EVALUATION OF ALLERGIC DISPOSITION IN IVF-CONCEIVED MICE AND THE EXPRESSION OF IMMUNE CHECKPOINTS IN PBMCS OF WOMEN WITH RPL

Ph.D. Thesis

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INTRODUCTION

During ART procedures, factors including hormone treatment, gamete and embryo manipulation, chemical and mechanical stress, and interference in the natural selection of gametes, lead to genomic and epigenetic alterations, which probably enhance the risk of disorders throughout the later life of ART-conceived offspring (1).

Embryos are susceptible to environmental cues during the preimplantation period. IVF is performed during the preimplantation period when the genome undergoes drastic epigenetic remodeling, and any environmental alterations can affect normal developmental programming (2,3). IVF-conceived babies might be at higher risk of developing diseases, e.g., high blood pressure and metabolic and epigenetic disorders, compared to their naturally conceived counterparts (4,5).

Immunological changes in ART-conceived offspring

Immunological changes in ART-conceived offspring have been evaluated in humans and animal models. Several studies demonstrated the changes in immune system functionality and increased rates of immune-related disorders in ART-conceived babies. ART-conceived mice exhibited less effective immune responses against the BCG vaccine, as indicated by a skewing toward T-helper (Th) 2-dominated responses. The enhanced prevalence rates of asthma, allergies, metabolic syndrome, and childhood diseases also indicate possible alteration of the immune system in IVF-conceived offspring (6-8).

Although the exact etiology is not completely understood, the interactions between genetics and environment appear to play a crucial role in the development of allergy and asthma (9,10). The biological effect of IgE is complex and is exerted by affecting the functions of several immune cells involved in the pathogenesis of allergic inflammation. The binding of IgE to FCεRI expressed

on the surface of dendritic cells (DCs) enhances their antigen-presenting capability. It has also been confirmed that IgE captures allergens and facilitates their presentation to memory Th2 cells (11). Allergic diseases are mainly mediated by Th2 lymphocytes. Once activated by allergens, specific Th2 cells not only activate B cells to produce IgE but also recruit and activate basophils, mast cells, and eosinophils by secreting IL-4, IL-5, and IL-13. IL-4 is involved in IgE production and the pathogenesis of several aspects of allergic disease (12,13).

Evaluating the risk of atopic disorders among ART-conceived babies

Several studies have evaluated the risk of atopic disorders among ART-conceived babies. It is concluded that the data regarding the risk of allergic diseases and asthma among ART-conceived offspring are inconsistent. Otherwise, some systematic reviews and meta-analyses, as well as population-based studies, have shown an increased rate of atopic disorders among ART-conceived offspring (14,15).

Several possible confounding factors probably influence the results of epidemiological studies, and adverse outcomes have not been completely attributed to ART procedures. Since animal studies can modulate infertility as a potential confounding factor, they may provide useful information to facilitate the authentication of human ART-related findings. Accordingly, we conducted the current study using IVF-conceived male mice to measure the serum levels of IgE and the weights of the lung and spleen following injection with ovalbumin (OVA), in comparison with naturally conceived counterparts.

Recurrent pregnancy loss (RPL) and the immune system

Recurrent pregnancy loss (RPL) is defined as two or more consecutive pregnancy losses before the 20th week of gestation and affects about 1 to 3 % of pregnancies. Parental chromosomal abnormalities, endocrine factors (e.g., untreated hypothyroidism), anatomic abnormalities of the

