Original Article

Sperm DNA Integrity in Leukocytospermia and Its Association with Seminal Adenosine Deaminase

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Abstract

Background: The study aimed to examine the effect of leukocytospermia on sperm quality and the levels of seminal adenosine deaminase (ADA) enzyme in males attending an infertility clinic in a tertiary hospital and to detect the association, if any, between seminal ADA and sperm quality. **Methodology:** Consenting male subjects, between 21 and 45 years, attending the infertility clinic and qualifying the eligibility criteria were recruited following informed consent. The collected semen samples were analyzed for the routine parameters based on the WHO protocols and for sperm DNA fragmentation. The seminal leukocyte count was detected using the peroxidase method, and the seminal ADA was assessed using spectrophotometry. **Results:** Samples from 110 participants were included in the study; leukocytospermia was detected in 33% of the samples. A significant reduction in the sperm quality with respect to conventional semen parameters (sperm motility and sperm vitality) and sperm DNA fragmentation index (SDFI) was noted in the presence of leukocytospermia. Furthermore, a significant positive correlation between the levels of seminal ADA and SDFI was noted ($P = 0.000$, $r = 0.412$). **Conclusion:** The sperm motility and DNA integrity are significantly compromised in the presence of leukocytospermia when the leukocyte count is > 1 million/mL of semen as well as 0.5–1 million/mL of semen. The positive correlation noted between seminal ADA levels and increased sperm DNA damage paves way for the possibility of seminal ADA to be an indicator of silent male genital tract inflammation as well as low-quality semen.

Keywords: *Adenosine deaminase, leukocytospermia, male infertility, sperm DNA*

Introduction

Leukocytospermia is the presence of leukocytes in the semen, and the presence of >1 million leukocytes per mL of semen is considered as pathological.[1] The incidence of pathological leukocytospermia in the general population is 10%–20%, and this percentage is higher in infertile men.[2-4] The evidence regarding the role of leukocytes in semen is complex. In the past, the presence of leukocytes in the semen was reported to be physiological, with a role in surveillance and causing phagocytosis of abnormal and dead sperms. Recent studies indicate that leukocytes are major contributors of reactive oxygen species (ROS) production in semen.[5,6] An excess of ROS and resultant-free radicals in the seminal plasma interferes with normal sperm function by causing peroxidation of its lipid component, disruption of proteins, RNA, or DNA, or inhibition of mitochondrial activity.[4-9]

The detection of sperm DNA fragmentation is an independent predictor of male fertility potential, more predictive than the routine semen analysis.[10,11] Studies have detected associations between increased sperm

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DNA damage and male infertility, abortion, recurrent pregnancy loss, and high failure rate following assisted conception.[10,12,13] Hence, detecting sperm DNA damage can complement the information obtained by the semen analysis. The sperm chromatin dispersion (SCD) assay is a simple and reproducible technique with results comparable to the gold standard test – Sperm Chromatin Structure Assay (SCSA).[14,15]

Adenosine deaminase (ADA) is an enzyme of purine metabolism that is produced largely by lymphocytes and macrophages and plays an integral role in inflammation.[16,17] Adenosine exerts a significant positive role in sperm capacitation and process of fertilization, and an elevated ADA activity, which results in a decrease in adenosine levels, causes a reduced fertilization rate.[18] A higher level of this enzyme has been detected in the serum of infertile men compared to fertile men, indicating the enzyme's possible association with infertility.[19] However, there is a definite paucity of evidence regarding its presence in the seminal plasma or its correlation with sperm quality.[19,20]

The present study aims to examine the effect of leukocytospermia on sperm quality and seminal ADA enzyme in males attending an infertility clinic in a tertiary hospital in South India and to detect the association, if any, between seminal ADA and sperm quality. The study could provide evidence in support to a theoretical assumption regarding the significance of the measurement of ADA in seminal plasma as a possible indicator of inflammation in the male genital tract and a marker of sperm quality or male fertility. In addition, it could help in filling the existing lack of data on the effect of leukocytospermia in a South Indian population.

