Gene expression analysis of membrane progesterone receptors in women with recurrent spontaneous abortion: a case control study

Reyhane Rahnama1, Mitra Rafiee1, Saloomeh Fouladi1, Maryam Akbari-Fakhrabadi2, Ferdos Mehrabian3 and Abbas Rezaei1*

Abstract
Objective: Recurrent spontaneous abortion (RSA) is a condition which is defined as three consecutive pregnancy losses prior to 20 weeks from the last menstrual period. Progesterone is a steroid hormone that has an essential role in the implantation and maintenance of pregnancy. The progesterone signaling is performed by nuclear progesterone receptors (NPRs) and membrane progesterone receptors (mPR). The aim of this study was to analyze gene expression of mPR-α, mPR-β and NPR in the endometrium of patients with a history of RSA compared to normal fertile women.

Results: In this study, endometrial samples were obtained from 10 women with a history of RSA and 10 fertile women during days 10–14 of menstrual cycle. Relative expression of mPR-α, mPR-β and NPR genes were studied by a quantitative real time polymerase chain reaction (qRT-PCR) and compared between the two groups. The mean relative expression of mPR-β gene was significantly lower in the case group compared to the fertile women (p < 0.05). However, the gene expression of mPR-α and NPR showed no significant difference between two groups. The findings suggest a reduction of endometrial gene expression of mPR-β in RSA patients may play an important role in pathogenesis of RSA.

Keywords: RSA, Progesterone, mPR, Endometrium, NPR

Introduction
Recurrent spontaneous abortion is one of the complications during pregnancy which occurs among 2–5% of couples [1, 2]. This condition is defined as three consecutive pregnancy losses prior to 20 weeks from the last menstrual period. Various factors are involved in the occurrence of abortion which include genetic, anatomical abnormalities of the uterus and also thrombophilic, endocrine, environmental, infectious and immunological factors [3, 4].

However, the cause of RSA remains un-known in around 50% of the patients. Since human endometrium is considered an important determining factor in fertility, it has been proposed that an unsuitable endometrium could be an effective factor leading to RSA [5].

Progesterone is a steroid hormone that is primarily secreted under influences of human chorionic gonadotropin (hCG) by corpus luteum [6]. This hormone has an essential role in reproduction which is involved in the menstrual cycle, implantation and maintenance of pregnancy. Progesterone insufficiently in the luteal phase and early pregnancy could be one of the causes of RSA [7]. Progesterone has an important immunomodulatory role during pregnancy by regulating mother immune responses in preventing fetal rejection. The progesterone physiologic effects are mediated by an interaction with its receptors called progesterone receptors (PRs) [8].
The interaction of progesterone with PRs at the decidua level plays an important role in regulating the maternal immune responses [9]. The Progesterone receptor signaling is performed by both genomic and non-genomic pathways. The genomic pathway is related to nuclear progesterone receptors (NPRs) and the non-genomic pathway is related to membrane receptors (mPR) such as mPR-α and mPR-β that bind progesterone at the cell surface and rapidly generate intracellular second messengers [10]. Furthermore, the other mechanism of progesterone in maintaining pregnancy is declining the uterine contractility and improving the utero-placental blood circulation [11, 12].

Therefore, the therapeutic application of progesterone is targeted to prevent pregnancy complications such as recurrent miscarriage [12]. There is yet controversy in the usage of this clinical method. Some studies have indicated the benefit of progesterone in treatment of RSA [13, 14]; whereas other studies revealed negative results. The latter assert that the inefficiency of progesterone is due to its responsiveness rather than its availability. In fact, in these cases the expression or function of progesterone receptors is involved [15]. A study accomplished in a PR knockout mice model showed that mutation in PR represented defects in all reproductive organs. This included a dysfunction in ovulation, hyperplasia and inflammation in uterine, defect in mammary gland, and incapability in sexual performance [16]. In addition, some specific PR polymorphisms have been reported in women with RSA [17, 18].

Despite the importance of progesterone receptors in determining the correct function of this hormone in preserving pregnancy, so far, no study has been done to investigate the role of these receptors in abortion. Therefore, considering the importance of progesterone in preserving pregnancy and confirming its interaction with its membrane receptors, this study aimed to evaluate for the first time the gene expression of progesterone membrane receptors in endometrial tissue of RSA patients compared to normal controls.

Endometrial samples were taken from both groups during the late proliferative phase of the menstrual cycle (days 10–14) by Pipelle Endometrial Suction Curette (Cooper Surgical Medical Devices, USA). A written consent form approved by the ethical committee of Isfahan University of medical sciences was obtained from all participants.

