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Successful pregnancy after Intralipid addition to sildenafil and enoxaparin in woman with history of recurrent pregnancy loss (RPL)

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Abstract

OBJECTIVES: Does addition of Intralipid to sildenafil and enoxaparin immunotherapy improve pregnancy outcome?
MATERIALS AND METHODS: Report of a striking case of a patient with history of 4 recurrent pregnancy losses (RPL) and IVF failures.
RESULTS: Adding of Intralipid resulted in giving birth to a healthy male baby in the 3th IVF cycle.
CONCLUSION: Combination therapy that includes Intralipid may generate successful IVF outcome, although this problem merits further study, especially regarding safety issues.

Abbreviations:

RPL	- recurrent pregnancy loss
IVF	- *in vitro* fertilization
PCOS	- polycystic ovary syndrome
OHSS	- ovarian hyperstimulation syndrome
TSH	- thyroid-stimulating hormone
Anti-TG	- thyroglobulin antibodies
ESHRE	- European Society of Human Reproduction and Embryology
ASRM	- American Society for Reproductive Medicine
MTHFR	- methylenotetrahydrofolate reductase
AMH	- anti-Müllerian hormone
PAI1	- plasminogen activator inhibitor-1

CASE REPORT

INTRODUCTION

The exact etiology of recurrent pregnancy loss (RPL) remains unresolved thus, the treatment modalities have not been clearly established in this setting (Kumar & Mahajan 2013; Kuon *et al.* 2015). Recurrent implantation failure is defined as unsuccessful conception following three cycles of IVF or ET. Recurrent miscarriage is described as two or more failed pregnancies according to the American Society for Reproductive Medicine (Kumar & Mahajan 2013; Kuon *et al.* 2015). Management of RPL is an important issue with high impact on global human health. No gold standard of such therapy has been established to date. Neither aspirin combined with heparin, nor aspirin alone improved the live birth ratio among women with unexplained RPL according to a recent PREFIX (Prevention of Unexplained Recurrent Abortion by Enoxaparin) study and Cochrane database (Pasquier *et al.* 2015; de Jong *et al.* 2014). Moreover, the Promise study was also disappointing – progesterone is of no use in this disease (Coomarasamy *et al.* 2015).

The prognosis is good in some cases even without treatment, although when the number of reproductive failures exceeds four, successful outcome is rather unlikely. Therefore, it is necessary to introduce new treatment modalities in selected cases with extremely bad prognosis (Kumar & Mahajan 2013; Kuon *et al.* 2015).

Parenteral administration of Intralipid (lipid emulsion) is capable of lowering the activity of NK cells (Kumar & Mahajan 2013; Kuon *et al.* 2015). Potential mechanism of action might involve regulation of NK cell function through decreasing cytotoxic activity and promotion of trophoblastic invasion. Intralipid is effective in suppression of abnormal NK cell activity in vivo, as well as in vitro (Meng *et al.* 2015; Fatemi & Popovic-Todorovic 2013; Coulam & Acacio 2012; Clark 1994). According to Roussev *et al.* (Roussev *et al.* 2007) study, suppression of NK cell activity was demonstrated in 50 patients: in 39 subjects (78%) suppression was observed during the first week of infusion, in 11 patients (12%) during the second infusion 2–3 weeks later, and the third dose was necessary to normalize NK cell activity only in case of the last 4 patients. In most patients the suppression effect lasted for a total of 6–9 weeks (Roussev *et al.* 2007; Roussev *et al.* 2008)

If this indeed proves to be correct, this drug might be effective in treatment of RPL among patients with NK cell activation. Elevated numbers of peripheral blood NK cells and increased infiltration of endometrial NK cells have been reported as related to pregnancy complications, such as miscarriage (Ntrivalas et al.2001; Aoki *et al.* 1995). Although immunophenotypes of the majority of peripheral blood NK cells are different from endometrial NK cells, the peripheral blood NK cells seem to be tightly related to decidual NK cells and may reflect the decidual NK cell functional status (Ntrivalas et al.2001; Aoki *et al.* 1995; Santoni *et al.* 2008). It is also currently accepted that uterine NK cells are at least partially derived from blood (Santoni *et al.* 2008). Additionally, some data suggest that women with RM are characterized by altered peripheral blood NK parameters and NK cells as a percentage of lymphocytes best discriminate between patients with RM and control group (Yamada *et al.* 2003; King *et al.* 2010). From a clinical point of view, NK cells are described by numbers or percentages of the total circulating lymphocyte count and/or characterized according to their activity. Two populations can be distinguished: peripheral (pNK) and uterine (uNK). NK proportion exceeding 18% of total peripheral blood lymphocytes was determined to be highly specific for RPL (Jerzak *et al.* 2008; Sacks 2015; Moffet & Shreeve 2015; Seshadri & Sunkara 2014). Recent meta-analysis by Seshadri and Sunkara (2014) indicated that RPL patients have higher levels of pNK cells (expressed as numbers as well as percentages) compared to healthy controls, while no differences in uNK levels were noted. However, testing should be repeated in the context of clinical research.

