Role of Activated Natural Killer Cells (CD3, CD56, CD16) in Repeated Implantation Failure in Women Undergoing IVF/ ICSI Cycles

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Abstract

Objective : to compare the level of peripheral blood natural killer (NK) cells (CD3, CD56, CD16) in cases of RIF and women with history of at least one successful ICSI trial.

Methods : A prospective cohort study conducted on 50 women underwent ICSI trial classified into 2 groups. Group I included 25 women repeated (2 or more) ICSI failures and group II included 25 women who previously achieved clinical pregnancy at least once in previous trial. Peripheral blood NKCs was assessed in all women. The primary outcome parameter was the number of NKCs in women of both groups. Other outcomes included the number and quality of retrieved oocytes, the number and quality of obtained embryos, fertilization rate, implantation rate, chemical pregnancy rate, clinical pregnancy rate, ongoing pregnancy rate and live birth rate.

Results: No significant difference between women with previous IVF failure and those with previous IVF success regarding number and quality of retrieved oocytes, number and quality of embryos, fertilization, implantation, clinical pregnancy, ongoing pregnancy and live birth rates. However, the chemical pregnancy rate was significantly higher in women with previous IVF success.

Conclusion: NKCs (CD56. CD16.CD3.) levels did not differ significantly between recurrent implantation failure cases and recurrent successful implantation controls.

INTRODUCTION

Since the first born IVF girl “Louise Brown” in 1978, more than 8 million live birth were achieved through assisted reproduction (1).

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IVF success is dependent on many factors, the most important one is successful implantation. Implantation success is achieved through a precise synchronization between endometrial and blastocyst maturity and development (2).

Modifications to the cellular, vascular, and immunological systems are necessary for optimal endometrial growth (3).

These modifications include the development of the stromal cells of the endometrium to decidual cells with pinopodes (apical projections) associated with growth of the endometrial glands, and the appearance of microvilli on the epithelial luminal surface of the endometrium (4).

Vascular invasion and endometrial immune cell infiltration are caused by alterations in adhesion molecules, cytokines, growth factors, and inhibitory mediators that are linked to these cellular changes (5).

Recurrent implantation failure (RIF) is a main cause of repeated IVF failure (6).

RIF has several definitions. Some consider it as failure to achieve clinical pregnancy after the transfer of at least 6 good quality embryos in fresh or frozen IVF cycles, at least 4 embryos in two egg donations, or after transfer of 10 or more embryos in multiple transfers, or the absence of a gestational sac on ultrasound at 5 weeks after embryo transfer (ET) following 3 ET with high-quality embryos (7).

Among the various reasons of RIF, uterine variables, including thin endometrium, poor endometrial receptivity, and immunological factors have drawn increased attention. Numerous factors are involved in the process of implantation, including embryo quality, endometrial receptivity, and immunological factors(6).

Natural killer (NK) cells are lymphocytes generated from bone marrow that work to eliminate foreign, infectious, and cancerous cells as well as to enhance immune response (8).

By virtue of their expression of the CD56 and CD16 cell surface antigens, they are subtyped. Most pNK cells express CD16, but have less CD56 surface antigens; as a result, they are frequently referred to as CD56dim/CD16+ cells. Cell lysis is caused by CD16 (9)

Both peripheral blood and the uterine mucosa contain NK cells. However, the NK cells at the two sites exhibit significant phenotypic and functional variations (10).

While uterine NK cells are primarily CD56 bright/CD16+ and primarily cytokine producers, peripheral blood NK cells are predominately CD56 dim/CD16+ and are cytotoxic and in direct contact with chorionic villi at inter billows space (11).

Importantly, however, it is believed that identical mechanisms govern peripheral and uterine NK cells. Therefore, determining the degree of NK cell activation in peripheral blood provides insight into the condition of uterine cells (12). In addition, some claim that pNK cells migrate into the uterus before becoming uNK cells. **(9)**.

Rai et al., reported an association between absolute count of activated NKC and reduced implantation rate in IVF cycles, which may suggest being a useful test to discriminate women who will benefit immune-modulation therapeutic intervention (10).

