Clinical Chemistry 65:1
161–169 (2019)

Reduced Testicular Steroidogenesis and Increased Semen Oxidative Stress in Male Partners as Novel Markers of Recurrent Miscarriage

Channa N. Jayasena,[1,2,3] Utsav K. Radia,[1] Monica Figueiredo,[1,2] Larissa Franklin Revill,[3] Anastasia Dimakopoulou,[1,2] Maria Osagie,[3] Wayne Vessey,[2] Lesley Regan,[3] Rajendra Rai,[3] and Waljit S. Dhillo[1*]

BACKGROUND: Recurrent pregnancy loss, (RPL) affecting 1%–2% of couples, is defined as ≥3 consecutive pregnancy losses before 20-week' gestation. Women with RPL are routinely screened for etiological factors, but routine screening of male partners is not currently recommended. Recently it has been suggested that sperm quality is reduced in male partners of women with RPL, but the reasons underlying this lower quality are unclear. We hypothesized that these men may have underlying impairments of reproductive endocrine and metabolic function that cause reductions in sperm quality.

METHODS: After ethical approval, reproductive parameters were compared between healthy controls and male partners of women with RPL. Semen reactive oxygen species (ROS) were measured with a validated inhouse chemiluminescent assay. DNA fragmentation was measured with the validated Halosperm method.

RESULTS: Total sperm motility, progressive sperm motility, and normal morphology were all reduced in the RPL group vs controls. Mean ±SE morning serum testosterone (nmol/L) was 15% lower in RPL than in controls (controls, 19.0 ± 1.0; RPL, 16.0 ± 0.8; $P < 0.05$). Mean ±SE serum estradiol (pmol/L) was 16% lower in RPL than in controls (controls, 103.1 ± 5.7; RPL, 86.5 ± 3.4; $P < 0.01$). Serum luteinizing hormone and follicle-stimulating hormone were similar between groups. Mean ±SE ROS (RLU/sec/10^6 sperm) were 4-fold higher in RPL than in controls (controls, 2.0 ± 0.6; RPL, 9.1 ± 4.1; $P < 0.01$). Mean ±SE sperm DNA fragmentation (%) was 2-fold higher in RPL than in controls (controls, 7.3 ± 1.0; RPL, 16.4 ± 1.5; $P < 0.0001$).

CONCLUSIONS: Our data suggest that male partners of women with RPL have impaired reproductive endocrine function, increased levels of semen ROS, and sperm DNA fragmentation. Routine reproductive assessment of the male partners may be beneficial in RPL.

Recurrent pregnancy loss (RPL[4]; recurrent miscarriage) may be defined as the loss of 3 or more consecutive pregnancies before 20 weeks' gestation, and affects 1%–2% of couples *(1, 2)*. Women with RPL are routinely screened for etiological factors such as antiphospholipid syndrome and thrombophilia *(3, 4)*. However, approximately 50% of RPL have been reported as idiopathic *(2, 5)*, which precludes development of targeted therapies. It is therefore imperative to identify novel markers associated with the pathogenesis of RPL to improve the management of affected couples.

Sperm DNA plays a critical role in placentation *(6)*, so it is biologically plausible that impairments in male reproductive function could increase the risk of RPL. Recent studies suggest that male partners affected by RPL have impaired sperm quality with reduced total motility and morphology *(7)* and increased sperm DNA damage *(8–12)*; however, the reasons underlying are not well understood. High levels of intratesticular synthesis of testosterone are required for spermatogenesis. Therefore, impairment of the reproductive endocrine axis could feasibly impair sperm function in male partners of women with RPL. Reactive oxygen species (ROS) are unstable metabolic by-products containing unpaired outer shell electrons, causing oxidative cellular damage *(12)*. Spermatozoa and semen polymorphonuclear leukocytes are

[1] Section of Investigative Medicine, Department of Medicine, Imperial College London, Hammersmith Hospital, London, UK; [2] Department of Andrology, Hammersmith Hospital, London, UK; [3] Department of Obstetrics & Gynecology, St. Mary's Hospital, London, UK.
* Address correspondence to this author at: Department of Investigative Medicine, 6th Floor Commonwealth Building, Imperial College London, Hammersmith Hospital, London W12 0NN. Fax 44-208-383-3142; e-mail w.dhillo@imperial.ac.uk.
Disclaimer: The views expressed are those of the authors and not necessarily those of the funders, the NHS, the NIHR, or the Department of Health.

Received March 8, 2018; accepted September 17, 2018.
DOI: 10.1373/clinchem.2018.289348

[4] Nonstandard abbreviations: RPL, recurrent pregnancy loss; RLUs, relative light units; ROS, reactive oxygen species; DFI, DNA fragmentation index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AUC, area under the curve.

both sources of ROS generation. ROS, therefore, have the potential to impair sperm function and cause sperm DNA damage.

We hypothesized that male partners of women with RPL have significant abnormalities in reproductive endocrine and metabolic function that may impair sperm quality, when compared with the general male population. We therefore investigated serum levels of reproductive hormones, semen ROS, sperm DNA fragmentation, and sperm function in men affected and unaffected by RPL in a female partner.

