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RESEARCH ARTICLE

The Correlation between Serum Levels of HLA-G and Recurrent Implantation Failure During Implantation Window in Women Undergoing In Vitro Fertilization

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ABSTRACT

Background: **Recurrent implantation failure (RIF) during artificial reproductive techniques (ARTs) remains a health problem. Many physiopathological pathways have been proposed for RIF, but the causes are not yet clear. Thus, the purpose of the current study was to evaluate the possible correlation between human leucocyte antigen-G (HLA-G) serum levels and recurrent implantation failure during the implantation window in women undergoing In vitro fertilization (IVF).**

Materials and Methods: **In this prospective case-control study, 39 patients with RIF and 20 cases with successful implantation were enrolled as the control group. HLA-G serum levels were measured using the sandwich ELISA method in both groups.**

Results: **HLA-G serum levels in the RIF group were significantly higher than in controls (0.085 ± 0.019 compared to 0.073 ± 0.005, $p = 0.024$). Furthermore, by grouping patients based on age and BMI, we found that in the cases with ages above 30 years and with BMI more than 25 (kg/m^2), HLA-G serum levels in the RIF group were remarkably greater than controls (0.088 ± 0.019 compared to 0.072 ± 0.004, $p = 0.002$ and 0.083 ± 0.012 compared to 0.072 ± 0.003, $p = 0.021$, respectively).**

Conclusion: **Serum levels of HLA-G increase in the RIF patients, especially in cases of patients above 30 years old and those with BMI $\geq$ 25, which remains significant after eliminating confounding variables. These results may indicate the involvement of physiopathological pathways of HLA-G in the increasing risk of RIF.**

Keywords: Human leucocyte antigen-G (HLA-G), in vitro fertilization (IVF), recurrent implantation failure (RIF).

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1. Introduction

Infertility is one of the major growing problems in reproductive-aged couples around the world (the frequency is 8%–12%) [1]. Therefore, artificial reproductive techniques (ARTs) enhance the chance of conceiving a child in couples with infertility problems. However, in vitro fertilization (IVF) failure can be observed in 10% of women undergoing ARTs due to recurrent implantation failure (RIF) [2], [3]. The etiology of RIF is complex and often is not clear. Several factors have been introduced for the successful implantation during IVF-ET, such as factors related to the embryo (genetics and sperm defects, and immunological factors) and maternal factors (uterine abnormalities and thrombophilia) [4]. Moreover, multiple studies have demonstrated significant associations between some genetic polymorphisms such as methylenetetrahydrofolate reductase (MTHFR), P53, cyclooxygenase-2 (COX-2), thymidylate synthase (TS), nuclear factor kappa B (NF-κB), vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1, and leukocyte antigen-G and the risk of RIF [5]–[11]. However, the human leucocyte antigen-G (HLA-G) gene was recently studied more as a risk factor for RIF [12]. Moreover, we need a non-invasive biomarker to predict the result of embryo

transfer and to identify complications associated with the abortion [13].

HLA-G is expressed by extravillous trophoblast cells and has an immunomodulatory role (impact on the natural killer cells in the decidua, T, and macrophages). HLA-G protects the fetus against the attack by the maternal immune system by regulating cell migration during placental development. Therefore, secretion of HLA-G inhibits trophoblast invasion in the decidua [14]–[16] and enhances the diameter of spiral arteries to provide proper fetal nutrition and ensure pregnancy outcomes. Hence, it seems that serum HLA-G levels may predict the risk of RIF [17]. There seem to be insufficient prospective studies on the role of serum HLA-G levels in RIF patients. Hence, the aim of the present study was to investigate the correlation between the serum HLA-G levels and RIF during the implantation window in women undergoing IVF treatment.

