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EXPLORING THE ASSOCIATION OF VITAMIN D RECEPTOR GENE VARIANTS AND MULTI-LOCUS HAPLOTYPES WITH RECURRENT PREGNANCY LOSS IN WOMEN FROM KAZAKHSTAN

Ainur Amanzholkyzy[1], http://orcid.org/0000-0002-1980-9032

Talshyn M. Ukybassova[2], http://orcid.org/0000-0002-5098-0727

Aizada M. Marat[3], http://orcid.org/0000-0002-3935-3351

Akbayan B. Turesheva[1], http://orcid.org/0000-0002-4176-4467

Samat S. Saparbaev[4], http://orcid.org/0000-0002-9570-4240

[1] West-Kazakhstan Marat Ospanov Medical University, Aktobe, Republic of Kazakhstan;
[2] CF "University Medical Center", Astana, Republic of Kazakhstan;
[3] NJSC "Astana Medical University", Astana, Republic of Kazakhstan;
[4] Medical Center "Al-Zhami", Astana, Republic of Kazakhstan.

Abstract

Relevance. Reproductive medicine is still faced with a significant problem of recurrent pregnancy loss (RPL), with over half of diagnoses unexplained. Vitamin D deficiency is linked with poorer pregnancy outcomes and polymorphisms in the vitamin D receptor (VDR) gene can affect reproductive health. Evidence from various populations is not uniform; however, no studies have yet been conducted among women in Central Asia.

Objective. The aim of the present study was to examine whether ten common polymorphisms found in the multi-locus haplotypes of the vitamin D receptor (VDR) gene predicted the risk of recurrent pregnancy loss among Kazakhstani women.

Materials and methods. These include 200 women with RPL and 200 multiparous healthy controls of the population from the Republic of Kazakhstan. Genotyping of polymorphisms rs739837, rs7975232, rs1544410, rs7975128, rs2228570, rs731236, rs7967152, rs2238140, and rs2853564 was carried out via high-precision real-time PCR.

Results. All SNPs analyzed were at Hardy–Weinberg equilibrium among healthy controls. No SNPs indicated associations were found between increased or decreased risk of RPL and minor allele frequencies were not different in RPL cases and controls. Genotype frequencies were also comparable across groups in all conditions (codominant, dominant, recessive, overdominant, log-additive). Using the most common GCCGGAAGA haplotype as a reference (OR = 1.00), haplotype analysis showed a negative association between the TATAGGCAG haplotype and RPL (P = 0.001) at baseline, which was held constant after correcting for major covariates (P = 0.006). In an experimental study, after Bonferroni correction, however, the association was less significant (P = 0.059).

Conclusion. We established no significant association between VDR gene polymorphisms and recurrent pregnancy loss among Kazakhstan women.

Keywords: *genotypes; haplotypes; recurrent pregnancy loss; vitamin D receptor.*

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Резюме

ИЗУЧЕНИЕ СВЯЗИ ВАРИАНТОВ ГЕНА РЕЦЕПТОРА ВИТАМИНА D И МУЛЬТИЛОКУСНЫХ ГАПЛОТИПОВ С ПРИВЫЧНЫМ НЕВЫНАШИВАНИЕМ БЕРЕМЕННОСТИ У ЖЕНЩИН КАЗАХСТАНА

Айнур Аманжолқызы[1], http://orcid.org/0000-0002-1980-9032

Талшын М. Укыбасова[2], http://orcid.org/0000-0002-5098-0727

Айзада М. Марат[3], http://orcid.org/0000-0002-3935-3351

Акбаян Б. Турешева[1], http://orcid.org/0000-0002-4176-4467

Самат С. Сапарбаев[4], http://orcid.org/0000-0002-9570-4240

[1] НАО «Западно-Казахстанский медицинский университет им. Марата Оспанова», г. Актобе, Республика Казахстан;
[2] Корпоративный Фонд "Университетский медицинский центр", г. Астана, Республика Казахстан;
[3] НАО "Медицинский университет Астана", г. Астана, Республика Казахстан;
[4] Медицинский центр «Аль-Жами», г. Астана, Республика Казахстан.

Актуальность. Привычное невынашивание беременности (ПНБ) признано серьезной проблемой в области репродуктивного здоровья, более половины случаев имеют неизвестные причины. Плохой статус витамина D у матери связан с неблагоприятным течением беременности, а мутации в гене VDR могут изменять репродуктивную функцию. Однако доказательства различий между популяциями непоследовательны, и исследования в контексте женщин Центральной Азии еще не проводились.

Цель. Данное исследование направлено на оценку потенциальных ассоциаций между десятью общими полиморфизмами и многолокусными гаплотипами гена рецептора витамина D (VDR) и материнским риском повторной потери беременности среди женщин, проживающих в Казахстане.

Материалы и методы. Исследование было проведено на основе выборки включавший 200 женщин с ПНБ и 200 многорожавших женщин, составивших контрольную группу из населения Республики Казахстан. Генотипирование полиморфизмов rs739837, rs7975232, rs1544410, rs7975128, rs2228570, rs731236, rs7967152, rs2238140 и rs2853564 проводилось с использованием высокоточной ПЦР в реальном времени.

Результаты. Однонуклеотидные полиморфизмы (SNPs) у здоровых женщин в контрольной группе находились в равновесии Харди-Вайнберга. Ни один из исследованных SNPs не был связан с более высоким или низким риском ПНБ, и частоты минорных аллелей (MAF) не различались у женщин с ПНБ и здоровых женщин. Аналогично, частоты генотипов всех SNPs не варьировались между двумя группами, с учетом соответствующих модификаторов и независимо от используемой генетической модели (кодоминантной, доминантной, рецессивной, сверхдоминантной или лог-аддитивной). Для наиболее распространенного гаплотипа GCCG-GAAGA (OR = 1.00) анализ гаплотипов предполагал отрицательную связь между гаплотипом TATAGGCAG и ПНБ (P = 0.001) изначально, которая сохранялась с учетом важных ковариант (P = 0.006). Однако при контроле на множественные сравнения, проведенные методом Бонферрони, эта ассоциация стала статистически незначимой (P = 0.059).

Заключение. Полученные данные демонстрируют, что полиморфизмы гена VDR не были статистически значимо связаны с восприимчивостью к привычному невынашиванию беременности у женщин Казахстана.