The aim of the study was to evaluate the pregnancy outcome after addition of Intralipid to enoxaparin and sildenafil immunotherapy in a woman with 4 RPL and 2 failed transfers during IVF.

MATERIALS AND METHODS

The subject of the study was a 36-year-old pregnant woman in her 5th pregnancy following cryotransfer in IVF procedure due to ovarian hyperstimulation syndrome (OHSS). Her medical history included four first-trimester miscarriages, including one after IVF performed due to subsequent difficulties conceiving. Long protocol was introduced, resulting in OHSS due to PCOS in our patient. Therefore, cryotransfer was performed and the third procedure resulted in successful pregnancy. Gestational age was established based on the last menstrual period and ultrasound examination.

The study was approved by the Bioethical Committee of the Military Institute of Medicine as well as the patient, including sildenafil therapy before pregnancy and addition of enoxaparin during pregnancy. Study protocol was described previously (Jerzak *et al.* 2008). Consent for Intralipid therapy was obtained from the patient only.

Before pregnancy, the patient was examined with regard to any possible causes of infertility and no apparent reason for previous failures was found. Anatomical, genetic, microbiological, autoimmune and hormonal causes of abortions were studied. Complete medical, surgical and social histories were obtained. Also, the couple underwent peripheral blood chromosome assessment. Hysterosalpingography did not reveal any abnormalities of patient's uterus. Luteal phase defect was excluded. Levels of TSH and prolactin were normal. However, the presence of anti-thyroid antibodies (anti-TG) was confirmed. Ultrasound examination of the

thyroid gland confirmed the diagnosis of Hashimoto disease. However, there were no other autoantibodies.

The Patient fulfilled the diagnostic criteria for PCOS according to the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) consensus workshop in Rotterdam (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). Moreover she presented with impaired OGTT with insulin resistance and high AMH levels (12.6 ng/ml).

No blood group incompatibility was noted. Semen analysis did not reveal any abnormalities. The existence of inherited thrombophilia (protein C, protein S, and antithrombin III deficiency, resistance to activated protein C, including Factor V Leiden mutation, hyperhomocysteinemia, MTHFR mutations, prothrombin gene mutation) was not detected. Patient presented at our department after the fourth pregnancy loss. She was administered prednisolone due to the presence of anti-TG antibodies until the 10th week of gestation, levothyroxine and vitamin D3. Moreover, because of insulin resistance and PCOS, she also received metformin, aspirin (75 mg) until 36–38 weeks of gestation.

NK assay

Natural killer cell activity was measured using flow cytometry. The methodology was described previously (Jerzak *et al.* 2008). Peripheral blood NK cells' surface antigens, CD16 and CD56, were also determined using flow cytometry (Simultest CD3/CD16+CD56, FACS-Calibur, Cell Quest software, Becton-Dickinson).

Separation of effector cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized patient blood by Ficoll gradient centrifugation. The mononuclear cells were adjusted to 1x10^7 cells/2.6ml in culture medium (RPMI 1640, 10% FBS). Target cells were human erythroleukemia K562 (ATTC, England). The K562 human erythroleukemia cell line was used as a standard target for human NK assays. K562 cells were washed in PBS 120g and labeled with 1.2ml DIO (3,3-dioctadecyloxacarbocyxacarbocyanine perchlorate, (Sigma)/1ml PBS and incubated for 20min in 37^{0}C CO_2. After two washes in PBS, cell concentration was adjusted to 1x10^6 cells/ml in medium (RPMI 1640, 10% FBS) and used for cytotoxicity assay.

Cytotoxicity assay

Groups of 8 replicate wells were incubated for 4h in 37^{0}C 5%CO_2: PBMC in medium, K562 in medium, mixed PBMC with target cells in ratios: 50:1 and 12:1. 25ml propidium iodide solution (0.1mg/ml in water, Sigma) was added to each sample to stain dead cells (total volume in each sample – 0.2 ml). Live target cells (T) were identified by strong green fluorescence, whereas dead target cells (Td) showed strong green and red fluorescence. The percentage of dead target cells (%Td) was calculated according to the following formula: %Td=(Td / T) x 100%. Specific lysis was calculated as: %Td (cultured with effector cells – %Td (cultured without effector cells).

