DOI: https://doi.org/10.53350/pjmhs2023175652

ORIGINAL ARTICLE

DNA Damage in Predatory Insects Comet Assay and Different Bioassay for Insecticides in Insects

HAFZA IRSHAD[1], SAAD IRFAN[2], ASIA NOUREEN[3], ARSHAD ALI[4], INAMULLAH[5], FARHAD BADSHAH[6], MUHAMMAD BABAR MALOOK[7]
[1]*Department, Center for Interdisciplinary Research in Basic Science, University of International Islamic University Islamabad, Pakistan*
[2]*Department of Zoology, Bachelor of Science in Zoology, University of Central Punjab, Lahore*
[3]*Center for Interdisciplinary Research in Basic Science, International Islamic University Islamabad*
[4]*Department of Zoology, Entomology laboratory, Faculty of Biological Sciences, Qauid-i-Azam University, Islamabad*
[5,6]*Department of Zoology, Abdul Wali Khan University Mardan*
[7]*Department Of Plant Pathology, Institute of Plant Protection, Muhammad Nawaz Sharif University of Agriculture Multan, Pakistan*
Correspondence author: Hafza Irshad, Email:hafsa_shah@outlook.com

ABSTRACT

Aim: This study aims to quantify DNA damage in predatory insects by the use of a comet assay or a bioassay designed for insecticides, and to identify the primary parameters that influence the efficacy of a bioassay or a comet test.

Methods: The present study involved the implementation of a qualitative analysis of the comet assay as a methodological approach. A group of scientists has studied DNA damage in predatory insects. Their research focused on understanding the impact of environmental factors on the DNA of these insects.

Results: By analyzing the DNA damage, they were able to identify potential threats to their survival and develop strategies for their conservation. The comet assay has revealed both the overall extent of damage in cells of predatory insects and the severity of damage incurred by individual cells. The comet assay is a field that can and should expand and diversify.

Practical Implications: Recent advances in the area include high throughput variants of the method, investigations into epigenetics via methylation, chromosome-separated comets, and the isolation of individual genes from inside the comet.

Conclusion: The problem at hand is and has been the laborious process of rating comets. The field of cometology is on the verge of adopting a fully automated photo analysis system, but first they need one. Meanwhile, once every two years, researchers from all around the world gather to share their latest findings and debate the latest developments in their field at the International Comet Assay Workshop.

Keywords: Bioassay, Cometassay, DNA, Insects

INTRODUCTION

Comet assay is a common method for measuring DNA damage in predatory insects brought on by variables like pesticide exposure. However, it is important to complement this assay with different bioassays specifically designed to evaluate the effectiveness and toxicity of insecticides on insects. These bioassays provide a comprehensive understanding of the impact of insecticides on predatory insects, helping researchers and policymakers make informed decisions regarding pest control strategies. The comet assay is favored by researchers because to its straightforward methodology, high level of sensitivity, wide range of applications, and overall appeal. The images it generates bear resemblance to heavenly entities, therefore simulating the act of observing compromised DNA molecules via the microscope. The process of extracting histones from DNA results in the disruption of the nucleosome structure.

Background: In the 1970s, Cook et al, suggested the idea of a nucleoid structure, in which individual DNA molecules construct a sequence of structural loops by linking at regular intervals to a nuclear matrix or scaffold[(1)]. The application of an electrophoretic field induces the extension of a single loop towards the anode subsequent to the relaxation of supercoiling caused by a DNA strand break (SB). Fractures induce the unwinding of DNA loops, hence leading to an increase in the amount of DNA present in comet tails. The quantification of DNA break frequency may be inferred by assessing the relative intensity of tail DNA fluorescence subsequent to the process of dye labelling. The initial comet test was conducted by Johanson utilizing lysis and electrophoresis techniques under a pH of 9.5[(2)]. The migration of DNA towards the anode was elucidated as a consequence of the relaxation of supercoils under cellular bombardment. The prevailing method for comet testing in contemporary times was established by the elevation of electrophoresis pH to a value exceeding 13, accomplished by employing a 0.3 M NaOH solution. The comprehensive methodology, which is now employed in our laboratory, was recently documented in a publication by Collins and Azqueta[(3)].

RESEARCH QUESTION

The following examination questions are what this research aims to answer:

- How far does DNA damage in predatory insects occur?
- What are the critical factors that influence bioassay or comet assay performance?

Aim and Scope: The objective of this study is to quantify DNA damage in predatory insects by the use of a comet assay or a bioassay designed for insecticides. Additionally, the study aims to identify the primary parameters that influence the efficacy of a bioassay or a comet test.

