The Role of Endometrial $CD16^{+}$ Natural Killer Cells in Unexplained Infertility A Controlled Clinical Trial

Thesis
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Contents

List of Abbreviations

ACOG	:	American College of Obstetricians and Gynecologists.
AFC	:	Antral Follicle Count.
AMH	:	Antimullerian Hormone.
APA$	:	Antiphospholipid Antibody Syndrome
ASRM	:	American Society of Reproductive Medicine.
BAFF	:	B-cell activating factor
BD	:	Becton-Dickinson Company
BMI	:	Body Mass Index.
C.pneumoniae	:	Chlamydia pneumoniae
CAP	:	Critical Analysis of Population
CASA	:	Computer –Aided Sperm Analysis
CAT	:	Chlamydia antibodies test.
CC	:	Clomiphene citrate
CCCT	:	Clomiphene citrate challenge test.
CCR	:	CC chemokine receptor
CD	:	Cluster of differentiation .
COH	:	Controlled ovarian hyper stimulation.
Crry	:	Complement Receptor Related Gene Y
CXCL	:	CXC chemokine ligand
CXCR	:	CXC chemokine receptor
DAF	:	Decay accelerating factor
DcR	:	Decoy receptor
DDCs	:	Decidual dentritic cells
DES	:	Diethylstilbestrol
DNA	:	Deoxyribose Nucleic Acid
E2	:	Estradiol.
ELISA	:	Enzyme-Linked Immunosorbent Assay Test
FACS	:	florescence –activated cell sorting
FasL	:	Fas ligand
FcRyIII	:	Fc receptor (receptor for immunoglobulin G), CD16.
FCS	:	Fetal calf serum.
FDA	:	Federal Drug Administration
FITC	:	Fluorescein isothiocyanate

FLAME	:	Flow analysis with automated multivariant estimation.
FSH	:	Follicular stimulating hormone.
GIBCO	:	Grand Island Biological Company
GnRH	:	Gonadotropin releasing hormone.
HCG	:	Human chorionic gonadotropin.
HLA	:	Human Leukocyte Antigen
HLA-A	:	Human Leukocyte Antigen A
HLA-B	:	Human Leukocyte Antigen B
HLA-C	:	Human Leukocyte Antigen C
HLA-E	:	Human Leukocyte Antigen E
HLA-F	:	Human Leukocyte Antigen F
HLA-G	:	Human Leukocyte Antigen G
HSG	:	Hysterosalpingogram.
HyCoSy	:	Hysterosalpingo-contrast sonography
ICI	:	Intracervical insemination of sperm.
ICSI	:	Intracytoplasmic sperm injection.
IDO	:	Indoleamine 2, 3_dioxyoxygenase
IL	:	Interleukin
INF	:	Interferon
IUI	:	Intrauterine insemination.
IVF	:	In vitro fertilization.
KIRs	:	Killer cells immunoglobulin-like receptors
LH	:	Luteinized hormone.
LIF	:	Leukemia inhibitory factor.
LBR	:	live birth rate
LIR	:	Leukocyte inhibitory receptors
MCP	:	Membrane cofactor protein
MHC	:	Major histocompatibility complex
MIRL	:	Membrane inhibition of reactive lysis
MRI	:	Magnetic resonance imaging
mRNA	:	Messenger Ribose Nucleic Acid
NICE	:	National Institute for Clinical Excellence (UK)
NIH	:	National institute of health.
NK	:	Natural killer cells.
NKG2A/C	:	Known as CD159a (Cluster of Differentiation 159a) is an activating receptor for natural killer cells.

PAP smear	:	Papanicolaou (Pap test or Pap smear; named for George Papanicolaou.
PCOS	:	Polycystic ovary syndrome.
PGE2	:	Prostaglandin E2
PNK	:	Peripheral natural killer cells
RCT	:	Randomized controlled trial
RPL	:	Recurrent pregnancy loss.
RSA	:	Recurrent spontaneous abortion.
TGF	:	T-cell growth factor
Th	:	T helper
Th2	:	T helper 2
TNF	:	Tumor necrosis factor
Treg	:	Regulatory T cells
TSLP	:	thymic stromal lymphopoietin
UI	:	Unexplained infertility
U/S	:	Ultrasonography
UNK	:	Uterine natural killer cells
WHO	:	world health organization.

