Editorial Article

Leukocyte immunotherapy improves live delivery rates following embryo transfer in women with at least two previous failures: A retrospective review

J.H. Check, *M.D., Ph.D.*; **J. Liss,** *M.L.T.*; **M.L. Check,** *B.A.*; **A. Diantonio,** *B.S., M.T. (ASCP)*; **J.K. Choe,** *M.D.*; **V. Graziano,** *B.A.*

The University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School at Camden, Cooper Hospital/University Medical Center, Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology & Infertility, Camden, NJ (USA)

Summary

Purpose: To determine whether leukocyte immunotherapy (LIT) could improve live delivery rate following embryo transfer (ET) in women who were not successful in prior attempts.

Methods: Paternal leukocytes were intradermally injected in some women who had failed to have a successful pregnancy following at least two prior ETs approximately two weeks prior to fresh or frozen ET and repeated at the time of the 3rd rising serum beta human chorionic gonadotropin level and at eight weeks if a pregnancy occurred. Clinical pregnancy and live pregnancy rates (PRs) were compared to those women having ETs during the same time period not receiving LIT.

Results: Thirty-six of 94 (38.3%) patients receiving LIT (group 1) conceived following fresh or frozen ET vs 98 of 341 (28.7%) for women not receiving LIT (group 2) (p = NS). The live delivery rate per ET cycle was 30.8% (39/94) vs 19.7% for group 2 (p = .02). For the subset of women failing despite five previous ETs 17 of 37 (45.9%) group 1 women had a clinical pregnancy vs 18 of 64 (28.1%) group 2 women (p = .07%) and live delivery rates were 35.1% (13/37) vs 15.6% (10/64) (p = .024).

Conclusions: These retrospective data encourage a prospective study of LIT combined with progesterone vs controls receiving progesterone only for recalcitrant patients having ETs.

Key words: Embryo transfer; Recalcitrant; Immunotherapy; Lymphocytes; Live deliveries; Progesterone.

Introduction

Leukocyte immunotherapy (LIT) for recurrent spontaneous abortion (RSA) is quite controversial. Studies suggesting that LIT reduces miscarriage rates go back to 1986-1990 [1-11]. Only one of these studies was randomized [1]. However, other controlled studies failed to find a significant reduction in miscarriage rates following LIT [12-15].

In fact, the Cochrane Database Systematic Review for immunotherapy for recurrent miscarriage reviewed 11 clinical trials considered to be of high quality and did not show significant differences between treatment and control groups [16].

Properly designed trials included in the Cochrane analysis would only include studies that would assess the efficacy of allogeneic LIT exclusively without any other concomitant therapy [15-17]. However, the possibility exists that some studies failed to show any benefit of LIT because its efficacy is enhanced by some other factor which was eliminated in efforts to retain purity of the study. For example, concomitant progesterone therapy has been excluded in the studies comprising the Cochrane Database Systematic Review. There are data that one mechanism of how LIT may decrease miscarriage rates is through the induction of progesterone induced immunomodulatory proteins, e.g., the progesterone induced blocking factor (PIBF) [18]. There are data suggesting that this 34 kDa protein inhibits natural killer (NK) cell activity [19]. Furthermore, PIBF may induce a shift from TH1 to TH2 cytokines [20, 21]. Data has been presented showing a significantly higher percentage of ongoing pregnancies at the end of the first trimester in primary aborters with recurrent miscarriages (3 or more) treated by the combination of leukocyte immunization and progesterone (P) supplementation vs P supplementation alone [22].

There are some data suggesting that PIBF may be also involved in the implantation process [23]. We hypothesized that some women who fail to have a live baby following the transfer of normal appearing embryos may be related to immune rejection. Thus some women in our in vitro fertilization (IVF) program who failed to have a successful pregnancy following at least two embryo transfers (ET) at the Cooper Center for IVF were offered LIT on an empirical basis prior to another transfer. The present study retrospectively evaluated IVF outcome according to whether LIT was performed or not in these refractory couples.

Revised manuscript accepted for publication January 10, 2005

Materials and Methods

No patients were offered LIT unless they had a minimum of two embryo transfers (ETs) at Cooper Center for IVF that failed to produce a live delivery (irrespective of IVF attempts at other centers).