All procedures performed in studies involving human participants were in accordance with the Ethics Committee of Isfahan University of Medical Sciences (Code of Ethics: IR.MUI.REC.1395.3.057) and with the Helsinki declaration and its later amendments or comparable ethical standards.

Recurrent spontaneous abortion cases
The case group included patients with at least two consecutive incidents of miscarriage prior to the 20th week of gestation with no identifiable cause which had been aborted for 3–6 months. All the cases were diagnosed with RSA; being previously evaluated for anatomical, chromosomal, genetic and hormonal abnormalities which had no detectable disorder.

Control group
Women who had at least one successful term pregnancy and visited for routine gynecological checkup diagnosed with no specific disorder, or who had undergone operations for unrelated procedures were included in our study as normal controls.

RNA isolation and cDNA synthesis
Endometrial samples were washed with Phosphate-Buffered Saline (PBS) and immediately stored in RNAlater (Sigma, USA) in −20 °C. After defrosting the frozen samples, tissues were removed from RNAlater and then total RNA was extracted using MN NucleoSpin® RNA kit (MACHEREY–NAGEL, Germany) according to the Kit instructions. Thereafter, cDNA synthesis was conducted by using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher, USA) according to the kit protocol. Consequently the cDNA was then kept at −20 °C.

Quantitative real time PCR (qRT-PCR)
Quantitative real-time PCR (qRT-PCR) was performed by the BioFACT™ 2X real-time PCR Master Mix (Biofact, Korea) on the cDNA samples by an Applied Biosystems StepOne™ machine (ABI Step One, CA, USA). The primers were designed by the Allele ID 7.0 software (Premier Biosoft, USA) which are listed in Table 1. Amplification was performed under the following conditions: 15 min at 95 °C, 45 cycles of 95 °C for 15 s and 60 °C for 60 s. For all genes a negative control consisting of non-template water instead of cDNA was used in each run of qRT-PCR. The
Table 1 Sequences of primers used in real time-PCR

| Gene     | Forward primer | Reverse primer |
|----------------|----------------|----------------|
| GAPDH       | 5'-GGAATCCCATCCATCTTCA-3' | 5'-CAATGAGCCCGCAGCTTC-3' |
| MPRα       | 5'-CTGAAGTTGGCTGACACCA-3' | 5'-AATAGGCGCAGCTGCA-3' |
| MPRβ       | 5'-CACGAAAGGACCCCAAACT-3' | 5'-CAATCCAAGACCCACCAT-3' |
| NPR        | 5'-GCTCAGGAAGTCAACCCAGT-3' | 5'-CACCATCCCTGCAAATAC-3' |

GAPDH glyceraldehyde-3-phosphate dehydrogenase, MPRα membrane progesterone receptor α, MPRβ membrane progesterone receptor β, NPR nuclear progesterone receptor

Table 2 Demographic and clinical characteristic of RSA and normal group

| Variable            | RSA patients (n = 10) | Healthy controls (n = 10) |
|---------------------|-----------------------|---------------------------|
| Age (years)         | 31.9 ± 1.32           | 32.9 ± 1.12               |
| Number of abortion  | 3.2 ± 2.42            | 0                         |
| Number of successful pregnancies | 0 | 2.82 ± 1.23          |
| BMI (kg/m²)         | 25.6 ± 0.98           | 26.8 ± 2.14               |

BMI body mass index, RSA recurrent spontaneous abortion

Discussion

Our study showed that the expression of progesterone membrane receptor (mPR-β) in the endometrial tissue of patients with recurrent spontaneous abortion was significantly lower in comparison with the normal control group. Progesterone hormone is an important steroid hormone which plays an important role in maintaining pregnancy. Progesterone activity depends on progesterone receptors (PRs) which include nuclear progesterone receptors (NPRs) and membrane progesterone receptors (mPRs) [19, 20]. Progesterone hormone plays an important role in the implantation process and maintenance of pregnancy. Therefore, its deficiency and a diminished luteal phase may result in disturbances in endometrium development which is related to RSA. However, a number of studies have demonstrated that progesterone supplementation for RSA patients does not improve pregnancy outcomes in some cases [14, 21].

