Role of endometrial cytokines of the female genital tract tuberculosis in the context of infertility

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Abstract:

BACKGROUND: Nowadays, one of the most common vulnerable sites of extrapulmonary tuberculosis is female genital tract tuberculosis (FGTB) leading to infertility. As FGTB produce clinical symptom quite late, its detection is very difficult to the health-care providers and according to some experts in this field; FGTB has no confirmatory investigation procedure. FGTB can cause other form of reproductive failure through ectopic pregnancy, tubal block, and implantation failure. Their presence may alter the cytokines level in endometrium.

METHODS: A total of 300 cases in our clinic had undergone polymerase chain reaction (PCR) for detection of tubercle bacillus (TB-PCR). Among of them, 91 individuals fulfilling all inclusion and exclusion criteria were included in this study. In our study, we measured cytokines, two from pro-inflammatory group (interleukin-6 [IL-6] and IL-10) and three from inflammatory group (IL-2, interferon gamma [IFNγ], and tumor necrosis factor alpha [TNFα]).

RESULTS: Out of 91 participants, 45 (49.46%) cases were TB-PCR positive, and 46 (50.55%) cases were TB-PCR negative. It has been observed that the value of IL-10 and TNFα did not fit any statistical parameter. The result showed that pro-inflammatory indicators IL-6 and inflammatory indicators IL-2 and IFNγ had significant different values between TB-PCR-positive and TB-PCR-negative groups. P value of this cytokines has statistically significant.

CONCLUSION: From our study, we conclude that cytokine study was undertaken, of which IFNγ showed a possibility to become an important clinical indicator of endometrial hostility followed by IL-2.

Keywords: Cytokine, tubercular infestation, tuberculosis-polymerase chain reaction, unexplained infertility

Female genital tuberculosis (FGTB) remains undetected for a long time because of its silent nature and lack of confirmatory diagnostic parameter. The average global incidence of FGTB in infertility clinics is 5%-10% (from 0.69% in Australia to 17.4% in India[1] and 9.6% in our clinic.[2] FGTB may damage fallopian tubes minimally leading to ectopic pregnancy or damage of endometrium severely leading to sterility.[3] Extrapulmonary tuberculosis (EPTB) is gradually becoming prevalent nowadays.[4] Tubercle bacilli may remain latent or asymptomatic for several years with low virulence.[5] Mere presence of this bacteria, otherwise called tubercular infestation, may lead to reproductive failure by altering the immunoochemical environment.[2] From literature, we got only specific immune mechanism effective is cell-mediated type. When any type of bacterial infection occurs, humoral immunity appears to be irrelevant. The key cell is activated CD4+ helper T cell, which can develop along with two different paths that are TH1 and TH2 cells. If immunomodulatory response is favorable, helpful cytokines, and growth factors remain latent or asymptomatic for several years with low virulence. Mere presence of this bacteria, otherwise called tubercular infestation, may lead to reproductive failure by altering the immunoochemical environment. From literature, we got only specific immune mechanism effective is cell-mediated type. When any type of bacterial infection occurs, humoral immunity appears to be irrelevant. The key cell is activated CD4+ helper T cell, which can develop along with two different paths that are TH1 and TH2 cells. If immunomodulatory response is favorable, helpful cytokines, and growth factors...
will appear, and successful implantation takes place. Immunologically, this is known as TH2 response. Release of harmful, inflammatory cytokines such as interleukin (IL-2), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα) may lead to implantation failure or recurrent miscarriage, if the host tissue fails to resist this trauma. This sort of local inflammatory impact if untreated, and sometimes, due to excellent host resistance, may pass off, leading to curing of the condition. The paucibacillary form of the disease, where culture and smear are always negative, the primary focus is rarely found outside the genital tract. Routine screening tests which are used clinically, are usually negative. Hence, they are detected by polymerase chain reaction (PCR). In selected patients, cytokines, some from pro-inflammatory and some from anti-inflammatory groups, were studied in our laboratory to clarify this view.

**Materials and Methods**

A total of 300 patients had undergone PCR for the detection of tubercle bacillus (TB) (TB-PCR study) over a period of 9 months, during the last quarter of 2014 and third quarter of 2015. Within 1 week from the day of collection, sample processing and analysis (both PCR and cytokine) have been done. However, the statistical analysis took a long time and report was available in the end of 2016. We again started this work from the end of 2016 and till now the work is continued. Among them, 91 participants fulfilling all inclusion and exclusion criteria were included in the study. Out of 91 participants, 45 (49.46%) cases were TB-PCR positive (+ve) and 46 (50.55%) cases were TB-PCR negative (-ve).