There was no age restriction nor any exclusion for increased early follicular phase serum follicle stimulating hormone (FSH) level. There was no exclusion reason for IVF, i.e., tubal factor, male factor or, unexplained infertility. There was no exclusion for intracytoplasmic sperm injection including sperm obtained from testes.

Methodology

Eight to ten 10 ml tubes of heparized blood were obtained from the male partner. No one using leukocytes from donors other than the male partner was included. Male partners were excluded if pre-testing suggested a risk for infection with a disease to the female partner or if immunologic testing suggested that the use of paternal lymphocytes might result in lymphocyte or platelet alloimmunization.

The blood was diluted with normal saline. Diluted blood was layered over Isoprep and was centrifuged at ~200 RPM in a large centrifuge for 30 minutes. The mononuclear cells form a distinct band at the interface between the sample layer and the lymphoprep solution. The band of cells is best removed using a sterile pasteur pipette without distrubing the layers. Removed mononuclear cells were now washed and resuspended in saline. A tuberculin syringe was then filled with 0.6 to 0.75 ml of the white cell suspension and 0.15 ml injected intradermally until all white cell suspension was used. The patient received the LIT at the very beginning of the menstrual cycle. If they conceived it was repeated with the third consecutive doubling of the beta-hCG level taken two days apart and again at eight weeks.

Study Design: Retrospective Analysis

The study patients included all women who failed to have a live delivery after at least two prior IVF cycles from January 1, 1997 to June 31, 2001. Women who received immunotherapy were advised of the experimental nature of the procedure and advised that we were not aware of a previous study demonstrating efficacy for IVF-ET. They were advised that from our personal experience LIT was beneficial for women with recurrent abortions but it was controversial. Women who attempted multiple cycles with immunotherapy were not excluded from the analysis or the subgroup analysis. Women informed of the option of LIT could decide to have this treatment or not. Not all women were advised of the option depending on which physician did the consultation.

Treatment Protocols

For frozen ET, all women were treated with the same regimen: graduated oral estradiol 2-6 mg over 14 days followed by progesterone vaginal suppositories 200 mg twice daily and progesterone in oil 100 mg daily. The progesterone therapy would be delayed and the estrogen therapy increased by 2 mg orally or vaginally, if the mean endometrial thickness was < 8 mm on day 14. The estrogen and progesterone therapy was continued during the first trimester if pregnancy occurred.

For fresh ET cycles, there were four controlled ovarian hyperstimulation regimens used: 1) luteal phase leuprolide started at the mid-luteal phase with a dosage of 0.5 mg for ten days and was decreased to 0.25 mg if serum estradiol was less than 50 pg/ml and the serum progesterone < 1.5 ng/ml. Gonadotropins (usually 225 IU FSH and 75 IU human menopausal gonadotropins) were usually given for about ten days when 10,000 units of human chorionic gonadotropins were given (with 2 lead follicles at 20 mm); 2) stop protocol – similar to luteal phase leuprolide except the 0.5 mg dosage was stopped after ten days and no more leuprolide was given; 3) short flare protocol - follicular phase leuprolide – started at 0.5 mg on day 2 and gonadotropins starting day 5; and 4) microdose flare – where the leuprolide dosage was diluted 1:20.

Statistical Analysis

Chi-square analysis was performed to compare clinical and delivered pregnancy rates according to the transfer cycle and for the total of all cycles. Since at the Cooper Center the pregnancy rate per cycle is equal for fresh and frozen transfer no distinction was made as to fresh or frozen ET.

Results

The clinical pregnancy rates and delivery rates were computed for patients undergoing their third or higher ET cycle. Patients were stratified by whether or not they had LIT prior to that transfer. It is possible that a woman began LIT on her fifth or sixth cycle. Some patients had LIT prior to the third transfer. The outcome according to whether LIT was performed or not is shown in Table 1. If you compare the pregnancy rates for cycle 3, there is no difference in the clinical pregnancy rate but the delivery rate is higher for the LIT group (p = .059). For all cycles combined, there is also no difference in the clinical pregnancy rate but the delivery rate is higher for LIT (p = .020).

