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New aspects of immunological risk factors in the treatment of recurrent pregnancy loss

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Abstract

Recurrent Pregnancy Loss (RPL) is diagnosed in 3% of all patients of reproductive age and is of high interest to reproductive medicine specialists. Immunological predispositions are among the crucial risk factors for RPL development in female patients suffering from RPL. Therefore, the purpose of this work is to investigate the role and effectiveness of individualised immunological treatment approaches by analysing several clinical cases with diagnosed pregnancy loss and the results of tailored immunological therapies. Based on 25 years of clinical experience and scientific research the paper analysed the relationship between Human Leukocyte Antigens (HLA) tissue compatibility and ABO blood group system incompatibility in immunological pregnancy failure. The connection of these factors was shown in 2 clinical cases suffering from RPL who agreed to participate in research.

Research was performed at Nakhchivan Birth Center and Nakhchivan State University. The results of the study provided evidence based on these cases of RPL that ended with successful childbirth after the suggested genetic assessment and immunological treatment. Individual treatment of immunological pregnancy failure with HLA and ABO blood group incompatibilities showed more effective outcomes if compared to previous schemes of treatment. HLA compatibility in loci 3 combined with affinity on the ABO system strongly demanded immunological therapy prescription for successful implantation of the blastocyst. The outcomes obtained from the study can be implemented in obstetrics and gynecology for the improvement of clinical cases suffering from RPL.

Introduction

Recurrent Pregnancy Loss (RPL) is a reproductive disorder that is diagnosed after 2 or more registered cases of impaired and terminated pregnancies. There are different causes of recurrent miscarriages. Recurrent early miscarriages (within 5-6 weeks) are most commonly due to genetic or immunological reasons.[1,2] Autoimmune problems cause miscarriage usually during 9-10 weeks of pregnancy. As mentioned by Chester *et al*.,[3] recurrent late miscarriages (after 16 weeks) can be the result of uterine abnormalities, autoimmune problems, an incompetent cervix, infectious diseases, hemostatic dysfunctions, and others. In Azerbaijan, RPL is a leading reproductive problem that demands specific scientific investigations in the population.[4]

A variety of RPL causes are being discovered, and it can be treated in many cases. It is still reported that over 50% of cases with RPL are developing due to unknown reasons. As several studies conducted in Azerbaijan show, RPL among Azeri women is often associated with genetic and immunological pathologies, which is why it is important to investigate immunological ways of RPL treatment.[5,6] Among all clinically recognised pregnancies, 15% end in miscarriage, according to

Vomstein *et al*.,[7] and approximately 16-20% of all conceptions are lost and the majority occur before even being noticed. Often, the failure of repeated pregnancies is considered a pathological syndrome caused by a genetically determined polyethiological complex of symptoms. Research conducted in Azerbaijan shows the implementation of classical protocols for RPL treatments that do not always show successful results, as mentioned by Yousefian *et al*.[5]

In the US, screening for thrombophilias and other genetic, anatomical, endocrine, and immunologic factors is advised by the American Society for Reproductive Medicine's guidelines.[8] The course of treatment for a certain condition may involve the use of low-dose aspirin, heparin, progesterone supplements, and intravenous immunoglobulin. According to guidelines from the Royal College of Obstetricians and Gynaecologists in the United Kingdom, women who are afflicted with antiphospholipid syndrome should be offered aspirin and heparin.[9] Certain cases additionally call for progesterone supplements and immunotherapies such as intravenous immunoglobulin. In China, conventional Western treatments for RPL are occasionally combined with traditional Chinese medicine techniques including acupuncture and herbal formulations. According to a study by Zhao *et al*., live birth rates increased when prednisone and aspirin were used with Chinese herbal medicine.[10]

