Eignung der high throughput Version des Comet Assays als Screening-Verfahren

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Abkürzungsverzeichnis

AT	Ames-Test
CA	Comet Assay
CT	Chromosomenabberationstest
MCP	Multichamberplate
MT	Mikronukleustest
OECD	Organisation for Economic Co-operation and Development
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals

1. Zusammenfassung

1.1 Zusammenfassung

Es besteht ein hoher Bedarf, die steigende Anzahl an gentoxischen Verbindungen in der Umwelt und bei der Entwicklung neuer Substanzen zu erfassen. Hierzu wurde der häufig angewandte Comet Assay als high throughput-Verfahren weiterentwickelt (Witte et al., 2007; Stang, 2006 (unveröffentlicht)). In der vorliegenden Arbeit wurde untersucht, inwieweit sich dieses neue high throughput Verfahren für ein Screening auf Gentoxizität von Umweltproben und Chemikalien während der Wirkstoffentwicklung eignet.

Die high throughput Version des Comet Assay detektierte die DNA-schädigende Wirkung von Mutagenen mit unterschiedlichen Wirkmechanismen sensitiv, mit geringem Fehler und sehr guter Reproduzierbarkeit (A. Stang & I. Witte, Performance of the comet assay in a high-throughput version. Mutat Res. 675 (2009) 5-10). Ein Vergleich zum Standardverfahren des Comet Assay nach Tice et al. (2000) zeigte, dass die Wirkung von Mutagenen konzentrationsabhängig und vergleichbar sensitiv nachgewiesen werden konnte. Die Integration eines Zytotoxizitätstests, der beim konventionellen Verfahren separat durchgeführt werden muss, veränderte nicht die Kometenbildung. Die high throughput Version des Comet Assay ermöglichte die Steigerung des Probendurchsatzes ca. um das 20fache im Vergleich zum konventionellen Comet Assay.

Eine weitere Erhöhung des Probendurchsatzes wurde durch eine schnellere Datenauswertung möglich (A. Stang, M. Brend´amour, C. Schunck & I. Witte, Automatic Analysis of Comets in the High Throughput Version of the Comet Assay. (eingereicht)). Hierzu wurde in Zusammenarbeit mit der Firma Metasystems ein voll automatisiertes Auswertungssystem entwickelt. Ein Vergleich mit interaktiven (manuellen) sowie mit automatisierten Auswertungssystemen für den konventionellen Comet Assay ergaben vergleichbare Ergebnisse mit geringem Fehler. Dadurch ergab sich eine zusätzliche Steigerung der Durchsatzrate um den Faktor 10 im Vergleich zur manuellen Auswertung.

Im Standard-Comet Assay werden - je nach Fragestellung - unterschiedliche Zellarten eingesetzt. Es wurde überprüft, ob dies auch im high throughput-Verfahren unter Verwendung von MMS, H_2O_2 und PCP (nach metabolischer Aktivierung) möglich ist (A. Stang and I. Witte, Ability of the high throughput comet assay to measure comparatively the sensitivity of five cell lines toward methyl methanesulfonate, hydrogen peroxide and pentachlorophenol. (eingereicht)). Alle untersuchten Zelllinien (adhärente Fibroblasten, HeLa, V79, Hepatozyten (HepG2) und nicht adhärente Lymphozyten) zeigten unterschiedlich sensitiv gentoxische Effekte. Im Vergleich zu den anderen getesteten Zelllinien reagierten Lymphozyten am Empfindlichsten.

Die Kombination des high throughput Comet Assay mit dem Ames II-Test für eine high throughput Testbatterie wurde anhand von 9 Standardmutagenen und 8 Umweltproben untersucht (M.B. Heringa, A. Stang, C.A.M. Krul, A.A. Reus, A.P. van Wezel, I. Witte, A high-throughput genotoxicity testing strategy for screening of (drinking) water. (eingereicht)). Der Vergleich der Standardmutagene zeigte, dass einige Verbindungen im Comet Assay, andere im Ames II Test sensitiver waren, sodass sich beide Assays sehr gut ergänzten. Bei der Detektion des gentoxischen Potentials von den Umwelt- (Wasser-)Proben erfasste der high throughput Comet Assay bei 3/8 Proben ein gentoxisches Potential, wohingegen der Ames II Test nur für 1/8 Proben ein gentoxisches Potential detektierte. Im Gegensatz dazu konnte durch den Mikronukleustest in keiner der Umweltproben ein gentoxisches Potential nachgewiesen werden.

1.2 Summary

The ever increasing number of foreign substances to be released into the environment demands the development of reliable evaluation systems for genotoxic assessment. One of the common tests for genotoxic measurement is the comet assay, which was further developed to the high throughput comet assay (Witte et al., 2007; Stang, 2006 (unpublished)). The aim of this dissertation was to examine if this high throughput version is suitable for genotoxic screening of environmental compounds and chemical development during drug design.

With the high throughput version of the comet assay DNA damages of mutagenic agents were detected sensitively, with low standard deviations and high reproducibility (A. Stang & I. Witte, Performance of the comet assay in a high-throughput version. Mutat. Res. 675 (2009) 5-10). A comparison with the conventional comet assay described by Tice et al. (2000) showed that the DNA damaging effects were detected in a concentration dependent way with similar sensitivity. The integration of a cytotoxicity assay, which has to be executed separately in the conventional comet assay, did not influence the comet formation in the high throughput comet assay. The high throughput version of the comet assay increased the throughput of samples by about 20fold compared to the conventional comet assay.

An additional enhancement of the throughput was gained by a new and faster evaluation system of the comet data (A. Stang, M. Brend´amour, C. Schunck & I. Witte, Automatic Analysis of Comets in the High Throughput Version of the Comet Assay. (submitted)). In cooperation with the company Metasystems a fully automated evaluation system was developed. A comparison with the interactive (manual) as well as the existing automated evaluation systems for the conventional comet assay showed similar results with low standard deviations and standard errors. Here, an additional throughput enhancement was gained by a factor of ten compared to the interactive evaluation.

A variety of different cell types are used in the standard comet assay depending on the scientific question. The high throughput comet assay was evaluated for usage of various cell types, using MMS, H_2O_2 and PCP (with metabolic activation system) as mutagenic compounds (A. Stang and I. Witte, Ability of the high throughput comet assay to measure comparatively the sensitivity of five cell lines toward methyl methanesulfonate, hydrogen peroxide and pentachlorophenol. (submitted)). All cell types used (adherent fibroblasts, HeLa, V79 cells and hepatocytes (HepG2) and non adherent lymphocytes) were able to express genotoxic potential, although with different degrees of sensitivity. The highest sensitivity was observed for human lymphocytes.

9 standard mutagens and 8 environmental probes were tested for their genotoxic potential with the high throughput comet assay, and simultaneously with the Ames II test for high throughput screening (M.B. Heringa, A. Stang, C.A.M. Krul, A.A. Reus, A.P. van Wezel, I. Witte, A high-throughput genotoxicity testing strategy for screening of (drinking) water. (submitted)). The comparison of the 9 standard mutagens showed that some agents were more sensitive in the comet assay and others in the Ames II test, so that both assays complemented each other. The detection of the genotoxic potential of 8 environmental water probes showed that the high throughput comet assay detected genotoxic potential in 3 probes and the Ames II test detected 1 genotoxic potential. In contrast to the high throughput comet assay, the micronucleus test did not detect any genotoxic potential in the environmental probes.

2. Einleitung

Der Mensch ist täglich bis zu 70.000 Chemikalien (EINECS, **E**uropean **I**nventory of **E**xisting Commercial **C**hemical **S**ubstances, Datenbank), welche die unterschiedlichsten Wirkungen auf den Menschen haben, ausgesetzt. Neben Industriechemikalien wie Lösungsmitteln und Petrochemikalien handelt es sich vor allem um pharmazeutische- und Pflegeprodukte bis hin zu Bioziden. Nur für ca. 4 % dieser Umweltchemikalien liegen toxikologische Befunden bezüglich ihrer Einzelwirkung vor (BUND 2008).

Insbesondere die Gruppe der gentoxischen Umweltchemikalien verfügt über ein hohes schädigendes Potential, da sie in der Lage sind, das menschliche Genom nachhaltig zu beeinflussen und so Mutationen oder Krebs auszulösen. Mit Hilfe der REACH (**R**egistrierung, **E**valuierung, und **A**utorisierung von **Ch**emikalien) Verordnung (verabschiedet am 1. Juni 2007) soll dieses schwer zu kalkulierende Gefahrenpotential für Umwelt und Mensch strengeren Richtlinien unterzogen werden. Die Chemikalien werden in der REACH Verordnung in verschiedene Klassen, welche sich an den Produktionsmengen (1-10 t,≥10 t, ≥100 t und ≥1000 t) orientieren, eingeteilt. Für jede dieser Klassen werden bestimmte Testverfahren hinsichtlich toxikologischer Eigenschaften vorgeschrieben. Ein Problem zeigt sich jedoch darin, dass in der REACH Verordnung kleine Produktionsmengen (< 1 t) aus dem Testverfahren entfallen und auch bei größeren hergestellten Mengen nur die Einzelwirkungen untersucht werden. Hömme et al. (2000) und Sommer (2006) zeigten, dass zwischen den Einzelsubstanzen zytotoxische und gentoxische Kombinationswirkungen auftreten können, obwohl die Konzentrationen der Einzelsubstanzen unterhalb ihres NOECs (**N**o **O**bserved **E**ffect **K**onzentration) liegen. Als Ursache hierfür wird die „Türöffner-Hypothese“ angenommen (Witte et al., 2000; Sommer, 2006; Henrichs, 2008). So konnten Sommer (2006) und Heinrichs (2008) zeigen, dass Gemische aus gentoxischen hydrophilen Substanzen und nicht gentoxischen lipophilen Substanzen zu einer erhöhten DNA Schädigung führen. Als Ursache wird angenommen, dass die lipophilen Substanzen die Membranstruktur verändern und die hydrophilen Substanzen dadurch stärker aufgenommen werden und die gentoxische Wirkung verstärkt wird. Dieser Prozess wäre durch eine Evaluierung der Einzelsubstanzen nicht nachweisbar.

Aufgrund der immensen quantitativen Möglichkeiten verschiedener Kombinationswirkungen ist eine Untersuchung von Stoffgemischen sehr aufwendig. Häufig finden hier chemisch analytische Verfahren Anwendung, die jedoch keine Aussage über die toxische Wirkung des untersuchten Gemisches zu lassen. In seltenen Fällen ist es möglich eine Aussage über die Einzelwirkung eines Gemischbestandteils zu treffen, da für diesen Stoff ein Grenzwert vorliegt. Daher ist die Entwicklung eines sensitiven, high throughput Testverfahrens wichtig, um sowohl die Quantität, als auch Qualität der möglichen Kombinationswirkungen schnell und sicher zu bestimmen.

Für die Untersuchung der gentoxischen Potenziale stehen der Toxikologie verschiedene Methoden zur Verfügung. Hierzu zählen unter anderem der Ames Test (AT), der Chromosomenaberrations Test (CT), der Mikronukleus Test (MT) und der Comet Assay (CA), auch unter Einzel-Zell-Gel-Elektrophorese Test (Singel cell gel electrophorese assay) bekannt. Diese Testverfahren können in zwei Gruppen eingeteilt werden. Zum Einen in Mutagenitäts- und zum Anderem in Indikatortestverfahren. Während Mutagenitätstests zeit- und arbeitsaufwändige Untersuchungen der möglichen mutagenen Wirkung eines Xenobiotikums sind, stellen die Indikatortestverfahren vereinfachte Testmethoden dar. Zu den Mutagenitätstestverfahren zählen der MT, CT und der AT, wohingegen der Comet Assay zu den Indikatortestverfahren gehört.

Der MT, CT und der AT detektieren fixierte DNA-Schäden in Form von Gen- oder Chromosomenmutationen. Mit dem Mikronukleus Test (MT) können sowohl chromosomenbrechende (klastogener Effekt) als auch chromosomenfehl-verteilende (aneugener Effekt) Eigenschaften verschiedenster Xenobiotika nachgewiesen werden (Miller *et al.* 1997). Der Chromosomenaberrationtest (CT) weist wie der MT Mutationen durch Doppelstrangbrüche nach. Beide Testverfahren können jedoch nur bei sich teilenden Säuger-Zellen eingesetzt werden und müssen einen Zellzyklus durchlaufen, damit die gentoxische Wirkung sich als Mutation manifestieren kann.

Der Ames Test (AT) ist eine der ältesten und etabliertesten Mutationstestverfahren (Ames et al., 1973). Mit Hilfe des AT können anhand von Bakterienstämmen

gentoxische Potenziale festgestellt werden. Hierzu werden verschiedene sensitive auxothrophe Salmonellen-Bakterienstämme, welche auf Grund einer Punktmutation nicht in der Lage sind eine bestimmte Aminosäure zu synthetisieren, mit dem Xenobiotikum behandelt und die resultierenden Revertanten erfasst.

Der Comet Assay (CA) detektiert keine Mutationen, sondern DNA-Schäden. Im CA werden direkte Strangbrüche bzw. DNA-Schäden, die in Strangbrüche überführt werden können, erfasst. Dies lässt jedoch noch keine direkten Rückschlüsse auf mutagene Wirkungen eines Xenobiotikums zu, da erst eine Zellteilung die reversiblen DNA-Schäden in eine Mutation überführt. Da jedoch die DNA Reparatur nicht immer vollständig und fehlerfrei abläuft, ist davon auszugehen, dass im CA detektierte DNA schädigende Eigenschaften in der Regel mit mutagenen Eigenschaften gleich zusetzen sind. Der CA weist jedoch einige Vorteile gegenüber dem MT, CT und AT auf. Zum Einen kann ein eventuell vorhandenes gentoxisches Potential (im Gegensatz zu den anderen Methoden) direkt nachgewiesen werden, wobei bei den anderen Methoden nur unmittelbar manifestierte Mutationen nachgewiesen werden können. Dies schlägt sich in einer höheren Sensitivität nieder, da der DNA-Schaden und dessen Reparatur einer manifestierten Mutation vorausgehen müssen. Zum Anderen ist es mit dem CA möglich, nicht nur sich teilende Zellen, sondern auch sich nicht teilende Zellen zu untersuchen, da nicht erst eine Zellteilung zur Manifestation der Mutation stattgefunden haben muss. Daraus folgt, dass auch andere Zelllinien wie Nervenzellen (Neuronen), welche nicht teilungsfähig sind, im CA untersucht werden können. Da im CA keine Zellteilung notwendig ist, ist dessen Durchführung im Gegensatz zum MT, CT und AT schneller, wodurch sich der CA sehr gut für die Entwicklung zum high throughput Screeningverfahren eignet.

Für den Einsatz des CA als Screeningverfahren ist eine gute Korrelation mit Mutagenitätstestverfahren wichtig. Hierzu zeigte Hartmann *et al.* (2001) in einer *in vitro* Studie, dass die Ergebnisse des MT und des Comet-Assay gut miteinander korrelieren. Es wurden 39 Substanzen, darunter 3 Standardmutagene im CA und MT getestet. Substanzen bei denen im CA keine Wirkung zu detektieren war, zeigten auch im MT keinen positiven Befund. Jedoch zeigten 9 im MT positive Substanzen im CA keine DNA-Fragmentierung. Dies war auf den Effekt einer hohen Zytotoxizität zurückzuführen, da der MT eine höhere Anzahl an falschen positiven Befunden

aufgrund hoher Zytotoxizität liefert als der CA, da sowohl über nekrotische als auch apoptotische Ereignisse die DNA geschädigt bzw. abgebaut wird.

Weitere Studien, die den CA mit anderen Mutagenitätstestverfahren verglichen, wurden von Hartmann et al. (2003) und Giannotti et al. (2002) durchgeführt. Beide Studien zeigten ebenfalls eine gute Korrelation zwischen CA und dem CT.

Für das Screening einer Vielzahl von Chemikalien während der frühen Phase der Wirkstoffentwicklung in der pharmazeutischen Industrie werden der MT und CT eingesetzt, erscheinen jedoch ungeeignet, da sie zeitaufwändig sind (Hartmann et al., 2001; Giannotti et al., 2002). Zudem werden große Mengen der meist begrenzt vorliegenden Wirkstoffe benötigt, um das gentoxische Potential zu bestimmen (Hartmann 2004).

So ist auch das Screening im Rahmen der REACH Verordnung, in der der MT und CT als Säugertest vorgeschrieben sind, mit dem MT und CT nur unter hohem Zeit- und Kostenaufwand zu bewerkstelligen. Neben den Säugertestverfahren wird in der REACH Verordnung auch der bakterielle Ames Test vorgeschrieben. Der Ames Test benötigt jedoch auch lange Versuchszeiten und ist daher auch nur unter hohem Zeitaufwand durchführbar.
Der Comet Assay ist in seiner Durchführung deutlich schneller wie die anderen Testverfahren (MT, CT und AT) und zeigte in Studien eine sehr gute Korrelation mit dem MT, CT und AT. Jedoch ist bislang der Comet Assay nur als Ergänzung zu den anderen Testverfahren in der REACH Verordnung vorgesehen. Aufgrund der guten Korrelation zwischen DNA-Schäden und Mutagenität, der hohen Sensitivität und einfachen Handhabung ist der Comet Assay jedoch sehr gut als Screening-Methode in der Wirkstoffentwicklung oder im Rahmen der REACH Verordnung geeignet.

Diese Eigenschaften des Comet Assay führten dazu, dass in der Arbeitsgruppe Witte der Universität Oldenburg im Rahmen eines EU-Projektes (Project EVK1-CT-2002-30027) der Comet Assay im high throughput Verfahren entwickelt wurde (Witte et al., 2007).

Der Comet Assay ist eine einfache, sensitive und schnelle Methode, um eine DNA-Fragmentierung auf Einzelzellebene nachzuweisen (Singh *et al.* 1988; Tice *et al.* 2000) und wurde erstmalig von Östling und Johanson (1984) entwickelt, um Doppelstrangbrüche in Säugerzellen zu detektieren. Dieser Assay unter neutralen Bedingungen wurde von Singh et al. (1988) zur alkalischen Version weiter entwickelt. Mit der alkalischen Methode können neben Doppelstrangbrüchen auch Einzelstrangbrüche in der DNA nachgewiesen werden. Dies geschieht durch die Überführung von alkalilabilen Stellen in der DNA in Einzelstrangbrüche. Der zusätzliche Einsatz von DNA-Reparaturenzymen ermöglicht zudem eine Steigerung der Sensitivität.

Aufgrund der einfachen Durchführung und hohen Sensitivität findet der konventionelle Comet Assay Anwendung in der Genotoxizitätsprüfung von Wirkstoffen (Witte *et al.* 2007). Weitere Anwendungsbereiche liegen in der Grundlagenforschung zu DNA-Schäden und deren Reparatur (Collins 2004), in der Umweltmutationsforschung (Lee und Steinert 2003) und in der Pharmaindustrie zur Risikobewertung von Wirkstoffen (Hartmann 2004), sowie im Umweltmonitoring (Møller P, 2005).

Im herkömmlichen Comet Assay nach den Richtlinien von Tice et al. (2000) werden die Zellen in Petrischalen ausgesät und mit der zu testenden Substanz inkubiert. Anschließend werden die adhärenten Zellen gewaschen, trypsiniert und in Low Melting Agarose (LMP) aufgenommen und auf mit Agarose beschichtete Objektträger überführt. Nach dem Erhärten der LMP werden die Zellen lysiert und einer Elektrophorese unterzogen. Dies ermöglicht die Wanderung der DNA Fragmente durch die Agarosematrix, wodurch der Kometenschweif entsteht. Die Analyse erfolgt mit Hilfe einer Färbung der DNA und einer mikroskopischen Auswertung einzelner Zellen. Der Unterschied zwischen dem Verhältnis der DNA im Kopf (Head (Nukleus) - Region) und im Kometenschweif (Tail-Region]) stellen so die DNA schädigende Wirkung einer Substanz auf Einzelzellebene dar (Abb.1).

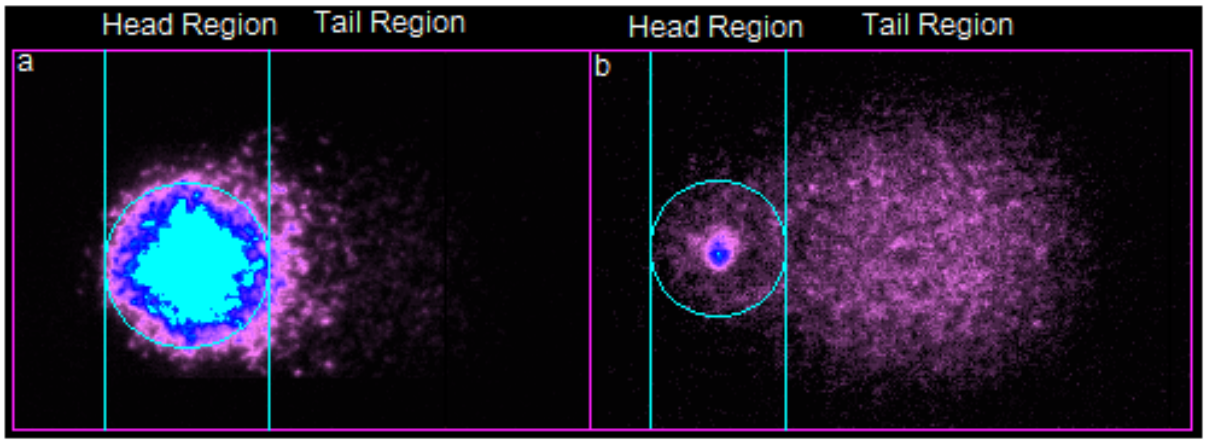

Abb.1: (a) Zelle mit geringen DNA Schäden und (b) mit starken DNA-Schäden und großen Kometen-Schweif

Das Verfahren ist durch den Trypsinierungsschritt, das Überführen auf Objektträger und der Analyse der Zellen jedoch zeitaufwendig, da die Bearbeitung für jede Probe einzeln erfolgt. Eine Erleichterung stellt die Methode von Kiskinis *et al.* (2002) auf einer 96´Well-Platte dar, bei der die Inkubations bzw. Waschschritte für alle Proben gleichzeitig erfolgen können. Jedoch bleibt der Arbeitsschritt des Trypsinierens und das Auftragen auf die Objektträger für jede Probe erhalten. So ist es kaum möglich, mehr als 24 Proben/Person/Tag zu untersuchen.

