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FIRST LINE MALE INFERTILITY DETECTION METHOD

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ABSTRACT

Infertility is a well-known terminology worldwide, and infertility affects both males and females. Routine semen analysis was one of many time-consuming and labor-intensive methods used to address the issue of male infertility. Shifting the focus to understand the DNA state in sperms; Using free DNA estimate methods and comet assay to assess fertility in sperm samples may be the most efficient way to accomplish it. The principal purpose was to use the free DNA estimate technique and the comet assay to measure the DNA damage in the sample. Correspondingly, with a sample size of 70, free DNA was estimated using DPA reagent and an alkaline comet assay, as per standard comet assay technique and DPA procedure. The not-proven fertile groups display higher comet tails and absorbance values at 600nm than the fertility groups. The alkaline comet assay's comet-like tails and high absorbance values at 600nm by free DNA quantification verified the underlying hypothesis that DNA damage could underlie male infertility.

Keywords: Infertility, Free DNA estimation, Comet assay, Sperm, DPA reagent

Introduction

Infertility is prevalent that has evolved into a severe medical issue. Many researchers have attempted to define infertility, and there are numerous definitions. Infertility affects half of the world's population, men and females, equally. Many technologies or procedures for detecting infertility and strategies for combating it have been developed during the last decade (Farris, E. J, 1951). Only a few investigations or alternative treatments for male infertility have been studied, and they would only be temporary solutions. Various factors play a role in male infertility, ranging from sperm damage to structural issues (Hopkinsmedicine.org, (article)). To comprehend the volume, motility, viability, or aberrant functions of sperms, rigid and arbitrary benchmarks have been used. Given the hundreds of sperms per ml, it is evident that one cannot correctly foresee the random character of all sperms (Farris, E. J, 1951).

Many research trends have shifted the attention from sperm quantity, motility, and other characteristics to the quality of sperm's DNA to understand the etiology of male infertility (Campbell M 2019). TUNEL, comet assay, and Acridine orange flow cytometric assays, among others, are used to identify DNA damage and its link to infertility (Zini A et al., 2009). The intact DNA in the chromatin of sperm helps in synapses with other DNA and helps form a viable zygote, whereas damaged DNA cannot build a healthy zygote. Many semen samples, even with average sperm count, have shown great sperm damage in them. The DNA damage in infertile males was 40 percent greater than infertile males. From that understanding, the DNA of sperm has become a biomarker in understanding male fertility (Mastroianni, B., 2019)

The comet test has always been a trailblazer for detecting DNA fragmentation in samples. The comet study is premised on denatured cleaved DNA fragments or damaged DNA's ability to travel out of the cell under electrophoresis, resulting in a "comet tail." On the other hand, the undamaged DNA stays within the cell membrane, forming the "comet head," and the range of DNA in the tail is linked to DNA damage. The perception of DNA damaged comet-like tail is utilized to determine the DNA condition of sperm (Ribas-Maynou J et al.2012, Collins AR et al.2004, Pereira AF et al.2017, Simon L et al.2011).

DPA indicator, which significantly binds to DNA compared to proteins or RNA, is another approach for determining the quantity of free DNA in a sample. The DPA method's conception might be a targeted approach to diagnosing DNA damage at its most fundamental and early stages. The DPA technique is based on the idea that in acidic conditions at 100°C, Deoxyribose in DNA produces hydroxy levulinic aldehyde, which reacts with Diphenylamine to produce a blue color. The amount of DNA

present is proportional to the color intensity, measured between 595 and 600 nm (Estimation of DNA by diphenylamine method(internet), Hasanain S 2019(internet))

Employing the intuitive understanding with the DPA colorimetric estimation approach and comet assay aids in knowing sperm sample fertility and can be a quick step toward establishing the cause of infertility and may provide a faster clinical decision than formerly.

Material and Methods

Samples

Seventy sample sizes were acquired and split into two groups, with five samples indicating established fertility and 65 samples extracted personal experiencing difficulty convincing progeny. From the sample collection point, informed consent was acquired.

Fertile control groups

The fertile control groups of 5 semen samples, labeled as fertile, had minimal DNA damage. The comet assay revealed modest amounts of DNA damage.

