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# Using of Comet Assay to detect DNA Damage in Infertility Men

**Ghassan M. Hassan**

College of Agriculture - University of Wasit, Iraq

Corresponding author: gmhassan@uowasit.edu.iq

**Abstract**:

Male infertility is a major health concern and is often accompanied by abnormal semen parameters. The detection and understanding of these abnormalities are critical in the improvement of fertility treatments and outcomes. This study aims to compare semen quality between men with infertility and healthy controls, focusing on sperm count, motility, morphology, and other important semen parameters.

A cohort of infertile men and healthy control donors were evaluated for semen parameters. Semen volume, sperm count, motility, vitality, and morphology measurements and the analysis were performed by using standardized laboratory procedures. The statistical differences were determined by applying independent t-test, Mann-Whitney U test, and descriptive analysis.

The semen volume in men with a diagnosis of infertility was significantly reduced compared to the control group (1.17 ± 0.50 ml versus 3.39 ± 0.56 ml, $p < 0.0001$). The sperm count in infertile men was also significantly lower (8.15 ± 4.02 x$10^6$/ml versus 79.33 ± 9.20 x$10^6$/ml, $p < 0.0001$). Moreover, sperm motility and vitality were significantly reduced, with motility being 24.75 ± 10.10% and active sperm at 1.73 ± 0.86%, compared to controls (76.26 ± 9.05% and 21.33 ± 9.02%, $p < 0.0001$). Abnormal sperm morphology was increased in infertile men (91.03 ± 3.25% vs. 10.46 ± 3.97%, $p < 0.0001$), while normal morphology was significantly lower. Semen morphology parameters (Q2 and Q3) showed wide variability within the infertility group, though differences between groups were not statistically significant.

Men with a diagnosis of infertility showed significant semen-quality anomalies, presenting decreased sperm count, motility, and vitality, and an increased incidence of atypical sperm morphology. Our findings underline the necessity for developing better diagnostic tools in terms of DNA integrity assessment and tailored therapeutic interventions for male infertility treatment. Further studies are required on large populations to investigate the clinical consequences of these observations.

**Keywords**: *Comet assay, DNA damage*

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## استخدام اختبار المذنب (Comet Assay) للكشف عن تلف الحمض النووي لدى الرجال المصابين بالعقم

غسان محمد حسم

قسم الإنتاج الحيواني ـ كلية الزراعة ـ جامعة واسط / العراق

**الخلاصة**

ان مشكلة وامراض العقم الذكري تعتبر من الأمور البالغة الاهمية وغالباً ما يصاحبه اضطرابات في معايير السائل المنوي. إن الكشف عن هذه الاضطرابات وفهمها أمر بالغ الأهمية لتحسين علاجات الخصوبة والنتائج العلاجية. تهدف هذه الدراسة إلى مقارنة جودة السائل المنوي بين الرجال المصابين بالعقم والأفراد الأصحاء، مع التركيز على عدد الحيوانات المنوية، والحركة، والشكل، والمعايير المهمة الأخرى للسائل المنوي.

في هذه الدراسة تم تقييم مجموعة من الرجال المصابين بالعقم ومتطوعين أصحاء كمتبرعين في معايير السائل المنوي. تم قياس حجم السائل المنوي، عدد الحيوانات المنوية، الحركة، الحيوية، والشكل، وأُجريت التحليلات باستخدام إجراءات مخبرية معيارية. تم تحديد الفروقات الإحصائية من خلال تطبيق اختبار (t) المستقل، واختبار مان-ويتنيU ، والتحليل الوصفي.