In einem Drittmittelvorhaben wurde in einer vorangegangen Doktorarbeit in der Arbeitsgruppe von Witte der Universität Oldenburg die Entwicklung einer high throughput Version des Comet Assay begonnen (de Wall, 2008) und von mir weiterentwickelt (Stang, 2006). Diese Methode ermöglicht eine gleichzeitige Bearbeitung von 96 Proben auf einer Multiwellplatte (MCP). Kern dieser Entwicklung stellt eine 96'er Wellplatte mit abnehmbaren Wänden dar, sodass der plane Boden einer Elektrophorese unterzogen werden kann. Dies gelingt durch eine spezielle Agarosebeschichtung der Bodenplatte, auf der die Zellen ausgesät werden (Abb.2). Diese Beschichtung ermöglicht die Arbeitsschritte für alle Parallelen gleichzeitig auszuführen, da das Trypsinisieren und das Einbetten, bzw. Überführen der Zellen auf die Objektträger entfällt. Dadurch ist eine Behandlung und Analyse aller Proben auf einer Platte mit Hilfe des Comet Assay möglich, was eine Vereinfachung des herkömmlichen Comet Asssay´s darstellt.

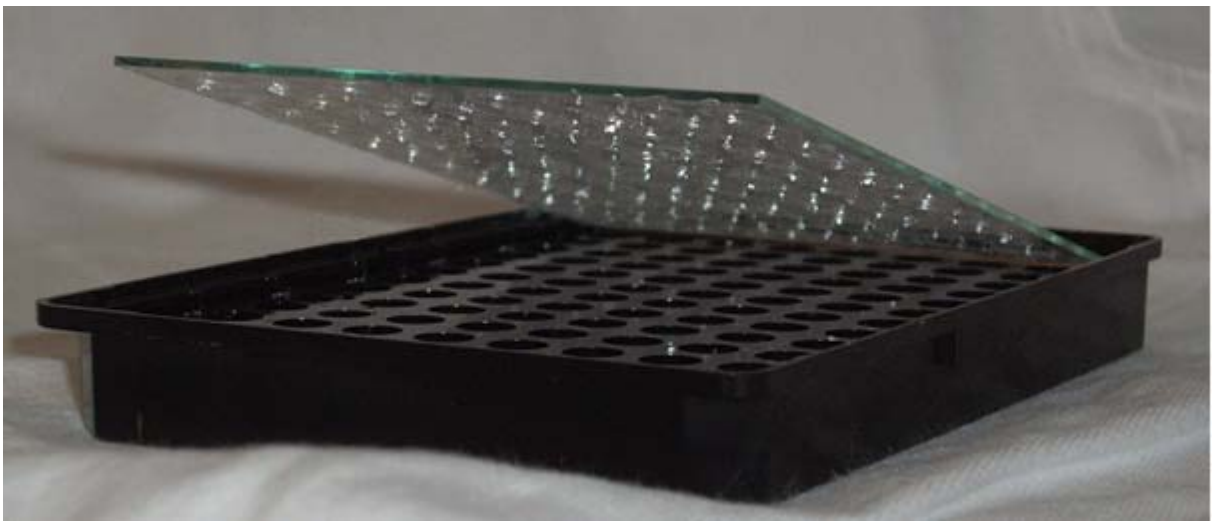

Abb.2: Darstellung einer MCP Platte mit angehobener beschichteter Bodenplatte

Eine Grundvoraussetzung für den Comet Assay ist die runde Form der Zellen, was im konventionellen CA durch das Trypsinieren erreicht wird. Im high throughput Verfahren wird die runde Form der Zellen dadurch erhalten, dass die Zellen sich zwar leicht anhaften jedoch nicht ausbreiten, wie es die Zellen bei einer Aussaat in der Petrischale tun würden. So werden die Arbeitsschritte des Trypsinierens und das einzelne Übertragen der Zellen auf Objektträger vermieden. Es wird sowohl eine Zeitersparnis (Präparation der Proben), als auch eine Senkung des Chemikalienverbrauchs (Probensubstanz, Lyselösung, Elektrophoresepuffer und DNA-Färbemittel) erzielt.
Zur Etablierung der Methode ist es jedoch unerlässlich, dass vergleichbare Ergebnisse im Vergleich zu der herkömmlichen Methode erzielt werden.

Zur Sicherstellung eines einheitlichen Protokolls wurde eine Richtlinie für die Durchführung des Comet Assay für Untersuchungen der Gentoxizität durch ein Gremium verfasst (Tice et al., 2000). In der Richtlinie wurden sowohl die Methode und deren Durchführung, sowie die Auswertung und Bewertung der Resultate beschrieben. Dadurch war eine Durchführung des Comet Assay nach einer einheitlichen Richtlinie möglich.
Dieser Richtlinie liegt der alkalische Comet Assay nach Singh et al. (1988) zu Grunde, welcher ebenfalls die Basis des high throughput Verfahren darstellt. Hierzu wurden die Arbeitsschritte der Lyse der Zellen, das alkalische Entwinden der DNA, die Elektrophorese unter alkalischen Bedingungen, die Neutralisation der Objektträger, sowie die Anfärbung und das Auswerten der Kometen adäquat zur

Richtlinie durchgeführt (Tice et *al.* 2000). Der so nach Tice et al. (2000) durchgeführte Comet Assay kann somit als qualitativ hochwertiger und sehr gut reproduzierbarer Gentoxizitätstest angesehen werden, was seine Einsatzmöglichkeit in großen Studien und in der Pharmaindustrie ermöglicht.

Zusätzlich zu der Untersuchung der Gentoxizität einer Chemikalie mit Hilfe des Comet Assay ermöglicht die high throughput Version auch eine Messung der Zytotoxizität. Dies ist wichtig, da es bei einer Behandlung von Säugerzellen mit stark zytotoxischen Substanzen zu einer DNA Schädigung aufgrund von nekrotischen und apoptotischen Ereignissen kommen kann (Henderson et al., 1998). Es wird daher empfohlen, bei einem Gentoxizitätstest eine parallele Bestimmung der Zytotoxizität durchzuführen, um falsch positive Resultate durch zytotoxische Wirkungen auszuschließen (Tice et al., 2000). Dies erfordert im konventionellen CA einen weiteren Test und somit zusätzliche Testsubstanz, da die zytotoxischen Testverfahren nicht in die Durchführung der Gentoxizitätstestverfahren integriert sind. Zur Zytotoxizitätsbestimmung werden häufig die Testverfahren FDA-Assay, MTT-Assay und der ATP-Assay angewandt, da sie schnell und einfach in ihrer Durchführung sind.

Das high throughput Verfahren ermöglicht, mit identischen Zellen den zytotoxischen Test (hier der FDA-Assay nach Rotman and Papermaster (1966)) und die Gentoxizitätsuntersuchung durchzuführen. Dies gelingt dadurch, dass der Zytotoxizitätstest in die Durchführung des Gentoxizitätstest integriert ist. Hierzu erfolgt vor der Lyse der Zellen die Anfärbung mit Fluoreszeindiacetat (FDA), wobei vitale Zellen das Membran-permeables, nicht fluoreszierendes Fluoreszeindiacetat aufnehmen. Intrazellulär werden die Acetatgruppen des Moleküls durch Esterasen im Cytosol zu Acetat und dem lipophilen, grün fluoreszierenden Xanthin-Farbstoff Fluoreszein hydrolysiert. Das resultierende Fluoreszein akkumuliert in der Zelle mit intakter Zellmembran, und das nunmehr geladene Molekül kann nur noch langsam aus der Zelle diffundieren. Anschließend erfolgt die Messung der 96‘er Wellplatte im Fluoreszenz-reader, um so die Vitalität der behandelten Zellen zu erfassen. Darauf folgt die Demontage der Wände und es wird mit der Methode des Comet Assay fortgefahren.

Ziel dieser Arbeit war es zu untersuchen, inwieweit der optimierte high throughput Comet Assay den Anforderungen für ein Screening-Verfahren zum Nachweis der Gentoxizität entspricht. Aus den erzielten Ergebnissen entstanden 4 Publikationen, die im Folgenden zusammenfassend dargestellt werden.

3. Darstellung der Ergebnisse

3.1 Durchführung des high throughput Comet und Vergleich mit dem standardisierten Comet Assay

(A. Stang and I. Witte, Performance of the comet assay in a high-throughput version. Mutat Res. 675 (2009) 5-10)

Das high throughput Verfahren ermöglichte eine erhebliche Steigerung der zu untersuchenden Probenanzahl pro Tag. In dieser Studie wurde die high throughput Version des Comet Assay hinsichtlich ihrer Qualität und Anwendbarkeit in der Praxis untersucht. Die Evaluierung mit Hilfe von 5 Standardmutagenen mit unterschiedlichen DNA-schädigenden Potential (Methylmethansulfonat [MMS], Ethylnitrosoharnstoff, 4-Nitroquinolin-1-oxide, Wasserstoffperoxid [H_2O_2] und Cis-platin) zeigte, dass das high throughput Verfahren mit den vorgenommenen Optimierungen, einschließlich integriertem Zytotoxizitätsverfahren, geeignet ist und mit hoher Sensitivität und geringen Fehler konzentrationabhängige Effekte detektiert. Zusätzlich zeigte das Verfahren im Vergleich zum Standardverfahren nach Tice et al. (2000) bei der Untersuchung von MMS und H_2O_2 quantitativ gleiche Ergebnisse.
Der Comet Assay im high throughput Verfahren erzielte so eine Steigerung der Durchsatzrate um den Faktor 20 im Vergleich zum Standardverfahren. Dies ermöglichte eine Vereinfachung der Messungen großer Probenzahlen bei der Untersuchung von Industriechemikalien, im Umweltbiomonitoring und Screening von Verbindungen in der frühen Phase der Wirkstoffentwicklung in der Pharmaindustrie vorkommen.

3.2 Automatische Auswertung von Kometen im high throughput Comet Assay

(A. Stang, M. Brend´amour, C. Schunck and I. Witte, Automatic Analysis of Comets in the High Throughput Version of the Comet Assay. Mutat Res. Submitted)

Die Methode des Comet Assay im high throughput Verfahren ist eine schnelle, einfache und sensitive Methode zur Ermittlung der Gentoxizität. Jedoch bleibt der limitierende Faktor die manuelle Auswertung der Kometen. In dieser Arbeit wurde in Zusammenarbeit mit der Firma Metasystems, eine Automatisierung der Auswertung für den Comet Assay im high throughput Verfahren entwickelt und die automatisierte Auswertung mit 2 interaktiv arbeitenden Auswertungssystemen verglichen. Die automatisierte Messung der Verbindungen MMS und H_2O_2 zeigte in geringen Konzentrationsbereichen, eine vergleichbare Sensitivität zu den inter-aktiven Messungen. Die automatisierte Auswertung erzielte so eine Steigerung der Geschwindigkeit der Auswertung um den Faktor 10 im Vergleich zur interaktiven Auswertung.

Die Automatisierung der Auswertung der Kometen ermöglicht eine zusätzliche Steigerung der Probenzahl, wodurch eine Gesamtsteigerung des Proben-durchsatzes in der high throughput Version um den Faktor von bis zu 180 im Vergleich zum konventionellen Verfahren erreicht wird.

3.3 Anwendbarkeit des high throughput Comet Assay unter der Verwendung 5 verschiedener Zelllinien

(A. Stang and I. Witte, Ability of the high throughput comet assay to measure comparatively the sensitivity of five cell lines toward methyl methanesulfonate, hydrogen peroxide and pentachlorophenol. Mutat Res. submitted)

Gentoxische Untersuchungen werden mit verschiedenen Zellarten durchgeführt. Daher sollte ein high throughput Verfahren auch mit vielen Zellarten anwendbar sein. Ziel dieser Arbeit war es, das high throughput Verfahren hinsichtlich der Verwendung verschiedener Zellarten zu testen und ein Vergleich der Sensitivität der Zellarten zu ermitteln. Hierzu wurden die adhärenten Fibroblasten, HeLa-, V79- und HepG2-Zellen und nicht adhärente Lymphozyten verwendet. Alle Zellen wurden mit MMS, H_2O_2 Pentachlorphenol (PCP), welche erst nach der Metabolisierung mit Cytochrom P450 ein gentoxisches Potential aufweist, behandelt. Die Untersuchung zeigte, dass unter Berücksichtigung der individuellen Anheftzeit alle getesteten Zellen im high throughput Verfahren getestet werden können. Ebenso konnten unterschiedliche Sensitivitäten zwischen den einzelnen Zellarten festgestellt werden, wobei menschliche Lymphozyten am sensitivsten reagierten.

Die erweiterte Anwendung des high throughput Verfahrens für verschiedene, spezialisierte Zellarten ist für die Forschung, das Screening und Monitoring interessant. So ist es z.B. jetzt möglich bei Arbeitsplatzmonitoring, oder Unfällen mit Chemikalien oder Strahlung größere Untersuchungen zur Auswirkung auf den Menschen mit Hilfe geringen Mengen menschlicher Lymphozyten durchzuführen.

3.4 Strategie für das Screening von Umweltproben im Hochdurchsatzverfahren

(Minne B. Heringa, Andre Stang, Cyrille A.M. Krul, Astrid A. Reus, Annemarie P. van Wezel, Irene Witte, A high-throughput genotoxicity testing strategy for screening of (drinking) water. Mutat Res. Submitted)

Für „Biomonitoring-Studien“ sind high throughput Verfahren für das Screening und der Evaluierung von gentoxischen sowie mutagenen Wirkungen sinnvoll, da es eine Vielzahl von Proben zu testen gilt. Aus diesem Grund wurde in dieser Arbeit untersucht, ob das high throughput Verfahren des Comet Assay mit dem Ames II Test korreliert. Zusätzlich wurde die Sensitivität der high throughput Version, des Ames II Test und des Mikronukleustest verglichen. Dazu wurden Umweltproben mit geringen gentoxischen Wirkungen untersucht.

Die Untersuchung von 9 verschiedenen gentoxisch wirkenden Substanzen ergab, dass die Ergebnisse vom Ames II Test und Comet Assay sich sehr gut ergänzten. Ebenso zeigte der Comet Assay bei der Untersuchung der Umweltproben eine höhere Sensitivität als der Ames II Test (3 von 8 Proben positiv im Comet Assay getestet, im Vergleich 1 Probe im Ames II Test positiv getestet), während mit dem MT keine Mutationen festgestellt werden konnte.

Die Untersuchungen zeigte, dass der Ames II und der Comet Assay im high throughput Verfahren sich ergänzten und so eine gute Kombination für eine Testbatterie zur Untersuchung von Umweltproben im Rahmen des Biomonitoring wäre. So ist es nun möglich, schnell und sensitiv gentoxisch wirkende Proben zu identifizieren.

4. Ausblick

Die stetig wachsende Anzahl an Xenobiotika, die in die Umwelt gelangen, verursacht ein erhebliches Risikopotential für den Menschen. Dieses Risikopotential entsteht nicht nur durch die Einzelsubstanzen, sondern hauptsächlich durch Kombinationseffekte. Bis heute stehen zur Ermittlung von Schadstoffen in Umweltproben chemisch analytischen Verfahren im Vordergrund. Die Ergebnisse hieraus erlauben jedoch noch keine Aussage über das gentoxische Potential des Gemisches oder der Umweltprobe.

Mit dem in dieser Arbeit entwickelten und auf Praxistauglichkeit getesteten high throughput Verfahren ist es möglich, mit vergleichsweise geringem Aufwand und geringen Kosten Kombinationswirkungen zu untersuchen bzw. ein Screening auf mögliche gentoxische Substanzen durchzuführen. Jedoch liegt die Durchführung des Comet Assay noch nicht wie andere Testverfahren (Ames-, Mikronukleus- und Chromosomenaberrations-Test) als OECD (**O**rganisation for **E**conomic **C**o-operation and **D**evelopment) Richtlinie vor. Möglicherweise liegt das daran, dass Mutationstests höher bewertet werden als der Nachweis DNA-schädigender Wirkungen. Da in verschiedenen Studien inzwischen gezeigt wurde, dass der Comet Assay gut mit den anderen Testverfahren korreliert (Hartmann et al., 2003; Giannotti et al., 2002), sollte dieses Argument entfallen. Da der Comet Assay sensitiver, schneller in seiner Durchführung und breiter in seiner Anwendung als die genannten Mutationstests ist, könnte der high throughput Comet Assay als Screening-Test den Mutationstests vorangestellt werden. In den (seltenen) Fällen eines positiven Befundes wäre der Einsatz von Mutationstests gefordert. Damit könnten alle Substanzen schneller und sensitiver auf Gentoxizität, wie es in der REACH Verordnung oder bei der Evaluierung der Phototoxizität notwendig ist, geprüft werden.

Der AT, MT, CT und der konventionelle Comet Assay sind „hightech“ Verfahren und benötigen zur Durchführung ein komplett eingerichtetes Zellzuchtlabor, wodurch eine Anwendung in Entwicklungsländern oder Schwellenländern nur bedingt möglich ist. Der high throughput Comet Assay hingegen ermöglicht die Anwendung in „lowtech“ - Regionen, da er bei der Durchführung mit humanen Lymphozyten keine Zellzucht

benötigt und auch unter semi-sterilen Bedingungen funktioniert, da die Durchführung innerhalb von Stunden erfolgt.

Die erweiterte Anwendung des high throughput Verfahrens auf verschiedene Zellarten ermöglicht eine Vielzahl von Tests im Comet Assay. So könnte mit Hilfe des high throughput Comet Assay organspezifische gentoxische Wirkungen an den Zellarten des jeweiligen Zielorganes untersucht werden. Auch ermöglicht der high throughput Comet Assay eine bessere Untersuchung der DNA-Reparaturkinetiken gegenüber dem konventionellen Assay, da der Trypsinierungsschritt entfällt und somit im Gegensatz zum konventionellen Comet Assay auch der Beginn der Reparatur erfasst werden kann.

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5. Publikationen der Ergebnisse

5.1 Performance of the Comet Assay in a High Throughput Version

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Abstract

The high throughput comet assay was developed to reduce the processing time and to increase sample throughput of the comet assay as described by Tice *et al*. [1]. This high throughput version allows for the processing of up to 400 samples per day. The basis of the new assay is a 96 well plate (multichamber plate, MCP) suitable for electrophoresis. After exposure of the cells to genotoxic agents, the walls of the MCP are separated from the bottom plate. All 96 samples together then go through lysis, alkaline unwinding, electrophoresis, neutralization, and staining.

In this study, the first concentration-dependent results obtained with the high throughput version are shown and a comparison with the standard version of the comet assay is made using five representative chemicals with different genotoxic properties. These genotoxic chemicals were methylmethane sulfonate (MMS), and ethylnitroso urea for small alkylation adducts, 4-nitroquinoline-1-oxide for bulky adducts, cisplatin for DNA crosslinks, and H_2O_2 for direct DNA breakage. For medium and high effective concentrations a standard deviation of 3-20 % for three replicates (25 comets per sample) was determined. A comparison of the standard assay and the high throughput version revealed similar results shown for MMS and H_2O_2. The integrated viability assay (FDA assay), which was performed after chemical treatment and before detachment of the bottom from the walls of the MCP, did not influence the outcome of the comet formation.

In conclusion, the high throughput version of the comet assay facilitates determining genotoxicity in cases where large numbers of samples have to be measured, such as testing industrial chemicals, biomonitoring of environmental samples, and early genotoxicity/photogenotoxicity screening of drug candidates. For such applications the cost- and time-saving of the high throughput method provides substantial advantages over the standard comet assay.

Keywords

comet assay; high throughput; MCP; multichamber plate; adherent cells

1. Introduction

Measuring genotoxicity is an important step in the complex procedure of determining the carcinogenic potential of chemicals. In areas such as environmental monitoring or in early drug candidate selection a high throughput method for screening genotoxicity would be beneficial. However, among current methods frequently used for determining genotoxicity (e.g. the micronucleus test (MNT), the chromosome aberration (CA) test, the comet assay), no high throughput version exists. The microscopical evaluations of these tests are very time consuming. In addition, in each genotoxicity test, it is necessary to evaluate the cytotoxicity of the test compounds in parallel to exclude false positive results. An excessive cytotoxicity leads in some cases to positive outcomes in these assays [2].

The comet assay and the MNT are potential methods for increasing the screening rate of substances. Automatically working evaluation systems exist for both methods [3-5]. Nevertheless, the performance of these assays remains time consuming and is not suitable for screening a large number of samples per day. The comet assay has some advantages over the CA and MNT. The CA and MNT require proliferating cells, while in the comet assay genotoxicity can be detected also in non-dividing cells. In addition, the CA [6] and MNT [7] show higher frequencies of false positive results in comparison with the comet assay. Therefore, these assays are either less specific, or more sensitive towards positive effects associated with cytotoxicity than the comet assay.

