UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

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INVOLVEMENT OF *KDR*, *iNOS* AND *p53* GENE POLYMORPHISMS IN RECURRENT MISCARRIAGE

THESIS SUMMARY

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INTRODUCTION

Recurrent spontaneous abortion (recurrent pregnancy loss) is defined as loss of three or more consecutive pregnancies. Epidemiological studies have shown that pregnancy pathology affects approximately 1% of all women, the risk of losing another pregnancy after three consecutive miscarriages is 55%. Several hypotheses have been proposed and analyzed various etiologies for recurrent abortion but many authors still consider about 50% of recurrent abortions as unexplained.

Current research had as main objective understanding at the molecular level the complex process of implantation, before improving diagnosis and treatment of infertility. Implantation human embryo is a unique process and no other mammal can not be considered as a true model.

Although there is progress in terms of assisted human reproduction, lack of control of implantation remains a major obstacle in the development of a successful pregnancy. Since periods of preimplantation and implantation are the source of various problems in pregnancy, it was tried understanding the mechanisms of implantation, to facilitate the discovery of new treatments in the most appropriate applicable for both the embryo and the endometrium.

Scientific observations accumulated over the past two decades have allowed to knowledge the physiology of preimplantation and implantation. The implantation process vary from species to species and ethical considerations and experimental difficulties did not allow this process to directly assaying human species. Although the studies were conducted, additional data are needed to clarify the molecular interaction processes through new technologies that bring new information.

Miscarriage is one of the most complex problems of modern medicine and consists of spontaneous interruption of pregnancy in which the embryo or fetus if it is unable to survive independently, generally before 20 weeks of gestation. Miscarriage is the most common complication of pregnancy, being a negative experience for both patient and doctor. It noted that the most common complication of human gestation is miscarriage.

Most miscarriages are unrecognized and may occur before or with the next menstruation. 15-20% of the recognized tasks may progress to miscarriage or ectopic pregnancy. Population studies also showed that about 5% of couples attempting a pregnancy, have two consecutive miscarriages and about 1% of couples have 3 or more consecutive pregnancy losses.

Recurrent spontaneous abortion is a delicate clinic issue, since advances in immunology and genetics have not elucidated the causes of it. The important problem that arises is to make couple, that

waiting for answers and solutions, to understand that this pathology can be frustrating even for a doctor.

Whereas the phenomena occurring at the site of implantation during normal and pathological development of pregnancy are extremely complex, as this study we considered it appropriate to refer to survey the site of implantation. If we can help develop approaches and existing knowledge in the field and the research results we could clarify some of the cellular and molecular processes that contribute to this pregnancy pathology, pathology-reaching psychosocial on couples, we think to the future, to determine a screening algorithm investigation and the appropriate therapy to prevent this complication preconception of pregnancy.

Keywords: Polymorphisms, VEGFR2 / KDR, iNOS, p53

I. CURRENT STATE OF KNOWLEDGE

Chapter I - "Epidemiology and etiopathogenesis recurrent abortion" presents recent data on the incidence and prevalence of this pathology and the study of etiologic factors involved in recurrent abortion.

Chapter II - "Angiogenesis of implantation" reviews the main factors mediating angiogenesis in implantation and the role of vascular endothelial growth factor in regulating vasculogenesis and angiogenesis. In-depth understanding of embryonic development during preimplantation and implantation process, will help identify aberrant mechanisms that lead to miscarriage, as well as offering new strategies to improve prevention of infertility and pregnancy complications.

General part continue with *"Genetic susceptibility to recurrent abortion"- Chapter III*, where they present the latest data on the molecular mechanisms and major susceptibility genes studied in recurrent abortion. Several studies suggest that polymorphisms in genes encoding different mediators may represent a susceptibility factor for unexplained recurrent miscarriages.

Chapter IV - "Genetic counseling and genetic counseling in recurrent abortion" the general concluded. Genetic counseling along with psychological support are essential steps that must be offered to all couples experiencing recurrent miscarriage, for a term pregnancy is reached and not result an anomaly.

II. PERSONAL CONTRIBUTIONS

Chapter V - AIMS AND OBJECTIVES OF THE STUDY

The aim of this study is to optimize methods of prevention of miscarriage and further development of medical therapies, to understand the molecular and genetic level, the complex process of implantation, before improving diagnosis and treatment of infertility.

The objectives of our study started from evaluation of the main polymorphisms located in genes encoding cytokines and receptors, in lots of patients with and without recurrent miscarriage and correlate genetic variants detected susceptibility to recurrent miscarriage in the Dolj county, a region where these genetic variants not they have been investigated so far.

Fulfilling this purpose in the scientific research project was carried out through the following specific objectives :

- ➢ determining the frequency of genotypes main genes encoding cytokines ;
- ➢ determining the frequency of genotypes for genes encoding receptors VEGFR-2 (KDR), iNOS and p53 ;
- ➢ establish possible associations between genes involved and susceptibility to recurrent abortion ;
- ➢ establish possible associations between genes involved.