**Selection criteria**

Human endometrial aspirates and menstrual blood of participants (2d day of cycle) were collected aseptically from infertile healthy woman belonging to unexplained infertile group (age group 22–40 years, i.e. reproductive age group), and their cycle lengths were within normal range (27–29 days). Only unexplained infertile woman having normal ovulation cycle, normal sperm count of counterpart, normal hormonal profile, normal tubal status and function, and normal body mass index (BMI) were considered for the study. A specific written consent was designed according to the Ethical Guidelines of Helsinki declaration, 1975 and approved by the Institutional Ethics Committee, were obtained from the selected participants after duly signed by each study participant.

**Inclusion criteria**

The women with unexplained infertility and sexually active were considered for this study. The complete clinical examination and investigation of all infertile participants were carried out. The female patients had normal functioning fallopian tubes which would be confirmed by hysterosalpingography, and if required, diagnostic laparoscopy, and dye test were performed. Normal ovulatory function and absence of any local and systemic bacterial infection have been confirmed and also the couples have been trying to conceive for at least last 1 year. Thus, the infertility investigation had not revealed any cause of the infertility at this stage.

**Exclusion criteria**

Individuals with any previous history of pulmonary/ extrapulmonary tuberculosis (TB) infection or taking antitubercular drug (ATD) would be excluded from this study.

Any case suffered from ovulatory diseases was excluded from the study. Cases with tubal defects, endometriosis, recurrent spontaneous abortions, pelvic inflammatory diseases, pelvic adhesion, and any endocrinological, anatomical, or immunological defect were excluded from this study.

**Collection of sample for polymerase chain reactions**

DNA study– The participants were advised to attend the clinic on the 2d day of menstrual cycle due to heavy blood flow. In some of the cases, endometrial aspirates were taken between 21st and 23rd day of the same cycle (mid-luteal phase), by the curetting the endometrial wall using cannula with or without anesthesia. After collecting menstrual blood along with endometrial tissue, the contents were transferred to the lysis buffer (50 mmol/l KCl, 10 mmol/l’TrisHCl pH 8.3, 1.5 mmol/l MgCl2, 0.1% Nonidet P-40, 0.5% Tween-20) for DNA extraction followed by multiplex PCR. After DNA extraction, within 1–2 days, PCR study was done. Due to short-time period, DNA was stored in the −20°C. Following PCR, DNA samples, as per standard protocol, were preserved at −80°C for long time for future use.

In cytokine study, its levels were checked in TB-PCR-positive and TB-PCR-negative groups, where only DNA PCR was performed. As the focus of our present investigation was to check the correlation between endometrial cytokine levels in both TB-PCR positive and TB-PCR negative groups, only DNA PCR study was undertaken. In future, we will consider TB-PCR for mRNA expression.

**Cytokine study**

Those participants, who were already enlisted in this study, requested to attend the clinic on the 22nd or 23rd day from the starting day of menstrual cycle. For collection the endometrial sample, an autoclaved speculum was placed on the vagina for visualizing external os of
cervices, without touching the vaginal wall or vulva, sterile cannula was allowed pass through the internal os and aspirate the small piece of the endometrial tissue by suction, transferred to the sterile cryovial containing the 1 ml normal saline solution and placed as soon as possible to the −20°C for a maximum 1 week. Within 1 week, on the day of cytokine extraction, at first, samples were transferred to the 4°C to avoid the heat shock, after some time samples were allowed to attain the room temperature

**DNA extraction**

DNA was extracted from selected endometrial tissues or menstrual blood samples using QIAamp Viral DNA Mini Kit (Qiagen, Hilden, Germany) and eluted in 200 µl of elution buffer and was stored at −20°C for further study within 1-2 days.

**Multiplex polymerase chain reaction**

PCR was performed after genomic DNA extraction. Multiplex PCR was carried out with three sets of primers (a) 1st set of primers expressing 165BP of a portion of a gene encoding for kDa antigen; (b) 2nd set of primers required for expression of 365BP portion of DNA gene; and (c) 3rd set of primers expressing 541BP region of insertion sequence, IS6110. Master Mix and condition of multiplex PCR were done accordingly, to follow our previous paper.[2,10]

From the obtained PCR products, 15 to 16 µl of each product were run on 2% agarose gel stained with ethidium bromide and visualized under Gel Documentation System (Bio-Rad, USA). The length of the PCR products was estimated by pGEM or ΦX174/Hae markers (Promega Corporation, Madison, Wisconsin, USA).

**Cytokine extraction**

At first for endometrial samples, tissues were diced into pieces by the scalpel, cleaned, with ethanol after trimming each samples. Each trimming samples and menstrual blood were transferred to the new Eppendorff microcentrifuged tube and allowed to pour 200 micro liters ice cold HEPES extraction buffer and samples were incubated for ½ h at 4°C. After incubation of samples, containing Eppendorffs were centrifuged at 13, 000 r. p. m for 10 min at 4°C. Then, elutes were tested for hemoglobin levels using Hemastix strips and then tested immediately for check cytokine level by ELISA method.

