A significant increase in PD-1 level of CS delivery may be explained by the fact that at labor and delivery, the increased PD-1 level indicates an immunosuppressive process. PD-1 causes inhibitionto T-cell propagation, downregulation of pro-inflammatory T-cell action and reduction of cytokine production. Also, it encourages the establishment of Treg cells, raises their function and causes inhibition of Teffector cells [25].

A significant rise of PD-L1 level in the CS delivery group agrees with [26] who recorded an increase in PD-L1 levels in females during the third phase of gestation and suggested that the origin of the prevalent PD-L1 molecules may be from the CD4+T and CD8+T cell surface or from external layer of placenta. Interestingly, the PD-L1 is not only immune checkpoint protein inhibitor that helps in the maintenance of gestation and the induction of immune tolerance for mother and fetus, but is also an inhibitor for internal pain that regulates the excitability of ganglion neurons in the spinal and peripheral nervous systems.

A highly significant increase was found in PD-1/PD-L1 level of the CS group compared with the ND group. Certainly, the PD-1/PD-L1 pathway is an essential regulator of immune system homeostasis by enhancing Treg immunity and suppressing Teffe like Th17 cell responses [27]. A previous study reported that PD-1/PD-L1 induces the immune homeostasis by regulating the Treg/Th17 balance through encouraging the distinction of Treg cells instead of Th17 cells in normal pregnancy, while the abnormal pathway of PD-1/PD-L1 might disturb the Treg/Th17 balance and at the latest cause pregnancy complications such as preeclampsia development, dystocia or prolonged labor [28]. That study also mentioned that Treg cells underneath inflammatory circumstances must transform into pro-inflammation cytokine-producing cells, thus leading to enriching the inflammation cell pool. This illustration may explain the current result of the increase PD-1/PD-L1 in the CS group that indicates the occurrence of an immunosuppression process by increasing Treg cells instead of Th17 cells in this group.

A highly significant decrease in IL-6 levels of the CS group compared to the ND group is consistent with Nandanan *et al.* [29] which reported lower IL-6 level in pregnant women who underwent CS labor compared to women who had ND. These findings demonstrated a strong association among the method of delivery and cytokine levels. Also, the present result is in agreement with HaghshenasMojaveri *et al.* [30] who stated a significant relation among the type of delivery and maternal IL-6 level. According to a previous study, increasing *OXTR* mRNA expression in uterine tissue of a pregnant woman is related to physiological variations in IL-6 concentrations, and that pregnant uterus treatment by IL-6 showed a third to five-foldrise in OXTR mRNA expression suggesting that IL-6 positively regulates OXTR mRNA expression and binding ability[31].

Levels of the Molecular Biomarkers:

In the current study, two SNPs of *OXTR* were determined, one of them (rs53576) by using AP- PCR method and gel electrophoresis of PCR product of gene fragment size (amplicon size = 224 bp), as shown in Figure1. Another SNP (rs2254298) was determined by using RFLP-PCR method and gel electrophoresis of PCR product of gene fragment size (amplicon size = 307 bp) as illustrated in Figure 2.

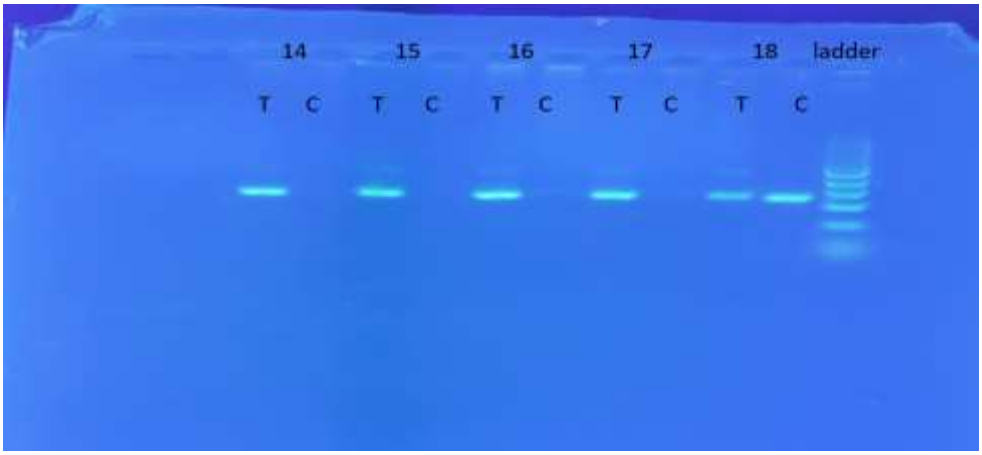

Figure 1: Gel electrophoresis of AS- PCR for detection of OXTR-SNP (rs53576) T/C, product size: 224 bp, sample 14-17: TT, sample 18: TC. Ladder: 100 bp.

