Original Research Paper

Volume - 11 | Issue - 03 | March - 2021 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Physiology

A STUDY OF CELLULAR ELEMENTS OTHER THAN MATURE SPERMS, ASCORBIC ACID AND PROTEIN LEVELS IN THE SEMINAL FLUID OF NORMAL AND INFERTILE MEN.

Dr Archana Agarwal*

PhD Associate Professor- Physiology, SAHS, Sharda University, Greater Noida, Uttar Pradesh, India.*Corresponding Author

ABSTRACT **Background:**Male infertility accounts for 40% of infertility problems in the society. Sperm morphology is assessed routinely as part of a standard laboratory analysis in the diagnosis of human male infertility. Beside spermatozoa human semen contains a population of round cells and leuocytes which are considered as pus cells. **Aim:** This study is an attempt to identify the nature of the abnormal cells other than spermatozoa and to explore their significance in relation to human infertility. In addition to also explore the correlation between round cells (pus cells and immature germ cells), seminal ascorbic acid, protein and albumin levels in the seminal fluid. **Material & methods:**The study was carried out on 40 patients (30 infertile and 10 normal) age ranging between 24-45 years. Routine examination for volume, pH, viscosity, sperm count, motility and biochemical estimation of ascorbic acid, protein and albumin in seminal fluid by colorimetric method was determined. Seminal fluid smear stained with modified Bryan Leishman's stain was examined for the presence of abnormal sperms and round cells (pus cells & immature germ cells).**Result:**On statistical analysis of the data, no specific trend could be observed in any of the biochemical parameters (Ascorbic acid, protein, albumin concentration) of seminal fluid studied but a high percentage of round cells were found in infertile group. And they can be differentiated fairly from pus cells by applying Bryan Leishman's stain. **Conclusion:** By applying this modified staining technique (Bryan Leishman's stain) the immature germ cells can be fairly differentiated from pus cells.

KEYWORDS : Sperm Morphology, Sperm Count, Male Infertility Ascorbic Acid, Semen Protein.

INTRODUCTION

Infertility is a challenging problem both for clinicians and bio medical scientists. In most societies, the female partner has been judged culpable for the childless home and therefore the investigations and treatment of sterility have focussed mainly on women. Only in the past 30-40 years, has the contribution of the male to infertility been seriously examined and found a male factor to be responsible in 40-50 % of infertile couples. Semen analysis is a major and important tool for the diagnosis of male infertility. Out of this morphology is an important factor because in some infertility cases they shows high sperm count but less motility and more abnormal sperms , which are responsible for low fertility . Beside spermatozoa human semen contains a population of round cells. These round cells may be pus cells or immature germ cells. But it is really difficult to differentiate pus cells from immature germ cells.[1] The WHO manual for semen analysis quotes that if the round cells are more than 1×10^6/mL, they should be differentiated to see for leucocytes.[2] Review of the literature clearly indicates that a differentiation of “round cells” into cells of spermatogenic and non-spermatogenic origin is important for a more accurate semen report.[3,4,5] The inclusion of all “round cells” into one group may increase the risk of misinforming the treating clinician. [6] Hence, there is a need for initial screening and differentiation of round cells into immature germ cells and leucocytes with a simple and cost-effective method. The aim of the present work was to study the round cells by a simple staining method and their differentiation and quantification into leucocytes and immature germ cells and then comparing these with sperm counts and motility. On the other hand, a sperm concentration of $20x10^6$/ml, the 'normal' or 'reference' value cited by WHO [7,8,9], has been considered too low for a lower reference limit because the probability of pregnancy is essentially linear with sperm concentrations up to $40–50x10^6$/ml.[10,11] Conversely, sperm concentrations above this value are repeatedly observed in infertile patients. [12] There may be no upper limit of any semen characteristics since pregnancy rates increase with superior sperm morphology and motility. [13] The then-current normal morphology value of WHO [7] was considered inadequate [14] as it did not distinguish between fertile and infertile men whose partners were healthy. With uncertain reference values, over- or under diagnosis may result. Although much of the investigation conducted to date has considered the WHO 'normal' or 'reference' values as cut-off limits separating fertile from infertile populations, doubts have been raised about the validity of this approach.

In the present study therefore specific staining method have been tried to study the nature of these abnormal cells, other than mature sperms in the semen and to explore their significance in relation to human infertility.

METHODOLOGY

For the present study 40 subjects (30 infertile and 10 normal) of age 25 to 45 years, where preliminary examination revealed a possible male factor and female partners were found to normal after a routine examination were selected for the study. A written, informed consent from the patients and ethical committee approval was obtained prior to the collection of sample and evaluation of patients.