uterus, and autoimmune diseases are possible aetiologies for RPL (16). Moreover, infectious diseases, thrombophilia, immunologic disorders, and certain environmental factors are associated with RPL (17). In more than 50 % of RPLs, even after a comprehensive investigation, no exact cause can be detected, and thus, they are considered unexplained RPLs (16). It has been suggested that disorganized maternal immune responses might contribute to the incidence of unexplained RPL (17). Myeloid cells, innate lymphoid cells (ILCs), T cells, and B cells induce tolerance during pregnancy (18). Decidual CD4/CD8 T cells recognize fetal antigens and activated CD4/CD8 T cells and regulatory T cells (Treg) play important roles in mediating immunological tolerance to the foetus by modulating the local immune microenvironment. CD4 T cells, particularly T helper (Th1, Th2), Th17, and Treg cells, are involved in fetal-maternal immune responses (19). By producing anti-inflammatory cytokines such as IL-10 and TGF-β, Treg cells modulate immune responses during pregnancy (20). These functional alterations include the increased expression of the inhibitory checkpoint receptors programmed death-1(PD-1) and T cell immunoglobulin and mucin domain 3 (Tim-3). T cell exhaustion is associated with decreased effector function and cytokine production, as well as increased expression of inhibitory receptors such as PD-1 and Tim-3 on their cell surfaces (21,22). T cell exhaustion and PD-1 and Tim-3 signaling probably play significant roles in inducing maternal immune tolerance and the establishment and maintenance of successful pregnancy; hence, the number of CD8+ Tim-3+ PD-1+ may be altered in RPL (23). Exhausted Treg cells are characterized by the expression of PD-1 molecules on their surface, and the interaction of the PD-1 molecule with its ligand (PD-L1) results in the increased suppressive function of Treg cells in vitro (24).

The role of MiRNAs in RPL and early pregnancy

MiRNAs are involved in various disease pathogeneses and may be utilized as prognostic and diagnostic biomarkers (25,26). Studies have investigated the role of miRNAs, such as miR-138 and miR-155, in regulating immune cells, embryo morphogenesis, cell proliferation, cell invasion, stem cell differentiation, and development (27). MiR-138-5p is a transcriptional regulator of the G protein-coupled receptor 124 (GPR124), which regulates the activation of NLRP3 inflammasome (27). MiR-138-5p was shown to be downregulated in the decidua from miscarriages compared to normal pregnancy, suggesting its role in embryo implantation and early pregnancy events. It has also been suggested that miR-138-5p is downregulated in spontaneous miscarriage compared to normal pregnancy (27). By targeting Foxp3, the main transcription factor of Treg cells, miR-155 can regulate the differentiation and functions of Treg cells (26). Downregulation of miR-155 results in the alteration of Treg cells frequency, whereas their function remains unchanged. The expression profiles of miRNAs in embryo implantation and early pregnancy may indicate pathological modifications specific to RPL. The contribution of miRNAs in modulating underlying signaling pathways related to abnormal embryo implantation and pregnancy needs attention. Our study aimed to characterize the expression of Tim-3 and PD-1 on CD4 T cells in the peripheral blood of women with RPL compared to healthy control women. In this section of our work, we also investigated the expression of mir-138 and mir-155 in PBMCs and the level of TGF-β and IP-10 in the sera of women with RPL compared to healthy control subjects.

OBJECTIVES

This study consists of two distinct sections. In our study, both human samples and mice were used to meet our objectives. Accordingly, this study aimed to evaluate the following goals comprehensively:

In mice:

- to evaluate the tendency of IVF-conceived male mice to develop allergic responses in comparison to their naturally conceived counterparts
- The serum levels of total IgE and IL-4 in IVF and naturally conceived male mice before and after sensitization with ovalbumin (OVA)
- The total body weight and weight of the lung and spleen in IVF and naturally conceived mice before and after sensitization with OVA

In the case of a human sample, the objectives were as follows

- to evaluate the expression of PD-1 and Tim-3, besides their related miRNAs in PBMCs of women with RPL
- The expression of PD-1 and TIM-3 on CD4+ and CD8+ T cells from women with RPL in comparison to their healthy pregnant counterparts
- The transcriptional levels of PD-1 and TIM-3 in the PBMCs of RPL patients and controls
- The transcriptional levels of mir-138 and mir-155 in the PBMCs of RPL patients and controls
- The serum concentration of TGF-β and IP-10 in women with RPL and healthy controls

MATERIALS AND METHODS

Animals

To conduct the experiments on mouse models, six-week-old male and female CBA and B6 mice were purchased from Charles River. This part of the study was approved by the Animal Research Ethical Committee of the University of Pécs and the Hungarian National Scientific Ethical Committee on Animal Experimentation.