كان حجم السائل المنوي في الرجال المصابين بالعقم منخفضًا بشكل كبير مقارنة بمجموعة التحكم (1.17 ± 0.50 مل مقابل 3.39 ± 0.56 مل، $p < 0.0001$). كما كان عدد الحيوانات المنوية في الرجال المصابين بالعقم أقل بشكل ملحوظ (8.15 ± 4.02 ×$10^6$/مل مقابل 79.33 ± 9.20 ×$10^6$/مل، $p < 0.0001$). علاوة على ذلك، كانت الحركة والحيوية في الحيوانات المنوية منخفضة بشكل كبير، حيث كانت الحركة 24.75 ± 10.10% والنسبة النشطة 1.73 ± 0.86%، مقارنة بالتحكم (76.26 ± 9.05% و21.33 ± 9.02%، $p < 0.0001$). كما كانت نسبة التشوهات في شكل الحيوانات المنوية أعلى في الرجال المصابين بالعقم (91.03 ± 3.25% مقابل 10.46 ± 3.97%، $p < 0.0001$)، بينما كانت نسبة الشكل الطبيعي أقل بشكل كبير. أظهرت معايير شكل السائل المنوي Q2وQ3 تفاوتًا واسعًا ضمن مجموعة العقم، على الرغم من أن الفروقات بين المجموعات لم تكن ذات دلالة إحصائية.

حيث أظهر الرجال المصابون بالعقم وجود شذوذات هامة في جودة السائل المنوي، حيث كان هناك انخفاض في عدد الحيوانات المنوية، والحركة، والحيوية، وزيادة في حدوث التشوهات في الشكل. تؤكد نتائجنا على ضرورة تطوير أدوات تشخيصية أفضل فيما يتعلق بتقييم سلامة الحمض النووي وتدخلات علاجية موجهة لعلاج العقم الذكري. تتطلب الدراسات المستقبلية عينة كبيرة للتحقيق في العواقب السريرية لهذه الملاحظات.

**الكلمات المفتاحية:** *اختبار المذنب (Comet Assay)، تلف الحمض النووي (DNA Damage).*

## Introduction

Approximately 50% of infertility cases in couples are attributed to male factor infertility [1]. Various etiologies have been identified as potential causes, including gene mutations, viral infections, ejaculatory duct obstruction, varicocele, radiation, chemotherapy, and erectile dysfunction [2]. DNA damage levels are significantly elevated in individuals exposed to environmental pollutants such as radiation, pesticides, and other reagents [3]. Parameters such as sperm concentration, motility, and morphology are commonly used to assess the fertilization potential of an ejaculate. While these metrics provide a general overview of sperm quality, they fail to capture one of the most critical factors affecting reproductive outcomes: DNA integrity. The presence of single- or double-strand DNA breaks is a key differentiator between fertile and infertile males [4]. Additionally, DNA damage tends to increase with age, as evidenced by comet assay results showing elevated levels of single-strand breaks and/or oxidized bases in older individuals [5].Over the past two decades, research has demonstrated that sperm carrying DNA damage can transmit this damage to the oocyte during fertilization, leading to negative pregnancy outcomes or genetic disorders in offspring [6]. Conventional techniques used to assess somatic cell DNA damage may not be suitable for evaluating sperm DNA integrity due to the unique structure, compaction, and integrity of sperm DNA. Notably, the sperm genome is the sole germline transmitter of genetic damage or mutations to subsequent generations.The comet assay, an efficient and cost-effective in vitro test system for detecting and quantifying DNA damage at the individual cell level, has gained prominence in recent years [7]. The measurement of sperm

DNA damage is a valuable tool for evaluating male infertility. The sperm nucleus lacks protection against oxidative stress, making it highly susceptible to oxidation-mediated DNA damage. The comet assay, also known as single-cell gel electrophoresis, is a relatively simple and sensitive technique used to detect strand breaks in DNA at the individual sperm level [8]. During electrophoresis, DNA fragments migrate away from the central DNA core, forming a characteristic "comet" shape. DNA damage is then quantified by measuring the displacement between the genetic material in the comet head and the resulting tail [9]. The alkaline comet assay has demonstrated high diagnostic value in assessing male reproductive health and offers significant prognostic potential [10]. This article reviews the methodology, principles, and current applications of the comet assay, emphasizing its utility in detecting variations in germ cells [11]. In line with recent advancements in molecular genetics, the objective of this study is to investigate the correlation between semen parameters, semen morphology, and the percentage of DNA damage in infertile. The aim of the present study is to compare and contrast the semen parameters of men with infertility and those of healthy control donors. The present study, in particular, tries to compare sperm volume, count, motility, vitality, and morphology in an attempt to find significant differences between the two groups. The study also seeks to analyze the morphological variation in semen among the infertility group and to explain possible pathophysiological mechanisms that could be involved in male infertility. Through the comparison of parameters, this study tries to give insights into the role of seminal parameters in male infertility and the requirement felt by professionals for advanced diagnostics to better evaluate and manage such cases.