These selected patients were evaluated in terms of:-

(a) Clinical history—personal history and history of past illness.
(b) Local examination – condition of testis, epididymis, & vas deferens, varicocele etc.
(c) Semen examination – samples were collected by masturbation into a clean wide-mouthed plastic semen container followed by five days abstinence period. Semen is collected under similar conditions and subjected for macroscopic (volume, liquefaction & pH) and microscopic (sperm count, motility & morphology) examination. For morphology semen smear were prepared on a clean glass slide by the feathering method given in the 5 th edition of the WHO Manual for semen analysis & then air dried. [7] For evaluation of all cellular elements found in the seminal fluid, a combination of modified BRYAN'S STAIN AND LEISHMAN'S STAIN was used. [16] The round cells were counted and differentiated into immature germ cells and leucocytes. [15&17]

RESULTS :

After detailed history and thorough clinical examination , semen samples was collected & routine semen parameters like volume, pH, viscosity, sperm count & motility were recorded. Semen smears were stained with Bryan leishman's stain in duplicate & examined under oil immersion lens of microscope for differential counts of abnormal spermatozoa and round cells into immature germ cells and leucocytes. Cells were identified and differentiated according to their size, shape, and morphology. [3,6]

Immature germ cells mainly seen were primary spermatocytes, identified by their large cellular diameter (10-11µ), large spherical homogeneous nucleus (8-9µ) with woolly appearance and evenly distributed chromatin granules.

The next common were spermatids (Sab & Scd type) which were identified by smaller cellular diameter (4-5 µ) , round to oval cells which lacks granular cytoplasm with a dark nucleus.

Acrosome cap can sometimes be seen as a small magenta crescent shaped protrusion on one side of the cell. Sometimes occasional bi-nucleate cells— secondary spermatocytes—were also seen. Leucocytes were differentiated by their smaller size and multilobed nuclei. Epithelial cells shows no unusual characteristics.

The cases were divided into four groups based on total sperm counts as shown in Table 1.

Table 1: Distribution of cases according to fertility status & total sperm count .

Group	Fertility status	Total sperm count
I	Normal	≥20 million /ml
II	Infertile & Normospermic	≥20 million /ml
III	Infertile & Oligospermic	≤20 million / ml
1V	Infertile & Azoospermic	Zero / azoospermic

DISCUSSION:

Investigators examining seminal fluid for the clinical evaluation of males with fertility related problems are very often confused by the number of abnormal looking cells, other than mature sperms, which are present in sample. Routinely these are put down as pus cells. But are they really pus cells or immature germ cells or something else? This is an intriguing question. It is difficult to say what these cells really are in fresh unstained semen samples. The clinical implications of this question is considerable. If we presume that all these cells other than sperm cells are pus cells then the conclusion is that there is an infection and antibiotics should be prescribed to the patients. But very often there is no other evidence that the clinician can find out any infection in the epididymis or testis. On the other hand if we presume that these abnormal cells are all immature germ cells, from spermatogonia to spermatids, then the treatment may be different.

In the present study attempts have been made to suitably stain the semen smears. So that pus cells, immature germ cells, epithelial cells or any other abnormal cellular elements, can be clearly identified.

Various staining methods had been tried to study the nature of these abnormal cells such as:-
Papanicolaou staining method, Haematoxylin & Eosin staining method, Giemsa staining method, fast green staining method, Peroxidase stain using orthotoluidine blue and Bryan Leishman staining method (modified Leishman stain)

Finally Bryan Leishman's staining method was found suitable and was used in the present study.

The most common round cells found were the immature germ cells. All forms of the immature germinal cells found do not have tails and thus have not completed their development process. In general their nuclei stain violet to purple and the cytoplasm is grey.

Different forms of immature germ cells and pus cells found were as follows: [16]

1) IMMATURE GERM CELLS

A- Spermatogonia – cellular diameter-12 µ, nuclear diameter 6-7 µ, nuclei-round or slightly ovoid, one or two nucleoli were seen resting on the edge of the nucleolus, within characteristics "halos". (Image 1,2,3)
B- Primary spermatocyte—cellular diameter 10-11 µ, nuclear diameter 8-9 µ, Large spherical nucleus which is homogenous. (Image 4,5)
C- Secondary spermatocyte – cellular diameter 8-9 µ, nuclear diameter 7 µ, nucleus is spherical, cell is smaller than primary spermatocyte.
D- Sab spermatid—cellular diameter 4-5 µ, lacks granular cytoplasm, acrosome cap can sometimes be seen as a small magenta crescent shaped protrusion on one side of the cell. (Image 5)
E- Scd spermatid – resembles a mature sperm head without the tail.(Image 4)

2) PUS CELLS

A- Polymorphonuclear leucocyte—nuclear diameter- 8-12 µ, purple to violet nuclei and pale blue granular cytoplasm.
B- Lymphocyte – nuclear diameter-7-15 µ, nucleus is non-homogeneous & non- spherical in shape, nucleus stains violet and cytoplasm pale blue. (Image 3)

3) EPITHELIAL CELLS

Have no unusual characteristics.

