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CD4 Cells, Haemopoietic Cells and Factors as Veritable Factors on Reproductive Hormones and Fertility

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Abstract

Immune system contributes to infertility, where T Reg play major role. Paucity of research in this crucial area, deems pertinent refocusing attentions on. CD4, haemopoietic cells (HC) and haemopoietic factors (HF) as veritable factors on fertility & reproductive hormones [Follicles stimulating hormone (FSH); Luteinizing hormone (LH); Prolactin (PRL); Estradiol (EZ); Progesterone (PROG); Testosterone (TESTO)] were investigated at a Teaching Hospital in Enugu, Nigeria between Feb – Oct 2022 on a pilot scale. Patients on doctor's provisional diagnosis of infertility were examined for their hormones' levels, CD4 & primary immune cells (PIC) counts. Analysis, using Paired Students t-test at 5% alpha level; & p value of test statistics was used for confirmation. All the patients have low CD4 counts (Range: 157-490). Our hypotheses were largely rejected: (i.e., Yes), CD4 & PIC have impact on the hormones; p values confirmed negative correlative association between CD4 & the six hormones; all the PIC have impact on PRL; except on EZ, neutrophil (N) has very significant impact on all the hormones; except on FSH, lymphocytes (L) has very significant impact on all the hormones; only on PRL that all the PIC have impact; except on PRL & TESTO, WBC (T) has no significant impact on the hormones except on PRL and TESTO; only N & L have significant impact on the PROG; only WBC (T), N & L have impact on TESTO; except L none of the PIC has impact on the EZ. Monocyte, Eosinophil & Basophils has impact only on FSH, LH & PLT. So, under no hormonal abnormality, the work strongly suggests that the inability to conceive resides on the low CD4 cells, plus impacts of it & PIC on hormones; hence, they are veritable factors. CD4 & PIC cells count should be a paradigm in infertility investigations.

Keywords: reproductive hormones, primary immune cells, CD4 cell count, flow cytometry, fully automated blood cells counter, fertility

1. Introduction

Author's interest on immunology as a feasible or veritable factor in fertility dated back to Anyiwo (1983) in a lecture in immunology on an M.Sc. degree class. It was then asserted that "speculation was in vogue that infertility could also arise from incompatibility between the sperms and the ova." In an understanding from Garcia-Velasco (2017) and Bashiri et al. (2017) that fact had been confirmed, and also explained part-failures in In Vitro Fertilization (IVF).

Recently, it is now known, immunologically, that a father and a mother have different parental major histocompatibility complex (MHC) factor, as well as different Human leucocyte antigens (HLA). Further, a foetus is half a factor from a father (spermatozoa) and half a factor from a mother (ovum); consequently, a foetus should hence be expected to have different MHC and HLA from and implantation mother. Indeed, immune system of embryos is different from that of the pregnant woman, as it as well contains genes from the father as stated above, which are strange to the immune system of the mother. Therefore, immunologically, the mother should regard the foetus as an antigen, to which her immune components will attack and destroys. But this is always not so; because the Regulatory T cells (T Reg) of the mother creates immunologically tolerance state between the mother and the foetus, so the foetus stays, grows and mature. The T-Reg are CD4 and CD25.

In a more definite explanation by Robertson, *et al.*, (2018), the immune system of embryos is different from that of the pregnant woman, as it contains genes from the father as well, which are unknown to the immune system of the mother. For a pregnancy to be normal, the woman's immune system develops a ***mechanism of immune tolerance*** in order not to attack the embryo. One of the most important immune cells is the ***lymphocytes or white blood cells***, capable of recognizing their own structures and also of producing antibodies that recognize foreign substances.

In actual fact, it is the embryo itself that "warns" the pregnant woman through the expression of the HLA-G Antigen, which function is to erase the cells of the immune system in order for the embryo to continue growing in the womb (Shushan & Schenker, 1992).

1.1 Justification of the Research

Role of immunology in fertility is not well-known by many medicos; to date, some still reflects only on reproductive hormones to investigate infertility. This usually ends up in enigma, especially in some cases of persistent infertility in situations of apparent adequate hormonal balances. Such unexplained or idiopathic infertility is a *condition, in which couples are not able to conceive without any definite causes* (Paraiso, et al, 2022).

