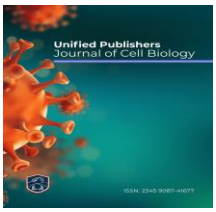

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Association Between Oligospermia and Sperm DNA Fragmentation: A Case-Control Study of Men Attending A Fertility Clinic

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ABSTRACT

A prevalent form of male factor infertility is oligospermia, often attributed to underlying factors such as DNA damage. The preferred treatment option for men with oligospermia is intracytoplasmic sperm injection and there are concerns about the cycling of male-factor infertility in the offspring of affected males because of heritable DNA damage. There is, therefore, a need to further investigate the association between DNA damage and oligospermia to determine the proportion of infertile men exhibiting both conditions. The aim of this study is to provide more evidence on the extent of DNA fragmentation in the sperms of oligospermic men. Semen samples were obtained from twenty-five (25) cases and ten (10) recent fathers as control. Routine semen analysis and preparation were performed according to WHO guidelines. Sperm DNA fragmentation was assessed using single-cell gel electrophoresis (Comet) assay. 100 cells were analysed per sample. The data was subjected to analysis for descriptive statistics and Pearson correlation. Cases with severe, moderate and mild oligospermia were 52%, 32% and 16%, respectively. The olive tail moment in 92% of males with oligospermia exceeded the control average of 5.91% and showed a strong inverse correlation with sperm concentration (r = -0.733), motility (r = -0.847), and morphology (r = -0.687). Age, sperm motility and sperm morphology also had strong correlations to DNA fragmentation indices. These indicators confirmed that a large proportion of oligospermic men attending fertility clinics have DNA damage and can benefit from interventions aimed at reducing DNA damage.

Keywords: Oligospermia, Infertility, Male, DNA, Fragmentation

INTRODUCTION

Male infertility accounts for about 50% of all causes of infertility globally, with a prevalence of 56,530 (Huang et al., 2023). It has been reported that in 30% of infertile couples, male factors occur as the singular cause of infertility (Morell, 1997). In Nigeria, the statistics for male factor infertility are quite alarming (Akang et al., 2023). For example, male factors have been reported to be responsible for 42.4% of infertility cases in the southwest of the country, while the prevalence of male factor causes was over 55% in southeastern populations (Ikechebelu et al., 2003; Uadia and Emokpae, 2015). The causes of male infertility are broadly classified due to their general underlying etiology into endocrine disorders, usually due to hypogonadism (2% to 5%), sperm transport disorders such as vasectomy (5%), primary testicular defects including abnormal sperm parameters without any identifiable cause (65% to 80%), and idiopathic where an infertile male has normal sperm and semen parameters (10% to 20%) (Leslie et al., 2024). The cases of male infertility due to low sperm counts, poor sperm quality or both are even reported to be as high as 90% of all causes of male infertility (Zakaria et al., 2022).

These abnormal sperm parameters are classified as Oligosospermia, Azospermia, Asthenozospermia and Teratozospermia.

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Oligospermia or Oligozoospermia refers to a condition resulting in male infertility or sub-fertility where there is a significantly reduced concentration of sperm cells in the ejaculate (Sami et al., 2015; Schlegel et al., 2020). For several years, the consensus definition for oligospermia was any concentration less than 20 million spermatozoa/ml of ejaculate (WHO, 1999). However, the World Health Organization (WHO) later redefined the reference values for oligospermia to be values below 15 million sperms/ml (Cooper et al., 2010). Oligozoospermia can be further classified as mild (between 10 and 15 million sperm/ml), moderate Oligozoospermia (between 5 and 10 million sperm/ml), and severe Oligozoospermia (less than 5 million sperm/ml) (Zakaria et al., 2022).

The causes of oligospermia may be broadly categorized into testicular and non-testicular causes. The testicular causes include those factors that cause the testes to produce low semen quality, where all other factors (e.g., hormonal factors) are normal. Examples include varicoceles, Y chromosome microdeletions, abnormal set of chromosomes (e.g., Klinefelter syndrome), idiopathic testicular failure, cryptorchidism, trauma and mumps (Sharma et al., 2023; Graziani et al., 2024). The non-testicular factors are those that do not necessarily affect testicular function directly. And these may include defects of the genital tract as well as problems in ejaculation, congenital absence of the vas deferens (CAVD), obstruction of the vas deferens, infections (e.g., prostatitis) and retrograde ejaculation. It is reported that about 30% of all cases of oligospermia are idiopathic and cannot be traced to a particular cause (Fainberg et al., 2019). Infertile couples should be counselled that high levels of sperm DNA fragmentation are positively associated with miscarriage. Environment and Genetics have also been established to contribute to male factor infertility (Selvaraju et al., 2020; Graziani et al., 2024).

In fact, based on evidence, the American Urological Association and American Society for Reproductive Medicine have recognized the role of genetics in Oligozoospermia and have provided detailed guidelines on the use of different genetic tests as part of the management of Oligozoospermia in males (Schlegel et al., 2020). These tests include karyotypic analysis to detect sperm aneuploidy, Y-chromosome microdeletions and other structural abnormalities, and sperm DNA fragmentation. DNA fragmentation is the most common manifestation of nuclear DNA damage, and in many cases, this refers to either single-strand or double-strand breaks that have gone beyond the repair mechanism of the cell (Shamsi et al., 2011). Abnormal sperm chromatin/DNA structure is thought to arise from several sources. These include deficiencies in recombination during spermatogenesis, abnormal spermatid maturation, inadequate ratio of the DNA packaging proteins (Protamine 1 and 2), abortive apoptosis, and oxidative stress.

