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Original Article

Sperm DNA fragmentation abnormalities in men from couples with a history of recurrent miscarriage

Mikaela LEACH,[1] Robert J. AITKEN[2] and Gavin SACKS[1,3,4,5]★

[1]*University of New South Wales, Sydney, NSW, Australia,* [2]*Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW, Australia,* [3]*St George Hospital, Kogarah, Sydney, NSW, Australia,* [4]*Royal Hospital for Women, Randwick, NSW, Australia and* [5]*IVFAustralia, Sydney, NSW, Australia*

Background: Previous studies have described an association between sperm with DNA damage and a history of recurrent miscarriage (RM), although it is not clear whether there is benefit in screening for sperm DNA fragmentation and to what extent DNA fragmentation impacts upon RM.

Aims: To identify what proportion of couples experiencing RM are affected by DNA fragmentation abnormalities.

Materials and Methods: In this retrospective study, between 2008 and 2013, couples with a history of recurrent miscarriage (≥3 first trimester miscarriages) were investigated comprehensively for known causes (karyotype, uterine, antiphospholipid syndrome, thrombophilia) and also by semen analysis, including DNA fragmentation [sperm chromatin structure analysis (SCSA)]. Statistical analysis was performed on SPSS software with significance taken as $P < 0.05$.

Results: There were 108 couples with a median sperm DNA fragmentation index (DFI) of 9.50%. Normal levels were found in 70.5% of men (DFI < 15%), 23% had high levels (DFI 15–30%), and 6.5% had very high levels (DFI > 30%). Couples with otherwise unexplained recurrent miscarriage had significantly higher DFI than those with other causes identified on routine screening ($P = 0.012$).

Conclusions: In couples experiencing RM, 30% (32/108) of men had sperm with high levels of DNA fragmentation (DFI > 15%). This may be a contributing factor to the clinical syndrome of RM, and future clinical trials of therapies for these couples are warranted.

Key words: DNA Fragmentation, sperm, recurrent miscarriage, recurrent spontaneous abortion, DNA damage.

Introduction

Recurrent miscarriage (RM) is commonly defined as three or more successive miscarriages[1] and is experienced by 1–3% of women seeking pregnancy.[2] In about 50% of cases, the cause is unable to be identified[3] and this can leave couples anxious and uncertain.

Known causes of recurrent miscarriages include genetic, anatomical, endocrine, immune and thrombophilic[3,4] factors although in some cases, it is multifactorial. Historically, research has primarily explored the role that the female contributes to RM, with the male contribution most commonly attributed to karyotype abnormalities (e.g balanced translocation).

Abnormalities in standard semen analysis parameters such as count, motility and morphology have been associated with miscarriage, but studies have been inconsistent.[5] A newer test of sperm quality, DNA fragmentation, assesses DNA damage in sperm (single- or double-stranded breaks, produced either pre- or post-ejaculation).[6] DNA fragmentation testing is a more consistent measure of sperm quality than classical parameters such as motility, morphology and count.[7,8] The origins of DNA damage are not fully understood,[6] but the main mechanisms are believed to be oxidative stress, apoptosis and chromatin remodelling.[9] Causal factors include ageing, infection and smoking,[9,10] varicocele[11] or laptop use of Wi-Fi.[12] One of the commonest methods to assess DNA damage is the sperm chromatin structure assay (SCSA), which measures DNA's susceptibility to acid denaturation.[4] This test is reliable and relatively accessible in many andrology units.

Normal levels of DNA fragmentation are strongly associated with fertility[7,8] (in one study of prevalence using SCSA, 83.6% of fertile men had DNA fragmentation index (DFI) < 15%, 16.4% had DFI 15–30%, and none had DFI > 30%) and high levels

Correspondence: Gavin Sacks, University of New South Wales, 1601 Westfield Tower 2, 101 Grafton St, Bondi Junction, Sydney, NSW, 2022, Australia. Email: gsacks@ivf.com.au

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with infertility[13] (prevalence of unexplained infertility couples with males DFI 20–30% was 17.7%, and DFI > 30% was 8.4%). Numerous studies have shown an association between DNA fragmentation and RM, these have used varied definitions of RM, involved relatively low numbers of cases (n = 21–85), and none have investigated an Australian population.[2,3,5,14–19] The aim of this study was to define the prevalence of sperm DNA fragmentation abnormalities in couples from an Australian population suffering from recurrent miscarriage.

Materials and Methods

Study design and population

This was a retrospective study. Women were selected from an IVF Australia database for recurrent miscarriage (≥3 successive miscarriages) and 108 whose partners had DNA fragmentation testing were identified. Case notes from 2008 to 2013 were analysed to define patient characteristics and results of all recurrent miscarriage investigations, including karyotype (male and female), uterine and tubal anomalies, antiphospholipid syndrome (APS), thrombophilia, polycystic ovarian syndrome, insulin resistance, thyroid function, anti-Mullerian hormone, natural killer cells, sperm count, motility, morphology and DFI. Other baseline data such as age, height, weight, BMI, smoking and alcohol history, medical history, occupation, number of miscarriages, number of live births, treatment and outcome were recorded. Subjects had three or more successive miscarriages before presentation to the clinic. This study was approved by the IVF Australia R&D and Ethics Committee (no. 093).