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Protocol of thesis:

It is estimated that infertility affects 1 in 7 couples in the UK, since the original NICE guideline on fertility published in 2004 there has been a small increase in the prevalence of fertility problems, and a greater proportion of people now seeking help for such problems **(NICE guidelines, 2011).**

Unexplained infertility is one of the most common diagnoses in a fertility clinic **(Hull et al., 1985; Adamson and Baker, 2003; Brandes et al., 2010).** In the absence of a specific medical cause a specific treatment for unexplained infertility is lacking. Instead, these couples are exposed to several empirical treatments, among which medical assisted reproductive treatments such as, Clomiphene Citrate (Hughes et al., 2010), controlled ovarian hyperstimulation combined with intrauterine insemination (COH/IUI) (**Verhulst et al., 2009),** and/or in vitro fertilization (IVF) **(Pandian et al., 2009)** with or without intracytoplasmic sperm injection (ICSI) **(Steures et al., 2006)**.

Couples were diagnosed as unexplained infertility after the standard work-up was normal. Even, who conceived an ongoing pregnancy before fertility work-up was completed, were also diagnosed with unexplained infertility, if at least no anatomical, ovarian, tubal or male factor could be detected. Hence correct etiological diagnosis of infertility is essential to improve the outcome of assisted reproduction treatments, taking into account that in up to 50% of Cases, the cause of infertility cannot be identified **(Hum Repod, 2009; Matthiesen et al., 2012).**

Standard work up of infertility consists of: history taking and physical examination, Semen analysis,

Assessment of ovulatory status by history or laboratory testing, Ultrasonography, Determination of tubal patency and presence or absence of abnormalities of the uterine cavity, usually by Hysterosalpingogram, and laparoscopy with dye, A positive CAT (Chlamydia antibodies test) was overruled by normal laparoscopic findings**(Coppus, 2011)**.

As several studies suggest that immunological testing is necessary for the management of infertility **(Kwak Kim, 2009),** natural killer (NK) cells are being included as they constitute the most abundant leukocyte population in the deciduas (**Moffett-king, 2002)**. These cells can be classified into. Two subsets that differ in their cell surface markers as well as in functional properties: the cytotoxic $CD56^{dim}$ $CD16^{+}$ subset and the angiogenic $CD56^{bright}$ CD16- **(Starkey et al., 1988).**

While 90% of peripheral blood NK cells belong to the cytotoxic subset **(Cooper et al., 2001),** CD16- NK cells predominate in the endometrium during the secretory menstrual phase. Migration of NK cells to the uterus as well as endometrial differentiation from CD34+ stem cell precursors has been postulated as of CD16- origin **(Vacca et al., 2011), (Dosiou et al., 2005)**.

Although NK cell counts in peripheral blood of women with infertility have become a common practice in many centers of reproductive medicine, there are still few clinical studies that evaluate the correlation of endometrial with peripheral NK cell counts or establish normal values. Additionally, studies performed in implantation failure patients are scarce, whereas most studies have been undertaken in women who have undergone recurrent spontaneous abortions (**Park et al., 2010).**

Considering that peripheral blood NK cell numbers do not necessarily correlate with increased NK activity **(Kwak-**

Kim and Gilman-Sachs, 2008), further investigation is needed to determine endometrial activity in infertility. A proper balance between inflammatory/anti-inflammatory cytokine expression and the association with CD 16^{+}/CD16- NK cell counts could be a potential tool for immunological diagnosis of infertility **(Guzeloglu-Kayisli et al., 2009)**.

The deficient levels of total and angiogenic endometrial NK ($CD56^{bright}$ $CD16^{-}$) cells observed in Junovich et al., study during implantation window of ovarian stimulated cycles indicating their possible role in implantation failure in ovarian stimulated patients with consequent high estradiol serum levels, herein, comes the importance of understanding their role in unexplained infertility **(Junovich et al., 2011)**.