Methods

PARTICIPANT RECRUITMENT AND SAMPLE COLLECTION

Ethical approval was granted by the West London and GTAC Local Research Ethics Committee (Ref 14/LO/1038), and the study was performed in accordance with the Declaration of Helsinki. The study protocol is summarized in Fig. 1 in the online Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue1. Cases were recruited from the recurrent miscarriage clinic at St. Mary's Hospital, between September 2016 and May 2017. RPL was defined by the Royal College of Obstetrics and Gynaecologists criteria *(5)*. Exclusion criteria were history of anemia, current symptoms of genitourinary tract infection, alcohol excess, active treatment for severe systemic disease, antioxidant nutritional supplement use within the previous 6 months, recent febrile illness, and female cause of RPL. Healthy male controls were recruited through local advertisements and completed a questionnaire to screen for conditions impairing their fertility, including the following: testicular surgery; orchidopexy; varicocele; history of systemic illness or sexually transmitted infection; medications; smoking; recreational drug use. Following informed consent, participants attended a single study visit to complete a questionnaire, undergo height and weight measurement, and provide semen and blood samples. Five study participants were excluded after the first study visit owing to excess alcohol intake >21 U/week. Two participants were excluded following study recruitment owing to azoospermia and active hepatitis B virus infection. To enable age-matched comparisons, subanalyses were performed between the Recurrent Miscarriage Clinic (RMC) group (n = 50) and all control participants more than 30 years of age (n = 33; mean age 36.4 ± 0.9, $P = 0.43$ vs RMC group).

SEMEN ANALYSIS

All samples were analyzed within the Department of Andrology, Hammersmith Hospital, UK, according to WHO 2010 guidelines and UK NEQAS accreditation *(13)*. All samples were produced on site following 2–7 days of sexual abstinence and incubated at 36 ± 1 °C for liquefaction, up to 60 min before analysis. Sperm morphology was analyzed on Papanicolaou prestained slides with Kruger strict criteria. Reference intervals were as follows: volume ≥1.5 mL; sperm concentration ≥15 million/mL; total motility ≥40%; progressive motility ≥32%; normal morphology ≥4%; total motile count ≥20 million.

MEASUREMENT OF SEMINAL ROS LEVELS

ROS were measured according to a previously described method *(14)*. In brief, 400 μL of undiluted (native) semen was mixed with 100 μL of stock solution containing 5-mmol/L luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which is oxidized, resulting in chemiluminescence. Each sample was gently mixed immediately before taking luminometer readings (GloMax® Promega Corporation). Chemiluminescence was measured as relative light units per second (RLU/sec), as measured over 10 min at 1-min intervals, reported as a mean. Negative control was 400 μL of PBS with 100 μL of luminol working solution. Positive control contained 395 μL of PBS, 5 μL of 30% H_2O_2, and 100 μL of luminol working solution. Methods for the initial assay validation are described in Vessey et al. *(15)*. Inhouse validation was performed daily to ensure consistent positive and negative calibration. Before commencing the study, the assay had been run daily for over a year. All analysis runs contained negative and positive control samples. The reference interval for semen ROS was <3.8 RLU/sec/million sperm.

DNA FRAGMENTATION ANALYSIS

DNA fragmentation was measured with the Halosperm G2 kit (Halotech DNA SL) according to the method described by Fernández et al. in 2005 *(16, 17)*. In brief, semen samples were mixed with heated inert agarose and cooled on pretreated glass slides. A denaturant agent and lysis solution were added, followed by staining with eosin and thiazine. Slides were subsequently viewed under bright-field light microscopy to assess sperm chromatin dispersion. With this method, a large halo is seen around sperm without substantial DNA breakage, due to spreading DNA loops emerging from a central core. However, no halo or a minimal halo is seen around sperm containing fragmented DNA. The Halosperm test kit was internally validated in the Andrology department at Hammersmith Hospital. Data were accumulated from QC tested sample using lot numbers G21701026 and G21701026 and analyzed between December 1, 2016, and February 23, 2017. Negative internal QC imprecision based on 28 analyses resulted in a CV of 2.6%. Positive IQC imprecision based on 64 analyses resulted in a CV of 2.7%. Samples with a DNA fragmentation index (DFI) <15% were considered normal, as directed by the kit *(18)*.

ENDOCRINE BIOCHEMISTRY

Morning blood samples were analyzed for serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, testosterone, and sex hormone–binding globulin in the clinical biochemistry department of Charing Cross Hospital, by use of the automated immunoassay platforms under UK Accreditation System standards of quality control and reporting. Reference intervals were as follows: LH, 2–12 IU/L; FSH, 1.7–8 IU/L; estradiol, <190pmol/L; sex-hormone–binding globulin, 15–55nmol/L; testosterone, 10–30nmol/L; free androgen index, 30–150.

STATISTICAL ANALYSIS

Data analysis was performed with GraphPad Prism v.7. Quantitative data were assessed for normality with the D'Agostino–Pearson normality test, followed by appropriate parametric (unpaired *t* test) or nonparametric (Wilcoxon rank-sum test) analysis. Group comparisons with respect to categorical variables were performed with the Fisher exact test or chi-squar test. All hypothesis testing was 2 tailed; $P < 0.05$ was considered statistically significant. Data are presented as either mean (SE) of mean (SE) or median and interquartile range, as applicable.