Extrinsic causes include lapse of time from the ejaculation, collection methods, sperm Preparation techniques, storage conditions, post-testicular oxidative stress, varicocele, bacterial Infections, age, abstinence, temperature of testis, reaction to clinical procedures, medicines or vaccines, and exposure to environmental chemicals. Usually, this is associated with apoptotic mechanisms in the cell because DNA fragmentation is a biochemical indicator for apoptosis. (Sakkas and Alvarez, 2010; González-Marín et al., 2012; Agarwal et al., 2020)

Several tests have been developed to analyze and determine the extent of DNA damage in sperm cells. The choice of assay to employ depends on the aspects of DNA damage intended to be investigated, as the current range of sperm DNA tests make use of different principles and have different sensitivities (Sharif, 2013; Esteves et al., 2020). The most commonly used tests include the Comet assay, the Sperm Chromatin Structure Assay (SCSA), the terminal transferase dUTP nick end labeling (TUNEL) assay, and the Sperm Chromatin Dispersion (SCD or Halo) test. In the comet assay, sperm are sandwiched between Agarose layers and then lysed and electrophoresed. The movement of fragmented DNA from a damaged sperm chromatin becomes visible as a comet with a tail. The assay is a microscopic variant of the normal electrophoresis in which the smaller DNA fragments migrate farther than the larger fragments. The staining intensity and length of the comet tail represent the amount of migrated DNA, indicating different degrees of DNA fragmentation. Of these four tests, the Comet Assay has been reported to possess greater sensitivity than other DNA fragmentation assays and is highly reproducible (Speit and Rothfuss, 2012; Dunkenberger et al., 2022; Collins et al., 2023; Walsh and Kato, 2023). It is the only test that measures the

actual damage in individual sperm cells. It can detect damage in sperm of 80% of couples previously diagnosed with idiopathic infertility (Simon et al., 2016). Comet assay has been used in vivo and in vitro in a variety of cells, including sperms, to study the response to genotoxic stimuli such as UV radiation, carcinogen, radiotherapy and chemotherapy (Akang et al., 2022). An additional advantage of comet assay is that it requires fewer sperm (as low as 50 cells) for analysis, so it is particularly useful for men with oligospermia as well as for DNA damage analysis on testicular sperm.

The measure of the extent of DNA fragmentation in sperm cells is called the DNA Fragmentation Index (DFI), and this refers to the percentage of DNA strand breaks in the total sperm. Several studies have tried to propose predictive threshold levels for DNA fragmentation index (DFI) for positive outcomes during ART procedures. For example, Duran et al. (2002) reported low rates of pregnancy after Intra intra-uterine insemination (IUI) in cases where the DFI was greater than 12%. Shamsi et al. (2011) reported DFI >24% in couples experiencing idiopathic recurrent spontaneous abortions. Similar studies (Carrell et al., 2003; Virro et al., 2004) showed sperm DNA fragmentation index to be approximately 38% as compared to about 22% in fertile controls. Furthermore, Shamsi et al. (2009) reported a high DFI for sperms from men with Oligozoospermia (20%) when compared to fertile controls (8%).

Intracytoplasmic Sperm Injection (ICSI) is often the preferred treatment option for men with oligospermia (Haddad et al., 2020; Esteves, 2022; Xu et al., 2022). In this method, a single sperm is selected based on morphology and injected directly into the cytoplasm of the oocyte (WHO, 2021). This direct, artificial selection and injection may be regarded as a shunt of the natural selection process and may increase the potential risk of fertilizing the oocyte with sperms having a defective genome (Siquera et al., 2020). The likelihood of achieving fertilization with DNA-damaged sperm, especially from oligospermic men during ICSI, has provoked extensive investigations on the possible consequences on embryo culture, implantation and, more importantly, the health of resultant offspring (Sharma et al., 2004; Sciorio and Esteves, 2022).

The main objective of this study is to provide more evidence on the extent of DNA fragmentation in the sperms of oligospermic Nigerian men who would normally be scheduled for assisted conception procedures such as ICSI. The study also aims to discuss and highlight the clinical significance of sperm DNA fragmentation testing in the light of assisted reproductive technology. The justification for this is that if the extent of DNA fragmentation in these subjects is beyond the safe reference range, then the prognosis for IVF with ICSI treatment in these patients is poor and more suitable treatment options should be made available for these patients.

MATERIALS AND METHODS

PARTICIPANTS' RECRUITMENT

Twenty-five (25) participants currently attending fertility clinics were recruited for this study after signing an informed consent document. This study was approved by the Bridge Clinic Ethics committee with reference 2016/01. Semen samples were obtained from patients beginning ART treatment at The Bridge Clinic, Lagos, Nigeria. Members of the control group consist of men who have recently become fathers (within the last 8 months), with evidence of established fertility. All semen samples were obtained by masturbation after 3-5 days of abstinence and were collected directly into a sterile plastic container.

SEMEN ANALYSIS AND SPERM PREPARATION

Routine semen analysis and preparation were performed according to WHO guidelines (WHO, 1992). In all cases, semen was prepared by density gradient centrifugation using a commercially manufactured colloidal silica suspension in an isotonic salt solution (PURESPERM 100 Nidacon International AB). The semen samples were layered carefully over the gradient and centrifuged at 1,300 rpm for 20 minutes in a conical tube. The pellets were then re-suspended in pre-equilibrated Human Tubal Fluid with HEPES buffer (LifeGlobal® Group) and centrifuged for 10 minutes. The supernatant was decanted out, and the sperm-rich pellet incubated at 37oC. Final sperm concentration/mL was determined, as well as total sperm motility.