As mentioned before, it is asserted that the problem is not just the hormone availability; but the abnormality of PRs is also involved. Decreased PR expression by the embryo and the endometrium has been associated with RSA [15]. Su et al. reported a specific PR polymorphism in women with a history of RSA [12, 17]. Furthermore, a correlation between RSA and polymorphism in intron G of the PR gene is related to implantation failure [22]. Due to the importance of PRs in progesterone therapy in avoiding preterm birth and recurrent spontaneous abortion [23], the better understanding of PR function in pregnancy complications will be helpful in better diagnosis and therapies in this context [19]. Studies show that the expression of mPR-α and mPR-β (two isotypes of mPRs) modifies during pregnancy in human endometrium. A decrease in expression of mPR-α during preterm and term labors has been determined, whereas mPR-β expression decreases only in term labor [24, 25]. mPR-α and mPR-β activate the p38 MAPK signaling pathway and induce phosphorylation which down regulate SRC2 expression at the end of pregnancy and onset of delivery [25, 26]. In this regard, other mechanisms include Ca²⁺ mobilization, opening of Na⁺ and Cl⁻ channels and the activation of phospholipase C which is involved in mPR activation process [27, 28].

relative quantitative gene expression was normalized by GAPDH, the internal control gene. Furthermore, the 2^ΔΔCt equation was considered for the calculation of relative mRNA levels.

Statistical analysis

The data was analysed by SPSS 24 software (IBM, Chicago, IL, USA). The Shapiro–Wilk test was used for evaluating the normal distribution of quantitative data. The genomic factors were analysed by the non-parametric Mann–Whitney test. p value less than 0.05 was considered statistically significant in this study.

Results

In this study, 10 RSA patients and 10 fertile healthy women were participated. Demographic and clinical characteristics of two groups are presented in Table 2. No significant difference was detected for age and body mass index between the two study groups (BMI, p > 0.05).

The results of this study showed that mPR-α gene was expressed higher in endometrium of the RSA group but this finding was not statistically significant compared to the control group (Fig. 1a). In addition NPR gene expression did not significantly differ between two study groups (Fig. 1b).

The results of qRT-PCR revealed that the mean relative expression of mPR-β gene was significantly lower in endometrium of women with RSA compared to normal fertile women (Fig. 1c).
Based on our knowledge, this is the first study to compare the expression of progesterone receptors in women suffering from recurrent spontaneous abortion with normal subjects. As previously mentioned, the results of our study showed a decrease in the gene expression of mPR-β in women with RSA compared to healthy subjects and if this problem occurs during pregnancy, it is likely to affect the normal pregnancy process. In addition, one of the routine treatments implied in RSA patients is progesterone therapy, and insufficiency of progesterone receptors may pose a problem with the treatment process.

**Conclusion**
The data of the present study suggest that reduction in expression of mPR-β is likely to contribute to the etiology of RSA. However, the definite role of membrane progesterone receptors in pathogenesis of RSA needs to be better investigated.

**Limitation**
There were some limitations in our study that are suggested to be addressed in future studies in order to better understand the role and function of progesterone membrane receptors. These limitations include: (1) limited number of samples, (2) our study was limited to the late proliferative phase. Since the expression of different progesterone receptors changes during different days of the menstrual cycle, studying the expression of these receptors in other phases of the menstrual cycle is important to better understand their function. (3) Our study has been carried out only at the gene expression level; evaluation of protein expression will show more accurate results. (4) Evaluating the expression of these receptors in the peripheral blood of people with recurrent abortions and comparing them with normal people can also provide valuable information.

**Abbreviations**
- RSA: recurrent spontaneous abortion
- NPRs: nuclear progesterone receptors
- mPR: membrane progesterone receptors
- qRT-PCR: quantitative real time PCR
- hCG: human chorionic gonadotropin
- PRs: progesterone receptors

**Acknowledgements**
The authors wish to thank the authorities in the research council of Isfahan University of Medical Sciences. The authors also would like to thank all participants who entered our study.
Authors' contributions

RR and MR participated in study design and data collection and evaluation. RR conducted molecular experiments. FM visited controls and diagnosed RSA patients and prepared endometrial samples. SF contributed to data and statistical analysis, and interpretation of data. RRAs and MA drafted the manuscript. AR supervised the study, contributed to study design and edited the manuscript. All authors performed editing and approving the final version of this paper for submission. All authors read and approved the final manuscript.

Funding

This work was supported by Grant 395057 from Isfahan University of Medical Sciences. The project was critically reviewed by review board of Isfahan University of Medical Sciences. The funding body was not involved in collection, analysis, interpretation of data and in writing of the manuscript.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on a reasonable request.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the Ethics Committee of Isfahan University of Medical Sciences (Code of Ethics: IR.MUI.REC.1395.3.057) and with the Helsinki declaration and its later amendments or comparable ethical standards.

Consent of publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1 Department of Immunology, School of Medicine, Isfahan University of Medical Sciences. 2 Department of Nutrition, School of Public Health, Isfahan University of Medical Sciences. 3 Department of Obstetrics and Gynecology, Al-Zahra Hospital, Isfahan University of Medical Sciences, Isfahan, Iran.