Methodology

The present cross-sectional study in infertile men was carried out in the Department of Anatomy in collaboration with the Departments of Obstetrics and Gynaecology, Pathology, and Biochemistry after obtaining approval from the Institute Scientific Advisory and Human Ethical Committee (JIP/IC/SC/2014/1/495).

Patient selection

Male subjects aged 21–45 years attending the infertility clinic at JIPMER were recruited for the study after obtaining informed written consent. Males with conditions that are likely to affect the sperm DNA integrity such as significant medical illness, history of smoking/alcohol intake, history of mumps, varicocele, cancer, or history of/ongoing radiotherapy or chemotherapy were excluded from the study.

Collection of samples

The semen samples were collected, based on the standards prescribed by the WHO,[1] by the method of masturbation into a sterile and labeled container following an abstinence period of 2–7 days. The samples were allowed to liquefy at room temperature for 30 min before analysis.

Semen analysis

The semen analysis was performed manually based on the based on 2010 WHO standard protocols.[1] The parameters – semen volume, pH, sperm motility and vitality, and sperm concentration – were analyzed. The peroxidase test/Endtz test was utilized to detect granulocytes in the semen.

Assessment of sperm DNA damage

The SCD assay, subjected to a few modifications to suit the present laboratory conditions, was used to detect the percentage of sperm DNA damage.[14] 50 μL of the diluted sample mixed with 50 μL of low melting agarose was pipetted onto a slide precoated with normal melting agarose. The slides were immersed in a mild acid solution to generate restricted single-stranded DNA motifs from the sites of DNA breaks and followed by treatment with neutralizing and lysing solutions to remove nuclear proteins and lyse cell membranes. The resultant sperms' nucleoids, with a central core and a peripheral halo of dispersed DNA loops, were subjected to sequential dehydration in 50%, 70%, and 95% of ethanol and air-dried. The slides were then stained with Wright stain, and the percentage of nucleoids with fragmented DNA was assessed by brightfield microscopy. The sperm nucleoids on each slide were grouped based on the comparison of the halo radius (r) to the diameter of the core (d) into four of the following patterns:

a. Nucleoid with large-sized haloes (r > d)
b. Nucleoid with medium-sized haloes (r = d)
c. Nucleoid with very small-sized haloes (r < d)
d. Nucleoid with no halo (the only core of nucleoid present).

Two hundred spermatozoa were assessed in each slide and the percentage of nucleoids belonging to each of the four patterns was noted. Those with absent haloes and small-sized haloes were grouped under spermatozoa with the presence of DNA damage, and those with medium-sized and large-sized haloes were grouped under spermatozoa without DNA damage. Sperm DNA fragmentation index (SDFI) is calculated using the formula:

SDFI = 100 × number of sperms with DNA damage/number of sperms counted.

Enzyme adenosine deaminase

The enzyme ADA in seminal plasma was assessed by the enzyme analysis kit named ADA–MTB (Tulip Diagnostics, Goa, India) by spectrophotometry.

Statistical analysis

The IBM SPSS Statistics (Version 19.0, Armonk, NY, USA) was used for the statistical analysis of the collected data. The distribution of the data of variables such as semen parameters, leukocyte count, ADA levels, and DNA damage was expressed as median with range after testing with the Kolmogorov–Smirnov test of normality. The comparison of these variables between the groups was carried out by the Mann–Whitney test, and the Kruskal–Wallis test was used to compare the variables between the subgroups. The linear relationship between the variables was carried out using the Spearman correlation analysis. The statistical analysis was carried out at 5% level of significance and a $P < 0.05$ was considered statistically significant.

Ethical considerations

Informed consent was obtained from the participants of the study. The study was approved by the Institute Scientific Advisory and Human Ethical Committee (JIP/IC/SC/2014/1/495).

Results

In the present study, the semen samples of 120 males who had attended the infertility clinic were assessed for routine semen parameters (semen volume, seminal pH, sperm concentration, sperm motility, and sperm vitality) as per the WHO guidelines 2010.[1] Table 1 shows the distribution of the conventional semen parameters of the samples. The data of 10 samples were excluded from the statistical analysis as they were samples with azoospermia in which sperm DNA fragmentation cannot be assessed for. The remaining 110 samples were subjected to peroxidase staining for leukocyte detection, SCD assay, and ADA enzyme estimation. Based on the number of leukocytes detected, the 110 samples were categorized into the following three groups: A, samples without leukocytospermia; B, samples with leukocytospermia of (0.5–1) million/mL; and C, samples with leukocytospermia of > 1 million/mL for analysis.