The standard procedure of the comet assay according to the recommendations of Tice *et al*. [1] is not suitable for screening a large number of samples for genotoxicity. Even if the throughput in the comet assay could be increased by using standard 96 well plates [8], time limiting steps like trypsinization of adherent cells of each sample and the subsequent individual treatment of all samples remain. These include mixing of the cells with agarose, spreading them on precoated slides, followed by lysis, electrophoresis, and staining. To avoid these time- and sample number-limiting steps, a high throughput version of the comet assay was developed [9].

The basis of the high throughput version of the comet assay is a 96-well plate (multichamber plate, MCP) suitable for electrophoresis. An agarose containing surface on the bottom of the MCP allows the cells to attach. After chemical treatment of the 96 samples, the walls of the MCP are removed from the bottom plate. The subsequent steps can be performed for all 96 samples at the same time. In addition to an assessment of DNA damage, the viability of the cells can be determined after chemical treatment by staining with fluorescein diacetate and evaluation by a fluorescence reader. Thereafter, the walls of the MCP are removed and the procedure of the comet assay can be continued. Thus, cell viability can be measured under identical conditions and with the same cells with which the comet formation is determined.

In this study we present our first results of the comet assay performed with the MCP. We determined the extent of comet formation in human fibroblasts induced by five DNA damaging chemicals with different DNA damaging properties: direct strand breakages (H_2O_2), damages with small conformational changes of the DNA (MMS, ethylnitroso urea ENU), large conformational changes of DNA caused by bulky adducts (4-NQO), and crosslinking (cisplatin). We compared the conventional comet assay with the new high throughput version. Further, we compared the comet formation with and without integrated cytotoxicity testing.

2. Materials and methods

2.1 Cell culture

Human fibroblasts cell line NHDF-p were purchased from Promochem (Heidelberg, FRG). Monolayer cultures (passage 8-15) were grown in D-MEM at 37 °C in an atmosphere of 5 % CO_2 and 95 % air with > 95 % humidity.

2.2 Multichamber plate (MCP)

The MCP is a specially coated 96 well plate purchased from Intox, Oldenburg, FRG, which allows the electrophoresis of cellular DNA. The surrounding walls of the wells

can be separated from the plate of the MCP. The cells of 96 wells remain on the flat plate and all samples together can be electrophorized.

2.3 Chemical treatment of the cells

Cells were treated with MMS (> 99 %, from Sigma, Deisenhofen, FRG), ENU (from Sigma, Deisenhofen, FRG), 4-NQO (98.2 %, from Sigma, Deisenhofen, FRG), cisplatin (Medoc, Hamburg, FRG), or with H_2O_2 (37 % aqueous solution from Acros Organics, NJ). All chemicals were used in non cytotoxic concentrations. For H_2O_2 maximum of DNA damage was observed after a 15 min incubation because of its short half life. The other chemicals required an incubation of 1.5 hours to exert a high level of DNA damage. MMS, ENU, cisplatin and 4-NQO were freshly dissolved in serum free medium (sfm) at pH 7.2 and 37 °C directly before cell treatment. H_2O_2 was diluted from the 37 % aqueous solution with sfm also immediately before cell treatment. DNA damage induced by the crosslinker cisplatin was measured indirectly. It was detected according to Pfuhler and Wolf [10] by previous treatment with MMS (2.1 mM for 1 hour) and subsequent addition of cisplatin for 1.5 hours. The % reduction of DNA strand breaks induced by MMS, named “relative DNA damage [%]” in the graphs, quantitatively reflects the DNA crosslinks induced by cisplatin.

2.4 Comet assay performed in the standard mode

The comet assay was performed according to Tice *et al.* [1]. In brief, 30,000 cells seeded into Petri dishes (5 cm in diameter) were treated with chemicals as described above. Thereafter the cells were washed with ice-cold phosphate buffered saline (PBS), trypsinized (0.125 % trypsin), and resuspended in 100 µl ice-cold PBS. A volume of 20 µl of the resuspended cells was mixed with 80 µl 0.5 % low melting agarose at 37 °C and applied to pretreated microscope slides. Pretreatment of slides involved coating with 1.5 % agarose, diluted in Ca^{2+} and Mg^{2+}- free PBS, pH 7.4. Each concentration was performed in duplicate. The slides mounted with cells were covered with coverslips and kept in the refrigerator for 3-5 min to solidify the low melting agarose. The following steps were performed under dim-light to prevent additional UV-induced DNA damage. After removing the coverslips, slides were immersed in 4 °C cold lysing solution pH 10.0 (2.5 M NaCl, 100 mM EDTA, 10 mM

Tris, 1 % N-lauroyl sarcosine, 1 % Triton X100, 10 % DMSO; the last two compounds were added freshly). Slides were kept at 4 °C for 1 h. After lysis, the slides were placed on a horizontal electrophoresis box. The unit was filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM EDTA, pH 13), until slides were completely covered with buffer. After an incubation for 40 min at 4 °C in alkaline buffer, to allow DNA unwinding and DNA breakage at alkali labile sites, DNA electrophoresis was performed in an ice bath at 25 V and 300 mA for 20 min. After electrophoresis, the slides were covered with neutralization buffer (0.4 M Tris HCl, pH 7.5) for 5 min. This step was repeated twice. Thereafter, the slides were briefly dipped into water and dried by air overnight. Finally, 40 µl ethidium bromide (20 µg/ml) was added to each slide. Slides were covered with a coverslip and kept for 5 min in the dark for DNA staining. DNA migration was analyzed by fluorescence microscopy (Nikon, Eclipse E600W). The tail moment (tm) as well as % DNA tail was determined using the software ”Lucia comet assay Single Stain” (Nicon). For each concentration, 100 randomly selected cells (50 cells each from two duplicate slides) were analyzed, and the medians of the non-normally-distributed values were determined.

2.5 Comet assay in the high throughput version

The comet assay in the high throughput version was performed on the basis of the guidelines of Tice *et al.* [1] with two important differences. First, the treatment of the cells with the genotoxic agents started 2-4 hours after seeding of the cells. Secondly, trypsinization of adherent growing cells was avoided. In addition, cell viability was measured with the same cells that were subsequently used for the comet assay.

Fibroblasts were seeded into the wells of the MCP (3,000 cells/well) two or four hours before treatment of the cells. Thereafter, treatment with the genotoxic chemicals followed as described above. For each concentration, 3-6 parallel replicates were performed. Then, the walls surrounding the wells of the MCP were separated from the bottom plate. The plate with the cells was covered with a 37 °C warm solution of 0.5 % low-melting agarose (type Sea Plaque from Biozym Diagnostik, Hessisch Oldendorf, FRG). The plate was kept in the refrigerator for 5 minutes to solidify the low melting agarose. The following steps were performed according the procedure recommended by Tice *et al.* [1] (see section 2.4). In brief, the plate was covered with

refrigerated lysing solution pH 10.0 and kept at 4 °C for 1 hour. After lysis the plate was placed on a horizontal electrophoresis box. The box was filled with freshly prepared alkaline buffer whereby the plate was completely covered with the buffer. During 40 minutes at 4 °C the alkaline treatment allowed alkaline unwinding of the DNA and DNA breakage at alkali labile sites. After electrophoresis the plate was covered three times with neutralization buffer, washed with aqua bidest., and stained with ethidium bromide. Fluorescence microscopy was used to determine the median DNA migration in each of the 3-6 parallel samples per concentration (25 comets/well). From these 3-6 values the mean and standard deviation was calculated. Statistical significance of the differences between the DNA migration (% DNA tail) induced by the mutagens in the standard assay and the high throughput method was determined by the non-parametric Mann-Whitney test ($p < 0.01$).

2.6 Measurement of cell viability integrated in the comet assay procedure

For measurement of the cell viability, we used the FDA assay according to Rotman and Papermaster [11]. The FDA assay measures the cell viability by the activity of cytosolic esterases converting fluorescein diacetate (FDA) to the fluorescent dye fluorescein.

After treatment of the cells with the genotoxic agent MMS, the fibroblasts were incubated for 10 minutes with 72 µM FDA (from 12 mM stock solution in acetone, freshly diluted with sfm) at 37 °C in the dark. Thereafter, the dye was removed and the fluorescence of the enzymatically formed fluorescein was measured in a fluorescence reader (FLUOstar, Offenbach, FRG) with an excitation of 485 nm and an absorption at 520 nm. No significant differences between the untreated controls and the MMS treated samples were found. Therefore the MMS treatment was not cytotoxic. After fluorescence measurement the walls and the bottom plate of the MCP were separated and the comet assay procedure was continued as described above.

3. Results

3.1 Influence of the time interval between seeding of fibroblasts and experiment on comet formation

To perform the comet assay with adherent cells it is necessary that cells are embedded in the coating layer in a rounded form. This is achieved in the standard comet assay by trypsinization after chemical treatment of adherent cells. On the MCP where trypsinization does not occur, the cells have to remain in their rounded morphology after seeding. This is necessary because in the comet assay the intact DNA is spread after lysis within the volume of the former cell. We seeded trypsinized cells on the MCP and performed the comet assay of these non-treated cells 2-8 hours after seeding. In Fig.1a-1d the “comets” of the cells without chemical treatment are shown. Up to 4 hours after seeding the DNA was found in a rounded shape. After this time the DNA began to spread on the MCP. This means, chemical treatment should occur 2-4 hours after seeding. During this period, statistical significant differences in the comet formation induced by chemicals were not observed (data not shown).

3.2 Comet formation on the MCP induced by DNA damaging chemicals

Human fibroblasts were seeded into the wells of the MCP and treated after 4 hours with increasing concentrations of the DNA damaging agents MMS, ENU, 4-NQO, and cisplatin for 1.5 hours. At least 5 parallel samples per concentration were made. In Fig. 2a-2d, the resulting comet formation is shown. For all chemicals a concentration dependent increase in DNA damage was observed while the untreated controls did not show any DNA migration. The lowest concentration of MMS to show DNA damage was 0.2 mM, of ENU 0.5 mM, of 4-NQO 0.125 µM, and of cisplatin 15.8 µM.

In Table 1, the tail moments (tm) and the % DNA tail obtained after treatment of fibroblasts with 4-NQO are listed. The standard deviations were calculated from 3-6 identically treated samples in the same MCP. 25 comets were evaluated in each well. The standard deviations were similar regardless of the number of (3, 4, 5 or 6) wells evaluated.

3.3 Comparison of the comet formation with and without integrated viability assay

The comet assay in the high throughput version was quantitatively compared with and without integrated FDA assay. After incubation with MMS, the fibroblasts were treated for 10 minutes either with the staining agent FDA, or with FDA-free sfm. After removing the FDA solution, or the sfm, the fluorescence was measured in the fluorescence reader. None of the MMS concentrations tested, revealed any cytotoxicity (data not shown). This is in accordance with data from literature where highly genotoxic concentrations of MMS, measured in the standard comet assay were not cytotoxic in human fibroblasts [12]. After fluorescence measurement the bottom plate was demounted from the walls, covered with low melting agarose and the comet assay procedure was continued. In Fig. 3 the results of comet formation with and without FDA staining is shown. There were no differences between the two approaches.

3.4 Comparison of the comet formation in the standard assay and on the MCP

The comet assay in the standard mode according to Tice *et al.* [1] and in the high throughput version was directly compared using the genotoxic chemicals MMS and hydrogen peroxide. The results of MMS are shown in Fig. 4, in Fig.5 those of H_2O_2. Between both methods no statistical significance was measured by the non-parametric Mann-Whitney test ($p < 0.01$) (exception: 20 µM H_2O_2).

4. Discussion

One of the more time consuming steps in the standard comet assay with adherent cells, except for scoring the comets, is the trypsinization step. To avoid this step a necessary precondition for experimentation with adherent cells is that the cells remain in their rounded shape after seeding. Fibroblasts, which assume an elongated shape when spread on the bottom of a Petri dish, remained on the MCP in their rounded form up to 4 hours after seeding. Thereafter, they slowly started to spread. It was investigated, if a short time period of 2-4 hours of attachment would elevate the sensitivity of untreated cells caused by damages via trypsin. This was not the case

because the untreated cells on the MCP did not show any comet formation just as in the standard assay where cells were seeded one day before the experiment. Singh *et al.* [13] first reported the performance of the comet assay 4 hours after seeding the cells. They successfully demonstrated DNA migration after irradiation with x-rays or treatment with H_2O_2. These and our observations show that seeding and performance of the experiment on the MCP can be done on the same day. Preliminary results with other adherent cell types suggest, the time between seeding and chemical treatment may be vary dependent on the properties of the cell types to adhere. This time has to be established for each cell type, individually. With non adherent cells, like lymphocytes, the high throughput version of the comet assay can also be performed. In this case the suspension cultures already pipetted into the wells of the MCP have to be centrifuged before and after chemical treatment (publication in preparation).

A concentration-dependent increase in comet formation was demonstrated with the newly developed high throughput version of the assay. This was independent of which kind of DNA damaging agent was used. A comparison of the standard assay and the high throughput mode revealed similar results. This means that the extra time needed for trypsinization in the standard assay is too small to detect additional DNA repair resulting in reduced DNA damage.

The calculation of the standard deviation revealed the homogeneity of the parallel samples on the MCP, because the values were similar between n=3 and n=6. This is important because it means that on one MCP, 32 different samples can be measured with a high degree of confidence.

It is known that the comet formation generally does not follow a Gaussian distribution [14]. That means, that from one sample with 50 or more comets evaluated, the medians (with percentiles) have to be calculated. The mean and the standard deviation can only be obtained by measuring at least three, better more parallels. In contrast to the standard assay, the performance of parallel samples in the high throughput version is easy and rapid, and allows the determination of the mean with standard deviation.

The measurement of the cell viability by FDA as part of the high throughput procedure did not influence the comet formation. This means that the vital dye fluorescein diacetate does not possess genotoxic properties. Under our conditions the lower detection limit was reached at about 300 untreated cells/well (data not shown). Using 3000 cells/well, a reduction of cell viability > 50 % was detectable. This is extremely helpful to know, because false positive results of genotoxicity due to high cytotoxicity can be determined by the integrated cytotoxicity [15] measurement.

Most chemicals have to be metabolically activated to exert their genotoxic potential. It is generally achieved in the *in vitro* comet assay by adding cofactor-supplemented postmitochondrial (S9) fraction to the incubation mixture [1]. This can also be done in the high throughput version of the comet assay. In first experiments with indirectly acting carcinogens comet formation was observed in the presence of S9 mixture, while S9 mixture alone did not provoke DNA migration (publication in preparation).

We have developed this high throughput assay for *in vitro* testing great numbers of samples. But it is feasible to also apply it for *in vivo* experimentations. However, for *in vivo* testing there are generally few samples available at the same time and trypsinization is omitted, so that the advantages of the high throughput method are reduced.

In conclusion, the high throughput version of the comet assay is useful for screening large numbers of samples. A comparison with the standard assay yielded similar results. An automatic evaluation system for the comets will further accelerate the speed with which the assay can be done. Such a prototype is under investigation in our laboratory. This evaluation system microscopically analyses the entire MCP counting 50 comets/ well within about 2 hours (publication in preparation). The combination of the MCP and the new automatically working evaluation system enables the measurements of about 400 samples per day. No other mammalian test system for genotoxicity permits a similar high throughput. Therefore, the high throughput version of the comet assay presented here will be of great value for screening genotoxicity.

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Abbreviations

CA	chromosome aberration test
D-MEM	Dulbecco's modified Eagle medium
ENU	ethylnitroso urea
FDA	fluorescein diacetate
MCP	multichamber plate
MMS	methyl methanesulfonate
MNT	micronucleus test
4-NQO	4-nitroquinoline-1-oxide
sfm	serum free medium
tm	tail moment

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Figures and Tables

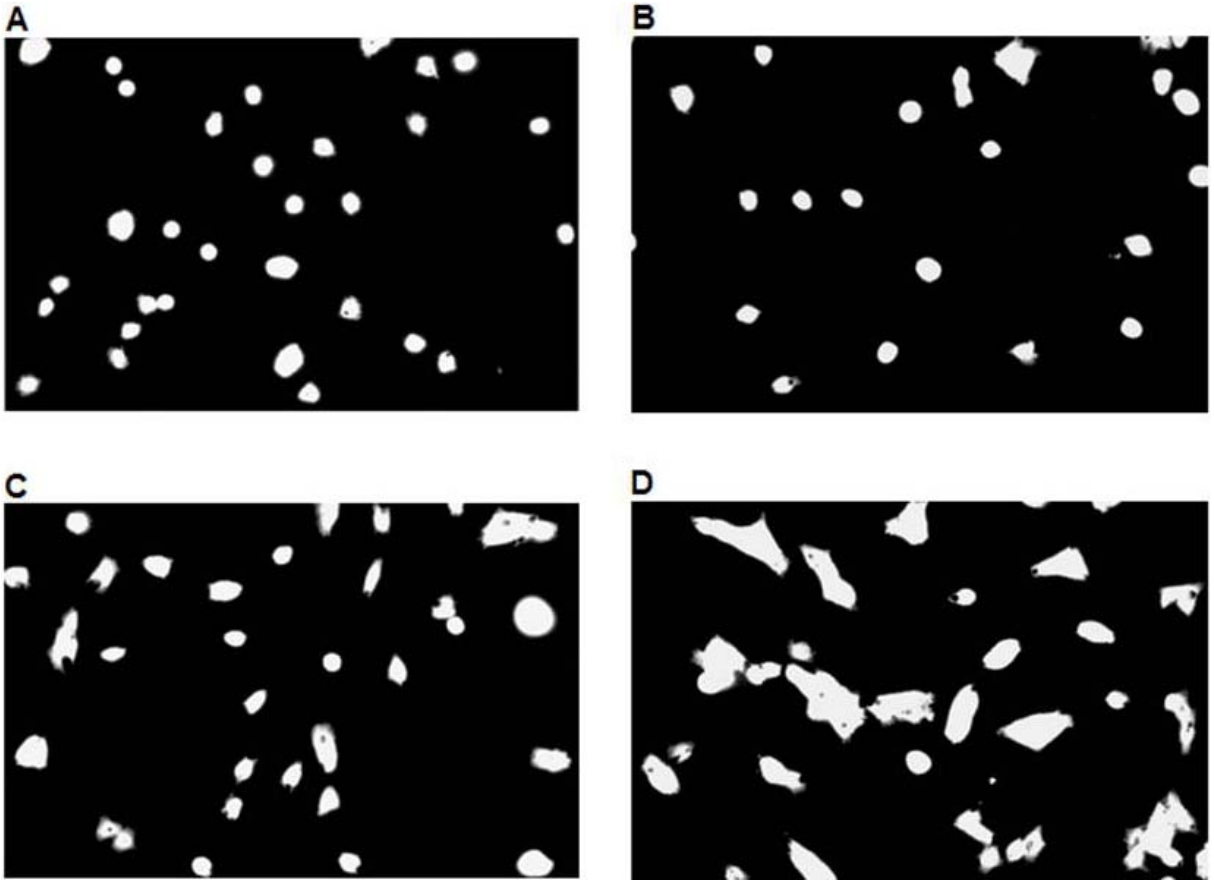

Fig. 1: Shape of “comets” obtained from untreated cells a) 2h b) 4 h c) 6h d) 8 h after seeding on the MCP and performance of the comet assay

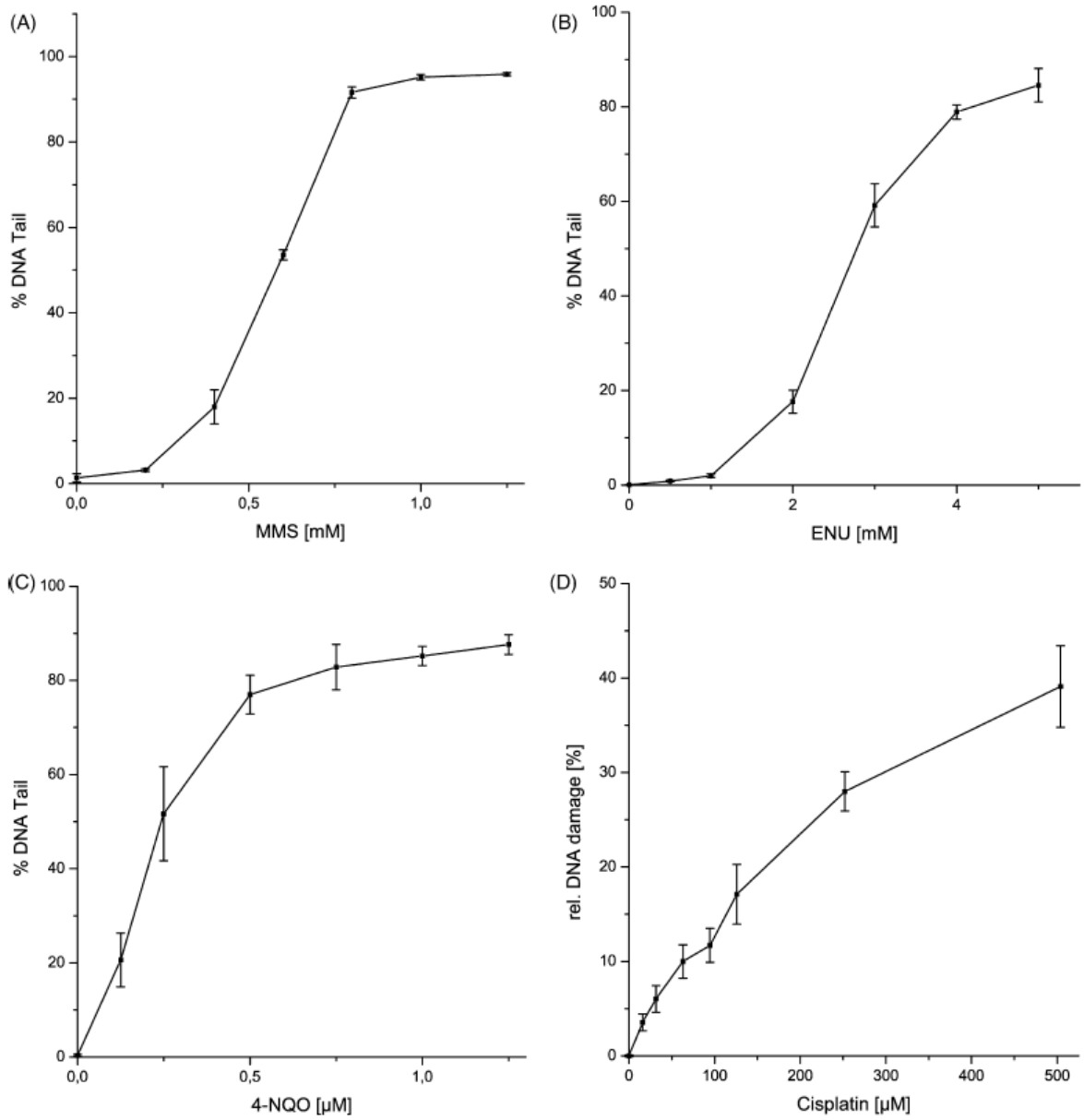

Fig. 2: Concentration dependent DNA damage induced by MMS (a), ENU (b), 4-NQO (c),and cisplatin (d) measured by the high throughput version of the comet assay, presented as the mean of 4 parallel samples (25 comets evaluated/sample) with standard deviation. The DNA damage induced by cisplatin, named “relative DNA damage [%]”, shows the % reduction of DNA strand breaks induced by MMS