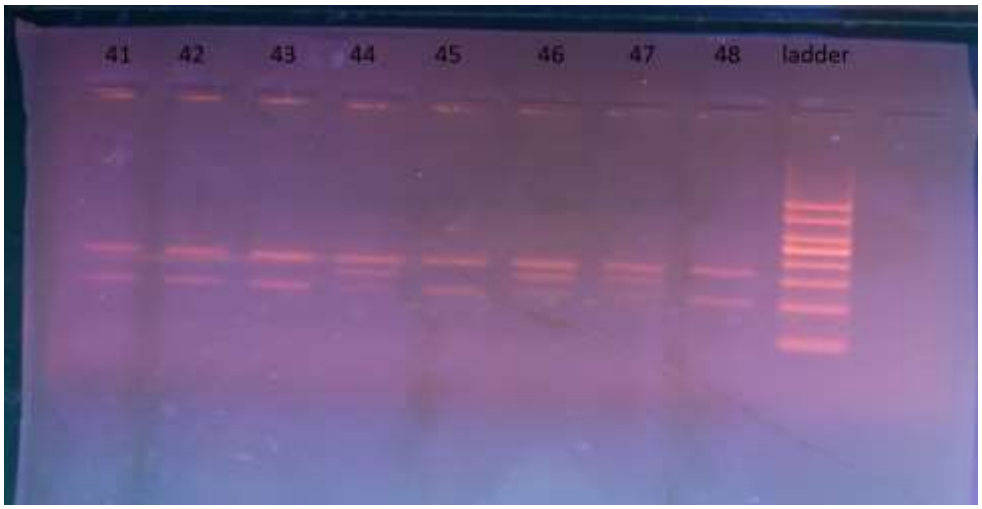

Figure 2: Gel electrophoresis of RFLP-PCR for detection of OXTR-SNP (rs2254298) A/G, product size: 307 bp, restriction enzyme: BsrI, samples 41-43, 45 and 48: G 9/163/34/101 bp, samples 44, 46 and 47: AG 9/101/163/135 bp. ladder 50 bp.

The results summarized in Tables 3 and 4 show the frequency of genotype and alleles in *OXTR* (rs53576) for studied groups. As shown in Table 3, the homozygous genotype CC (27.1 %) was lower while the heterozygous TC (72.9 %) was higher in CS group when compared with their values (41.6 % and 54.2 %)in ND group. The statistical results however showed no significant changes (P>0.05) among them. On the other hand, no significant change (P>0.05) was found in the genotype TT between CS group (0.0%) and ND group (4.2%).

Table 3: Comparison of the genotype of *OXTR* (rs53576) polymorphism between the studied groups by AP-PCR

Genotype	Study Groups		*P-value*
	CS	ND	
CC	13 (27.1 %)	20 (41.6 %)	
TC	35 (72.9 %)	26 (54.2 %)	0.090 NS
TT	0 (0.0 %)	2 (4.2 %)	
Total	48 (100.0 %)	48 (100.0 %)	

NS: Non-Significant (P>0.05)

In addition, the present results revealed a non-significant (P>0.05) difference in C mutant allele between CS group (63.5%) compared with ND group (68.8%), OR = 1.26 (0.68-2.3%). Also, T wild allele revealed non-significant difference (P>0.05) between CS and ND groups [(36.5%) and (31.2%)], respectively (Table 4).