**Cytokine study**

It is well known that there are multiple cytokines which help in implantation of embryos in the endometrium, and many other inflammatory cytokines which prevent implantation. All these cytokines are produced by T-helper (Th) lymphocytes and pro-inflammation group called Th-2 cytokines whereas inflammatory cytokines preventing implantation are called Th-1 cytokines. In our cytokine study, we selected two cytokines from pro-implantation group—IL-10 and IL-6, and three cytokines from inflammatory group—IL-2, TNFα, and IFNγ. All these cytokines (IL-2, IL-6, IL-10, TNFα, and IFNγ) estimation were performed by a solid phase sandwich enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody specific for the particular cytokine assayed has been coated. Cytokines were estimated by a solid phase sandwich ELISA procedure, using the kit from Diaclone SAS, France. The wells of the microtiter plate provided for the assay purpose was coated with a monoclonal antibody specific for the particular cytokine assayed. Samples, including standards of known cytokine concentration control specimens and unknowns, were taken into these wells along with a biotinylated monoclonal antibody specific for the cytokine being estimated, and then the strips were incubated for ½ h at room temperature (10°C–25°C). After the incubation period was over, the contents of each well were discarded, and wells were washed thoroughly, dried, and then streptavidin-peroxidase enzyme was added and the wells incubated for ½ h at room temperature. Following second incubation, also, the wells were thoroughly washed to discard any unbound enzyme. Next, a substrate solution tetramethylbenzidine that would act as bound enzyme was added to each well to induce the formation of a colored reaction product. The reaction was allowed to take place in dark at room temperature for 15 min, following which sulfuric acid (H2SO4) was added as stop reagent to stop the reaction. As the intensity of the color developed was directly proportional to the concentration of the particular cytokine being assayed in the samples, the absorbance of each well was read using Monobind Acculite ELISA reader (Eldex 3.8) against a 450 and 630 mm filter, immediately after stopping after stopping the reaction.

Cytokine minimal detectable dose:
- IL-2 < 7.0 pg/ml
- IL-6 < 2.0 pg/ml
- IL-10 < 5.0 pg/ml
- IFNγ <5.0 pg/ml
- TNFα 8.0 pg/ml

Gene-Probe, Diaclone SAS, France.

**Statistical analysis**

The data obtained from each sample group would be expressed as mean ± standard deviation (SD). The histograms of different inflammatory cytokines suggested that none of the indicators follow normal distribution. Hence, we used the nonparametric test to do find the differences between population averages of two groups. The Mann–Whitney test was performed to find the difference in median of the subsequent two groups. \( P \leq 0.05 \) was used as the criterion for statistical
significance. All the statistical analysis was performed using Statistical Package for Social Sciences (SPSS) program (Version 16.0, SPSS, Inc, Chicago, IL, USA).

Results

Out of 91 participants, 45 (49.46%) cases were TB-PCR-positive and 46 (50.55%) cases were TB-PCR-negative. The range, mean, median, and SD along with maximum and minimum values of all cytokines studied were tabulated. It has been observed that the value of IL-10 and TNFα did not fit in any statistical parameter, instead showed bizarre pattern. Hence, these two cytokines were excluded from the study. The remaining cytokines IL-6 (Th-2 group) and IL-2 and IFNγ (Th-1 group) could be tabulated and comparison was made between TB-PCR-positive and TB-PCR-negative groups according to these cytokine levels [Table 1]. In Table 2, P values were calculated and they showed significant results when positive and negative groups of TB-PCR were considered.

It was observed that as compared to TB-PCR negative group [Figure 1], the mean and median values of cytokines (IL-2, IL-6, and IFNγ) were higher in TB-PCR positive group [Figure 2]. The result showed that all the selected pro-inflammatory indicators (IL-6)\(^{[11]}\) and anti-inflammatory indicators (IL2 and IFNγ)\(^{[12]}\) had significant different values of median (\(P \leq 0.05\)) between TB-PCR-positive and TB-PCR-negative [Figure 3].

The bar chart [Figure 3] shows the median of each cytokine against TBPCR status. IL-6 has higher median value when TBPCR negative whereas IL-2 and IFNγ have higher median values when TBPCR positive. The correlation graphs [Figure 4] of cytokines indicate a significant relationship between the IL-6 and IL2 with TB-PCR status.

