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Original Article

Effect of leucocytospermia on seminal parameters of human male infertile subjects

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ABSTRACT

Introduction: Leucocytospermia is recently recognized factor of male infertility. Leucocytes when exceeds the threshold of 1 x106/ml of semen contribute significantly to production of ROS (reactive oxygen species) and thereby cause sperm damage leading to male infertility. Aims : To study the effect of leucocytospermia on seminal parameters. Methods: The study was conducted at the Reproductive Biology Unit in the Department. of Physiology, Mahatma Gandhi Institute of Medical Sciences Sevagram; Wardha. Routine semen analysis was done by SQA II C-P (Sperm Quality Analyser) [Medical Electronic System Ltd. Israel]. The presence of leukocyte concentration in 140 semen samples were assessed by myeloperoxidase staining technique [Endtz test] and samples were classified as leucocytospermic [≥ 1x106/ml] (L group) and nonleucocytospermic [<1x106/ml] (NL group). Nitroblue Tetrazolium (NBT) Reduction Test was used to detect ROS generation in leucocytes and spermatozoa. Results: Out of 140 semen samples 20 samples were leukocytospermic and 120 were nonleukocytospermic. Mean count of percentage of NBT positive leukocyte was more in leukocytospermic group (16.20 ± 6.51) than nonleukocytospermic group (12.21 ± 6.10) and was found to be statistically significant. In leukocytospermic group, we obtained significant negative correlation between percentage of NBT positive lekcocytes and sperm count ($r = -0.49$, $p < 0.05$), % sperm motility ($r = -0.51$, $p < 0.05$) and % morphologically normal spermatozoa ($r = -0.45$, $p < 0.05$) respectively. In nonleucocytospermic group, the coefficient of correlation between percentage of NBT positive leucocyte and sperm count ($r = -0.16$, $p > 0.05$), % sperm motility ($r = -0.16$, $p > 0.05$) & % morphologically normal spermatozoa ($r = -0.14$, $p > 0.05$) were not significant. Conclusion: Leukocytospermia adversely affect the seminogram parameters leading to male infertility.

1. Introduction

The impact of seminal leucocytes on sperm parameters and sperm dysfunction is controversial. Some investigators have reported a negative impact of leucocytospermia on semen parameters[1,2,3] and some have concluded that leucocytes may play possible role in male infertility[4] while still others suggested leucocytes and leucocyte subpopulation in semen are not a cause of male infertility[5,6,7]. From the most recent literature it is now clear that the main negative effect of leucocytospermia is the production of Reactive Oxygen Sepcies (ROS) causing DNA Aim: To study the effect of leucocytospermia on seminal parameters in male infertile subjects. fragmentation and damage of spermatozoa [7]. According to World Health Organization (WHO), leukocytospermia is defined as leucocytes concentration more than one million per ml of semen [8]. Activated leukocytes are capable of producing 100-fold higher amounts of ROS than non-activated leukocytes [9]. Leukocytes may be activated in response to a variety of stimuli including inflammation and infection [10]. Esfandari N et al, in his study suggested that the NBT test can be used as a simple alternative to ROS-TAC score for assessment of levels of seminal oxidative stress and may have important implication both for the andrology research and clinical practice. It is readily available, easy to perform and has high sensitivity. This

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test is used for assessment of seminal oxidative stress and differential contribution of cells to ROS generation and to determine the activation of seminal leucocytes [11].

2. Materials and Methods:

The study was conducted at the Reproductive Biology Unit in the Dept. of Physiology, MGIMS Sevagram; Wardha. Subjects were referred from the Dept. of Obstretics & Gynaecology. Detailed history of present and past illness as well as medical and surgical treatment was taken. Selected male partners underwent through surgical examination of genito-urinary system to rule out the exclusion criteria. Semen samples were obtained from 140 male patients complaining of infertility (both primary and secondary) of age 20-58 yrs. All the tests were done with due permission of the ethical committee of the Institute and informed written consent was taken from each patient. Semen samples were delivered by masturbation after 3 days of sexual abstinence. After complete liquefaction at room temperature, each sample was tested.

Exclusion criteria: Subjects with varicocoele, hydrocoele, undescended testes or any other structural abnormality or any history of surgical intervention in genitourinary tract which may interfere with male fertility were excluded from the study. Subjects with any acute febrile (>38 0C body temperature) illness or a history of similar episode in last six months or treatment history with drugs like cancer chemotherapy, nitrofurantoin, niridazole, colchicine or any hormonal preparation which may directly suppress the spermatogenesis were excluded from the study. Subjects having severe Oligoasthenoteratozoospermia (<1million/ml) and Azoospermia were also excluded from the study.

Routine Semen Analysis: Routine semen analysis was done by SQA II C-P (Sperm Quality Analyser) [Medical Electronic System Ltd. Israel] for sperm concentration (millions/ml), sperm motility(%), sperm morphology(%) and according to WHO guideline 1992 subjects were categorized as Normozoospermics, Oligoasthenoteratozoospermics [OAT] Asthenoteratozoospemics [AT] and Azoospermics. Semen samples were utilized for further biochemical assessment.