The aim of this study is to analyse and compare the level of peripheral blood natural killer (NK) cells (CD3, CD56, CD16) in cases of RIF and women with history of at least one successful ICSI trial.

Material and Methods

This prospective cohort study was conducted on 50 women who underwent IVF/ ICSI cycle at IVF unit of the department of obstetrics and gynecology, Cairo university between ……. and …….

An informed written consent was signed by all participating women after explanation of

the aim, procedure, risks and benefits of the trial. The study was approved by kasr Alainy ethical committee on ……. with number ……

Fifty women who underwent an ICSI trial were arranged into two groups, group I of women who experienced repeated (2 or more) ICSI failures and group II of women who previously achieved clinical pregnancy at least once in previous trial.

Inclusion criteria included age between 20 and 35 years. Women in group I inclusion criteria were unexplained infertility, mild male factor, tubal factors (not including hydrosalpinx), anovulation (not PCOS nor POI) with normal anatomical, hormonal and gynecological profile and had at least two failed attempts of ICSI (fresh or frozen) with total embryo transfer of six embryo of good quality. Women in group II had a minimum of one successful (with confirmed clinical pregnancy) ICSI trial with no history of repeated miscarriage not due to abnormal hormonal, gynecological nor anatomical causes (unexplained).

Exclusion criteria included women with immunological diseases such as anti-phospholipids proven by (normal anticardiolipin IgG & IgM and lupusanticoaglant antibodies) previously done by the patient already, chronic diseases such as DM, uterine anomalies (fibroid, polyp, septum), documented chromosomal rearrangement in either parent, hydrosalpinx, endometriosis, endocrinological and metabolic disease, gynecological intervention: endometrial polypectomy, myomectomy) and severe male factor (severe oligo-atheno-terato zoospermia).

All participants were evaluated through full history (especially the details of previous IVF cycles) and examinations (general, abdominal and pelvic) Laboratory and ultrasonographic evaluation were done to ensure stickiness to inclusion and exclusion criteria.

During the preparatory follow up visit peripheral blood NKC assessment using 3 mL of blood using flow cytometry (13).

At the Flow- Cytometry Laboratory, the flow-cytometric analysis was completed. Peripheral blood samples weighing three millilitres were drawn into heparinized tubes. Before staining, anticoagulated blood can be kept at room temperature (20°C–25°C) for up to 6 hours. 10 ml of each of the following monoclonal antibodies were pipetted into 100 l of each specimen before being tagged with the patient's name. Anti-CD3 antibody FITC conjugated; clone SK7, is composed of mouse IgG1 heavy chains and kappa light chains, , Anti-CD56 antibody R phycoerythrin-cyanin 5 (PC5) conjugated; clone MY31, is composed of mouse IgG1 heavy chains and kappa light chains and, Anti-CD16 antibody R phycoerythrin-cyanin 5 (PC5) conjugated; clone B73, is composed of mouse IgG1 heavy chains and kappa light chains, (all supplied by BD Biosciences, Becton, Dickinson and Company, USA). Reagents were provided in 1 mL of buffered saline with gelatin and 0.1% sodium azide.

Incubation for 10 to 12 minutes at room temperature (20°C–25°C) in the dark. Immediately after incubation, tubes were centrifuged at 300g for 5 minutes at room temperature (20°C–25°C) and the supernatant was discarded. The cell pellet in the residual fluid was Re-suspended, and then 2 mL of PBS was added with 0.1% sodium azide to each tube. Flow cytometric analysis was performed on the Becton Deckinson FACS calibre flow cytometer using. Cells negatively stained for CD3, positively for CD56 were selected and CD16 expression was analyzed.