## Materials and methods

Sixteen infertile men and twenty healthy control donors were included in this study. Semen samples were collected by masturbation following 72 hours of abstinence. The semen analysis was conducted at Kamal Al-Sameree' Hospital. After liquefaction for 30 minutes at 37°C, the semen samples were evaluated according to the World Health Organization (WHO) criteria [12]. The routine semen variables studied included sperm count, sperm motility, semen volume, and the percentage of normal and abnormal sperm, comparing infertile men and control donors.Sperm DNA integrity was assessed using the Comet Assay, as outlined in previously published procedures [13][14]. This part of the experiment was conducted according to the manufacturer's instructions (Comet Assay Kit) under standard laboratory conditions. Sperm DNA was subjected to single-cell gel electrophoresis (Comet Assay) as described by [15]. Briefly, sperm cells were rapidly thawed at room temperature, embedded into miniature agarose gels on microscope slides, and lysed in situ to remove DNA-associated proteins. This process

allowed the compacted sperm DNA to relax. The lysis buffer (Tris 10 mmol/l, 0.5 mol/l EDTA, and 2.5 mol/l NaCl, pH 10) contained 1% Triton X-100, 40 mmol/l dithiothreitol, and proteinase K (100 µg/ml). The microgels were electrophoresed (20 minutes at 25V/0.01A) in neutral buffer (Tris 10 mmol/l containing 0.08 mol/l boric acid and 0.5 mol/l EDTA, pH 8.2). During electrophoresis, damaged DNA migrated from the nucleus toward the anode. DNA damage was visualized by staining the slides with SYBR Green I, and sperm was identified by its size and the presence of a tail.

Comet measurements, including tail length, tail moment, and percentage of tail DNA, were performed using a fluorescence microscope [16]. Data analysis was conducted using SAS software [17]. Notched boxplots were generated to identify significant differences in medians and measure the correlation between control donors and infertile men in terms of semen morphology. A heatmap was also generated to visualize the correlation matrix between DNA damage in the head, tail, and neck of sperm cells.

**Results and Discussion**

The study depicted significant differences in semen parameters between control donors and infertile men, thereby proving the statement that infertility could have marked effects on seminal parameters. Specifically, sperm volume was significantly reduced in infertile men (1.17 ± 0.50 ml) compared with control donors (3.39 ± 0.56 ml), indicating possible dysfunction of the seminal vesicles or some form of endocrine disturbances. Likewise, sperm count was also significantly reduced in infertile subjects: 8.15 ± 4.02 $x10^6$/ml vs. 79.33 ± 9.20 $x10^6$/ml. Alongside the count, there were also marked reductions in sperm motility and vitality. In infertile men, each motility was 24.75 ± 10.10% and the percentage of active sperm was 1.73 ± 0.86% compared to the control donors 76.26 ± 9.05% and 21.33 ± 9.02%, respectively. Moreover, the percentage of normal sperm morphology was significantly lower in infertile men (6.70 ± 3.24%) compared with controls (22.60 ± 3.41%), while abnormal sperm morphology increased correspondingly (91.03 ± 3.25% vs. 10.46 ± 3.97%) showing in (Table 1). These findings are supportive of the view that infertility is coincident with widespread semen quality defects, which might be provoked by factors such as oxidative stress, DNA damage, and morphological abnormalities in spermatozoa. The abnormalities identified suggest that advanced diagnostic tools, including the comet assay for the assessment of DNA integrity, are required to provide appropriate and targeted therapy in the improvement of reproductive outcomes.