Ключевые слова. *Генотипы; гаплотипы; привычное невынашивание беременности; рецептор витамина D.*

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Түйіндеме

ҚАЗАҚСТАН ӘЙЕЛДЕРІНДЕГІ ҚАЙТАЛАМАЛЫ ЖҮКТІЛІКТІҢ ҮЗІЛУІМЕН D ДӘРУМЕНІ РЕЦЕПТОРЫ (VDR) ГЕНІНІҢ ВАРИАНТТАРЫ МЕН КӨПЛОКУСТЫ ГАПЛОТИПТЕРДІҢ АССОЦИАЦИЯСЫН ЗЕРТТЕУ

Айнур Аманжолқызы[1], http://orcid.org/0000-0002-1980-9032

Талшын М. Укыбасова[2], http://orcid.org/0000-0002-5098-0727

Айзада М. Марат[3], http://orcid.org/0000-0002-3935-3351

Акбаян Б. Турешева[1], http://orcid.org/0000-0002-4176-4467

Самат С. Сапарбаев[4], http://orcid.org/0000-0002-9570-4240

[1] «Марат Оспанов атындағы Батыс Қазақстан медицина университеті», Ақтөбе қ., Қазақстан Республикасы;
[2] Корпоративтік қор «Университеттік медициналық орталық», Астана қ., Қазақстан Республикасы;
[3] «Астана медицина университеті», Астана қ., Қазақстан Республикасы;
[4] Медициналық орталыгы «Аль-Жами», Астана қ., Қазақстан Республикасы.

Өзектілігі. Қайталамалы жүктіліктің үзілуі (ҚЖҮ) — репродуктивті денсаулық саласындағы өзекті мәселелердің бірі болып табылады, әрі оның себептері жағдайлардың жартысынан астамында анықталмаған күйінде қалып отыр. D дәруменінің тапшылығы жүктіліктің қолайсыз нәтижелерімен байланысты, ал D дәрумені рецепторын (VDR) кодтайтын геннің генетикалық вариациялары репродуктивтік функцияға әсер етуі мүмкін. Алайда әртүрлі популяциялар бойынша алынған мәліметтер бір-біріне қарама-қайшы және Орталық Азия әйелдері арасында мұндай зерттеулер бұрын жүргізілмеген.

Мақсаты. Қазақстан әйелдері арасында D дәрумені рецепторы (VDR) генінің он кең таралған полиморфизмдері мен көплокусты гаплотиптерінің қайталамалы жүктіліктің үзілу қаупімен ықтимал байланысын анықтау.

Материалдар мен әдістері. Зерттеуге ҚЖҮ диагнозы бар 200 әйел және бақылау тобы ретінде 200 көп босанған әйел қатыстырылды. Барлық қатысушылар Қазақстанның әйелдерінен іріктеліп, rs739837, rs7975232, rs1544410, rs7975128, rs2228570, rs731236, rs7967152, rs2238140 және rs2853564 полиморфизмдері нақты уақыттағы ПТР әдісі арқылы жоғары сенімділікпен генотиптелді.

Нәтижелері. Барлық зерттелген SNP-тер бақылау тобындағы сау әйелдер арасында Харди–Вайнберг тепе-теңдігіне сай болды. Ешбір полиморфизм ҚЖҮ даму қаупімен немесе оны төмендетумен статистикалық тұрғыдан байланысты болған жоқ. Минорлық аллель жиілігі мен генотиптер үлестері екі топ арасында айтарлықтай

айырмашылық көрсетпеді, генетикалық модельдерге (кодоминантты, доминантты, рецессивті, артық-доминантты, лог-аддитивті) қарамастан. Гаплотиптік талдау кезінде ең жиі кездесетін GCCGGAAGA гаплотипі эталон ретінде алынғанда, TATAGGCAG гаплотипі мен ҚЖҮ арасында бастапқыда теріс байланыс байқалды (P = 0,001), бірақ Бонферрони түзетуінен кейін маңыздылығын жоғалтты (P = 0,059

Қорытынды. Зерттеу нәтижелері Қазақстанның әйелдер популяциясында VDR генінің полиморфизмдері мен қайталамалы жүктіліктің үзілу қаупі арасында айқын байланыс жоқ екенін көрсетті.

Түйінді сөздер: *генотиптер; гаплотиптер; қайталамалы жүктіліктің үзілуі; D дәрумені рецепторы.*

Дәйексөз үшін:

Аманжолқызы А., Укыбасова Т.М., Марат А.М., Турешева А.Б., Сапарбаев С.С. Қазақстан әйелдеріндегі қайталамалы жүктіліктің үзілуімен D дәрумені рецепторы (VDR) генінің вариантттары мен көплокусты гаплотиптердің ассоциациясын зерттеу // Ғылым және Денсаулық сақтау. 2026. Vol.28 (1), Б. 55-65. *doi 10.34689/SH.2026.28.1.007*

Introduction

Spontaneous pregnancy failure is currently one of the major barriers in reproductive medicine. Approximately 15-25% of all clinically registered pregnancy loss [23, 29]. The recurring pregnancy loss (RPL), which is commonly known as habitual abortion and recurring miscarriage, has been recognized as a massive reproductive challenge that occurs in 1-2% of married couples globally [23, 34]. In Kazakhstan, the population incidence of RPL has been estimated at 3.2 per 1,000 pregnancies, highlighting the clinical and public health relevance of this condition in the region [29]. According to the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE), RPL consists of 2 or more failed pregnancies determined via ultrasonography or histopathologic examination [23], and, according to the Royal College of Obstetrics and Gynecology (RCOG), RPL is also defined as 3 and more consecutive pregnancy losses before 24 weeks [34, 19]. RPL is a condition with a number of etiologies. Numerous papers on inherited and acquired factors predisposing the patient to RPL have been published. These include: anti-phospholipid antibodies [31, 32], Rh incompatibility [8], inherited thrombophilia resulting from factor V-Leiden and prothrombin G20210A mutations [25, 38], uterine anatomical abnormalities [34, 12], and chromosomal aberrations in any partner. Even though these and the other probable causative factors identified, more than 50% of registered RPL cases do not reveal the exact causes [34, 3]. Nowadays, there is an apparent interest in the fat-soluble vitamin D (calciferol) and its role in reproductive health. Current evidence indicates about its involvement in implantation, placental development, and immune regulation throughout the pregnancy period [27, 41]. It does promote innate immunity, as well as suppresses adaptive immune responses [15]. The key role that the Vitamin D plays in regulating pregnancy processes were proved by the studies showing links of Vitamin D deficiency with undesirable pregnancy outcomes including intrauterine fetal growth restriction, preeclampsia, gestational diabetes, and spontaneous miscarriages [27, 14, 28]. Tin Vitamin D binds it nuclear receptor, the vitamin D receptor (VDR). It functions as a ligand-stimulated transcription factor to modulate the expression of many targeted genes taking part in different vitamin D-regulated implantation processes. [20, 36].