In Vitro Fertilization (IVF) and Natural Conception

CBA female mice were injected with 7.5 IU of pregnant mare serum gonadotropin (PMSG), followed by 7.5 IU of human chorionic gonadotropin (hCG) with 48 48-hour interval. Cumulus–oocyte complexes (COCs) were harvested from the oviducts 12–15 h after hCG injection by oviduct recession after scarification.

To produce IVF-conceived mice, IVF was performed as previously described with minor adjustments. Briefly, 1×10^5 capacitated sperm cells were added to oocytes in KSOM medium and incubated at 37 °C for 6 h. Subsequently, the fertilized zygotes were washed serially in M2 medium to remove the excess spermatozoa, debris, and cumulus cells. Then they were transferred into an equilibrated drop of KSOM, covered with paraffin oil. The embryos were cultured at 37 °C with 5% CO_2 until they reached the blastocyst stage. About 7–10 embryos at the blastocyst stage were transferred into each side of the uterus. Seventeen days following embryo transfer, the pups were delivered naturally.

To produce naturally conceived offspring, one or two female mice were mated with a male mouse. The next morning, they were checked for the presence of vaginal plugs, and those with vaginal plugs were kept individually in separate cages until the day of delivery.

Ovalbumin-induced inflammation model in mice

To produce an OVA-induced allergic model, male mice at 8 weeks were injected intraperitoneally on days 1 and 8 with 20 μg of OVA (Sigma, St. Louis, MO, USA) mixed with 1 mg of aluminum hydroxide (Sigma-Aldrich, Seoul, Republic of Korea) dissolved in a total volume of 200 μL of saline. On days 15, 16, and 17, after the initial sensitization with OVA, the mice were injected intranasally with 20 μg of OVA dissolved in 40 μL of saline. In the NC and IVF groups, mice were injected with an equal volume of normal saline.

Experimental Groups

Animals were divided into 4 groups:

- naturally conceived non-OVA-treated (NC) (n = 8)
- naturally conceived OVA-sensitized (OVA-NC) (n = 13)
- IVF-conceived non-OVA-treated (IVF) (n = 8),
- IVF-conceived OVA-sensitized (OVA-IVF) (n = 13).

Measurement of Total Body and Organ Weights in Mice

The total body weights of the mice were measured and recorded before sacrifice. Mice were matched based on their weight and sacrificed by cervical dislocation at 10 weeks of age. The spleen and lungs were removed; their weights were measured using a scale and recorded.

Measurement of Total IgE and IL-4 serum levels in mice

The total serum IgE and IL-4 concentrations were measured by ELISA kits according to the manufacturer's instructions. The optical density was determined by measuring the absorbance at 450 nm using an ELISA reader.

Patients

This part of the study (Human experiments) was approved by the Research Ethics Committee of Tabriz University of Medical Sciences.

Samples were collected from 50 women suffering from RPL between the 10–12 weeks of gestation and from 50 gestation-age-matched healthy pregnant women.

Isolation and culture of peripheral blood mononuclear cells from RPL patients and controls

Fifteen ml of peripheral blood were collected from both RPL patients and healthy volunteers under sterile conditions and supplemented with anticoagulants (heparin) for the isolation of peripheral blood mononuclear cells (PBMCs). Finally, the frequency of PD-1 and Tim-3 on CD4+ T cells and CD8+ T cells was detected using flow cytometry.

