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Research Article

Semen parameters and sperm DNA damage and its impact on birth

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Abstract

This case-control study, conducted from July 2021 to August 2022 in Duhok, Kurdistan Region, Iraq, investigates the semen parameters and sperm DNA damage in male partners of couples experiencing recurrent pregnancy loss (RPL) compared to fertile couples without pregnancy loss. The study, with participants gathered from various gynaecological clinics, sheds light on the implications for mammalian reproductive health and wildlife conservation such as captive breeding programs. Semen indices, analyzed according to World Health Organization (WHO) standards, revealed that couples with RPL exhibited significantly lower rates of progressive motility (45.62 ± 16.01 vs. 53.08 ± 10.11, P=0.037) and normal morphology (2.88 ± 1.5 vs. 3.48 ± 1.2, P=0.048) compared to the control group. Furthermore, the study employed the alkaline comet assay to assess the magnitude of sperm DNA damage. Results indicated a significantly higher degree of sperm DNA damage in couples with RPL (21.99 ± 1.13 vs. 17.42 ± 1.39, P=0.018), along with a notable increase in DNA Fragmentation Index (DFI) (43.46 ± 20.39 vs. 29.83 ± 16.25, P=0.0048) compared to the control group. These findings not only contribute to our understanding of human reproductive health but also have broader implications for mammalian fertility, potentially influencing wildlife populations. Further research is warranted to unravel the underlying mechanisms and explore interventions for couples experiencing RPL, with potential applications in mammalian and wildlife conservation. The semen of male partners of couples with RPL have lower progressive motility and a higher percentage of abnormal morphology with a significantly higher degree of DNA damage when compared with their age-matched control group, therefor sperm DNA damage may be included as an important step in the evaluation of couples with RPL.

Keywords: recurrent pregnancy loss, comet assay, sperm DNA damage, semen analysis

Introduction

Recurrent Pregnancy Loss (RPL) is a distressing condition marked by the spontaneous loss of two or more pregnancies within the first 24 weeks. In 50 % of cases, the exact cause could not be identified. Existing research primarily focuses on maternal factors, exploring various etiological elements such as anatomical issues, infections, autoimmune factors, chromosomal abnormalities, and thrombophilia, among others (Kolte et al.,2015). Given that half of the embryo's genetic makeup originates from the male partner, the normal paternal DNA content is crucial not only for initiating pregnancy but also for its progression into later stages (Robinson et al.,2012). When sperm with damaged DNA fertilises a healthy egg, it results in an embryo with abnormal genetic content, potentially affecting its development at any stage. The severity of transmitted DNA damage determines whether it leads to embryo death or manifests later in the neonatal period or childhood (Aitken,2017). Despite the significance of male factors, their role in RPL development has not been extensively studied. Currently, karyotype analysis is the widely accepted and proven test for investigating the male partner in RPL cases (Kamkar et al.,2018; Ibrahim, and Johnstone, 2018).

Initial assessments of semen parameters, including concentration, morphology, and motility, are routine. This assay is utilized for the assessment of males with infertility and may give a clue why some of these couples cannot achieve pregnancy yet their role in evaluating RPL remains controversial due to conflicting results in related studies (Benagiano et al.,2017).Interestingly, men with normal productivity and standard semen analysis often exhibit higher genetic purity, while those facing infertility issues, especially with abnormal semen indices, tend to have lower DNA integrity. Consequently, infertile males may exhibit significant DNA damage despite seemingly normal semen examinations (Schulte et al.,2010).The assessment of sperm DNA damage involves testing for DNA fragmentation, the separation or breakage of DNA strands into pieces. Various methods, such as Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), sperm chromatin structure assays (SCSA), sperm chromatin dispersion test (SCDT) and the Comet assay, have been employed for this purpose. Notably, the Comet assay, also known as single cell gel electrophoresis assay (SCGE), has been used to identify significant DNA damage in couples with infertility and RPL (Simon et al.,2011; Collins, 2004).Numerous studies have established a direct link between the extent of DNA damage in sperm and both infertility (Lewis et al.,2010; Zhao et al.,2014; Cho, and Agarwal, 2018; Oleszczuk et al.,2013) and and RPL (Niederberger, 2016; Ribas-Maynou et al.,2012; Absalan et al.,2012). In addition, it was documented that the less