The VDR gene is localized on chromosome 12 (12q13) and is highly polymorphic, with over 470 variants distributed in eight coding exons and promoter regions [21]. Sever-al functional VDR SNPs associated with various pathologies were described, of which four were extensively studied. These were the Taq I (rs731236; 3'-UTR), Bsm I (rs1544410; intron-8), ApaI (rs7975232; intron-8), and the FokI (rs2228570; exon-2) variants [9-24]. Both ApaI and BsmI variants alter VDR gene expression without altering the VDR coding sequence but by disrupting mRNA splice sites or altering intronic regulatory elements [35] and, subsequently, VDR mRNA stability [35]. However, the FokI mutation is linked with a protein altered both structurally and functionally. The C allele (major) is resulting in 424-amino acid active protein, whilst the T allele (minor) is encoding a less active 427-amino acid VDR protein [6, 7]. The investigations of variations in the VDR gene had been conducted in such reproductive disorders as gestational diabetes mellitus [24, 39], preterm birth [9], preeclampsia [14, 16], intrahepatic cholestasis of pregnancy [37], and endometriosis [22].

While it is considered there is a probability of the VDR genetic variants role in the etiology of RPL, few investigations have shown the association between VDR polymorphisms and the risk of RPL. The results are often contradictory [28, 36, 21, 7]. This was attributed to the small sample size and the focus on specific ethnic groups, both of which hampered the generalization of this association. The genetic architecture of RPL susceptibility shows significant population-specific variation, with studies in European populations showing conflicting results for VDR polymorphisms [36, 7]. Central Asian populations, including those from Kazakhstan, represent a unique genetic admixture with contributions from European, Asian, and Middle Eastern ancestries, yet they remain largely understudied in reproductive genetics studies.

This study aims to investigate the potential associations between the presence of 10 genetic variants of the VDR gene and their respective 10-locus haplotypes with RPL in reproductive-age Kazakhstani (Central Asian) women. The study hypothesis: there is a link between genetic variants of the VDR gene with the risk of RPL in reproductive - age Kazakhstani women.

Materials and Methods

A case–control study involving 200 women confirmed with recurrent pregnancy loss (RPL) by the ESHRE criteria was performed. The RPL case group included women with confirmed recurrent pregnancy losses and was not a population-based sample.

Participants were recruited from outpatient gynecology clinics in a number of cities in Kazakhstan, including

Astana, Shymkent, Taraz, Aktobe, Oral (Uralsk), and Atyrau, between June 2022 and January 2024. Patient enrollment was carried out at public and private healthcare facilities. Recurrent pregnancy loss (RPL) was defined according to ESHRE as two or more failed pregnancies confirmed by ultrasonography or histological examination. Inclusion criteria for the case group were age ≥18 years, a past history of two or more unexplained first-trimester pregnancy losses, and documented informed consent to participate in the study. Exclusion criteria included a history of only one spontaneous miscarriage, age of the mother at first pregnancy (>40 years), Rh blood incompatibility, hypertensive disorders of pregnancy (systolic >140 mmHg and diastolic >90 mmHg), and anatomical defects in the uterus. Women with systemic autoimmune diseases, endocrinopathies (such as diabetes mellitus and thyroid diseases), liver disorders, controlled ovarian hyperstimulation, and pregnancies with assisted reproductive technology (ART) were also excluded.

Biodata was obtained during patients' outpatient obstetrics and gynecology clinic visits and from the medical records. It included the age at the entry into the study, pregnancy history, confirmation of RPL, and tabulation of potential risk factors of RPL. RPL-specific data represents the age at first loss, gestation age at each loss, time intervals between losses, RPL classification (primary, secondary). Family and maternal age at first loss (RPL risk factors) are presented as well. Patients' demographic and clinical data have been also collected in a questionnaire while enrolling.

Two hundred multiparous healthy women without a personal or family history of recurrent pregnancy loss served as controls. Control patients were from the same outpatient gynecology clinics and cities as the RPL subjects at the same time period during the study. Control women had no history of pregnancy loss, stillbirth, or neonatal death and the overall pregnancies were equal to the total number of live births. Inclusion criteria for the control group were ≥18 years of age, history of two or more pregnancies with term deliveries, and a written informed consent to participate in the study.

VDR Genotyping

Genomic DNA was extracted from 200 µL peripheral blood samples using the Thermo Scientific™ GeneJET Whole Blood Genomic DNA Purification Mini Kit (Fisher Scientific UK, catalog #K0781) following the manufacturer's protocol. NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for DNA concentration determination. DNA purity was assessed by taking A260/A280 ratios (acceptable range: 1.8-2.0) and A260/A230 ratios (acceptable range: 2.0-2.2). DNA integrity was checked using agarose gel electrophoresis. Working DNA concentrations have been adjusted to 10 ng/µL and stored at -20°C until the usage. The nine VDR SNPs selection was guided by:

1. correlations of reproductive disorders and/or vitamin D metabolism,
2. frequencies of minor allele exceeding 5% frequency in European and Central Asian countries population,
3. functional significance or position in the regulation regions,
4. linkage disequilibrium patterns, reflecting haplotype diversity in VDR gene region.

Tio Among the SNPs that have been selected are the following variants: FokI (rs2228570; exon-2), BsmI (rs1544410; intron-8), ApaI (rs7975232; intron-8) and TaqI (rs731236; 3'-UTR), and five other variants (rs739837, rs7975128, rs7967152, rs2238140, rs2853564) in regulation regions, or having associations with other phenotypes cited above. The variants are placed within or in similar proximity to the VDR gene on a chromosome 12q13 (rs10783291 RefSNP Report - dbSNP - NCBI).