It was on premise of this known role of T-Reg, and on a tripod that immune reactions are interplays of many combined factors *Pari passu* fertility hormones, that the authors sort to investigate the direct relationship of CD4 and other primary immune cells on cases of infertility.

1.2 Aim and Objectives

Aim: The aim of this research is to investigate the role of CD4 and primary immune cells as veritable factors on reproductive hormones and fertility.

1.3 Specific Objectives

1) Select patients on medical doctors' provisional diagnosis of infertility.
2) Collect blood samples from them.
3) Analyse each of the blood samples for the levels of: (a) CD4 using a Partec Flow Cytometer; (b) the five primary immune cells using Mindray BC-5150 Fully Automated Blood Cells Analyzer; (c) the six fertility hormones (Follicle Stimulating hormone, Luteinizing hormone, Prolactin, Progesterone, Testosterone and Estradiol), using I-Chroma^{TM} Reader made by Boditech Med Inc.
4) Analysing the result using Paired Students t-test at 5% (0.05) alpha or significant level; and p value of test statistics used for confirmation.

1.4 Null Hypothesis

Ho1: Effect of hormones and CD4 are in equilibrium

Ho2: Effect of hormones and primary immune cells are in equilibrium

Ho3: Effect of hormones and haemopoietic factors are in equilibrium

2. Materials and Methods

2.1 Collection of Blood Samples for Analysis

The patient's sleeve was raised above the left elbow, and a tourniquet tied to the upper arm. With the patient's fist clenched, the area where the needle will be inserted was swabbed with methylated spirit soaked in a cotton wool, then the cover of the hypodermic needle was removed without touching the needle tip, then the now sterilely opened needle, in a slanting position, was gently inserted into one of the most prominently displayed veins in the arm. Then the syringe was gently drawn up to suck in the blood. After about10ml of blood sucked, the tourniquet was loosened, and the hypodermic needle gently withdrawn from the patient's arm. The blood sample were then evenly share into three different respective bottles for the hormonal assays, the CD4 counts and the full blood counts. The methylated spirit soaked in a cotton wool was also used to cover the point of

insertion of the needle for few minutes to control bleeding.

2.2 Analysis of Primary Immune Cells

The primary immune cells analysis was done with Mindray BC-5150 Fully Automated Blood Cells Analyzer. 2.5ml of the venous blood collected in an EDTA bottle was placed in a mixer until ready for use. The haematology auto-blood analyser was put on. Then the probe of the analyser was inserted into the bottle with the blood sample to allow the analyser to pick the well-mixed blood sample. The result is displayed on the autoanalyzer's screen, read, printed out and recorded.

2.3 CD4 Cells Count

2.3.1 Principle of Flow Cytometry

The basic principle of flow cytometry is the passage of cells in a single file in front of a laser so they can be detected, counted and sorted. Cell components (such as CD_4^+ TEST cells) are labelled and then excited by the laser to emit light at varying wavelengths.

2.3.2 Procedure

The CD4 cells count was done with Partec Flow Cytometer Code No. CY-S-3022.

The instrument main power was switched on at the back of the instrument and then the green button was pushed on the left of the instrument.

2.3.3 Cleaning

Sample tube was first plugged with cleaning solution and inserted into the sample port, then the bottom of the flow cytometer was pressed to start the measurement; after the measurement had stopped automatically, another sample tube containing a decontamination solution was plugged into sample port. When the cleaning procedure had stopped automatically the process was repeated with 1.6ml of sheath fluid in order to remove residual cleaning solution.

2.3.4 Quality Control (Count Check Beads Green)

A sample tube with well mixed 850µl count check beads green was plugged into the sample port and the start button pressed to begin measurement. When the measurement and cleaning procedure have stopped automatically, the result is indicated at the result area on the screen. This was compared with the lot specific number and check if it was within the allowed 10% range.