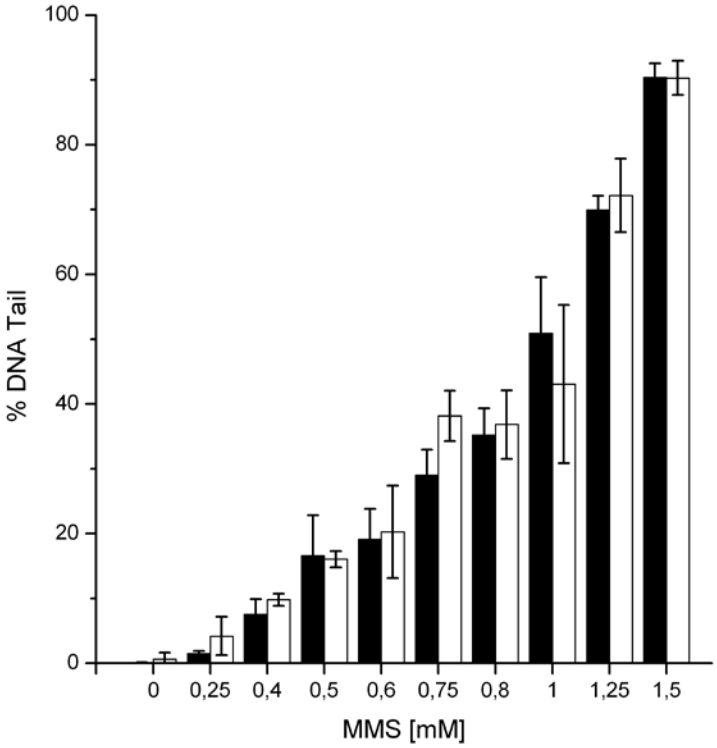

Fig. 3: High throughput comet formation induced by MMS with (black bars) and without (white bars) integrated viability assay. The data represent the mean of 4 parallel samples (25 comets evaluated/sample) with standard deviations

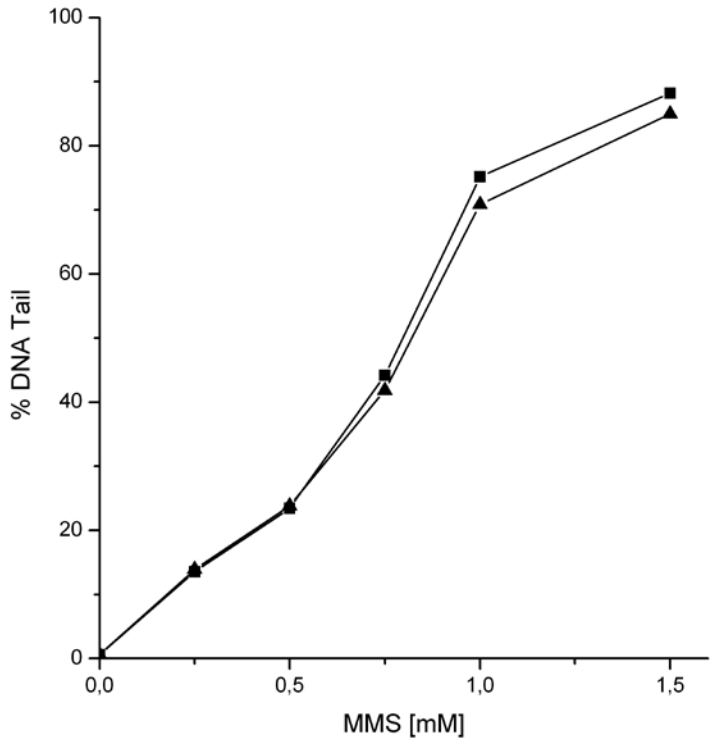

Fig. 4: DNA strand break induction by MMS determined in the standard assay according to Tice *et al.* [1] (■, median of 100 comets) and on the MCP (▲, median of 100 comets in 4 parallel samples); between both methods, no statistical significance was measured using the Mann-Whitney test ($p < 0.01$).

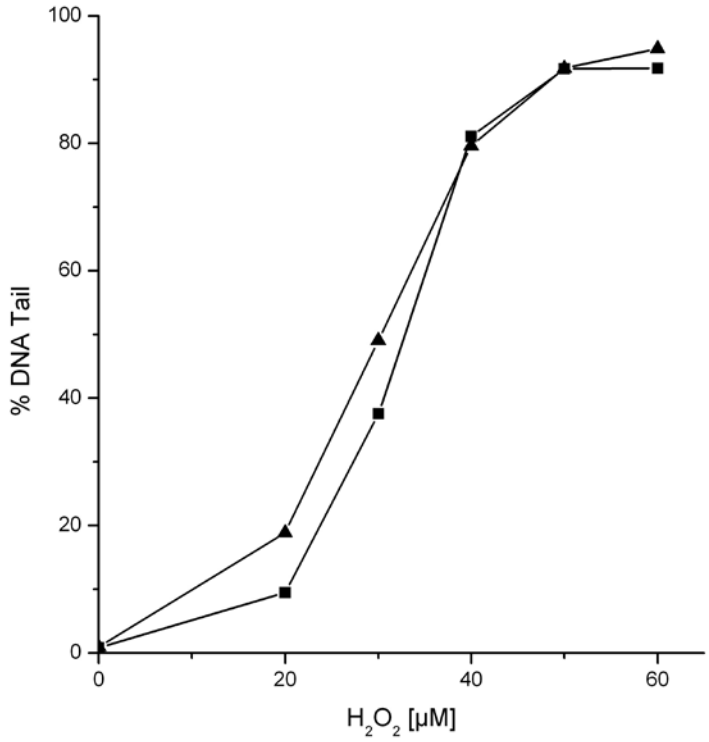

Fig. 5: DNA strand breaks induction by H_2O_2 determined in the standard assay according to Tice *et al.* [1] (■, median of 100 comets) and in the MCP (▲, median of 100 comets in 4 parallel samples); between both methods no statistical significance was measured by the Mann-Whitney test ($p < 0.01$) except 20 µM H_2O_2

Tab. 1: Comet formation in the high throughput comet assay induced by 4-NQO evaluated by the (a) tail moment (tm) or (b) % DNA tail with the standard deviations in dependence on the number (n) of wells. In each well 25 comets were measured.

(a)

Tail moment

4-NQO (µM)	n=3		n=4		n=5		n=6	
	Mean	SD (%)	Mean	SD (%)	Mean	SD (%)	Mean	SD (%)
0	0.15	80	0.13	82.1	0.11	93.4	0.1	97.8
0.13	9.04	38.4	8.79	32.8	9.2	28.9	9.24	25.7
0.25	25.66	20.8	27.29	19.9	27.88	17.6	27.54	19.2
0.5	50.57	4.1	50.12	3.8	50.48	3.6	51.62	6.3
0.75	67.85	1.1	68.04	1.1	67.91	1.0	68.18	1.3
1	78.74	5.2	78.7	4.3	79.49	4.3	78.86	4.4
1.25	97.61	0.58	97.44	0.6	95.78	3.9	95.11	3.9

(b)

% DNA tail

4-NQO (µM)	n=3		n=4		n=5		n=6	
	Mean	SD (%)	Mean	SD (%)	Mean	SD (%)	Mean	SD (%)
0	0.36	86.1	0.4	67.9	0.37	64.9	0.35	62.2
0.13	22.83	28.4	20.19	34.3	19.93	34.6	21.36	29.2
0.25	57.54	21.0	56.87	17.5	55.06	17.3	53.73	17.0
0.5	77.87	4.6	75.78	6.7	76.63	6.3	77.15	5.8
0.75	80.46	1.6	81.45	1.3	81.82	1.4	82.32	1.6
1	86.14	2.6	85.91	2.2	85.07	2.9	85.16	2.6
1.25	87.63	3.4	87.93	2.9	87.39	2.7	87.11	2.6

5.2 Automatic Analysis of Comets in the High Throughput Version of the Comet Assay

(submitted)

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Abstract

Recently a high throughput version of the comet assay was developed using a special 96-well plate (MCP, multichamber plate) [1]. In this version, the electrophoresis is performed directly on the MCP which makes transferring of cells to microscope slides unnecessary.

In order to facilitate the scoring procedure we adapted an automated slide scanning system (Metafer MetaCyte with CometScan) to enable unattended analysis of comets on the MCP. The results of the system were compared with the data obtained with two interactive comet assay analysis systems. For induction of comets in human fibroblasts methyl methanesulfonate (MMS), or H_2O_2 was used. The three systems revealed similar, concentration dependent results for all parameters tested: tail moment, % DNA tail and Olive tail moment. Near the detection limit of 5-6 % tail DNA a significance of $p \leq 0.01$ was obtained using 4 parallel samples. Additionally, after evaluation of either 50 or 100 comets, the standard errors were similar for either treatment with MMS, or H_2O_2, thus showing that the method is suitable to reveal the crucial low-dose effects with high precision. The results also showed that the time needed for automatic evaluation of comets on the MCP was reduced by a factor of 10 when compared to the time required for interactive evaluation. In summary, the high throughput version of the comet assay combined with the automated evaluating system increased the output by a factor up to 180 compared to the standard method.

Keywords

comet assay, high throughput, automated analysis

1. Introduction

Biomonitoring of environmental probes, or examining the genotoxic potential of chemicals according to REACH (**R**egistration, **E**valuation and **A**uthorisation of **Ch**emicals), or pre-screening of pharmaceutical candidates demands the measurement of large numbers of samples. Therefore, a high throughput method for mammalian genotoxicity is desirable necessity for evaluation.

For determining DNA damages the comet assay is a well established genotoxicity test, which enables the possibility of measuring in a high throughput mode. The comet assay allows testing of a broad spectrum of DNA damages with high sensitivity, *in vitro* as *in vivo* [3-4].The comet assay was first introduced by Östling and Johanson [5] and was further refined by a number of laboratories. Singh et al. [6] developed the more versatile alkaline method of the comet assay. Based on this assay and the guidelines of Tice et al. [7] a high throughput version of the conventional comet assay was recently developed [1-2]. This method enables to test 96 samples at one time by using a modified 96-well plate (MCP). The innovation of the MCP allows to perform the electrophoresis directly on the plate, without transferring the cells to slides [1-2].

So far, the evaluation of comets is a very time consuming step, which is done by microscopic fluorescence analysis of individual comets, thus taking several hours for each single experiment. In the past, some automated analyzing systems were developed for the conventional comet assay [8-10], which reduced the comet scoring time by approx. 50% compared to the manual evaluation, and made unattended overnight evaluation possible.

To analyze the comets on the MCP we developed a method to score comet assay samples using the fully automated slide scanning platform Metafer and the MetaCyte CometScan software. In this publication we present data from the comparison of scan results obtained by automatic analysis with the results obtained with two interactive comet assay analysis systems. We measured the genotoxic effects of two DNA damaging chemicals, methyl methanesulfonate (MMS) and hydrogen peroxide (H_2O_2) in human fibroblasts.

2. Materials and methods

2.1 Cell cultures

Human fibroblasts from the cell line NHDF-p were purchased from Promochem (Heidelberg, FRG). The cells were grown in D-MEM, supplemented with 12 % fetal calf serum, vitamins, non essential amino acids, 100 U/ml of penicillin, and 100 µg/ml streptomycin at 37 °C in an atmosphere of 5 % CO_2 and 95 % air with more than 95 % humidity. The human fibroblasts were used in passage 8-15.

2.2 Multichamber plate (MCP)

The MCP is a specially coated 96 well plate purchased from Intox, Oldenburg, FRG. This coating and its specific design makes it suitable for the electrophoresis of cellular DNA. The surrounding walls of the wells can be separated from the flat base plate of the MCP. The cells remain on the plate, and all samples can be electrophorized together in a single step.

2.3 Chemical treatment of the cells

Cells were treated with methyl methanesulfonate (MMS, > 99 %, from Sigma, Deisenhofen, FRG), or H_2O_2 (37 % aqueous solution from Acros Organics, NJ). MMS was freshly dissolved in serum free medium (sfm) at pH 7.2 and 37 °C directly before cell treatment. H_2O_2 was diluted in serum free medium (sfm) also immediately before cell treatment.
The cells were exposed to MMS for 1 hour, the treatment time was reduced to 15 min for H_2O_2 because of its short half life.

2.4 Comet assay in the high throughput version (MCP)

The comet assay in the high throughput version was performed on the basis of the guidelines of Tice *et al.* [7], described by Stang and Witte [1] in detail. Before seeding the cells, the base plate of the MCP was covered with poly-L-lysine for 30 minutes

and washed 3 times with D-PBS-buffer. 3000-5000 cells were seeded into each well of the MCP. The adherent cells were allowed to attach to the bottom of the MCP for 4 hours. Afterwards the MCP was centrifuged (Labofuge 400; rotor: 8177; Heraeus®, FRG) for 2 minutes at 900 rpm. The cells were washed with sfm, treated with the genotoxic chemicals as described above, and centrifuged again.

The genotoxic chemicals were removed, and the walls surrounding the MCP were separated from the plate. The plate with the cells was covered with a solution of 0.5 % low-melting agarose (type Sea Plaque agarose from Biozym Diagnostik, Hessisch Oldendorf, FRG) preheated to 37 °C. The plate was kept in the refrigerator for 5 minutes to solidify the low melting agarose. The following steps were performed according the procedure recommended by Tice *et al.* [7] and described in Stang and Witte [1]. In brief, the plate was covered with refrigerated lysis solution, pH 10.0, and kept at 4 °C for 1 hour. After lysis the plate was placed on a horizontal electrophoresis box. The box was filled with freshly prepared alkaline buffer whereby the plate was completely covered with the buffer. During 40 minutes at 4 °C the alkaline treatment allowed alkaline unwinding of the DNA and DNA breakage at alkali labile sites. After electrophoresis the plate was covered three times with neutralization buffer, washed with aqua bidest. and stained with SYBR Green (Sigma, Deisenhofen, FRG). Fluorescence microscopy was used to determine the median DNA migration in each of up to 12 parallel samples per concentration (100 comets/well). The mean, the standard deviation (SD), and the standard error (SE) of the medians of the parallel samples were calculated. Statistical significance of the differences between the DNA migration induced by low concentrations of the mutagens and the untreated controls was determined by the non-parametric Mann-Whitney test and the parametric t-test if the basic assumption for parametric tests were met.

2.5 Automated comet assay analysis

Automated analysis of the Comet assay was performed using a MetaCyte CometScan system based on the slide scanning platform Metafer (MetaSystems, Altlussheim, FRG). The system consists of a motorized microscope (AxioImager Z1, Carl Zeiss, Jena, Germany) with fluorescence illumination, a motorized X/Y scanning stage (Maerzhaeuser, Wetzlar, Germany) with a range of 225 x 76 mm, a high

resolution monochrome megapixel charge coupled device (CCD) camera (M4+; JAI AS, Glostrup/Copenhagen, Denmark), and a Windows^{TM} compatible PC (DELL, Langen, Germany) running the Metafer software. Hardware components (e.g. the microscope focusing motor, the fluorescence filter turret, and the motorized stage) are directly driven by the software. The Maerzhaeuser stage has been modified to adapt it to the 96 well plates used in this study.

Parameters for slide scanning and automated Comet assay analysis were set according to the experiment's requirements by selecting the matching settings file (classifiers) in the Metafer software. Classifiers define details on image acquisition, number of captured fields at each well position, cell selection, image analysis procedures, and more. In addition, layout settings files defining the number and positions of wells to scan on the plate were created.

For analysis the classifier and layout files were selected in the setup dialogue of the Metafer software, and a file name for the results file was entered. Unattended MCP scanning was started subsequently using a final magnification of 10x. The plane of best focus was determined automatically at each captured field inside the wells. This is done by automatically moving the stage in the z-direction, capturing images in different focus planes, and analyzing the focus quality based on a local contrast criterion. Subsequently the exposure time for the final image was automatically adjusted avoiding saturated pixels. Each image was then analyzed for the presence of target Comet cells. Target cells are defined in the classifier by morphology criteria such as size, aspect ratio, concavities, and other parameters.

Cells being initially detected by the system were rejected if they were subject to the following conditions: a) another object was present in the close neighborhood, that might interfere with the measurements, b) the background around the candidate comet showed significant inhomogeneities, and c) the tail intensity of the comet did not decrease to the background level inside the measurement rectangle, indicating that the comet is larger than the region of interest.

Once a comet was finally accepted by the system, its intensity profile was automatically analyzed within a measurement rectangle defined by the software. Head and tail of the comet were determined based on the intensity levels. The background levels were subtracted from the intensity values obtained. Different comet features (e.g. intensity of head and tail, comet shape, tail moment, Olive tail moment) were measured, and an image of each cell was stored in a gallery. Overlays

within these cell images show borderlines between head and tail, and the head and tail regions, as they were defined by the analysis algorithms. Depending on the classifier setup, selected cell features (e.g. tail moment and percentage of DNA in the tail) were displayed in the gallery image.

2.6 Manual comet assay analysis

Samples analyzed with the MetaCyte CometScan Software were subsequently analyzed with conventional evaluation systems. Each sample on the MCP was interactively evaluated using the Lucia Comet Assay Single Stain software (Laboratory Imaging s.r.o, Czech Republic), which is a separate stand-alone imaging system, and the MetaSystems CometImager, which represents the interactive evaluation system of MetaCyte CometScan. The MetaSystems CometImager use the same imaging hardware and scoring algorithms like Metafer. For the interactive evaluation 100 comets / well were randomly selected and measured.

3. Results

The comet formation of identical samples was measured automatically (MetaCyte CometScan Software) and compared to the measurements of the interactive evaluation systems. In order to directly compare the impact of automation on the results, we used the MetaSystems CometImager, which was installed on the Metafer system working with the same imaging hardware and scoring algorithms like the automated system. In addition, comets were interactively scored by the independently working system Lucia Comet Assay Single Stain, which was installed on a different hardware platform. Within 3 hours the MetaCyte Scan System evaluated fully automatically a MCP with 96 samples, counting 100 comets per well, which was equivalent to a scoring speed of 50 comets per minute.
To evaluate comets, human fibroblasts were treated with MMS for 1 hour, or with H_2O_2 for 15 minutes on the MCP. The results of the parameters tail moment (tm), % DNA tail, and Olive tail moment are shown in Fig. 1 for MMS, and in Fig. 2 for H_2O_2.

The comet formation for both chemicals showed concentration dependent induction of DNA damages. The quantitative results were similar for automatic and interactive

measurements either for MMS (Fig. 1), or for H_2O_2 treatment (Fig. 2). They were also similar regarding the parameter tail moment (tm), % DNA tail, and Olive tail moment. In the untreated control samples no or only a very low DNA migration was observed.

In early drug candidates selection or biomonitoring it is necessary to detect low DNA damaging effects. Therefore the high throughput comet assay has to be reliable and highly sensitive. In order to test the sensitivity of the assay we measured DNA damage induced by H_2O_2, and MMS at low effective concentrations and compared the standard errors in dependence to the number of analyzed samples. The results of tm and % DNA tail are presented in Tab.1. In general, the standard error (SE) was slightly lower for the parameter % DNA tail than for tm, especially at the lower concentration of the two tested chemicals. In samples with about 7-8 % of DNA tail (tm: 0.6-0.9), four parallels were sufficient to reach a significance level of $p \leq 0.01$ or $p \leq 0.001$. This was shown for both DNA damaging agents (MMS, H_2O_2). The evaluation of the tm of four identical samples gave a significance level of $p \leq 0.05$.
The values shown in Tab.1 were the result of the analysis of 100 comets per sample. Additionally, we tested the effect of reducing the number of comets evaluated in respect to the standard error (SE). Tab. 2 shows the results for 25, 50, or 100 comets per sample (4 parallels measured). The SE for 50 or 100 comets determined were similar, both for, MMS and H_2O_2, while scoring of 25 comets resulted in a higher SE.

4. Discussion

The MCP-based comet assay clearly increases the throughput of number of samples [1], when compared to the conventional standard assay [7]. The problem which still remains is the time-consuming analysis of comets if no automated evaluation system is available. To interactively analyze the huge volume of data produced by the high throughput comet assay a technician usually has to invest more than one complete working day to score a single MCP. In addition, interactive analysis compared to automated systems results in a higher number of errors due to scoring biases [8].