Problem Statement: The main concern is on identifying the key elements that exert the greatest influence on the efficacy of a comet test or bioassay for pesticides in assessing the extent of DNA damage in predatory insects. It is of utmost importance to correctly evaluate DNA damage in predatory insects by identifying the fundamental parameters that impact the efficacy of a comet test or bioassay for pesticides. The acquisition of this knowledge will not alone augment our comprehension of the ramifications of pesticides on these species, but also facilitate the formulation of efficacious approaches for their preservation and administration.

Significance of the Study: Cultured cells, hemolymph cells from predatory insects, sperm cells, disaggregated animal tissues, yeast cells, and nuclei discharged from plant tissue are just some of the many cell types that have been analyzed with the comet assay. It would appear that the comet test is applicable to the study of every eukaryotic cell type that can be acquired as a single cell or nuclear suspension. The production of comets from single chromosomes has been demonstrated to be feasible[(4)]. In addition to quantifying DNA single-strand breaks (SBs), the technique has been adapted to identify impaired bases by subjecting the DNA to enzymatic digestion following lysis, utilizing a lesion-specific enzyme that induces a DNA breakage at the site of damage (refer to the section under "Measuring various types of DNA damage"). The investigation of oxidized and alkylated bases, as well as the examination of cyclobutane pyrimidine dimers generated by UV irradiation, has been conducted in this manner.

The comet test has been implemented in four primary research domains. Genotoxicity testing is employed to evaluate the possible carcinogenic capabilities of newly developed medications, cosmetics, or other chemical substances. This screening process can be conducted either in vivo, including the examination of different tissues obtained from experimental animals, or in vitro,

utilizing appropriate cultivated cell lines. Equally prevalent is the utilization of human biomonitoring in various contexts. This includes investigating the impact of occupational or environmental exposure to hazardous agents on DNA, examining the factors contributing to disease or its effects, exploring individual variations in DNA repair capacity or antioxidant resistance, and monitoring alterations resulting from dietary intervention involving antioxidant micronutrients. Ecogenotoxicology represents a field of study that involves the utilization of diverse creatures such as mussels, earthworms, snails, and plants, among others, to serve as indicators for assessing genetic harm caused by pollutants. This assessment can be conducted either in natural environments or controlled laboratory settings. The comet assay is an invaluable instrument in fundamental scientific investigations pertaining to the processes behind DNA damage and repair.

Literature Review: The Comet test works best with cells in suspension, such as those found in peripheral blood mononuclear (PBMN) cells or cultured cells that have been grown in suspension or are easily accessible in suspension. Although tissues provide a bigger challenge, scientists have devised methods, such as the use of enzymes and/or physical maceration, to successfully extract cells or nuclei of excellent quality from a wide range of animal tissues[5]. The formation of ice crystals can cause physical shearing of DNA, rendering frozen cells or tissues unsuitable for preservation. However, DNA can be preserved by a gradual freezing process if cells are suspended in a freezing solution made of culture medium supplemented with 10% foetal bovine serum and 10% dimethylsulfoxide (DMSO).

The researchers demonstrated that entire blood may be effectively cryopreserved at a temperature of -80 °C. Nevertheless, according to a recent study conducted by Al-Salmani, it was shown that credible data on DNA strand breaking upon thawing may be obtained by using tiny aliquots without the presence of DMSO and without previous separation of peripheral blood mononuclear (PBMN) cells[6]. This discovery has significant utility; nevertheless, the long-term viability of preserving such samples remains uncertain.

Special attention is necessary while doing the comet assay on plants. The release of nuclei from plant tissue is achieved by mechanically disrupting the tissue with a sharp blade, while maintaining a low temperature environment[7]. According to Pourrut, it is advisable to conduct nuclear isolation in conditions of darkness or under non-actinic red light in order to prevent any potential harm caused by exposure to white light[8].

Yeast cells possess a cellulose cell wall that necessitates disruption in order to liberate the nucleus. However, the comet test has demonstrated effective execution as evidenced by the work of Oliveira and Johansson[9].

The detection of mitochondrial DNA is challenging due to its tiny size, leading to its rapid disappearance from the gel upon cell lysis[10]. In theory, the study of mitochondria and bacteria using the comet test may not be feasible due to their lack of the conventional organisation of eukaryotic DNA on a nuclear matrix. The formation of comet tails in this assay is dependent on the anchoring of DNA loops in the comet head, which is believed to represent the residual matrix.

When comparing different doses of ionizing radiation that might cause DNA damage, the comet test has a limited dynamic range, measuring only from 0.1 to 10 Gy. There are hundreds to thousands of fractures in a normal human cell, and this phenomenon includes the whole range. Radiation exposure of 10 Gy causes these gaps to spread out to around 1 mega base. Therefore, thinking about this process in terms of DNA fragmentation alone is inadequate. Since DNA breaks within the physiological dynamic range often have the capacity to mend, this reduces worries about cytotoxicity as a possible contributor to DNA damage.