Chapter VI - MATERIALS AND METHODS

VI.1. Establishing lots and inclusion of patients in study

In this study included 139 patients diagnosed with recurrent miscarriage based on history, clinical examination, ultrasound and histological examination. Patients included in this study were diagnosed in Obstetrics and Gynecology Clinics of Philanthropy Craiova Municipal Hospital, between October 2009 - December 2014.

For comparative analysis was constituted a control group (patients with recurrent miscarriage history and who had at least one birth) consisting of 145 patients hospitalized and investigated for miscarriage/abortion, in the Obstetrics and Gynecology Clinics of the same hospital.

Both large plots were established on the basis of inclusion and exclusion. Both patients with recurrent miscarriage patients in the control group and signed an informed consent for inclusion in the study and a detailed questionnaire to obtain demographic data important.

VI.2. Harvesting samples and biological material

Regarding patients in the group with recurrent miscarriage, biological material was represented by blood (about 2.5 - 5 mL venous blood) collected in tubes with EDTA and maintained at 4°C until isolation of DNA and placental tissue harvesting in RNA later solution and kept at - 20°C. For patients in the control group, the biological material was represented by blood collected in tubes with EDTA. Samples were coded with letters and numbers in the order given harvest.

VI.3. Identification of genetic polymorphisms

Identification of polymorphisms of genes VEGFR-2 / KDR, iNOS and p53 was performed in the Laboratory of Cellular and Molecular Biology at the University of Medicine and Pharmacy of Craiova.

Protocol to identification of genetic polymorphisms included the following steps : isolation of genomic DNA from blood and tissue, spectophotometric assessment, identification of allelic variants with technique Real Time PCR, interpretation of results.

VI.4. Biostatistical analysis of study groups

Biostatistical analysis of experimental data followed by descriptive statistics, charts, statistical tests of interference studies of the correlation parameters in the spontaneous and induced abortion on demand, which is normal pregnancy. It has also been assessed Hardy-Weinberg equilibrium - X^2 test, correlation, and establishing an association between the parameters studied and the results of genetic investigations - odds ratio (OR), confidence intervals (95% CI) and p.

Chapter VII – RESULTS

VII.1. CHARACTERISTICS OF THE STUDY GROUPS

The group with recurrent miscarriage abortion (RMA) has been classified and studied, depending on the patient's obstetrical history:
- Primary RMA - 76 cases, when the patient has no history of birth;
- Secondary RMA - 50 cases in patients where there was an initial rise followed by miscarriages;
- Tertiary RMA - 13 cases, when the patient had three non-consecutive recurrent abortions, and these should be considered as separate entities, perhaps representing different pathophysiological mechanisms leading to miscarriage.

VII.2. STATISTICAL ANALYSIS OF THE STUDY GROUPS

Analysis of cases studied, watching history and clinical examination of patients allowed us to highlight selected data sought in the study. Data processing was performed by the statistical analysis performed using statistical indicators applied to the studied cases.

- We have introduced as a parameter tracking and investigating a series *of demographic indices.*

- Depending on *the type of RMA,* we met 54.68% of the primary RMA, secondary RMA in 35.97% and tertiary RMA in 9.35% of the cases, out of a total of 139 cases.

- Demographic characteristics of the study group has shown non-significant difference in *the demographic distribution* of the cases studied, with a proportion of 58.27 cases in urban areas and a proportion of 41.73 cases in rural areas, in cases with RMA.

- I correlated the *residence areas with type of RMA*, to see if there is a distribution algorithm to type of RMA, depending on the residence areas. The test result was *p = 0.935, p> 0.05*, so there is a statistically significant relationship between the residence areas and type of current RMA.

- The distribution of patients by *age at the time of taking in the study*, showed in both groups that most patients with recurrent miscarriage were aged between 25 and 34 years (62.59%) and the control group percentage being 59.31 in the same age group. This interval corresponds to age range in which women are most high fertility rate.

- I tried to see if *maternal age* has influence and correlate with the type of RMA. I found that in the primary RMA cases, mean age ± SDV was 28.66 years. In the secondary RMA cases mean age ± SDV was 31.02 years and in the tertiary RMA was 31.08 years, so the same age as the secondary RMA.

- Regarding the *obstetrical history*, we considered to the group with RMA, number of abortions and the number of births, their type, complications. I noticed that only a percentage of 14.39 of cases have a history of obstetric RMA, referring to cases in previous births and related complications.

- We statistically correlated *type of RMA and obstetrical history* - we obtained a significant difference between actual RMA types, in the share obstetrical complications of history, pChi square <0001 which shows a highly statistically significant result.

- We tried to *combine two risk factors* for miscarriage production and to see what is the future of these obstetrical cases.

- I correlated *maternal age and number of abortions in history* → the result ANOVA test was *p = 0.000106 <0.001*, so there is a highly significant difference between the three groups of patients : those patients with 3 abortions has significantly less mean age than patients with 4 abortions, which, has significantly less mean age than group witn 5 abortions.

- We analyzed the possible *correlation between the number of abortions in the history and the residence areas* - thinking that in rural areas probably access to specialized services is more difficult → The test result was *p = 0.099> 0.05*, therefore, despite differences percentage observed (in urban areas are more abortions than 3 cases in contrast to rural areas), there is no statistically significant link between the residence areas and the number of previous miscarriages.