The genotyping of rs739837, rs7975232, rs1544410, rs7975128, rs2228570, rs731236, rs7967152, rs2238140, and rs2853564 *VDR* gene variants was performed by the allelic-discrimination procedure on QuantStudio 6/7 PCR systems, pursuant to the instrument's guides (Applied Biosystems; Waltham, US), with pre-designed probe sets (assay IDs for each SNP can be accessed at https://www.thermofisher.com/taqman/snp/assay/query?keyword=). Each 10 µL PCR reaction contained TaqMan® Genotyping Master Mix (5.0 µL; Applied Biosystems, #4371355), TaqMan® SNP Genotyping Assay (0.5 µL; 20X), genomic DNA (1.0 µL at 10 ng/µL), and nuclease-free water (3.5 µL). Thermal cycling conditions include denaturation in the beginning (95°C for 10 minutes), then by 40 cycles of denaturation (95°C for 15 seconds) and annealing/extension (60°C for 1 minute), and a final post-read (60°C for 30 seconds) steps. Quality control measures included inclusion of both positive controls (samples with known genotypes), and negative controls (no-template controls with nuclease-free water), and re-genotyping duplicate samples (10% of total samples); genotyping success was 100%. For the association analysis, the major allele homozyg genotype was used as the reference category (OR = 1.00) when ORs for overall RPL risk were derived.

Raw genotyping data from QuantStudio 6/7 PCR systems were exported as SDS files and converted to Excel format for analysis. Quality control included manual review of amplification plots, cluster plots, and allelic discrimination plots for all samples. Genotype calls with confidence scores <95% were excluded and reanalyzed. Population allele frequency comparisons were performed using data from the 1000 Genomes Project Phase 3 and gnomAD database v3.1.2, with specific attention to Central Asian populations (CAU) when available.

Statistical analysis

Demographic and clinical characteristics and VDR allelic and genotypic frequency were contrasted between RPL women and control subjects by using SPSS version 29 (IBM, Armonk, NY) and R version 4.1.2 Windows with "nnet," "SNPassoc," and "Hmisc" packages. Sample size calculations have been provided on 80% power to indicate odds ratios ≥1.5 with minor allele frequencies ≥10% at α=0.05. The final sample size of 400 participants (200 cases, 200 controls) have shown adequate power for the primary analysis. Hardy-Weinberg equilibrium (HWE) was rated for all the variants in RPL cases and controls with usage of chi-square goodness-of-fit tests.

Minor alleles were defined according to allele frequencies reported in the 1000 Genomes Project Phase 3 and gnomAD v3.1.2 reference datasets. HWE calculations were performed using the built-in feature of SNPStats software, as per: $\chi^2 = \Sigma[(\text{Observed} - \text{Expected})^2/\text{Expected}]$,

where expected genotype frequencies are calculated as $p^2 + 2pq + q^2$ for AA, Aa, and aa genotypes respectively (where p and q represent allele frequencies). All tested VDR variants were in HWE among control women ($p > 0.05$).

The analysis was two-staged: (i) unadjusted comparisons of unselected RPL cases with control subjects, and (ii) adjustment analyses with adjustment for age, BMI, menarche, and gravida. Continuing variables have been reported as mean ± standard deviation (SD), categorical variables as number (percentage). The comparison of means was completed with the help of Student's t-test and proportions with the usage of Chi-square test or Fisher's exact test where detected.

These same statistical procedures were applied to the comparisons reported in Table 1.

Multinomial logistic regression was employed in the analysis of outcomes with respect to carriage of specific VDR variants. Linkage disequilibrium and haplotype reconstruction were performed in Haploview 4.2 (Broad Institute, Cambridge, MA) with the expectation–maximization algorithm, estimating only those haplotypes with >1% frequency. Spearman's rank correlation was also applied in examining associations between VDR genotypes and participant characteristics. A p-value of < 0.05 was considered statistically significant.

Ethical approval

The study was performed according to the rules of Helsinki declaration. The study was verified and approved by the Institutional Research Ethics Committee of Nazarbayev University (NU-IREC) on 29/09/2022, #599/05092022.

The study participants have been informed about the potential study and its goals, methods applied and anticipated risks or benefits before signing the consent.

Written consent was obtained from all participants of the study (cases and controls).

Results

Demographic and clinical characteristics.

Demographic and clinical characteristics. The content of the study participants, both demographically and clinically, is shown in Table 1.

Table 1.

Demographic and clinical characteristics of study participants.

	RPL Cases	Controls	*P****
Age at inclusion in study (years) *	31.4 ± 6.3	32.2 ± 6.4	0.170
Higher/University Education level **	130 (61.9)	118 (58.7)	0.507
High Family economic status**	29 (13.8)	27 (13.4)	0.841
Body mass index (kg/m2) *	24.9 ± 4.7	24.5 ± 3.8	0.225
Obesity **	4 (6.5)	3 (2.5)	0.644
Smokers **	4 (1.9)	1 (0.5)	0.379
Normal Pap smear **	154 (73.3)	134 (66.7)	0.218
Hypertension **	5 (2.4)	3 (1.5)	0.729
Earlier use of oral contraceptives **	23 (11.5)	21 (10.5)	0.752
Miscarriages *	3.7 ± 1.2	0.0 ± 0.0	<0.001
Menarche (years) *	13.1 ± 1.1	13.3 ± 1.1	0.212
Irregular menstrual function **	16 (7.6)	4 (2.0)	0.029
Women with no live birth **	96 (48.0)	0 (0)	<0.001
Number of pregnancies **	3.7 ± 1.7	3.1 ± 1.1	0.241
Live birth **	0.5 ± 0.8	3.1 ± 1.1	<0.001
Age at first pregnancy loss (years) *	26.8 ± 4.2	N/A	N/A
Age >35 years at first loss **	23 (11.5)	N/A	N/A
Family history of RPL **	18 (9.0)	N/A	N/A

Table footnotes. ** Mean±SD.*

*** Numbers in parentheses represent percentages, which were calculated relative to the available data for each variable (denominators may vary slightly if data were missing). RPL risk factors assessed based on medical history and clinical examination.*

**** Student t-test for continuous variables, Pearson chi square (or Fisher's exact test where appropriate) for categorical variables).*

Various parameters were examined among the 200 women with RPL and an equal number of matched control counterparts. Control women had no pregnancy losses as all pregnancies resulted in live births, while RPL cases averaged 3.7 ± 1.2 miscarriages with only 0.5 ± 0.8 live births despite similar total pregnancy numbers. Specifically, factors such as age, body mass index (BMI), educational background, household income, smoking habits, prior use of oral contraceptives, Pap smear results, and presence of hypertension were all similar between the two groups (all $P > 0.05$). On the contrary, the data about lifestyle and eatery habits has not been collected and represents some limitations. Information about the first menstruation ($P = 0.212$) and overall number of pregnancies ($P = 0.241$) had been indistinguishable in RPL cases and controls. However, disparities emerged in certain aspects: RPL cases exhibited a higher incidence of irregular menstrual cycles ($P = 0.029$) and a greater number of miscarriages ($P < 0.001$), while their count of live births was notably lower ($P < 0.001$) compared to controls. In addition, a comparable proportion of patients above the age of 40 year old was clear in both groups (11.7% of RPL cases and 10.4% of controls, $P = 0.750$).