Hydrogen peroxide treatment on samples

Hydrogen peroxide causes oxidative stress and fragmentation of samples by influencing the redox status and temperature state (Mesa, A.M et al.2017); thereby, intending to develop DNA breaks in samples, Different concentrations of hydrogen peroxide were harnessed at room temperature for 60 minutes; for instances, the concentrations include 0.025, 0.05, 0.075, 0.1, and 0.125mM. The five fertile samples were diluted to 10X107 spermatozoa per ml and then treated with various concentrations of hydrogen peroxide.

Comet assay for Hydrogen peroxide treated samples.

DNA fragmentation caused by hydrogen peroxide can be detected in alkaline circumstances (Villani. P et al. 2010). According to the comet assay protocol, the cells are thawed and washed in 1 percent phosphate buffer under alkaline conditions with modest changes. (Ribas-Maynou J et al.2012). The 10µl mixture was then embedded in 1% agarose and mounted on a slide, after which it was rinsed with tris borate EDTA (0.445 M tris HCL, 0.445M boric acid, and 0.01M EDTA). The DNA is then denatured under alkaline conditions and electrophoresed once the cells have been lysed. The cells were stained with ethidium bromide dye and photographed after being analyzed under ultraviolet light.

Free DNA estimation of the Hydrogen peroxide treated samples.

The DPA technique examined the samples with fragmented molecular weight in the supernatant. The tubes were filled with 3ml of DPA reagent. Then they were immersed in a boiling water bath for 10 minutes at 100℃, and the absorbance (O.D.) was

measured with a spectrophotometer at a wavelength of 600nm.

Comet assay and Free DNA estimation by DPA method of the semen samples

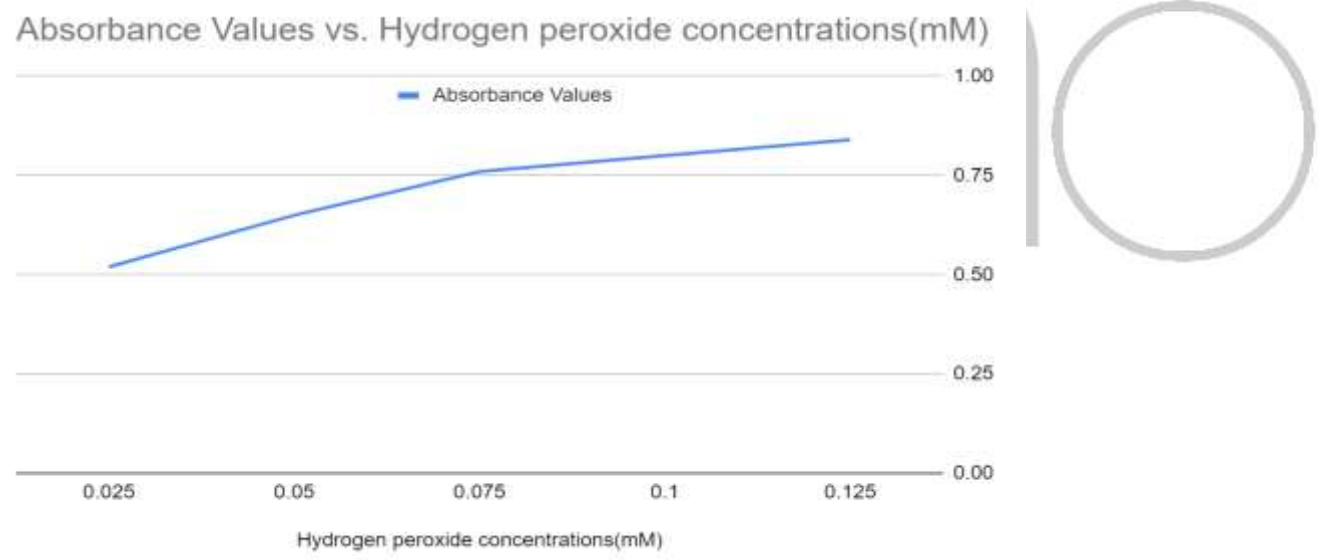
It is acknowledged as the easiest and most convenient method for identifying DNA damage in semen samples. First, 10X107 spermatozoa per ml were taken; cells and nuclear membrane were washed with 1% SDS and high molecular weight genomic DNA was preferentially precipitated with 3 M sodium acetate.