Table 1. Clinical characteristics of participants.[a]

Parameter	Controls (*n* = 63)	Partners of women with RPL (N = 50)[b]
Age, years	30.8 ± 1.0	37.3 ± 0.7[c]
BMI, kg/m^2	24.8 ± 0.4	27.6 ± 0.6[c]
Ethnicity		
White	43	34
Asian, Indian	5	5
Asian, Other	6	3
Afro-Caribbean	4	3
Other	5	5
Smoker, %	14	12
Alcohol, %	73	66
Alcohol intake (units/week)[d]	11.4	13.6
Previous children	9	18

[a] Data for age and body mass index presented as mean ± SE.
[b] RPL, recurrent pregnancy loss; BMI, body mass index.
[c] $P < 0.05$, vs with healthy controls, with unpaired Student *t* test or Wilcoxon rank-sum test.
[d] Alcohol intake presented as mean.

Results

CLINICAL CHARACTERISTICS OF MALE PARTNERS OF WOMEN WITH RPL

Controls and the RPL group had similar clinical characteristics with regard to ethnicity, smoking, and alcohol intake (Table 1). Furthermore, the RPL group had no apparent increase in exposure to comorbidities known to be associated with seminal ROS generation, including genitourinary diseases such as sexually transmitted infection, orchidopexy, or varicocele (see Table 1 in the online Data Supplement). Mean age and body mass index were higher in the RPL group than in the controls. However, neither age nor body mass index were associated with seminal ROS levels, sperm DNA fragmentation, serum testosterone, or serum estradiol in the control or RPL groups (see Fig. 2 and Table 2 in the online Data Supplement). Nine of the control group and 18 of the RPL group had fathered children previously (Table 1).

REPRODUCTIVE HORMONE PROFILING OF MEN WITH RPL

Serum reproductive hormone levels in both groups are shown in Table 2. Levels of serum morning testosterone were approximately 15% lower in the RPL group than in the control group (mean ±SE serum testosterone in nmol/L: control, 19.0 ± 1.0; RPL, 16.0 ± 0.8; $P < 0.05$). Furthermore, levels of serum estradiol (which is predominantly synthesized in the testes with testosterone) were 16% lower in the RPL group than in the control group (mean ±SE serum estradiol in pmol/L: 103.1 ± 5.7, control; 86.5 ± 3.4, RPL, $P < 0.01$). Serum levels of LH were lower in the RPL group than in the controls, but this difference was nonsignificant (mean ±SE serum LH in iU/L: control, 3.9 ± 0.7; RPL, 2.7 ± 0.2; $P = 0.10$). Serum FSH levels were similar in both groups (mean ±SE serum FSH in iU/L: control, 3.3 ± 0.2; RPL, 3.6 ± 0.2; $P = 0.30$). Sex hormone–binding globulin levels were similar between men with RPL and healthy controls.

Table 2. Endocrine parameters of participants.[a]

Parameter	Controls (*n* = 63)	Partners of women with RPL (*n* = 50)[b]
LH, IU/L	3.9 ± 0.7	2.7 ± 0.2
FSH, IU/L	3.3 ± 0.2	3.6 ± 0.2
Estradiol, pmol/L	103.1 ± 5.7	86.5 ± 3.4[c]
SHBG, nmol/L	32.7 ± 1.6	29.4 ± 1.3
Testosterone, nmol/L	19.0 ± 1.0	16.0 ± 0.8[d]
Free androgen index	60.8 ± 2.8	56.7 ± 2.6

[a] Free androgen index calculated as (serum testosterone × 100)/SHBG. Data presented as mean ± SE.
[b] RPL, recurrent pregnancy loss; LH, luteinizing hormone; FSH, follicle stimulating hormone; SHBG, sex hormone binding globulin.
[c] $P < 0.01$.
[d] $P < 0.05$.

