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ORIGINAL ARTICLE

Polish experiences with paternal lymphocyte immunization in women with recurrent miscarriages

Hanna Motak-Pochrzęst [1], Andrzej Malinowski [2]

1 The Opole University of Technology, Faculty of Physical Education and Physiotherapy, Opole, Poland
2 Department of Operative and Endoscopic Gynaecology and Oncological Gynaecology, Polish Mother's Memorial Hospital Research Institute, Łódź, Poland

Correspondence to: Hanna Motak-Pochrzęst MD., PhD.
The Opole University of Technology
Faculty of Physical Education and Physiotherapy
Prószkowska 76, 45-758 Opole, Poland.
tel: +48 695 761 771; e-mail: hannamotak@o2.pl

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Abstract

OBJECTIVE: This study aimed to evaluate the efficacy of PLI and results of subsequent pregnancy in women with RM showing alloimmune response.
MATERIALS AND METHODS: Immunological investigations were performed in patients with RM. Subsequently, PLI was administered to 241 patients at their request. Of these, 202 conceived between September 2005 and September 2012.
RESULTS: Of the 202 women, 169 pregnancies resulted in term delivery; the remaining 33 resulted in subsequent miscarriages (success rate = 83.7%). During seven-years observations of 202 tested individuals, 114 women were pregnant again for the second time and 92 pregnancies of them resulted in the next term delivery (success rate =80.7%).
CONCLUSIONS: Alloimmune background indicated that PLI might improve pregnancy outcome in patients suffering from RM. Long-term monitoring did not reveal any negative effects of PLI on the immunological system in the treated women or newborn babies.

INTRODUCTION

Recurrent miscarriages (RM) affect 2–5% of women of reproductive age and present an important reproductive health issue. The etiology of RM is often unclear and may be multifaceted and the diagnosis and treatment are the subject of considerable controversy (Coulam *et al.* 1994). Alloimmunization with paternal lymphocytes (PLI) has been used for the prevention of unexplained recurrent miscarriage. Although this approach has been controversial for many years (Peña *et al.* 1998; Porter *et al.* 2006), it is continues to be proposed as a mode of treatment (Nonaka *et al.* 2007; Takeshita 2004). In addition to reports demonstrating the high efficacy of alloimmunization (Carp *et al.* 1992; Peiyan *et al.* 2012; Wilczyński *et al.* 1992), there are also studies reporting no significant differences between women who underwent alloimmunization and control subject (Christiansen *et al.* 2004; Gatenby *et al.* 1993; Jauniaux *et al.* 2006; Scott 2003).

The discrepancies in the reports on the effectiveness of alloimmunization depend not only on the heterogeneity of the immunological disturbances in women with RM but also on the differ-

ent prognostic factors of successful pregnancy outcome. The following factors are known to have prognostic values: the number of miscarriages (including pregnancies confirmed by biochemical tests), maternal and paternal age, the interval between alloimmunization and the subsequent pregnancy, the mode of immunization and the occurrence of the same type I and type II HLA antigens in both partner (Cowchock *et al.* 1990; Daya & Gunby 1994; Gharesi-Fard *et al.* 2008; Kwak *et al.* 1994).

The objective of this study was to evaluate seven-years observations of the efficacy of the pregnancies in women with RM who underwent PLI.

MATERIALS AND METHODS

241 patients with a history of three or more primary recurrent miscarriages were retrospectively evaluated. The present retrospective study was performed between September 2005 and September 2012. Patients were registered at the Department of Operative and Endoscopic Gynaecology at the Polish Mother's Memorial Hospital-Research Institute, Lodz. The patients gave written consent for participation in the study and the approval of the Ethics Committee was obtained. RM was defined as the occurrence of three or more clinically or biochemically proven pregnancy failures before 20 weeks of gestation from the last menstrual period or less than 500 g of foetal body weight (Coulam 1991). All reported pregnancies were confirmed by a positive HCG test of urine or serum, ultrasonic examination or/and histology of aspirated tissue from the uterus documented in the hospital's records.

The tested group consisted of nonpregnant women who had experienced RM with the same partner and for whom we could find no explanation for the abortion in the case histories or in the clinical and laboratory investigations. The patients' ages ranged from 23 to 45 years and the number of abortions ranged from 3 to 7 (mean 3.8). The participants had normal standard medical and gynaecological examinations.