After that, the fragmented low molecular weight is analyzed using the DPA approach in the semen samples, the blue color intensity at 600nm was detected, and absorbance (O.D.) readings were taken. The comet assay procedure was carried out in alkaline settings as described above.

Results and Discussion

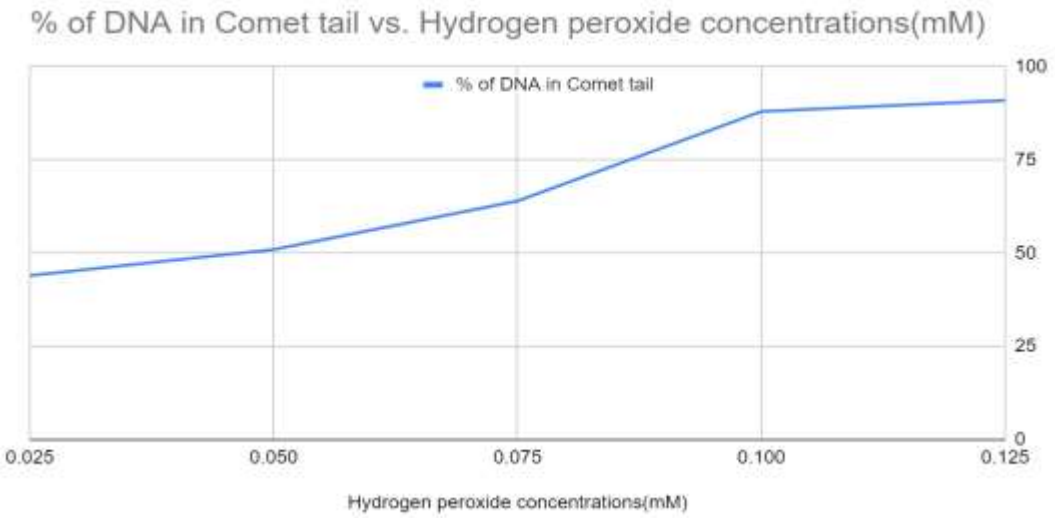
Comet assay and Free DNA estimation for hydrogen peroxide treated samples.

Hydrogen peroxide treated fertile samples were employed as a controlled group to study the DNA damage. Different concentrations of hydrogen peroxide were used. O.D. measurements were taken at 600nm by the DPA method. A standard graph was also constructed on the "Y" axis for absorbance at 600 nm vs. the concentration of hydrogen peroxide treated samples on the "X" axis.



Graph 1: Depicts the free DNA or DNA damage recorded at various hydrogen peroxide concentrations.

The comet assay was performed in the same sequence based on protocol, and a graph was devised by plotting the "Y" axis for DNA damage against the concentration of hydrogen peroxide treated samples on the "X" axis.



Graph 2: shows the percentage of DNA in comet tails at various hydrogen peroxide concentrations.

It can be deduced from graphs 1 and 2 that when samples were treated with hydrogen peroxide, a certain percentage of DNA was fragmented and visible in the comet test as comet tails, which is understood from the principle of the comet assay, that displays DNA damage