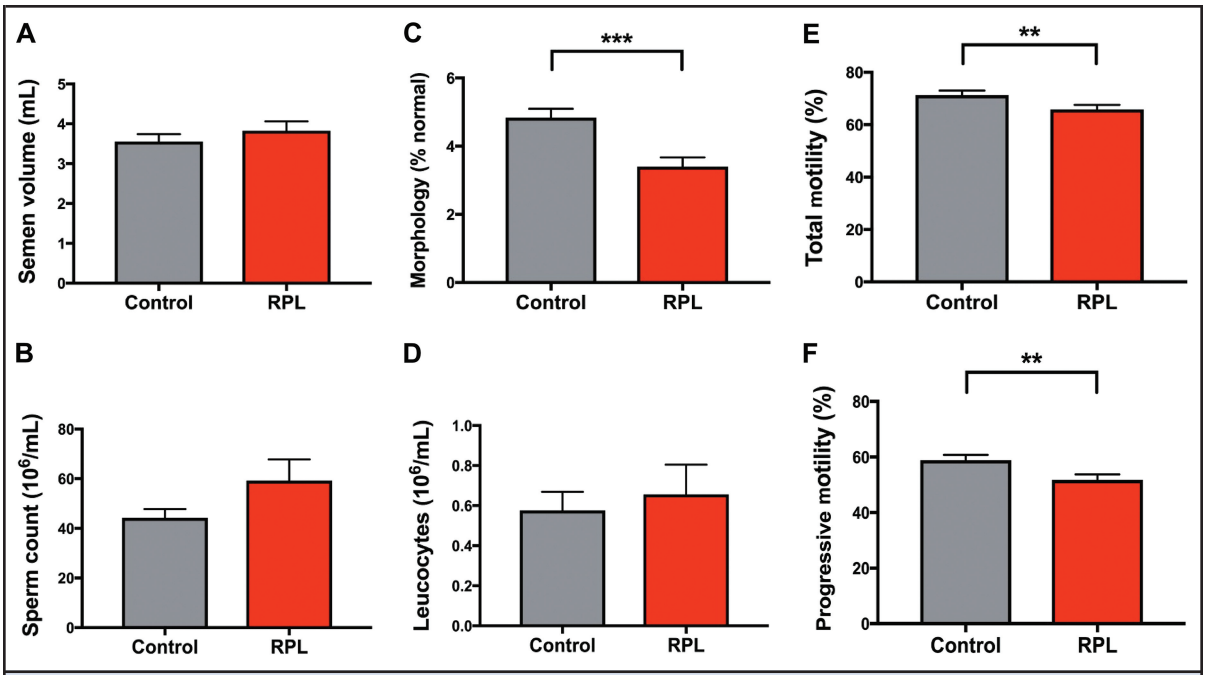

Fig. 1. Sperm characteristics of male partners of women with recurrent pregnancy loss.

Bar graphs compare semen volume (A), sperm count (B), normal sperm morphology (C), leukocyte count (D), total sperm motility (E), and progressive sperm motility (F) in recurrent pregnancy loss (RPL) group *versus* control group. Data are mean ±SE. $^{**}P < 0.01$; $^{***}P < 0.001$.

SPERM FUNCTION IN MEN WITH RPL

Semen analysis parameters are summarized in Fig. 1 and see Table 1 in the online Data Supplement. Reduced levels of sperm motility, progressive motility, sperm morphology, ejaculate volume, and sperm count are established markers of the failure to conceive (i.e., infertility) in affected couples *(13)*. We were therefore interested in investigating whether these factors also were reduced in male partners of women with RPL. Ejaculate volume and sperm count were not significantly different between the study groups. Men in the RPL group had significantly fewer motile sperm than controls (mean ±SE total percentage of total sperm motility: control, 65.8 ± 1.7; RPL, 71.3 ± 1.7; $P < 0.01$) and fewer progressively motile sperm than controls (mean ±SE percentage of progressively motile sperm: control, 58.9 ± 1.8; RPL, 51.8 ± 2.0; $P < 0.01$). The RPL group had a significantly lower proportion of morphologically normal sperm according to WHO criteria (reference range is 4% or above) than controls (% sperm ±SE with normal morphology: control, 5.0 ± 0.3; RPL, 3.0 ± 0.3; $P < 0.001$). Latent genitourinary infection may cause sperm damage through semen ROS generation; it is therefore important to note that levels of semen leukocytes (which are an important source of semen ROS) were similar between control and RPL groups (Fig. 1D; see Fig. 3D in the online Data Supplement).

MOLECULAR SPERM CHARACTERISTICS IN MEN WITH RPL

Having observed that male partners of women with RPL had significant impairments in sperm function, we investigated whether these men also had abnormally increased levels of semen oxidative stress and sperm DNA damage, which are known to impair sperm function. Mean semen ROS levels were more than 4-fold higher in the RPL group than either controls (mean semen ROS in RLU/sec/10^6: control, 2.0 ± 0.6; RPL, 9.1 ± 4.1; $P < 0.01$; Fig. 2A). Male partners of women with RPL were 4-fold more likely to have abnormally increased levels of semen ROS than either controls [proportion (%) of men with semen ROS above reference interval: control, 5/63 (7.9); RPL, 16/50 (32.0); $P < 0.0001$; Fig. 2B].

Mean levels of sperm DNA fragmentation were more than 2-fold higher in the RPL group than in either controls (mean DFI: control, 7.3 ± 1.0; RPL, 16.4 ± 1.5; $P < 0.0001$; Fig. 2C). Furthermore, male partners of women with RPL were 4-fold more likely to have abnormally increased levels of sperm DNA fragmentation than controls [proportion (%) of men with DFI above refer-