Many misunderstandings have developed around the comet assay. Among the most widely held beliefs is that a pH of 7 in the lab can only detect double-strand breaks (DSBs), whereas a pH of 9 is necessary to detect single-strand breaks (SSBs). Alkaline elution and alkaline unwinding, which need a high pH to assist the separation of DNA strands, provide a misleading parallel. Each single-stranded break facilitates denaturation during alkaline unwinding. The number of fractures determines how much of the molecule will unwind upon neutralization of the alkali after a certain incubation period. Depending on their size, these DNA fragments might be either single- or double-stranded. One way to gauge how often SBs occur is by measuring how much DNA is single-stranded. While certain alkali-labile sites may convert into breaks under alkaline pH circumstances, the comet test depends on the relaxing of supercoils and hence minimizes the influence of pH. Is there evidence to back up this theory, and if so, how strong is it? Single-strand breaks (SSBs) are 20 times more common than double-strand breaks (DSBs) when it comes to ionizing radiation[11]. Hydrogen peroxide (H2O2), according to their findings, is even more effective than ozone at inducing SSBs. Johanson were the first to publish results from experiments using ionizing radiation, detailing things like dosage responses and limitations of detection[2].

Notably, despite differences in the pH conditions employed, with one approach conducted at a neutral pH and the other at a strong alkali, both studies yielded highly comparable results. The study conducted by Collins, in which he demonstrated comparable levels of sensitivity across neutral and alkaline tests in the detection of fractures generated by H2O2 and methylmethanesulphonate (MMS)[3]. He did not see any migration under neutral circumstances following irradiation with X-rays up to a dose of 1 Gy, which is known to induce comet tails at high pH. This observation, however, raises curiosity.

Another prevalent misconception is to the notion that comets with diminutive heads and substantial tails are indicative of apoptosis. This topic will be further addressed and explored in a subsequent section of this article titled "Hedgehogs and viability."

One practical constraint associated with the comet assay is to the maximum number of samples that may be effectively processed within a single experimental setup. In general, it is common practice to employ a conventional big electrophoresis tank and utilize 2 gels per slide, resulting in a maximum of 40 gels. However, it is worth noting that more efficient procedures for higher throughput, such as those discussed in the next section titled "High throughput comet assay methods," have been developed.

The process of scoring comets can be laborious, and the appeal of the comet assay may diminish with time, particularly when there is an increase in throughput. This is due to the additional time required for microscopic examination. Thus far, the achievement of complete success in the realm of completely automated scoring has remained elusive.

The original version of the comet test is capable of detecting strand breakage and alkali-labile sites. Sugar-sweetened beverages (SSBs) undergo prompt repair mechanisms and are generally considered to have minimal impact on the cell's survival and genetic integrity. DNA damage encompasses a range of distinct forms, including oxidized and alkylated bases, adducts, and cross-links, which are considered to be of significant interest and importance. These forms of DNA damage have the ability to induce mutations, and as a result, the test was subsequently adapted to identify and measure certain types of these damages.

METHODOLOGY

This research study aims at exploring the DNA damage in predatory insects using Comet assay. For this purpose, the relevant research study is disclosed in this section.

Study Design and Setting: The present study involved the implementation of a qualitative analysis of the comet assay as a methodological approach. The objective of this study was to examine the degree of DNA damage in predatory insects that were subjected to specific environmental variables. The comet test, a commonly utilized method for evaluating DNA damage, was

implemented inside a controlled laboratory environment to precisely quantify the extent of genetic harm in these insects.

Study Participants: A group of scientists has studied DNA damage in predatory insects. Their research focused on understanding the impact of environmental factors on the DNA of these insects. By analyzing the DNA damage, they were able to identify potential threats to their survival and develop strategies for their conservation.

Comet Assay: In order to investigate DNA damage, the team used the comet test technique. DNA damage in single cells may be quickly and accurately measured using single cell gel electrophoresis (SCGE), often known as the Comet Assay. Cancer researchers use this technique, among others, to evaluate the effectiveness and genotoxicity of potential cancer-prevention chemotherapy. Swedish researcher Johanson, developed this technique in 2014[13]. To improve upon this technique, he created the Alkaline Comet Assay. The end result resembles a "comet" with a "head" and "tail." Broken or damaged strands make form the DNA molecule's tail, whereas intact strands make up the molecule's head. Successful applications of the Comet Assay to the study of plant cells are somewhat uncommon, although its principal employment have been in the study of eukaryotic animals.