2.3.5 Sample Preparation for Absolute CD4 Count (Wet)

20 µL of antibody m Ab PE was pipetted into Partec tube. 20 µL of whole blood was then added into the tube containing the antibody. This was mixed gently and incubated at 15 minutes in a dark field. Then 800µL of no lyse buffer solution was added and analyzed with the C.Y flow counter. For the analysis, the script for CD4 measurement was loaded. Then sample tube with the prepared blood sample was inserted into the machine. Before measurement, the gain value and gating for proper CD4 T-cell measurement is selected. Then the measurement started. After the measurement, the machine cleans automatically. The CD4 Count result is then displayed on the screen, read, printed out and recorded.

2.4 Reproductive Hormones Analysis

The six reproductive hormones (Follicle Stimulating hormone, Luteinizing hormone, Prolactin, Progesterone, Testosterone and Estradiol) were analysed with I-Chroma™ Reader made by Boditech Med Inc.

2.4.1 Procedures

a. *Luteinizing hormone*: 150*u*L of serum was transferred to the detection buffer. The tube was covered and then mixed by shaking about 10 times. 75uL of the mixture was then loaded into the sample well on the cartridge, incubated at room temperature for 15 minutes, and scanned using the I-Chroma scanner. The results were displayed on the screen, read and recorded.
b. *Follicle Stimulating hormone*: Same as in the Luteinizing hormone.
c. *Prolactin*: 150uL of diluent was added to the granules on the sample tube, then 75uL of the blood sample was added to the sample tube, incubated at room temperature for 10 minutes, and scanned using the I-Chroma scanner. The results were displayed on the screen of the scanner, read and recorded.
d. *Progesterone*: 150uL diluent was added to the granules on the sample tube, then 30uL of the blood sample was added to the sample tube, mixed by shaking for about 10 minutes. 75uL of the mixture was pipetted into the sample well on the cartridge, incubated at room temperature for 15 minutes, then scanned using the I-Chroma scanner. The results were displayed on the screen, red and recorded.
e. *Testosterone*: 30uL displacing reagent was added to a tube. 75uL of the blood sample was added to the

displacing reagent, mixed and incubated for 3minutes. 75uL of to the mixture was transferred to the detection buffer tube. Mixed again. 75uL of this mixture was put into the sample well on the cartridge, incubated the loaded cartridge at room temperature for 12 minutes, then scanned on the I-Chroma scanner. The results were displayed on the I-Chroma scanner screen, read and recorded.

2.5 Statistics

The results were analysed using Paired Students t-test at 5% (0.05) alpha or significant level; and p value of test statistics used for confirmation.

3. Results

Table 1. Impact of CD4 Cells on the Reproductive Hormones

Parameters	**RR**	**AM 1**	**AM 2**	**AM 3**	**AM 4**	**AM 5**	**AM 6**	**AM 7**	**Pairs**	**P-value**	**Sig?**
CD4	500-1500	198	284	375	490	157	353	341			
FSH (mIU/ml)		5.26	5.62	4.35	8.41	7.25	3.53	5.62	CD4-FSH	0.0003	**Yes**
LH (mIU/ml)		7.52	6.19	2.67	11.87	8.13	2.53	7.36	CD4-LH	0.0004	**Yes**
PRL	5-25	19.93	16.05	7.04	8.14	16.72	10.5	32.55	CD4-Prl	0.0004	**Yes**
(n/mL)											
EZ (pg/ml		71.5	0	0	0	47.15	0	0	CD4-Ez	0.0003	**Yes**
PROG (nmol/L)		0.46	0	7.91	0	16.37	0	12.48	CD4-Prog	0.0003	**Yes**
TESTO (ng/mL)	0-8	0	4.19	0	4.38	0	6.22	0	CD4-Testo	0.0003	**Yes**

Key: FSH = Follicles Stimulating Hormone; LH = Luteinizing hormone; PRL = Prolactin; EZ = Estradiol; PROG. = Progesterone; TESTO = Testosterone; CD4 = Cluster of Differentiation; AM 1 – AM 7 = Patients; RR = Reference Range.

Table 1 showed that all the patients have low CD4 counts (Range: 157- 490). Our hypothesis ($_{Ho1}$) was rejected: (i.e., Yes) that CD4 cells have impact on the reproductive hormones; p values confirmed negative correlative association between CD4 and the six hormones.