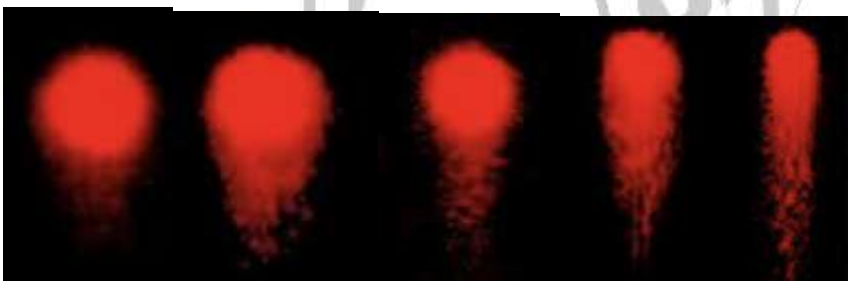
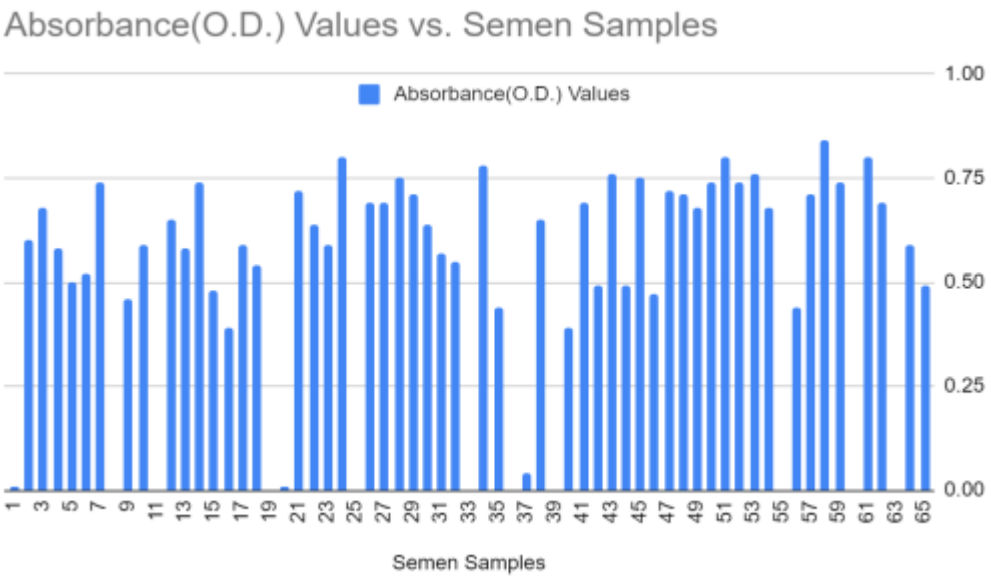


Figure 1: Comet assay findings with varied hydrogen peroxide concentrations, depicting the comet-like tails of varying lengths reflecting the extent of DNA damage in samples.

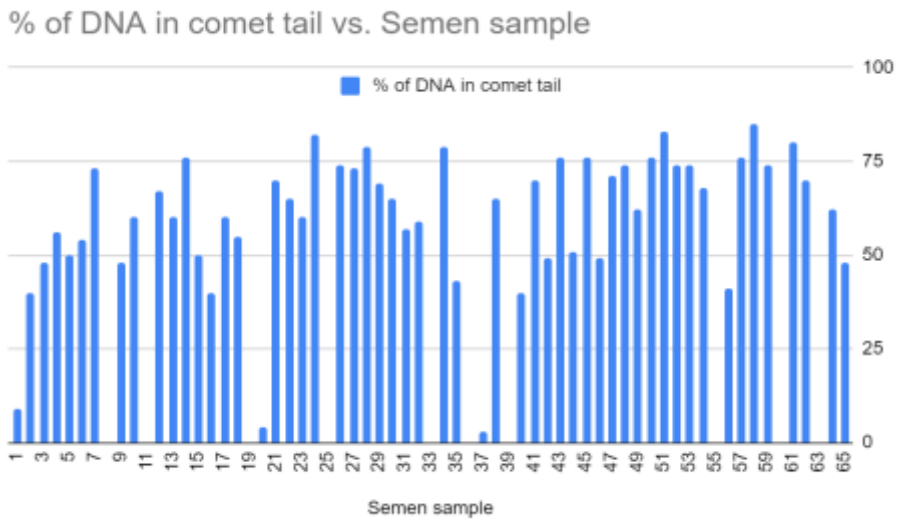
Detection of DNA damage in semen samples by DPA method and comet assay. The comet assay, which used semen samples, produced a comet-like tail, demonstrating DNA damage while displaying a standard structure in healthy samples. The overall concentration of DNA is represented by the semen samples exposed to the DPA procedure, which yields O.D. readings. With a sample size of 70, apart from 5 control groups. DNA damage was found in 80% [52] of the samples, whereas DNA damage was not found in 20% [13]. A standard graph was also displayed for absorbance at 600 nm on the "Y" axis vs. semen sample on the "X" axis was displayed.