In cycle 3, there were 98 (51%) fresh transfers and 94 frozen ETs (49.0%) in the untreated group, compared to 13 (38.2%) fresh and 21 (61.8%) frozen ETs in the LIT group. Overall, of the 341 standard cycles there were 175 (51.3%) fresh and 166 (48.7%) frozen ETs. In the LIT groups there were 52 (55.3%) fresh and 42 (44.7%) frozen ETs. The mean age for cycle 3 was 34.9 ± 4.2 and 35.1 ± 5.0, respectively. The mean number of embryos transferred was 3.5 ± 1.1 in the non-LIT group and 3.8 ± 1.3 in the LIT group.

Table 1. — *Pregnancy rates according to embryo transfer cycle and use or non-use of LIT.*

Transfer No.	LIT performed prior to transfer		No LIT performed prior to transfer	
	Clinical	Delivered	Clinical	Delivered
3	38.2% (13/34)	32.3% (11/34)	25.0% (48/192)	18.2% (25/192)
4	26.1% (6/23)	21.7% (5/23)	37.7% (32/85)	25.9% (22/85)
5	42.9% (9/21)	38.1% (8/21)	27.5% (11/40)	20.0% (8/40)
6	50.0% (4/8)	37.5% (3/8)	33.3% (4/12)	16.7% (2/12)
7	40.0% (2/5)	20.0% (1/5)	25.0% (2/8)	0.0% (0/8)
8	66.7% (2/3)	33.3% (1/3)	25.0% (1/4)	0.0% (0/4)
Totals	38.3% (36/94)	30.8% (29/94)	28.7% (98/341)	19.7% (67/341)

Discussion

In vitro fertilization is very expensive, labor intensive for both patient and physician, and is potentially risky especially for the development of ovarian hyperstimulation syndrome. Patients with multiple failures despite multiple transfers of apparently normal embryos are frequently willing to try unproven but relatively non-risky modifications to the procedure if there are at least theoretical benefits to the proposed therapy. These data suggest that LIT can improve the likelihood of success following another ET in this refractory group. An extensive search of the English literature did not show any previous publications pro or con about using LIT to improve outcome following IVF-ET.

Though the present study suggests that LIT improves outcome following ET at least in women who previously failed to have a live delivery following ET, one has to be careful about conclusions reached from a retrospective study. Even though there did not appear to be any significant confounding variables to explain the improvement in those treated vs not treated by LIT one should still be cautious about the conclusions from a retrospective study. However, these data should encourage a prospective study.

There have been many theories of how leukocyte injection may decrease miscarriage rates (at least by those research centers finding positive results) [2, 6, 11, 23-26]. The theory that LIT may act by inducing P receptors in gamma/delta T cells through allogeneic stimulation, possibly with certain HLA antigens, e.g., HLA-G or HLA-E, was only presented to explain why in our studies we have combined aggressive P therapy with leukocyte immunization [28-32]. The hypothesis continues that these P receptors on the gamma/delta T cells when exposed to very high concentrations of P (which may possibly only be achieved at the maternal/fetal interface) causes these cells to secrete PIBF [18-20, 33, 34]. This immunomodulatory protein, in turn, inhibits the cytolytic activity of NK cells [19, 20]. The data presented is consistent with but does not provide evidence that supports this hypothesis. These data merely show that LIT may improve the likelihood of a successful pregnancy following fresh or frozen ET in women who failed to have a success following at least two previous embryo transfers.

Not only should a prospective study of LIT include supplemental P administration, but the study should be careful to use fresh leukocytes rather than ones stored overnight and refrigerated. The only study that suggested a harmful effect of LIT was that by Ober *et al.* [15] and that was one of the few human studies using paternal leukocytes that had been stored overnight at 1-6°C rather than fresh leukocytes. It was the Ober study, that made the difference between the meta-analysis published in 1994 showing a benefit of LIT in preventing miscarriages [17] and the opposite conclusion reached by the Cochrane Database Systematic Review of 2003 which found no benefit [16]. Furthermore, it was the Ober study that influenced the United States FDA to disallow LIT without a new drug approval. The use of refrigerated leukocytes for pre-pregnancy immunization was one of several concerns expressed by Carp and Toder who were asked to provide commentary on the manuscript by Ober *et al.* [35].