sperm DNA damage present, the greater the chance of achieving a remarkable grade of success in infertility management with a higher birth rate. Despite these documented studies, routine assessment of infertility and RPL does not include an assessment of the degree of sperm DNA damage or fragmentation. The underutilization of this important method is probably due to lack of sufficient data, high cost and meticulous equipment required for these tests (Harlev, 2017).Despite increasing attention in the last two decades on how the genetic composition of the male partner influences reproductive diseases and RPL, data on the association between sperm DNA damage and RPL in Iraq are scarce. Thus, this study aims to investigate the potential relationship between sperm DNA damage, as detected by the Comet assay in males with normal sperm count in couples experiencing RPL when compared with the normal control group in Duhok City, Iraq.

Martial and methods

From the period from July 2021 to August 2022, information from ninety couples with RPL was gathered from different gynaecological clinics in Duhok province in Iraq. Detailed history, examination with emphasis on previous obstetrics and gynaecological history for females and appropriate laboratory investigation(including abdominal ultrasound, TORCH screening Toxoplasma IgG/IgM, Rubella IgG/IgM, Herpes IgG/IgM, and CMV IgG/IGM, thyroid hormone, HbA1c, Vitamin D, prolactin, thrombophilia screening for mutation detection, protein C and S, and lupus anticoagulant and anti-cardiolipin antibodies) were performed to diagnose the cause of RPL specifically concentrating on major causes of maternal miscarriage like anatomical, endocrinological, immunological and infectious causes. Only fifty couples with no identifiable obvious cause for RPL were eligible to be included in this study as a patient group. The control group, which consisted of 30 couples (ages were matched), had normal pregnancy till delivery without any complications.

After receiving permission from both the patient and control group, semen samples were obtained from the male partners of both groups via masturbation after two to five days of ejaculatory abstinence and were analyzed within one hour of collection. After seminal liquefaction, a routine semen analysis was performed using computer-aided semen analysis (WLJY-9000 WEILI Color Sperm Analysis System, China) according to the 2010 World Health Organization (WHO) specification (Edition, 2010). Semen samples of the two groups were kept in the freezer at -20 Celsius degree for DNA measurement by alkaline comet assay. The collection of samples was completed within one year. The patient and control group were age-matched and both were the

same alcoholic and smocking behaviours.

DNA Damage Measurement:

Alkaline Comet Assay (ACA): DNA damage in sperm cells from both patient and control groups was assessed using the Alkaline Comet Assay (ACA) technique. The ACA involved several steps, beginning with the preparation of slides, gel formation, electrophoresis, and scoring the results. Initially, 10 µL of sperm cells were mixed with 180 µL of pre-warmed low melting point agarose (LMP) solution in an Eppendorf tube. Subsequently, 80 µL of the mixture was distributed onto pre-coated microscopic slides, covered with coverslips, and allowed to solidify. After solidification, slides were transferred to pre-cold lysis buffer, washed, and then underwent electrophoresis in an alkaline buffer solution. The slides were incubated in an ice-cold alkaline buffer, allowing DNA to unwind. Electrophoresis was run for 20 minutes, and the slides were then washed and left to dry overnight. Staining with Syber Green dye followed, enabling examination under a fluorescence microscope. Under a fluorescence microscope, multiple pictures were taken for different sections on each slide. DNA damage was assessed by observing comets, where intact DNA with only a circular head indicated no damage, while a long-migrated tail represented the extent of DNA damage as shown in Figure 1. Casp Lab Software was utilized to measure the DNA fragmentation, when a sperm with a ratio of tail DNA exceeds 25 % to head it will be considered a damaged sperm. Fifty comets from each gel were selected and captured at different magnifications (20x, 40x, 100x), their mean will be calculated and recorded. In addition, the DNA fragmentation index (DFI) was calculated as indicated below:

DFI (%) = 100 × (No. of spermatozoa with fragmented DNA/ No. of spermatozoa counted)

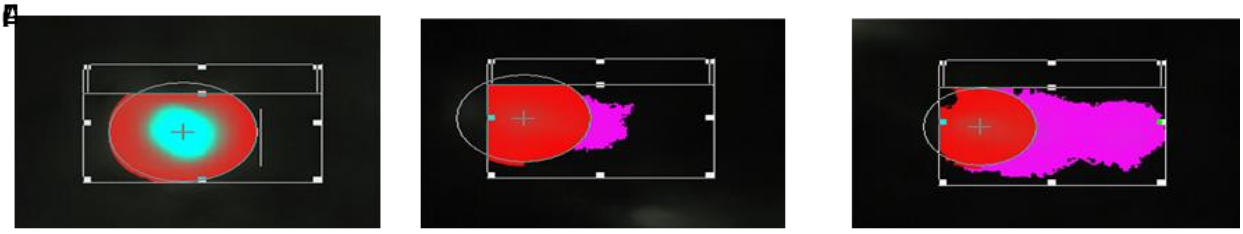

Figure 1. Measurement of tail DNA migration detected through and assessed by using Casp Lab Software. (A- no tail represents no damage; Short tail represents mild to moderate damage; C- long tail represents severe DNA damage)

Statistical analysis

The statistical analysis was performed using Graph Prism Pad software (version 4.0). the data were

coded appropriately and the qualitative variables were given as frequency and percentage. An unpaired t-test was used to detect deviations in means between the two groups. Regression analysis was performed to identify the effect between two different variables. A significance level of $p<0.05$ will be considered statistically significant. This study was approved by the scientific committee of the College of Medicine\University of Duhok and got ethical approval from the general directorate of the health of Duhok province-Iraq. This study was conducted during (2021-2022)

Results

A total of 50 patients from the RPL group and 30 males from the control group were included in this study. The mean age of both groups is similar. Indices of Semen analysis in the unexplained RPL group and control group are shown in Table 1. The means of ejaculate volume, sperm count, sperm concentration and PH of RPL group and controls were similar. However, the percentage of morphologically normal sperm and the progressive motility were significantly lower in the unexplained RPL group versus the control (P= 0.0487 and P= 0.0371 respectively). The number of immotile sperms was found to be higher in the RPL group in comparison to the control group but it was not statistically significant (P= 0.0558).

Table 1. Sperm DNA Damage

Variable	Cases	Control	Value
Age	39.98 ± 6.729	35.80 ± 6.124	0.4635
Volume (ml)	3.35 (0.93)	3.4 (1.02)	0.99 (NS)
PH	7.45 (0.19)	7.40 (0.15)	0.22 (NS)
Sperm concentration (million /ml)	64.4 (49.64)	62.6 (38.52)	0.86 (NS)
Normal morphology (%)	2.881 ± 1.51	3.480 ± 1.229	0.0487
Immotile (%)	21.22 ± 14. 31	15.56 ± 3.33	0.0558
Progressive Motility (%)	45.62 ± 16.01	53.08 ± 10.11	0.0371

Regarding DNA damage level detected by comet assay, the mean of DNA damage in the patient group was higher than the control group (21.99±1.130 vs 17.42±1.391) and with significant P value (0.0189), similarly the DFI was significantly higher in the patient group than the control (43.46 ±20.39 vs 29.83 ±16.251) which was significant (P =0.0048) as shown in figure 2.

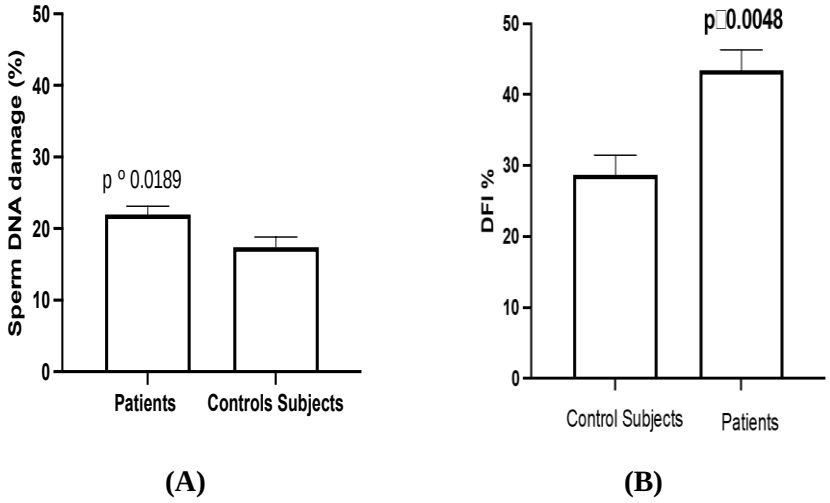

Figure 2. Relation of sperm DNA damage (A) and DNA fragmentation index (B) between RPL group and control

The relation of sperm DNA damage with abnormal sperm morphology was examined and revealed significant association (P =0.0341), but relation with percentage of immotile sperm was nonsignificant (P=0.164) as shown in figure 3.

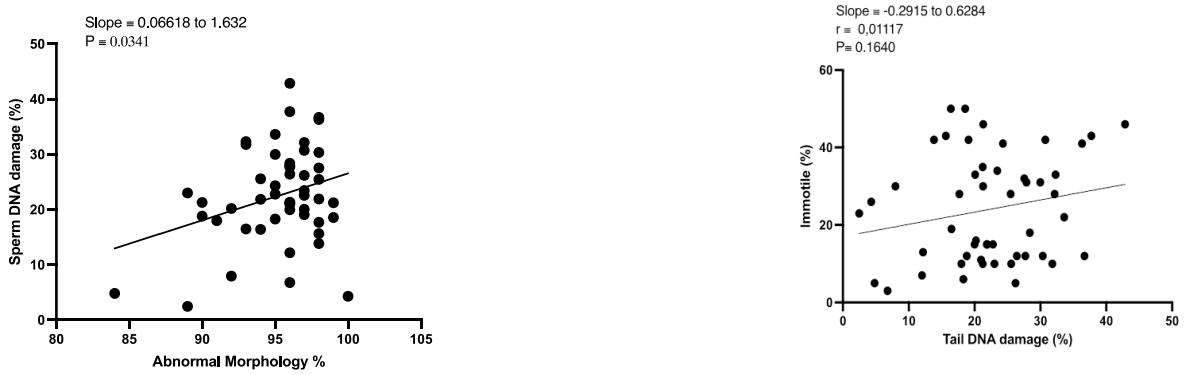

Figure 3. Correlation between Sperm DNA damage measured by Alkaline comet assay and sperm abnormal morphology(A) and immotile sperm(B) in patients with RPL.

Discussion

Gestation and subsequent embryo development depend on the integrity of the gamete's DNA. Both sperm and oocyte contribute equally to the formation of the embryonic DNA; therefore, normal sperm chromatin structure is essential for the safe and healthy transmission of parental genetic compositions. The defect in sperm DNA structure compromises fertilization and subsequent development of the embryo (Agarwal et al.,2016). This study analyzed the semen of male partners of couples with recurrent pregnancy loss after the exclusion of key maternal causes and displayed that those sperms had significantly higher abnormal morphology and lower progressive motility. Furthermore, these sperms had a high sperm DNA damage in contrast to the control group, which may have contributed to RPL. This study used the Single cell gel electrophoresis (Comet assay) to measure the amount of DNA damage per spermatozoon, as a single cell that can be detected over the gel. This method is highly sensitive because it can measure and assess variable types of DNA damage (single or double-stranded) in a single cell (Alahmar et al.,2022).In the present study, it was an obligation to freeze the semen samples for the comet assay because it was impossible to perform the comet assay every day, as it was done twice weekly. The semen samples were frozen using an ultra-rapid freezing method to decrease the injury to the specific characteristics of sperm DNA that might have been induced by freezing (Duty et al.,2022). This result is supported by the observation that the DNA of the spermatozoa obtained from fertile men was found to be unaffected by the cryopreservation process (Donnelly et al.,2001).