Ovarian stimulation was performed using GnRH antagonist protocol. On day 2 of the therapy cycle, rFSH (Gonal-f; Merck Serono) subcutaneous injections were initiated daily. The daily subcutaneous administration of cetrorelix (Cetrotide; Merck Serono) at 0.25 mg was started as soon as one or more of the following conditions—one or more follicles reaching a diameter of 14 mm, the blood level of estradiol reaching 2203 pmol/L, and the serum

level of LH reaching 10 IU/L—were met. The GnRH antagonist and rFSH were given every day up until the triggering day. Ovum collection was carried out under transvaginal ultrasound supervision 34–36 hours following hCG triggering. Under ultrasound guidance, a Day 3 embryo transfer was carried out utilising a Labotect semirigid catheter (Labotect, Göttingen, Germany). From the day of ovum collection until serum -hCG testing, luteal phase support is provided by daily intramuscular injections of 100 mg progesterone (Prontogest; Amsa, Rome, Italy) (14).

The primary outcome parameter was the number of NKCs in women of both groups. Other outcomes included the number and quality of retrieved oocytes, the number and quality of obtained embryos, fertilization, implantation, chemical pregnancy, clinical pregnancy, ongoing pregnancy and live birth rates.

Sample size calculation was done using the comparison of CD56/16 between Cases with recurrent implantation failure and those with no implantation failure in couples doing IVF-ET, as it was the primary outcome of our study. As reported in previous publication (15), the mean ±SD of CD56/16 level in recurrent implantation failure group was approximately 13.62 ± 4.6, while in control group it was approximately 4.51 ± 0.98. Accordingly, we calculated that the minimum proper sample size was at least 21 women in each group to be able to detect a real difference of 9.1 with 99% power at $\alpha = 0.05$ level using Student's t test for independent samples. Sample size calculation was done using Stats Direct statistical software version 2.7.2 for MS Windows, Stats Direct Ltd., Cheshire, UK.

Data analysis was done using IBM SPSS statistics (V. 26.0, IBM Corp., USA, 2019). For quantitative non-parametric measures, data were expressed as median and percentiles, and for categorised data, both number and percentage were used. For non-parametric data, the Wilcoxon Rank Sum test was used to compare two independent variables, and the Kruskall Wallis test was used to compare more than two patients. Chi-square test to investigate the relationship between each of the two variables or to compare the two independent variables in relation to the categorised data. At 0.05, the likelihood of mistake was regarded as significant, whereas at 0.01 and 0.001, it is highly significant.

Results

We assessed 721women for eligibility, 615 of them did not fulfill our inclusion criteria and 56 declined to sign the informed consent. So, 50 women were included in our study (25 in each group). All of them underwent the treatment and followed up till completion of the study and none were excluded from analysis.

No significant difference between women with previous IVF failure and those with previous IVF success regarding age, BMI, AMH or the number of NKCs. However, the duration of infertility was significantly longer in women with previous IVF failure (table 1).

No significant difference between women with previous IVF failure and those with previous IVF success regarding number and quality of retrieved oocytes, number and quality of embryos, fertilization, implantation, clinical pregnancy, ongoing pregnancy and live birth rates. However, the chemical pregnancy rate was significantly higher in women with previous IVF success (table 2).

When comparing women who achieved pregnancy in the current cycle and other women, we found no significant difference between them regarding age, BMI, duration of infertility, AMH, the number of NKCs (table 3), number and quality of retrieved oocytes (table 4) but women with successful current pregnancy showed a significantly higher number of embryos especially of good quality (A and B)(table 4).

When comparing women who underwent fresh ET to those who underwent frozen ET, there was no significant difference between them regarding age, BMI, duration of infertility while AMH and the number of NKCs were significantly higher in women with frozen ET (table 5).

All outcome parameters named the number and quality of embryos, fertilization, implantation, chemical pregnancy, clinical pregnancy, ongoing pregnancy and live birth rates were not statistically different between women with fresh and frozen ET (table 6).

Discussion

Our study found no statistical significant difference between recurrent implantation failure (RIF) history group and patients with history of successful ICSI trial as regard natural killer cells population, this agrees with Zhang et al., (16), Kolanska et al., (17) and Tohma et al., (18) in the same time Mardanian et al., (15), Wafa et al., (19) and Azargoon et al., (20) found that the level of NKC is higher in the RIF, while Prado-Drayer et al., (21) and Ghafourian et al., (22) reported remarkably low proportions of NK cells in healthy controls. Moreover Sacs et al., (23) found a decreased proportion of NK cells in an infertile population compared with the control group.