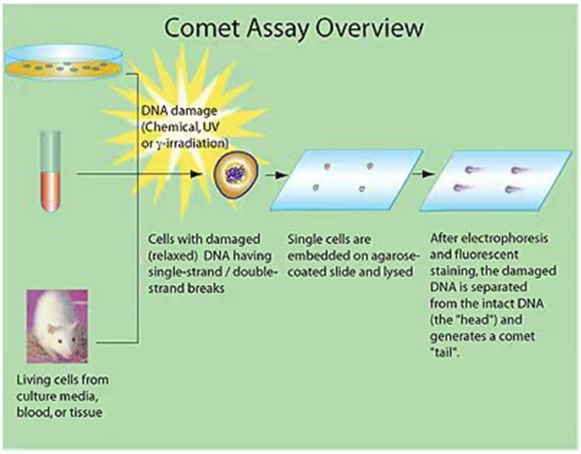

Figure 1: Comet Assay Technique

Microscopic slides of cells preserved in agarose gel. All of the cell's proteins are drained out by lysing the cells. DNA can unravel in neutral or alkaline environments. Unwinding the DNA strands allows for electrophoresis, which removes any faulty or broken DNA from the nucleus. After staining the gel with a DNA-specific fluorescent dye such ethidium bromide or propidium iodide, the amount of fluorescence in the head and the length of the tail are read. Damage to DNA is directly proportionate to the amount of DNA shed by the comet's nucleus. The Comet Assay is a very sensitive method for detecting various forms of DNA damage, such as double- and single-strand breaks, alkali-labile sites, oxidative base damage, and DNA cross-linking with DNA or protein. The Comet Assay is also useful for monitoring DNA repair in real-time in live cells[12].

Data Analysis: The data has been examined using qualitative analysis, including thematic analysis techniques. Hence, certain observations necessitate the utilization of a quantitative methodology for their representation. Thematic analysis was employed to analyze the data. Thematic analysis is a qualitative research approach that entails the identification and examination of recurring patterns and themes within the collected data. Through the utilization of this methodology, scholars are able to acquire a more profound comprehension of the fundamental significances and interpretations embedded within the collected data. Nevertheless, to enhance the comprehensiveness of the findings, it may be imperative to integrate quantitative methodologies for the purpose of quantifying and assessing certain dimensions of the discovered themes.

RESULTS

Measuring DNA Damage in Predatory Insects: Enzymatic digestion of the nucleoid DNA soon after lysis is generally sufficient for the identification of modest changes to DNA bases. An enzyme specific to the lesion removes the changed base during this digestion, creating an apurinic/apyrimidinic (AP)-site. A subsequent action of an AP lyase activity converts the AP-site into a DNA break. Alternatively, if the AP lyase activity is slow-acting, the DNA's exposure to high pH circumstances after lysis might cause the AP-site to convert into a DNA break (as seen in Figure 2).

Collins et al. used the Escherichia coli-derived DNA repair enzyme Endonuclease III (EndoIII; thymine glycol DNA glycosylase, EC 4.2.99.18)[14]. Duthie et al.'s research entailed the first-ever controlled trial of a dietary intervention in humans[15]. These results represent the first clinical research to show that antioxidant supplementation can reduce endogenous DNA damage. In 2010, Collins reported the use of Formamidopyrimidine DNA glycosylase (FPG, EC)[14]. Removed by FPG are oxidized purines such as 8-oxo-7,8-dihydroguanine (8-oxoGua) and open-ringed adenine and guanine molecules known as formamidopyrimidines. Alkylating chemicals can cause ring-opened N7 guanine adducts, which Li and Speit showed FPG can attack. 8-oxoGua DNA glycosylase 1 (OGG1) (EC 4.2.99.18) is the mammalian homologue of FPG[12][16]. Since OGG1 does not function on alkylated purines, Smith claim that it is more specific than FPG[17]. AlkA, also called 3-methyladenine DNA glycosylase II (EC 3.2.2.21), uses 3-methyladenine as its major substrate. But it's worth noting that the enzyme may serve as a non-specific nuclease, although a weak one. Collins et al. utilized the comet test to detect and measure the extent of MMS damage[3]. According to the study's findings, there is a significant amount of damage in human PBMN. In 2017, McMillan introduced uracil DNA glycosylase (UDG: EC 3.2.2.3) as a method for detecting misincorporated uracil in DNA [15]. UV radiation, especially at shorter wavelengths like UVC, causes adjacent pyrimidines inside the DNA molecule to dimerize. T4 endonuclease V, also called pyrimidine dimer DNA glycosylase (EC 3.1.25.1), then converts these pyrimidine dimers into breaks. Collins et al. 2013b showed that this enzyme was useful in the study and characterisation of cell lines with increased sensitivity to UV light.

