

INTRODUCTION

Miscarriages are the most common complication in human pregnancies. Approximately 0.5 to 1.0% of couples (1) experience recurrent pregnancy loss (RPL), which is defined as three or more consecutive miscarriages (2). Pregnancy losses are classified as pre-embryonic (<5 weeks), embryonic (5–10 weeks) or fetal (>10 weeks).

The various etiologies like chromosomal, anatomic, hormonal, immunological have been studied extensively in females but sperm characteristics which are likely to influence the quality of the conceptus have not been examined in detail till now. Recently, in a few IVF studies, male factors are being recognized as a possible cause associated with repeated pregnancy failure in the female (3, 4, 5, 6, 7). This may imply that male factor may play a role in recurrent pregnancy loss (8, 9). However, the information related to male factor and its possible linkage with RPL is grossly inadequate.

Although, the cause & effect relationship is difficult to establish, a strong frequent association of abnormal sperm quantity and quality in male partners of RPL couples can be attributed to the repeated episodes of pregnancy loss. Abnormal sperm morphology in few instances has been attributed as a possible factor (3) since fertilization with an abnormal sperm may not always result in the healthy maintenance of the conceptus. Increased incidence of spontaneous abortions reported from IVF centers in couples having severe sperm head abnormality suggest that it could be responsible for repeated abortions (4) although a few other studies have shown

no significant correlation between abnormal sperm concentration/sperm morphology with recurrent spontaneous abortions (10, 11). It has been observed that in spite of apparently normal sperm count subtle membrane defect in the spermatozoa and sub-optimal hypoosmotic swelling score have been implicated as factors in the RPL (4).

The present study is important in the sense that both male and female partners of the RPL group were simultaneously evaluated to detect any genetic, anatomic, immunological or infective factors which may cause recurrent abortions. Routine seminal parameters and sperm function tests of the RPL group were compared with the control group to determine whether there was an increased frequency of abnormal factors in the RPL group.

METHODS

The study was conducted in a tertiary care referral centre after obtaining Institutional Ethical Committee clearance. Seventy-five (75) couples with a history of three or more consecutive pregnancy loss in the first or early second trimester were recruited in the study after signing a written informed consent. Women above 40 years of age, male partners above 45 years, women with premature ovarian failure, couples with one or more living issue, or having less than 3 consecutive abortions or with abortions occurring after 20 weeks or with history of preterm labor and known diabetics were excluded from the study. Twenty (20) male volunteers with recently proven fertility were also included for detailed sperm morphology and sperm functions test as controls.

Thorough clinical examination (general, systemic and local) was carried out. All female subjects underwent investigations to look for chromosomal, anatomic, hormonal, immunological and infective pathologies. Investigations done for the female partners included hematological and biochemical investigations including haemogram with ESR, Urine analysis. Blood Sugar, Blood Group and Rh and VDRL. Hormonal investigations including Serum FSH, LH, Estradiol, Prolactin and TSH were performed on day 6, 7 or 8 of cycle; Serum Progesterone and premenstrual endometrial biopsy were done on day 21 of cycle. Endocervical secretions of female patients were tested for Chlamydia, Bacterial Vaginosis, Mycoplasma and Ureaplasma by bacteriological culture. TORCH, Karyotyping (by extended banding), antiphospholipid antibodies (Anticardiolipin Antibody, Lupus Anticoagulant) antisperm antibodies (agglutinating and immobilizing) tests in cervical mucus, semen and blood of both partners were done for all female patients. Ultrasonography/hysterosalpingography was done postmenstrually before day 10 of cycle.

Laboratory investigations were carried out on all males of RPL and control group. Routine semen analysis was done. Semen samples were obtained by masturbation after 3–4 days of abstinence. Samples were allowed to liquefy at 37°C for 30 minutes. All samples were evaluated (for sperm volume, concentration, motility) according to WHO criterion (12). A minimum of two seminograms were obtained at a two week interval. All samples were then processed for sperm morphology within 1 hour of collection. The assessment was carried

out by a single experienced observer, who was blind to the clinical status of the samples.

To test the quality of spermatozoa hypo-osmotic swelling test, nuclear chromatin condensation and decondensation test, seminal total leukocyte count estimation and acrosomal reaction test by methods described previously (13, 14) were done for male patients of the study and the control group.