To understand the etiology of RPL and investigate appropriate treatment tools, it is essential to estimate the predispositions, fertilisation, and implantation processes impairment. To achieve successful implantation of a fertilised egg that is at stage of blastocyst, the endometrium of the uterus should undergo structural and functional remodelling for the maintenance of the embryo, as mentioned by Moqadami *et al*.[4] Immunological preparation of the endometrium takes place from the 4-5th day of fertilisation to blastocyst implantation and is essential for early gastrulation and embryogenesis. The blastocyst enters the uterine cavity and penetrates the endometrium on around 7th to 8th days after conception. After implantation, the immune response to the pregnancy that occurred at the humoral level in the endometrium begins. As mentioned by Tomkiewicz and Darmochwał-Kolarz, different immunological processes during implantation in the endometrium

may cause tolerogenic conditions, in which Human Leukocyte Antigens (HLA) factors play a crucial role in RPL.[11] Over 30% of healthy females develop anti-HLA antibodies through different gestation periods, as said by Shields *et al*., which makes this issue important for novel methods of treatment.[12] Connection of antibodies to paternal HLA agents of the embryo may result in complement binding and antibody-driven rejection of the embryo.[13,14] In female patients suffering from RPL detection of anti-HLA antibodies is strongly linked with a decreased possibility of a live birth. Therefore, the importance of HLA compatibility is a topical issue for the current obstetrics and gynecology field in the focus on maintaining healthy pregnancies.[15]

By offering insightful information about the function of immunological variables, particularly HLA compatibility and ABO blood group incompatibility, in the management of RPL, this study closes a significant gap in the body of current literature. Through the examination of clinical cases with differing levels of ABO blood group (in)compatibility and HLA compatibility, the study highlights the necessity of customised immunological treatment strategies based on these unique immunological profiles. A thorough analysis of ABO and HLA variables leads to a more sophisticated comprehension of the immunological processes behind RPL and provides direction for individualised therapeutic approaches. This research emphasises the interaction between HLA compatibility and blood group incompatibility and their combined impact on treatment results, whereas other studies have examined the individual contributions of these 2 parameters.

This study aimed to investigate new aspects of immunological treatment for RPL by examining clinical cases with diagnosed RPL and the results of individualised immunological treatment approaches. The main objectives were the following: i) to assess the impact of targeted immunological therapies, such as lymphocyte immunisation therapy (LIT) and desensitisation treatment, on improving pregnancy outcomes for couples with RPL; ii) to examine the relationship between ABO blood group incompatibility and HLA tissue compatibility as immunological risk factors for RPL.

Materials and Methods

In order to evaluate effectiveness of immunological treatment of RPL, the research involved the examination and treatment process adjustment of 2 couples both diagnosed with RPL. RPL is estimated as two or more consecutive, spontaneous miscarriages occurring during the first trimester, with the same partner. Because increased maternal age and number of previous miscarriages are shown to increase the risk of further RPL the study involved only patients of middle or young age. More thorough testing should be taken into consideration if the woman is older than 30 years and has experienced 3 losses, or if she is younger than 30 years and has experienced 2 unexplained, recurrent miscarriages.

Patients for the research were introduced among those applied to Nakhichevan Birth Center suffering from spontaneous miscarriages and with no successful pregnancies in anamnesis. Patients and their partners were required to take blood tests to exclude infectious diseases such as Acquired Immune Deficiency Syndrome (AIDS) (condition caused by the Human Immunodeficiency Virus, HIV, which attacks the immune system of the body, making it difficult to fight off infections and diseases). Additional demands for inclusion in the research included: participants had negative cervical mucus culture and cellular results, healthy karyotype assessment results; harmonised hormonal status without signs of hyperprolactinemia (abnormally high levels of the hormone prolactin in the blood) or hyperandrogenaemia (excessive levels of androgens - male sex hormones such as testosterone - in the body); absence of diagnosed endocrine pathologies including diabetes mellitus and thyroid pathology; negative antinuclear antibody and anticardiolipin antibody results; semen analysis results without pathologies. The main demand for the couples who were suffering from unexplained RPL diagnosis was to have corresponding records of treatment and outcome. To get involved in the study, informed consent was mandatory to be signed by both representatives of the couple. Every procedure used in the study complied with the 1964 Helsinki Declaration and its subsequent revisions, as well as the institutional research committee’s ethical requirements.