Though automated analysis systems for conventional comet assay samples exist on the market [10], the automated screening of MCPs has not yet been established. The aim of our study was to develop a method for the automated and rapid analysis of

MCP based comet assay samples, and to test this method with focus on high throughput screening of potentially genotoxic chemicals with high precision.

Typical values for interactive scoring of comets are in the range of about 5 comets / minute. Automated analysis systems developed for conventional comet assay samples were described to evaluate about 7 comets / min [8-9]. The highest time-saving potential lies in the possibility to score samples unattended and overnight. However, the requirements for real high-throughput analyses are not met with these automated evaluation systems. The MetaCyte CometScan system, when used with MCPs, reduced the evaluation time for comets dramatically by a factor of 10, so that 50 comets / min can be measured. Reasons for the higher efficiency of the automated analysis of MCPs can be found in the layout of the samples. All 96 samples on the MCP are located in direct proximity to one another on the same plate, while the automatically working systems of Frieauff et al. [9] and Böcker et al. [8] were designed for microscope slides with 1-2 samples / slide. The automatic change of the slides is time-consuming. In addition, the scan can be performed with lower magnification compared to Frieauff et al. [9] and Böcker et al. [8]. Both, 96 samples on one plate and the use of lower magnification, increase the scanning speed dramatically.

Our results showed an excellent concordance of the fully automated analysis MetaCyte CometScan software with the two interactive scoring systems MetaSystems CometImager and the Lucia Comet Assay software. This was shown for two carcinogens with different mutagenic pathways, either causing preferentially single strand breaks (H_2O_2), or methylations of the DNA (MMS). All three systems revealed similar concentration-dependent results for all parameters tested: tail moment, % DNA tail, or Olive tail moment. At low effective DNA damaging concentrations the parameter % tail DNA showed lower standard deviations than the parameter tail moment. The standard error of the samples was low (about 10%) even when low effective genotoxic concentrations were used (5 % DNA tail). Thereby DNA damage was detected using 4 parallel samples with a significance of $p \leq 0.01$. This demonstrates the high sensitivity of our automatic evaluation system for scoring low levels of DNA damage which is especially of importance in biomonitoring studies or pharmaceutical early drug selection where only low genotoxic effects are expected.

With the automatic evaluation system presented here at least 6 different compounds can be tested on one MCP. In this case each sample could be measured with three concentrations using 4 parallels per concentration. Under these conditions the throughput on the MCP is accelerated by a factor of about 20 compared to the conventional method. So 4-6 MCPs with 36 different compounds versus 2 compounds in the conventional comet assay along the guidelines of Tice et al. [7] can be tested at one day. The automatic analysis further enhances the throughput of samples by a factor of 10 compared to interactive evaluation or by a factor of 6-7 compared to automatic analysis systems developed for the standard comet assay. In summary, the time needed to perform the comet assay and to evaluate the comets in our high throughput version is reduced by a factor of 70-130 when compared to previously existing automatic analysis methods, and by a factor of 120-180 when compared to interactive analyses. Additionally, time can be saved by the high throughput version due to integrated measurement of cytotoxicity [1], whereas in the standard assay cytotoxicity has to be measured separately in a parallel experiment. Thus, our data presented hear indicate clearly that genotoxic candidates can be tested by a real high throughput.

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Abbreviations

D-MEM	Dulbecco's modified Eagle medium
D-PBS-buffer	Dulbecco’s Phosphate Buffered Saline buffer
FDA	fluorescein diacetate
MCP	multichamber plate
MMS	methyl methanesulfonate
REACH	**R**egistration, **E**valuation and **A**uthorisation of **Ch**emicals
SD	standard deviation
SE	standard error
sfm	serum free medium
tm	tail moment

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Figures and Tables

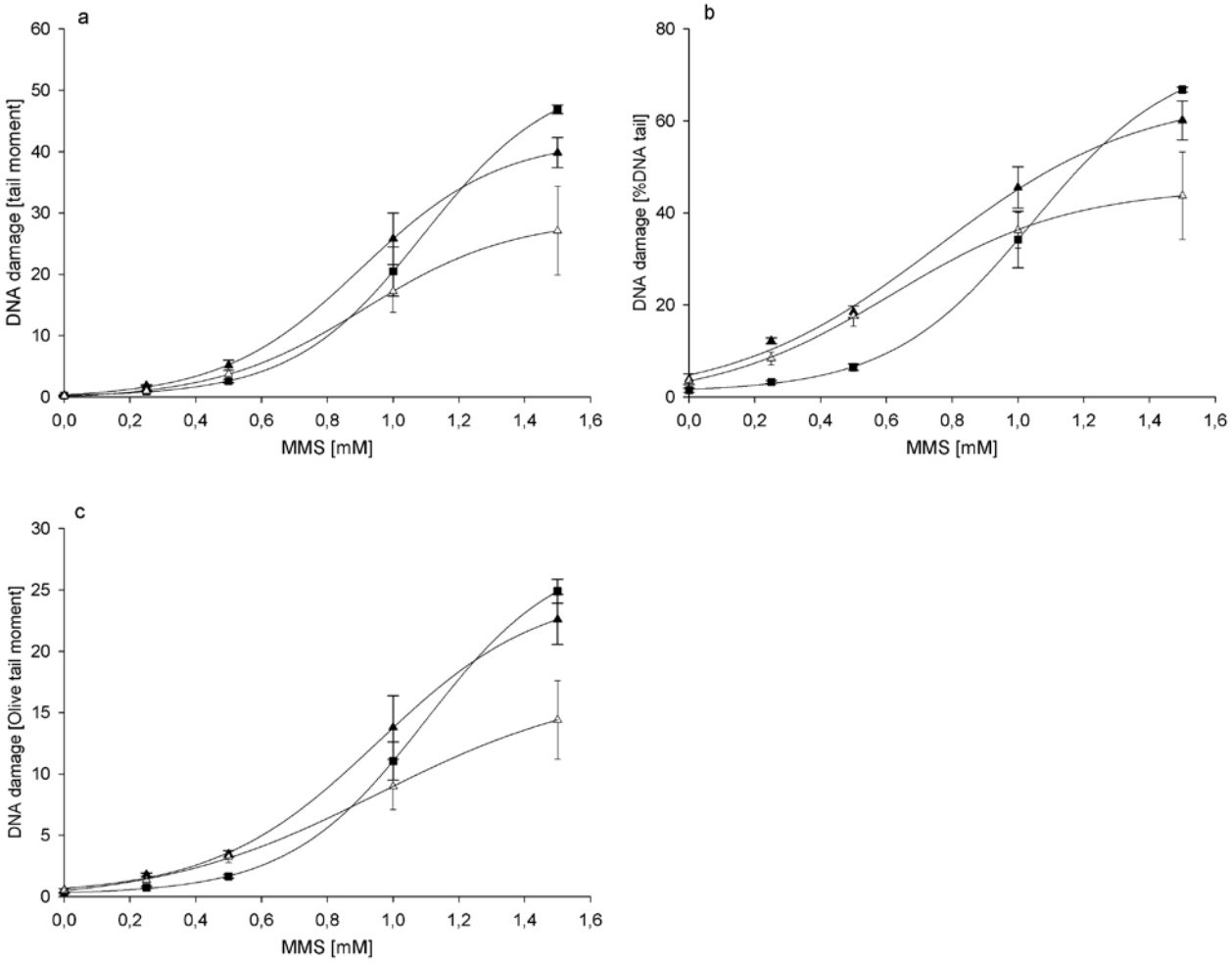

Fig. 1: Concentration dependent DNA damage in human fibroblasts induced by MMS; (a) tail moment, (b) % DNA tail, and (c) Olive tail moment. Microscopic analysis was performed by (■) Lucia Comet Assay Software (interactive), (▲) MetaSystems CometImager Software (interactive), and (Δ) MetaCyte CometScan Software (automated). The medians were calculated from each of 4 parallel samples per concentration (100 comets/well). From these 4 values the mean and standard deviation was determined

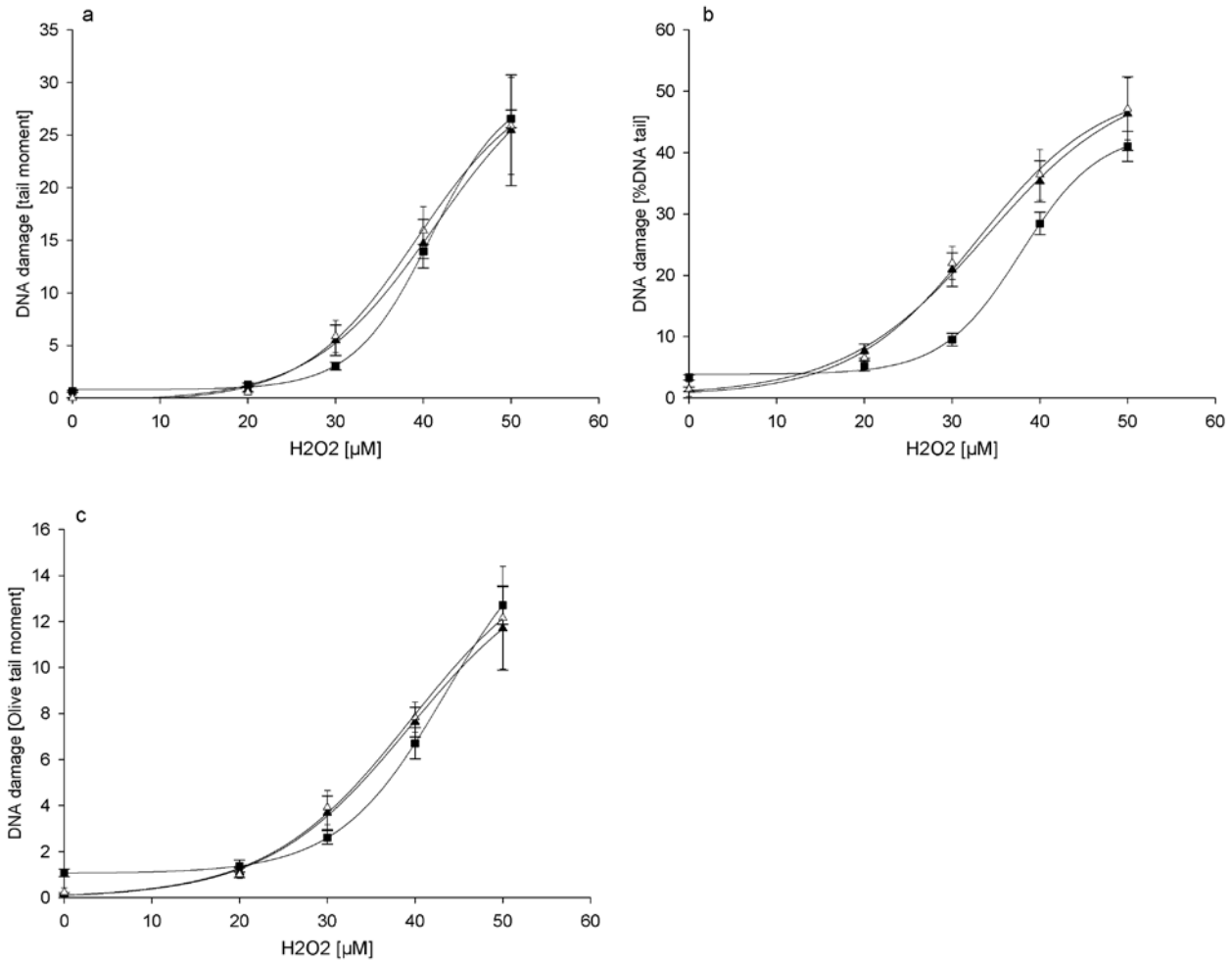

Fig. 2: Concentration dependent DNA damage in human fibroblasts induced by H_2O_2; (a) tail moment, (b) %DNA tail, and (c) Olive tail moment. Microscopic analysis was performed by (■) Lucia Comet Assay Software (interactive), (▲) MetaSystems CometImager Software (interactive) and (Δ) MetaCyte CometScan Software (automated). The medians were calculated from each of 4 parallel samples per concentration (100 comets/well). From these 4 values the mean and standard deviation was determined

Tab.1: DNA damage induced by MMS, or H_2O_2 at low effective concentrations dependent on the number of parallels; tm and % DNA tail of identical samples are given 100 comets per well were evaluated; significant differences to the untreated control were:* p ≤ 0.05; **p ≤ 0.01; *** p ≤ 0.001 [1] = p > 0,05; SE= standard error

a)	tail moment [µm]					
number of wells	MMS [mM]			H2O2 [µM]		
	0	0,25	0,5	0	20	30
	mean±SE	mean±SE	mean±SE	mean±SE	mean±SE	mean±SE
n=3	0.18±0.02	0.93±0.16 *	3.75±0.49*	0.02±0.02	0.49±0.16[1]	6.06±1.03**
n=4	0.17±0.01	0.96±0.12*	3.76±0.35**	0.01±0.01	0.54±0.12*	5.88±0.75**
n=5	0.15±0.02	0.88±0.12**	3.79±0.27***	0.01±0.01	0.62±0.13***	6.01±0.60***
n=6	0.15±0.02	0.89±0.10***	3.40±0.45**	0.01±0.01	0.61±0.10**	5.82±0.53***
n=9	0.18±0.05	0.91±0.12***	3.70±0.41***	0.01±0.01	0.72±0.18***	6.63±1.04***
n=12	0.24±0.08	0.90±0.10***	3.85±0.29***	0,00±0.01	1.10±0.30***	6.56±0.66***

b)	%DNA tail					
MMS [mM]	MMS [mM]			H2O2 [µM]		
	0	0,25	0,5	0	20	30
	mean±SE	mean±SE	mean±SE	mean±SE	mean±SE	mean±SE
n=3	3.62±0.28	8.39±0.99*	17.86±1.50**	1.50±0.79	6.46±0.66**	22.74±1.62**
n=4	3.62±0.20	8.42±0.70**	17.60±1.09***	1.44±0.57	6.55±0.48***	22.02±1.36***
n=5	3.34±0.31	7.98±0.70***	17.73±0.86***	1.31±0.46	6.51±0.37***	22.26±1.08***
n=6	3.25±0.27	8.17±0.60***	16.39±1.52***	1.43±0.39	6.72±0.37***	21.83±0.98***
n=9	3.53±0.49	8.38±0.82***	17.49±1.35***	1.49±0.34	7.12±1.00***	23.58±2.83***
n=12	3.81±0.67	8.25±0.64***	17.85±1.18***	1.34±0.25	8.54±1.05***	23.39±1.28***

Tab. 2: DNA damage induced by MMS, or H_2O_2 at low effective concentrations dependent on the number of comets evaluated; the mean was given by the medians of 4 parallels

tail moment [µm]						
evaluated comets	MMS [mM]			H_2O_2 [µM]		
	0	0.25	0.5	0	20	30
	mean±SE(%)	mean±SE(%)	mean±SE(%)	mean±SE(%)	mean±SE(%)	mean±SE(%)
25	0.31±0.11(35)	1.33±0.19(14)	4.35±0.71(16)	0.00±0.00(0)	0.98±0.48(48)	5.47±1.11(20)
50	0.21±0.04(19)	1.20±0.20(17)	4.24±0.57(13)	0.00±0.00(0)	0.45±0.07(16)	4.09±1.20(29)
100	0.17±0.01(6)	0.96±0.12(13)	3.76±0.35(9)	0.01±0.01(100)	0.54±0.12(22)	5.88±0.75(13)

% DNA tail						
evaluated comets	MMS [mM]			H_2O_2 [µM]		
	0	0.25	0.5	0	20	30
	mean±SE(%)	mean±SE(%)	mean±SE(%)	mean±SE(%)	mean±SE(%)	mean±SE(%)
25	5.09±1.07(21)	10.58±1.24(12)	19.35±1.17(6)	0.83±0.50(60)	7.49±2.03(27)	22.19±3.80(17)
50	3.91±0.89(23)	9.52±1.25(13)	19.88±0.79(4)	1.08±0.64(59)	6.68±0.30(5)	20.74±3.28(16)
100	3.62±0.20(6)	8.42±0.70(8)	17.60±1.09(6)	1.44±0.57(40)	6.55±0.48(7)	22.02±1.36(6)

5.3 Ability of the high throughput comet assay to measure comparatively the sensitivity of five cell lines toward methyl methanesulfonate, hydrogen peroxide and pentachlorophenol

(submitted)

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Abstract

To test a newly developed high througput version of the comet assay human fibroblasts were used (Stang and Witte, 2009). In this study we examined if this high throughput comet assay works also with other adherent and non-adherent cell lines. In addition to human fibroblasts we showed that V79, HeLa, Hep G2, and lymphocytes can be used. The time intervals needed for attachment on the agarose coated 96-well multichamber plate (MCP, specially developed for the high throughput comet assay) was different for the adherent cell lines used. These were for V79 cells 6 h, for fibroblasts 2-4h, for Hep G2 18 h, and for HeLa 16 h. After attachment chemical treatment occured. Non-adherent lymphocytes could be treated with the chemicals directly after they had been pipetted into the wells of the MCP () and centrifuged.

We compared the sensitivities of the cell lines toward the directly DNA damaging compounds methyl methanesulfonate (MMS) and hydrogen peroxide (H_2O_2) and toward the indirectly acting pentachlorophenol (PCP). Except of Hep G2 cells PCP was metabolized in all tested cell lines in the presence of postmitochondrial S9 fraction. In all cell lines DNA damage occured in a concentration dependent manner. Human lymphocytes were the most sensitive cells toward the three chemicals tested, fibroblasts showed a similar sensitivity toward the directly acting MMS and H_2O_2, but were less sensitive toward PCP. HeLa, V79, and Hep G2 reacted with similar sensitivity.

Keywords

high throughput; comet assay; V79; Hep G2; HeLa; fibroblasts; lymphocytes

1. Introduction

A high throughput version of the comet assay was developed for screening large numbers of samples such as for screening industrial chemicals, for biomonitoring tasks, or for prescreening of pharmaceutical candidates [1,2].The core of the high throughput comet assay is a specific 96-well plate (multichamber plate, MCP) where the treated cells are maintained throughout the whole comet assay procedure on the MCP. So, the time-consuming steps of trypsinisation, transfer of cells to microscope slides and individual processing of each sample is avoided. This is possible because the walls of the MCP can be separated from the plate and thus the 96 samples of the MCP plate can be electrophorized all together.

First experiments using the MCP were performed with human fibroblasts [2]. A short time interval of 2-4 hours after seeding, the fibroblasts were slightly attached keeping their rounded form (obtained by trypsinisation). During the comet assay procedure no further spreading of the cells was observed with the consequence that after lysis the heads of the comets were also in a round shape. This is a prerequisite for comet evaluation. In this study we examined if other cell types than fibroblasts can also be used in the high throughput version of the comet assay. We tested in addition to fibroblasts non adherent lymphocytes, and cell lines often used in the standard comet assay such as V 79, HeLa and Hep G2 cells.

The determination of genotoxicity in several cell lines in parallel enables the direct comparison of the sensitivities of these cell lines in the comet assay. From our knowledge this was not done systematically so far. We measured the comet formation induced by the methylating methyl methanesulfonate (MMS), and the hydroxyl radical producing hydrogen peroxide (H_2O_2). In addition, we tested the effect of pentachlorophenol (PCP). PCP needs metabolic activation via Cyt P450 to be converted into the highly genotoxic metabolite tetrachlorophenol [3,4,5]. Here a comparison of the metabolic competent Hep G2 cells and non-competent cell lines in the presence of postmitochondrial S9 fraction was made.

2. Materials and methods

2.1 Cell cultures

Human fibroblasts, cell line NHDF-p were purchased from Promochem (Heidelberg, FRG), hamster fibroblasts cell line V79 were a gift of Dr. Speit, University of Ulm, FRG., human hepatocyte cell line Hep G2 and, HeLa cell were a gift from Dr. Janssen-Bienhold, University of Oldenburg, FRG. The cells were grown in D-MEM, supplemented with 12 % fetal calf serum, vitamins, non essential amino acids and 100 U/ml of penicillin, and 100 µg/ml streptomycin at 37 °C in an atmosphere of 5 % CO_2 and 95 % air with more than 95 % humidity. The human fibroblasts were used in passage 8-15.

The donor of the lymphocytes was a non-smoking volunteer. Lymphocytes were separated on a Ficoll gradient, resuspended in D-MEM, seeded into the wells of the MCP and thereafter treated with the genotoxic agents.

2.2 Multichamber plate (MCP)

The MCP is a specially coated 96 well plate purchased from Intox, Oldenburg, FRG, which allows the electrophoresis of cellular DNA. The surrounding walls of the wells can be separated from the plate of the MCP. The cells remain attached on the flat agarose coated plate and all samples can be electrophorized together. The following procedure can also be performed for all 96 samples in one [2].

2.3 Chemical treatment of the cells

3000-5000 cells were seeded in D-MEM into each well of the MCP. The adherent cells were allowed to attach to the bottom of the MCP while the lymphocytes were centrifuged (Labofuge 400; rotor: 8177; Heraeus, FRG) for 2 minutes at 900 rpm. Then the cells were washed with sfm, centrifuged and treated with methyl methanesulfonate (MMS, > 99 %, from Sigma, Deisenhofen, FRG), pentachlorophenol (PCP, recrystallized, a gift of Dr. Butte University of Oldenburg,

FRG), or H_2O_2 (37 % aqueous solution from Acros Organics, NJ) diluted to the desired concentration with sfm. MMS and PCP were freshly dissolved in serum free medium (sfm) at pH 7.2 and 37 °C directly before cell treatment. H_2O_2 was diluted with sfm also immediately before cell treatment.