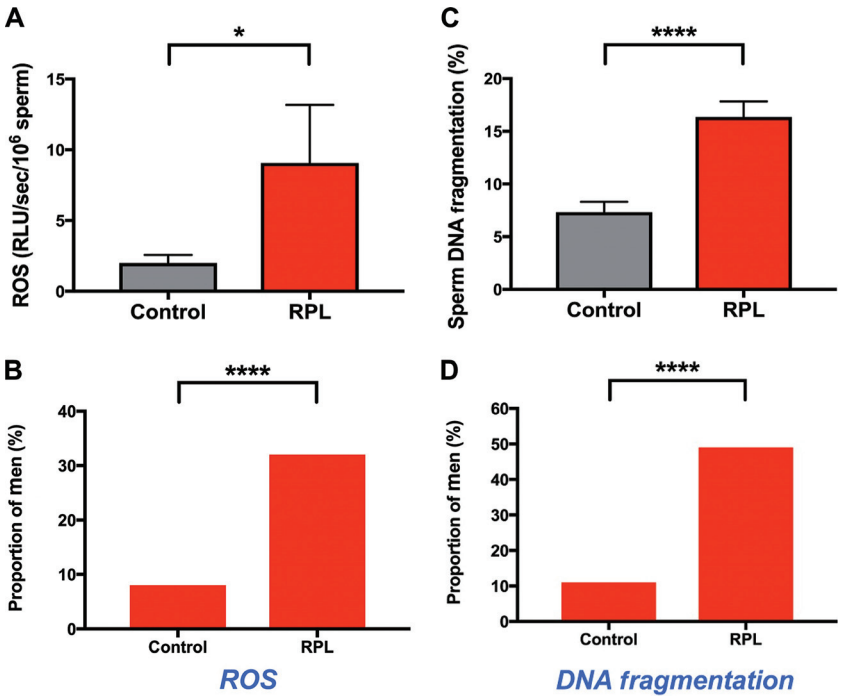

Fig. 2. Sperm DNA damage and oxidative stress in male partners of women with recurrent pregnancy loss.

Bar graphs compare semen reactive oxygen species (ROS; A) and sperm DNA fragmentation (B) in recurrent pregnancy loss (RPL) versus controls. Data are mean +SE. Histograms compare the proportion of participants with increased semen ROS (C) and sperm DNA fragmentation (D). *$P < 0.01$; ****$P < 0.0001$.

ence range: control, 7/63 (11.1); RPL, 22/50 (44.0); $P < 0.0001$; Fig. 2D].

COMPARING REPRODUCTIVE PARAMETERS BETWEEN CONTROLS AND MEN WITH RPL

Of the investigated markers, sperm morphology, semen ROS, and sperm DNA fragmentation had the greatest mean or median difference (>30%) between controls and RPL cases. We therefore investigated the potential of these factors to distinguish men with ROS from controls in the study, using ROC analyses (Fig. 3). ROC analysis suggested that sperm morphology, semen ROS, and sperm DNA fragmentation each discriminated significantly between controls and men with ROS. The greatest discriminator between control and ROS groups was sperm DNA fragmentation, which had an ROC curve area under the curve (AUC) value of 81% ($P < 0.0001$ vs line of nondiscrimination).

SUBANALYSIS WITH CONTROL PARTICIPANTS OLDER THAN 30 YEARS

Similar patterns in hormone analysis were observed when analysis was restricted to the 30 control participants older than 30 years of age; mean serum testosterone and estradiol were higher than in the RPL group, although neither comparison reached statistical significance (see Table 3 in the online Data Supplement). No significant differences in semen volume, sperm concentration, total motility, or progressive motility were observed (see Fig. 3 in the online Data Supplement). However, the RPL group had a significantly lower proportion of morphologically normal sperm than age-matched controls (% sperm ±SE with normal morphology: controls >30 years, 5.0 ± 0.4; RPL, 3.0 ± 0.3; $P < 0.001$). Mean semen ROS levels were more than 4-fold higher in the RPL group than in age-matched controls (mean ±SE semen ROS in RLU/sec/10^6: controls >30 years, 2.0 ± 0.8; RPL, 9.1 ± 4.1, $P < 0.05$; see Fig. 4A in the online Data Supplement). Mean ±SE levels of sperm DNA fragmentation were more than 2-fold higher in the RPL group than in age-matched controls (mean DFI: controls >30 years, 7.7 ± 7.0; RPL, 16.4 ± 1.5, $P < 0.0001$; see Fig. 4C in the online Data Supplement). Furthermore, the RPL group were 3-fold more likely to have abnormally increased ROS or sperm DNA fragmentation than age-matched controls (see Fig. 4, B and D, in the online Data Supplement). ROC curve analyses suggested that total motility, morphology, ROS, and DNA fragmentation were all dis-