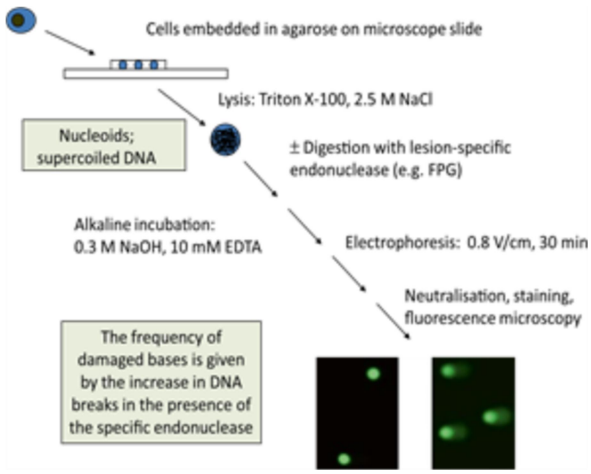

Figure 2: Measuring DNA in Predatory Insects

The incorporation of lesion-specific enzymes has significantly enhanced the use of the comet test, particularly in the context of human biomonitoring. Notably, the utilization of FPG has been extensively employed in several studies investigating

oxidative DNA damage. Nevertheless, there are some crucial factors to take into account while employing the enzyme technique.

Purification of enzymes can provide products with reduced stability compared to crude extracts. The presence of non-specific nucleases in a crude extract is not a significant concern, as the enzymes used are often derived from bacterial strains that over-produce the enzyme and have it as a major portion of the overall protein content.

The concentration at which the enzyme is used has significant importance. The detection threshold should be set at a level that is sufficiently high to identify all existing lesions, while ensuring that nonspecific nucleases do not cause interference. It is imperative to conduct a titration experiment utilizing cells that are confirmed to possess the specific lesion under investigation. Ideally, these cells should be treated with a suitable agent, such as UVC (for T4 endonuclease V), MMS (for AlkA), or photosensitizer Ro 19-8022 in combination with light (for FPG and OGG1). When considering endonuclease III (or FPG in the absence of the photosensitizer), cellular treatment with H2O2 and subsequent incubation can facilitate the repair of single-strand breaks (SBs), resulting in oxidized bases that can serve as a substrate for enzyme testing. An ideal detection of lesions can be achieved when a plateau is attained at a specific enzyme concentration.

In the typical experimental procedure, it is customary to include sample gels that are treated with enzyme buffer in addition to the gels that are incubated with the enzyme. The score obtained from the buffer incubation is deducted from the score obtained with the enzyme, resulting in the determination of "net enzyme sensitive sites," which is reported as a percentage of DNA in the tail.

As previously noted, it is important to consider the lack of complete specificity exhibited by enzymes when interpreting experimental findings. The presence of apurinic/apyrimidinic (AP) lyase activity alongside the glycosylase enzyme should not compromise specificity. This is because any AP-sites that are already present in the DNA would theoretically be converted to strand breaks (SBs) under alkaline conditions, and thus be accounted for in the buffer incubation score. The presence of cross-links between DNA molecules, or between DNA and protein, elicits an opposing influence compared to single-strand breaks (SBs), since they impede the elongation of fragmented DNA loops into a comet tail. Consequently, their examination may be conducted by assessing their capacity to impede the typical translocation of DNA subsequent to ionizing radiation or H2O2 exposure.

The critical factors that influence bioassay or comet assay performance: With the goal of promoting standardization of comet assay protocols, two research groups (one of which was our own) set out to define the factors that are most important in determining comet assay performance and influencing results; their conclusions were very similar[18][19].

There is a discernible variation depending on the agarose concentration. Although a concentration of 0.4% may provide some tail DNA (with cells harboring DNA breaks), this is far too low to be useful. As the agarose concentration increases to 1.3%, less and less tail DNA is present in the sample.

There appears to be no correlation between lysis time and solubility in Triton and high salt solutions. Although overnight lysis is preferred, it is common practice, and keeping gels in lysis solution for weeks has been proven to have no appreciable effect on results.

There must be a period of incubation in an alkaline environment. The percentage of tail DNA either gradually grew from 10 to 60 minutes[18] or began to level out at 40 minutes[19]. The response of untreated cells to incubation was time dependent.

Variations in voltage and electrophoresis time elicited similar responses in comets originating from treated and untreated cells[18]. The relative tail intensity rises dramatically with both voltage and duration, as seen in Fig. 3. The voltage gradient is the most important element, namely the voltage gradient across the slides' base. There are just a few millimeters of solution over the slides, thus the voltage drops between the electrode and the edge of the platform is negligible for electrophoresis. An increase in the voltage gradient can partially negate the effect of a decrease in electrophoresis time.