Sperm morphology

Smears prepared from semen were stained by Papanicolaou stain and examined under microscope. Head, neck, midpiece and tail defects were specifically examined and recorded.

Hypo-osmotic swelling test

A modified hypo-osmotic swelling (HOS) test protocol was adopted as developed in our laboratory and described (13). In brief 50 µL of semen was incubated in 500 µL of hypo-osmotic swelling solutions for 5 minutes at room temperature. One drop of the incubation mixture was placed on a clean glass slide and examined under a microscope. Percentages of spermatozoa with tail coiling were recorded.

Nuclear chromatin decondensation test

The nuclear chromatin decondensation (NCD) test was performed as described earlier. In brief, liquefied semen sample was centrifuged and sperm pellet was washed in 0.05 M borate buffer. One volume of sample was incubated with nine volumes of

ethylenediaminetetraacetic acid (EDTA) (6 mM) and sodium dodecyl sulfate (SDS) (1%) mixture at 37% for 60 minutes. The reaction was stopped by addition of an equal volume of 2.5% glutaraldehyde in borate buffer. One drop of the mixture was then placed on a clean glass slide and examined under a microscope. Percentage of sperms with swollen chromatin was recorded.

Acrosomal status test

Acrosomal status (AS) test was carried out as described. In brief, semen was diluted in phosphate-buffered saline (PBS) D-Glucose (1:20) and equilibrated at 37°C for 30 minutes. A smear of the diluted mixture was gently placed on a gelatin coated glass slide and excess water was evaporated. The slides were incubated at 37°C for 120 minutes in a humid chamber, air dried, and examined under the microscope. Percentage of spermatozoa with halos surrounding the sperm head was recorded.

Urethral smear was taken from all males of the RPL group after prostatic massage and was tested for the presence of Chlamydia, Gardenella, Mycoplasma and Ureaplasma by anaerobic bacteriological culture. If pus cells were present in semen analysis or if seminal total leukocyte count was higher than 0.6×10^6 , aerobic culture of the semen was done. All male patients underwent VDRL test and karyotyping and antisperm antibody detection in semen and blood.

Statistical analysis was performed using unpaired t-tests. Results are reported as mean \pm standard error of mean.

RESULTS

Mean Age of the females in this study was 28.4 ± 4.2 (Range: 21 to 38), Mean age of the males of the RPL group was 31.8 ± 4.7 (Range: 24 to 43) while mean age of the males of the control group was 29.6 ± 3.2 (Range: 22 to 40). Mean number of abortions in the RPL group was 3.8.

Male factors associated with RPL are depicted in Table I. The commonest factor associated with male partners of RPL group was infection in 23(30.6%) followed by anatomical in 3(4%), genetic in 1(1.3%) and immunological in 1(1.3%) patient out of a total of 75 males.

TABLE I: Male factors associated with RPL.

	<i>Factors</i>	<i>N=75</i>	<i>%</i>
Anatomical factor	Varicocele	3	(4.0%)
Genetic factor	(46XYd ⁺)	1	(1.3%)
Immunological factor	Antisperm antibody	1	(1.3%)
Infective factor		23	(30.6%)
	Ureaplasma	15	(20%)
	Chlamydia	7	(9.3%)
	Mycoplasma	1	(1.3%)
	VDRL	Nil	

Abnormal parameters in routine semen analysis were present in only 18(24%) individuals while subnormal sperm function were present in 34(45.3%).

Comparison of routine semen analysis of RPL and the control groups is shown in Table II. On comparing the routine semen analysis in males of RPL group with the control group, semen volume, count, motility and viability were within normal range in both

TABLE II: Routine semen analysis.

Parameter	RPL N=75	Control N=20	P value
Volume (Mean±S.E.) (Median)	2.7±0.10 (2.5)	2.8±0.10 (2.9)	0.453
Count (Mean±S.E.) (Median)	64.9±2.22 (69.0)	72.0±1.06 (70.0)	0.117
Motility (Mean±S.E.) (Median)	52.1±1.50 (50.0)	58.5±0.49 (57.5)	0.036
Viability (Mean±S.E.) (Median)	65.9±1.36 (70.0)	72.4±0.47 (71.5)	0.019

groups. Sperm motility (P=0.036) and viability (P=0.019) were found to be significantly lower in the RPL group.