DNA fragmentation (SCSA)

Semen samples were collected from multiple laboratories and diluted with TNE buffer (pH 7.4) to 3–4 M/mL, and two straws were stored and frozen in liquid nitrogen. Samples were analysed centrally. Thawed samples (50 µL) were combined with 100 µL of acid detergent (pH 1.2) for 30 sec. The sample was then stained with 300 µL of acridine orange (AO) staining solution (600 µL AO 1.0 mg/mL to 100 mL staining buffer pH 6.0) and rested for a total of three minutes.

Samples were run using a BD FACSCalibur flow cytometer, with an air-cooled argon laser and a red diode laser, recording 5000 events. A reference sample was used prior to testing and repeated every five samples to check accuracy of data. Each sample was run twice by an individual observer. Under AO, double-stranded DNA fluoresces green and single-stranded DNA fluoresces red. DNA fragmentation index (%DFI) was calculated by assessing the ratio of red to total fluoresced cells. This value was calculated, and if within 5% of repeated value, it was averaged and recorded.

Table 1 Demographics of the study population

	Females (n = 108)	Males (n = 108)
Age (years)	35.7 ± 4.9	37.8 ± 5.5
Nulliparous (%)	53.7	56.5
Number of live births	0.00 [0.00–1.00]	–
Number of recurrent miscarriages	3.00 [3.00–4.00]	–
Number of years trying	1.50 [1.00–2.00]	–
BMI (kg/m^2)	25.0 ± 3.5	–
IVF (prior to investigation) (%)	7.4	–
Concentration ($\times 10^6$/ml)	–	80.15 [47.90–115.40]
Motility (% progressive)	–	63.75 [57.5–72.00]
Morphology (% normal)	–	9.00 [5.00–16.00]
SCSA (% DFI)	–	9.50 [5.00–16.28]
Alcohol – None (%)	50	34.4
Moderate (%)	50	59.4
Heavy (%)	0	6.3
Smoking – Never (%)	93.8	78.1
Present (%)	1.9	18.8
Past (%)	0	3.1

Values are expressed as means ± SD or median [interquartile range].
Percentages are used for categorical variables.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics Standard Grad Pack 21.0. Categorical data were expressed as percentages and numerical data as mean± standard deviation or median (interquartile range). Statistical significance amongst two groups' was tested for using the nonparametric Mann–Whitney U-test, or three groups using the Kruskal–Wallis test. Correlations were performed on paired numerical data using the Pearson correlation coefficient. Cross-tabs were performed for qualitative analysis. Significance was defined as $P < 0.05$.

Results

This study included 108 couples with RM (Table 1). Males had a higher mean age than females (37.8 versus 35.7, $P = 0.003$), and male and female age was positively correlated ($r = 0.601$, $P < 0.001$). Mean female BMI was 25.0 and male BMI was not recorded. The median number of miscarriages was 3.0, with median of 1.5 years trying for pregnancy. Median values for semen parameters were within WHO classifications for count (15 million/mL), progressive motility (32%) and normal morphology (4%).[20] Alcohol use was more prevalent in males (59.4% moderate, 6.3% heavy) than in females (50% moderate, 0% heavy), similarly with smoking status, males (18.8% present, 3.1% past) and females (1.9% present, 0% past).

DNA fragmentation (SCSA)

Median SCSA was 9.50%. Normal levels (DFI 0–15%) were found in 70.5% of men, 23% had high levels (DFI 15–30%), and 6.5% had very high levels (DFI ≥ 30%). There was no significant different in age between the three groups.

No significant difference in SCSA was found between primary and secondary miscarriage groups, or between IVF or spontaneous conception groups, and there was no correlation with age, smoking or alcohol status demonstrated in this study. Increased DNA fragmentation index was weakly associated with lower sperm concentration ($r = -0.307$, $P = 0.009$), motility ($r = -0.335$, $P < 0.001$) and morphology ($r = -0.240$, $P = 0.017$).

After extensive investigation, 59/108 (54.63%) of couples had no known causes of RM (excluding sperm DNA fragmentation). This group had a significantly higher DNA fragmentation than the group of couples with an identifiable cause for RM ($P = 0.012$). DFI > 15% was found in 21/59 (35.6%) of those with unexplained cause of RM and only 11/49 (22.4%) of the explained group.

From the group with very high DFI (>30%), there were seven subjects, five of these were in the unexplained group. In this group, the average male age was 37 years and female age was 34.7 years.

Discussion

This study aimed to investigate the association between recurrent miscarriage and male sperm DNA fragmentation in an Australian population. We found that 23% of men had 'high' levels and 6.5% had 'very high' levels. This can be compared to a normal fertile population with 16.4% and 0%, respectively.[7] These data confirm an association of increased sperm DNA fragmentation in couples with RM,[2,3,5,14–19] and more detailed analysis supports the hypothesis of a causal effect. It was shown that couples with unexplained RM had significantly higher DFI than those in whom another identifiable cause was found. Of the cases with 'very high' DFI (>30%), five of seven were in the unexplained group.