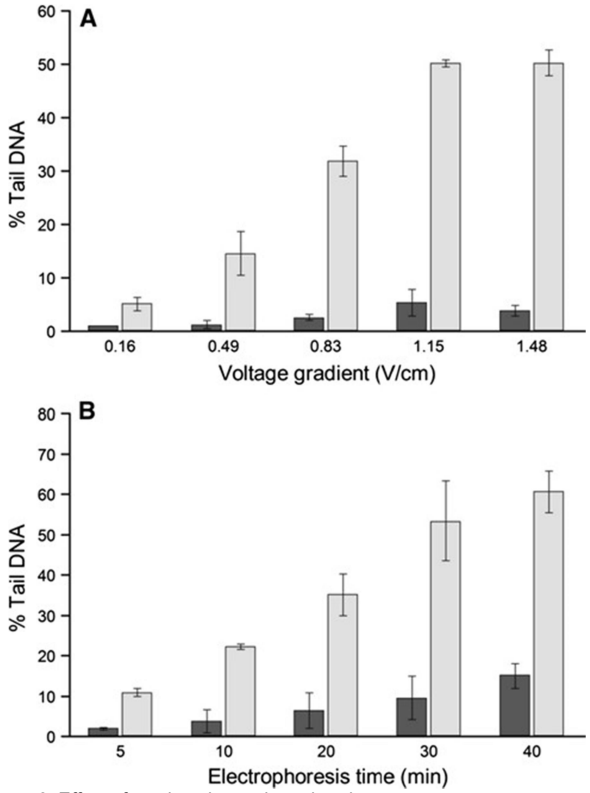

Figure 3: Effect of varying electrophoresis voltage

Many researchers utilize 300 mA, adjusting the current by adding or subtracting electrophoresis fluid. Changing the current has a minor effect on the comet's external appearance since a change in the depth of solution causes a change in the voltage gradient. The temperature may rise dramatically if a very high current is used. The electrophoresis is typically performed in a cold environment, with temperatures at or below 15 °C. The availability of temperature-controlled tanks should facilitate greater reproducibility of results both within and between laboratories that employ the same instruments.

Incorporating an enzyme digestion stage requires careful consideration of enzyme concentration and incubation duration. Non-specific breakdown can occur at too high of a concentration (particularly when a crude enzyme extract is utilized, as additional nucleases are likely to be present at low concentrations), as was described above. Once the proper concentration of the enzyme has been determined by titration or is that specified by the supplier, the enzyme should be tested using a variety of incubation durations. After a particular amount of time has passed (usually about 30 minutes), the number of breaks should level off[18]. In the event that normal cells possess an adequate baseline amount of damage, such as base oxidation, peripheral blood mononuclear (PBMN) cells may be employed for the examination. The experimentation can be conducted by subjecting cells to chemical treatment or radiation exposure, therefore inducing the necessary damage.

Various stains are employed, leading to different levels of fluorescence intensity. Factors such as the quality and adjustments of the microscope, the age of the UV lamp, and the settings of the image analysis software (e.g., fluorescence detection threshold) might influence comet scores. The presence of several factors may

account for a significant portion of the variability reported in the trials conducted by the European Comet Assay Validation Group (ECVAG), as suggested by Ersson et al.[18]. The results showed that automated image analysis had the highest accuracy in comet detection and analysis, followed by conventional computerized image analysis. Visual scoring had the lowest accuracy and was subject to operator bias. These findings suggest that automated image analysis is a more reliable and objective method for comet analysis compared to conventional computerized image analysis and visual scoring. It has been observed that visual grading tends to consistently exaggerate mild degrees of damage, whereas automated image processing has a propensity to ignore comets with significant damage. Nevertheless, a Bland-Altman analysis conducted by Azqueta revealed that the outcomes obtained from the three scoring techniques exhibit comparable validity and interchangeability[19]. This finding implies that there is minimal variation in sensitivity across these approaches.

The importance of cell/comet density cannot be overstated. While having too few cells in the gel would be disastrous, having too many would also be problematic. Comets that overlap each other are extremely challenging to grade, therefore high-quality picture analysis software will often filter them out. Finding the optimal density requires practice, but we've provided some pointers.

- Principles (A condensed, illustrative list based on the material presented above)
- Aim for 104 cells per gel when employing the traditional approach, and 250 cells per gel when employing minigels. Keep in mind that there is a chance that some cells will die off in the processing steps[20].
- Bring the final concentration of cells in agarose to somewhere between 0.6% and 0.8%. Since the stock agarose solution tends to become more concentrated via evaporation, it is recommended that it not be used more than three or four times and instead be stored in tiny aliquots.
- You can lyse for 1 hour, overnight, or even longer at 4 degrees Celsius (but watch out for gel detachment after lengthy lysis times).
- The optimal incubation conditions for enzymes should be strictly followed. Gels that have had enzyme added to them while still at room temperature will need to be incubated at 37 °C for at least 10 minutes.
- Incubate in alkaline solution at 4 C for ideally 40 minutes (but no less than 20).
- Perform electrophoresis in a cool environment for 20 minutes at 1.15 V/cm (across the platform), or for 30 minutes at 0.8 V/cm.
- The gels should dry at room temperature after neutralization and washing. Drying the gel makes it thinner, allowing for easier comet focus.