ALKALINE COMET ASSAY

Sperm DNA fragmentation was assessed using single cell gel electrophoresis (Comet) assay, previously optimized for human sperm by Ribas-Maynou et al.

(2012) and stained according to Osipov et al (2014). Briefly, 15µL of each semen sample was mixed with 25µL of low melting point agarose and allowed to solidify with a coverslip on glass slides. After coverslips were removed, slides were incubated in lysis solutions for half an hour. The slide designated for alkaline Comet was denatured in an alkaline buffer for 2.5 minutes and electrophoresed for 20 minutes. Finally, slides were washed in neutralization buffer and ethanol series. After drying, it was followed by staining using Giemsa dye and images were captured with a photomicroscope.

The comets in the images were then analysed using the open-source software OPEN COMET as a plug-in to IMAGE J image-editing software. The following comet parameters are generated on a spreadsheet by the software program. Comet Area, Comet Length, Comet DNA Content, Comet Average Intensity, Head Area, Head Diameter, Head DNA Content, Head Average Intensity, Head DNA %, Tail Area, ail Length, Tail DNA Content, Tail Average, Tail DNA, Tail Moment, Olive Moment. 100 cells were analysed per sample and the mean score of each sample was used as the representative score for that sample in further computations.

STATISTICAL ANALYSIS

The data generated by OPEN COMET were subjected to analysis for mean and standard deviation in SPSS statistical software, version 22.0. Additionally, correlation analysis was performed using Pearson's correlation coefficient (r) to evaluate the strength and direction of linear relationships between variables, such as sperm DNA fragmentation levels and other parameters like sperm concentration, motility, and morphology.

Table 1: Summary of Semen Data for the samples used in the study

CLASS OF OLIGOSPERMIA	NO. OF INDIVIDUALS	AVERAGE AGE	MOTILITY (%)	NORMAL MORPHOLOGY (%)
SEVERE (<5Mio/mL)	13	42.7	29.3	0.0
MODERATE (5-10Mio/mL)	8	34.8	34.3	0.0
MILD (10-15Mio/mL)	4	31.0	45.5	0.5
MILD (10-15Mio/mL)	10	33.7	59.4	3.5

Semen analysis results were classified into three categories – Mild, Moderate and Severe Oligospermia (Table 1). Cases with sperm concentration between 10 - 15 million/mL were classified as mild oligospermia. Semen samples with concentrations 5>10 million/mL were considered to be in the moderate oligospermia category, while sperm concentrations <5 million/mL made up the severe oligospermia category. Out of 25 patients, 13 have severe oligospermia, 8 with moderate and 4 with mild oligospermia. All subjects in this study were observed to have severe teratozoospermia, as all sperm cells in their ejaculate had grossly abnormal morphology. The average age range of participants affected with oligospermia was between 31 – 44 years old. The average age for the participants with normal sperm cells was 33 – 34 years old.

Table 2: Comparison of the mean values for comet parameters between the test cases and the control group

COMET PARAMETER	CONTROL (Mean ±SD)	CASES (Mean ±SD)
COMET AREA	842.11±467.07	903.22±628.23
COMET INTENSITY	47.68±30.00	53.78±36.51
COMET LENGTH	31.26±9.62	32.46±11.33
COMET DNA	46656.55± 50871.1	49246.95±45654.46
HEAD AREA	669.02±425.97	421.35±498.37
HEAD INTENSITY	60.28±33.15	54.34±39.60
HEAD LENGTH	20.30±8.39	18.93±12.27
HEAD DNA	26709.80±47854.14	26483.34±36069
HEAD DNA PERCENT	55.52±17.88	54.78±43.07
TAIL AREA	420.99±138.91	481.86±558.40
TAIL INTENSITY	43.70±25.64	34.62±37.60
TAIL LENGTH	11.78±5.77	13.5±15.09
TAIL DNA	19681.93±9545.04	22763.61±36665.9
TAIL DNA PERCENT	44.48±17.88	45.21±43.07
TAIL MOMENT	9.03±3.19	11.87±14.54
OLIVE MOMENT	5.91±2.62	6.82±7.99

DNA fragmentation was observed in all cases in this study. All comet parameters indicated that there was greater DNA damage in the case group compared to the controls (Table 2). The comet area, intensity, length, Head DNA quantity, tail area, tail length, tail DNA quantity, tail DNA percent, and tail and olive moment were all significantly increased in the cases compared to the controls, indicating an increase in the quantity of fragmented DNA.

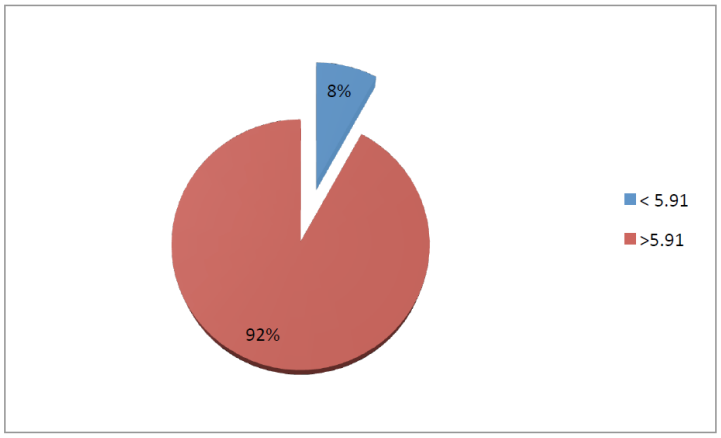


Figure 1: 92 % of sperms from Oligospermia-affected males who participated in the study had an Olive moment higher than the control average of 5.91.