These conflicted results may be explained by the heterogeneity in population recruited characteristics between studies especially as regard recruited population type, age, NKC subpopulation measured and method of analysis.

As regard number and quality of oocyte retrieved, no statistical significant difference was found between both groups this is supported by the findings of Ocal et al., (24) and Roy Choudhury et al., (25). This may be due to the age convergence between both groups (age min is 22 and age max is 35 with average 31.6 and 31 for group with history of successful ICSI and RIF group respectively), as the female age is known to be an important determinant of the ovarian reserve which is an independent factor for the no. and quality of oocyte retrieved as well established by de Bruin et al., (26), Amanvermez and Tosun (27) and Shahrokh Tehraniejad et al., (28).

Our current study showed a statistically significant difference between recurrent implantation failure (RIF) history group and patients with history of successful ICSI trial as regard duration of infertility which is consistent with findings observed by Ocal et al., (24), meanwhile, a conflict appear with the later study regarding the absence of statistical significant difference for age, BMI and AMH (as a marker for ovarian reserve). This is goes with the observations of Roy Choudhury et al., (25). This can be explained by the wider range of patients demographic data in Ocal et al.,(24) and the similarity of patients characteristics between our study and that of Roy Choudhury et al.,(25) and reflects the homogeneity of patients distribution rather than a true contradiction.

Multiple influential factors are involved in such situation among which the immunological factors (29), which have been proven to be a prominent cause for recurrent implantation failure through testing the efficacy of multiple immune-modulator interventions like; intravenous immunoglobulin (IVIG) (30), intralipid (31). Granulocyte colony-stimulating factor (G-CSF) intrauterine administration (29) and endometrial scratch (32).

Natural killer cells role in human fertility in general and recurrent implantation failure in specific has been discussed extensively (32) to make use of the gained knowledge to improve the assisted reproduction outcomes especially the implantation rate which has been described as the barrier in assisted reproduction to be overcome (33).

Also there was no statistical significant difference between the two groups as regard number and quality of embryo resulted and

transferred which goes in a line with Ocal et al., (24) and Roy Choudhury et al., (25) results. Oocyte quality affects the quality of embryos as suggested by Khalili et al., (34); Balaban and Urman (35) and since the two groups were comparable to each other as regard the oocyte quality, so it was logic to be again comparable to each other as regard embryo quality.

Multiple interrelated reasons may determine the number of resultant embryos such as the preference of physicians to transfer more than one embryo, that become a policy in many centers now wide world to gain higher implantation and pregnancy rate (33), and make every effort to produce more oocytes and subsequently more embryos to be transferred using for such purpose protocols and stimulation drug doses able to achieve this to maximize the chances of pregnancy (24).

Single embryo transfer is becoming more and less popular nowadays and only 15% of doctors are still choosing single embryo transfer in their practice especially in the presence of signs of good prognosis (36).

In the same contest, our study showed no statistical difference between RIF group and successful ICSI history group regarding fertilization and implantation rates. Oocyte quality plays a major role in fertilization process and embryo development in ART program as suggested by Khalili et al., (34) and in the same time it also determines the implantation potential of the derived embryo as reported by Balaban and Urman (35). So having no statistical difference regarding oocyte and embryo quality between the two groups resulted in having such a similar indifference regarding implantation and fertilization rates.

As regard achieving pregnancy, a statistical significant difference was found between the two groups regarding chemical pregnancy (which may represent the overall pregnancy rate) in the favor of the successful ICSI group, while when it comes to achieving clinical pregnancy and detection of a pulsating G.S. in ultrasound no statistical significant difference was detected this is agrees with Sacs et al., (23) and partially disagrees with Ocal et al., (24).

Our study also showed no statistical significance between the two groups as regard continuation of pregnancy past 1st trimester, miscarriage rate and live birth rate. This came in contrast to Sacs et al., (23) who recorded that RIF women had a higher miscarriage rate and lower live birth rate while Chin et al., (37) found that RIF patients' obstetric and perinatal results are comparable to those of control IVF-ET patients, indicating that they do not experience overtly negative effects when compared to controls.