Given HLA is a genetic incompatibility related to the leukocyte antigen, both couples have undergone HLA genetic assessment. HLA genes are crucial regulatory factors in controlling the immune system during pregnancy and organ transplantation, which was of interest for RPL in both investigated couples. The greater immunogenic dissimilarity of maternal-fetal (HLA haplotype, inherited from the father) HLA compatibility increases the chances of successful, naturally conceived pregnancies, the accomplishment of which was the target for the treatment.

If the fetus receives 50% of allogeneic information from the father, it means that the successful morphogenesis of the fetus relies on HLA incompatibility. Analysing the precise maternal immunological tolerance (the ability of the maternal immune system to tolerate and not reject the semi-allogeneic fetus during pregnancy) towards the embryological tissue is essential for patients with PRL in order to provide implantation and a further healthy pregnancy. Here, in contrast to organ transplantation, the high level of mismatch of protein structure in maternal-fetal HLA antigens is a main criterion of clinical research. Normally, starting from the initial week of gestation, the mother's body secretes antibodies against the foetal antigen, produced by embryological tissue. In most cases, these antibodies are established against Class 2 antigens. It means that if partners' Class 2 antigens are similar, the women's body accepts it as compatible and suspends forming protective, blocking immune clones. Thus, insufficient antibody-antigen stimulation results with spontaneous miscarriage or severe toxicosis. The risk of miscarriage is 100% if partners share the same HLA Class 2 antigens. HLA typing is important to confirm the diagnosis. For this purpose, blood cells that perform immunological function – leukocytes are separated from the blood taken from the elbow veins of all participants. Additionally, HLA-A, -B, -DR, -DQ and -C personal material typing was done with the help of polymerase chain reaction.

Results

Clinical Case I (Patient I)

Clinical Case I which has been evaluated and managed is represented by a couple with burdened clinical anamnesis. Due to privacy issues, the names and personal data of patients are hidden. Patient I was 30 years old and had experienced 3 clinically confirmed failed pregnancies. All 3 pregnancy losses were diagnosed and undergone at early gestational weeks (from 6^{th} to 7^{th} weeks of pregnancy). Patient I has undergone uterine curettage and had in total 5 years of unprotected sex life with the same partner, who is her spouse. The partners share the same blood group which is O (I) Rh+. The couple has undergone HLA tissue testing, indicators of which can be found in Table 1.

When analysing the final outcomes of the HLA tissue type examinations from Clinical Case I of Patient I, one may observe the match for 3 antigen types. These antigens include A+-04; B+-51 and CW+-15 which are clearly seen as positive. If partners have 2 or more gene loci matching, the probability of pregnancy miscarriage reaches up to 100%.

Medical management of Patient I in Clinical Case I was performed by active and passive immunisation prescription. Active immunisation was carried out with the concentrated white blood cell mass of the father (donor), with immunised leukocyte solutions. In LIT, which stands for immunological treatment with activated lymphocytes, the concentrated lymphocyte mass of the partner (or the donor, in case of diagnosed incompatibility) is inoculated to the woman (patient with RPL). In Clinical Case I, Patient I was receiving the concentrated lymphocyte mass of the spouse partner under standard clinical protocol. During the conduction of the LIT method, the woman's immune response (Patient I) was clinically established to increase by 10,000 times (if compared to initial data). Immunological treatment with lymphocytes was repeated several times strictly within the period from 6^{th} to 8^{th} day of the menstrual cycle and throughout clinically approved pregnancy. No side effects have been recorded or reported by Patient I. During the implementation and conduction of this treatment method, the main aim was to somehow teach a woman's body (Patient I) to recognise her partner's sex cells in order to achieve successful fertilisation, implantation, and further intrauterine development of the embryo.