Abnormal sperms were also very clearly stained by this method. (Image 1,4,6)

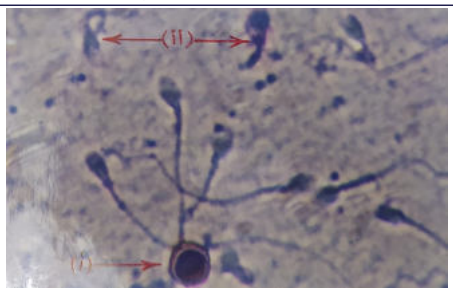

Image 1

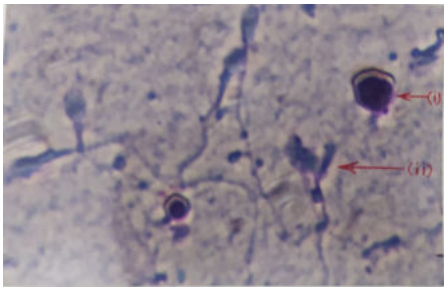

Image 2

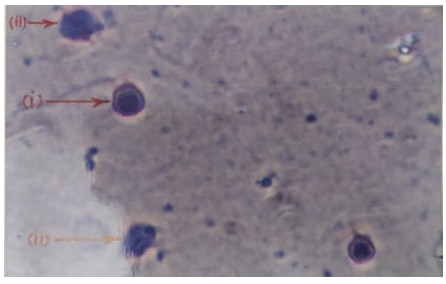

Image 3

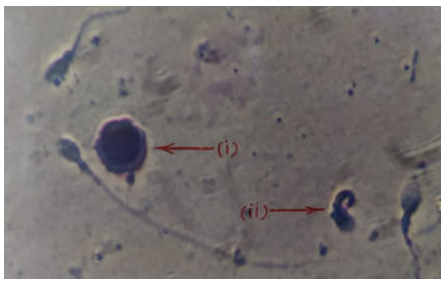

Image 4

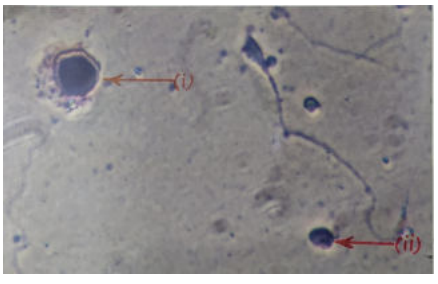

Image 5

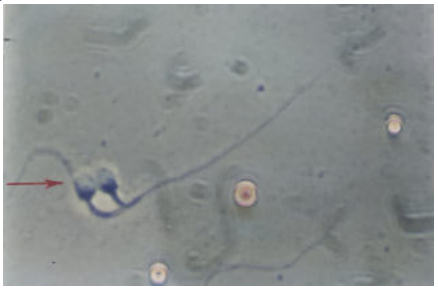

Image 6

In the present study the control group seminal fluid did not contain significant number of pus cells and immature germ cells/HPF(round cells/HPF). While in infertile patients eleven patient have significant number of pus cells and immature germ cells/HPF.

By comparing the statistical analysis of normal and infertile patients, we found a highly significant correlation between the number of pus cells and immature germ cells/HPF present in seminal fluid and infertility.

In smears prepared by the ejaculation of normal subjects, nearly 3% of total sperm count consist of spermatogonia, spermatocytes and spermatids. The maximum percentage being 5%. In some pathological cases this number increases up to 40 % or more. [18]

The semen of infertile male often contains significant number of immature germinal cells. As also observed in present study, If number of immature germ cells increases, there has probably been primary or secondary damage to the seminiferous tubules. [19]

It is not clear why immature germ cells should be present in large number in some subjects & the cells getting exfoliated from the seminiferous tubules ?