The olive moment from 92% of oligospermia-affected males was higher than the control average of 5.91 (Fig. 1 & 2). When compared with the mild and moderate group, sperms of men with severe oligospermia had significantly increased DNA damage as observed using tail percent DNA, tail moment and olive moment (Fig.

3a). The DNA damage, however, did not show a clear trend, as the level of DNA damage in the mild and moderate group were not significantly different from each other.

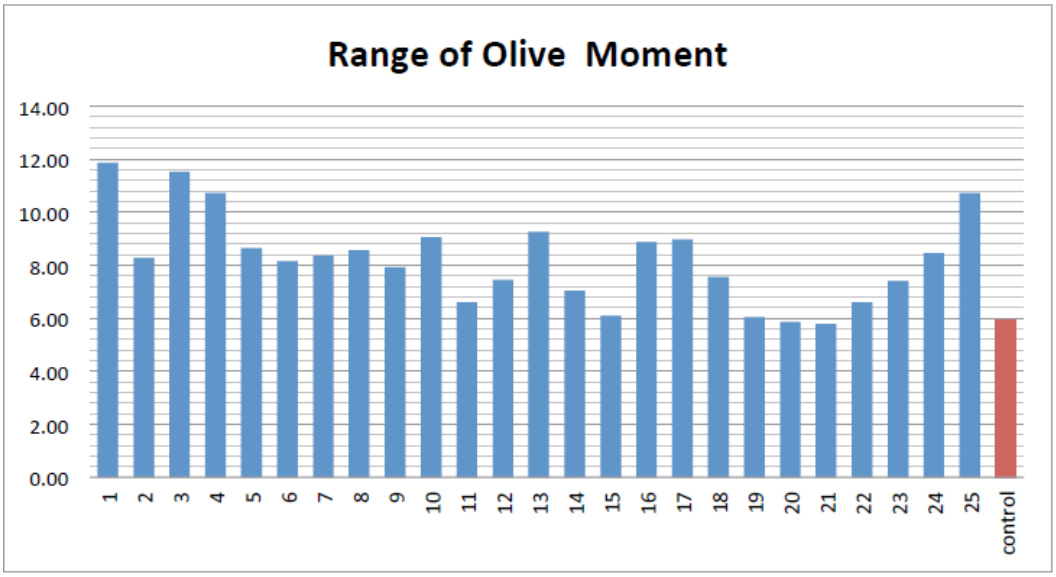
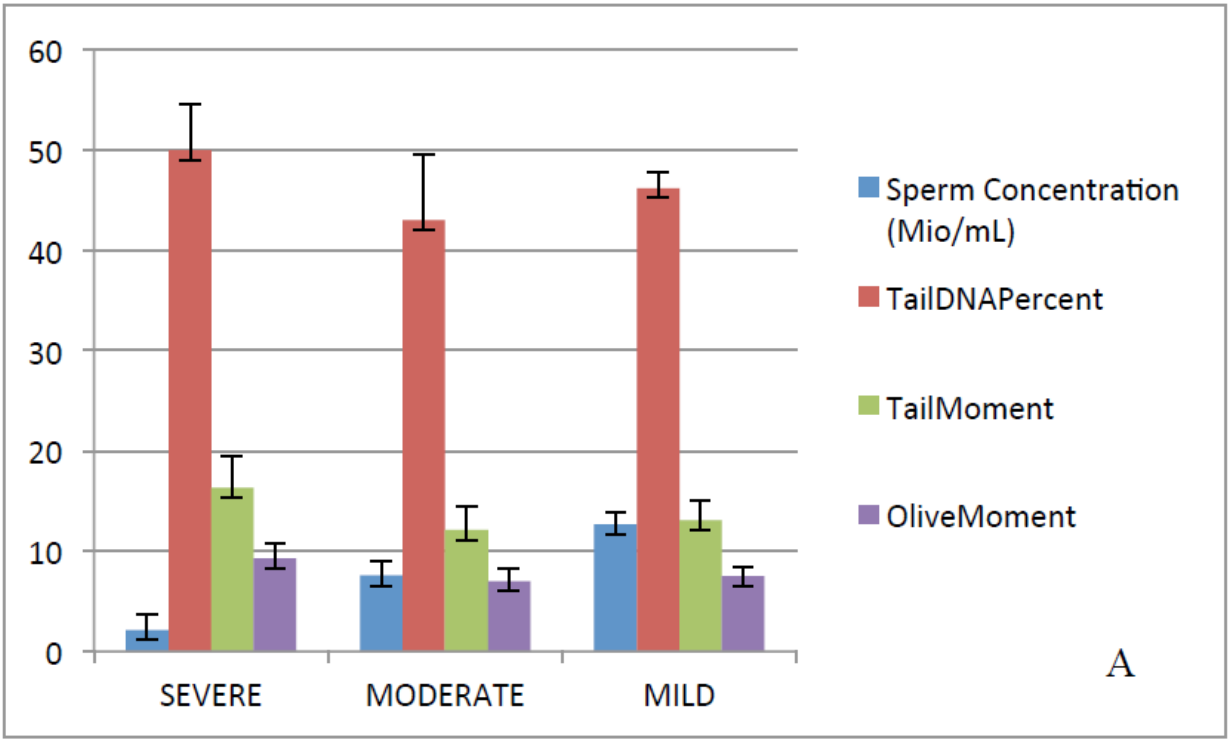


Figure 2: Olive moment in Cases compared with the control average. The range was from 5.77 (Case 21) to 11.87 (Case 1)



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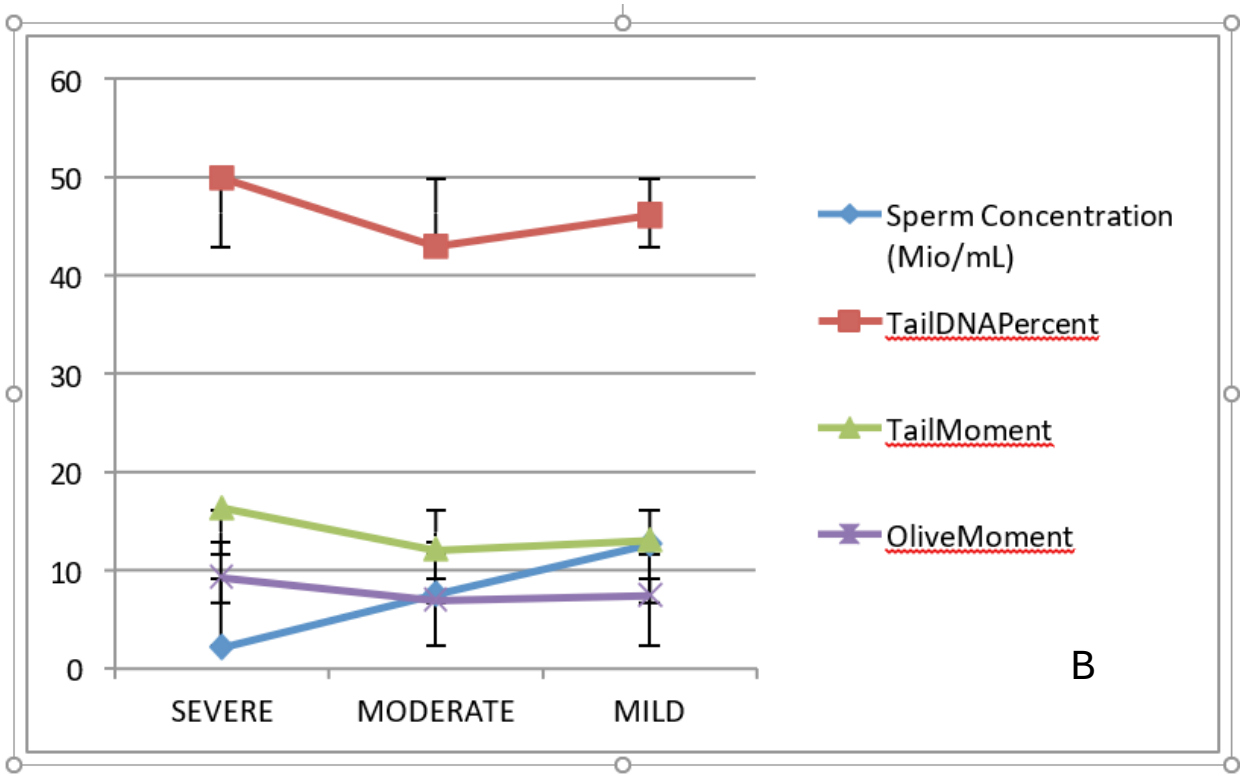

Figure 3: Graphs A and B showing an increase in the values of comet parameters with severity of oligospermia. However, the relationship is not linear.

Sperm concentration had negative, non-significant correlations with Tail DNA Percent, (r (2) = .358, p = .642); Tail Moment, (r (2) = .823, p = .177); and Olive Moment, (r (2) = .733, p = .267) (fig 4a, b, c). These correlations were, however, strong with Tail moment and Olive moment and medium with Tail DNA percent.

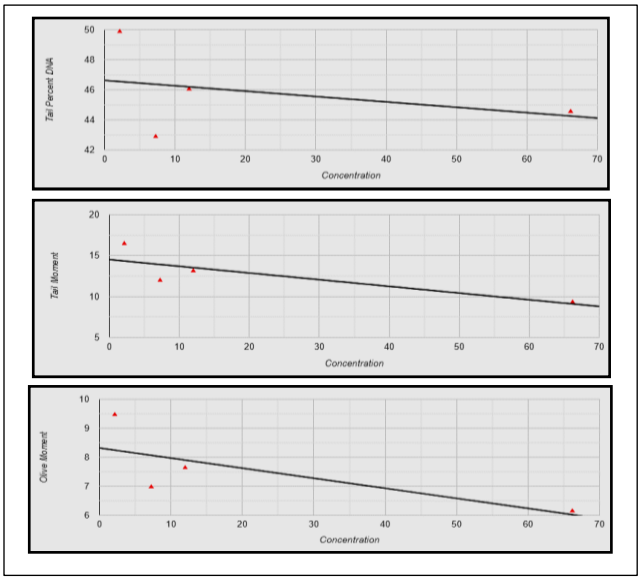

Figure 4a, b, c: Pearson correlation of sperm concentration with (a) Tail DNA Percent, (b) Tail Moment and (c) Olive Moment.

Large positive non-significant relationships were observed between Age and Tail DNA Percent, (r (2) = .719, p = .281); Age and Tail Moment, (r (2) = .711, p = .289) as well as Age and Olive Moment, (r (2) = .767, p = .233) (fig 5a, b, c).

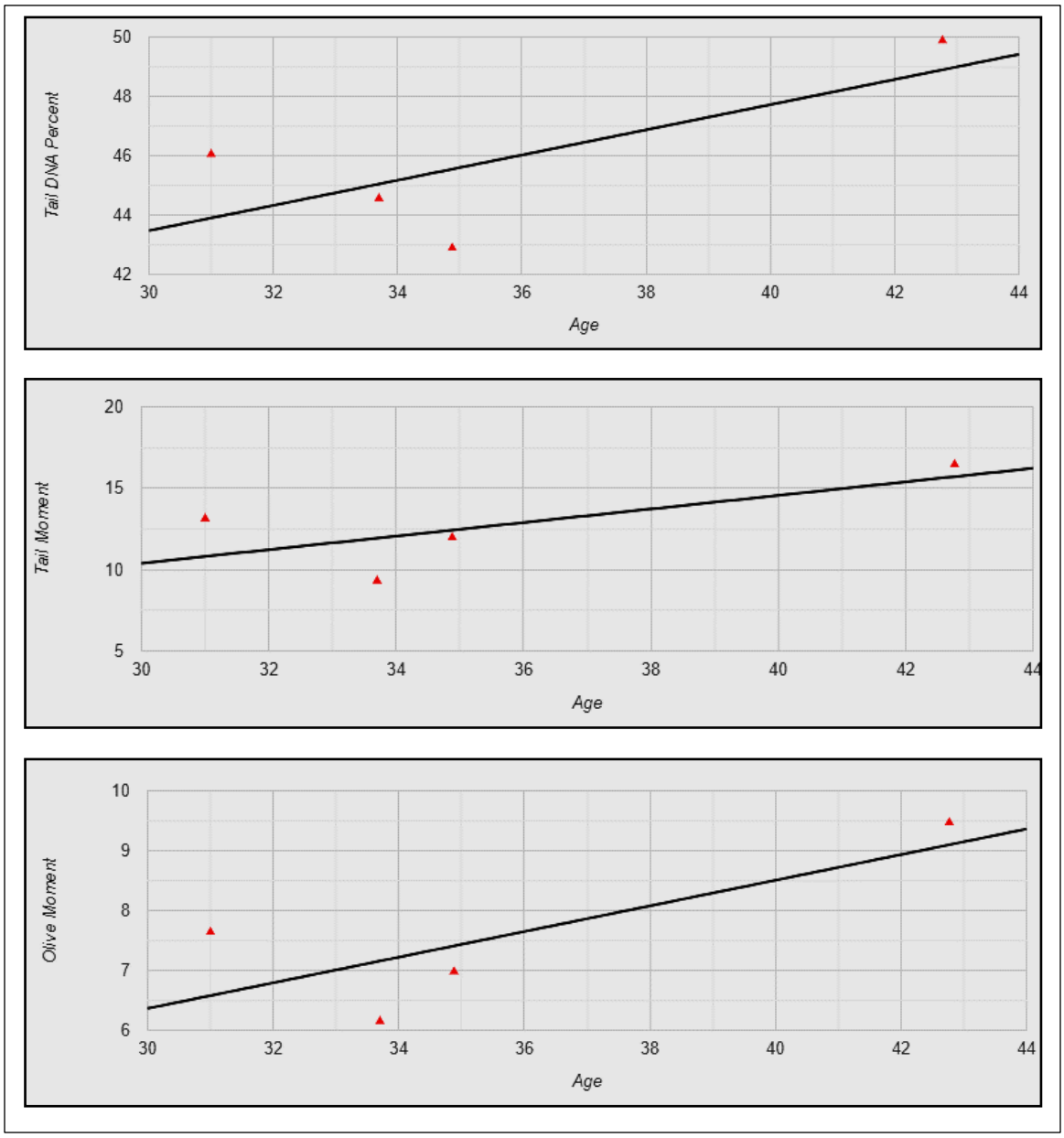

Figure 5a, b, c: Pearson correlation of age with (a) Tail DNA Percent, (b) Tail Moment and (c) Olive Moment.

The results of the Pearson correlation indicated that the relationship between motility and the measures of DNA fragmentation is strong and negative but non-significant. Motility and Tail Percent DNA, (r (2) = .536, p = .464); Motility and Tail Moment, (r (2) = .888, p = .112); and Motility and Olive Moment, (r (2) = .847, p = .153) all had strong correlation as evidence by their coefficients (fig 6a, b, c).

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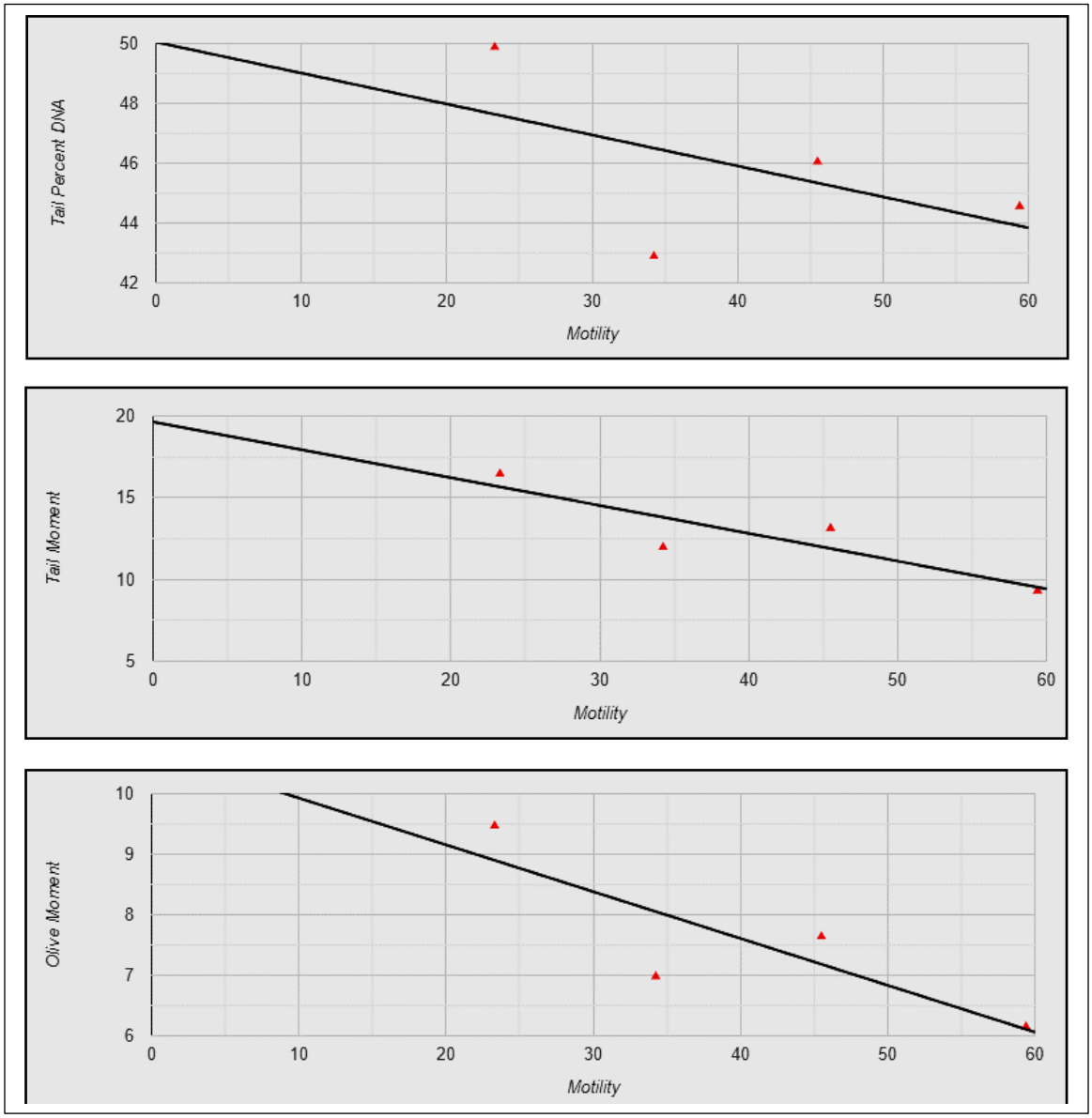

Figure 6a, b, c: Pearson correlation of sperm motility with (a) Tail DNA Percent, (b) Tail Moment and (c) Olive Moment.

Sperm morphology for the mild, moderate and severe groups correlated strongly with Tail Percent DNA, (r (2) = .292, p = .708); Tail Moment, (r (2) = .783, p = .217; and Olive Moment, (r (2) = .687, p = .313). These DNA fragmentation indicators increased with poor sperm morphology (fig 7a, b, c).

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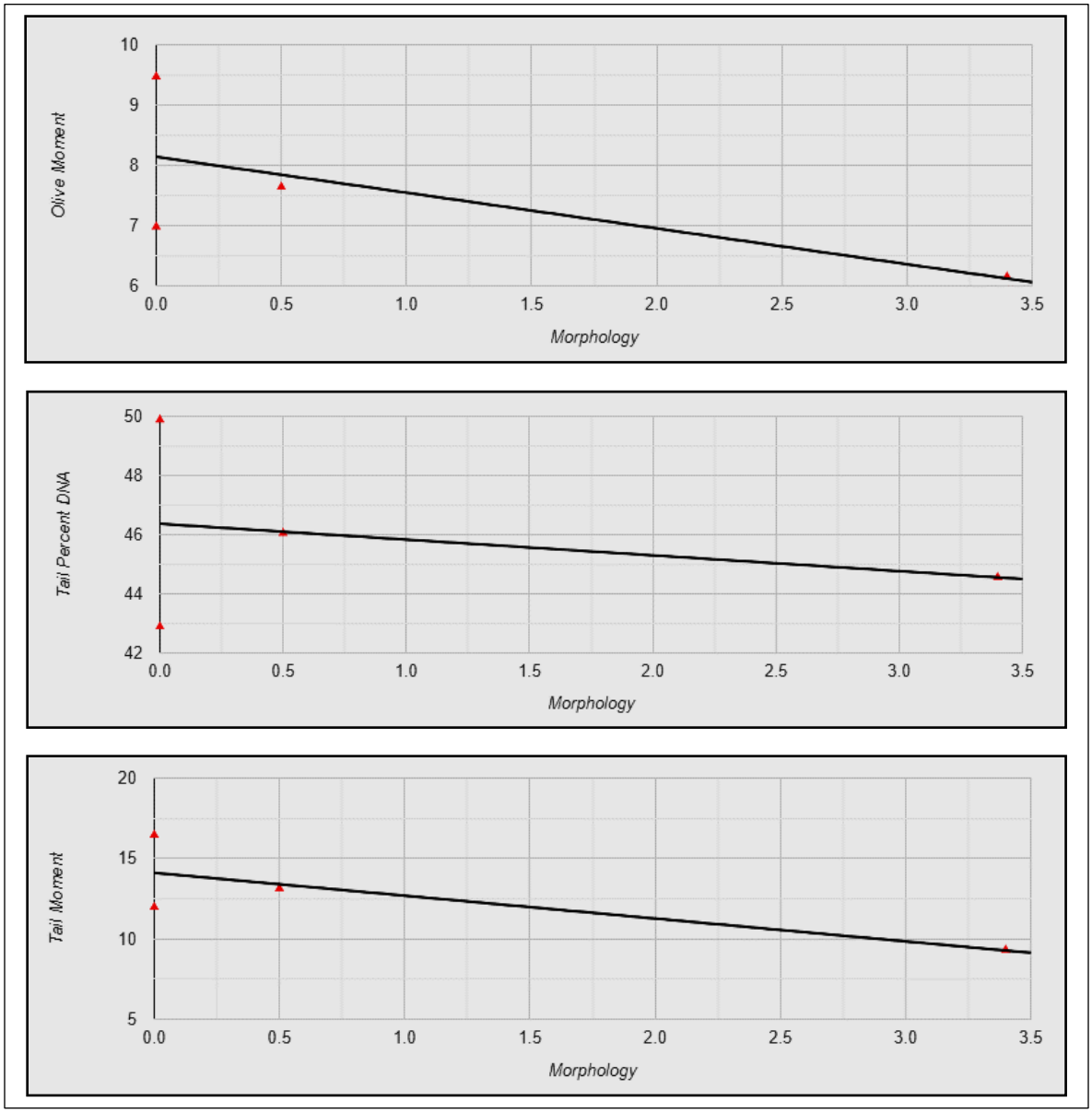