Most studies on endometrial NK cells have been performed by histological evaluation, where differences in NK cell subsets are limited while studies using flow cytometry provide a much better analysis of NK cell populations (**Starkey et al., 1988).**

Aim of the work

This study is designed to investigate the relation between increased endometrial NK cells count and unexplained infertility.

Research question:

Is there an increase in NK cells count In women with unexplained infertility compared to matched fertile women?

Research hypothesis:

Increased endometrial natural killer cells don't correlate with unexplained infertility.

Patient and methods

1) Type of the Study:

A prospective controlled clinical trail

2) Study Settings:

The study will be conducted at Ain-Shams University Maternity Hospital, starting from November 2013 till completion of number of study population.

3) Sample size justification

Assuming an average absolute count for endometrial CD16 as 5.1±6.1 for infertile & 2.0±1.5 in fertile women based on **(Gisela et al study, 2013)** results, a sample size of 33 to 50 in each group (to exclude drop outs), is enough to detect such difference at 0.05 alpha & 0.80 power of the test calculated using **Sata®lO** software program.

4) Study Population:

100 consenting women will be recruited in this study and divided into two groups; 50 female patients will represent cases suffering from primary unexplained infertility visiting our infertility clinic, and the other 50 normal fertile female will represent controls recruited from the contraception clinic for IUCD replacement for measuring endometrial CD 16+ NK cells.

The study group:

Fifty women will be recruited in this group diagnosed with unexplained infertility after fulfilling those criteria:

- Primary unexplained infertility.
- 21_38 years old.
- Regular menstrual cycles (24_35 days).
- FSH <10 mIU/mL.
- LH < 10 mIU/mL.
- Prolactin level < 29 ng/mL.
- E2 <50 pg/ml on day 2-3 of the menstrual cycle.
- Normal thyroid profile.
- Normal semen analysis according to WH criteria 2010 (total sperm concentration $15x10^6$ per ml , volume 1, 5ml, total motility (PR+ NP) is 40%, Progressive motility (PR) is 32%, normal morphology is 4%).
- Normal Hysterosalpingogram.
- A laparoscopy with chromotubation using methylene blue was done and was normal.

Exclusion criteria:

- Endometriosis evidenced by laparoscopy ±histopathology.
- Tubal factor.
- Ovulatory dysfunction.
- Anatomical uterine pathology.
- Abnormal male factor according to the reference values for healthy human semen established m World Health Organization guidelines).

The control group:

Fifty normal fertile females matched in all possible variables to the cases after explanation the purpose of the study and obtaining their consents, who have the following criteria:

- 21-35 years old.
- ≥1 live birth younger than 2 years old.

- Regular menstrual cycle (24-35 days).

Matching will include the following variables:

- Age: 21-38 years old.
- BMI:kg/m^2
- Regular menstrual cycle (24-35 days).
- Time of endometrial biopsy from day 19 to 23 (implantation window).
- Using hormonal therapy in the last cycle.

Ethics:

The study will be approved from the Ethical Committee of the Department of Obstetrics and Gynecology, Faculty of Medicine, Ain-Shams University.

Informed written consent will be taken from all participants before recruitment in the study, and after explaining the purpose and procedures of the study.

5) Methodology:

After taking informed verbal consent, the recruited patients will be subjected to the following:

- History taking, with particular emphasis on menstrual, sexual, gynecological, medical as (T.B, cancer, thyroid disease, galactorrhea, hirsutism, pelvic or abdominal pain, dysmenorrhea), previous pelvic surgery, induction of ovulation, vaginal infection, abnormal Pap smears, family, and life style habits as alcohol, smoking.

- Physical examination, including general examination as BMI, signs of excess androgens, signs of thyroid toxicity, galactorrhea, and local examination for abnormal uterine size, mobility, infections, tenderness, masses, vaginal discharge.

- Diagnostic tests: