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Alkylation of sperm DNA is associated with male factor infertility and a reduction in the proportion of oocytes fertilised during assisted reproduction

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ABSTRACT

Approximately one-third of IVF cases in the UK are attributed to male factor infertility and in the majority of cases the origin of male infertility is unknown. The integrity of sperm DNA is important both for the success of assisted reproduction and the implications for the off-spring. One type of DNA damage that has not been investigated with respect to fertility outcomes is the adduct N7-methyldeoxyguanosine (N7-MedG), a biomarker for exposure to alkylating agents. A prospective cohort of couples attending for IVF had their N7-MedG levels in sperm measured using an immunoslot blot technique to examine whether sperm N7-MedG levels are associated with male factor infertility, semen quality measures or assisted reproduction outcomes. Sufficient DNA for analysis was obtained from 67/97 couples and N7-MedG was detected in 94% of sperm samples analysed. Men diagnosed with male factor infertility had significantly higher mean levels of N7-MedG in their sperm DNA ($P = 0.03$). Logistic regression analysis showed that N7-MedG levels were significantly negatively associated with the proportion of oocytes successfully fertilised irrespective of the method of fertilisation used (IVF or intra-cytoplasmic sperm injection; ICSI, $P < 0.001$). Therefore exposure to DNA alkylating agents is significantly associated with male infertility and the proportion of oocytes fertilised during assisted reproduction. Reducing such exposure may improve male fertility but further work is required to determine the relative importance of exogenous and endogenous sources of exposure.

1. Introduction

According to NHS data compiled from Office for National Statistics estimates (2001) one in six or seven couples in the UK are likely to experience problems in conceiving [1] and during 2006 32.5% of IVF cases were attributed to male factor infertility, an increase of approximately 5% since 2000 [1]. In the majority of cases the origin of male factor infertility is unknown [2]. However, there is increasing evidence that exposure to DNA damaging agents may be a contributing factor as infertile men have higher levels of sperm DNA damage, either strand breaks or specific base modifications such as 8-oxoguanine, compared with fertile men [3–5]. Furthermore, studies of the effect of DNA damage on assisted reproduction (ART) outcomes using the Comet assay [3], TUNEL [6] or SCSA [7] show varying associations with fertilisation rates, embryo quality and pregnancy rates but no consistent relationship [7–9]. A recent meta-analysis concluded that DNA damage reduces the chance of pregnancy during ART [10]. Therefore DNA damage in sperm is of concern not only in treating infertility [11], but has implications for ART where there is significant concern about an increased risk of birth defects [12].

So far, studies of sperm DNA damage have largely been limited to measurement of strand breaks (and other measures of DNA integrity) and oxidative DNA damage [4,13]. However, it is known that human tissues contain variable levels of other types of DNA damage including the alkyl DNA adduct, N7-methyldeoxyguanosine (N7-MedG) [14–17]. N7-methyldeoxyguanosine (N7-MedG) is a relatively stable adduct ($t_{1/2} = 144$ h) both *in vitro* and *in vivo* [18] and has been used as a biomarker of exposure to alkylating agents such as *N*-nitroso compounds [19] that can arise through endogenous mechanisms [20] or exogenous sources [21]. Environmental exposures include diet, particularly preserved foods or well-cooked meat, smoking [22,23] and alkylating drugs. Some occupations carry a risk of exposure such as the rubber, metal and leather industries. Although N7-MedG is not mutagenic itself, levels are often correlated with the presence of other pro-mutagenic and carcinogenic adducts such as O^6-methyldeoxyguanosine (O^6-MedG) [24]. N7-MedG levels in

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cervical DNA have been associated with increased risk of failure of treatment for cervical intraepithelial neoplasia [15]. N7-MedG adducts are poorly repaired in somatic cell extracts [25] and during late spermatogenesis DNA repair ceases alongside down-regulation of apoptosis mechanisms [11]. For example the half-life of 7-MeGua in mice testicular DNA was found to be considerably shorter than in sperm DNA suggesting the lack of excision-repair capability in sperm [26]. Therefore N7-MedG levels in sperm would be expected to reflect inter-individual differences in intensity of exposure and metabolism and provide an estimate of the levels of other mutagenic adducts such as O^6-MedG.