Discussion

The indication for treating asymptomatic subclinical tubercular involvement or otherwise called tubercular infestation is not yet standardized. Many authorities do not accept positive TB-PCR as a standard parameter for diagnosing tubercular involvement and depending on that they deny to initiate any antitubercular treatment, as false positivity of TB-PCR rate is high. It is to be mentioned here with strict asepsis and multiple PCR testing, as it is performed here with more than one set of primer either from menstrual blood or endometrial sampling, the error can be avoided. Any insidious, low-grade inflammation is quite likely to alter the function of Fallopian tubes, uterus, and of the endometrium, as it happens in *Mycobacterium tuberculosis* (MTB) infestation, which leads to subtle damage and functional loss of the uterus and the tubes. Molecular mechanisms constitute the important causes of implantation failure in gynecologic diseases.\(^{[13]}\) Such mechanisms, however, have not been studied or reported in the case of GTB. Mycobacterial presence may also alter endometrial receptivity and cause implantation failure through mechanisms such as disturbed immunomodulation and cytokine overburden, endocrine disruption, activation of antiphospholipid antibodies, and micro thrombosis without the presence of an obstetric complication.
of overt clinical disease.\(^{[14-17]}\) Latent TB was also shown to be responsible for unexplained infertility and repeated IVF failures in women with apparently normal pelvic and non-endometrial tubal factors.\(^{[18]}\) There are multiple studies in literature which have highlighted a very high diagnostic specificity, sensitivity, and clinical correlation of TB-PCR with GTB.\(^{[19-22]}\) Even in normal pelvis detected by laparoscopy, tubercular infestation otherwise called latent TB detected by positive TB-PCR study, instituting antitubercular treatment improves pregnancy outcome. Two other studies also support this information.\(^{[23,24]}\)

To solve this dilemma, we have studied the concentration of cytokine in endometrium, which might influence reproductive performance. We studied five cytokines to start with– IL-2, IL-10, IL-6, TNF\(_{\alpha}\), and IFN\(_{\gamma}\). Of all these, three cytokines showed reproducible results with statistically significant differences in positive and negative TB-PCR cases. The inflammatory cytokines such as IFN\(_{\gamma}\) and IL-2 are significantly high in TB-PCR-positive cases indicating hostility of endometrium, possibly leading to implantation failure. Many of the patients showed prolonged luteal phase followed by onset of period in ovulatory cycles, detected by ultrasonography-guided ovulation monitoring. It is observed that IL-6, which is a Th-2 cytokine helps in implantation, is also high in TB-PCR-positive cases. This is possibly because of the body environment trying to induce favorable endometrium for implantation, which is an independent of endometrial inflammation and possibly initiates implantation process, but subsequently due to harmful cytokines IL-2 and IFN\(_{\gamma}\) implanting embryos are rejected by the endometrial wall, leading to implantation failure, causing so-called unexplained infertility. The other two cytokines IL-10 and TNF\(_{\alpha}\) show bizarre concentration between positive and negative TB-PCR cases and could not be corroborated statistically.

It is well recognized that reproductive failure may happen due to endometrial hostility. In assisted reproductive program me (ART) ,amount of administrated to retrieve more eggs which can affect not only follicular environment but also the endometrial behavior. This may result in increased implantation failure in ART program me. It is difficult to standardize any parameter to detect that. It is also difficult to estimate the endometrial cytokines in day-to-day practice. What appears from the present study is that IFN\(_{\gamma}\) may come out as an important indicator of endometrial hostility. There is an established relation between interferon-gamma and TB, as evidenced in literature review.\(^{[24]}\) This interferon gamma can be detected in blood when the question of generalized infection comes. In case of infestation, as we suggest, IFN\(_{\gamma}\) can also become an indicator of local presence of MTB and their harmful influences. We believe that any form of MTB bacillus may influence the endometrial environment by mere presence and manipulate cytokine release.

**Conclusion**

The ill effect of MTB infection on reproductive function is long-established when it comes from the tubercular infection. Our finding in the present study suggests that mere presence of mycobacterium colonization in endometrial or tubal wall surface, what we mention as tubercular infestation, may cause reproductive failure as
well. The most offensive cytokine for pregnancy wastage is TNF-α.\textsuperscript{[25]} This was a pilot study. In this study, we got the prevalence of IFN-γ and not TNF-α, as measurement of interferon gamma in serum in case of PTB or EPTB infection is one of the predictive parameters.\textsuperscript{[26]} The findings of increased prevalence of IFN-γ in endometrial aspirate in TB-PCR-positive cases probably corroborate the above finding from serum to determine that cytokine studies was undertaken of which IFNγ showed a possibility to become an important clinical indicator of endometrial hostility followed by IL2. Treating this infestation by ATD improved reproductive outcome indicating the ill effect is reversible, as in contrast to tubercular infection, were the destructive nature of this disease leads to irreversible infertility in many cases. The diagnosis of infestation is made mostly by multiplex TB-PCR using multiple primer sets. The sample size of this study is relatively small, and further study with large samples size are to be undertaken to confirm the findings.

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Conflicts of interest

There are no conflicts of interest.

References

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