2. Method

2.1. Study Design

This prospective case-control study was performed in a private infertility clinic in Mashhad from November 2019 to May 2020. The serum HLA-G concentrations from patients with RIF after IVF (case group) were compared to individuals with successful implantation (control group). The inclusion criteria for the case group consisted of patients referred to a private infertility clinic in Mashhad with a diagnosis of RIF, which means having more than one failed IVF attempt, failure in transfer of more than six good quality cleavage stage embryos, patients with implantation failure which was defined as serum levels of βhCG < 5 mIU/mL 14 days after embryo transferring, signing a consent form for participation and age lower than 40 years. The inclusion criteria for the control group were successful implantation for the first IVF-embryo transfer. Individuals with known uterine disorders, polycystic ovary syndrome, couples carrying autoimmune disease and thrombophilic mutations and with abnormal karyotype or genetic disorders, having an anatomical and hormonal cause for abortion, endometriosis, age below 20 years or more than 40 years, and any dissatisfaction for participation and those with incomplete information were excluded from the study. The study flowchart is shown in Fig. 1.

45 individuals with RIF diagnosed by obstetricians and informed according to their clinical and para-clinical experiments and based on the inclusion and exclusion criteria were included. Moreover, 45 individuals who achieved successful embryo implantation at first embryo transfer, matched with case groups on age, were enrolled. The Ethics Committee of the Azad University of Mashhad (IR.IAU.MSHD.REC.1398.213) approved the study, and participants signed a consent document. All cases were checked using ultrasonography for the presence of genitourinary tract defects and endometriosis. Moreover, infection with the toxoplasma gondii, cytomegalovirus, and German measles and the existence of antiphospholipid antibodies and anti-DNA antibodies were checked by the immunological examinations. Five cc of heparinized blood samples from patients in each group were received during the luteal implantation window (on days 19 to 23 of the menstrual cycle, especially on day 21). They were frozen at −70 °C. After collecting the samples, HLA-G serum concentrations were examined using the sandwich ELISA method through the manufacturer's kit instructions (NorthAmerica Europe/International EBioscience). Data were recorded in each group, and HLA-G concentrations were compared and evaluated.

2.2. Data Analysis

Data was analyzed using SPSS v22 (SPSS Inc., Chicago, IL, USA). The normality of the data was checked using the Kolmogorov-Smirnov test. A comparison of qualitative variables between the groups was carried out using the Chi-square test. Data with normal distribution were analyzed using the Student T-test, while Mann-Whitney and Wilcoxon tests were used for non-parametric analyses. P values less than 0.05 were regarded as statistically significant.

3. Results

Age ($p = 0.904$) and BMI ($p = 0.9$) in control and case groups were matched (Table I). Thirty-nine individuals did not participate, and 59 individuals entered the study (39 in the RIF group and 20 in the control group). Moreover, 45% of patients in the RIF group had implantation failure three times, and 55% had it more than three times. Results showed that HLA-G serum levels in the RIF group were remarkably greater than controls (0.085 ± 0.019 vs. 0.073 ± 0.005, $p = 0.024$). Furthermore, by grouping patients based on age and BMI, we found that in cases with ages above 30 years and with BMI more than 25 (kg/m^2), HLA-G serum levels in the RIF group were noticeably greater than controls (0.088 ± 0.019 vs. 0.072 ± 0.004, $p = 0.002$ and 0.083 ± 0.012 vs. 0.072 ± 0.003, $p = 0.021$, respectively). However, there was no remarkable difference in the RIF group in terms of number of implantation failures ($p = 0.97$, Table I).

A covariance analysis model was used to examine the studied variables with the HLA-G serum concentrations. Modulating the effect of age and BMI variables, a significant relationship still can be found between the two groups in terms of HLA-G serum levels ($p < 0.05$, Table II).

4. Discussion

In this study, higher serum levels of HLA-G were associated with RIF, especially in cases over 30 years old and with a BMI over 25, which remains significant after eliminating confounding variables. Enghelabifar *et al*. showed that the HLA-G insertion/deletion genotype is associated with a higher risk of RIF and can be used for the prediction of implantation failure following ART [18]. In another study, Nardi *et al*. showed that HLA-G allelic distributions differ among women with implantation failure and controls. They indicated higher HLA-G*01:03:01 alleles in the women with implantation failure. At the same time, they did not find a significant difference in the sHLA-G serum concentrations among the women with implantation failure and controls [11]. Monti *et al*. demonstrated that