Leukocytospermia

Of the total 120 samples, leukocytospermia was present in 33 (27.5%) of them, of which 15 had a leukocyte count of 0.5–1 million/mL (Group B) and 18 had a leukocyte count of >1 million/mL of semen (Group C). Two of the samples with a leukocyte count of >1 million/mL of semen had azoospermia.

Conventional semen parameters

The comparison of semen parameters between the three groups is shown in Table 2. There is no significant difference in values of seminal volume, seminal pH, or sperm concentration between them, but a significant difference in sperm motility and sperm vitality was noted.

A significantly lower sperm motility (progressive motility, $P = 0.002$ and total motility, $P = 0.008$) and sperm vitality ($P = 0.016$) was recorded in Group B (samples with leukocytospermia of 0.5–1 million/mL) in comparison to Group A (samples without leukocytospermia). In comparing Groups A (samples without leukocytospermia) and C (samples with leukocytospermia of >1 million/mL), a significantly lower sperm motility (progressive motility, $P = 0.002$ and total motility, $P = 0.003$) was noted in the latter with no significant difference in sperm vitality. However, there was no significant difference in conventional semen parameters between Groups B (samples with leukocytospermia of 0.5–1 million/mL) and C (samples with leukocytospermia of >1 million/mL).

Sperm DNA damage

Among the 110 samples, 74 (67.27%) had an SDFI >30%. The median SDFI value of the samples was 45% with a range of 10%–95%.

The comparison of SDFI between the three groups is shown in Table 2. A significantly higher SDFI, $P = 0.000$ and $P = 0.049$, was noted in Group C (samples with leukocytospermia of > 1 million/mL) when compared

Table 1: Distribution of routine semen parameters within the sample population

Parameter	Lower reference value of the 2010 WHO guidelines	Number of samples (*n*)	Median	Range	Number of samples below lower reference limit	Percentage of samples below lower reference limit (%)
Semen volume (mL)	1.5	120	3	1-8	3	2.5
Seminal pH	7.2	120	8	6.5-9	7	5.8
Sperm concentration ($\times 10^6$/mL)	15	110	47.50	5-150	24	20
Progressive sperm motility (%)	32	110	20	0-80	72	65.4
Total sperm motility (progressive + nonprogressive) (%)	40	110	66	0-95	17	14.2
Vitality (%)	58	110	80	0-95	18	16.4

Table 2: Comparison of semen parameters between samples with no leukocytes, samples with leukocytes of 0.5-1 million/mL of semen, and samples with leukocytes of>1 million/mL

n	Parameter	Samples with no leukocytes (*n*=79) Group A	Samples with leukocytospermia of 0.5-1 million/mL (*n*=15) Group B	Samples with leukocytospermia of >1 million/mL (*n*=16) Group C	AB	BC	AC
1	Age (years)	33 (25-45)	30 (26-40)	32 (26-45)	0.153	0.922	0.231
2	Semen volume (mL)	3 (1-6)	3.5 (1.5-6)	3 (1.5-8)	0.163	0.711	0.254
3	Seminal pH	8 (7-9)	7.5 (7-8.5)	7.5 (7-9)	0.135	1.000	0.214
4	Sperm concentration ($\times 10^6$)	50 (8-150)	50 (10-80)	37.5 (5-80)	0.926	0.379	0.195
5	Progressive motility (%)	30 (0-80)	15 (5-35)	15 (0-30)	0.002*	0.922	0.002*
6	Nonprogressive motility (%)	30 (0-80)	30 (10-70)	25 (0-60)	0.909	0.423	0.189
7	Total motility (%)	70 (0-95)	50 (15-85)	42.5 (0-85)	0.008*	0.495	0.003*
8	Immotility (%)	30 (5-100)	50 (15-85)	57.5 (10-100)	0.010*	0.495	0.003*
9	Vitality (%)	80 (5-100)	60 (30-90)	60 (20-90)	0.016*	0.953	0.054
10	SDFI (%)	40 (10-95)	55 (20-80)	62.5 (30-95)	0.052	0.049*	0.000*
11	ADA (U/L)	10.024 (0.538-60.883)	10.258 (0.80-43.41)	22.161 (3.982-55.23)	0.849	0.401	0.097