Figure 7a, b, c: Pearson correlation of sperm morphology with (a) Tail DNA Percent, (b) Tail Moment and (c) Olive Moment.

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Discussion

There is a growing body of literature that establishes the involvement of sperm DNA in male infertility (Aitken and de Iuliis, 2007; Evenson et al., 2007). However, the extent of occurrence of male infertility due to DNA damage in different populations is the subject of several research (Lewis & Agbaje, 2008; Esteves et al., 2020, 2021; Go & Shim, 2024; Graziani et al., 2024). Comet assay accurately measures sperm DNA damage (Ribas-Maynou, Garcia-Peiro, et al., 2012; Ribas-Maynou, García-Peiró et al., 2012; Simon & Carrell, 2013; Simon et al., 2017). This study has, therefore, computed the proportion of men with cases of oligospermia with detectable sperm DNA damage and evaluated the level of association of sperm DNA damage with different semen parameters in these men.

This study identified that of the 25 men with oligospermia who reported for assessment, 92 % had varying degrees of sperm DNA damage as measured by the Olive Moment (>5.91) higher than the mean values in the control group. The Olive moment was greater with reduced sperm concentration ($r(2) = .733$, $p = .267$), reduced motility ($r(2) = .847$, $p = .153$), and poor morphology ($r(2) = .687$, $p = .313$) confirming that DNA fragmentation may be responsible for the male-factor infertility (Boushaba and Belaaloui, 2015 Belva et al., (2016). This also increased with increasing age ($r(2) = .767$, $p = .233$) and possibly a result of increasing oxidative stress because of decreased epididymal antioxidant capacity with increasing age in animal models (Graziani et al., 2024).

Similar association between all semen parameters (concentration, motility and morphology) and Tail moment and Tail Percent DNA. Defective sperm DNA, including mitochondrial DNA, is known to affect sperm motility by interfering with energy production in the sperm cells. Also, the impact of DNA on sperm morphology is directly a result of errors during cell division (Maamar, 2022). Several factors cause DNA damage in sperm, including nuclear re-modeling, apoptosis and seminal oxidative stress in the ejaculate. Seminal oxidative stress is mainly caused by activated leucocytes, sperms with abnormal morphology, redox cycling of metabolites or xenobiotics or exposure to radiofrequency radiation or even anti-retroviral therapy might have a critical effect on the etiology of infertility (Graziani et al., 2024; Akang et al., 2022; Selvaraju et al., 2020). The role of Y-chromosome microdeletions can also not be overlooked in cases of oligospermia.

The proportion of men with infertility is maintaining an upward trajectory (Agarwal et al., 2015; Inhorn & Patrizio, 2015; Vollset, 2020; Huang et al., 2023). Therefore, evaluation of oxidative status, antioxidant defense systems and DNA damage, together with sperm parameters, might be a useful tool for diagnosis and treatment of male infertility. Fertility treatment centers can, therefore, be improved by assessing the extent of sperm DNA damage in oligospermic men, as about 70% of men attending fertility clinics are reported to have oligospermia (Uadia and Emokpai, 2015).

The prognostic value of DNA-damaged sperms in ART has been extensively discussed, and DNA damage in human spermatozoa has been identified as a major cause of male infertility and has been used to predict the success of assisted reproductive technologies (ART) (Lin et al., 2008; Esteves et al., 2015; Muratori et al., 2020; Graziani et al., 2024). This DNA damage has also been identified as a major factor responsible for the decline in male fertility indicators in many countries over the past few decades (Carlsen et al., 1992; Faduola and Kolade, 2015; Muratori et al., 2020; Obukohwo et al., 2024).

With the development of intra-cytoplasmic sperm injection (ICSI) and higher magnification sperm selection, more sub-fertile couples with oligospermia seemingly have a better chance at conception than before. And this is even more evident with the proliferation of ART centres in developing countries, including Nigeria (Ajayi et al., 2003; Ola, 2012; Aydos & Aydos, 2021). However, sperm DNA fragmentation has been found to correlate with poor outcomes during ART procedures, including the newly developed intra-cytoplasmic morphologically selected sperm injection (IMSI). Such negative outcomes may include low fertilization rates, cleavage failures, poor embryo quality, implantation failures and early pregnancy loss (Shamsi et al., 2011).

Declarations

Ethics approval and consent to participate

Approval of the research design, with the consent forms and biosample collection methods, was obtained from the Ethics Review Board of the Bridge Clinic Ethics committee

Consent for publication

All the authors provided consent for publication.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

The authors received no funding for this research

Authors' contributions

All authors conceived and designed the research. JO and MUA obtained and analysed the semen samples. UUA performed the Comet assay, and analysed the data. UUA and JHO wrote the manuscript. All the authors read and approved the final manuscript.

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None

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