Flow cytometric detection of PD-1 and Tim-3 on $CD4^+$ T cells and $CD8^+$ T cells

After washing the PMBCs, 1 million cells were re-suspended in 100 µl of washing buffer and incubated with specific monoclonal antibodies for 60 min, in the dark at 4 °C. The following antibodies were used: anti-CD4-FITC, anti-PD-1-PerCp/Cy5.5, anti-Tim3-APC, and anti-CD8-PE. The proportion of T CD4 cells and T CD8+ + cells expressing PD-1 or Tim-3 was measured using a FACS Calibur flow cytometer.

Analysis of messenger RNA transcripts

RNA isolation from PBMCs was performed using the Ytzol pure RNA extraction kit. The mRNA expression levels of PD-1 and Tim-3 were evaluated using the RT-PCR method and CYBER Green Master Mix. For each sample, relative mRNA levels were standardized to β-actin

mRNA expression level. Mir-138 and mir-155 were evaluated in PBMCs using RT-PCR and Light Cycler 2.0 RT-PCR System machines.

Determination of TGF-β and IP-10 in the serum of RPL patients and controls

ELISA kits were used to measure the serum levels of TGF-β and IP-10. The minimal detectable concentrations for TGF-β and IP-10 were 1 pg/ml and 2.5 pg/ml, respectively.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 and SPSS version software. Data are expressed as the Mean ± SD. The Unpaired *T*-test was used to calculate the mean difference between the groups. Pearson's r correlation test was used to calculate the correlation coefficients. *P*-values of less than 0.05 were considered significant. The ANOVA statistical test was also used to compare the mean difference in the markers studied among the four animal groups. $p \leq 0.05$ was considered statistically significant.

RESULTS

Serum Levels of Total IgE in Naturally and IVF-Conceived Mice

The basic IgE levels in IVF-conceived mice were slightly higher than those in naturally conceived ones. OVA sensitization in both naturally and IVF-conceived mice resulted in significantly increased serum IgE levels ($p = 0.012$ and $p = 0.029$, respectively). The enhanced IgE levels following OVA treatment were more pronounced in IVF-conceived than in naturally conceived mice. Our results also show significantly higher serum levels of total IgE in OVA-IVF mice than OVA-NC counterparts ($p = 0.004$).

Serum Levels of IL-4 in Naturally and IVF-Conceived Offspring

The basic IL-4 levels in IVF-conceived mice were significantly ($p = 0.048$) higher than those in naturally conceived ones. OVA sensitization in both naturally and IVF-conceived mice resulted in higher serum IL-4 levels than non-OVA-sensitized counterparts ($p = 0.0148$ and $p = 0.0189$).

Measurement of Total Body and Organ Weights in Mice

Following OVA injection, the lung weights of both naturally and IVF-conceived animals were significantly higher compared to those of their non-injected counterparts ($p = 0.034$ and $p = 0.013$, respectively). The results demonstrated significantly increased lung weights among N-IVF and OVA-IVF mice compared to their N-NC and OVA-NC counterparts ($p = 0.0335$ and $p = 0.001$, respectively). Lung weights were normalized to body weights by multiplying the lung weight by 100 and then dividing the result by the total body weight. Our results demonstrated that sensitization with OVA resulted in increased lung index values in both IVF- and naturally

conceived mice compared to their non-sensitized counterparts (0.88 vs. 0.68 and 0.76 vs. 0.625, respectively). The increase was more pronounced in IVF-conceived mice.

Our results demonstrated that OVA injection resulted in increased spleen weights in both naturally conceived and IVF-conceived offspring compared to their non-injected counterparts (p = 0.007 and p = 0.008). We also demonstrated that the spleen weight in N-IVF mice was higher than that in N-NC ones (p = 0.013). The spleen weight was significantly higher in OVA-IVF mice than in OVA-NC counterparts (p = 0.041).