**Table 1 : Comparison of semen characteristics between Control donors and Infertility**

| Variable | Control donors | Infertility | p-value |
|---|---|---|---|
| **Sperm Volume ml** | 3.39 ± 0.56 A | 1.17 ± 0.50 B | < 0.0001 |
| **Sperm Count x$10^6$/ml** | 79.33 ± 9.20 A | 8.15 ± 4.02 B | < 0.0001 |
| **Motility %** | 76.26 ± 9.05 A | 24.75 ± 10.10 B | < 0.0001 |
| **Active %** | 21.33 ± 9.02 A | 1.73 ± 0.86 B | < 0.0001 |
| **Normal Sperm** | 22.60 ± 3.41 A | 6.70 ± 3.24 B | < 0.0001 |
| **Abnormal Sperm** | 10.46 ± 3.97 A | 91.03 ± 3.25 B | < 0.0001 |

Semen morphology parameters showed wide variability in the infertility group, while clear differences were seen between the donor control and infertility groups. Descriptive statistics showed that the infertility group presented higher mean values for Q2 (11.67 ± 7.64) and Q3 (9.33 ± 9.45) when compared with the donor control group (1.67 ± 0.58 and 1.33 ± 0.58, respectively). However, such differences were not statistically significant by either an independent t-test (t = -2.26, p = 0.1507) or a Mann-Whitney U test (U = 0.0, p = 0.0765). These findings suggest that semen morphology parameters may be highly variable in men with infertility and might reflect underlying pathophysiological heterogeneity. The lack of statistical significance may, however, be attributed to the small sample size, and further studies with larger numbers of participants are therefore needed to define the clinical implications of these observed trends. The data were further visualized using boxplots and line charts, demonstrating wide variability in values within the infertility group for the Q2 and Q3 parameters, thus further underlying the need for more extensive studies to understand in detail the reasons underlying these differences.