However, during the inpatient management of Clinical Case I, it was important to balance between 2 strategies of the LIT method, more importantly considering immunological aspects. To achieve maximal treatment effects, the protocol included both immunotherapy with lymphocytes due to its high effects reported,[13] as well as passive immunisation. Such tactics were supported by literature research: Carbonnel *et al.*[13] refer to the high effect of immunotherapy with lymphocytes, yet Habets *et al.*[14] claim that the usage of immunological methods is not of such importance, and that it is impossible to achieve a successful pregnancy result for patients with RPL with passive immunisation only. During passive immunisation in Clinical Case I, the protocol was adjusted to current demands and guidelines. Passive immunisation of Patient I was conducted strictly on the day of ovulation which was approved by ultrasound investigation and basal temperature. In order to perform passive immunisation through inpatient conditions, the doctors performed intravenously transferring of human immunoglobulin material 3-4 times during pregnancy which stimulates the woman's immune response. In the case of HLA matching (which was the case for Patient I), 50 ml of 3 human normal immunoglobulin daily injections were recommended to transfer every month. The 50 ml dosage was selected to keep the patient's systemic immunoglobulin levels steady and sufficient throughout the day. The immunoglobulins may remain longer in the body as a result of the numerous daily injections, which may improve their capacity to interact with pertinent antigens and influence the immune response.

For Patient I passive immunisation did not show any positive effects. Since passive immunisation was ineffective, LIT therapy was administered to Patient I. Lymphocyte immunisation therapy was prescribed as a monotherapy without passive immunisation. After treatment with LIT, Patient I gave birth to her first child. The newborn was a healthy girl, with no congenital malformations, weighed 3,100 gr, and was born on 19 March 2013.

Clinical Case II (Patient II)

Clinical Case II of proposed investigation included Patient II. Patient II was 31 years old and met all requirements of the research as she had experienced 4 pregnancies in total, all of which failed during

the early gestational weeks (in the time frame from the 6th to the 7th weeks of pregnancy). Patient II has undergone uterine curettage and has reported 5 years of unprotected sex life with the same partner (who is her official spouse). The couple has undergone HLA tissue testing, and their outcomes can be found in Table 2.

As seen from the results of diagnostic HLA testing, since there was a 50% match between 4 compatible antigens in the HLA material type assessment, it was highly recommended to perform an immunotherapeutic LIT clinical therapy for representatives of Clinical Case II. Because LIT therapy is quite an expensive method for RPL treatment and is not covered by state insurance, the couple of Clinical Case II refused this offer and decided to continue their treatment at Nakhchivan Birth Center. In addition to detected 4 similar Alloimmune markers, which are 4'11, 0301 and 05-1, 301-4, evidence of additional infections (Herpes, Toxoplasmosis, Chlamydia) and results of ABO-system indirect Coombs test 1:256 detected, which was a reason to latent sensitisation (process by which the immune system becomes activated and primed to respond to a particular antigen (foreign substance)) between partners. Neamţu *et al.*[16] suggest that incompatibility on the ABO-blood group system becomes dominant during the incompatibility on the Rh system, and fetal hemolytic disorder becomes predictably milder. Specially designed treatment for Clinical Case II was commenced, after the authors of the current study proved to deliver positive treatment results in case partners have 3-4 loci HLA tissue compatibility in the relationship with AB-O system incongruence. During the treatment course the fact that some microorganisms (viral and bacterial infections) cause hidden sensitisation, trigger isoimmune, autoimmune and alloimmune processes during pregnancy, and genetically mimic blood groups of some pathogens was considered.[17]

The Clinical Case II couple underwent a rehabilitation program before planning pregnancy. A course of desensitising treatment was prescribed for the elimination of ABO blood type incompatibility marks. As a result, several causes of early pregnancy losses were detected: post-implantation pregnancy failure, endometrial insufficiency (for feasible implantation of the first-week embryo), and rejection of primary embryologic tissue by the mother's organism.[18,19] These factors were eliminated

but continued to be possible risk factors for early stages of potential pregnancy, stillbirth or childbirth with low viability. The couple received specific therapy for viral and bacterial infections, immunosuppressive therapy, desensitising treatment for ABO-system incompatibility before conceiving. The treatment ended successfully and Patient II delivered a healthy newborn girl (3,500 gr of weight). Patient II currently has 2 healthy daughters.