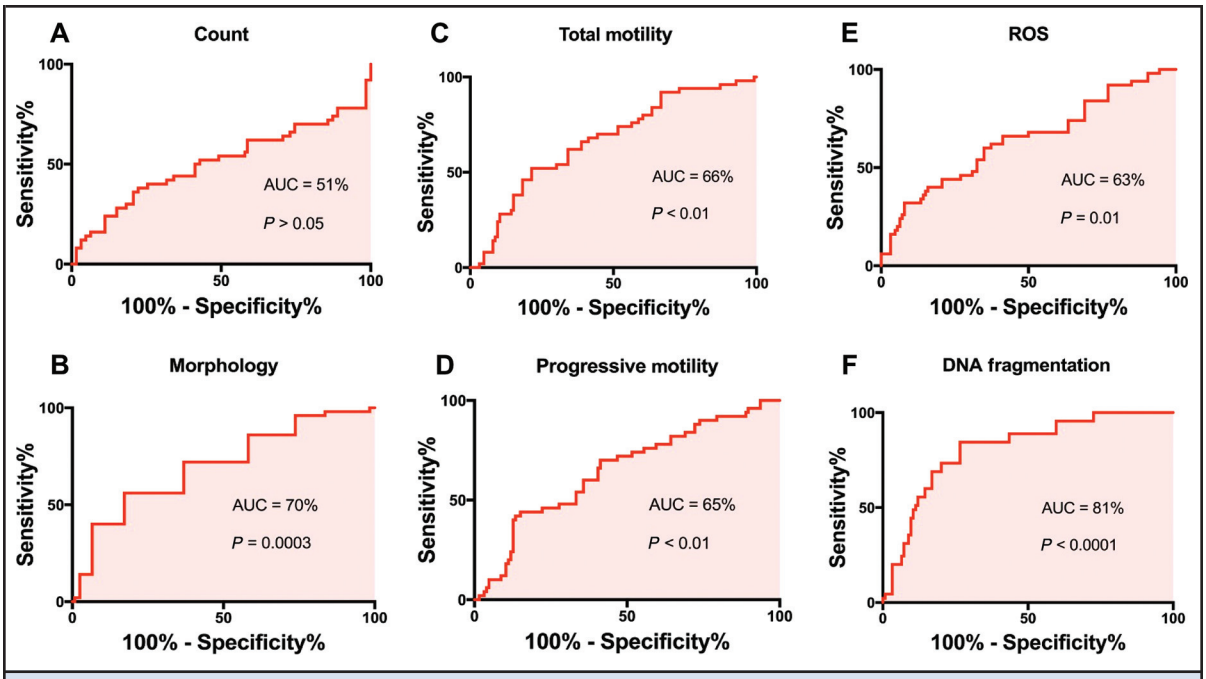

Fig. 3. Receiver operation characteristics of reproductive parameters in male partners of women with recurrent pregnancy loss.
ROC analyses for sperm count (A), normal sperm morphology (B), total sperm motility (C), progressive sperm motility (D), semen reactive oxygen species (E), and sperm DNA fragmentation (F). Area under curve (AUC) values are presented for each parameter.

criminatory between controls older than 30 years and the RPL group (see Fig. 5 in the online Data Supplement); the greatest discriminator was sperm DNA fragmentation, which had an ROC curve AUC value of 79% ($P <$ 0.0001 vs line of nondiscrimination).

Discussion

No underlying cause can be found in half of all couples with RPL *(19)*, and current guidelines do not recommend the routine diagnostic investigation of male partners. We have performed the first study evaluating reproductive endocrine and metabolic sperm function in male partners of women with RPL. We report that male partners of women with RPL have reduced concentrations of serum testosterone and estrogen when compared with controls, which warrant further investigation. We also report markedly increased levels of semen ROS and sperm DNA fragmentation and reduced functional sperm parameters when compared with control participants. Our data suggest that male partners may benefit from diagnostic assessment in the routine management of couples with RPL.

No previous study has investigated endocrine function in male partners of women with RPL. Intratesticular production of testosterone is critical for the final stages of spermatogenesis, and testosterone deficiency is associated with male infertility *(20)*. Testosterone and estrogen synthesis from testicular Leydig cells is driven by the pulsatile secretion of LH from the pituitary gland *(20)*. We observed that testosterone and estradiol were reduced by 15% and 16%, respectively, in the RPL group when compared with controls, although these differences became nonsignificant when excluding controls older than 30 years. Primary hypogonadism (i.e., low testicular production of testosterone) tends to increase serum LH levels owing to reduced feedback. However, levels of serum LH were not increased in the RPL when compared with the control group, which might be consistent with a partial secondary hypogonadism due to hypothalamopituitary impairment. It would be important to confirm these data with more detailed endocrine and metabolic phenotyping in a large age-matched cohort.

Paternally imprinted genes play an important role in the regulation of placentation, which is critical to embryo viability *(21)*. This characteristic is illustrated by observing that mouse embryos from 2 paternal genomes (androgenotes) have deficient embryo formation but relatively preserved placental formation; conversely, mouse embryos from 2 maternal genomes (parthenogenotes) have deficient placental formation with relative sparing of embryo formation *(6)*. It is therefore clinically important to investigate whether novel diagnostic markers of sperm function may cause miscarriage. Furthermore,

these studies may enable an improved understanding of how the paternal genome regulates placentation and embryo development. Multiple studies have reported that sperm DNA fragmentation is increased in male partners affected by RPL when compared with unaffected men *(7–12, 22, 23)*, although failure to demonstrate this relationship has also been reported *(24)*. Our results are in agreement with these previous studies by observing that men with RPL had a much higher risk of increased sperm DNA fragmentation than controls. The mechanisms underlying increased sperm DNA fragmentation and reduced sperm function in couples with RPL have been poorly understood. Previous studies have implicated oxidative stress as a major cause of sperm DNA fragmentation *(12, 25, 26)*. We used a previously described and validated chemiluminescent assay using luminol, which detects both intracellular- and extracellular-produced ROS, including superoxide, hydrogen peroxide, hydroxyl, and hypochlorite *(15)*. We observed that mean levels of ROS were 4-fold higher in men with RPL than in controls. Furthermore, one-third of men with RPL had increased ROS, whereas only 10% of controls had increased ROS. Mean levels of seminal leukocytes were similar in men with RPL and controls. However, some of the included participants may have had asymptomatic infection, which may occur in the absence of leukocytospermia. It would be interesting to investigate if the relatively high levels of semen ROS observed in men with RPL are sperm or leukocyte derived. Varicocele and genitourinary infection are 2 major known causes of semen ROS elevation; we did not observe any increased risk of varicocele or genitourinary infection in men with RPL when compared with controls, although our sample size was small. Interestingly, a randomized controlled trial by Ghanaie et al. observed that varicocele repair significantly improved pregnancy rates and reduced miscarriage risk when compared with nontreatment of varicocele in couples with recurrent miscarriage *(27)*. Furthermore, a retrospective analysis by Negri et al. has suggested that miscarriage rates were similar to the general population following varicocele repair for the male partners of couples with infertility *(28)*. In addition, Kanakas et al. performed a case–control study in couples undergoing IVF after the male partner had been tested for seminal *Ureaplasma urealyticum* infection; abortion rates following IVF were significantly higher in the infected group than in the noninfected group *(29)*. Future studies should investigate whether male partners affected by RPL are more likely to have varicocele and genitourinary infection than other men. It would also be interesting to further investigate whether treatment of varicocele and genitourinary infection in male partners of women with RPL reduces the risk of future miscarriage. Several ongoing studies are investigating whether the administration of dietary or pharmaceutical antioxidants in men who had a complete clinical investigation, excluding infectious or surgical causes of increased ROS and DNA damage, could be used to improve clinical outcomes in couples with infertility *(30, 31)*. It is therefore possible that seminal ROS measurement has diagnostic and therapeutic potential for couples with RPL, which warrants further investigation.