The treatment with PCP requires metabolic activation by cytochrom P450 present in Hep G2 cells. For all other cell lines postmitochondrial S9 fraction from Aroclor 1254 induced rat liver was used for metabolic activation. S9 mix was prepared immediately before use. The S9-fraction (final concentration 0.8mg protein / ml) was mixed with a solution containing 8 mM $MgCl_2$, 33 mM KCl, 4 mM NADP, 5 mM glucose-6-phopshate, pH 7. The treatment with MMS and PCP was 1 hour, with H_2O_2 15 min because of its short half life. After chemical treatment the MCP was centrifuged, the agents removed and the walls of the MCP detached.

2.4 Comet assay in the high throughput version (MCP)

The comet assay in the high throughput version was performed on the basis of the guidelines of Tice *et al.* [6], described by Stang and Witte [2] in detail.

After chemical treatment, as described above, the cytotoxicity of chemical exposure was measured according to Stang and Witte (2009). Thereby the fluorescein diacetate assay (FDA assay) was used [7]. The FDA assay measures the cell viability by the activity of cytosolic esterases converting fluorescein diacetate to the fluorescent dye fluorescein. The cells were incubated for 10 minutes with 72 µM FDA (from 12 mM stock solution in acetone, freshly diluted with sfm) at 37 °C in the dark. Thereafter the dye was removed and the fluorescence of the enzymatically formed fluorescein was measured in a fluorescence reader (FLUOstar, Offenbach, FRG) with an excitation of 485 nm and an absorption at 520 nm.Thereafter the walls and the plate of the MCP were separated and the comet assay procedure was continued as described in detail by Stang and Witte [2]. In brief, the plate with the cells was covered with a 37 °C warm solution of 0.5 % low-melting agarose (type Sea Plaque from Biozym Diagnostik, Hessisch Oldendorf, FRG) and kept in the refrigerator for 5 minutes to solidify the low melting agarose. Then the plate was covered with refrigerated lysing solution pH 10.0 and kept at 4 °C for 1 hour. After lysis the plate was placed on a horizontal electrophoresis box. The box was filled with freshly

prepared alkaline buffer whereby the plate was completely covered with the buffer. During 40 minutes at 4 °C the alkaline treatment allowed alkaline unwinding of the DNA and DNA breakage at alkali labile sites. After electrophoresis the plate was covered three times with neutralization buffer, washed with aqua bidest and the DNA was stained with ethidium bromide. DNA migration was analyzed by fluorescence microscopy (Nikon, Eclipse E600W) using the software ”Lucia comet assay Single Stain” (Laboratory Imaging, Prague, Czech Republic). The median DNA migration determined by the parameter “% DNA tail” was calculated from each of 4 parallel samples per concentration (25 comets/well). From these 4 values the mean and standard deviation were calculated.

3. Results

A precondition to perform the comet assay on the MCP is that the cells seeded remain in their rounded form obtained after trypsinisation. Thereby the attachment of the cells has to be strong enough that they do not detach from the bottom during chemical treatment. The suitable time for slight attachment was individual for each cell line as shown in Tab.1.

The cells were treated after their individual attachment time with increasing concentrations of the directly acting DNA damaging agents MMS for 1 hour and H_2O_2 for 15 minutes. At least 4 parallel samples per concentration were prepared. None of the concentrations tested reduced cell viability by≥ 30 % when determined by the FDA assay (data not shown). So, comet formation caused by high cytotoxicity could be excluded [8]. In Fig.1 and Fig.2 the resulting comet formation induced by MMS and H_2O_2 is shown. For both chemicals a concentration dependent increase in DNA damage was observed while the untreated controls show none or only a low DNA migration. Human lymphocytes and the adherent human fibroblasts were the most sensitive cell lines while the other three adherent cell lines V 79, HeLa and Hep G2 were significant less sensitive. This was shown in Tab.2 for low effective concentrations (10 % DNA tail), moderate (30 % DNA tail) and higher effective concentrations (50 % DNA tail). V79, HeLa, and Hep G2 cells were similar sensitive at least in low and moderate effective concentrations.

The indirectly acting genotoxic PCP was tested in fibroblasts, V79, Hela and lymphocytes in the presence of postmitochondrial S9 fraction and additionally in Cyt P450 containing Hep G2 cells without S9 fraction. The results are shown in Fig.3 and Tab.2. Again, lymphocytes were the most sensitive cells at least in low and moderate concentrations. Human fibroblasts with the lowest sensitivity of all cell lines tolerated the highest PCP concentrations up to 1mM without any DNA damage. In effective concentrations PCP revealed similar DNA damage in V79, HeLa, fibroblasts and Hep G2 shown by similar slopes of the curves. A direct comparison with the ultimate PCP metabolite tetrachlorohydrochinone in fibroblasts showed the high DNA reactivity of this metabolite (Fig. 3). The use of S9 fraction alone did not elevate comet formation during the incubation time of one hour compared to the control.

4. Discussion

In this study it was shown that the high throughput version of the comet assay can be performed with cell lines which were often used in the conventional comet assay. While non-adherent lymphocytes could be treated immediately after having been pipetted into the wells of the MCP with subsequent centrifugation the adherent cells had to attach on the surface of the wells up to 18 hours. The cell attachment is mediated through different families of receptors such as integrins or proteoglycans [9,10]. The expression of cell receptors depends on the particular type of cell [9]. So time needed for attachment as well as for detachment via trypsin which cleaves receptor proteins [11] are cell type specific. This time for attachment was either short enough to perform the comet assay at the same day when cells had been seeded (fibroblasts, V79) or seeding had to be performed the late afternoon before the experiment (Hep G2, HeLa). For other cell lines than those used in our study, the individual attachment time which is needed to avoid spreading had to be determined before the comet experiment. For strongly attached cell lines where long trypsinisation provoke non physiological conditions for the cells the high throughput version of the comet assay may be of advantage compared to the conventional assay.

In all cell lines a concentration dependent DNA damage induced by MMS, H_2O_2, or PCP (after metabolic activation) was observed. S9 fraction as well as the Cyt P 450 containing Hep G2 cells transformed the non-genotoxic PCP to a highly DNA damaging metabolite identified as tetrachlorophenol [3,5]. Directly acting genotoxic chemicals provoked less DNA damage in Hep G2 cells than in the other cell lines which we have tested. In the organism hepatocytes are generally more exposed to xenobiotics than other cell types. A high level of scavenger molecules and repair enzymes is necessary to ensure their survival.

Indirectly acting genotoxic chemicals were detected in Hep G 2 cells as well as in all other cell lines in the present of S9 fraction of Aroclor 1254 induced rat liver. It cannot be decided what kind of activation will be better for genotoxicity screening. Metabolites of genotoxic chemicals produced by S9 fraction can be quite different from those produced in Hep G2 cells [12]. Some of them are genotoxic in Hep G2 cells and non genotoxic using S9 fractions and vice versa. [13].

A variety of different cell types are used in the comet assay depending on the scientific question. A comparison of the sensitivity of several cell lines in the comet assay was not done so far. In some biomonitoring studies hepatocytes and metabolic non competent cells were used to detect directly as well as indirectly acting genotoxic agents [14, 15, 16]. For screening a high number of unknown chemicals or samples it is necessary to use one or two cell lines with high sensitivity in the comet assay. In addition an easy handling/cultivation of the cells would be of advantage. In our experiments human lymphocytes were the most sensitive cells toward the three genotoxic agents followed by human fibroblasts which were similar sensitive toward the directly acting MMS and H_2O_2 but less sensitive toward PCP.

In summary, we could show that the high throughput comet assay can be performed using adherent and non adherent cell lines. Thereby the effects of directly acting genotoxic compounds as well as of indirectly acting compounds can be determined in a concentration dependant way. It was shown that lymphocytes revealed the highest sensitivity. The advantage of lymphocytes is that no time is needed for attachment of the cells. This accelerates the comet assay. Additionally, time can be saved by the

combination of the MCP with a newly developed automatically working evaluation system for the comets on the MCP, which enhances the throughput of samples by a factor of more than 150 compared to the conventional comet assay [17 (submitted)].

Acknowledgements

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Abbreviations

D-MEM	Dulbecco's modified Eagle medium
D-PBS-buffer	Dulbecco’s Phosphate Buffered Saline buffer
FDA	fluorescein diacetate
MCP	multichamber plate
MMS	methyl methanesulfonate
PCP	pentachlorophenol
SD	standard deviation
sfm	serum free medium
tm	tail moment

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Figures and Tables

Tab.1: time needed for slight attachment of 5 cell lines on the bottom of the MCP

celltype	time for attachment
human lymphocytes	no attachment
Hep G2	18 h
human fibroblasts	2-4 h
Hela	16 h
V79	6 h

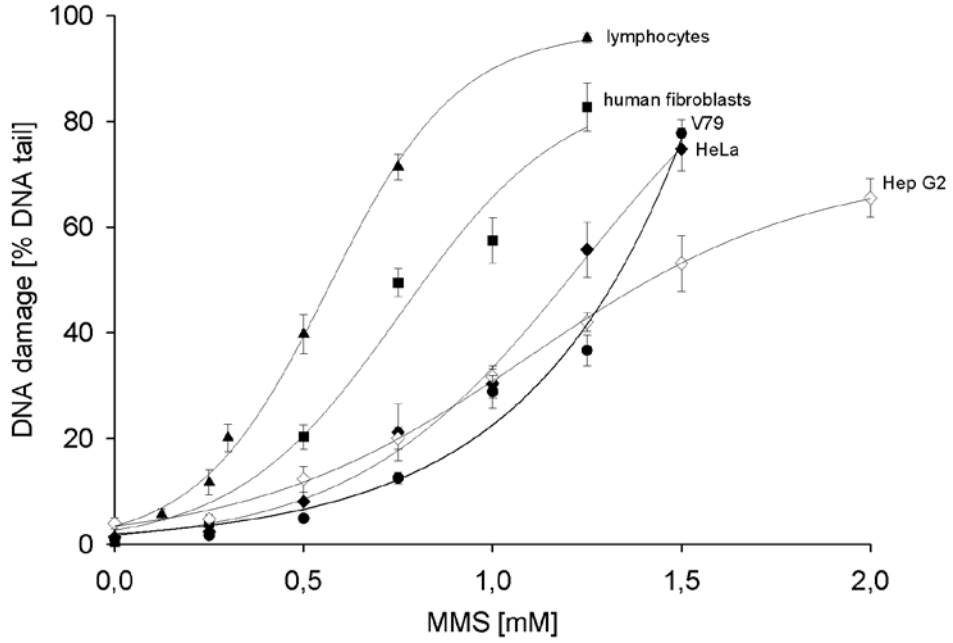

Fig. 1: Concentration dependent DNA damage induced by MMS in human fibroblasts (■), human lymphocytes (▲), Hep G2 cells(◊), V79 (•) and HeLa cells (♦); the standard deviation was calculated from the mean of 4 parallel samples

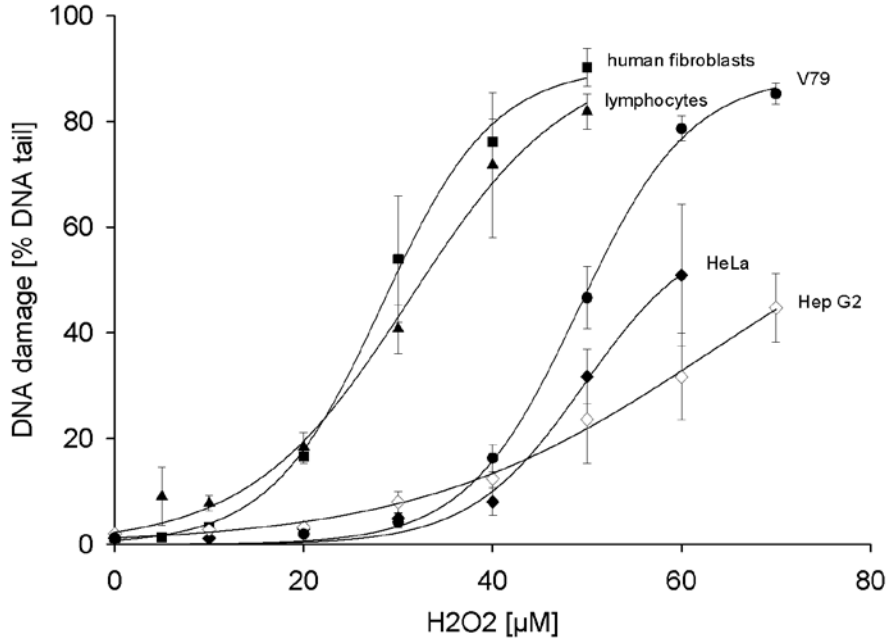

Fig. 2: Concentration dependent DNA damage induced by H_2O_2 in human fibroblasts (■), human lymphocytes (▲), Hep G2 cells(◊), V79 (•) and HeLa cells (♦);the standard deviation was calculated from the mean of 4 parallel samples

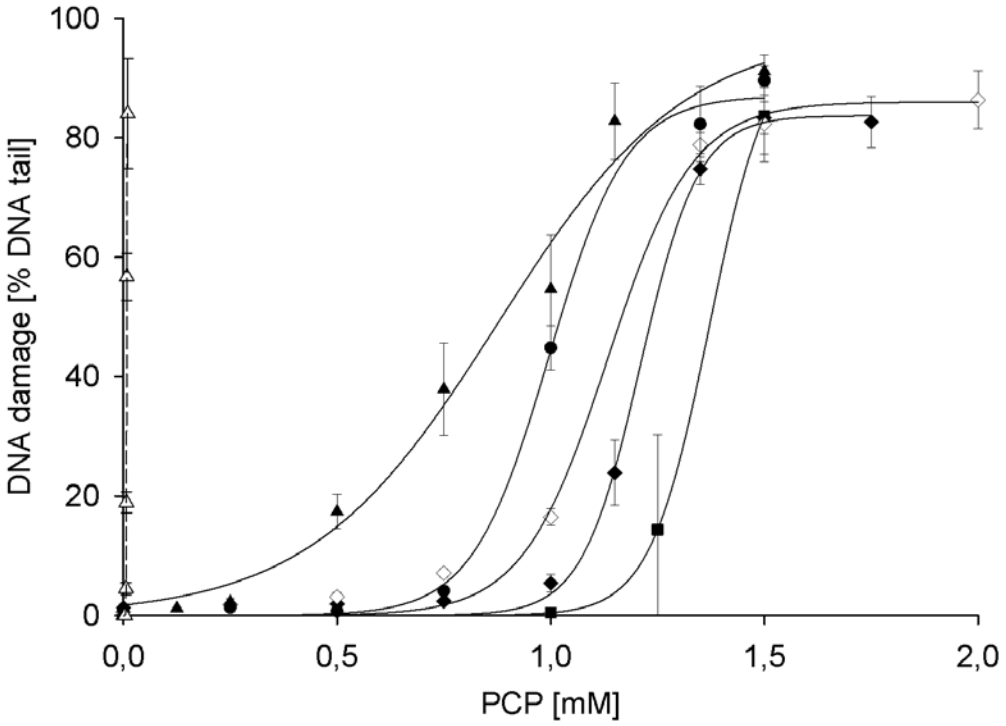

Fig. 3: concentration dependent DNA damage induced by PCP in human fibroblasts (■), human lymphocytes (▲), Hep G2 cells(◊), V79 (•) and HeLa cells (♦). ∆ = effect of the ultimate PCP metabolite tetrachlorhydroquinone (TCHQ) on human fibroblasts; the standard deviation was calculated from the mean of 4 parallel samples

Tab.2: Sensitivity of 5 cell lines toward MMS, H_2O_2, and PCP; the data were picked up from the curves in Fig.1-3

celltype	MMS			H2O2			PCP		
	% DNA Tail [µM]			% DNA Tail [µM]			% DNA Tail [µM]		
	10%	30%	50%	10%	30%	50%	10%	30%	50%
lymphocytes	235	400	600	13	24	31	400	700	850
Hep G2 cells	500	1000	1450	36	55	nd	900	1100	1200
fibroblasts	350	650	800	20	28	36	1200	1250	1350
HeLa	600	900	1200	41	50	60	1100	1200	1250
V79	750	1200	1400	38	44	50	750	900	1050

5.4 A high-throughput genotoxicity testing strategy for screening of (drinking) water

(submitted)

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Abstract

The sources of our drinking water can be polluted with anthropogenic chemicals. To assess the health hazard of this pollution, *in vitro* genotoxicity tests are of great importance. With the many systems available, it is still unclear which battery to choose. We applied a strategy of high-throughput *in vitro* genotoxicity testing to nine water extracts from different types of sources in the Netherlands with the Ames II, high-throughput comet assay and the micronucleus test. In addition, a validation set of eight well-known genotoxicants and one non-genotoxic carcinogen was tested with the Ames II and the comet assay. The aims of this study were:

1. To compare the outcome of the Ames II with either the comet or the micronucleus test with respect to their complementarity
2. To determine the suitability of using the high-throughput Ames II and comet assay for water extracts;
3. To compare the comet assay and micronucleus test in the detection of genotoxic compounds present in water.
4. To evaluate the occurrence of genotoxic compounds in Dutch waters.

We found that the comet assay and micronucleus test are complementary to the Ames II test, as the Ames II was more sensitive for some tested compounds, but did not detect the genotoxicity of all well-known genotoxins and of all water samples positive in the comet assay. The micronucleus test did not detect any genotoxicity in the water samples. The high-throughput Ames II and comet assay were selected for future testing strategy of water samples.

Keywords

Ames II, comet, micronucleus, monitoring

1. Introduction

In the sources for drinking water, thousands of industrial chemicals can be present [1]. In the EU, 30.000 to 70.000 chemicals are in daily use (EINECS database), varying from industrial chemicals (such as solvents, petrochemicals), consumer chemicals (such as pharmaceuticals, personal-care products) to biocides. Due to global trends such as a growing and ageing population, increasing prosperity, and urbanization [2], there is an increasing chemical pressure on drinking water sources. For example, only about 10% of European river water samples could be classified as 'very clean' [3]. Contaminants have been found throughout Europe up to high ng/L median concentrations [3], many entering the water cycle via wastewater [4]. This occurrence of chemicals in sources of drinking water is, depending on the treatment processes, sometimes reflected in (much lower) occurrence of these chemicals in finished drinking water [5]. Thusfar, where there was sufficient information, concentrations of individual contaminants in drinking water in the Netherlands do not exceed their individual health-based guideline values (Schriks *et al*., in preparation).

However, despite rapidly evolving chemical analytical techniques [6], it is impossible to analyze and identify all different chemicals present in the aqueous environment. In addition, for many identified chemicals no statutory health-based (drinking) water quality guideline values have been established and no or scarce toxicological information is available to estimate human health risks (e.g. [7]). Finally, the understanding of the combined mixture effects in these complex mixtures of many different chemicals occurring in low concentrations is still limited [8]. The use of *in vitro* effect-directed bioassays can be an additional tool to interpret health risks of complex mixtures in drinking water and its sources. *In vitro* bioassays do not determine the presence of single or groups of compounds directly, but determine their collective effect in a biological system such as cultured cells. Chemicals that cannot be revealed by analytical techniques but do attribute to the toxicological effects are included in the bioassays, and thus, the assays give a clue of the toxicity of the total mixture of chemicals present in the water sample.

As the concentrations in the water are relatively low, but drinking water is consumed life-long, only effects occurring at relatively low, but chronic exposure are relevant. One of the most relevant toxic effects in this case is therefore genotoxicity, as genotoxic compounds can be effective at very low dosages. *In vitro* genotoxicity analyses on all types of water have been performed for many years all around the world already, with a variety of test

systems (e.g. [9-14] [15]). With the many test systems available and applied, it is unclear which to choose in order to best detect all genotoxic compounds present in a water sample. Genotoxicity can be tested by measuring the formation of DNA interactions (DNA adducts) themselves, DNA damage (i.e. gene mutations or chromosomal aberrations), or the induction of the DNA repair. As DNA damage can occur through different mechanisms, a battery of tests is necessary. However, for the DNA adducts and repair enzymes, no full battery covering all adduct and repair types for the different types of damage is available. Therefore, for genotoxicity testing of drinking water and its sources, we have chosen a strategy applying an *in vitro* test battery consisting of a gene mutation test, and a test detecting chromosomal damage (e.g. chromosomal breaks) in mammalian cells, following the recommendations of e.g. [16-18].

For the choice of tests among the *in vitro* gene mutation and chromosomal damage tests, we have considered whether the test could be used for Toxicity Identification and Evaluation (TIE), among other common factors such as sensitivity, wide acceptance, costs, etc.. TIE is applied when an environmental sample is found (geno)toxic and one wants to identify the responsible compound(s) (e.g. [19]). The sample is then fractionated, and the (geno)toxicity test is performed on the different fractions. Subsequently, the (geno)toxic fraction is chemically analyzed, containing fewer compounds than the original sample, leading to a simpler analysis. To be able to apply TIE if desired, we selected genotoxicity tests that were preferably performed in multi-well plates to be able to handle large numbers of fractions with relatively small volumes in the future. Additionally, a multi-well plate format requires much less sample, which saves labour and costs in the sample preparation.

For the gene mutation test, we found only one method applying multi-well plates: the Ames II. This modern version of the very well-known classic Ames test is performed in liquid medium instead of agar plates, and uses a colour change as indicator of growth instead of visual colony counting [20]. In principle, any Ames test strain can be applied with this method, but the Ames II kit applies TAMix, a mixture of 6 base-pair mutated strains, instead of TA100 [21,22].

For the test detecting chromosomal damage, a high throughput version of the comet assay has recently been introduced [23,24], using a special 96-well plate of which the bottom can be removed after the exposure step. Nesslany and Marzin [25] also developed a 96-well version of the micronucleus test, where the exposure step took place in 96-well plates, but the subsequent analysis steps were still performed on microscope slides in this method.