Notably, most cases (93.8%) and controls (98.5%) identified themselves as ethnically Kazakh.

Distribution of VDR alleles and genotypes.

The alleles distribution of rs739837, rs7975232, rs1544410, rs7975128, rs2228570, rs731236, rs7967152, rs2238140, and rs2853564 VDR SNPs in RPL cases and control subjects are described in Table 2.

Table 2.

Minor allele frequencies of VDR variants in women with RPL and controls*.

SNP	Location **	Minor Allele	HWE	Cases	Controls	Chi square	p	OR (95% CI) ***
rs739837	47844438	T	0.9891	169 (0.42)	167 (0.42)	0.001	.887	1.02 (0.77, 1.35)
rs731236	47844974	A	0.4583	81 (0.20)	94 (0.24)	1.408	.265	0.83 (0.59, 1.16)
rs7975232	47845054	C	0.5145	170 (0.42)	176 (0.44)	0.304	.671	0.94 (0.71, 1.24)
rs1544410	47846052	C	0.6375	88 (0.22)	101 (0.25)	1.347	.279	0.84 (0.60, 1.16)
rs7967152	47850401	A	0.5921	171 (0.43)	176 (0.44)	0.231	.718	0.95 (0.72, 1.26)
rs7975128	47852045	G	0.6375	88 (0.22)	101 (0.25)	1.347	.279	0.84 (0.60, 1.16)
rs2238140	47858881	A	0.7854	173 (0.43)	171 (0.43)	0.020	.887	1.02 (0.77, 1.35)
rs2228570	47879112	A	0.2945	148 (0.37)	136 (0.34)	0.611	.374	1.14 (0.85, 1.52)
rs2853564	47884704	A	0.0989	131 (0.33)	152 (0.38)	2.279	.121	0.79 (0.59, 1.06)

Table footnotes. *HWE, Hardy-Weinberg Equilibrium (calculated using SNPStats built-in chi-square goodness-of-fit test); RPL, recurrent pregnancy loss.Study subjects included 200 women with RPL, and 200 multiparous control women*
** Based on GRCh38.p14 release.* *** Adjusted for age, BMI, menarche, and gravida.*
**** Number of alleles (frequency).*

All nine *VDR* polymorphisms were in Hardy-Weinberg equilibrium (HWE) among multiparous healthy controls (all $P > 0.05$), indicating no significant deviation from expected genotype frequencies and confirming the absence of population stratification in our control group. The typed SNPs were not associated with increased and decreased risks of RPL. The minor allele frequency (MAF) of SNPs did not differ significantly between RPL and controls. This was lasting after the age control, BMI, menarche and gravida as the main covariates.

Similar to the allelic distribution, the genotype frequencies of all types of SNPs were not significantly different between women with RPL and control women, even after controlling for age, BMI, menarche, and gravida as key modifying variables (Table 3).

Table 3.

Frequencies of *VDR* Genotypes in women with RPL and control women.

SNP	Genotypes	RPL Cases *	Controls*	p	OR (95% CI) **	p	OR (95% CI) ***
rs739837	G/G	65 (0.32)	70 (0.35)	.73	1.00 (Reference)	.57	1.00 (Reference)
	G/T	101 (0.50)	93 (0.46)		1.17 (0.75, 1.82)		1.07 (0.67, 1.72)
	T/T	34 (0.17)	37 (0.18)		0.99 (0.56, 1.76)		0.77 (0.41, 1.46)
rs731236	A/A	127 (0.64)	114 (0.57)	.39	1.00 (Reference)	.20	1.00 (Reference)
	A/G	65 (0.32)	78 (0.39)		0.75 (0.49, 1.13)		0.67 (0.42, 1.06)
	G/G	8 (0.04)	8 (0.04)		0.90 (0.33, 2.47)		0.63 (0.19, 2.06)
rs7975232	C/C	63 (0.32)	62 (0.31)	.80	1.00 (Reference)	.49	1.00 (Reference)
	C/A	104 (0.52)	100 (0.50)		1.02 (0.66, 1.60)		0.94 (0.58, 1.53)
	A/A	33 (0.16)	38 (0.19)		0.85 (0.48, 1.53)		0.69 (0.36, 1.31)
rs1544410	C/C	122 (0.61)	109 (0.55)	.39	1.00 (Reference)	.12	1.00 (Reference)
	C/T	68 (0.34)	81 (0.40)		0.75 (0.50, 1.13)		0.64 (0.40, 1.01)
	T/T	10 (0.05)	10 (0.05)		0.89 (0.36, 2.23)		0.60 (0.20, 1.76)
rs7967152	A/A	63 (0.32)	62 (0.31)	.87	1.00 (Reference)	.59	1.00 (Reference)
	A/C	103 (0.52)	100 (0.50)		1.01 (0.65, 1.58)		0.93 (0.58, 1.51)
	C/C	34 (0.17)	38 (0.19)		0.88 (0.49, 1.57)		0.72 (0.38, 1.36)
rs7975128	G/G	122 (061)	109 (0.55)	.39	1.00 (Reference)	.13	1.00 (Reference)
	G/A	68 (0.34)	81 (0.40)		0.75 (0.50, 1.13)		0.64 (0.41, 1.01)
	A/A	10 (0.05)	10 (0.05)		0.89 (0.36, 2.23)		0.60 (0.20, 1.76)
rs2238140	G/G	62 (0.31)	66 (0.33)	.84	1.00 (Reference)	.73	1.00 (Reference)
	G/A	103 (0.52)	97 (0.48)		1.13 (0.73, 1.76)		1.03 (0.64, 1.67)
	A/A	35 (0.18)	37 (0.18)		1.01 (0.57, 1.79)		0.82 (0.43, 1.54)
rs2228570	G/G	78 (0.39)	83 (0.42)	.53	1.00 (Reference)	0.11	1.00 (Reference)
	G/A	96 (0.48)	98 (0.49)		1.04 (0.69, 1.58)		1.07 (0.67, 1.69)
	A/A	26 (0.13)	19 (0.10)		1.46 (0.75, 2.84)		2.12 (1.03, 4.38)
rs2853564	A/A	94 (0.47)	81 (0.40)	.33	1.00 (Reference)	.62	1.00 (Reference)
	A/G	81 (0.40)	86 (0.43)		0.81 (0.53, 1.24)		0.90 (0.57, 1.44)
	G/G	25 (0.12)	33 (0.16)		0.65 (0.36, 1.19)		0.73 (0.38, 1.38)

Table footnotes: ** Number of alleles (percent total);* *** Crude (unadjusted) analysis;*
**** Adjusted for age, BMI, menarche, and gravida.*

To place our findings in a broader genetic context, we compared the allele frequencies of the studied *VDR* variants in our Kazakhstani cohort with reference populations from the 1000 Genomes Project Phase 3 and the gnomAD v3.1.2 databases. The observed distribution of minor alleles (Table 4) showed similarities to those of European and African populations for several variants, while differing markedly from those of East Asian populations. The full dataset is also provided in Supplementary File S1 (Excel format). These comparisons offer insight into the unique genetic admixture of the Kazakhstani population, helping to interpret the lack of association observed in our cohort (Table 4).