We finally investigated the performance of the 3 most promising potential diagnostic factors to distinguish men with RPL from controls by using ROC analysis. All factors had significant diagnostic performance (i.e., ROC AUC significantly different from the line of nondiscrimination), although sperm DNA was the best-performing test to distinguish men with RPL from controls. Large prospective cohort studies are required to further investigate if sperm indices influence the risk of miscarriage in couples.

It is important to consider limitations of the study. Since commencement of the current study, new guidelines have been released by the European Society of Human Reproduction and Embryology *(32)*, defining RPL as 2 rather than 3 consecutive miscarriages. It is therefore important to consider that accurate comparison with future studies may be limited by heterogeneity in the definition of RPL. Several methods of sperm DNA fragmentation measurement are available. We used the Halosperm method, which is as an index of abnormal chromatin packaging rather than a direct assessment of DNA damage itself *(33)*. It would be interesting to compare results from the current study using Halosperm with other methods such as SCSA, TUNEL, and COMET, which more directly measure sperm DNA damage and have higher reported sensitivities for detecting sperm DNA fragmentation *(22, 34)*. It is important to consider that mean concentrations of serum testosterone and estradiol in the RPL group were within the reference interval for men. Furthermore, levels of sperm DNA fragmentation and semen ROS associated with male infertility are usually much higher than the mean levels reported in the RPL group of the current study *(35, 36)*. Further studies are required to determine if the observed abnormalities of endocrine and sperm function in RPL translate to pathogenic changes leading to pregnancy loss. Finally, we chose not to stipulate fatherhood as an inclusion criterion within our control group; it is therefore plausible that a small minority of our controls might later experience reproductive disorders. Selecting fathers as controls may have increased the power of the current study. However, one could argue that our choice of control group with unproven fertility increases the robustness of our conclusions, by revealing genuine abnormalities in the reproductive physiology of men affected by RPL when compared with the general male population, rather than just fathers *(37)*.

In summary, DNA fragmentation and ROS are recently identified markers of male reproductive dysfunction *(38, 27)*. We report that male partners of women with RPL have multiple abnormalities in reproductive function including testicular steroidogenesis, sperm function, sperm DNA damage, and semen oxidative stress. Our data have important implications for the management of couples with RPL. Endocrine and molecular sperm profiling may offer a potential novel approach to stratifying future miscarriage risk. Further studies will investigate whether endocrine and molecular sperm abnormalities may be ameliorated by lifestyle, dietary interventions, and hormonal interventions, to optimize chances of successful conception in couples with RPL.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements:* (*a*) *significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data;* (*b*) *drafting or revising the article for intellectual content;* (*c*) *final approval of the published article; and* (*d*) *agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

C.N. Jayasena, financial support, statistical analysis; U.K. Radia, statistical analysis, administrative support, provision of study material or patients; M. Figueiredo, statistical analysis, administrative support, provision of study material or patients; L.F. Revill, administrative support, provision of study material or patients; M. Osagie, administrative support; L. Regan, provision of study material or patients; R. Rai, provision of study material or patients.

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: The Section of Investigative Medicine is funded by grants from the MRC, BBSRC and is supported by the NIHR Imperial Biomedical Research Centre Funding Scheme. C.N. Jayasena, NIHR Post-Doctoral Fellowship; U.K. Radia, SRF Vacation Grant; M. Figueiredo, Imperial College Healthcare Charity / Imperial BRC; W.S. Dhillo, NIHR Research Professorship.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Acknowledgments: The authors are indebted to all the staff of The Recurrent Miscarriage Team at St. Mary's Hospital, London, and staff members within Department of Andrology, Hammersmith Hospital, UK, for their help with patient recruitment and sample analysis, respectively.