We have applied this strategy of high-throughput *in vitro* genotoxicity testing to nine water extracts from different types of sources in the Netherlands with the Ames II, high-throughput comet assay and the micronucleus test. These samples were taken from the most polluted sources of drinking water (rivers and polluted ground waters) and a typical sewage water treatment plant effluent including hospital wastewater, representing worst case water samples relevant for the watercycle. In addition, a set of eight well-known genotoxicants and one non-genotoxic carcinogen was tested with the Ames II and the comet assay.

The aims of this study were:

1. To compare the outcome of the Ames II with either the comet or the micronucleus test with respect to their theoretical complementarity
2. To determine the suitability of using the Ames II and high-throughput comet assay for water extracts;
3. To compare the comet assay and micronucleus test in the detection of genotoxic compounds present in water in order to make a choice which to use in future monitoring.
4. To evaluate the occurrence of genotoxic compounds in Dutch waters.

2. Materials and Methods

2.1 Chemicals

The following chemicals were used to test as well-known genotoxic chemicals and one non-genotoxic carcinogen, covering different genotoxicity mechanisms: methyl methanesulfonate (MMS, > 99 %, from Sigma, St. Louis), N-Nitroso-N-ethylurea (ENU from Sigma, G), 4-nitroquinoline-1-oxide (4-NQO, 98.2 %, from Sigma), cisplatin (Cis, from Medoc, Hamburg, FRG), cyclophosphamid (CP,97 from Sigma,), benzo(a)pyrene (B(a)P, ≥ 96 % from Sigma, 2-acetyl aminofluorene (2-AAF, ≥ 90 % from Sigma), estradiol (E2, > 98 %, Sigma) and pentachlorophenol (PCP, recrystallized, a gift of Dr. Butte, University of Oldenburg). For all experiments, the tested chemicals had identical lot numbers. For the sample preparation, acetonitrile was purchased from Mallinckrodt Baker B.V. (Deventer, the Netherlands) and dimethylsulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium). For the Ames II, the positive control 2-aminoanthracene (2-AA) was purchased from Sigma (St. Louis). For the micronucleus test, the positives

controls cyclophosphamide, colchicines and mitomycin C were obtained from Asta Werke (CP) and Sigma (colchicines and mitC).

2.2 Preparation of water extracts (performed by KWR)

In May 2007, samples of 4 L were taken in extensively washed glass bottles from four Dutch groundwater pump stations where the ground was known to be contaminated, from three Dutch surface water sites where water is taken in to produce drinking water and from one effluent of a Dutch municipal sewage treatment plant (STP) which also treats hospital wastewater. Samples were cooled immediately and stored at 4 °C.
Within 24 hours after collection, the samples were filtered over 0.45 µm cellulose nitrate membrane filters (Sartorius; Goettingen, Germany). Within another 24 hours, four replicates of one liter of every sample were extracted by solid phase extraction (SPE) with pre-washed 200 mg Oasis® HLB cartridges (Waters Corporation, Milford, USA) at ambient pH. Elution was performed with 3 serial additions of 2.5 mL of acetonitrile. The 7.5-mL eluates were evaporated and taken up in 50 µL of DMSO yielding 20,000-fold concentrated extracts. All extracts were stored at -18°C until analysis.

2.3 S9 fraction

Treatment of bacteria or mammalian cells occurred with the indirectly acting chemicals or with water extracts in the presence of post mitochondrial S9 fraction from Aroclor 1254-induced rat livers. For the Ames II and comet experiments the S9 fraction with the same batch number was used (MP Biomedicals, Solon,U.S.A.), for the micronucleus test the S9 was produced in-house according to the procedure described by Ames *et al*. [26] and Maron and Ames [27]. The S9-fraction (final concentration 0,8 mg protein / mL) was mixed with a solution containing 8 mM $MgCl_2$, 33 mM KCl, 4 mM NADP, 5 mM glucose-6-phopshate, pH 7. S9 mix was prepared immediately before use.

2.4 Ames II test (performed by KWR)

The tested chemicals were dissolved and diluted in DMSO and added to form no higher concentrations than the solubility limit. The water extracts were diluted to 100 µL (1:1) with DMSO to obtain a sufficient amount of sample for all tests. The Ames II test strains (TA98

and TAMix) and media were purchased from Xenometrix (Basel, Switzerland). The test procedure provided by Xenometrix, also described by Fluckiger-Isler *et al*. [20], was followed, with minor modifications. Per well of a 24-well plate (Greiner Bio One), the following was added: 6 µL of test sample (compound or water extract) in 100% DMSO, 30 µL overnight culture, 10 µL of S9-mix if applicable and 264 or 254 µL of Exposure Medium, respectively, to obtain 300 µL in total. Thus, the bacteria were exposed to a 200-fold concentration of the water samples. Water extracts were tested in triplicate at one concentration, while compounds were tested in duplicate at nine different concentrations. A triplicate negative control (DMSO only), a triplicate positive control for genotoxicity (table 1), and a triplicate positive control for cytotoxicity (1 mg/mL 4-NQO in DMSO) were included as well.

A custom cytotoxicity test was performed with subsamples of the exposure cultures in medium with histidine, to check for possible artifacts due to effects on cell survival and growth. After an incubation of 90 minutes at 37 °C and 250 rpm, 10 µL from each exposure mixture was transferred to a well of a 96-well plate (Greiner Bio One) for a cytotoxicity measurement. To each well of the 96-well plate, 90 µL of Exposure Medium (containing histidine) was added and this was then left to incubate for another 3 hours at 37 °C and 250 rpm. Then, the OD at 595 nm of the 96-well plate was measured with an Opsys MR platereader (Clindia; Leusden, the Netherlands). Samples with OD-values significantly below that of the negative control (t-test, p=0.05), and below 90% of the OD-value of the negative control, were considered cytotoxic.

To the remaining exposure mixture in the 24-well plate, 2.61 mL of purple Indicator Medium (not containing histidine) was added. The total 2.9 mL was subsequently divided over 48 wells (50 µL per well) of a 384 well plate and left to incubate for 48 hours at 37 °C. Then, the number of yellow wells per 48 wells of one sample were counted manually.

As Ames test responses are not normally distributed, but follow a Poisson distribution [28], no standard statistical tests could be performed on the data. As an alternative, a water extract was determined to be genotoxic if the number of yellow wells exceeded the detection limit of the test. The detection limit ($DL_{response}$) was calculated using equation 1:

$$DL_{response} = \overline{NC} + 3 \cdot s_{NC} \qquad \text{equation 1}$$

In this equation, $\overline{NC}$ is the average response of the negative control in the same test as the sample and s_{NC} is the standard deviation in the responses of the negative control.

2.5 Cell culture

For the comet assay human fibroblasts (cell line NHDF-p, passage 8-15, purchased from Promochem, Heidelberg, FRG) and the permanent cell line HepG2 were used. Cells were grown in D-MEM supplemented with 12% fetal calf serum at 37 °C in an atmosphere of 5 % CO_2 and 95 % air with > 95 % humidity.

For the micronucleus test human lymphocytes were used. The human lymphocytes were obtained by venapuncture from a healthy, non-smoking male, not currently taking any medication. The same donor was used for the first and second assay. The blood was collected in sterile, heparinized vacutainer tubes and gently mixed to prevent clotting. The cultures were set up within 2 hours after withdrawal of the blood. The medium for culturing the human peripheral blood lymphocytes consisted of RPM1-1640 medium with Glutamax-1, supplemented with heat-inactivated fetal calf serum (20%), penicillin (100 units/mL medium), streptomycin (100 µg/mL medium) and phytohaemagglutinin (PHA-L, 10 (µL/mL medium). Whole blood (0.5 mL) was added to sterile screw-capped tubes containing 4.5 mL culture medium. The blood cultures were incubated for 48 hours at ca. 37° C in humidified air containing ca. 5% CO_2 to ensure

2.6 High-throughput comet assay (performed by Carl von Ossietzky Universität Oldenburg)

The principle of the high-throughput comet assay is a 96 well plate (multichamber plate, MCP purchased from Intox, Oldenburg, FRG) suitable for electrophoresis. After exposure of the cells to genotoxic agents, the walls of the MCP are separated from the bottom plate. All 96 wells are subjected to lysis, alkaline unwinding, electrophoresis, neutralization, and staining simultaneously.

The comet assay in the high-throughput version was performed according Stang and Witte [23]. Either fibroblasts (3,000 cells/well) or HepG2 cells (4,000 cells/well) were seeded 2-4 h (fibroblasts) or 16-24 h (Hep G2) before treatment of the cells into the wells of the MCP. Subsequently, the cells were treated with the genotoxic chemicals or the water extracts for 1.5 hours. The nine test chemicals were freshly dissolved directly in serum free medium (sfm) at pH 7.2 at which concentration?. The lipophilic B(a)P and 2-AAF were dissolved in DMSO/Cremophor EL® (1/1) and diluted with sfm directly before cell treatment (maximum

final concentration of DMSO/Cremophor: 2.5 %). The water extracts were diluted in sfm 50-800.times directly before cell treatment, to obtain a 25 to 400-fold concentration of the water samples in the assay. The solvents alone did not provoke any geno- or cytotoxicity.

After treatment of the cells with the mutagens or water extracts, the walls surrounding the wells of the MCP were separated from the bottom plate. The plate with the cells was covered with a 37 °C warm solution of 0.5 % low-melting agarose (type Sea Plaque from Biozym Diagnostik, Hessisch Oldendorf, FRG). The plate was kept in the refrigerator for 5 minutes to solidify the low melting agarose, and thereafter covered with refrigerated lysing solution at pH 10.0 (2.5M NaCl, 100mM EDTA, 10mM Tris, 1% N-lauroyl sarcosine, 1% Triton X100, 10% DMSO; the last two compounds were added freshly) and kept at 4 °C for 1 hour. After lysis, the plate was placed on a horizontal electrophoresis box. The box was filled with freshly prepared alkaline buffer whereby the plate was completely covered with the buffer. The 40 minutes of the alkaline treatment at 4 °C allowed unwinding of the DNA and DNA breakage at alkali labile sites. After electrophoresis (300 mA, 25 V for 20 minutes), the plate was covered three times with neutralization buffer, washed with aqua bidest., and stained with ethidium bromide. Fluorescence microscopy was used to determine the median tail moment (tm) in each of the 4 replicates per concentration (25 comets/well) using Lucia Comet Assay Single Stain software with an upper detection limit of tm = 100. From these 4 values the mean and standard deviation was calculated. Statistical significance of the differences between the DNA migration induced by the mutagens and the untreated controls was determined by the non-parametric Mann-Whitney test ($p < 0.05$ or $p< 0.01$).

DNA damage induced by the crosslinkers cis and CP was measured indirectly. It was detected according to Pfuhler and Wolf [29] by previous treatment with MMS (2.1 mM for 1 hour) and subsequent addition of Cis or CP for 1.5 hours. The reduction of DNA strand breaks induced by MMS quantitatively reflects the DNA crosslinks induced by cisplatin.

For measurement of the cell viability, we used the FDA assay according to Rotman and Papermaster [30] during the comet assay, in the same cultures used for comet determination. The FDA assay measures the cell viability by the activity of cytosolic esterases converting fluorescein diacetate (FDA) to the fluorescent dye fluorescein. After chemical treatment the cells were centrifuged in the MCP (2 min at 400 x g) and incubated for 10 minutes with 72 µM FDA (from 12 mM stock solution in acetone, freshly diluted with sfm) at 37 °C in the dark. Thereafter, the cells were shortly centrifuged again, the dye was removed and the fluorescence of the enzymatically formed fluorescein was measured in a

fluorescence reader (FLUOstar, Offenbach, FRG) with an excitation of 485 nm and an absorption at 520 nm. Cell viability was calculated as the percent ratio of the fluorescence values of the samples to the referring control. After fluorescence measurement the walls and the bottom plate of the MCP were separated and the comet assay procedure was continued as described above.

2.7 Micronucleus test (performed by TNO Quality of Life)

For each culture, 5 mL of whole blood was incubated with PHA-L for 48 hours at ca. 37° C in humidified air containing ca. 5% CO_2. After this incubation period, the cells (which are then in the exponential stage of their growth) were exposed to test substances, in both the absence and the presence of S9-mix. Single cultures were used for each water extract for each exposure period as well as for the positive and negative (vehicle) controls.

For the test water samples' preparation, DMSO was used as vehicle. Water extracts were diluted 2-fold (1:1) using DMSO, to yield 10.000-fold concentrated stock solutions. The final concentration of DMSO of water extracts in the cultures was 1% (v/v) and corresponded to 100-fold concentrated extracts. Cells were exposed to water extracts for 4 hours in the presence of S9-mix, or exposed for 24 hours in the absence of S9-mix. In the absence of S9-mix, Mitomycin C (at 10 and 5 µg/mL), a known clastogen, and colchicines (at 5 and 2.5 µg/mL), a known aneugen, were used as positive controls. In the presence of S9-mix, Cyclophosphamide, a known clastogen requiring metabolic activation, was used as positive control (at 2 and 1 mg/mL).

Micronucleus test in the absence of the S9-mix (24 hour exposure): The cells were harvested by low speed centrifugation and resuspended in freshly prepared tissue culture medium with fetal calf serum (20%). The test substance was added to the cultures and the cells were exposed to the test substance for 24 hours (continuous treatment). After the 24 hours treatment period, the cells were washed with phosphate-buffered saline (pH 7.4) and subsequently supplied with culture medium (containing 20% serum). Thereafter, Cytochalasin B was added to each culture, to a final concentration of 6 µg/mL. The cultures were incubated for an additional 20 hours at ca. 37 °C in humidified air containing ca. 5% CO_2.

Micronucleus test in the presence of the S9-mix (4 hour exposure): The cells were harvested by low speed centrifugation and resuspended in freshly prepared tissue culture medium without fetal calf serum. Thereafter, the test substance was added to the cultures and each culture was supplemented with 0.5 ml S9-mix (see paragraph 2.3). The cells were exposed to the test substance for only 4 hours, because of the toxicity of the S9-mix for the cells. After the 4 hours treatment period, the cells were washed with phosphate-buffered saline (pH 7.4) and subsequently supplied with culture medium (containing 20% serum). Thereafter, Cytochalasin B was added to each culture to a final concentration of 6 µg/mL. The cultures were incubated for an additional 20 hours at ca. 37 °C in humidified air containing ca. 5% CO_2.

Harvesting and slide preparation: Each culture was harvested and processed separately. The cells were harvested by low speed centrifugation, treated with a hypotonic solution (0.075 M potassium chloride), fixed three times with a freshly prepared mixture of methanol and acetic acid, spread on clean slides and air dried. All procedures were performed at room temperature. Two slides were prepared from each culture of the test substance and from the negative and positive controls. Slides were stained with a fluorescent DNA-specific dye (acridin orange) and coded by a qualified person not involved in scoring of the slides to enable "blind" scoring. Per slide, 1000 binucleated cells (2000 binucleated cells per culture) were examined for the presence of micronuclei.

3. Results and Discussion

3.1 Testing of nine well known carcinogens

As the Ames II and high-through put comet assay are relatively new, especially in the application for water sample analysis, a simple validation of these assays was performed. Comparing the classical Ames test as well as the conventional comet assay with their high-throughput versions, similar results have been obtained before [22,23]. Eight other well known standard mutagens and one non-genotoxic carcinogen (estradiol) were tested in the high-throughput comet assay and in the Ames II test with the same exposure time. Five of the eight genotoxins are on the recommended list of Kirkland *et al.* [31] for compounds for performance assessment of new tests. The results are shown in Fig. 1 for directly acting mutagens and in Fig. 2 for indirectly acting mutagens that need metabolic activation.

Indirectly acting mutagens were tested in the presence of S9 fraction, either in the Ames II test or with fibroblasts in the comet assay. In addition, the metabolic competent cell line Hep G2 was used in the comet assay.

In comparison to TA98, the TAMix was more effective in detecting mutations induced by the directly acting mutagens ENU, MMS and 4-NQO (Fig. 1a-1c), whereby the alkylating MMS and ENU even failed to show any mutagenicity in TA98 (Fig. 1a and 1b). This is also in accordance with Brams *et al.* [32] and Eder *et al.* [33], where MMS was negative in TA98, while positive in TA100. Alkylating substances such as MMS and ENU are also known to create mainly base-pair substitutions [34], which are detected by the TAMix or TA100. While in the comet assay DNA damage could be detected for all four directly acting chemicals, in the Ames II test the crosslinker Cis was not mutagenic in either TA98 or TAMix (Fig. 1d) in contrast to expectation. Also the indirectly acting crosslinker CP gave unexpected negative results in the Ames II test at the concentrations tested (Fig. 2d). While for the crosslinkers Cis and CP mostly positive results were described for the strains TA98 and TA100 in literature at similar concentrations [20,35-37], also negative results have been reported [20,38].

In the comet assay, both in fibroblasts with S9 fraction and in HepG2 cells DNA crosslinks were measured for CP. The DNA damaging properties of PCP were also detected in fibroblasts + S9 and in Hep G2 but no positive response was found in the Ames II test (Fig. 2 b). The carcinogenic PCP [39] is transformed to the highly genotoxic metabolite tetrachlorohydroquinone by S9 fraction enzymes [40]. The detection of the mutagenic potential of PCP (with S9 fraction) in the Ames test was described by Gopalaswamy and Nair [41], whereas Gichner *et al*. [42] as well as Moriya *et al*. [43] obtained negative results using TA98. B(a)P was more effective in the Ames test than in the comet assay (Fig. 2a) and 2-AAF only tested positive in the Ames II test (Fig. 2c). Thereby the strain TA98 was more sensitive than TAMix, indicating these bulky adduct–forming compounds form mainly frameshift mutations. 2-AAF was the only substance where no DNA damage was detected in the comet assay after a 90 min treatment. It is known that for 2-AAF longer incubation times of one day [44,45] or three days [46] are needed to detect genotoxicity in the comet assay. Estradiol was negative in both the Ames II test and high-throughput comet test, as expected (table 2), but appears to give positive results in the micronucleus test, due to aneugenic action (e.g. [47,48]. Estradiol therefore seems to be a genotoxic carcinogen after all. Despite some unexpected results, which also have been reported before,

however, we find the predictivity of the Ames II and high-throughput comet assay satisfactory.

It is impossible to decide which cell line (metabolic competent HepG2 cells or with fibroblasts in the presence of S9 fraction) may be the more effective one to detect indirect genotoxic compounds in the comet assay. Only three of our compounds were tested and detected in under these conditions, showing different trends. PCP was more effective in HepG2 cells by the factor 8, while the detection limit of CP was 4 times lower in fibroblasts with S9 fraction. This may be result of the difference between the metabolic system in both cultures, with one being an isolated mix of rat enzymes and the other an innate human enzyme system of the cell-line.

A comparison of the sensitivities of the Ames II test and the high-throughput comet assay in detecting the lowest effective concentrations of the mutagens is given in Table 2, as well as literature data on the sensitivity of the micronucleus test. The Ames II test was more sensitive than the comet or micronucleus test for three of the eight genotoxic compounds (MMS, ENU, 4-NQO). However, the Ames test did not pick up all compounds (CP, Cis, PCP) which induced comet or micronucleus formation. Thus, this gene mutation test and the tests for chromosomal damage complemented one another, as expected when testing genotoxic compounds with different mechanisms of action and applying tests with different genotoxicity endpoints.

3.2 Testing of water extracts

The Ames II, the high-throughput comet assay, and the micronucleus test were applied to nine extracts of water samples for genotoxic contaminants. In the Ames II (Fig. 3a), only the STP effluent showed genotoxicity, for all other samples the response did not differ significantly from that of the negative control. This is in concordance with past results of classic Ames tests on the Dutch Rhine and Meuse rivers, where over the years a decline in genotoxic activity was seen until very low, sometimes insignificant responses were obtained [49]. The STP effluent was positive in TA98 + S9, not in the other conditions. Filipic and Toman [50] found a mutagenic response in a municipal STP effluent in both TA98 and TA100 (+/- S9).

In the comet assay, three of the nine water samples tested positive in a concentration dependent way (Fig. 4 a-c and 3c). The DNA damage in these samples was low, but still statistically significant. This was also due to the small values of the untreated control with

low standard deviations of four parallel samples in the high-throughput version of the comet assay. Even when the cytotoxicity was high enough to be responsible for the genotoxic effects at the highest concentration tested [51], at lower, non-cytotoxic concentrations significant genotoxicity was also detected (Fig. 3c and 4a). In addition to the STP effluent, also surface water 3 and groundwater 4 tested positive. In these three samples metabolic activation reduced the genotoxicity, even though a significant DNA damage remained either in fibroblasts with S9 fraction or in HepG2 cells. Interestingly, in the Ames II test the metabolically activated STP sample was mutagenic while in the comet assay DNA damage was more pronounced in the absence of S9 fraction. In contrast, Zegura *et al*. [52] found genotoxicity in an effluent from a municipal STP with the SOS/*umuC* test (applying the same bacteria as in the Ames test) in absence of S9, and not with the comet assay in metabolically active HepG2 cells. Of course, STP effluents can differ in chemical load between locations and especially between countries.

As these tests detect different types of DNA damage, different compounds could be responsible for the different observed effects in these two assays. Another difference between these two assays is the occurrence of damage repair: in contrast to the Ames II test, in the comet assay the cells do not complete a cell division cycle including DNA damage repair during the incubation time of 90 min. Therefore the damage remains detectable by the comet assay, while it may be repaired in the Ames II test, where the bacteria fulfil around 2 cell divisions in 90 min.