Quantification of seminal leucocytes: The presence of leucocytes concentration in semen were assessed by myeloperoxidase staining technique (Endtz test) [12]. 20µl volume of liquefied semen specimen was placed in a corning 2.0 ml cryogenic vial and 20µl of Phosphate Buffer Saline (pH-7.0) and 40µl of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for five minutes. Peroxidase Positive WBCs staining dark brown were counted in all 100 squares of grid in a Makler's chamber [Sefi-Medical Instrument, Haifa, Israel] under the bright-field objective (magnification, 20X). The results after correction for dilution were recorded as counts x 106/ml Semen samples were classified as leucocytospermic [L] group (≥ 1x106/ml) and nonleucocytospermic [NL] group(<1x106/ml) .

Qualitative lipid peroxidation: ROS generating activity in leucocytes were determined in semen based on their morphological characteristics acquired by deposition of formazan granules in ROS positive cells. NBT test were used to detect ROS generation in seminal ejaculate [11]. NBT staining was done for whole ejaculate by adding equal volumes of 0.1% of NBT solution and incubated for 30 minutes at 37oC. The tubes were centrifuged at 250g for 5 minutes and smears were prepared from the pellet and air-dried. The air dried smears were stained with Wright's stain.

Wright's Stain Procedure: The slide was covered with Wright's stain for 2min, buffered water about double the volume of stain was added, staining was allowed to continue for 5-7min then stained off and smear was washed in a stream of buffered water. After air-drying total of 100 NBT stained leucocytes (Photomicrograph no. 1) and 100 NBT stained spermatozoa (Photomicrograph no. 2) were scored under microscope [100X magnification].

The data obtained was statistically analysed with the help of z test and Pearson's correlation by SPSSver17 software. P value <0.05 was considered significant.

3. Result

This study was conducted on 140 male partners of infertile couples with infertility duration more than 1 year without using any contraceptives. Semen samples were analysed for routine seminogram parameters. 47.85% were normozoospermics, 18.57% were OAT and 33.57% were AT. Out of 140 samples 20 were leucocytospermic(L) & 120 were nonleucocytospermic(NL) [Table no.1].

Table no. 1: Semen samples based on leucocyte count by Endz test

Categories	Group L	Group NL	Total
Normozoospermic	6(30%)	61(50.83%)	67(47.85%)
OAT	4(20%)	22(18.33%)	26(18.57%)
AT	10(50%)	37(30.83%)	47(33.47%)
Total	20	120	140

Table 2 : Semen characteristics in group L & group NL subjects:

Semen characteristics	L (n=20)* Mean ± SD	NL (n=120)* Mean ± SD	p value
Sperm Concentration (millions/ml)	49.85 ± 31.51	61.58 ± 39.68	0.14 (NS
% Motility	39.55 ± 13.33	43.20 ± 16.08	0.28 (NS)
% Normal Morphology	26.75 ± 8.78	29.92 ± 10.38	0.15 (NS)

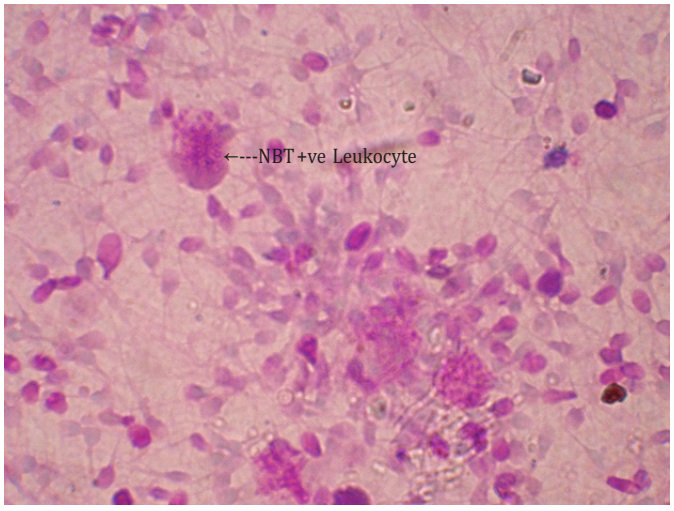
Note: All leucocytospermic samples exhibited NBT positive leucocytes and spermatozoa however all 120 nonleucocytospermic samples exhibited NBT positive spermatozoa and 38 samples exhibited NBT positive leucocytes.