Though only a small percentage of women used donor leukocytes, they were eliminated from the study for purposes of uniformity. Use of male partner's leukocytes is healthier from an infectious standpoint, but theoretically may be less efficacious than donor leukocytes.

For this retrospective study, all cycles from women having more than two previous ETs without success were included. Thus the same women could be used more than once. A woman could be counted in both groups if for example she failed to conceive without LIT on cycle 4 and then could have received LIT in cycle 5. There were 37 women included in this study whose outcome of their first ET cycle after LIT was compared to that of 37 matched controls (also included in this retrospective study) at the 2003 Pacific Coast Fertility Society Meeting [36]. The matched control study evaluating the first cycle of LIT not only showed a significantly better outcome in the LIT treated group vs. controls, but the magnitude of improvement was even greater when evaluating only first cycles in the subgroup [36].

Whenever there was frozen or fresh ET the women were aggressively treated with progesterone supplementation. The use of progesterone could explain why LIT was successful for recalcitrant patients having failed IVF-ET versus those studies failing to show any benefit of LIT for recurrent miscarriage [12-15, 37, 38]. It should be noted that in one randomized control study when one group was treated with P only and the other with LIT and P, significantly higher live delivery rates were obtained in the group treated with LIT and P [22]. Hopefully, these data will stimulate interest in re-evaluating the efficacy of LIT for recurrent miscarriage but to include a group with LIT and P therapy. Since most IVF centers use P in the luteal phase, a prospective study would merely have two treatment arms: LIT or not LIT (or possibly placebo LIT). Such a study could be performed by even one IVF center performing a lot of yearly IVF cycles. However, since P supplementation is not universally prescribed for those with recurrent miscarriages, a prospective study may require four treatment arms (progesterone and LIT, placebo P and real LIT, P and placebo LIT, and placebo P and placebo LIT). A prospective study with four treatment arms would probably require multi-center cooperation.

References

[1] Taylor C., Faulk W.P.: "Prevention of recurrent abortion with leucocyte transfusions". *Lancet,* 1981, *ii,* 68.

[2] Mowbray J.F., Gibbings C., Liddell H., Reginald P.W., Underwood J.L., Beard R.W.: "Controlled trial of treatment of recurrent spontanoues abortion by immunization with paternal cells". *Lancet,* 1985, *1,* 941.

[3] Mowbray J.F.: "Genetic and immunological factors in human recurrent abortion". *Am. J. Reprod. Immunol. Microbiol.*, 1987, *15,* 138.

[4] Mowbray J.F.: "Success and failures of immunization for recurrent spontaneous abortion". In: Beard R.W., Sharp F. (eds.): "Early Pregnancy Loss, Mechanisms and Treatment". Ashton-under-Lyme, Peacock Press, 1988, 325.