All of the women were investigated to exclude the following known causes of RM:

1. anatomical causes (congenital anatomic abnormalities of the utery such as: incomplete Müllerian fusion, septum resorption and cervical incompetence and acquired abnormalities such as: synechiae, leiomyomas, endometrial polyps) in the result of HSG and/or hysteroscopy and pelvic USG,
2. endocrinological causes (LH, FSH, TSH, PRL, oestradiol, testosteron) including luteal phase insufficiency, polycystic ovary syndrome, insulin resistance, hyperprolactinaemia, hyperandrogenism, diabetes mellitus,
3. infectious factors such as TORCH (Toxoplasmosis and other infectious microorganisms including rubella, cytomegaly and *Herpes simplex*), infections of *Mycoplasma hominis* et *Chlamydia trachomatis*,
4. heritable thrombophilias related to RM: activated protein C resistance associated with mutations in Factor V Leiden, deficiencies in protein C and S, mutations in prothrombin 20210 A, mutations in antithrombin III and hyperhomocysteinemia

The women and their partners had normal karyotypes (determined using peripheral blood leucocytes).

The partners of the women exhibited normal spermiograms. Chromosomal abnormality of the products of conception was excluded using FISH analysis for chromosomes 13, 16, 18, 21, X and Y in patients who suffered subsequent abortions beginning with the third pregnancy loss. Patients with confirmed embrional aneuploidy were excluded from the study. None of the participants had autoimmune disease.

All patients were qualified on the basis of the negative lymphocytotoxic test (LCT) – lack of HLA class I antibodies and lack of lymphocyte activity in mixed lymphocyte reaction test (MLR). Tests were performed before the next planned pregnancy.

Pregnancies were planned after achieving sufficient levels of MLR test. After immunization procedures allowing to become pregnant, patients called the department as soon as they had a positive pregnancy test. All registered women were contacting the clinic for ultrasound pregnancy and in cases of the complications during the pregnancy (haemorrage, prematures contractions, PIH, abnormal laboratory tests during pregnancy). The pregnancies following paternal lymphocyte immunization had been completed in all women included in the study. The data concerning the course of consecutive pregnancies were further collected and stored in the database.

If patients did not become pregnant after six months from PLI, the next MLR test was reiterated. Only women who become pregnant between September 2005 and September 2012 were enrolled in the study. The period of following up the patients, was seven years. Concerning the patients who enrolled in this study after September, 2005, the outcome of pregnancies was determined in September 2013. The course of the next pregnancies following PLI during seven years observation were also analysed using the questionnaire.

The control group consisted of 36 non pregnant women who had experienced primary RM, had negative lymphocyte activity in MLR test and who did not desire immunotherapy. The patients' ages ranged from 23 to 32 years and the number of abortions ranged from 3 to 4 (mean 3.1) The natural course of their pregnancies was observed.

Lymphocytotoxic test

For the measurement of serum anti-HLA class I IgG (ELISA lymphocytotoxic test) QUICKSCREEN Solid Phase HLA Antibody Screening Kit QS3G or QS12G (GTi Diagnostics, USA) was used. Lymphocytotoxic test (LCT) values of <0.30 U/ml were considered to be negative.

Mixed lymphocyte reaction test

The influence of the serum from the examined women on the proliferative response compared with that of the allogenic serum was defined as the percentage of MLR blocking (% MLR blocking) according to the following formula: % MLR blocking = (1–BI) × 100%, BI= (cpm in autologous serum–control cpm)/(cpm in allogenic serum–control cpm). A value above 30% for % MLR blocking was recognized as an indicator of the existance of antibodies that blocked the proliferative activity of lymphocytes (MLR-BAbs) (Takeuchi 1990; Zeman *et al.* 1988).

Immunization procedure

The consent of the Local Board for Scientific Research Ethics was obtained from both partners. Sera from both partners were tested for the presence of HIV, HBV and HCV infections and syphilis. All of the couples had negative results. After the presentation of negative results, PLI was proposed and thoroughly discussed with couples.

Samples containing 100 ml of blood were collected from both partners in heparinised tubes. The lymphocytes were isolated in the Gradisol G Gradient (Polfa, Kutno, Poland) according to the method described by Zeman (Zeman *et al.* 1988).

Paternal lymphocyte immunization was performed using 100–277×10^6 freshly isolated lymphocytes that had been washed three times in a buffer of NaCl solution, pH 7.4 and X-irradiated. Any erythrocytes still present were eliminated with lysise solution. The obtained lymphocytes were suspended in 2 ml of 0.9% NaCl. The homogeneity of lymphocytes administered to the women was 96–98%. The solution of lymphocytes was injected subcutaneously in eight places on the upper lateral surface of both forearms. Any short-time adverse effects after PLI were noticed, only local irritation on the surface of the forearms. As well long-time observations did not reveal any adverse effects in women who underwent PLI.

Alloimmunization was performed at two weeks intervals, twice to six times before next planned pregnancy. Two weeks after alloimmunization the MLR test was performed. Once appropriate blocking activity appeared in the sera following the series of vaccination, the patients were allowed to become pregnant. Immunization was considered successful when the level of blocking activity in MLR test in the serum was at least over 30%. Additional alloimmunization was performed once at the beginning of pregnancy (after positive pregnancy test) in every patients. In patients with the level of MLR test by the level of 30%, so called poor-responders, the vaccination was repeated twice: at the beginning of pregnancy and in 6–8 weeks of gestation. All of the immunized women conceived spontaneously.