It is well known that human seminal fluid contains a fairly large percentage of morphologically abnormal sperms. The percentage may be as high as 20 % in normal subjects. Phadke described a special type of cell, "spermiophage" in some patients. [6]

Table 2: Comparison of seminogram & Biochemical parameters of infertile patients with normal patients.

	Normal (N=10)		Infertile (N=30)		T	p
Semen Characteristics	mean ±SD	Range	mean ±SD	Range	value	value
volume (ml)	03.23±0.83	2.40-5.20	04.32±01.50	1.3-7.0	2.18	,.05
pH	07.87±0.43	7.00-8.50	08.03±0.39	7.00-8.50	1.1	NS
Total count ($x10^6$)	89.50±25.22	60-135	34.27±26.75	00-86	5.73	<.001
sperm motility (%)	72.00±12.06	50-90	38.67±24.28	00-70	4.15	<.001
round cells /HPF	3.55±1.59	0-6	05.30±05.10	00-20	6.19	<.001
protein gm/dl	5.75±1.32	3.76-8.21	06.36±02.50	3.07-10.61	0.88	NS
Albumin gm/dl	1.83±0.45	1.16-2.50	2.07±00.85	0.83-4.50	0.85	NS
A/G ratio	0.47±0.08	0.32-0.60	00.52±00.24	0.13-1.35	0.64	NS
Ascorbic acid mg/dl	4.18±1.41	1.46-5.96	04.33±02.30	1.38-10.50	0.19	NS

In the present study only one normal subject exhibit abnormal spermatozoa. While in infertile patients, even exhibit abnormal spermatozoa. The value obtained were compared by applying the student't' test. The number of round cells/HPF was high positive in infertile group in comparison to control group. The average value being 5.3±5.10 and 3.55±1.59/HPF respectively, suggesting that high percentage of these cells are associated with infertility.

Ascorbic acid is essential for the structural and functional integrity of reproductive organs and also has important role in motility of human spermatozoa. [20,21] Considering the importance of ascorbic acid in semen , seminal ascorbic acid was also estimated. In normal patients seminal ascorbic acid was found to be in the range of 1.46 – 5.96 mg/dl with the mean of 4.18±1.41mg/dl while in infertile group it varied between 1,38-10.50 mg/dl, having a mean of 4.33±2,30mg/dl. Statistical analysis did not show any type of correlation between seminal ascorbic acid level and other parameters studied i.e. sperm count, motility, viscosity, protein and albumin levels.

Large amount of protein is present as a component of normal seminal plasma, but it is difficult to assign any functional purpose. [23] To define the exact role of protein in seminal fluid, the seminal protein, albumin and A/G ratio is estimated in the present study. The seminal protein levels were 5.75±1.32g/dl and 6.36±2.05 g/dl in control and infertile group respectively. No relationship could be observed between protein and fertility status of patients. Moon and Bunge also determined protein contents of 159 male patients coming to infertility clinic and observed the mean values to be 3.9gm/dl of total protein in different groups of infertile semen samples, which ranged from 1.3-7.0 gm/dl.[23] There was no statistical differences between semen quality and the concentration of protein. Also the comparison of protein concentration in normal and abnormal semen revealed no significant difference.

Human seminal fluid also contains a considerable amount of albumin. In combination with Zinc, albumin serves to regulate sperm metabolism. [24] An attempt is made here to explore the possible role of albumin in evaluation of fertility status of infertile males. The seminal albumin levels in normal subjects ranged between 1.16- 2.0 gm/dl with a mean average value of 1,83±0.45 gm/dl. While in infertile patients the albumin concentration was 2.07±0.85 gm/dl with a range of 0.83-4.50 gm/dl. No significant trend could be observed in this case. But some researchers had found some correlation between the serum albumin like electrophoretic fraction of bull seminal plasma and the pathological characteristics of spermatozoa. Also the presence of traces of serum albumin in seminal plasma could be due to an inflammatory tissue exudate. [25]

Analysing the data it is observed that there is some association of infertility with high percentage of round cells. In smears stained with Bryan- Leishman's stain , the immature cells can be fairly satisfactorily differentiated from pus cells. However, there is need of more elaborate work to establish this staining technique in routine semen examination of infertile males and also to correlate the percentage of these cells with ascorbic acid and protein, particularly albumin levels.