Table 4.

Global Distribution of Studied *VDR* Polymorphisms *.

No.	SNP	Kazakhs	Europeans	Northern Europeans	Africans	Asians	East Asians	South-Central Asians	Latinos	Middle Eastern	Ashkenazi Jews
1	rs739837	0.42	0.486	0.472	0.483	0.07	0.08	0.24	0.488	0.281	0.416
2	rs731236	0.219	0.398	0.354	0.281	0.05	0.048	0.333	0.369	0.288	0.47
3	rs7975232	0.432	0.463	0.463	0.369	0.313	0.291	0.385	0.415	–	0.430
4	rs1544410	0.236	0.399	0.356	0.263	0.060	0.056	0.362	0.299	0.366	–
5	rs7967152	0.434	0.470	0.469	0.405	0.282	0.280	0.459	0.429	0.431	–
6	rs7975128	0.236	0.401	0.358	0.271	0.064	0.062	–	0.286		0.361
7	rs2238140	0.430	0.399	0.490	0.229	0.120	0.225	0.450	0.000	–	
8	rs2228570	0.355	0.387	0.372	0.275	0.438	0.432	0.309	0.390	0.339	0.435
9	rs2853564	0.354	0.397	0.408	0.129	0.312	0.329	0.202	0.271	0.309	0.324
* Performed using SNPstats software.											

The observed differences between Kazakhstani cohort and other Asian populations likely reflect the unique genetic admixture of Central Asian populations, which have historical contributions from European, Middle Eastern, and Asian ancestries, as documented in previous population genetic studies of Kazakhstan [17, 18]. Allele frequencies were obtained from the official dbSNP Reference SNP (rs) database of NCBI (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/snp), which provides curated population-level frequency data for each variant analyzed in this study.

The lack of association of the typed *VDR* SNPs with altered risk of RPL was established, irrespective of the genetic model used (codominant, dominant, recessive, over-dominant, log-additive) (Table 5).

Table 5.

Association of *VDR* variants with RPL susceptibility according to specific genetic models *.

	Dominant		Recessive		Over-dominant		Log-additive	
SNP	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)
rs739837	0.60	1.12 (0.74, 1.69)	0.69	0.90 (0.54, 1.51)	0.42	1.17 (0.79, 1.74)	0.89	1.02 (0.77, 1.35)
rs731236	0.18	0.76 (0.51, 1.14)	1.00	1.00 (0.37, 2.72)	0.17	0.75 (0.50, 1.14)	0.25	0.82 (0.58, 1.16)
rs7975232	0.91	0.98 (0.64, 1.49)	0.51	0.84 (0.50, 1.41)	0.69	1.08 (0.73, 1.60)	0.66	0.94 (0.71, 1.25)
rs1544410	0.19	0.77 (0.51, 1.14)	1.00	1.00 (0.41, 2.46)	0.18	0.76 (0.50, 1.14)	0.27	0.83 (0.59, 1.16)
rs7967152	0.91	0.98 (0.64, 1.49)	0.60	0.87 (0.52, 1.46)	0.76	1.06 (0.72, 1.57)	0.72	0.95 (0.71, 1.26)
rs7975128	0.19	0.77 (0.51, 1.14)	1.00	1.00 (0.41, 2.46)	0.18	0.76 (0.50, 1.14)	0.27	0.83 (0.59, 1.16)
rs2238140	0.67	1.10 (0.72, 1.67)	0.79	1.93 (0.56, 1.56)	0.55	1.13 (0.76, 1.67)	0.89	1.02 (0.77, 1.35)
rs2228570	0.61	1.11 (0.74, 1.66)	0.27	1.42 (0.76, 2.66)	0.84	0.96 (0.65, 1.42)	0.36	1.15 (0.85, 1.55)
rs2853564	0.19	0.77 (0.52, 1.14)	0.26	0.72 (0.41, 1.27)	0..61	0.90 (0.61, 1.34)	0.14	0.61, 1.07)
** Performed using SNPstats software.*								

VDR haplotype distribution in RPL cases and controls

Table 6 lists the distribution of the nine-locus *VDR* rs739837-rs7975232-rs1544410-rs7975128-rs2228570-rs731236-rs7967152-rs2238140-rs2853564 haplotypes in women with RPL and healthy control women If to consider the most prevailing CGCCGGAAGA haplotype as reference (OR = 1.00). Considering the most prevalent GCCGGAAGA haplotype as the reference (OR = 1.00), haplotype analysis initially uncovered a negative link between the TATAGGCAG haplotype and RPL (P = .001), which was persisting after controlling for key covariates (P = .006). This association experienced a failure after the multiple comparisons correction using the Bonferroni correction method (P = .059) (Table 6).

Table 6.

Distribution of *VDR* haplotypes in RPL cases and control women.

Haplotype *	All	RPL cases	Controls	P **	P ***	P &	OR *** (95% CI)
1	2	3	4	5	6	7	8
G C C G G A A G A	0.2357 &&	0.1983	0.2634	0.092	0.031	0.314	1.000 (Reference)
G C C G A A A G A	0.1450	0.1649	0.1295	0.130	0.381	1.000	1.415 (0.651, 3.073)
T A T A G G C A A	0.0908	0.0968	0.0815	0.076	0.111	1.000	1.892 (0.863, 4.148)
G C C G G A A G G	0.0872	0.1132	0.0762	0.147	0.134	1.000	1.768 (0.838, 3.726)
G C C G A A A G G	0.0767	0.0732	0.0705	0.768	0.854	1.000	1.08 (0.478, 2.437)

Continuation of Table 6.