The data is expressed as median and range. The statistical analysis was done using Mann-Whitney U-test, *P<0.05 was considered as significant. A=Samples with no leukocytes, B=Samples with leukocytes of 0.5-1 million/mL of semen, and C=Samples with leukocytes of >1 million/mL. AB=Comparison between samples with no leukocytes and samples with leukocytes of 0.5-1 million/mL of semen, BC=Comparison between samples with leukocytes of 0.5-1 million/mL of semen and samples with leukocytes of >1 million/mL, AC=Comparison between samples with no leukocytes and samples with leukocytes of >1 million/mL. ADA=Adenosine deaminase, SDFI=Sperm DNA Fragmentation Index

with Group A (samples without leukocytospermia) or B (samples with leukocytospermia of 0.5–1 million/mL), respectively.

Adenosine deaminase

The median ADA level of the semen samples was 10.07 U/L with a range between 0.538 and 60.77 U/L.

There was no significant difference in ADA levels when the three groups were compared. However, on testing with Spearman's rho correlation analysis as seen in Figure 1, a significantly positive correlation of levels of seminal ADA with SDFI was noted (P = 0.000, r = 0.412) in the total sample (n = 110). A similar significant correlation between ADA and SDFI was noted when correlation analysis was applied separately to Group A (n = 79, P = 0.000, r = 0.444), but the same did not appear when applied to samples with leukocytospermia (n = 31, P = 0.050, r = 0.355).

Discussion

Based on the results in our study, there is a significant difference in the quality of sperms in leukocytospermic samples compared to nonleukocytospermic samples reflected in the conventional semen parameters (sperm motility and sperm vitality) and sperm DNA integrity. The samples with leukocytospermia of >1 million/mL showed a significant reduction in sperm motility and vitality, and the samples with leukocytospermia of 0.5–1 million/mL showed a significant reduction in the sperm motility. With respect to sperm DNA damage, both the above groups of samples with leukocytospermia had a significantly increased sperm DNA damage when compared to nonleukocytospermic samples.

The reduced sperm quality in the presence of a leukocytospermia of >1 million/mL has been observed previously by Ziyyat *et al*.[5] and Aziz *et al*.[21] and the same is designated by the WHO[1] as pathological leukocytospermia. However, our results show a decrease in sperm quality (sperm DNA integrity and sperm motility) in semen with leukocytospermia, irrespective of the number of leukocytes. Contrary to our findings, Ziyyat *et al*. described an unusual paradoxical increase in sperm motility when the leukocyte concentration in semen is <1 million/mL.[5] A similar positive effect of leukocytes (<1 million/mL) was noted by Lackner *et al*. on sperm motility and sperm morphology.[2] Ziyyat *et al*.[5] and Lackner *et al*.[2] attribute this paradoxical increase in sperm quality, when leukocyte count is <1 million/mL, to an increased level of ROS coexistent, but masked, by higher levels of total antioxidant capacity (TAC) in the seminal plasma. The seminal TAC is referred to the total antioxidant protection available in the semen to protect the sperms from ROS.[5] The production of excessive ROS has been frequently implicated as the mechanism of action for leukocytes to cause sperm damage in semen; it acts by causing lipid peroxidation of the sperm plasma membrane and the inhibition of mitochondrial activity which could affect sperm motility and vitality and can interfere with nucleic acid synthesis, leading to chromatin damage.[5,8,21] Sperm plasma membrane is particularly susceptible to lipid peroxidation as Poly