Up to 3% of couples trying to conceive experience recurrent miscarriage. It is commonly accepted that spontaneous miscarriages are most likely due to random aneuploidy[4]. Hence, the medical approach is one of reassurance and encouragement to try again. However, as the number of miscarriages increases, the likelihood of chromosomal errors decreases and there is a possibility of a recurring parental abnormality.[21] Traditionally, screening for such abnormalities becomes worthwhile in couples who have had three or more miscarriages, although even then only about 50% have defined abnormalities (e.g. cardiolipin antibodies or chromosomal translocations). It is acknowledged that chromosomal abnormality in previous pregnancies was not assessed in this study, as is the case in most clinical studies of the recurrent miscarriage syndrome.[22] The problem is that identifying enough truly unexplained cases would almost certainly need a far larger base population than any single RM clinic, the reality of clinical practice being most products of conception following miscarriage are not sent off for cytogenetic analysis. It is known that couples with higher numbers of successive miscarriages produce more karyotypically normal losses.[21] One study[21] showed women with four previous losses had a subsequent loss rate in which 55% had abnormal karyotype. This important issue undermines all research in couples with recurrent miscarriage and clearly needs to be borne in mind in all interpretations and clinical applications. But the observations reported here could be used to plan such a larger multicentre study and perhaps even stimulate studies using pregnancies achieved with IVF and preimplantation genetic diagnosis for aneuploidy screening.

If DFI abnormalities were considered as a potential causal factor for couples with RM, the traditional view of RM investigation would be substantially altered. This shift is along the lines of the appreciation of the male contribution to infertility investigation and is likely to be welcomed by the majority of couples faced with such a frustrating clinical problem. It has been shown that men in pregnancy loss often feel a lack of control, frustration and guilt[23] and therefore investigating males may help them to feel more included in the process.

The current study used the SCSA test, which is clinically accepted in testing male infertility[7] and has been shown to be reliable over time.[17] Previous studies have used a variety of assays, which cannot be directly compared as they have different thresholds[8] and different methodologies, although they all directly evaluate DNA damage.[24] Nevertheless, these different assays are correlated.[25] SCSA analysis in one laboratory can produce clinical values that are useful for greater populations, and hence we believe this study can be extrapolated to assess the prevalence of high DFI in RM couples more generally.

The SCSA is currently a costly test to perform and so there is discussion surrounding whether a standard semen analysis, a relatively cheaper and more accessible option, is a suitable marker for DNA fragmentation. This study and others[8] have demonstrated a weak correlation between semen abnormalities and high DNA fragmentation, although other studies have found no relation between standard parameters and DNA fragmentation.[10] Following on, in our study, there were 20/32 (63%) men who had high DNA fragmentation but totally normal semen parameters. Therefore, routine semen analysis would be a relatively poor screening test for DNA fragmentation abnormalities. Moreover, the purpose of such testing is for diagnosis and, more significantly perhaps, for treatment. Specific therapeutic measures to treat semen analysis results are only useful in limited cases,[13] and currently, we are not aware of any medical treatment for abnormalities of sperm concentration, motility and morphology for the purposes of reducing the risk of miscarriage.

A recent systematic review and meta-analysis has recommended that couples experiencing recurrent failure

to achieve pregnancy should be offered DNA damage testing,[24] as there are a number of potential interventions to improve DNA fragmentation abnormality. Use of oral antioxidant therapy has been shown to reduce DFI, specifically in the setting of oxidative DNA damage.[26,27] This supports the use of antioxidant therapy for men with oxidative DNA damage, but the benefit of antioxidant treatment in men with high DFI in a recurrent miscarriage population is still unknown.

A variety of other lifestyle factors have been shown to reduce DNA fragmentation, including daily ejaculation,[28] and it has been shown that non-smokers have significantly lower DFI than smokers.[10] It has been suggested that a healthy lifestyle, quitting smoking and alcohol, increasing fruit and vegetables, will improve sperm quality by decreasing reactive oxygen species.[29] Unfortunately, the men in this study did not have BMI recorded, but interestingly, weight loss in severely obese men has not been shown to result in DFI improvement.[30] For infertile males with varicocele, DNA fragmentation was significantly reduced after varicocelectomy and pregnancy rates higher in males with greater reductions in DFI.[11]

In conclusion, this study has demonstrated a significant number of men with potentially correctable sperm DNA fragmentation abnormalities in a population of couples with RM. The case for routine sperm DNA fragmentation testing in a recurrent miscarriage clinic is certainly not proven, as this study involved relatively small numbers of couples and did not exclude aneuploidy as the main underlying cause of miscarriage in the majority of cases. However, as treatment with antioxidants would appear to be safe and easily available, it would be relatively easy for many couples to simply take antioxidants on an empirical basis whenever they wish to start trying for a family. This approach is likely to overtreat the vast majority of couples. Far better would be diagnostic testing with sperm DFI and targeted therapy for those who might benefit. Hence, we would encourage more clinical trials to demonstrate the link between RM and sperm DNA fragmentation and to prove that intervention with antioxidants is beneficial.

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