Our results may be attributed to underlying parental risk factors that are more prevalent in patients suffering from infertility (38), taking into consideration the homogenous presence of the factors that influence miscarriage and live birth rates such as age (39).

We tried to look into our data from a different prospective, so we rearranged our cases into two new groups; the group of women who fail to achieve pregnancy in the current cycle and the one who got pregnant and we found that:

No statistically significant differences between both groups as regard demographic data (duration of infertility, age, BMI and AMH) which agrees with the findings of Roy Choudhury et al., (25) while Mardanian et al., (15) disagree with our results regarding the age.

Also no statistical significant difference was found regarding natural killer cells (CD56. CD16.C3.), this goes in line with Fukui et al., (40), Thum et al., (41) and Baczkowski et al., (42) while Mardanian et al., (15) found significant rise of The level of CD56dim CD16+ cells in women with IVF failure compared to successful IVF women but fail to record the same result for CD56bright CD16- cells levels.

This might be explained by differences in sample size, inclusion criteria or method of analysis between our study and the compatible studies from one side and Mardanian et al.,(15) on the other side.

A statistical significant difference was found between the two newly created groups as regard number and good quality embryos (A&B) resulted despite absence of any statistical difference has been found regarding number and quality of retrieved oocyte, however, the number of retrieved oocyte from the successful ICSI trial cases, although not statistically significant, were higher than those from failed ones which agree with Labarta et al., (43) and Vermey et al., (44) who confirmed a strong positive association between oocytes retrieved and top/good-quality embryos and that the group with the most oocytes aspirated had the most top/good-quality embryos

The previous result may spot some light on factors that lead to the success of the ICSI trial in these women.

The quality of transferred embryo, as well as the number of good quality embryos, is one of the most important determinants of for the success of ICSI and the chances of achieving clinical pregnancy (45).

Moreover, some studies in the literature stated that if an aged woman is producing a good number of good quality embryos, the pregnancy rate is almost same as that of young women (46).

A statistical significant increase in the NKC (CD56.CD16.CD3.) in the frozen embryos group compared to the fresh embryos patients; this finding seem to be strange and unrelated at the first instant . This provoked us to search for the possible relation between NKC and causes lead to freezing embryos.

By analyzing the causes of freezing embryos in our study, we found that in 11 patients out of 20, freezing the embryo was a necessity. Eight patients are for the fear of OHSS and three patients are for anticipation of poor endometrium receptivity. OHSS is then comprised around 70% of the inevitable causes of embryo freezing and 40% of all causes.

Digging deeper, cytokines (particularly IL2) are suggested to be the link between OHSS and NKC.

Exposure of hyper stimulated ovaries to hCG leads to the production of pro-inflammatory mediators. This includes vascular endothelial growth factor (VEGF) and varieties of cytokines among which is IL2.cytokines are likely to be involved in the pathogenesis and clinical features of OHSS (47).

In the same time, IL2, which is secreted predominantly from T lymphocytes, found to have a regulatory role in NKC function and number (48).

From the above it may be suggested that cytokine especially IL2 may be a common factor, a result or even a cause for increased NKC in the frozen embryo transfer group. However, more researches and studies are needed to confirm this result, figure out its impact on the success of IVF/ICSI and exclude the possibility of being a statistical coincidence rather than a true association.

The main strength of our study is its prospective nature, proper selection of participants and long term follow up duration while its main limitation was the relative small sample size

Our study concluded that NKCs (CD56. CD16.CD3.) levels did not differ significantly between recurrent implantation failure cases and recurrent successful implantation controls. This result is questioning their role in RIF and does not support the routine use of such marker in RIF outside the research work; accordingly, the use of immunomodulaors may be not justified based on this marker alone. Larger prospective studies are needed to confirm and extend our results. Further studies are needed to evaluate the correlation

between peripheral and endometrial NKC as the later role in cases of RIF is more evident in the literature.

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