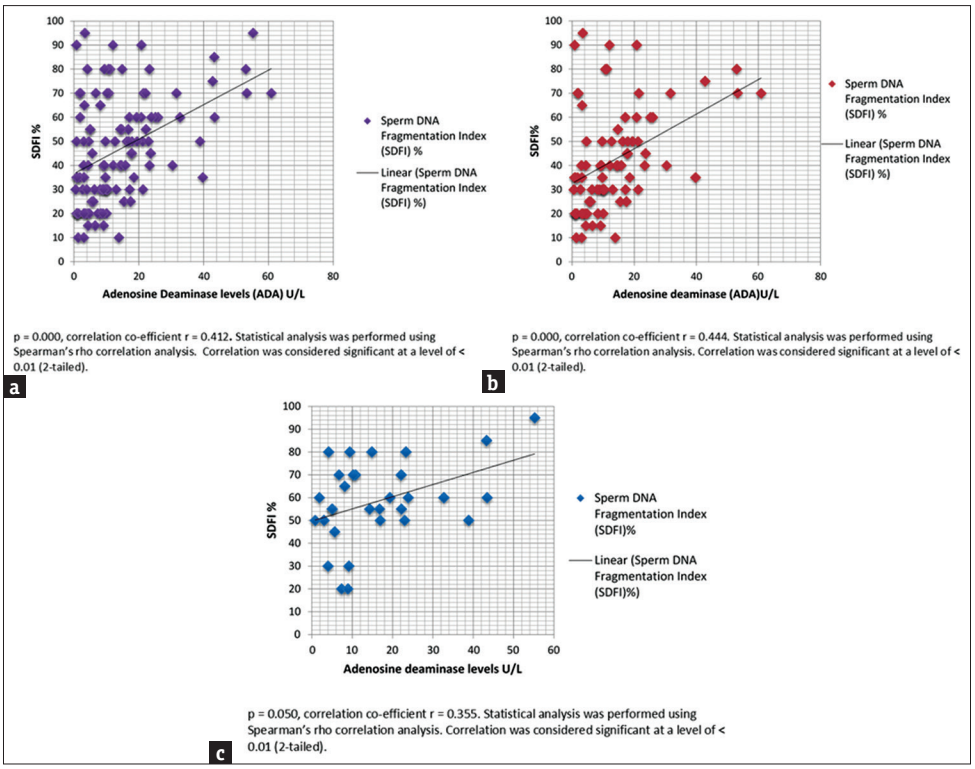

Figure 1: The correlation of sperm DNA fragmentation index and seminal levels of adenosine deaminase in (a) total sample, *n* = 110; (b) samples without leukocytospermia, *n* = 79; and (c) samples with leukocytospermia, *n* = 31. The statistical analysis was performed using Spearman's rho correlation analysis and the correlation was considered significant at a level of <0.01 (two-tailed)

Unsaturated Fatty Acid (PUFA) constitutes 60% of its plasma membrane lipids.[11] According to Sharma *et al*., the presence of leukocytes in semen, irrespective of number, has been associated with high levels of ROS.[4] In addition, evidence of a low TAC has been reported in infertile men.[10] Thus, an explanation to the absence of paradoxical improvement in sperm quality in our study, i.e., a decrease in % sperm motility and increase in sperm DNA damage, in the presence of a leukocyte count of <1 million/mL, could be due to a reduction in the seminal TAC in addition to high level of seminal ROS. It may be hypothesized that the samples with a leukocyte count of 0.5–1 million/mL in this study had a low TAC which led to an absence of the above-mentioned paradoxical effect and the presence of reduced sperm quality (sperm motility and sperm DNA integrity). A possible follow-up of this study would be to determine the TAC in the seminal plasma and to examine its correlation with sperm DNA damage.