To sum up, the examination of these 2 clinical instances emphasises how crucial it is to take into account both ABO blood group incompatibility and HLA compatibility while treating recurrent pregnancy loss immunologically. The findings show that more intense LIT is necessary for favourable treatment outcomes when partners show HLA compatibility at locus 3 together with ABO blood group compatibility. However, in cases where HLA compatibility is seen at the 4th locus coupled with ABO blood group incompatibility, a milder therapeutic approach incorporating desensitisation treatment may be appropriate. Notably, the results indicate that at the 3rd and 4th loci, ABO blood type incompatibility is more important than Rh incompatibility and HLA compatibility, requiring a gentler therapeutic approach specifically targeted to address the ABO incompatibility. Therefore, for couples experiencing recurrent pregnancy loss, the combination of ABO blood group (in)compatibility and HLA compatibility at specific loci serves as a guiding factor in determining the appropriate immunological treatment strategy, ultimately improving the chances of successful pregnancy and childbirth.

Discussion

RPL can be developed under the influence of chromosomal impairment, anatomical endometrial defects, autoimmune diseases, and uterine dysfunction.[20-22] In practical medicine, obstetrics insists that infectious causes of bacterial and viral diseases do not cause RPL, thus some researchers do not recommend undergoing relevant examinations. Still, it should be considered that endometrium, impaired by specific or non-specific inflammation, and damaged by viral and bacterial infections,

leads to incomplete nidation, poor quality progress of blasto- and embryogenesis, and disruption of embryo's nutrition that may lead to the development of RPL.[23,24] Thus, the chronic and acute inflammatory process have direct and indirect effects that as a result of triggering auto- and alloimmune processes in the disruption of pregnancy will cause an incomplete implantation and malnutrition in female patients. As mentioned by Ali *et al*., these may lead to RPL as well.[25] It becomes clear that above mentioned direct and indirect causes of RPL should be considered for achieving successful treatment results in patients with laden anamnesis.

Thus, when diagnosing RPL for immunological reasons as in the Clinical Case I, HLA tissue matching at 3-locus accompanied by ABO matching (both O(I) Rh+) required more intensive LIT therapy treatment. In addition to HLA, ABO, Rh and other rare blood system mismatches, latent sensitisation, and viral and bacterial infections should not be overlooked, as mentioned by Barbaro *et al*.[26] Depending on the etiological reason of pathological process in the endometrium and female reproductive tract, a specific factor creates corresponding cascades of non-specific inflammation – endometritis, disrupting the physiological preparation for pregnancy in the uterus.[27,28] A study by Thomsen *et al*.[29] was conducted with RPL patients who have undergone HLA-DRB-1 typing by DNA-based methods for detecting or HLA-DRB1*07 evaluation allele frequency. The authors statistically approved the association linking HLA-DRB1*07 with RPL which may be different from previous papers which established an association between HLA-DRB1*03 and RPL. These data differ from outcomes received in the current paper, but one may suggest the connection of genetic predispositions with RPL development.

The currently conducted study achieved successful childbirth after LIT prescription, creating a strong maternal immune response. In case II HLA material compatibility at the 4-locus and ABO erythrocytes system compatibility (ABO titter 1/256) was detected. Because bacterial and viral infections mimic blood groups, by treatment of dominant ABO incompatibility the immune status that leads to successful pregnancy and childbirth was established. Resembling paper was published by Aimagambetova *et al*.: the research has discussed the contribution of DRB-1, DQB-1, and DPB-

1 HLA class 2 alleles with final 3-locus haplovariants to the general possible risk of RPL development.[30] Authors deliver outcomes that show a relatively low influence of class 2 alleles and appropriate 3-locus haplotypes to the impaired development of RPL development among diagnosed female patients. As Ajmal *et al*. propose, antigen hiding is a process that leads towards immune evasion by the embryological cells of the blastocyst and fetus as the placental barrier lacks HLA expression.[31] Nevertheless, the HLA agents that are indicated by the extraembryonic trophoblastic cells influence maternal immune response towards a protective activation, primary to acceptance of the embryonic tissues and structures.