[5] Beer AE.: "Pregnancy outcome in couples with recurrent abortion following immunological evaluation and therapy". In: Beard R.W., Sharp F. (eds.): "Early Pregnancy Loss, Mechanisms and Treatment". Ashton-under-Lyme, Peacock Press, 1988, 337.
[6] Reznikoff-Etievant M.F., Durieux I., Huchet J., Slamon C., Netter A.: "Human MHC antigens and paternal leukocyte injections in recurrent spontaneous abortions". In: Beard R.W., Sharp F. (eds.): "Early Pregnancy Loss, Mechanisms and Treatment". Ashton-under-Lyme, Peacock Press, 1988, 375.
[7] Smith J.B., Cowchock F.S.: "Immunological studies in recurrent spontaneous abortion: Effects of immunization of women with paternal mononuclear cells on lymphocytotoxic and mixed lymphocyte reaction blocking antibodies and correlation with sharing of HLA and pregnancy outcome". *J. Reprod. Immunol.*, 1988, *14,* 99.
[8] Carp H.J.A., Toder V., Gazit E., Orgad S., Mashiach S., Nebel L., Serr D.M.: "Immunization by paternal leukocytes for prevention of primary habitual abortion. Results of a matched controlled trial". *Gynecol. Obstet. Invest.,* 1990, *29,* 16.
[9] Takakuwa K., Toto S., Hasegawa I., Ueda H., Kanazawa K., Takeuchi S, Tanaka K. Result in immunotherapy on patients with unexplained recurrent abortion: A beneficial treatment for patients with negative blocking antibodies". *Am. J. Reprod. Immunol.,* 1990, *23,* 37.
[10] Daya S., Gunby J.: "The effectiveness of allogeneic leukocyte immunization in unexplained primary recurrent spontaneous abortion". *Am. J. Reprod. Immunol.,* 1994, *32,* 294.
[11] Agrawal S., Kishore R., Halder A., Sharma A., Sharma R.K., Das V. *et al.*: "Outcome of pregnancy in women with recurrent spontaneous abortion following immunotherapy with allogeneic lymphocytes". *Hum. Reprod.,* 1995, *10,* 2280.
[12] Cauchi M.N., Lim D., Young D.E., Kloss M., Pepperell R.J.: "Treatment of recurrent aborters by immunization with paternal cells: Controlled trial". *Am. J. Reprod. Immunol.*, 1991, *25,* 16.
[13] Ho H.N., Gill T.J. III, Hsieh H.J., Jiang J.J., Lee T.Y., Hsieh C.Y.: "Immunotherapy for recurrent spontaneous abortions in a Chinese population". *Am. J. Reprod. Immunol.,* 1991, *25,* 10.
[14] Hwang J.L., Ho H.N., Yang Y.S., Hsieh C.Y., Lee T.Y., Gill T.J.: "The role of blocking factors and antipaternal lymphocytotoxic antibodies in the success of pregnancy in patients with recurrent spontaneous abortion". *Fertil. Steril.,* 1992, *58,* 691.
[15] Ober C., Karrison T., Odem R.R., Barnes R.B., Branch D.W., Stephenson M.D. *et al.*: "Mononuclear-cell immunisation in prevention of recurrent miscarriages: a randomised trial". *Lancet,* 1999, *354,* 365.
[16] Scott J.R.: "Immunotherapy for recurrent miscarriage". Cochrane Database Syst. Rev., 2002, 2, CD000112.
[17] Recurrent Miscarriage Immunotherapy Trialist Group: "Worldwide collaborative observational study and meta-analysis on allogeneic leucocyte immunotherapy for recurrent spontaneous abortion". *Am. J. Reprod. Immunol.,* 1994, *32*, 55.
[18] Check J.H., Arwitz M., Gross J., Peymer M., Szekeres-Bartho J.: "Lymphocyte immunotherapy (LI) increases serum levels of progesterone induced blocking factor (PIBF)". *Am. J. Reprod. Immunol.,* 1997, *37,* 17.
[19] Szekeres-Bartho J., Autran B., Debre P., Andreu G., Denver L., Chaouat G.: "Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction". *Cell Immunol.,* 1989, *122*, 281.
[20] Szekeres-Bartho J., Kilar F., Falkay G., Csernus V., Torok A., Pacsa A.S.: "The mechanism of the inhibitory effect of progesterone on lymphocyte cytotoxicity: I. Progesterone-treated lymphocytes release a substance inhibiting cytotoxicity and prostaglandin synthesis". *Am. J. Reprod. Microbiol.*, 1985, *9,* 15.
[21] Szekeres-Bartho J., Barakonyi A., Polgar B., Par G., Faust Z., Palkovics T. *et al.*: "The role of gamma/delta T cells in progesterone-mediated immunomodulation during pregnancy: a review". *Am. J. Reprod. Immunol.,* 1999, *42,* 44.