Concerning the major semen parameters in this study, with the exemption of sperm count, the group of RPL had lower rates of morphologically normal sperm with a significant value (P=0.048) in comparison to the control. This result was similar to other studies (Mohamed et al.,2007; Khadem et al.,2014; Cao et al.,2017; Dai et al.,2022).In addition to sperm morphology, progressive motility was found to be significantly lower in the RPL group than the control one (P=0.0371). This finding is consistent with findings of other conducted studies that showed male partner of couples with RPL have significantly impaired total and progressive motility than their fertile control (Mohamed et al.,2007; Khadem et al.,2014; Cao et al.,2017; Dai et al.,2022). These significant differences regarding sperm morphology and progressive motility were not found in other studies (Zhang et al.,2012; Carlini et al.,2017; Bhattachary, 2008). Despite that, the association of semen indices with sperm DNA damage is still not clear. In this study, semen samples that have a higher percentage of abnormal sperm morphology had significantly higher DNA damage (P=0.0341). This was also observed in other studies (Sivanarayana et al.,2014).

Additionally, similar findings were found by another study where they used a neutral comet assay for the detection of DNA damage (Chi et al.,2011) ,these findings may follow some observations that showed the association between the quality of the semen and degree of sperm DNA damage (34,35). Such a conclusion about this relation was not supported in other reports (Ferrigno et al.,2021; Xie et al.,2018).

The degree of DNA damage in the sperms of couples with RPL was significantly higher than the fertile control group as shown by the obvious change of mean DNA damage and DFI between the two groups (P= 0.009 and P=0.0048 respectively). This significant association was similarly seen in two large recent meta-analyses (Tan et al.,2019; Dai et al.,2022) which included more than 30 studies with enrollment of approximately four thousand participants in total as a control and couples with RPL. Both demonstrated significantly higher levels of sperm DNA damage detected in couples with RPL when compared to the fertile control group regardless of the type of genetic test used for the detection of sperm DNA damage. This relation of RPL with sperm DNA damage was also elucidated by an article from a nearby country, Iran, which used the SCD test for the detection of DNA impurity of the sperm (Khadem et al.,2014). In addition, the role and association of sperm DNA damage with RPL is strengthened with the evidence that embryo quality and survival are adversely affected by the degree of DNA damage in sperm and the use of alkaline comet assay is a useful method for predicting the success of pregnancy (Simon et al.,2011; Morris et al.,2022).

Accordingly, most of these aforementioned results demonstrated that women whose partners had a lower percentage of sperm DNA damage were more likely to have a successful pregnancy while women whose partners had a higher percentage of sperm DNA damage were more likely to experience pregnancy loss. However, some studies have reported no significant differences in sperm DNA damage between couples with and without RPL (Zhang et al.,2012; Eisenberg et al.,2017; Esquerré-Lamare et al.,2018).The inconsistency that might be found in the results of these studies could be largely due to differences in sample characteristics, standards that have been used for semen analysis, and the use of different techniques for the detection of sperm DNA damage with their variable sensitivities and threshold of detection. This study has many positive points as it is the first study according to prior observation to assess the degree of sperm DNA damage in couples with RPL in our area. It is also a case-control study with a relatively average sample size in comparison to many other studies. This study also relied on the detection of genetic damage in sperm with comet assay, which is considered to be a very sensitive method although not commonly

used in many parts of the world for various reasons. The limitations of this study include the need for the involvement of a larger number of patients from multiple centers with different ethnicity.

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