A successful pregnancy outcome was defined as a live birth, whereas the next spontaneous abortions were considered failures.

RESULTS

Paternal lymphocyte immunization was proposed and administered to 241 patients at their request, from among 202 become pregnant between September 2005 and September 2012. 39 patients who did not become pregnant in this time were excluded from the observation. 36 women with negative blocking activity did not desire the immunotherapy and were included in this study as the control group.

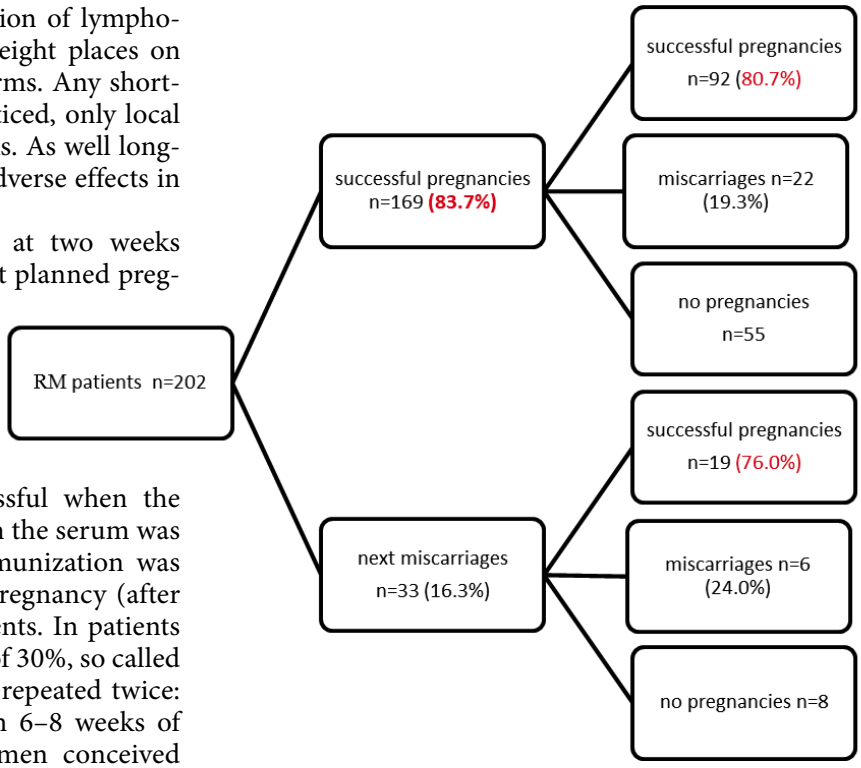
In the group of 202 women who received alloimmunization with paternal lymphocytes and become pregnant, 169 pregnancies resulted in term delivery (83.7%) and the remaining 33 resulted in subsequent miscarriages (16.3%)

For the 36 control patients who had negative blocking activity and who did not receive immunization, 13

Tab. 1. The outcome of the pregnancies subsequent PLI.

	RM patients n=202	control group n=36	*p*-value
successful pregnancies	169 (83.7%)	13 (36.1%)	<0.05
unsuccessful pregnancies	33 (16.3%)	23 (63.9%)	>0.05

RM-recurrent miscarriages



Tab. 2. The course of pregnancies following PLI.

term pregnancies resulted in deliveries (36.1%) and 23 pregnancies resulted in subsequent miscarriages (63.9%).

During seven-years observation of 202 tested individuals, 114 women were pregnant again for the second time and 92 pregnancies resulted in the next term delivery (success rate 80.7%).

Of 33 patients who experienced miscarriage after PLI, 25 patients become pregnant after next immunization procedure performed six months later. 19 pregnancies of them resulted in term delivery (76.0%), 6 women experiences next miscarriage (24.0%) and 8 patients did not become pregnant.

The rate of success for the first pregnaniesy subsequent PLI was significantly higher in the immunized group (group I) than in the non-immunized group (group II) (83.7% versus 36.1%, $p<0.01$). Taking into consideration the subsequent second 139 pregnancies following PLI we found successful deliveries in 111 (92+19) patients (success ratio 79.8%).

As mentioned above, 241 patients suffering RM have undergone the immunization with paternal lymphocytes. Appropriate blocking activity in MLR test allowing to become pregnant was detected after two vaccinations in 77.9% of these, after four vaccination in 15.9%. patients and after six and more vaccinations in 6.2% more patients. The outcome of pregnancy according to the number of vaccinations necessary is shown in Table 3.