Table 2. Impact of Primary Immune Cells on the Reproductive Hormones

PARAMETERS	**FSH**	**LH**	**PRL**	**EZ**	**PROG**	**TESTO**
WBC (T)	No	No	**Yes**	No	No	**Yes**
N	**Yes**	**Yes**	**Yes**	No	**Yes**	**Yes**
L	No	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
M	**Yes**	**Yes**	**Yes**	No	No	No
E	**Yes**	**Yes**	**Yes**	No	No	No
B	**Yes**	**Yes**	**Yes**	No	No	No

Key: FSH = Follicles Stimulating Hormone; LH = Luteinizing hormone; PRL = Prolactin; EZ = Estradiol; PROG. = Progesterone; TESTO = Testosterone; CD4 = Cluster of Differentiation; WBC (T) = White Blood Cells (Total Count); N = Neutrophil; L = Lymphocytes; M = Monocytes; E = Eosinophils; B = Basophils.

Table 2 showed that our hypotheses was rejected: (i.e., Yes), PIC have impact on the reproductive hormones. Table 2 also showed that all the PIC have impact on PRL. Except on EZ. Neutrophil (N) has very significant impact on all the hormones. Except on FSH. Lymphocytes (L) has very significant impact on all the hormones. Only on PRL that all the PIC have impact. Except on PRL & TESTO, WBC (T) has no significant impact on the hormones. Only N & L have significant impact on the PROG. Only WBC (T), N & L have impact on TESTO. Except L none of the PIC has impact on the EZ. Monocyte, Eosinophil & Basophils has impact only on FSH, LH & PLT.

Table 3. Mean of the % population of Primary immune cells (Total and Differential) in the7Nos specimens of the infertile patients from a teaching hospital in Enugu, Nigeria

Parameters/Specimens	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total**	**Mean**	**R/R.**
N (%)	**31**	**46**	**39**	**39**	**39**	**47**	73	314	**44.9**	50 - 70
E (%)	0	0	1	0	1	1	2	5	0.7	0.5-5.0
B (%)	0	0	0	0	0	0	0	0	0	0.0-1.0
L (%)	**67**	**51**	**58**	**60**	**58**	**49**	22	365	**52**	20-40
M (%)	2	3	2	**1**	2	3	3	16	2.3	2-12
WBC (T) x 10^9/L	**3.82**	4.01	4.32	4.85	4.36	6.30	7.55	35.21	5.03	4-10

Key: N = Neutrophil; L = Lymphocytes; M = Monocytes; E = Eosinophils; B = Basophils; WBC (T) = White blood cells (Total); 1 – 7 = Specimens; R/R. = Reference Range.

Table 3 showed that the neutrophil counts were *below* the reference range in the Mean and in all the patients, except Patient No. 7 which was above the reference range. Table 3 also showed that the lymphocyte counts were *above* the reference range in the Mean, and in all the patients, except Patient No. 7 which is within the reference range. WBC (T) were all within the reference range except in Patient number 1.

Table 4. Impacts of the haemopoietic factors on the six reproductive hormones of the infertile patients from a teaching hospital in Enugu, Nigeria

Pairs/Significance (P value < 0.05)	**FSH**	**LH**	**PRL**	**EZ**	**PROG**	**TESTO**
C (x 10^{12}/L)	No	No	**YES**	No	No	No
Hb (g/dL)	**YES**	**YES**	No	No	No	**YES**
PCV (%)	**YES**	**YES**	**YES**	No	**YES**	**YES**
MCV (FL)	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
MCH (pg)	**YES**	**YES**	**YES**	No	**YES**	**YES**
MCHC (g/dL)	**YES**	**YES**	**YES**	No	**YES**	**YES**
PLT (x 10^9/L)	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**

Key: RBC = Red blood cells; Hb = Haemoglobin; PCV = Packed cells volume; MCV = Mean Cell Volume; MCH = Mean Cell Haemoglobin; MCHC = Mean Cell Haemoglobin Concentration; PLT = Platelets; R/R. = Reference range; Follicles Stimulating Hormone; LH = Luteinizing hormone; PRL = Prolactin; EZ = Estradiol; PROG. = Progesterone; TESTO = Testosterone; CD4 = Cluster of Differentiation.