Some reasons that cause the RPL have been represented previously. Researchers prove a list of conditions, including uterine congenital malformations and topographical anomalies, endometrial insufficiency, and hormonal background as diseases that may be linked with RPL development as risk factors.[32] Elbaşı *et al*. emphasise that for RPL development a few immunological factors impairment should be combined.[33] Their research, conducted in a selected population with RPL, claims that male HLA-C2 homozygosity may influence RPL development. Authors have also established an incidental match between male patients' HLA-C2 and female patients' HLA-C1 ligand Killer-cell Immunoglobulin-like Receptors (KIR) might disturb the activatory and inhibitory homeostasis in KIR-ligand connections during different gestation periods in couples with RPL. By means of different diagnostic methods and specific treatment approaches, current research delivers immunological parameters that vary between partners, including HLA and ABO blood group incompatibilities. Referring to the deliverables of the examination, it is advisable to recommend immunological diagnostics and treatment therapies for patients suffering RPL that provide an overview of the promising immune alteration of RPL.[34,35]

When summarising the results of investigated cases considering therapeutic and diagnostic strategies of immunologic predisposing factors for RPL patients following can be concluded. First of all, the compatibility of 3-4 loci on the HLA genes between partners has specific patterns. The similarity of partners' class 2 antigens during the alloimmune process, the mother's body is similar to fetus, thus

clones of immune cells that protect and block pregnancy are not formed, and insufficient antibody-antigen stimulation causes miscarriage and RPL. Secondly, partners' HLA tissue compatibility at 3-4 loci, ABO erythrocytes method compatibility (ABO titter 1/128, 1/256), bacterial and viral infections mimic blood groups. In this case, the compatibility of the ABO blood system will be prior and therefore easily treated. And lastly, the dominant ABO incompatibility is treated by desensitisation therapy which recovers immune status and leads to successful pregnancy results.

It may be concluded that final data from the current research can be used in the fields of obstetrics, gynecology, and reproductive medicine for modernisation of individual treatment of patients and couples suffering from RPL, as well as better initial treatment of female patients with infertility risk factors. Presented data is useful for the field of reproductive medicine, obstetrics, and gynecology for better management of clinical cases with RPL.

Conclusions

The conducted research has provided comparative data of 2 couples, each suffering from clinically diagnosed RPL with HLA compatibility at 3rd and 4th loci. In provided Clinical Case I, partners were found to be compatible with 3 loci during HLA typing and compatible according to the ABO blood group system; both of them share the same blood group (O(I), Rh+). In Clinical Case I female patient was treated with LIT that has shown successful outcomes. Patient I resulted in the natural delivery of a healthy female newborn weighing 3,100 grams. In Clinical Case II, the couple has suffered from RPL (recorded 4 pregnancy failures within 6-7 weeks of pregnancy). During the HLA typing test compatibility with partners at loci 4 was discovered. Incompatibility detected in AB-O blood group system (title 1/256). The couple refused LIT therapy, but the woman received desensitisation treatment according to the AB-O system accompanied by infectious diseases treatment that is among the main causes of masked desensitisation, AB-O type mimicry. Despite the fact that Patient II did not receive LIT therapy, her desensitisation treatment resulted in the birth of a healthy newborn weighing 3,500 grams.

Therefore, it may be concluded that HLA affinity on loci 3 combinations including affinity on the AB-O system demands LIT treatment and can show positive clinical results. Compound treatment of incompatibility on the AB-O system, with HLA on loci 4 compatibility, demands softer therapy. As a result, incompatibility according to the ABO system, both with Rh incompatibility and with HLA affinity on loci 3-4, showed a controlling character with a need for milder therapy. Future steps of the research topic expansion can include a wider range of patients (including different age groups and preliminary anamnesis) and additional indexes for diagnostic analyses.

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Table 1. Diagnostics results of Patient I and her partner (spouse).

HLA results from a female patient			
A+	B++	C+	DR+
A+-02	B+-50	CW+-02	D RBI+-14
A+-03	B+-26	CW+-15	D RBI+-09
HLA results from a male patient			
A+	B++	C+	DR+
A+-04	B+-51	CW+-14	D RBI+16
A+-11	B+-51	CW+-11	D RBI+-03

HLA, Human Leukocyte Antigens

Source: composed by the authors

Table 2. Diagnostics results of the Patient II and her partner (spouse).

HLA Results from a female patient		
DR-B_1	DQ-A_1	DQ-B_1
4’11	0301-05-1	0305 301-4
HLA Results from a male patient		
DR-B_1	DQ-A_1	DQ-B_1
4’11	0301-05-1	03-02 301-4

HLA, Human Leukocyte Antigens

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