Received: 22 August 2019 Accepted: 5 November 2019

Published online: 04 December 2019

References

1. Pereza N, et al, Systematic review and meta-analysis of genetic association studies in idiopathic recurrent spontaneous abortion. Fertil Steril. 2017;107(1):150–9.
2. Wang SW, et al. The effect of intravenous immunoglobulin passive immunotherapy on unexplained recurrent spontaneous abortion: a meta-analysis. Reprod Biomed Online. 2016;33(6):720–36.
3. Practice Committee of the American Society for Reproductive M. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. Fertil Steril. 2012;98(S):1103–11.
4. Motedayen H, et al. Immunomodulatory effects of human amniotic epithelial cells on naive CD4(+) T cells from women with unexplained recurrent spontaneous abortion. Placenta. 2018;71:31–40.
5. Amichaghmaghi E, et al. Gene expression analysis of VEGF and its receptors and assessment of its serum level in unexplained recurrent spontaneous abortion. Cell J. 2015;16(4):538–45.
6. Walsh KT, Huber JC. Progesterone for recurrent miscarriage: truth and deceptions. Best Pract Res Clin Obstet Gynaecol. 2008;22(2):375–89.
7. Su MT, Lin SH, Chen YC. Association of sex hormone receptor gene polymorphisms with recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2011;96(6):1435–44.
8. Szekeres-Bartho J. Progesterone-mediated immunomodulation in pregnancy: its relevance to leukocyte immunotherapy of recurrent miscarriage. Immunotherapy. 2009;1(5):873–82.
9. Shah NM, et al. Progesterone-related immune modulation of pregnancy and labor. Front Endocrinol (Lausanne). 2019;10:198.
10. Smith JL, et al. Heterologous expression of human mPRalpha, mPRbeta and mPRgamma in yeast confirms their ability to function as membrane progesterone receptors. Steroids. 2008;73(11):1160–73.
11. Czyzyk A, et al. The role of progesterone therapy in early pregnancy: from physiological role to therapeutic utility. Gynecol Endocrinol. 2017;33(6):421–4.
12. Di Renzo GC, et al. Progesterone in normal and pathological pregnancy. Horm Mol Biol Clin Invest. 2016;27(1):35–48.
13. Carp H. A systematic review of dydrogesterone for the treatment of threatened miscarriage. Gynecol Endocrinol. 2012;28(12):983–90.
14. Coomarasamy A, et al. PROMISE first-trimester progesterone therapy in women with a history of unexplained recurrent miscarriages—a randomised, double-blind, placebo-controlled, international multicentre trial and economic evaluation. Health Technol Assess. 2016;20(1):1–92.
15. Hickman TN, et al. Decreased progesterone receptor expression in the intermediate trophoblastic cells of spontaneous abortions. Fertil Steril. 2002;77(5):1001–5.
16. Lydon JP, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 1995;9(18):2266–78.
17. Su MT, et al. Association of progesterone receptor polymorphism with idiopathic recurrent pregnancy loss in Taiwanese Han population. J Assist Reprod Genet. 2011;28(3):239–43.
18. Schweikert A, et al. Association of progesterone receptor polymorphism with recurrent abortions. Eur J Obstet Gynecol Reprod Biol. 2004;113(1):67–72.
19. Valadez-Cosmes P, et al. Membrane progesterone receptors in reproduction and cancer. Mol Cell Endocrinol. 2016;434:166–75.
20. Haas DM, Hathaway TJ, Ramsey PS. Progestogen for preventing miscarriage in women with recurrent miscarriage of unclear etiology. Cochrane Database Syst Rev. 2018;10:CD003511.
21. Haas DM, Ramsey PS. Progestogen for preventing miscarriage. Cochrane Database Syst Rev. 2013;10:CD003511.
22. Kramer DW, et al. Human progesterone receptor polymorphisms and implantation failure during in vitro fertilization. Am J Obstet Gynecol. 2003;189(4):1085–92.
23. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science. 2014;345(6198):760–5.
24. Fernandes MS, et al. Regulated expression of putative membrane progesterin receptor homologues in human endometrium and gestational tissues. J Endocrinol. 2005;187(1):89–101.
25. Karteris E, et al. Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term. Mol Endocrinol. 2006;20(7):1519–34.
26. Mesiano S, Wang Y, Norwitz ER. Progesterone receptors in the human pregnancy uterus: do they hold the key to birth timing? Reprod Sci. 2011;18(1):6–19.
27. Bramley T. Non-genomic progesterone receptors in the mammalian ovary: some unresolved issues. Reproduction. 2003;125(1):3–15.
28. Wartski K, et al. Androgen receptor-mediated non-genomic effects of vinclozolin on porcine ovarian follicles and isolated granulosa cells: vinclozolin and non-genomic effects in porcine ovarian follicles. Acta Histochem. 2016;118(4):377–86.

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.