Table 3: Percentage of NBT positive leucocytes and sperms in Group L and Group NL

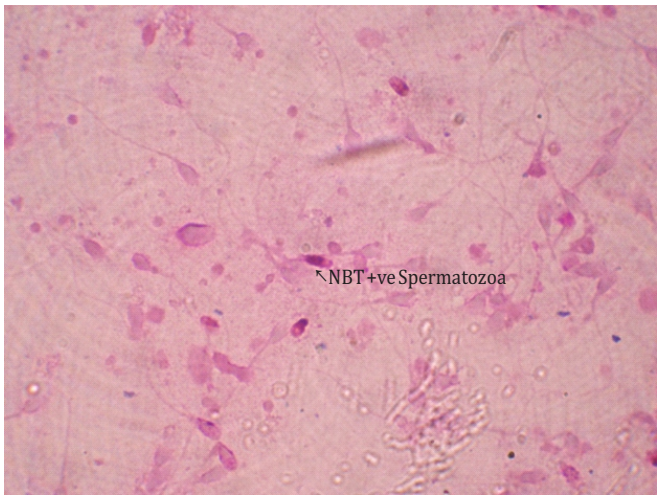
Categories	L(n=20)* Mean ± SD	NL(n=120)* Mean ± SD	p value
% of NBT positive Leucocytes	16.20 ± 6.51	12.21 ± 6.10	0.03 (S)
% of NBT positive Sperms	25.20 ± 10.56	24.30 ± 16.00	0.75 (NS)

Note: Among 120 nonleucocytospermic samples 38 samples had NBT positive leucocytes however sperms in all 120 samples were positive for NBT staining

Photomicrograph No. 1 showing Nitroblue Tertazolium(NBT) positive leucocyte [100X Wright's Stain]



Photomicrograph no. 2 showing Nitroblue Tertazolium(NBT) positive spermatozoa [100X Wright's Stain]



In nonleucocytospermic group, the coefficient of correlation between percentage of NBT positive leucocyte and sperm count (r = -0.16, p > 0.05), % sperm motility (r = -0.16, p > 0.05) & % morphologically normal spermatozoa (r = -0.14, p > 0.05) were not significant which were obtained by excluding NBT negative samples. The coefficient of correlation between percentage of NBT positive sperms and sperm count (r = -0.16, p > 0.05), % sperm motility (r = -0.13, p > 0.05) & % morphologically normal spermatozoa (r = -0.12, p > 0.05) were also nonsignificant.

Discussion:

In our study sperm characteristics was not significantly different between L and NL group. Our finding goes in favour of study by Esfandari N et al [11] however not in favour of Garg V. et al [13]. Esfandari N et al [11] in his study suggested that the NBT test can be used as a simple alternative to ROS-TAC score for assessment of levels of seminal oxidative stress. In our present study, we found significantly increased NBT positive leucocytes in group L compared to group NL however no significant difference of NBT positive sperms in both groups. Our finding goes in favour of the study by Esfandari N et al [11]. NBT test being indirect reflection of ROS generating activity in the cytoplasm of cells above finding suggest that leucocytes contribute more than that of sperms in leucocytospermic group.

We found significant negative correlation between % NBT positive leucocytes with sperm conc., % sperm motility and % normal morphology in group L suggesting that high leucocytes concentration level adversely affect the seminogram parameters and may lead to male infertility. Our findings do not agree with El-Demiry et al [14] who did not found association between conventional semen parameters and leucocyte concentration in human semen. However, we agree with findings of Ziyyat et al [15] and Lackner JE et al [16] that impairment of seminogram parameters are evident at leucocyte concentration greater than 1x 106/ml. In nonleucocytospermic group, there was no significant correlation between percentage of NBT positive leucocyte and seminogram parameters indicating that leucocytes does not affect seminal parameters adversely as long as leucocyte count is below 1 x 106/ml.

It had been suggested that partial ingestion of spermatozoa allows for white blood cell phagosome communication with the external milieu resulting in passage of lysosomal constituents into seminal fluid[17]. During phagocytosis polymorphonuclear leucocytes release myeloperoxidase and hydrogen peroxide which are inhibitary to sperm motility. Zalata AA et al[18] have documented an increase in lipid peroxidation after an invitro incubation of sperm with PMA-stimulated leucocytes, which suggest a decrease in biological value of sperm cell membranes in the environment of oxidative stress. It can not be excluded that the oxidative stress that appears in leucocytospermia is exerted by increased levels of cytokines themselves[19] . In this situation (ROS generated by leucocytes) acts synergistically with proinflammatory cytokines to exacerbate the destructive environment for spermatozoa. According to Armstrong et al [20] who use electron spin resonance analysis, chemiluminescence and NBT reduction indicated that the ROS-producing activity of spermatozoa may be different and significantly lower than the WBC-NADPH-oxidase. The mechanism of ROS generation in human

sperm recently was found to depend upon a novel NADPH-oxidase(NOX5) resembling the multicomponent NADPH-oxidase of white blood cells (WBC). Presence of leucocytes > 1 x 106/ml significantly increases the rate of spermatozoal lipid peroxidation. The degree of lipid peroxidation in leucocytospermic group reaches a level at which it affects the seminogram parameter adversely and thereby may contribute to male infertility.

Conclusion:

We concluded that leucocytospermia (leucocyte count > 1 x106/ml) significantly increased the rate of spermatozoa lipid peroxidation to the level which adversely affected the seminogram parameters (sperm concentration, % motility and % normal morphology) leading to male infertilit

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