Functional capacity of the sperms was tested by performing sperm function tests including the HOS, AR and NCD test. Mean test scores for HOS and NCD were below normal range in the RPL group. All sperm function scores in the control group were within normal range. Comparison of sperm function tests of RPL and the control group revealed that HOS (P=0.000), NCD (P=0.000) and AR (P=0.000) were significantly lower in the RPL group (Table III).

Seminal total leukocyte count (TLC) was measured to rule out infection in male partners. Seminal TLC was not significantly different (P=0.287) in the RPL group as compared to control group (Table III). If this count was more than 0.6×10^6 , aerobic semen culture and sensitivity was done. Thereafter patient was treated with appropriate antibiotics. Seminal TLC was used as a predictor of Male Accessory Gland infection (MAGI), as observed by Roy et al. (15). In a large series they have noted that a TLC of

TABLE III: Sperm function test.

Test performed	RPL N=75	Control N=20	P value
HOS (Mean±SE) (Median)	58.9±1.29 (64)	76.1±0.42 (77)	0.000
NCD (Mean±SE) (Median)	67.1±1.59 (71)	83.2±0.41 (84)	0.000
Acrosomal reaction (Mean±SE) (Median)	60.3±1.48 (62.5)	75.0±0.55 (76)	0.000
TLC (Mean±SE) (Median)	0.84±0.18 (0.5)	0.47±0.02 (0.5)	0.287

HOS: Hypo-Osmotic Swelling.

NCD: Nuclear Chromatin Decondensation test.

TLC: Total Leukocyte Count.

more than 0.6×10^6 WBC/ml. is associated with infection.

Prevalence of infection in the males of RPL group was 30.6% (23 subjects). Abnormal count/motility/presence of pus cells was present in 21.3% (16) subjects out of 75 males of the RPL group. Amongst anaerobes, infection was maximum with Ureaplasma (20%) followed by Chlamydia (9.3%) and Mycoplasma (1.3%). Aerobic culture revealed maximum prevalence of Staphylococcus (22.6%) followed by E. Coli (12%) and Pseudomonas (1.3%). In our study one or more male factors were present in 34 (45.2%) patients.

Of the 75 female partners, 39(52%) had associated hormonal, 38(50.6%) infective, 30(40%) anatomical, 9(12.0%) immunological factors that could be responsible for the repeated pregnancy loss. None had any genetic abnormality. Sixty three women (84%) had more than one associated abnormal factors.

DISCUSSION

The role of male factors in recurrent abortions remains largely unknown barring a few cytogenetic abnormalities (16). Good quality sperms yield good quality fetus. Any deviation from the normal or sub-standard sperms will affect its motility, penetration and decondensation, ultimately hampering the conception, implantation and nidation, as one of the limiting steps in the success of fertility is the quality of the fertilized ovum (16). Abnormal sperm morphology has been associated with increased miscarriage rates in couples undergoing IVF-ET (3).

Only 6(8%) subjects of the RPL group were detected to be having abnormal sperm morphology (>70% abnormal spermatozoa) and all of them had significantly lower motility and sperm function parameters. One patient with genetic defect (46XYq+) had normal semen parameters, normal morphology but subnormal HOS scores. This couple gave history of 4 abortions at 5–7 weeks all of whom were diagnosed as blighted ovum on ultrasonography. Although, minor chromosomal abnormalities may not result in gross morphological defects of the sperm, they may cause subtle alterations in the membrane constitution of the sperm resulting in functional defects. These defects can be evaluated by performing HOS, which tests the functional integrity of the cell membrane.

In our study, 24% (18) had history of abortions occurring before 5 weeks, 64% (48) between 5–10 weeks and 12% (9) after 10 weeks of gestation. Low sperm motility (<50%) was found in 16(21.3%) patients of the RPL group, all of these subjects had

subnormal sperm function. However, patients with normal motility also demonstrated subnormal sperm functions. This points towards the limitation of routine semen analysis in detecting abnormality in sperm function other than motility.