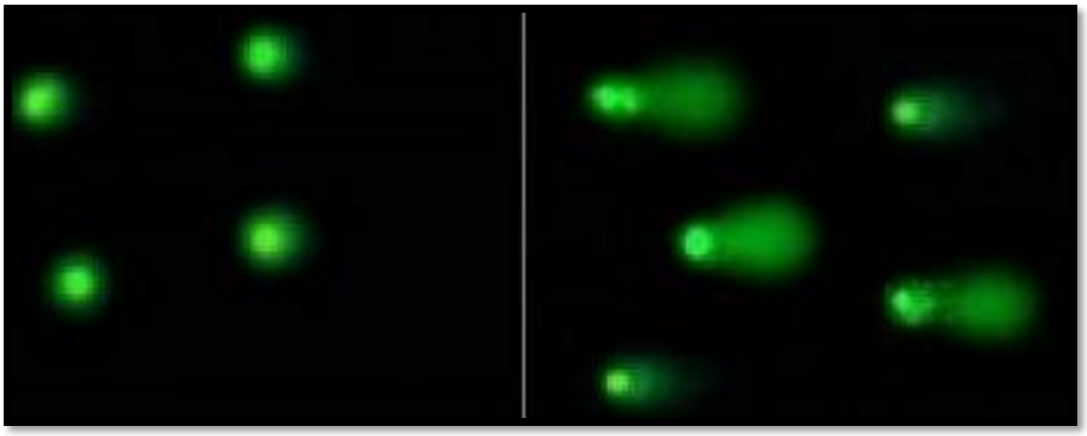

**Figure 1: Examples for comet assay in different DNA damage**

**(A: Non-fragmented, B: fragmented Comet assays)**

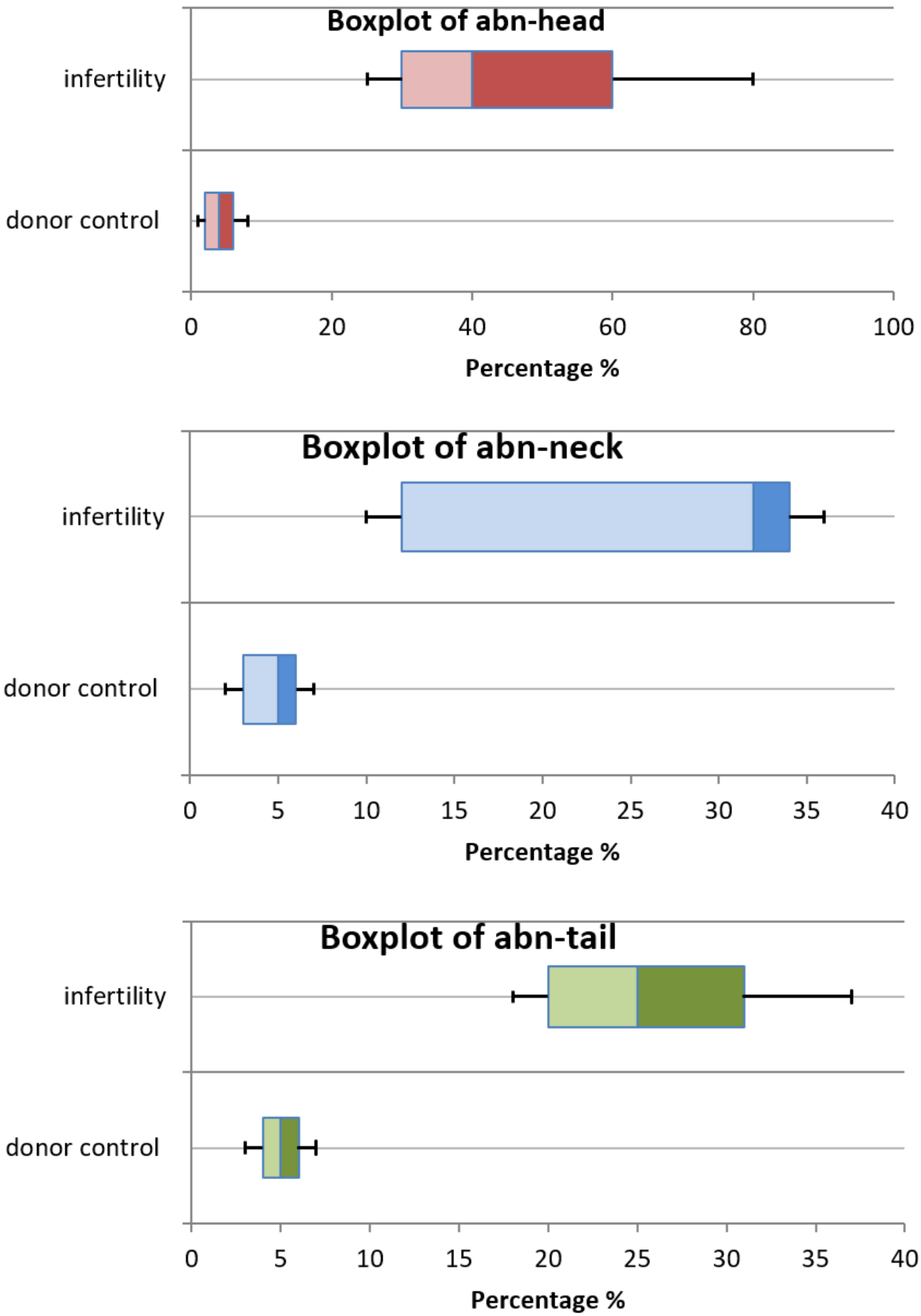


**Figure 2: (a,b,c)Compare between donor control and infertility of semen morphology**

Single cell gel (SCG) can used for detection of types DNA alternative, such as double and single strand breaks, alkali-labile sites, imperfect repair sites, cross links and repair in individual cells in this study we used the visual process because of is preferably than the automates method [18]. This assay was used by a researcher and investigators to trace the defects of DNA also used to determine the quantity of DNA by measuring the exchanges between the DNA of the nucleus and the consequent of tail as shown in Figure1, it showing fluorescent spheres without DNA damage in the control donor and showing a lot of fluorescent heads with tails indicating DNA damage infertility [19][20].