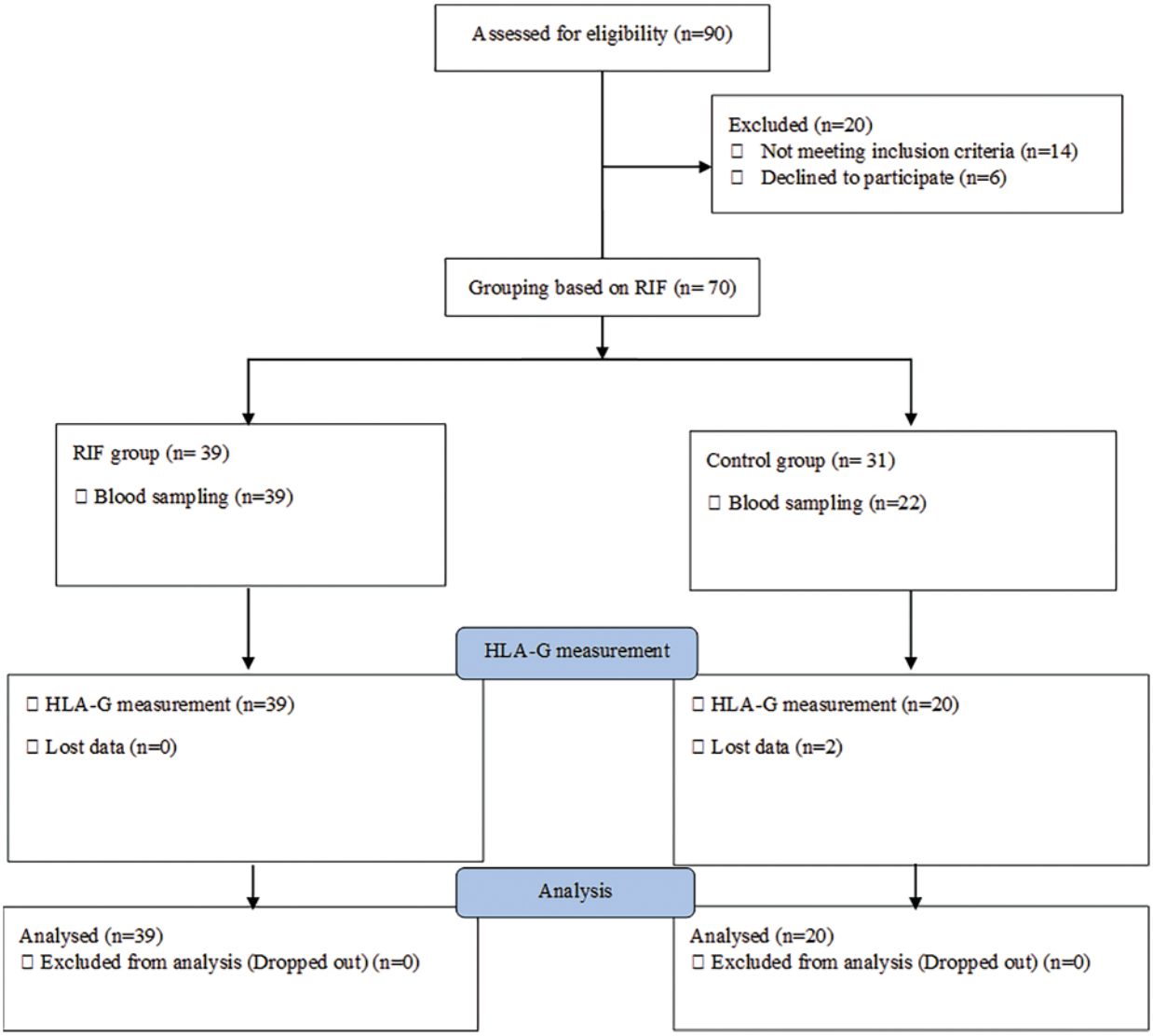

Fig. 1. Flowchart depicting the study design.