References

1. Stirrat GM. Recurrent miscarriage. Lancet 1990;336:673–5.
2. Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. Fertil Steril 1996;66:24–9.
3. Devi Wold AS, Pham N, Arici A. Anatomic factors in recurrent pregnancy loss. Semin Reprod Med 2006;24:25–32.
4. Byrn FW, Gibson M. Infectious causes of recurrent pregnancy loss. Clin Obstet Gynecol 1986;29:925–40.
5. Royal College Obstetricians and Gynaecologists (RCOG). The investigation and treatment of couples with recurrent first-trimester and second-trimester miscarriage. R Coll Obstet Gynaecol 2011;17:1–18. https://www.rcog.org.uk/globalassets/documents/guidelines/gtg_17.pdf (Accessed September 2018).
6. Brevini TAL GP. Gametogenesis, early embryo development and stem cell derivation. New York (NY): Springer; 2013.
7. Imam SN, Shamsi MB, Kumar K, Deka D, Dada R. Idiopathic recurrent pregnancy loss: role of paternal factors; a pilot study. J Reprod Infertil 2011;12:267–76.
8. Henkel RR. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. Asian J Androl 2011;13:43–52.
9. Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, et al. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 2004;81:965–72.
10. Ribas-Maynou J, Garcia-Peiro A, Abad C, Amengual MJ, Navarro J, Benet J. Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. Hum Reprod 2012;27:652–8.
11. Simon L, Lutton D, McManus J, Lewis SEM. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. Fertil Steril 2011;95:652–7.
12. Gupta S, Agarwal A, Banerjee J, Alvarez JG. The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: a systematic review. Obstet Gynecol Surv 2007;62:334–5.
13. WHO laboratory manual for the examination and processing of human semen. http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/ (Accessed September 2018).
14. Homa ST, Vessey W, Perez-Miranda A, Riyait T, Agarwal A. Reactive Oxygen Species (ROS) in human semen: determination of a reference range. J Assist Reprod Genet 2015;32:757–64.
15. Vessey W, Perez-Miranda A, Macfarquhar R, Agarwal A, Homa S. Reactive oxygen species in human semen: validation and qualification of a chemiluminescence assay. Fertil Steril 2014;102:1576–83.e4.
16. Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, et al. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 2005;84:833–42.
17. Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, et al. Halosperm is an easy, available, and cost-effective alternative for determining sperm DNA fragmentation. Fertil Steril 2005;84:860.
18. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, et al. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999;14:1039–49.
19. Ford HB, Schust DJ. Recurrent pregnancy loss: etiology, diagnosis, and therapy. Rev Obstet Gynecol 2009;2:76–83.
20. Fauser BC, Smals AG, Rolland R, Dony JM, Doesburg WH, Thomas CM. Testicular steroid response to continuous and pulsatile intravenous luteinizing hormone-releasing hormone administration in normal men. Andrologia 1986;18:89–96.
21. Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum Reprod 2004;19:611–5.
22. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 2012;27:2908–17.
23. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril 2014;102:998–1005.e8.
24. Coughlan C, Clarke H, Cutting R, Saxton J, Waite S, Ledger W, et al. Sperm DNA fragmentation, recurrent implantation failure and recurrent miscarriage. Asian J Androl 2015;17:681–5.
25. Puscheck EE, Jeyendran RS. The impact of male factor on recurrent pregnancy loss. Curr Opin Obstet Gynecol 2007;19:222–8.
26. Lewis SEM, John Aitken R, Conner SJ, Iuliis G De, Evenson DP, Henkel R, et al. The impact of sperm DNA damage in assisted conception and beyond: recent advances in diagnosis and treatment. Reprod Biomed

Online 2013;27:325–37.
27. Mansour Ghanaie M, Asgari SA, Dadrass N, Allahkhah A, Iran-Pour E, Safarinejad MR. Effects of varicocele repair on spontaneous first trimester miscarriage: a randomized clinical trial. Urol J 2012;9:505–13.
28. Negri L, Levi-Setti PE. Pregnancy rate after varicocele repair: how many miscarriages? J Androl 2011;32:1.
29. Kanakas N, Mantzavinos T, Boufidou F, Koumentakou I, Creatsas G. Ureaplasma urealyticum in semen: is there any effect on in vitro fertilization outcome? Fertil Steril 1999;71:523–7.
30. Ko EY, Sabanegh ESJ, Agarwal A. Male infertility testing: reactive oxygen species and antioxidant capacity. Fertil Steril 2014;102:1518–27.
31. Showell MG, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. Antioxidants for male subfertility. Cochrane Database Syst Rev 2014;(12): CD007411.
32. The ESHRE Guideline Group on RPL. ESHRE guideline: recurrent pregnancy loss, Human Reproduction Open 2018;2:hoy004. https://www.eshre.eu/Guidelines-and-Legal/Guidelines/Recurrent-pregnancy-loss.aspx (Accessed September 2018).
33. Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59–66.
34. Simon L, Liu L, Murphy K, Ge S, Hotaling J, Aston KI, et al. Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. Hum Reprod 2014;29:904–17.
35. Agarwal A, Ahmad G, Sharma R. Reference values of reactive oxygen species in seminal ejaculates using chemiluminescence assay. J Assist Reprod Genet 2015;32:1721–9.
36. Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod 2007;22:174–9.
37. Zondervan KT, Cardon LR, Kennedy SH. What makes a good case–control study? Design issues for complex traits such as endometriosis. Hum Reprod 2002;17: 1415–23.
38. Cho CL, Agarwal A. Role of sperm DNA fragmentation in male factor infertility: a systematic review. Arab J Urol 2017;16:21–34.