Table 4: Comparison of the allele of *OXTR* (rs53576) polymorphism between studied groups by AP-PCR

Allele	Study Groups		*P-value*	OR (CI: 95%)
	CS	ND		
C	61 (63.5 %)	66 (68.8 %)		
	Mutant			
T	35 (36.5 %)	30 (31.2 %)		
	Wild		0.542 NS	1.26 (0.68-2.3)
Total	96 (100.0 %)	96 (100.0 %)		

OR: Odds ratio; CI: Confidence interval, NS: Non-Significant (P>0.05).

The genotype and alleles frequency in OXTR (rs2254298) for studied groups are shown in Tables 5 and 6. A highly significant (P≤0.001) increase was found in the homozygous genotype GG in CS group (72.9 %) compared with ND group (31.2%), while a strongly significant (P≤0.001) decrease was found in the heterozygous AG in CS group (8.3%) compared with ND group (14.6%). Also, the homozygous genotype AA revealed a strong significant (P≤0.001) decline in CS group (18.8%) compared with ND group (54.2%) (Table 5).

Table 5: Comparison of the genotype of *OXTR* (rs2254298) polymorphism between studied groups by RFLP-PCR

Genotype	Study Groups		*P-value*
	CS	ND	
GG	35 (72.9 %)	15 (31.2%)	
AG	4 (8.3%)	7 (14.6%)	
AA	9 (18.8%)	26 (54.2%)	
Total	48 (100.0%)	48 (100.0%)	<0.001**

**Strong Significant (P≤0.001)

There was a strong significant (P≤0.001) increase in mutant allele G frequency found in CS group (77.1%) compared with ND group (38.5%).Whereas, a strongly significant (P≤0.001) decline was found for the frequency of A allele in CS group (22.9%) compared

with ND group (61.5%). That means pregnant females carry the mutant allele show 5.3 times potential for occurrence of CS when compared to wild type allele carriers, OR= 5.3 (2.8-976%) (Table 6).

Table 6: Comparison of the allele of OXTR (rs2254298) polymorphism between studied groups by RFLP-PCR

Allele	Study Groups			
	CS	ND	*P-value*	OR (CI: 95%)
G Mutant	74 (77.1%)	37 (38.5%)	<0.001**	5.3 (2.8-9.76)
A Wild	22 (22.9%)	59 (61.5%)		
Total	96 (100.0 %)	96 (100.0 %)		

OR: Odds ratio; CI: Confidence interval, **Strong Significant (P≤0.001)

It has been stated that the effectiveness of the uterus can be enhanced by synthetic OT to induce /augment labor, stimulate the delivery of the placenta and avoid bleeding that occurs after childbirth. During labor, endogenous OT binds to its receptor in the smooth muscle tissue of the uterus to cause uterus contractions [32]. Any dysregulation of the OT receptor, genetic variation and structural anomalies could play a significant role in the late-term gestation because the production of the endogenous OT in the myometrium of pregnant women increases synchronously with the rise in OXTR production at the beginning of labor which may be the essential step for the beginning of delivery [33]. A prior study found that the modifications in the OXTR discovered in the human population at large significantly changed the way OXTR functions [12]. Particularly, these variants change how OXTR localizes to the cell membrane, reduce the amount of $Ca2^{+}$ signaling caused by OT and modify the recruitment and signaling of β-arrestin.

Additionally, there are several signs that OT receptor single nucleotide variations might have an impact on how well OT treatments work in terms of labor initiation / augmentation, mode of delivery, and pre-term birth frequency [34]. Another study showed that the allele of the common rs53576 mutation is linked to a prolonged conversion to active phase of labor and that variations in OXTR can elevate the risk of preterm delivery [35]. Moreover, Akdemir *et al.* hypothesized that the OXTR (rs53576) is related to late-term pregnancy by directly modulating the levels of the OT and OXTR [33].

The current findings and the findings of other research support the concept that the variation in OXTR (rs53576 and rs2254298) mostly impacts the mother and fetus psychological and behavioral factors. Pregnant women who carry the mutant allele G were more likely to undergo a CS compared to those who carry the wild allele A, with an odds ratio of 5.3 (2.8-976%).

Conclusion

It can be concluded that monitoring the physiological and immunological biomarkers during pregnancy can avoid pregnancy complications, dystocia labor and thus avoid CS delivery. Additionally, it can also be concluded that pregnant women carrying the mutant G allele show 5.3 times potential for occurrence of CS delivery when compared with wild A allele carriers.

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