Tab. 3. Comparison of pregnancy outcome according to the number of immunizations necessary for appropriate blocking activity in MLR test.

	two PLI	four PLI	six and more PLI
number of patients (n=241)	165 (68.6%)	53 (21.9%)	23 (9.5%)
number of pregnancies (n=202)	157	31	14
successful pregnancy (n=169)	133	27	9
success rate (n=83.3%)	84.7%	87.0%	64.2%
next miscarriage (n=33)	25	5	3

The rate of successful pregnancy among the patients in whom the blocking activity appeared after two vaccinations was 84.7 % (133 of 157 patients), after four vaccinations was 87.0% (27 of 31 patients) and after six or more vaccinations was 64.2 %.(9 of 23 patients).

The number of previous abortions and the efficiency of PLI is presented in Table 4.

Taking into consideration the number of abortions, the success rate differed between patients with 3–4 abortions and those with 5 or more abortions, as well as in the immunized as non-immunized women. The lowest pregnancy success rate after PLI was observed in women with 5 and more abortions (75%, $p>0.05$).

Of 202 pregnant patients who underwent PLI, 169 have experienced delivery. 166 delivered normal healthy infants in the 37th week of gestation or later, three infants were born as premature delivery in the 32nd, 34th and 35th week of gestation.

After seven years a retrospective study was conducted. All previously registered patients were asked to answer if they become pregnant to present the course of their pregnancies and the conditions of their newborns. Among of patients who become pregnant for the second time following PLI, 114 delivered normal healthy infants in the 37th week of gestation or later.

As found in our previous study (Malinowski *et al.* 1997), all newborns of mothers treated with PLI did not reveal differences between birth weight, general condition at birth, occurrence of complications in the adaptation period and studied hematological and immunological parameters compared with randomly chosen control newborns. No differences were noted as to the duration of pregnancy.

DISCUSSION

Paternal lymphocyte immunization is believed to provoke immunological changes that improve the outcome of pregnancy. Controversy exists as to whether active immunotherapy with paternal lymphocyte immunization or passive immunotherapy with intravenous immunoglobulin (IvIg) treatment can improve the chance of live birth in women with unexplained recurrent miscarriages (Gatenby *et al.* 1993). In the literature, few authors (Szekeres-Bartho 2009; Szpakowski *et al.* 2001; Yang *et al.* 2008) have investigated the factors that influence the success of PLI as a treatment modality for RM.

Tab. 4. The efficiency of alloimmunization depending on the number of miscarriage.

number of miscarriages	RM patients n=202	successful pregnancies n=169/202 (83.6%)	control group n=36	successful pregnancies n=13 (36.1%)
3	173	147 (84.9%)	34	13 (38.2%)
4	21	16 (76.2%)	2	0
≥5	8	6 (75%)	0	0

RM-recurrent miscarriages

It has been shown (Cowchock & Smith 1992) that the risk of miscarriage of the subsequent pregnancy after PLI was associated with the number of previous failures and it was calculated that the chance for success of a current pregnancy was reduced by 15% after the occurrence of three or more previous abortion. In other studies (Coulam *et al.* 1994) this relationship was only observed after the loss of 5 pregnancies. In our own studies, the negative influence of a higher number of miscarriages on the final outcome was not observed. In women with five or more miscarriages, the success ratio was 75.0%, compared with 84.9% in women with three miscarriages, $p>0.05$. Similar results contradicting the significance of the number of pregnancy failures with regard to the efficiency of PLI have been reported in the studies by Carp (Carp *et al.* 1992) as well as Cowchock and Smith (Cowchock & Smith 1992).

It has been observed that the efficiency of PLI diminished with increasing time between vaccination and the conception. The recommendations suggest that the appropriate time for conception is as soon as possible after achieving necessary levels of MLR test. The additional PLI treatments should be given after 5–6 months if conception is not achieved. The effect of the gradually diminishing efficiency of PLI was reported by authors who applied only a single immunization before pregnancy (Cowchock & Smith 2009). The seven years follow-up study shows that only single immunization applied between first successful pregnancy after PLI and before next planned pregnancy is found to be enough to achieve necessary level of blocking activity in MLR test. The additional immunization performed at the beginning of pregnancy is recommended in every pregnancy after PLI.

The results of our own previous studies (Malinowski *et al.* 1997; Malinowski *et al.* 1998) allow to conclude that high efficiency of alloimmunization in preventing consecutive miscarriages does not seem to depend significantly on: the women's and her partner's age, number, type (primary versus secondary) and time of previous miscarriage and the number of lymphocytes used for the procedure. Alloimmunization with paternal lymphocytes correlates with the subsequent pregnancy success and seems to be also safe for the fetus and the newborn (Malinowski *et al.* 1997).

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