- We analyzed the *involvement of obstetrical history* in producing recurrent abortion and compared total RMA with births of live fetuses history at secondary and tertiary RMA → found that most cases of RMA had three previous abortions, 77.92% of the cases, and 77.08% of this cases abortions had one pregnancy with live fetus.

- We analyzed *gestational age at the time of the abortion* which coincided with the entry into the study groups → Most patients in the group with RMA had a miscarriage at the current gestational age of 6-9 weeks of gestation (103 cases, 74.10% of cases).

- I found that there was a significant difference (*p = 0.257> 0.05*) between the three types of RMA on the gestational age at which they were produced.

- When the study group with RMA, we found a number of *associated diseases,* in this category entered and non recurrent miscarriage (less than 3 previous miscarriages).

- The type of *associated diseases* was represented by the hematological diseases (here referring to cases with thrombophilia confirmed), pathology uterine represented by tumors and malformations of the uterus and pathology frequent among these patients was represented by local infection or general → tertiary RMA presents a much greater association with infections and uterine pathology than primary and secondary RMA (Chi square test, *p = 0.003 <0.05*).

- Thrombophilia was diagnosed in 25.97% of cases with primary RMA, who did not have previous births, in 10.42% of cases with one birth in history and just 7.14% of the cases of two previous births → primary RMA presented a more pronounced association with thrombophilia.

- We analyzed the relationship between the *presence/absence of thrombophilia and recurrent miscarriage type* → taking into account that the number of cases with tertiary RMA is very low and that those three cases presented thrombophilia in association with urinary pathology or infections, thrombophilia are likely to be associated significantly greater with primary RMA than with secondary or tertiary RMA.

- Given the statistical involvement of thrombophilia in cases studied, we analyzed the involvement of two factors in the cases studied, *maternal age and presence of thrombophilia* →

there is a highly significant difference between the ages of patients with or without thrombophilia, those with thrombophilia having higher average age than others (32.35 years than 29.13 years, *p = 0.004 <0.01*) → We proposed the assumption that the presence of two factors: age and thrombophilia, may be risk factors for recurrent miscarriage production.

VII.3. ANALYSIS GENE POLYMORPHISMS OF KDR, iNOS AND p53 IN THE POPULATION STUDIED

- We investigated polymorphisms **VEGFR-2 / KDR 604T→C (rs 2071559), iNOS 2087A→G (rs 2297518) and p53 360 C→G (rs 35993958)** in the two groups (the group of patients who have harvested product pathologically placental tissue and that patients who have venous blood collected);

- Regarding polymorphisms investigated in the control group, there were no deviations from Hardy-Weinberg equilibrium properly (*p> 0.05, x^2> 3.84*);

- We determined the frequency of genotypes of these genes and established the existence of possible associations between genes involved and susceptibility to RMA;

- Comparative analysis of genotypes (referring to international databases have taken as reference genotype - TT) and statistical data obtained, it was found that polymorphism *VEGFR2 (KDR) - 604T→C* it is not associated with an increased risk of recurrent miscarriage;

- Also comparing the genotypes (referring to international databases was taken as reference genotype - GG) and statistical data obtained showed that polymorphism *iNOS 2087A→G* is not associated with an increased risk of recurrent miscarriage;

- And polymorphism p53 -360 T→C did not notice association with an increased risk of recurrent miscarriage.

CONCLUSIONS

- This study constituted as a vanguard study, in an attempt to elucidate the causes of recurrent miscarriage abortions, represents a complex analysis of epidemiological point of view, phenotypic and genotypic of patients diagnosed with recurrent miscarriage in the Dolj county, a region where such analysis has not been done so far.

- The originality of this study consists in determine the phenotype profile of patients, associating certain phenotypic characteristics with different etiologic factors involved in recurrent miscarriage by assessing the main polymorphisms located in genes encoding cytokines and receptors in lots of patients with and without recurrent miscarriage and correlate genetic variants detected susceptibility to recurrent abortion.

- We found significant differences in terms of demographic distribution of cases by area of residence, aged between 25 and 34 years (62.59%), ages significantly lower in patients with primary RMA than with secondary or tertiary RMA, the average gestational age at which the abortion was about the same, with minimal differences between 8 weeks and 8.53 weeks.

- We also showed some correlation between maternal age and number of abortions in history, between the number of abortions in history and area of residence, the correlation background obstetric tasks with live fetuses and the number of recurrent miscarriages, presence of associated diseases and their correlation with type recurrent spontaneous abortion and the number of previous pregnancies with live fetuses, tertiary RMA presented a much greater association with infections and uterine pathology than primary and secondary RMA, primary RMA presented a more pronounced association with thrombophilia.

- Gene polymorphisms of VEGFR2 (KDR) -604T→C (rs 2071559), iNOS 2087A→ G (rs 2297518) and p53 -360C→G (rs35993958) does not affect recurrent pregnancy loss in a Romanian population, Dolj County.

- Further studies are needed to replicate our finding in different ethnic groups with a larger sample.

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