$CD4^+$ T cells and $CD8^+$ T cells expressing PD-1 and Tim-3 in RPL patients and controls

Surface expression of PD-1 and Tim-3 on CD4+T cells and CD8+T cells was determined by three-color flow cytometry. Immunophenotyping revealed a significantly decreased percentage of PD-1 expressing CD4+and CD8+T cells in RPL patients, compared to healthy controls. The frequency of CD8+ T cells expressing Tim-3 was significantly higher in the controls, compared to RPL patients. There was no significant difference in Tim-3 expression of CD4+T cells between the two groups.

The expression of PD-1 and Tim-3 mRNA in PBMCs from RPL patients and controls

The mRNA expression of both the PD-1 and Tim-3 genes was downregulated in PBMCs of RPL patients compared to controls; however, the difference was not statistically significant for the latter.

TGF-β and IP-10 concentrations in the sera of RPL patients and control subjects

The serum levels of TGF-β in RPL patients (25.11 ±15.08 pg/ml) were significantly lower than in the controls (36.78 ±21.96 pg/ml). By contrast, the serum level of IP-10 in the RPL patients (210.7 ±123.5 pg/ml) was significantly higher compared to the controls (143.1 ±81.76 pg/ml).

Tim-3 expression on both CD4 and CD8 T cells positively correlated with serum level of TGF-β ($r = 0.3526$, p 0.0120 and $r = 0.4514$, $p = 0.0010$, respectively), and negatively with IP-10 serum levels ($r = 0.3229$, $p = 0.0222$ and $r = 0.3929$, $p = 0.0048$, respectively).

Transcriptional levels of mir-138 and mir-155 in RPL patients and controls

The relative expression of miR-138 and miR-155 was significantly lower in PBMCs of RPL patients than in those of healthy pregnant women. We found a significant positive correlation between mir-155 and PD1 expression ($r = 0.3933$, $p = 0.0047$), and a positive correlation between mir-138 and PD-1 expression ($r = 0.4562$, $p = 0.0009$). There was also a positive correlation between mir-138 and Tim-3 ($r = 0.1172$, $p = 0.4176$) and a significant positive correlation between mir-155 and Tim-3($r = 0.3329$, $p = 0.0182$).

DISCUSSION

IVF is used to overcome both male and female infertility-related problems. Approximately 200,000 babies are born annually using ART around the world, contributing to 5% of all babies conceived in developed countries (1). In parallel with growing demands for ART, concerns about the long-term health status of ART-conceived babies have also increased. The underlying mechanisms of these potential health problems need to be clarified. Studies have confirmed a link between ART and imprinting disorders.

Several studies have evaluated the altered immune system functioning and an increased rate of immune-related diseases in ART-conceived offspring.

In our previous study, we showed that ART-conceived mice exhibit less efficient immune response against BCG vaccination by skewing toward Th2 and Th17 dominant responses (6). Since Th2 and Th-17-biased immune responses have been suggested as typical features of several allergies, autoimmune disorders, and inflammatory conditions, ART-conceived offspring may be more prone to developing allergic and inflammatory immune responses (28).

Our findings from animal experiments showed that sensitization of mouse offspring from both natural and IVF conception with OVA presumably resulted in an allergic reaction represented by enhanced serum levels of IgE and IL-4, as well as increased weights of the spleen and lungs. Previous studies have indicated that immunization with OVA can induce IgE-mediated splenocyte proliferation, as well as Th-2 dominant immune responses (29). In addition, OVA-sensitized mice develop a stronger inflammatory reaction in their airways (30).

A collective comparison of OVA-induced inflammatory responses among the four studied groups indicated that IVF procedures probably result in a higher tendency to

develop allergic reactions in offspring, as demonstrated by increased serum levels of IgE and IL-4 and greater lung and spleen weights among IVF-conceived mice compared to their naturally conceived counterparts.

During the IVF procedure, any alterations in either the contact materials (plasticware, metalware, glassware) or micro-environment conditions, such as culture media, incubator, oxygen, freezing media, carbon dioxide, and ambient air, may exert adverse effects on the gametes and resultant embryo quality, consequently enhancing the rate of genetic defects (1,31).