Besides routine semen analysis, qualitative tests like HOS, NCD and AR were also done to evaluate the functional capacity of the sperms of males whose wives suffer from RPL. Sperm function scores were subnormal in all 3(4%) patients with varicocele, 22(29.3%) patients with infection and in 1(1.3%) patient with antisperm antibody. In contrast, all subjects of the control group had normal motility, morphology and sperm function parameters.

HOS test measures the number of sperms with intact membranes. Recent studies have indicated that low HOS test scores in couples undergoing IVF do not affect rates of fertilization or pregnancy, but are associated with higher rates of spontaneous miscarriage (4, 5, 7, 8). Mean HOS tests scores of the RPL group were subnormal and twenty six (34.6%) patients of RPL group had low HOS scores. Photographs of HOS test in the control and RPL group are depicted in Figure 1a and 1b respectively.

The basis of the Acrosome reaction test is that the acrosome of spermatozoa contains proteases, which help in penetration of spermatozoa through outer membranes of oocyte. Mean AR scores in both RPL and control groups were within normal range. Subnormal scores were present in 20(26.6%) patients of the RPL group. Photographs of AR test in the control and RPL group are depicted in Figure 1c and 1d respectively.

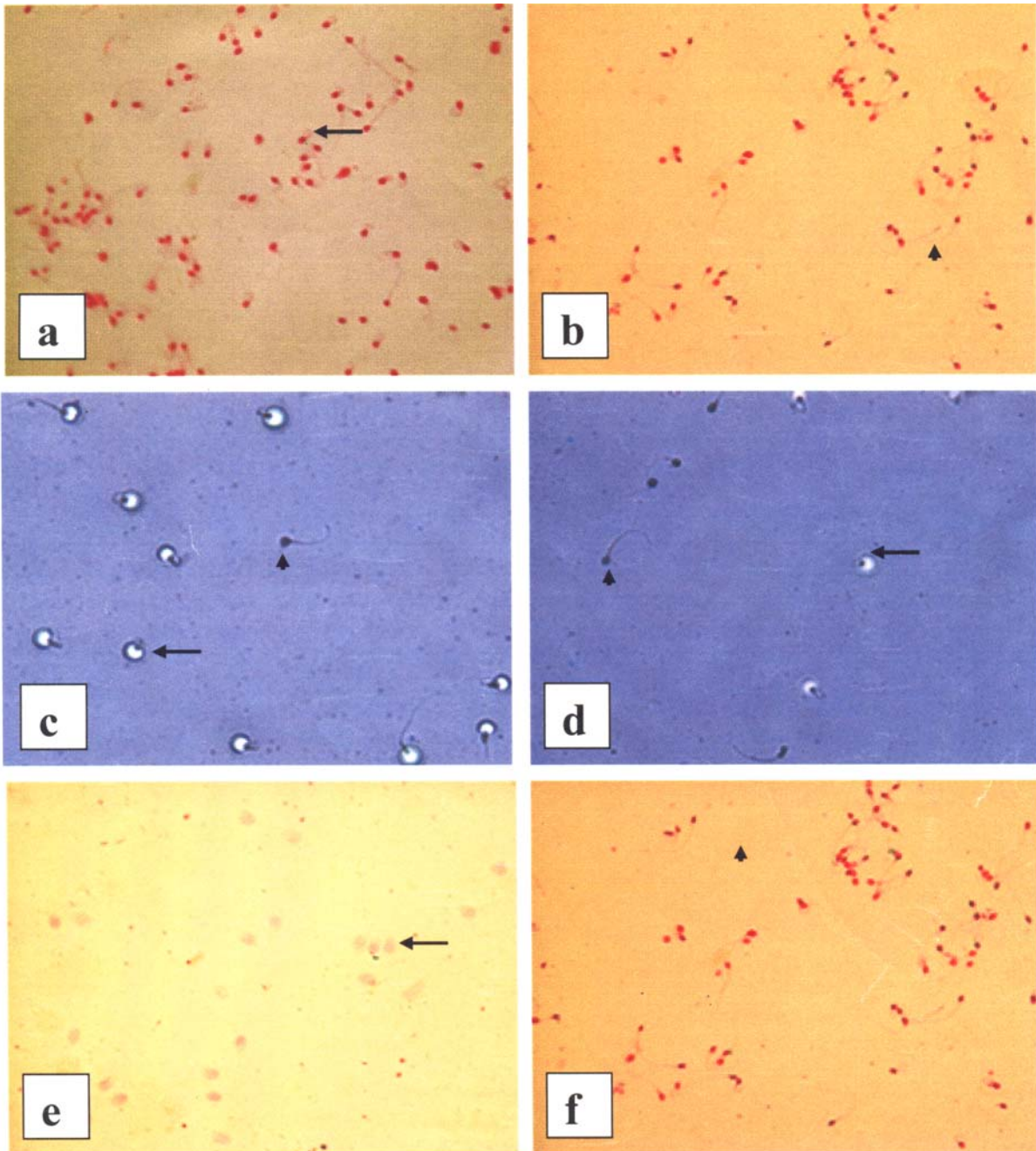


Fig. 1 : Sperm function tests of human subject from control and repeated abortion group. Large number of spermatozoa showing positive response (arrow) for hypo-osmotic swelling test in the form of tail bending (a), acrosomal reaction test with formation of halo surrounding sperm head (c) and nuclear chromatin decondensation in the form of swelled head (e) in control group compared to more negative response (arrow head) in repeated abortion group (b, d & f respectively). (n=75 for RPL group and 20 for control group, x400).

Other functional test done was Nuclear condensation and decondensation test. Significant decrease in nuclear chromatin to condense *in vitro* and sperm head abnormality has been reported in 32 men whose wife had more than three abortions (17). The structural study had shown defect of chromatin condensation and irregular nuclei with vacuoles. Loss of chromatin integrity as a possible contributing factor from males leading to early pregnancy loss has been pointed out (17). In this study mean NCD score of the RPL group was below normal range and subnormal scores were present in 31(41.3%) subjects. Photographs of NCD test in the control and RPL group are depicted in Figure 1e and 1f respectively.