REFERENCES:

1) Patil PS, Humbarwadi RS, Patil AD, Gune AR. Immature germ cells in semen–correlation with total sperm count and sperm motility. Journal of Cytology/Indian Academy of Cytologists. 2013 Jul;30 (3):185.
2) Cooper TG. Semen analysis: An overview. In: Kvist U, Bjo L, editors. Manual on Basic Semen Analysis. 4th ed. Cambridge: Cambridge University Press; 2002. p. 4.
3) Fedder J, Askjaer SA, Hjort T. Nonspermatozoal cells in semen: relationship to other semen parameters and fertility status of the couple. Archives of andrology. 1993 Jan 1;31(2):95-103.
4) Jassim A, Festenstein H. Immunological and morphological characterisation of nucleated cells other than sperm in semen of oligospermic donors. Journal of reproductive immunology. 1987 Jun 1;11(2):77-89.
5) Tomlinson MJ, Barratt CL, Bolton AE, Lenton EA, Roberts HB, Cooke ID. Round cells and sperm fertilizing capacity: the presence of immature germ cells but not seminal leukocytes are associated with reduced success of in vitro fertilization. Fertility and sterility. 1992 Dec;58(6):1257.
6) Phadke AM, editor. 1st ed. New Delhi: Jaypee Brothers; 2007. Clinical atlas of sperm morphology.
7) World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction, 2nd edn. Cambridge: Cambridge University Press, 1987, 80 p.
8) World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction, 3rd edn. Cambridge: Cambridge University Press, 1992, 107 p.
9) World Health Organization. Task Force on MethWorld Health Organization. World Health Organization laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Inc, Cambridge, UK. 1999:1-38.
10) Bonde JP, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Scheike T, Giwercman A, Skakkebæk NE, Henriksen TB, Olsen J. Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. The Lancet. 1998 Oct 10;352(9135):1172-7.
11) Slama R, Eustache F, Ducot B, Jensen TK, Jørgensen N, Horte A, Irvine S, Suominen J, Andersen AG, Auger J, Vierula M. Time to pregnancy and semen parameters: a cross-sectional study among fertile couples from four European cities. Human Reproduction. 2002 Feb 1;17(2):503-15.
12) Nallella KP, Sharma RK, Aziz N, Agarwal A. Significance of sperm characteristics in the evaluation of male infertility. Fertility and sterility. 2006 Mar 1;85(3):629-34.
13) Garrett C, Liu DY, Clarke GN, Rushford DD, Baker HW. Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. Hum Reprod 2003;18:1643– 1649
14) Check JH, Bollendorf A, Press M, Blue T. Standard sperm morphology as a predictor of male fertility potential. Archives of andrology. 1992 Jan 1;28(1):39-41.
15) Bartoov B, Eltes F, Pansky M, Lederman H, Caspi E, Soffer Y. Estimating fertility potential via semen analysis data. Hum Reprod 1993;8:65 – 70.
16) World Health Organisation. WHO Laboratory manual for the examination of human semen and sperm cervical mucus interaction. Ed. Belsey MA, Eliasson R, Gallegos, Moghissi, Paulsen CA & Prasad MRN. Singapur Press Concern 1980; p-18.
17) Chan SY, Tang LC, Tang GW, Ho PC, Wang C. Spermatozoal fertilizing capacity in polyzoospermia: a preliminary study. Andrologia. 1986 Mar 4;18(2):208-13.
18) Sperling K, Kaden R. Meiotic studies of the ejaculated seminal fluid of humans with normal sperm count and oligospermia. Nature. 1971 Aug;232(5311):481-.7
19) Endtz AW. A rapid staining method for differentiating granulocytes from" germinal cells" in Papanicolaou-stained semen. Acta cytologica. 1974;18(1):2.
20) Prasad MR, Chinoy NJ, Kadam KM. Changes in succinic dehydrogenase levels in the rat epididymis under normal and altered physiologic conditions. Fertility and Sterility. 1972 Mar 1;23(3):186-90.

21) Chinoy NJ, Buch RP. Metabolic significance of ascorbic acid in human semen. Indian journal of experimental biology. 1977 Oct;15(10):921-3.
22) Nun S, Musacchio I, Epstein JA. Variations in seminal plasma constituents from fertile, subfertile, and vasectomized azoospermic men. Fertility and sterility. 1972 May;23(5):357.
23) Moon KH, Bunge RG. Observations on the biochemistry of human semen: 5. Protein. Fertility and sterility. 1970 Mar 1;21(3):220-1.
24) Lindholmer CH. The importance of seminal plasma for human sperm motility. Biology of reproduction. 1974 Jun 1;10(5):533-42.
25) Bennett JP. Quantitative comparisons of the proteins of the seminal plasmas of bull, ram, rabbit and boar by agar gel electrophoresis. Reproduction. 1965 Apr 1;9(2):217-31.