RESULTS

Flow cytometry analysis revealed increased number of NK cells (Table 1). We introduced sildenafil suppositories intravaginally since such administration reduces NK cell activity (Jerzak *et al.* 2008). We administered 25-mg sildenafil suppositories four times a day for 6 days from day 3 of the cycle, followed by 40mg of enoxaparin and 75 mg of aspirin. However, the 2nd IVF failure occurred. Therefore, we introduced 100ml of 20% Intralipid two weeks after the third ET, followed by administration of 40mg of enoxaparin and 75mg of aspirin until the 36th week of gestation. Despite NK cell parameters leveling off at 8 weeks of gestation, there was a risk of preterm delivery over the course of this pregnancy. We decided to re-check the NK cells parameters, which increased again. Routine obstetrics care of high high-risk pregnancy, including periodic uterine ultrasonography and fetal heart monitoring, was provided. A cervical pessary for prevention of preterm birth was implanted due to suspected cervical incompetence. Pregnancy was also complicated by urinary tract Escherichia coli infection and toxoplasmosis. Therefore, we decided not to introduce the subsequent doses of Intralipid due to possible side effects. A normal, healthy boy weighing 3650g was delivered by caesarean section at 36 weeks of gestation and is now a normal, healthy six-year-old.

DISCUSSION

As is known, polycystic ovary syndrome is associated with significantly increased risk of miscarriage. Literature data suggest that as much as 30–40% of pregnancies in PCOS end in miscarriage during the first trimester (Gray & Wu 2000). Moreover, PCOS is responsible for about 32–82% of habitual miscarriages (Li *et al.* 2002). Literature suggests hyperinsulinemia

Tab. 1. Expression of NK cell markers and NK activity in the study patients.

NK assay	CD16+ (%)	CD56+ (%)	NK activity (%)
After 4th abortion	20	17	11
2nd cryotransfer after sildenafil therapy	22	5	9
5 pregnancy – 4 week of gestation	21	5	0
5 pregnancy – 8 week of gestation	14	3	9
5 pregnancy – 18 week of gestation	24	4	12
5 pregnancy – 22 weeks of gestation	21	9	0

Normal value for pregnant and not pregnant women <12%.

to be the direct cause behind pregnancy loss in PCOS through increase in the activity of plasminogen activator inhibitor-1 (PAI-1) (Sun *et al.* 2010). It also seems that high AMH level might suggest a more severe form of PCOS. Administration of metformin, aspirin and LMWH is based on the data from literature, confirming the efficacy of such treatments. Among women with PCOS administration of metformin in combination with enoxaparin reduces the risk of miscarriage as much as 3.4-fold (Glueck *et al.* 2004).

Glueck *et al.* (Glueck *et al.* 2006) demonstrated that administration of metformin in the preconception period as well as the first trimester of pregnancy reduces the activity of PAI-1 and increases the proportion of live births. Moreover, metformin and low-dose aspirin reduced uterine artery impedance and enhanced oocyte quality (Jamal *et al.* 2012; Zhao *et al.* 2014). However, administration of both aspirin as well as LMWH in unexplained recurrent miscarriages is controversial (de Jong & Kaandorp 2014; Pasquier *et al.* 2015).

There is limited in humans with regard to safety of Intralipid in pregnancy (Sacks 2015; Moffet & Shreeve 2015). To date, there has been no evidence that Intralipid is associated with embryonic toxicity, teratogenicity or increased risk of pregnancy loss (Sacks 2015; Moffet & Shreeve 2015). In the studied case, Intralipid therapy was introduced at the beginning of pregnancy to reduce NK cell levels to normal values. The association with successful correction of immune test abnormalities by Intralipid was also described by Roussev *et al.* (2007; 2008). In conclusion, addition of Intralipid (to sildenafil and enoxaparin) normalized NK cell parameters and led to a successful pregnancy. Effectiveness of such therapy may derive from regulation of immune, inflammatory, and/or procoagulation pathways that act synergistically in the pathogenesis of pregnancy failure. Since previous IVF attempts failed despite introduction of sildenafil, it would seem that a combination therapy was the key strategy leading to success. However, the clinical effect was not ever lasting, since the of NK cell parameters returned to abnormal levels at 18 weeks of gestation in the studied patient. We introduced routine obstetrics care of high-risk patient following a diagnosis of E. coli infection and toxoplasmosis in our patient. It cannot be excluded that such infection might have been connected to Intralipid therapy. However, some known side effects or adverse events due to Intralipid infusion were described: hepatomegaly, jaundice, cholestasis, splenomegaly, thrombocytopenia, leukopenia and fat overload syndrome occurring in < 1% in clinical trials according to the FDA, 2007 (Sacks 2015; Moffet & Shreeve 2015). In addition, a salutary report described a case of systemic candidiasis presenting at just 18 weeks of gestation, resulting in fetal loss following immunotherapy with Intralipid among other drugs, such as TNF-alpha inhibitor and prednisolone (Akhanoba *et al.* 2014). Therefore, it is hard to predict whether Intralipid might influence not only NK cells function, but also may have some negative systemic effects. We decided not to introduce the second infusion of Intralipid in order to avoid intensification of systemic sepsis.

CONCLUSIONS

Intralipid might be a promising therapy in selected cases with bad prognosis. Recurrent pregnancy loss and recurrent implantation failure are associated with abnormal NK cell function. However, the exact role of intralipids, especially in the context of additional and supportive therapy, should be further elucidated in a research setting. Combination therapy including Intralipid may allow successful IVF outcome, although this issue merits further studies, especially regarding safety problems.

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