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# Critical issues with the *in vivo* comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT)

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ABSTRACT

As a part of the 6th IWGT, an expert working group on the comet assay evaluated critical topics related to the use of the *in vivo* comet assay in regulatory genotoxicity testing. The areas covered were: identification of the domain of applicability and regulatory acceptance, identification of critical parameters of the protocol and attempts to standardize the assay, experience with combination and integration with other *in vivo* studies, demonstration of laboratory proficiency, sensitivity and power of the protocol used, use of different tissues, freezing of samples, and choice of appropriate measures of cytotoxicity. The standard protocol detects various types of DNA lesions but it does not detect all types of DNA damage. Modifications of the standard protocol may be used to detect additional types of specific DNA damage (e.g., cross-links, bulky adducts, oxidized bases). In addition, the working group identified critical parameters that should be carefully controlled and described in detail in every published study protocol.

*In vivo* comet assay results are more reliable if they were obtained in laboratories that have demonstrated proficiency. This includes demonstration of adequate response to vehicle controls and an adequate response to a positive control for each tissue being examined. There was a general agreement that freezing of samples is an option but more data are needed in order to establish generally accepted protocols. With regard to tissue toxicity, the working group concluded that cytotoxicity could be a confounder of comet results. It is recommended to look at multiple parameters such as histopathological observations, organ-specific clinical chemistry as well as indicators of tissue inflammation to decide whether compound-specific toxicity might influence the result. The expert working group concluded that the alkaline *in vivo* comet assay is a mature test for the evaluation of genotoxicity and can be recommended to regulatory agencies for use.



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## 1. Introduction

The comet assay is a test for the detection of DNA damage which is widely used in basic research, biomonitoring and genotoxicity testing. The working group of the 6th International Workshop on Genotoxicity Testing (IWGT) considered only the *in vivo* comet assay and its use in regulatory genotoxicity testing. The *in vivo* comet assay has become a component of some genotoxicity test strategies and generally accepted test protocols have evolved over the years. A large international collaborative trial sponsored by the Japanese Center for the Validation of Alternative Methods (JaCVAM) was recently completed [1–4] and approval of an OECD test guideline [5] is expected this year.

The first attempt to develop guidelines for the use of the comet assay in genetic toxicology was made at the IWGT (at that time still called IWGTP) held in 1999 [6]. The goal of the expert panel was to identify minimal standards for obtaining reproducible and reliable comet assay data. An *in vivo* comet assay working group was also part of the 4th IWGT held in 2005. The discussion topics covered at the 2005 workshop were: multiple dose levels versus limit dose, cell isolation process, image analysis or manual scoring, historical control data, minimal reporting standards and concurrent measures of cytotoxicity [7]. At the 5th IWGT held in 2009, there was no comet assay working group, but an "*in vivo* genotoxicity testing" working group discussed two topics related to the *in vivo* comet assay: combination of the micronucleus (MN) assay and comet assay into acute studies and integration of comet assays into repeated-dose toxicity (RDT) studies. The working group came to the conclusion that the combination/integration of the *in vivo* MN assay and the *in vivo* comet assay is scientifically justified for both acute and RDT studies. The report stated that there is a need to provide historical control data and that more data are needed for compounds with diverse modes-of-action, test compounds with extra-hepatic target tissues, and the use of frozen cell samples [8]. Based on this previous work, a comet assay working group convened in conjunction with the 6th IWGT held in Foz do Iguacu, Brazil in October/November 2013 and addressed the following topics: identification of the domain of applicability, identification of critical parameters of the protocol and attempts to standardize the assay, experience with combination and integration with other *in vivo* studies, demonstration of power and sensitivity of the test protocol and laboratory proficiency, use of tissues other than liver, freezing of samples and appropriate measures of cytotoxicity.

## 2. Domain of applicability and regulatory acceptance

Based on a general agreement of all members of the working group, the first fundamental conclusion was that the *in vivo* comet assay is mature enough to produce reliable results. However, for regulatory purposes, it should only be performed by laboratories that have demonstrated proficiency (see Section 4). The assay will be used in various contexts such as for screening compounds with unknown genotoxic potential, to follow up positive *in vitro* genotoxicity test results, or to follow up a finding of tumors in a particular tissue.