In a healthy pregnancy, the immune system is highly regulated to accept the semi-allogeneic developing fetus, while protecting the mother and conceptus from pathogens. Therefore, as the immune system is responsible for supporting pregnant mothers and their developing fetuses, incomplete adaptation of the immune response might be an underlying factor in RPL pathogenesis (32).

Galectin-9 and PD-L1, as ligands for Tim-3 and PD-1, respectively, are elevated during normal pregnancy and may play a role in mediating immune tolerance to the semi-allogenic fetus. Both PD-1 mRNA expression in PBMCs and the expression of the PD-1 protein on the surface of $CD4^+$ and $CD8^+$ T lymphocytes were downregulated in women with RPL (33,34). We demonstrated significantly downregulated Tim-3 mRNA expression in PBMCs, and Tim-3 protein expression in peripheral $CD8^+$, but not in $CD4^+$ T cells from women with RPL.

As regulators of gene expression, miRNAs can influence endometrial receptivity by affecting endometrial angiogenesis, energy metabolism, cell proliferation, decidualization, and the local cytokine balance (27). We found a significantly decreased expression of miR-138 and miR-155 in PBMCs of patients with RPL, and a positive correlation between miR-155 and the expression of PD-1 in PBMCs.

By regulating endometrial receptivity, TGF-β plays a critical role during embryo implantation (35). In addition, TGF-β plays a role in Treg cells differentiation, since $CD4^+$ T cells deficient in TGF-β signaling fail to differentiate into Treg cells either in vitro or in vivo. We demonstrated that the level of TGF-β is significantly decreased in the sera of RPL patients compared to those from healthy women. In line with our findings, other studies have reported a significantly lower expression of TGF-β1 in the decidual tissue of patients with RPL(36).

It has also been suggested that increased levels of IP-10 are closely associated with certain pregnancy-related pathologies. We observed significantly higher concentrations of IP-10 in the serum of women with RPL than in healthy pregnant women, and we demonstrated an inverse correlation between the expressions of Tim-3 and PD-1 on both CD4+ and CD8+ T cells, along with IP-10 concentrations in the sera of women with RPL. Although an inflammatory response involving IP-10 is necessary during implantation and delivery, excessive inflammation can lead to pregnancy-related complications.

In summary, we found downregulated expression of PD-1 and Tim-3 mRNA in PBMCs, and Tim-3 and PD-1 protein expression on CD8+ T cells from women with RPL. We also indicated lower expressions of mir-138 and mir-155 in PBMCs of patients with RPL. Furthermore, our results demonstrated a decreased concentration of TGF-β in sera from RPL women compared to their healthy counterparts.

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Total Impact Factor: 23.5

Impact Factor of publications included in the dissertation: 13.1

Own publications included in the dissertation:

1. **Ahmadi Hamid**, Zoltan Bognar, Timea Csabai-Tanics, Basil Nnaemeka Obodo, and Julia Szekeres-Bartho. 2024. "Allergic Disposition of IVF-Conceived Mice" *International Journal of Molecular Sciences* 25, no. 23: 12993.

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2. **Ahmadi H**., Soltani-Zangbar, M.S., Yousefi, M., Baradaran, B., Bromand, S., Aghebati Maleki, L., Szekeres-Bartho, J., 2024. The evaluation of PD-1 and Tim-3 expression besides their related miRNAs in PBMCs of women with recurrent pregnancy loss. Immunol. Lett. 266, 106837.

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3. **Ahmadi Hamid**, Leili Aghebati-Maleki, Shima Rashidiani, Timea Csabai, Obodo Basil Nnaemeka, and Julia Szekeres-Bartho. 2023. "Long-Term Effects of ART on the Health of the Offspring" *International Journal of Molecular Sciences* 24, no. 17: 13564.

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