The comet assay is an efficient tool to measure single and double-strand DNA breaks at the cellular level. Also, this assay has been widely applied as a "golden standard" in studies regarding genotoxicity and biomonitoring [21]

A sample of 80 individuals (20 donor control and 60 infertility) were used to comet study. Show the damaged of DNA increased in infertility than donor control as result of oxidative stress and produced free radicals or reactive oxygen species (ROS) that lead to base damage or breaks of strand, these results were reflected negatively of semen variable [4] [22].

Different studies have been proposed to explain the origin of DNA damage in mature spermatozoa from infertile men, including defective sperm chromatin packaging, apoptosis and oxidative stress, other studies tried to correlate the seminal plasma contents with the male factors of infertility [23].

Indices of Semen analysis in donor control and infertility are shown in Table 1. The means of ejaculate volume, count, motility, sperm activity, normal and abnormal sperm were similar. However, the semen volume ($3.39 \pm 0.56$ and $1.17 \pm 0.50$, $p < 0.0001$) for Control donors and Infertility respectively , high significant of Sperm Count 106/ mL in control donors (79.33) and infertility (8.15) , the percentage of motility in control donors and infertility were significantly higher ( 76.26 and 24.75 respectively).The percentage of active sperm was found to be higher in the donor control comparison to the infertility the means percentage of donor control ( 21.33) and (1.73) of infertility . The high significant of normal sperm and abnormal for donor control and infertility (22.60 vs 6.70 and 10.46 vs 91.03 respectively) [24]. It found a significant decline in sperm count and progressive motility over a decade, indicating worsening fertility parameters among men experiencing infertility [26].

However, the percentage of semen morphology, that show in figure 2, results were significantly more prevalent between control donors and infertility men [25]. The percentage of abn-head 3.80 % for donor control and 50% for infertility, The percentage of abn-neck of donor control and infertility respectively (4.40 and 26.13 %). The percentage of abn-tail of donor control (0.93%) and infertility (26.26 %).

In this study, using the boxplot to investigated the correlation between Control donors and Infertility men of semen morphology , it was higher than the infertility ($49.66 \pm 19.63$ vs $3.8 \pm 0$ 2.59) and with significant P value (0.0001) in the abn-head variable , similarly the results in abn-neck and abn-tail , it was significantly higher in infertility than the control donor ($26.13 \pm 10.45$ vs $4.40 \pm 0.50$ ) in abn-neck variable , ( $26.26 \pm 6.30$ vs $0.93 \pm 0.79$) in the abn-tail variable, which was significant ($P = 0.0001$) as shown in figure 2 [27].