The median seminal ADA level in the total sample size (*n* = 120) was 10.07 U/L. Though the median seminal ADA value was higher in cases with leukocytospermia >1 million/mL of semen (22.161 U/L), no significant difference was noted when it was compared with cases with leukocytospermia 0.5–1 million/mL of semen or without leukocytospermia. Rostampour *et al*. compared levels of the ADA enzyme in the serum of fertile and infertile men and detected significantly increased levels of the ADA enzyme in infertile men compared to the former.[19] A genetic variation in the genes coding for the ADA enzyme has been suggested as a cause for this increase in ADA activity in the serum of infertile men[19] and the same could possibly be reflected in the seminal plasma: an increase in seminal ADA enzyme levels in samples with leukocytospermia, as noted in our study. In the study by Rostampour *et al*., the mean ADA enzyme activity in the plasma of infertile men measured by colorimetric method was 43.4 ± 22.7 U/L.[19] According to the kit used in our study, a range of 0–30 U/L and 0–10 U/L is considered normal in serum or plasma and cerebrospinal fluid, respectively. However, to the best of our knowledge, there is a definite paucity in the literature that explores the levels of ADA enzyme or defines its normal range in seminal plasma, and hence, a cutoff level for ADA in seminal plasma could not be agreed upon. Though an increasing trend in the seminal ADA levels was noted with increasing leukocytospermia, further studies with a higher sample size and the determination of the normal range of seminal ADA would be required to establish these findings.

The seminal ADA levels showed a negative correlation with semen parameters and a positive correlation with

SDFI when correlation analysis was applied to the total sample size (n = 110) as well as in samples without leukocytospermia [Figure 1]. Thus, elevated seminal ADA levels may indirectly reflect a decrease in the quality of sperms. Rostampour *et al*., who detected higher ADA levels in the serum of infertile men, suggested that elevated ADA had a role in decreasing the fertility of infertile men.[19] The enzyme ADA is known to play an integral role in both acute and chronic inflammation.[16] As elevated levels of ADA point out to an inflammation, the above-mentioned correlation may suggest the possibility of an inflammation in the genital tract that is adversely affecting the quality of sperms. This inflammation, however, could not be picked up using the leukocyte detection screening by Endtz test as the enzyme ADA is primarily produced by monocytes, macrophages, and lymphocytes,[22] whereas the leukocyte detection by peroxidase method only identifies the activated granulocytes – mainly the neutrophils in the semen.[23] This view is in concordance with Sánchez *et al*., who studied the role of elastase in semen and described that mere absence of leukocytes does not rule out a seminal inflammation.[24] Hence, as seminal ADA levels correlate with semen parameters and sperm DNA damage, its use in the absence of leukocytospermia could help not only in detecting silent inflammation but also in detecting low-quality semen. However, when the correlation analysis was applied in the samples with leukocytospermia (n = 31), no significant correlation was noted between seminal ADA and SDFI, in spite of a significantly increased SDFI in this group. It may be assumed that in the presence of frank leukocytospermia, levels of various other chemical mediators are elevated and could have an additional role in causing damage to sperm DNA[25,26] and thus leading to a lack of correlation between seminal ADA levels and SDFI.

Clinical significance

The inflammation of the male genital tract is usually clinically silent, affects male fertility potential, and contributes to a significant proportion of cases of male infertility.[24] Hence, detecting a silent inflammation could be valuable in diagnosing male infertility.[9] As the absence of leukocytes does not rule out an inflammation,[24] the use of biochemical assays,[26] such as seminal ADA, could serve useful in this regard.

Studying the effects of male genital tract inflammation on sperm quality could assist in exploring its treatment options. For example, a significant decrease in semen leukocyte count and improvement in sperm motility were detected in subjects with pathological leukocytospermia[3] and abacterial leukocytospermia[27] following a course of a COX-2 inhibitor therapy. A detectable improvement in sperm quality could improve success rates of artificial reproductive techniques procedures such as intracytoplasmic sperm injection or allow selection of less invasive options such as monitored intercourse or intrauterine insemination.[13]

Conclusion

Based on our study, it may be concluded that the sperm motility and sperm DNA integrity are significantly compromised in the presence of leukocytospermia (when the leukocyte count is > 1 million/mL of semen as well as 0.5–1 million/mL of semen). With respect to ADA, an increasing trend in the seminal ADA in the presence of leukocytospermia was noted and further studies with a wider sampling would be required to substantiate these findings. In the absence of leukocytospermia, a positive correlation was noted between seminal ADA levels and sperm DNA damage and a negative correlation between the former and the semen quality. Hence, the seminal ADA levels could be an indicator of silent male genital tract inflammation as well as low-quality semen.

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Conflicts of interest

There are no conflicts of interest.

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