[22] Check J.H., Tarquini P., Gandy P., Lauer C.: "A randomized study comparing the efficacy of reducing the spontaneous abortion rate following lymphocyte immunotherapy and progesterone treatment versus progesterone alone in primary habitual aborters". *Gynecol. Obstet. Invest.*, 1995, *39,* 257.
[23] Check J.H., Szekerez-Bartho J., O'Shaughnessy A.: "Progesterone induced blocking factor seen in pregnancy lymphocytes soon after implantation". *Am. J. Reprod. Immunol.,* 1996, *35,* 277.
[24] Lubinski J., Vrdoljak V.J., Beaman D.K., Kwak J.Y., Beer A.E., Gilman-Sachs A.: "Characterization of antibodies induced by paternal lymphocyte immunization in couples with recurrent spontaneous abortion". *J. Reprod. Immunol.,* 1993, *24,* 81.
[25] Sugi T., Makino T., Maruyama T., Kim W.K., Iizuka R.: "A possible mechanism of immunotherapy for patients with recurrent spontaneous abortions". *Am. J. Reprod. Immunol.,* 1991, *25*, 185.
[26] Ito K., Tanaka T., Tsutsumi N., Obata F., Kashiwagi N.: "Possible mechanisms of immunotherapy for maintaining pregnancy in recurrent spontaneous aborters: analysis of anti-idiotypic antibodies directed against autologous T-cell receptors". *Hum. Reprod.,* 1999, *14,* 650.
[28] Chiu L., Nishimura M., Ishii Y., Wieda M., Maeshima M., Takedani Y. *et al.*: "Enhancement of the expression of progesterone receptor on progesterone treated lymphocyte after immunotherapy in unexplained recurrent spontaneous abortion". *Am. J. Reprod. Immunol.,* 1996, *35*, 552.
[29] Ellis S.A., Plamer M.S., McMichael A.J.: "Human trophoblast and the choriocarcinoma cell line Be Wo express a truncated HLA class I molecule". *J. Immunol.,* 1990, *144,* 731.
[30] Kovats S., Main E.K., Librach C., Stubblebine M., Fisher S.J., DeMars R.: "A class I antigen, HLA-G, expressed in human trophoblasts". *Science,* 1990, *248,* 220.
[31] Mandelboim O., Pazmany L., Davis D.M., Vales-Gomez M., Reyburn H.T., Rybalov B. *et al.*: "Multiple receptors for HLA-G on human natural killer cells". *Proc. Natl. Acad. Sci USA,* 1997, *94,* 14666.
[32] Rouas-Freiss N., Marchal R.E., Kirszenbaum M., Dausset J., Carosella E.D.: "The alpha 1 domain of HLA-G1 and HLA-G2 inhibits cytoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors?". *Proc. Natl. Acad Sci USA,* 1997, *94,* 5249.
[33] Szekeres-Bartho J., Csernus V., Hadnagy J.: "The blocking effect of progesterone on lymphocyte responsiveness is receptor-mediated". *Biol. Immunol Reprod.,* 1989, *15,* 36.
[34] Szekeres-Bartho J., Szekeres G.Y., Debre P., Autran B., Chaouat G.: "Reactivity of lymphocytes to a progesterone receptor-specific monoclonal antibody". *Cell Immunol.,* 1990, *125*, 273.
[35] Carp H.J.A., Toder V., Gazit E., Orgad S., Mashiach S., Nebel L., *et al.*: "Immunization by paternal leukocytes for prevention of primary habitual abortion: results of a matched controlled trial". *Gynecol. Obstet. Invest.*, 1990, *29,* 16.
[36] Check J.H., Liss J.R., Check M.L., DiAntonio A., Duroseau M.: "Lymphocyte immunotherapy can improve pregnancy outcome following in vitro fertilization (IVF)-embryo transfer (ET) in patients failing to successfully conceive after two previous ETs". *Clin. Exp. Obstet. Gynecol.* (in Press).
[37] Gatenby P.A., Cameron K., Simes R.J., Adelstein S., Bennett M.J., Jansen R.P. *et al.*: "Treatment of recurrent spontaneous abortion by immunization with paternal lymphocytes: results of a controlled trial". *Am. J. Reprod. Immunol.*, 1993, *29*, 88.
[38] Christiansen O.B.: "A fresh look at the causes and treatments of recurrent miscarriage, especially its immunological aspects". *Hum. Reprod.,* 1996, *2*, 271.

Address reprint requests to:
J.H. CHECK, M.D., Ph.D.
7447 Old York Road
Melrose Park, PA 19027 (USA)