It was observed that all the three test scores (HOS, NCD and AR) were significantly lower in the RPL group as compared to the control group ($P=0.000$). This seems logical as most of them presented with secondary infertility after three or more pregnancy losses.

Elevated concentration of white blood cells (WBC) in semen, termed leukocytospermia have been associated with infertility, poor semen parameters and decreased sperm function (18). Male accessory gland infection (MAGI) is associated with inflammatory changes in accessory organs leading to leukocytospermia. This produces oxidative stress which causes varying degrees of impairment of seminal parameters. Appropriate treatment with antibiotics, anti-inflammatory drugs and anti-oxidants improves the seminal parameters. The prevalence of leukocytospermia among male infertility patients is approximately 10% to 20%. There is controversy on the significance of WBC in semen. Whereas some authors did not observe sperm damage in the presence of leukocytospermia, others

have found evidence that WBC damaged sperm function and hamster ovum penetration *in vitro* and were important prognostic factors for IVF-ET failure (19). Leucocytospermia may occur due to anti sperm immunity and enhanced clearance of defective spermatozoa. High concentration of CD 4+ and CD 8+ lymphocyte concentrations is present in semen from men whose partners have cellular immunity to sperm antigens and is associated with higher incidence of RPL. Sperm damage by WBC can be mediated by reactive oxygen species, proteases and cytokines. Furthermore, genital tract inflammation facilitates the formation of sperm antibodies. These patients can be treated by specific immunosuppressive or anti-cytosine therapies, which may be useful in cytokine, mediated recurrent spontaneous abortions. Culture and sensitivity study of semen should be done and any infection should be treated with appropriate antibiotics as mentioned above. In patients with increased W.B.C. count in semen, where both aerobic and anaerobic culture is sterile, pus cells may still be present in the semen as a result of oxidative stress/inflammatory process. Transrectal digital examination of the prostate may be done to rule out chronic inflammatory disease.

In this study, male partners of RPL couples were simultaneously evaluated to determine any association of male factor with RPL. Though this may not show a cause and effect relationship, but the highly significant association of the abnormal factors in the male may suggest a possible role in etiology. Besides routine semen analysis, sperm function tests may be an informative tool in cases of idiopathic RPL. We recommend that in all cases of RPL both the partners should be simultaneously evaluated and treated in order to achieve the desired outcome.

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