1	2	3	4	5	6	7	8
T A T A G G C A G	0.0705	0.0595	0.0764	0.001	0.006	0.059	4.392 (1.531, 12.599)
T A C G G A C A A	0.0686	0.1062	0.0461	0.074	0.110	1.000	0.37 (0.11 , 1.251)
T A C G G A C A G	0.0397	0.0098	0.0615	0.983	0.615	1.000	1.331 (0.437, 4.050)
T A C G A A C A A	0.0387	0.0352	0.0357	0.243	0.199	1.000	1.979 (0.699, 5.602)
T A C G A A C A G	0.0356	0.0422	0.0239	0.649	0.719	1.000	0.771 (0.187, 3.183)
T A T A A G C A A	0.0264	0.0261	0.0290	0.092	0.031	0.314	2.25 (1.075, 4.708)
TOTAL	0.8210	0.8719	0.7915				

Table footnotes.

** rs739837-rs7975232-rs1544410-rs7975128-rs2228570-rs731236-rs7967152-rs2238140-rs2853564 haplotypes; underlined boldface indicates minor alleles.*

*** Crude (unadjusted).* **** Adjusted for age, BMI, menarche, and gravida.*

& Corrected for multiple comparisons as per Bonferroni method [Pc = 1 – (I – P)12]. *&& Haplotype frequencies.*

Discussion

Vitamin D deficiency in pregnancy is widespread globally, and a correlation between inadequate vitamin D levels and the incidence of RPL was suggested by most [15, 28, 26, 30] but not all [40] studies. Insofar as vitamin D mediates its effects through binding VDR, we investigated the association of the extensively investigated Taq I (rs731236), Bsm I (rs1544410), ApaI (rs7975232), and FokI (rs2228570) variants, along with five additional variants of the VDR gene, with the risk of RPL among Kazakhstani women. None of the tested variants was associated with an altered risk of RPL, regardless of the genetic model employed (codominant, dominant, recessive, etc.), and no multi-locus VDR haplotype was identified to be linked with RPL. This study is the first to identify the lack of association between VDR genetic variations and the risk of RPL among women in Kazakhstan (a Central Asian population), thus extending similar conclusions reached by studies on predominantly European populations [36, 1].

Kazakhstan, spanning an area of 2,724,900 km², lies in North-Central Asia and Eastern Europe, with a population of 20 million comprising 100 ethnic groups, including Kazakhs, Russians, Uzbeks, Ukrainians, Tatars, Germans, and many others [17, 18]. The distribution of the minor alleles of the tested variants reflects the ethnic diversity of the present-day Kazakhstani population. While the comparability of MAF most typed variants to Europeans and Northern Europeans was expected, it was of interest to note the closeness in MAF of rs739837, rs731236, rs7975232, rs1544410, rs7967152, rs7975128 among Kazakhstani population Africans, and the similarity in MAF of rs739837, rs7975232, rs1544410, rs7967152, rs7975128, and rs2228570 with Latin Americans (Table 4).

It was also noteworthy that, apart from rs2853564, the MAF of the genotyped variants was markedly higher than those established for Asians and East Asians but not South-Central Asians (Table 4). This highlights the input from different genetic pools to the makeup of present-day Kazakhstani, which in turn may influence disease associations of polymorphic variants [17, 18]. All RPL cases and control women were matched according to their ethnic background and geographical site to minimize the influence of ethnicity and residence, which are ingrained in genetic association studies [4, 5].

To ensure data quality and address potential concerns about genotyping accuracy, all raw SDS files from QuantStudio analysis have been made available for independent verification. The divergent allele frequencies observed in our Kazakhstani population compared to East Asian populations (Table 4) warrant careful consideration. These differences likely reflect the complex demographic history of Central Asia, where multiple migration events and population admixture have created unique genetic profiles distinct from both European and East Asian populations. Similar patterns have been documented in previous genetic studies of Kazakhstani populations [17, 18].

Earlier studies showed an association between genetic variations of the VDR gene with reproduction disorders in select ethnic groups, such as preterm births in Chinese [9], preeclampsia and gestational hypertension in Asians [16], gestational diabetes [2], and RPL in Slovenians [7]. An inadequate number of studies investigated the link between the carriage of VDR polymorphisms and RPL, often with contradictory conclusions, and an ethnic component to this association appears likely. This was highlighted by the association between the TaqI (rs731236) VDR variant and RPL in Slovenian women [7], but not in Polish [36] or Bosnian [1] women, and the association of FokI (rs2228570) with RPL in Slovenian women [7], but not in Polish women [36]. In addition, BsmI (rs1544410) was associated with RPL in Polish women [36] but not in Bosnian women [1]. We extended earlier findings by documenting the lack of association of common and novel VDR gene variants with RPL pathogenesis and susceptibility.

Population frequency data sourced from 1000 Genomes Project Phase 3 and gnomAD v3.1.2. The observed differences between our Kazakhstani cohort and other Asian populations likely reflect the unique genetic admixture of Central Asian populations, which have historical contributions from European, Middle Eastern, and Asian ancestries, as documented in previous population genetic studies of Kazakhstan [17, 18]. Allele frequencies were obtained through the Reference SNP (rs) Report included in the National Library of Medicine (https://ncbi.nlm.nih.gov/snp).

While the tested VDR variants were in LD, most haplotype diversity was captured in 12 of the possible 1024 haplotypes in 87.19% of cases and 79.15% of control women. While initial analysis identified significant association of TATAGGCAG haplotype with reduced risk of RPL (P = .001) that persisted after controlling for key covariates (P = .006), this association was lost after correcting for multiple comparisons (P = .059). No other haplotype combinations, including the well-studied 4-locus

rs2228570-rs1544410-rs7975232-rs731236 haplotype (p = 0.88), were linked with risk of RPL in the studied cohort. This was in contradiction to a recent Polish study on rs2228570 (FokI), rs1544410 (BsmI), rs7975232 (ApaI) and rs731236 (TaqI) variants, which identified the TTGT haplotype to be negatively associated with RPL, since it was more frequent among controls (frequency 0.09) than RPL cases (frequency 0.017) [36]. Factors such as differences in study populations, methodologies (PCR RFLP vs. real-time PCR), and controlling for potentially modifying factors could contribute to these discrepancies.

There are numerous factors as distinctions in study populations, methodologies (PCR RFLP vs. real-time PCR), controlling for modifying factors could have contributed in these discrepancies. The vitamin D and the VDR gene system interaction in RPL is complicated and affected by genetic, hormonal, immunological, and environmental factors that influence RPL [41, 42].