Graph 3: The graph depicts the free DNA recorded from semen samples using the DPA technique; the graph explains that the most significant absorbance values reflect the free DNA that is present in the mixture, with the damaged, free DNA being indicated by the highest absorbance values and low values or absence of column in graph denote the absence of free DNA.

The graph revealed that the higher the absorbance values, the more DNA damage was present in 52 samples, whereas the other 13 samples had negligible values, indicating minimal DNA damage. The results were compared to graph 1, and DNA damage was noted.

Then the comet assay is followed, and a graph was devised by plotting the "Y" axis for DNA damage against the concentration of hydrogen peroxide treated samples on the "X" axis.



Graph 4: The graph depicts the % DNA in comet tail recorded from semen samples using the comet assay; the graph explains that the greatest % of DNA in comet tail values reflect the damaged DNA that is present in the mixture according to the principle of the comet assay, with the damaged DNA being indicated by the highest % of comet tails and low values or absence of column in graph denote the absence of damaged DNA in the comet tail.

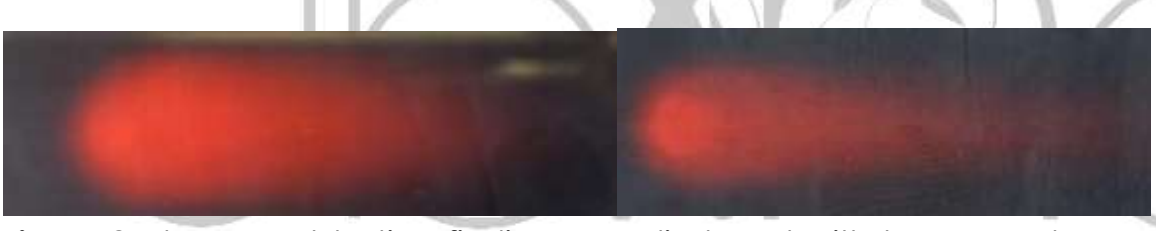


Figure 2: The comet testing findings are displayed with two DNA damaged sperm samples among the 62 samples. The comet-like tails indicate the DNA damage in samples.

Statistical analysis

Standard graphs for DNA fragmentation under hydrogen peroxide treatment by free DNA estimation technique using DPA reagent were drawn for a correlational comparison to identify the DNA damage in the semen samples. Furthermore, a comparable percentage was noticed from the graphs, which denotes the presence of DNA fragmentation in the sample.

From the study of comet assays with various doses of hydrogen peroxide and comet assays of sperm samples, the amount of damage done by Hydrogen peroxide was related to the amount of damage present in the semen samples. The technique is supported by in vitro DNA damage caused by hydrogen peroxide.

This study contributes to our understanding of many techniques for identifying DNA damage in sperm samples (Barratt C L 2017). Combining the DPA and the comet assay potentially has its own set of advantages and disadvantages. On the flipside, detecting male infertility could be a quick and straightforward test. The results reveal that DNA damage is more severe in infertile sperm samples than in viable sperm samples, and this approach might be a straightforward method to test the existence of DNA damage (Vasan S.S. 2011).

Damage to a male's DNA has the potential to affect his ability to reproduce (J. Ribas-Maynou etal.2012, Acharyya S, 2004). DPA (Hasnain S 2019) and comet assay(Collins A R 2004) discussed the simple methods separately, but a combination between them would be simple and clear to decide the procedures that needed to be run after that if a step was made to discover the exact reason. Rather than a long process to assess if a male is fertile or infertile, this approach may provide a speedy result toward assisted reproductive technologies (ATS) methods (maleinfertility.org(internet)).

The case study employing O.D. readings by free DNA estimate and comet test supports the underlying idea that DNA may be a factor in infertility. There will undoubtedly be a break in the usual semen analysis when the assumption is confirmed.

If the samples are not taken correctly or the materials used are not up to standard, the results will be limited. After a more thorough examination under ideal conditions, this test will be added as the first step in regular sperm analysis. Even said, bigger sample size and additional testing may make the method more reliable.

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