DISCUSSION

The comet assay can reveal both the overall extent of damage in cells of predatory insects and the severity of damage incurred by individual cells. Understanding the reaction of single cells to a toxic substance might be crucial in some situations. Some human PBMN cells are resistant to H2O2 treatment and stay unharmed even when exposed to amounts of H2O2 that cause a high percentage of tail DNA in the remainder of the cell population[19]. Although the phenomena have not been thoroughly studied, it may be due to differences in antioxidant levels among the various PBMN cell types.

In most circumstances, however, we may safely ignore comet-specific data in favor of the sample-wide mean or median comet score when determining the amount of the damage. For the purposes of statistical testing, the 'experimental unit' is defined at this stage. It is important to consider the context while choosing between mean and median. The median may be instructive if there are just a few comets with extremely high damage and there is reason to believe that they are outliers. In cases where dispersion is small, however, the mean can be useful. With the visual scoring system, you just sum up the points for each comet, so there's no need to make that call. To learn more about how to choose the right statistical tests to assess your comet assay results, we recommend the excellent publications by Lovell and Omori[21][22].

When evaluating base damage using lesion-specific enzymes, it is usual practice to subtract the score for buffer incubation from the score for enzyme incubation to determine the number of net enzyme-sensitive sites. Remove the buffer from the incubation process of nucleoids if the score for breakage increases significantly above the 'lysis only' value. Without initially translating all comet scores to DNA break frequencies using a calibration curve (see below), scores from the nonlinear part of the dose-effect curve may lead to an underestimate of enzyme-sensitive locations.

In most cases, the amount of DNA damage present in the tail is acceptable. However, the regularity of actual DNA breaks can be a useful means of conveying damage. For this, a calibration curve can be quite helpful. Ionizing radiation causes 0.3 DNA breaks per 109 Dalton per Gy in most cell types, according to alkaline sucrose sedimentation studies[23]. Exposure of cells to X- or -irradiation at doses up to around 10 Gy results in a linear relationship between the amount of radiation and the amount of tail DNA lost, up to about 75% tail DNA[24]. Breaks per 109 Dalton, breaks per 106 base pairs, and breaks per cell are all easily translated from % tail DNA values using a few easy conversion factors. Embedding cells in agarose (prior to lysis) and irradiating them can prevent breaks in the embedding from healing over during a calibration experiment on ice. According to Collins et al., calibration curves created in various laboratories are similar but not identical[24]. Recent comparative study coordinated by the EC-funded COMICS project (Brunborg et al. in preparation) suggests that errors in radiation source calibration account for part of the variation. Ionizing radiation is typically hard to come by in laboratories. The use of chemicals that can harm DNA is not acceptable in calibration. The degree to which H2O2 causes strand breakage varies substantially between cell types and even between individual cells, most likely as a result of changes in antioxidant levels. Frank fractures are more likely to form as a result of the repair process, which increases the potential variability and makes them unsuitable for calibration, despite the fact that alkylating agents produce more base damage than frank breaks.

CONCLUSION

The comet assay is a field that can and should expand and diversify. Recent advances in the area include high throughput variants of the method, investigations into epigenetics via methylation, chromosome-separated comets, and the isolation of individual genes from inside the comet.

Regulatory bodies are beginning to take notice of the test, which is still likely the most often utilized for genotoxicity evaluation. We think that the incorporation of lesion-specific enzymes significantly enhances its use as a "indicator assay," revealing insights into the mechanisms of action of putative carcinogens. It's possible that more enzymes will be added to the repertoire in the future, and that many of these enzymes have not yet been tested. The creation of an enzymatic method for recognizing big adducts is very desirable. Many influential research in human biomonitoring have relied on the comet test. While it would seem that using a consistent procedure and a comparable scoring mechanism would lead to improved uniformity, this is not the case.

It is possible that logistical challenges in collecting and transporting samples from far-flung locations are to blame for the comet assay's lack of widespread use in ecogenotoxicology. A 'field comet assay' that can do all the procedures, from sample collection to image analysis, in the back of a van would be incredibly useful.

The problem at hand is and has been the laborious process of rating comets. The field of cometology is on the verge of adopting a fully automated photo analysis system, but first they need one. Meanwhile, once every two years, researchers from all around the world gather to share their latest findings and debate the latest developments in their field at the International Comet Assay Workshop.