The most commonly used *in vivo* genotoxicity assay is the *in vivo* rodent MN assay in bone marrow or peripheral blood. When further *in vivo* testing was required, in the past the preferred second *in vivo* assay was the rodent liver unscheduled DNA synthesis (UDS) assay. Nowadays, the *in vivo* comet and transgenic rodent mutation assays have widely replaced the *in vivo* UDS assay due to the perceived lack of sensitivity of the UDS assay and the ability to sample a wide variety of tissues for the newer assays. A comparative analysis of the *in vivo* UDS and comet assays revealed that the *in vivo* comet assay had higher sensitivity for the detection of rodent carcinogens which gave negative results in the *in vivo* rodent MN assay [9]. This evaluation is supported by a recent survey among pharmaceutical companies [10]. Their experience with use of the *in vivo* comet assay for regulatory purposes in drug development was assessed. The comet assay was used as a second *in vivo* test, most often to follow up a positive finding in an *in vitro* test. Of the more than 100 comet assay studies reviewed, around 10% showed a positive result. This was a much higher incidence of *in vivo* positives when compared to use of the *in vivo* rat liver UDS test as the second *in vivo* test during regulatory drug testing. When used in this way the positive rate for the *in vivo* UDS test did not exceed the 1% level (P. Kasper, personal communication). Although studies using both the *in vivo* UDS and the comet assay were not available to provide a direct comparison, the >10-fold higher frequency of positive results of the comet assay suggests a higher sensitivity compared to the UDS test. The study authors assessed overall experience with the assay in safety evaluation of pharmaceuticals as satisfactory. In most cases interpretation of the results was straightforward, with only a few studies not clearly positive or negative. The need for expert judgment to interpret the results was relatively rare and consistent with experience with other *in vivo* assays. During discussion of this survey it was noted that some regulatory agencies in Europe are already beginning to train regulatory reviewers to assess comet assay study reports.

The comet assay is a test for the detection of DNA damage. The standard protocol for the alkaline version detects a broad spectrum but not all types of DNA damage. Use of this protocol enables detection of induced DNA strand breaks, alkali-labile sites and strand breaks associated with incomplete excision repair sites. Even when the lesion is unknown the domain of applicability is broad enough such that the assay can be useful as part of a battery of tests to detect DNA-damaging agents. DNA crosslinking may be detected by a decrease in migration compared to the concurrent control but the standard protocol is not designed for this purpose and may need to be altered to reliably detect this class of damage. Since chemicals which cause crosslinking can sometimes cause other types of DNA damage in addition to crosslinks, the reduced migration of crosslinked DNA and the increased migration of DNA induced by strand breaks may make it difficult to reliably identify some members of this class of chemicals. With blinded testing in the JaCVAM validation trial damage by one crosslinking agent, cis-platin, was detected, but for another, busulfan, it was not [2]. Modifications that improve the detection of crosslinking chemicals are well established for the *in vitro* comet assay [11–13] but published experience with crosslinking agents using the *in vivo* comet assay is limited [14]. Bulky adducts are another example of a lesion class which might be missed. Bulky adducts may lead to increased DNA migration during excision repair because incision of DNA in the course of excision repair may increase DNA migration [15] but in practice it is sometimes difficult to detect this increase. DNA strand breaks induced by reactive oxygen species (ROS) *in vivo* have proved hard to detect, perhaps because such damage is rapidly repaired. However, ROS also oxidize bases, and these can be readily detected by incorporating in the assay (after cell lysis) a step in which the DNA is incubated with a lesion-specific endonuclease that converts the altered bases to breaks. This approach has been successfully used in *in vitro* studies [16,17] but experience with the *in vivo* comet assay is limited. However, similar to the detection of crosslinks, it seems plausible to assume that this methodology will work *in vivo* also (and its use has been reported [18]) since the enzyme treatment is done *ex vivo*, after slides are prepared. However, some participants noted experiencing increased inter-sample variability when using lesion-specific enzymes. Furthermore, a selective application of this approach has practical limitations for safety testing where experiments must be conducted blind and the type of lesion that may be induced in tissues is typically not known in advance.



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