Table 4 showed that two of the haemopoietic factors, MCV and platelet have statistically significant impact ($p < 0.05$) on all the six reproductive hormones. PCV, MCH and MCHC that has statistically significant impact on all the reproductive hormones except on Estradiol. Red blood cells have statistical significance impact only on prolactin. HB that has impact on only three: Follicles Stimulating Hormone, Luteinizing hormone and testosterone.

Table 5. Mean of the Haemopoietic factors in the7Nos specimens of the infertile patients from a teaching hospital in Enugu, Nigeria

Parameter/Specimens	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total**	**Mean**	**R/R**
RBC (x 10^{12}/L)	4.37	4.42	4.51	**3.18**	5.09	4.31	4.11	29.99	4.82	**3.5-5.5**
Hb (g/dL)	11.9	12.3	12.1	**7.1**	14.4	11.5	11.2	80.5	11.5	**11-16**
PCV (%)	**36**	37	37	**22**	43	**35**	**34**	244	**34.86**	**37-54**
MCV (FL)	82.4	83.7	82.0	**69.2**	84.5	81.2	82.7	565.7	80.81	**80-100**
MCH (pg)	27.2	27.8	**26.8**	**22.3**	**26.7**	27.3	27.6	157.6	**22.51**	**27-34**
MCHC (g/dL)	33.1	33.2	32.7	32.3	33.5	32.9	32.9	230.6	32.94	**32-36**

PLT (x 10^9/L)	174	285	220	253	168	425	441	1713.25	244.75	**100-300**

Key: RBC = Red blood cells; Hb = Haemoglobin; PCV = Packed cells volume; MCV = Mean Cell Volume; MCH = Mean Cell Haemoglobin; MCHC = Mean Cell Haemoglobin Concentration; PLT = Platelets; R/R. = Reference range; 1-7 = Patients samples.

Table 5 showed that PCV and MCH fell below the reference range; all the other haemopoietic factors were within the reference range.

4. Discussion

Role of immunology in fertility is not well-known by all medicos; to date, many still concentrate only on reproductive hormones to investigate infertility (Robertson, *et al.,* 2018). This may end up in enigma, especially in cases of persistent infertility in situations of apparent adequate hormonal balances, as was seen in this work (Table 1). This is as a result of their basal knowledge in immunology, immunopathology, immunotherapy and immunodiagnosis/immunochemistry or immunoassay.

Regulatory T cells or Treg cells (i.e., $CD4^+$ $CD25^+$), have recently been implicated in human pregnancy as key players in protecting the conceptus from alloreactive immune rejection (Robertson, *et al.,* 2018). An increase in circulating Treg cells is evident in pregnancy from the first trimester until shortly after delivery (Ciraci1 *et al.,* 2019). From the low CD4 counts obtained in this work (Table 1) and compared with the adequate hormonal balances of all the patients, the cause of infertility is obviously immunologic infertility. Besides, our hypotheses were largely rejected: (i.e., Yes) that CD4 cells ($_{Ho1}$) and PIC ($_{Ho2}$) have impact on the hormones (Tables 1 and 2); and p values confirmed negative correlative association between CD4 & the six hormones (Table 1).

Regulatory T cells are specialized subpopulation of T-lymphocytes that act to suppress immune response, thereby maintaining homeostasis and self-tolerance. Further, it has been shown that T Reg are able to inhibit T cell proliferation and cytokine production as well as play critical role in preventing autoimmunity. That is the same way it creates an active state of maternal immune tolerance (e.g., of spermatozoa and foetus), thereby ensure a robust placenta and sustain pregnancy.

Thus, pregnancy is immunologically re-defined as a special, exceptional situation in a woman's body, as it is forced to be home for a "foreign body" for 9 months (Paraiso, et al, 2022). This is against the formal definition as a failure to conceive after a year of unprotected intercourse with the same partner.

Dysregulation in T Reg cell frequency [in number, as was seen in this work (Table 1)] and consequently in functions may lead to the development of autoimmunity, as well as immune imbalance or tolerance between the implantation mother and spermatozoa/foetus or embryo, as was perceived in this work — result of which would be maternal intolerance of foetus/spermatozoa as cause of inability to conceive. This situation is referred to as "immunologic infertility."