REFERENCES

1. Cook, PR. The organization of replication and transcription. Science (2nd Edition) 284:1790–1795, 2011
2. Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123:291–298, 2014
3. Collins, AR, Azqueta, A. Single-cell gel electrophoresis combined with lesion-specific enzymes to measure oxidative damage to DNA. Meth Cell Biol 112:69–92, 2012a
4. Cortés-Gutiérrez EI, Dávila-Rodríguez MI, Fernández JL, LópezFernández C, Gosálbez A, Gosálvez, J. New application of the comet assay: chromosomeGÇôComet assay. J Histochem Cytochem 59:655–660, 2011
5. Hagmar L, Bonassi S, Stromberg U, Brogger A, Knudsen LE, Norppa H, Reuterwall C. Chromosomal aberrations in lymphocytes predict human cancer: a report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). Cancer Res 58:4117–4121, 2010
6. Al-Salmani K, Abbas HHK, Schulpen S, Karbaschi M, Abdalla I, Bowman KJ, So KK, Evans MD, Jones GDD, Godschalk RW, Cooke MS. Simplified method for the collection, storage, and comet assay analysis of DNA damage in whole blood. Free Rad Biol Med 51:719–725, 2011
7. Gichner, T. Znidar, I. Wagner, ED. Plewa, MJ. The use of higher plants in the comet assay. In: Anderson D, Dhawan A (eds) The comet assay in toxicology. Royal Society of Chemistry, London, pp 98–119, 2009.
8. Pourrut B, Séverine J, Silvestre J, Pinelli E. Lead-induced DNA damage in Vicia faba root cells: potential involvement of oxidative stress. Mut Res Genet Toxicol Environ Mutagen 726:123–128. 2011
9. Oliveira R, Johansson B. Quantitative DNA damage and repair measurement with the yeast comet assay. In: Bjergbæk L (ed) DNA repair protocols, Meth Mol Biol 920. Humana Press, New York, pp 101–109, 2012
10. Shaposhnikov S, Larsson C, Henriksson S, Collins A, Nilsson M. Detection of Alu sequences and mtDNA in comets using padlock probes. Mutagenesis 21:243–247, 2006
11. Kohn KW (2019) X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution. Nucl Acids Res 7:793–804, 2019
12. Li Q, Laval J, Ludlum DB. FPG protein releases a ring-opened N-7 guanine adduct from DNA that has been modified by sulfur mustard. Carcinogenesis 18:1035–1038, 2013
13. Anderson SP, Dobson VL, Duthie SJ, Kyle JAM, Collins, AR. Absorption and DNA protective effects of flavonoid glycosides from an onion meal. Eur J Nutr 39:213–223, 2020
14. Collins A. Detection of oxidised purines and UVinduced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay. Alt Lab Animals 24:405–411, 2010
15. McMillan P (2017) Uracil misincorporation in human DNA detected using single cell gel electrophoresis. Carcinogenesis 18:1709–1714, 2017
16. Speit G, Schütz P, Bonzheim I, Trenz K, Hoffmann H. Sensitivity of the FPG protein towards alkylation damage in the comet assay. Toxicol Lett 146:151–158, 2004
17. Smith J, Dusinska M, Kuricova M, Soucek P, Vodickova L, Susova S, Naccarati A, Tulupova E, Vodicka P. Relationship between the capacity to repair 8-oxoguanine, biomarkers of genotoxicity and individual susceptibility in styrene-exposed workers. Mut Res Genet Toxicol Environ Mutagen 634:101–111, 2006
18. Ersson JF. & Meller C. Assessing the DNA methylation status of single cells with the comet assay. Anal Biochem 400:190–194, 2011
19. Azqueta A, Gutzkow KB, Brunborg G, Collins, AR. Towards a more reliable comet assay; Optimising agarose concentration, unwinding time and electrophoresis conditions. Mutat Res 724:41–45, 2011a
20. Ho CM. Lewis SEM, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Molec Human Repro 2:613–619, 2011
21. Lovell DP. Statistical analysis of comet assay data. In: Anderson D, Dhawan A (eds) The comet assay in toxicology. Royal Society of Chemistry, London, pp 424–450, 2008
22. Lovel YT, Benzie IFF, Collins AR, Choi SW, Cheng CY, Yow CMN, Tse MMY. A buccal cell model comet assay: development and evaluation for human biomonitoring and nutritional studies. Mutat Res 578:371–381, 2022
23. Ahnstorm, B. The in vitro and in vivo comet assays. In: Parry JM, Parry EM (eds) Genetic toxicology: principles and methods, pp 143–163, 2012
24. Collins AR. Increasing the resolution of the comet assay using fluorescent in situ hybridization: a review. Mutagenesis 24:383–389, 2008