TABLE I: Studied Variables in Both Control and RIF Groups

		Groups		
Variables		RIF group (n = 39)	Control (n = 20)	P-value
Age (year)		32.8 ± 4.5	32.6 ± 4.1	0.904
BMI (kg/m^2)		25 ± 1.7	24.9 ± 2.1	0.9
Number of RIF	Three	9 (45%)	–	–
	Four	9 (45%)	–	
	Five	2 (10%)	–	
HLA-G serum levels		0.085 ± 0.019	0.073 ± 0.005	0.024
HLA-G serum levels	<30 years	0.078 ± 0.015	0.077 ± 0.006	0.844
based on age	≥30 years	0.088 ± 0.019	0.072 ± 0.004	0.002
HLA-G serum levels	<25	0.088 ± 0.024	0.074 ± 0.006	0.105
based on BMI	≥25	0.083 ± 0.012	0.072 ± 0.003	0.021
HLA-G serum levels based	Three	0.084 ± 0.012	–	0.97
on the number of RIF	Four and Five	0.087 ± 0.023	–	

recurrent implantation failure is more prevalent among European women with HLA-G 14 bp insertion/insertion genotype [19]. Moreover, Fan displayed that Caucasian populations with the HLA-G 14-bp insertion allele have a higher risk of RIF [12]. Except for one study by Nardi *et al*. [11], the other studies showed a significant correlation between HLA-G and the risk of RIF. However, they did not find differences in serum levels; they found that HLA-G polymorphism is significantly associated with RIF.

HLA-G protects target cells against NK-cell-mediated cytolysis and prevents allorecognition by maternal cytotoxic lymphocytes [20]. More recently, studies focused on

TABLE II: Analysis of Variance of the Relationship between All Variables and Serum Levels of HLA-G

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	0.002	3	0.001	3.393	0.029
Intercept	0.002	1	0.002	11.373	0.002
Group	0.001	1	0.001	7.745	0.009
Age	8.546E−5	1	8.546E−5	0.459	0.502
BMI	0.0001	1	0.0001	2.166	0.150
Error	0.007	35	0.0001		
Total	0.254	39			
Corrected total	0.008	38			

immunomodulation through the inducing cytokine production display a chemical link between the maternal immune tolerance mechanisms and the embryo.

Recently, investigations on the recombinant sHLA-G and HLA-G proteins obtained from transfected cells display the role of these proteins in the regulation of T cells [21], antigen-presenting cells (APCs), and NK cells [22], [23]. Recombinant sHLA-G inhibits T cell proliferation and stimulates the production of tumor necrosis factor-α (TNF-α) and interferon-γ through NK cells. On the other hand, sHLA-G has no impact on the destructive action of mononuclear cells in the uterine [24]. Moreover, sHLA-G, through APCs, induces the secretion of TGF-β1 [25]. In vitro investigations revealed that HLA-G can regulate cytokine release from human allogeneic peripheral blood mononuclear cells (PBMCs) [26]. Taken together, HLA-G may be involved in the regulation of cytokine release in order to regulate trophoblast invasion, induce immunotolerance, and remodel uterine spiral arteries to provide successful graft acceptance and ensure pregnancy.

Some studies on the analysis of amniotic and serum concentrations of HLA-G demonstrate changes in the expression of HLA-G during pregnancy [27], which decreases in the amniotic fluid as pregnancy progresses. Therefore, immune tolerance toward the fetus reduces, resulting in fetal rejection. An abnormal decrease in HLA-G expression in the early months of pregnancy may lead to an increased risk of immunologic response of the mother's immune system against the fetus, resulting in an abortion [28]. Hence, HLA-G is a potential factor for diagnosing RIF in pathological conditions. However, accurate methods for measuring HLA-G concentrations and ROC curves for the diagnostic value of HLA-G for predicting RIF are strongly required. Additionally, accurate and reliable assessment of HLA-G serum levels may provide a strong tool for predicting a suitable immune tolerance status to achieve successful pregnancy or/and utilizing HLA-G antagonists for treating RIF.

In summary, an increase in the serum levels of HLA-G in the RIF patients, especially in cases over 30 years old and with BMI $\geq$ 25, remains significant after eliminating confounding variables. These results showed a possible physiopathological pathway of HLA-G in increasing the risk of RIF to prevent or detect RIF in the future. However, further investigations are suggested to evaluate potential causative effects.

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Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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