Hence, besides hormonal imbalance, immunologic infertility greatly contributes to inability to conceive. Consequently, CD4 cells count (by extension as T Reg, CD25 inclusive) should be a paradigm in the investigation of infertility.

Immunological infertility is a bit under-defined by Dondero, *et al* (1993, 2011) as the presence, in one or both partners, of an anti-sperm immune reaction capable of interfering with fertility variables; because other immunological situations of infertility exist. Indeed, according to Shibahara, (2022) and Tung, *et al.,* (2017), a significant number of infertile men show an autoimmunity to sperm, and that experiments have suggested that anti-sperm antibodies (ASA) can interfere with the fertilizing ability of spermatozoa.

ASA can act negatively on the motility of spermatozoa in semen, on their ability to pass through female genital secretions, or on the penetration of the oocyte. In particular, owing to *in vitro* fertilization techniques, it has been possible to demonstrate the effects of antibody-bound sperm directly, at the level of *in vitro* gamete interaction (Shibahara, 2022; McLachlan, 2002). Among factors responsible for this form of autoimmunity is the situation referred to as "expression of sequestered or occult or hidden antigens," (such as the antigens of the testis, eyeballs, ovary, etc.,) due to when there is break up of their protected tissue-blood barrier, are thereafter exposed to the body's immune cells which consequently starts destroying them. Infections and trauma are one the major causes of such break in tissue-blood barrier, for which infertility is the penalty.

The noted percent rise in lymphocyte in this work (Table 3) is that they must have come from other conventional lymphocytes (B-cell, Nk cells and other classes of CD cells) and not from the required $CD4^+$ and $CD25^+$ necessary to maintain immune tolerance to sustain implantation in the mothers; and the deficient CD4 detected from all the patients agreed with the assertion. The neutrophil low count agreed with our rejected Ho_2 hypotheses (i.e. Yes), PIC have impact on the reproductive hormones (Tables 2 and 3), and the P value further confirmed the negative correlative association between PIC and the six reproductive hormones (Table 3).

Our Ho_3 hypothesis was also largely rejected: (i.e. Yes), the haemopoietic factors has impact on the reproductive hormones. Two of the haemopoietic factors, MCV and platelet have statistically significant impact ($p < 0.05$) on all the six reproductive hormones (Table 4). According to National Institutes of Health (.gov) (a) capacity of platelets (or thrombocytes) to participate in innate immunity is largely due to their ability to release a myriad of inflammatory and bioactive molecules stored within granules or synthesized upon activation; these mediators attract and modulate the effector cells of the innate immune system. They also contribute to atherothrombosis (National Institutes of Health (.gov)); (b) if platelet count is too high, blood clots can form in the blood vessels and this can block blood flow through the body (National Institutes of Health (.gov)) (2022); our finding (Table 5), did not show any impairment of the immune system through thrombocytopenia nor any thrombocytosis to indicate a role in pregnancy. The statistically significant impact, however, is without doubt.

The only correlation to haemoglobin disorder in our work is fall in PCV and MCH below the reference range, but what role that played in infertility cannot offhand be discerned. And PCV, MCH and MCHC that has statistically significant impact on all the reproductive hormones except Estradiol. Red blood cells have statistical significance impact only on prolactin; reason for that cannot be explained. Corollary too to limited significance impact to the reproductive hormones is HB that has impact on only three: Follicles Stimulating Hormone, Luteinizing hormone and testosterone.

5. Conclusion

In conclusion, under no hormonal abnormality, this work strongly suggests that the inability to conceive resides on the low CD4 cells (and its consequent deficient immune regulation), plus statistically significant impacts of it and PIC on hormones; hence, they are veritable factors. Roles of other haemopoietic factors, such as PCV, MCH and MCHC that has statistically significant impact on all the reproductive hormones except Estradiol needs further analyses. CD4 cells counts (and primary immune cells counts) therefore should be a paradigm in investigations of infertility.

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Competing Interest

The authors declare no competing or conflicting interest whatsoever in this research.

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