The aims of this prospective study were therefore to determine whether sperm DNA contains detectable levels of the adduct N7-MedG, whether N7-MedG levels are associated with male infertility or semen quality and to examine the relationship between N7-MedG levels and ART.

2. Materials and methods

2.1. Study design

The prospective cohort study was conducted on 97 couples attending for infertility treatment (IVF or ICSI) at St Mary's Hospital, Manchester between November 2005 and September 2006. All couples had provided written informed consent for research, and the study had Local Ethics Committee Approval (Central Manchester REC ERP/91/078, HFEA research licence R0026). All women were aged between 23 and 39 years and men between 25 and 50 years. Of these couples 65 had IVF treatment, 31 had ICSI and one had no treatment with the semen sample analysed for N7-MedG, due to a failure to harvest oocytes during the treatment cycle. Men were diagnosed clinically with male factor infertility by a consultant in reproductive medicine usually on the basis of poor quality semen (count $<20 \times 10^6$/ml, motility <25% rapid progression or <50% forward progression, sperm morphology (strict) <2% normal). The assessment was carried out prior to any DNA adduct analysis.

2.2. Assisted reproduction treatment cycles

Ovarian stimulation was achieved using a conventional long protocol down-regulation involving pituitary desensitisation, described previously [27]. Semen samples were obtained between 9 and 10 am by masturbation into a wide-mouthed plastic container and analysed for volume, sperm concentration and motility. Sperm morphology data (strict criteria [28]) were available from a previous semen sample from the same individual. Duration of sexual abstinence was recorded. An aliquot was prepared for IVF or ICSI by density gradient centrifugation using standard protocols [29]. The remaining semen was stored at room temperature and became available for N7-MedG analysis at 3 pm. The semen was centrifuged at $2000 \times g$ for 20 min followed by separation of sperm and seminal plasma and storage at −80°. For the purposes of analysis oocytes with 2 pro-nuclei and 2 polar bodies were designated "successfully fertilised" and embryos suitable for transfer back to the patient between 48 and 72 h after oocyte retrieval were designated "high quality embryos". Embryos considered suitable for transfer had all the cells of equal size, were even and had less than 20% fragments, according to the routine clinical grading protocol [30].

2.3. Measurement of N7-MedG levels in sperm DNA

Genomic DNA was extracted from sperm cells using the Qiagen genomic DNA extraction kit (Qiagen, Crawley, UK) except that the proteinase K and ribonuclease A digestion was carried out at 4° overnight. The mean yield of DNA was 30 μg (range 0.8–135 μg). Levels of N7-MedG in the sperm DNA were measured using an immunoslot blot technique [17]. Sufficient DNA for N7-MedG analysis was extracted from 75 semen samples but 8 DNA samples were lost during immunoslot blot for technical reasons and insufficient DNA remained for a repeat experiment. Twenty-two semen samples did not yield sufficient DNA for analysis (<3 μg) resulting in 67 semen samples successfully analysed. The yield of DNA for the 67 analysed was 43 μg (range 3–135 μg) and for the 30 not analysed was 3 μg (range 0.8–6 μg). Of the 67 semen samples successfully analysed, 63 had detectable levels of N7-MedG (94%). N7-MedG analysis was carried out blind to fertility status, semen parameters and outcomes.