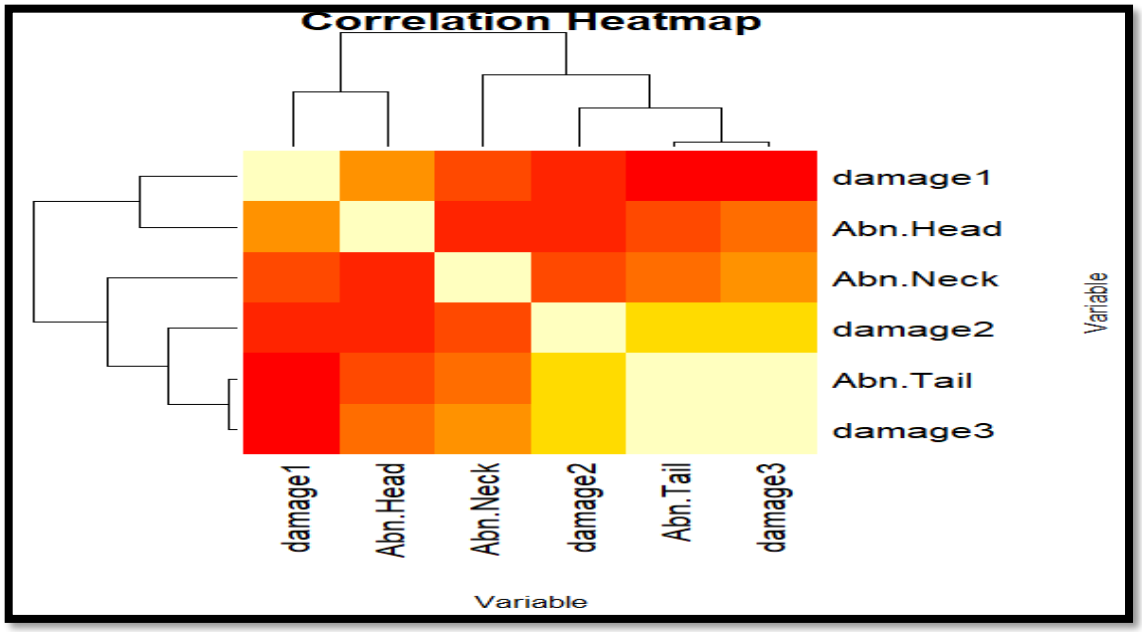


**Figure 3: The correlation matrix between DNA damage variables**

- Yellow: Represents moderate positive correlations.
- Orange-Yellow: Indicates strong positive correlations.
- Orange: Represents very strong positive correlations.
- Dark Red: Represents the strongest positive correlations

Heatmap cluster figures are often used to represent data sets in the omic sciences. The default option of the frequently used R heatmap function is to cluster data according to Euclidean distance, which groups data mainly to their numerical value and not to its relative behavior [28]. heatmap was employed to visualize the correlation structure among the variables of interest. The heatmap, depicted in Figure 3, presents a color-coded representation of the pairwise correlations between variables. Darker shades indicate stronger correlations, with positive correlations shown in warm colors and negative correlations in cool colors. The row and column labels denote the specific variables under consideration. Notably, the heatmap allows for the identification of patterns and relationships within the dataset, providing insights into the interplay between variables [29].

"Abn.Head" has a high positive correlation with "Damage1," which suggests that an increase in abnormal head measurements is associated with an increase in damage1 measurements.

Abn.Head vs. Damage1: The correlation between abnormal head measurements (Abn.Head) and Damage1 is depicted in varying shades of yellow, indicating a moderate positive correlation. Abn.Head vs. Abn.Neck: The heatmap reveals an orange-yellow hue, signifying a strong positive correlation between Abn.Head and abnormal neck measurements (Abn.Neck).

Abn.Head vs. Damage2: An orange shade suggests a very strong positive correlation between Abn.Head and Damage2. Abn.Head vs. Abn.Tail: The heatmap displays a dark red color,

representing the strongest positive correlation between Abn.Head and abnormal tail measurements (Abn.Tail). Abn.Head vs. Damage3: A very strong positive correlation is portrayed by the orange shade, highlighting the relationship between Abn.Head and Damage3.

Impaired semen quality infertility in both sexes and aneuploidies are all major health problems; they tend to cluster in individuals and families. I hypothesize a pathogenesis that underlies some cases of these conditions: environmentally caused germ cell genetic damage that becomes transgenerational. It starts with spermatid DNA damage that undergoes faulty

the resulting structural change becomes disruptive at meiosis because of cell cycle delay due to unequal lengths of the maternal and paternal chromosomes [30].

Sperm DNA damage is a significant biomarker of male infertility, correlating with increased miscarriage risk and affecting IVF and ICSI success. It can result from oxidative stress causing single strand breaks or dysfunction during spermatogenesis leading to double-strand breaks [31].

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