Our results suggest that there is no significant association between specific VDR variants and RPL susceptibility among Kazakhstani women. Vitamin D deficiency was previously implicated with an increased risk of RPL [28, 42, 10], and correcting it through supplementation was reported to result in better pregnancy outcomes in women with RPL [42]. This prompted the recommendation of vitamin D supplementation as part of a comprehensive approach to managing RPL [15, 13]. Others have found no similar association between vitamin D levels and miscarriage [40]. While not within the scope of the current study, the benefits (or lack thereof) of vitamin D supplements on pregnancy outcomes appear to be attributed to differences in study design, participant characteristics, including racial/ethnic background [16], dosage and duration of supplementation, and baseline vitamin D levels [10, 33].

Our findings have important clinical implications for RPL management in Central Asian populations. The shortage of links between VDR polymorphisms and RPL in the women of Kazakhstan suggests that there might be no clinical benefits for RPL risks assessment in this country. However, the probable significance of vitamin D status monitoring and supplementation must not be underrated, since the factors of environment and vitamin D levels may still affect pregnancy outcomes independently of genetic variation. The distribution of unique allele found in Kazakhstan cohort submits valuable population genetics information for such an understudied territory. The similarities between certain variants in both European and African populations, while differing markedly from those in East Asian populations, reflect the complex demographic history of Central Asia and have implications for personalized medicine approaches in this region.

This study has several strengths that strengthen the drawn conclusions. The selection criteria have been matched to the last ESHRE guidelines for the patients and control groups both and a thorough approach has minimized any probable confounding variables that are broadly associated with genetic studies. The use of various genetic models for genotype association seems to be another marked strength. However, there is a clear necessity in further investigations due to some limitations that have challenged current studies. Even though the sample size is sufficient for the initial analysis, some significant data about vitamin D levels and the administration of vitamin D supplementation was absent from both cohorts, thus it limits understanding of the impact of vitamin D on RPL. Furthermore, the study encompassed only 9 genetic variants of the *VDR* gene, prompting speculation about the involvement of other genetic polymorphisms in vitamin D metabolism, particularly those newly identified through next-generation sequencing or genome-wide approaches, may contribute to RPL susceptibility. There is a clear demand in future studies using expanded SNP panels and whole-genome methods that will warrant a better understanding of the role of VDR and related pathways in RPL. Such significant factors as dietary habits or lifestyle that are closely linked to nutrient intake and overall health behavior were not assessed. Abovementioned factors as well as socio-demographic data will contribute in a more comprehensive evaluation of RPL risk determinants.

Moreover, given that the study focused solely on Kazakhstani women, the generalizability of the findings to other populations is uncertain, thereby necessitating future research on diverse ethnic backgrounds to confirm or refute the link between *VDR* polymorphisms and the risk of RPL across different populations.

Conclusions

Kazakhstani case-control in women reveals no connection between ten common CDR gene polymorphisms or their multi-locus haplotypes and RPL risk. In agreement with recent European cohort findings our studies show that genetic variation in VDR is unlikely to be an important factor in RPL in this population. Yet the possibility that additional untested SNPs or rare variants may still play a role exists, the further investigations using broader variant coverage are needed to confirm or refute this possibility. While vitamin D deficiency has been linked to adverse pregnancy outcomes, our current results reveal that the contribution of VDR polymorphisms to RPL pathogenesis is not significant. The findings put a focus on the multifactorial etiology of RPL under the impact of complex genetic, immunologic, hormonal, and environmental determinants. Future investigations will have to include these observations with bigger study groups and also functional studies of vitamin D metabolism, and measurement of vitamin D levels and supplementation as potential modifiers of pregnancy outcome.

Moreover, investigation of studies in diverse populations will be critical to define the potential role of VDR polymorphisms in different ethnicities and geographic areas. Overall, current studies do offer informative proof that VDR gene polymorphisms are not involved in RPL in Kazakhstan women to a high extent, helping to focus the search for relevant biomarkers and guide the development of more targeted RPL prevention and treatment methods.

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Institutional Review Board Statement*: I All methods were carried out in accordance with relevant guidelines and regulations. The study was approved by the Institutional Research Ethics Committee of Nazarbayev University (NU-IREC) on 29/09/2022, #599/05092022. All participants were informed of the risks, benefits, goals, and methods of the study. Written consent was received from participants after they were informed about the volunteer and anonymous nature of the study. Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.*

Data Availability Statement: *The data that support the findings of this study are available from the Mendeley Data Repository at: https://doi.org/10.17632/jdvj22sbnr.1. Raw genotyping data files (SDS and Excel formats) from QuantStudio 6/7 PCR systems are available upon reasonable request for independent verification and reanalysis.*

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Contact information:

Ainur Amanzholkyzy– Ph.D., Associate Professor of the Normal Physiology Department, "West Kazakhstan Marat Ospanov Medical University" NCJSC Aktobe, the Republic of Kazakhstan, tel. 87132549813, e-mail: a.ainur.82@mail.ru, ORCID ID: https://orcid.org/0000-0002-1980-9032.

Talshyn Ukybassova- MD Professor Clinical Academic Department of Women's Health, CF "University Medical Center", Astana, Kazakhstan, e-mail: talshynu@yandex.ru ORCID https://orcid.org/0000-0002-50980727.

Aizada Marat - Ph.D., Obstetrician-gynecologist, Department of Obstetrics and Gynecology #1, NJSC "Astana Medical University", Astana, Kazakhstan; tel. 87057837472 , e-mail: marat.a@amu.kz ORCID https://orcid.org/0000-0002-3935-3351

Samat Saparbaev – Candidate of Medical Sciences, Ph.D., Associate Professor transplant doctor, surgeon of the highest qualification category, Al-Zhami Medical Center, Astana, Kazakhstan Republic; тел. 87012501873, e-mail: samat-saparbayev@mail.ru, ORCID ID: https://orcid.org/0000-0002-9570-4240

*** Corresponding Author:**

Turesheva Akbayan B. – 3rd year doctoral student, Master of Medical Sciences, senior lecturer at the Department of Normal Physiology, "West Kazakhstan Marat Ospanov Medical University" NCJSC. obstetrician-gynecologist of the highest qualification category, Aliya Medical Center. Aktobe, the Republic of Kazakhstan; https://orcid.org/0000-0002-4176-4467.

Postal code: The Republic of Kazakhstan, 030000, Aktobe, Gaziza Zhubanova 15/1 9 a.

E-mail: akbayan_85@mail.ru.

Phone: 87027905905