2.4. Statistical analysis

The values for N7-MedG analysis were matched to the treatment cycle in which the semen was used. Levels below the limit of quantitation for N7-MedG (0.1 μmol/mol dG) were given a value of 0.05 μmol/mol dG for statistical analysis. The *t*-test was used to compare N7-MedG levels according to criteria potentially related to infertility and treatment outcome. Linear regression analysis was used to examine the relationship between [N7-MedG] and sperm morphology and male age. Logistic regression was used to model the proportion of successfully fertilised oocytes or high quality embryos and the relation of these with [N7-MedG], morphology, total number of sperm and type of treatment (IVF or ICSI) was tested by comparing the reduction of $\Sigma(O_i - E_i)^2/E_i$ with the appropriate χ^2 distribution. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) version 15.

3. Results

Semen was available for analysis from 97 couples attending for fertility treatment. The yield of DNA was not associated with the N7-MedG levels ($R^2 = 0.003$, $P = 0.66$). The semen samples yielding sufficient DNA for analysis had significantly higher mean total number of sperm and mean volume of semen but not sperm concentration or percentage of motile sperm (Table 1). No significant

Table 1
Summary of semen quality and outcomes for men attending for infertility treatment with and without sufficient DNA extracted for N7-MedG analysis.

Fertility parameter	Sufficient DNA ($n = 67$)	Insufficient DNA ($n = 30$)	P (*t*-test or χ^2)
	Mean ± SD (range or %)		
[N7-MedG] (μmol/moldG)	1.16 ± 0.99 (0.05–4.06)	–	–
Male age (years)	35.7 ± 5.1 (25.7–50.1)	36.3 ± 5.0 (27.4–47.0)	0.62
Abstinence (days)	4.4 ± 3.5 (2–28)	3.8 ± 1.5 (1–7)	0.41
Semen volume (ml)	3.2 ± 1.1 (1.2–5.8)	2.5 ± 1.4 (1.0–7.2)	0.005
Sperm concentration ($\times 10^6$)	59.5 ± 54.4 (2.0–315)	54.5 ± 54.5 (0.3–197)	0.68
Total sperm	176.9 ± 164.7 (2.1–744.6)	110.7 ± 99.4 (0.8–404.8)	0.04
Motile sperm (%)	57.7 ± 15.8 (12–85)	59.3 ± 12.9 (27–76)	0.63
IVF/ICSI (%IVF)	45/21 (68%)[a]	20/10 (67%)	0.85
Female age	32.6 ± 3.4 (25–39)	33.1 ± 3.8 (23–39)	0.54
No. of eggs collected	11.0 ± 6.7 (0–33)	14.9 ± 7.8 (4–33)	0.01
No fertilised with IVF	5.5 ± 4.9 (0–21)	7.9 ± 6.1 (0–20)	0.10
No fertilised with ICSI	5.5 ± 4.2 (1–18)	5.5 ± 2.2 (2–10)	0.99
No embryo transferred	1.3 ± 0.8 (0–2)	1.2 ± 0.9 (0–2)	0.91
No embryo frozen	2.1 ± 4.5 (0–18)	3.3 ± 5.6 (0–20)	0.29
Pregnancy using this semen[a]	14/66 (21%)	5/30 (17%)	0.62
Live births using this semen[a]	13/66 (20%)	4/30 (13%)	0.47
Pregnancy over all cycles	14/67 (21%)	6/30 (20%)	0.92
Live births over all cycles	13/67 (19%)	4/30 (13%)	0.47
Male factor infertility	19/67 (28%)	7/30 (23%)	0.61
Mean no. of treatment cycles	1.6 ± 0.74 (1–4)	1.8 ± 0.93 (1–5)	0.33
Mean no. of cycles to pregnancy	1.79 ± 0.80 (1–3)	1.40 ± 0.55 (1–2)	0.34
No. of eggs collected in cycle 1	9.6 ± 6.7 (0–28)	11.6 ± 7.8 (0–28)	0.19
No. of eggs collected in cycle 2	11.5 ± 7.1 (0–33)	14.9 ± 8.5 (2–35)	0.13

[a] One woman had no eggs harvested in this cycle.