Dutch waters have only sparingly been analyzed with chromosomal damage tests, such as the comet assay, before. Alink *et al*. [53] found a significantly increased response in the comet assay and sister chromatid exchange (SCE) test in fish gill cells, after exposing the fish for 11 days in Rhine water in 2005. Applying much shorter exposure times, we have also found an increased comet assay response in one surface water, also for the first time in a Dutch groundwater and STP effluent. These results show that further, more extensive analysis of Dutch waters, with inclusion of a chromosomal damage test, are recommended for safeguarding the quality of Dutch drinking water.

In the micronucleus test none of the water samples tested positive (Fig. 3b).This may also be the result of a prolonged incubation time of 4 hours + 20 hours recovery time, allowing complete repair of DNA damages in the micronucleus test (necessity to pass through a cell cycle). As consequence, a lower sensitivity in comparison to the comet assay was observed. As the water samples represent different water types that are relevant to monitor

(STP effluent, surface water and groundwater) and represent worst cases (i.e most polluted) of these water types, the results are considered representative for all Dutch water samples. Also in other studies, the comet assay proved to be the better tool to detect low levels of genotoxic contaminants. This was shown for lake drinking water [9], river samples [14,54], water soil leachates [15], river sediments [55], chlorinated drinking water samples [9] and other aquatic environments [56]. Valentin-Severin *et al*. [44], however, found that the micronucleus test was more sensitive than the comet assay for a limited set of four of the five tested compounds in HepG2 cells.

It may be argued that the inclusion of the DNA repair step in the micronucleus test is an advantage, as this mimics real life. However, presence of DNA damaging compounds, causing DNA strand breaks as detected by the Comet assay, is not desirable for drinking water. For hazard identification it is important to use sensitive assays, and cover all genotoxic endpoints, therefore the comet assay and Ames II test are selected. The argument that the micronucleus test can also detect aneugens, while the comet can not, is of minor weight, as aneugens are rare [57]. Thus, high sensitivity is given priority and the comet is therefore preferred.

The Ames (II) test and the comet assay have been successfully combined for biomonitoring of environmental samples before (e.g. [55,58-60]). The Ames II is shown to be suitable for water analysis. Further, the first analysis of water extracts with the high-throughput comet assay has shown no practical problems and sensitivity for aqueous genotoxic substances was demonstrated. Therefore, we conclude that the high-throughput version of the comet assay is also suitable for water analysis.

Of course, the outcome of these *in vitro* tests do not provide a determination of a health risk for consumers, as the bacteria of the Ames II test are very different from human cells, and no information on absorption, distribution, metabolism and excretion in a human being is included. Therefore, test results should be regarded as signalling values, with negative results indicating no health hazard is expected, and positive results indicating there are genotoxic compounds in the water, and further research is necessary to determine the risks these compounds pose.

In summary, the Ames II test and the high-throughput comet assay are two sensitive assays which complement one another because of their different sensitivities toward different classes of mutagens. This sensitive complementary working of the two test systems combined with easy, quick and economic handling should be a favourite

combination for screening environmental probes with low levels of contaminants. It is recommended that Dutch drinking water and its sources are screened with this combination of tests.

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Abbreviations

2-AA	aminoanthracene
2-AAF	2-acetyl aminofluorene
B(a)P	benzo(a)pyrene
Cis	cisplatin
CP	cyclophosphamid
D-MEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
E2	estradiol
ENU	ethylnitroso urea
EU	European Union
FDA	fluorescein diacetate
MCP	multichamber plate
MMS	methyl methanesulfonate
4-NQO	4-nitroquinoline-1-oxide
PCP	pentachlorophenol
sfm	serum free medium
STP	sewage treatment plant
TIE	Toxicity Identification and Evaluation
tm	tail moment

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Figures and Tables

Table 1. Positive controls for the different strains and S9-conditions in the Ames II test

Strain and S9-condition	Positive control (in DMSO)
TA98 –S9	10 µg/mL 4-NQO
TA98 +S9	5 µg/mL 2-AA
TAMix –S9	5 µg/mL 4-NQO
TAMix +S9	100 µg/mL 2-AA

Table 2: detection limit of the genotoxicity/mutagenicity of 9 chemicals as tested in the Ames II test and the comet assay and as found in literature for the micronucleus test; nd: not determined; nf: not found; bold type: lowest detection limit of all test systems

Detection limit (µM)									
Substance	**Ames II**				**96-well Comet**			**Micronucleus**	
	TA98		**TAmix**		**Fibroblasts**		**HepG2**	**Variable celltypes**	
	0	**0**	**0**	**0**	**0**	**0**		**0**	**0**
4-NQO	0.21	< 11	**0.053**	<5.3	0.13	nd	nd	0.12-0.62[1,11]	55.611
MMS	>18,000	nd	**35**	nd	200	nd	nd	49.4-182[1,2,3,8,11]	14811
ENU	> 85,000	nd	**171**	nd	500	nd	nd	nf	nf
Cis	> 500	>500	>500	>500	**16**	nd	nd	nf	nf
CP	nd	>7,200	nd	>7,200	nd	125	500	100->380[6,9,11]	**11.3-200[7,8,11]**
PCP	> 19,000	> 19,000	> 19,000	> 19,000	nd	1000	**125**	nf	nf
BaP	nd	< 0,79	nd	0.79	nd	50	75	**0.26-91[1,4,5]**	12.5-200[7,8,10,11]
2-AAF	nd	02. Feb	nd	0.0088	nd	>600	cytotox	**0.1-333[1,6,9,11]**	14010
estradiol	>18	>18	>18	>18	nd	>9.2	>9.2	0.001 -29[12,13]	nf

1. Hep-G2; 4h; [44]. 2. L5178Y mouse lymphoma; 4 h; [61]. 3. L5178Y mouse lymphoma; 4 h; [62]. 4. HepG2 and Hep3B; 24 h; [63]. 5. V79-MZ; 24 h; [64]. 6. rat hepatocytes; variable exposure time; [65]. 7. human lymphocytes; 90 min; [66]. 8. CHL: 6 h; [67]. 9. rat hepatocytes; 48 h; [68]. 10. L5178Y mouse lymphoma; 4h; [25]. 11. CHO-K1; 3 h; [69]. 12. MCL-5 lymphoblast and WILL3 human fibroblasts; 24 h; [47]. 13. MCF-7 human breast cancer cells; 24 h; [48].

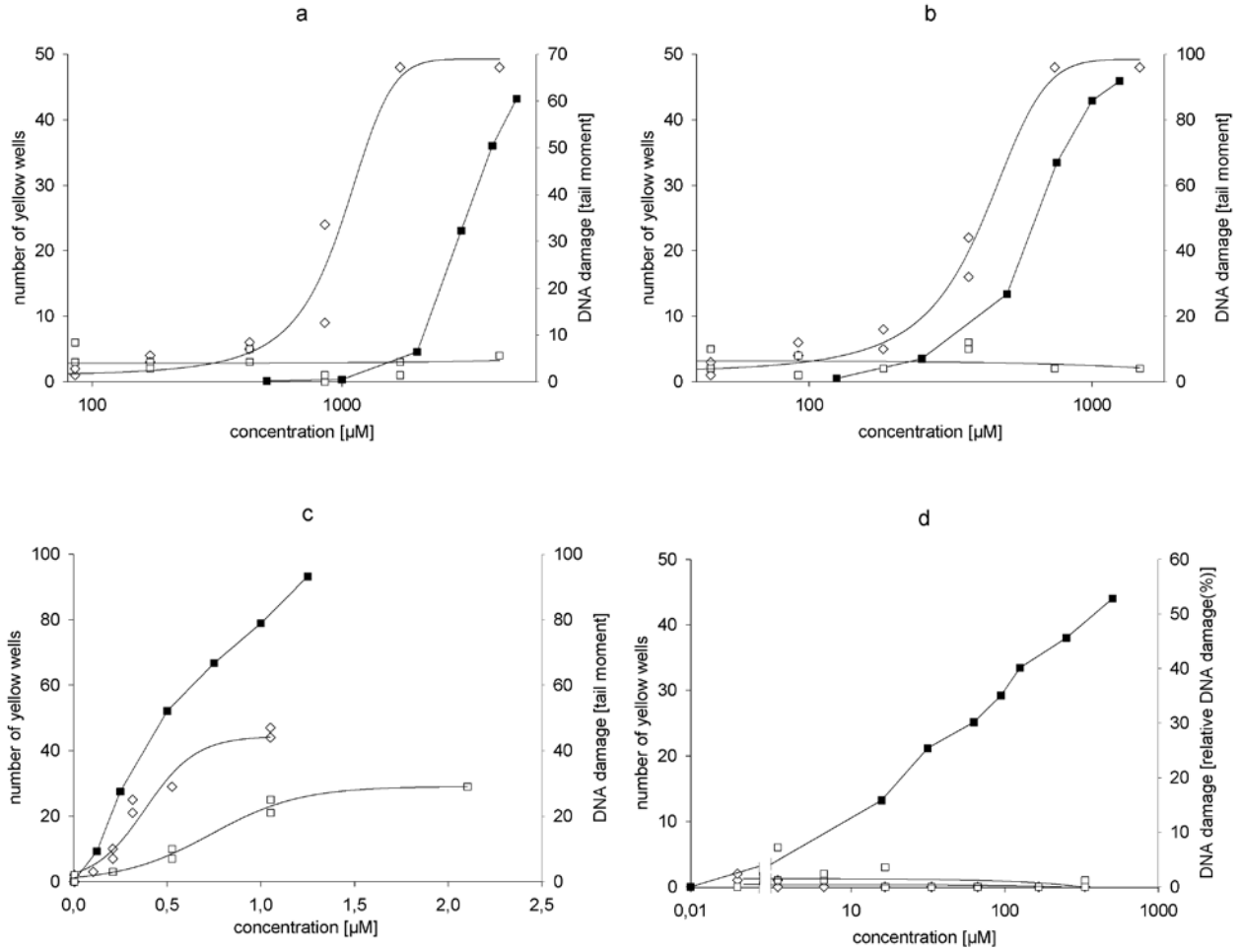


Fig. 1: concentration dependent DNA damage/mutagenicity of directly acting chemicals (a) ENU, (b) MMS, (c) 4-NQO and (d) Cis in the Ames II (□TA98, ◊ TAMix; n = 3) and the comet assay (■human fibroblasts , n = 4). The standard deviation of 4 parallel samples in the comet assay was about 10 %.

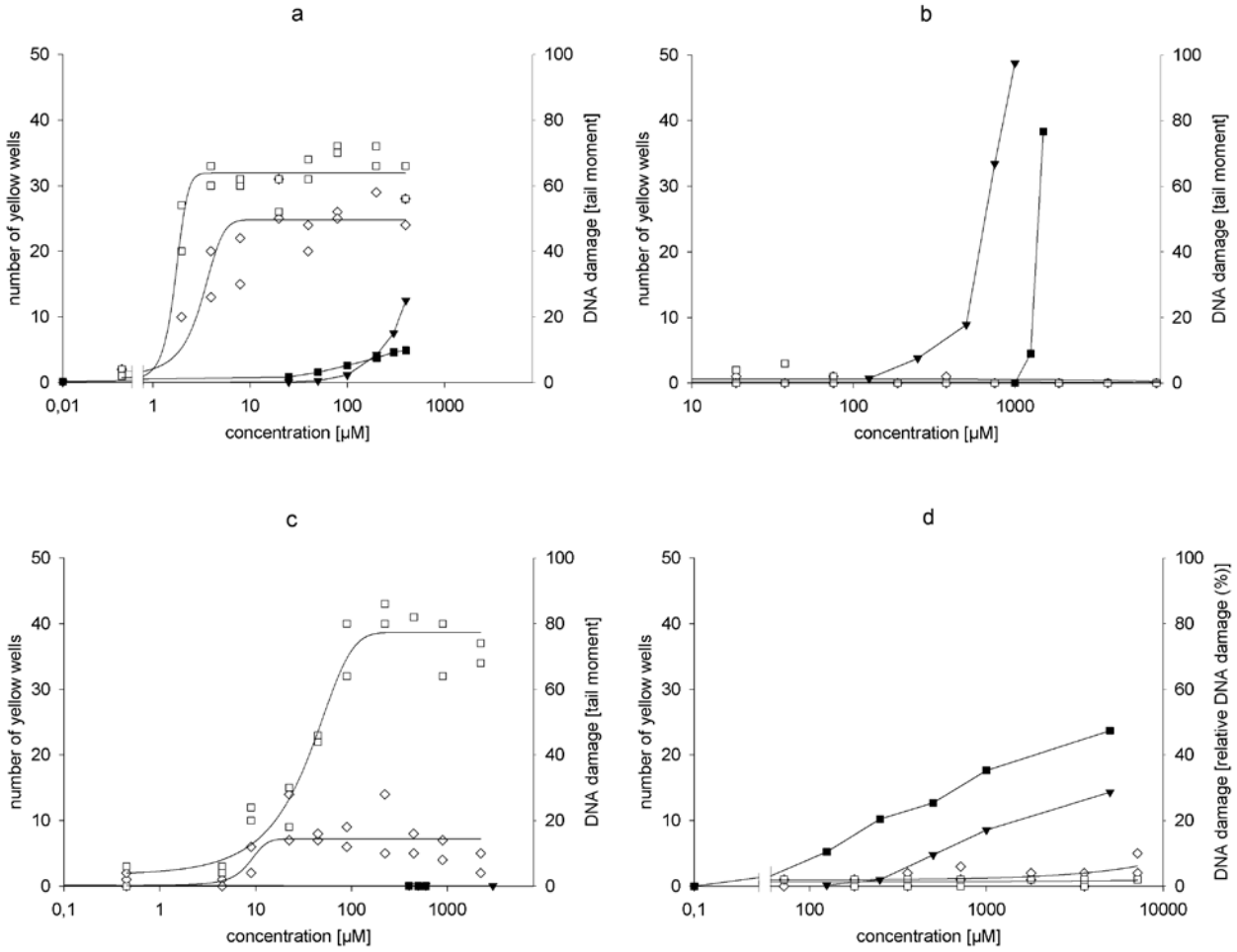
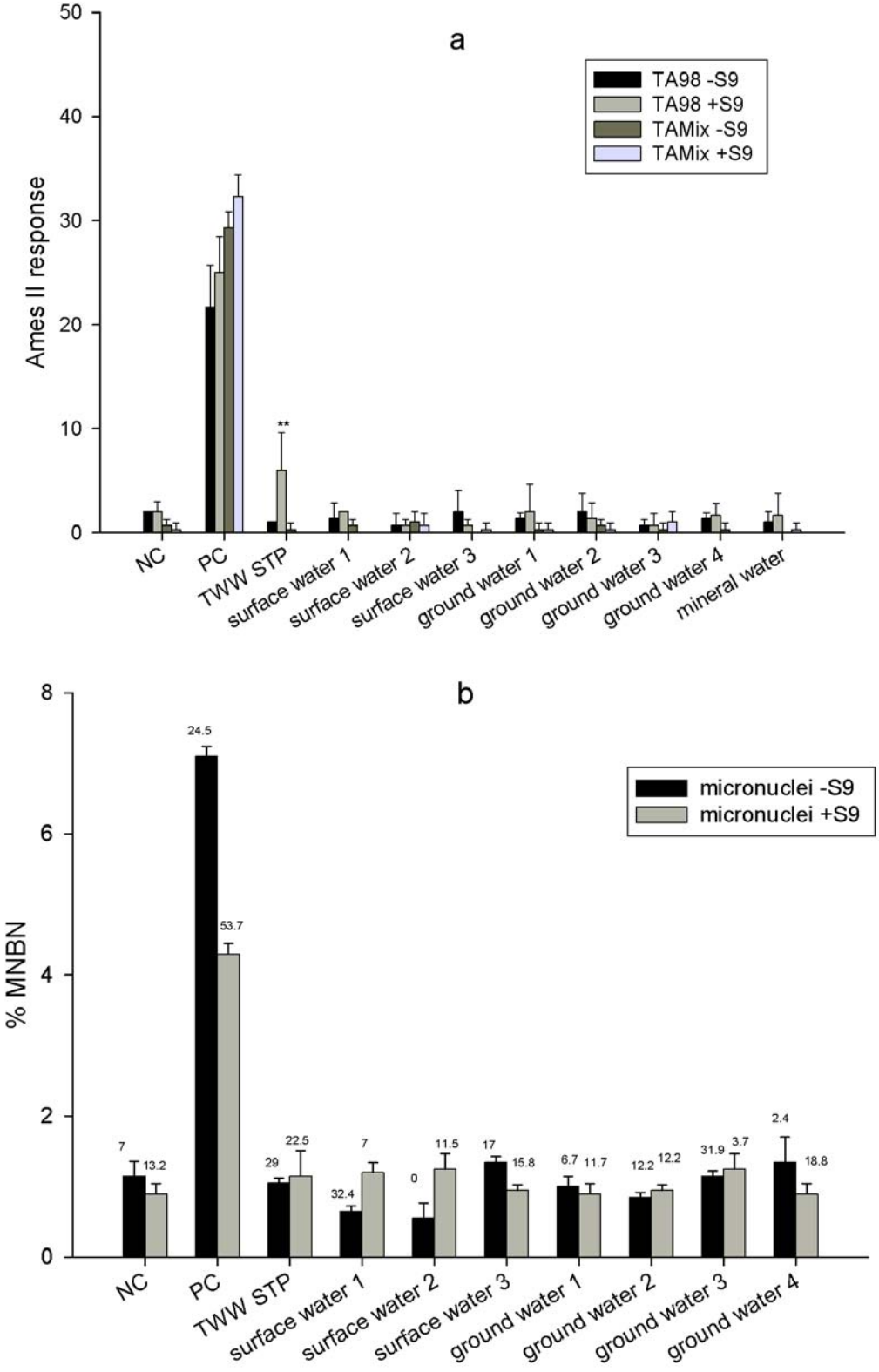


Fig. 2: concentration dependent DNA damage/mutagenicity of indirectly acting chemicals (a)B(a)P, (b)PCP, (c)2-AAF and (d) CP in the Ames II with S9 fraction (□TA98, ◊ TAMix; n= 3) and the comet assay (■human fibroblasts with S9 fraction, ▼Hep G2 cells; n = 4). The standard deviation of 4 parallel samples in the comet assay was about 10%.


a
TA98 -S9
TA98 +S9
TAMix -S9
TAMix +S9
Ames II response
50
40
30
20
10
0
**
NC
PC
TWW STP
surface water 1
surface water 2
surface water 3
ground water 1
ground water 2
ground water 3
ground water 4
mineral water
b
micronuclei -S9
micronuclei +S9
% MNBN
8
6
4
2
0
24.5
53.7
7
13.2
29
22.5
32.4
7
0
11.5
17
15.8
6.7
11.7
12.2
12.2
31.9
3.7
2.4
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TWW STP
surface water 1
surface water 2
surface water 3
ground water 1
ground water 2
ground water 3
ground water 4

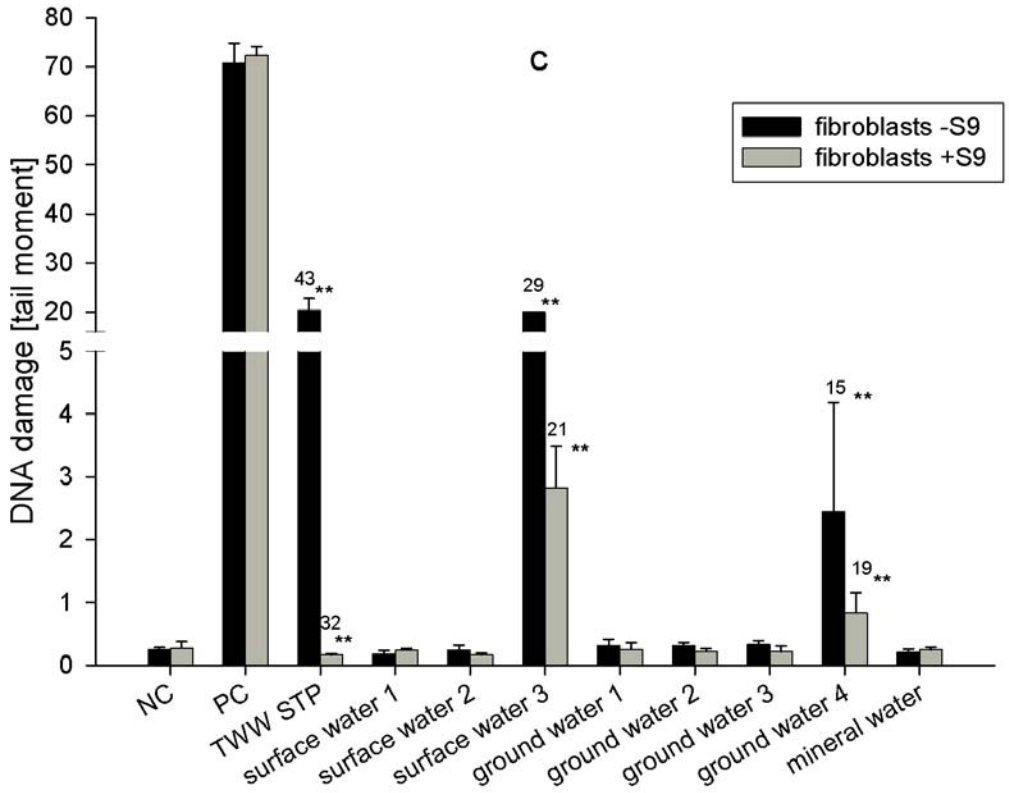

Fig. 3: mutagenicity/genotoxicity of environmental samples measured (a) in the Ames II test (200-fold concentrated samples), (b) in the micronucleus test (100-fold concentrated samples) and (c) in the comet test (400-fold concentrated samples); numbers above the bars: % cytotoxicity in the micronucleus test and comet assay; in the Ames II test the cytotoxicity did not exceed 8%; **significance level compared to the untreated control $p \leq 0.01$

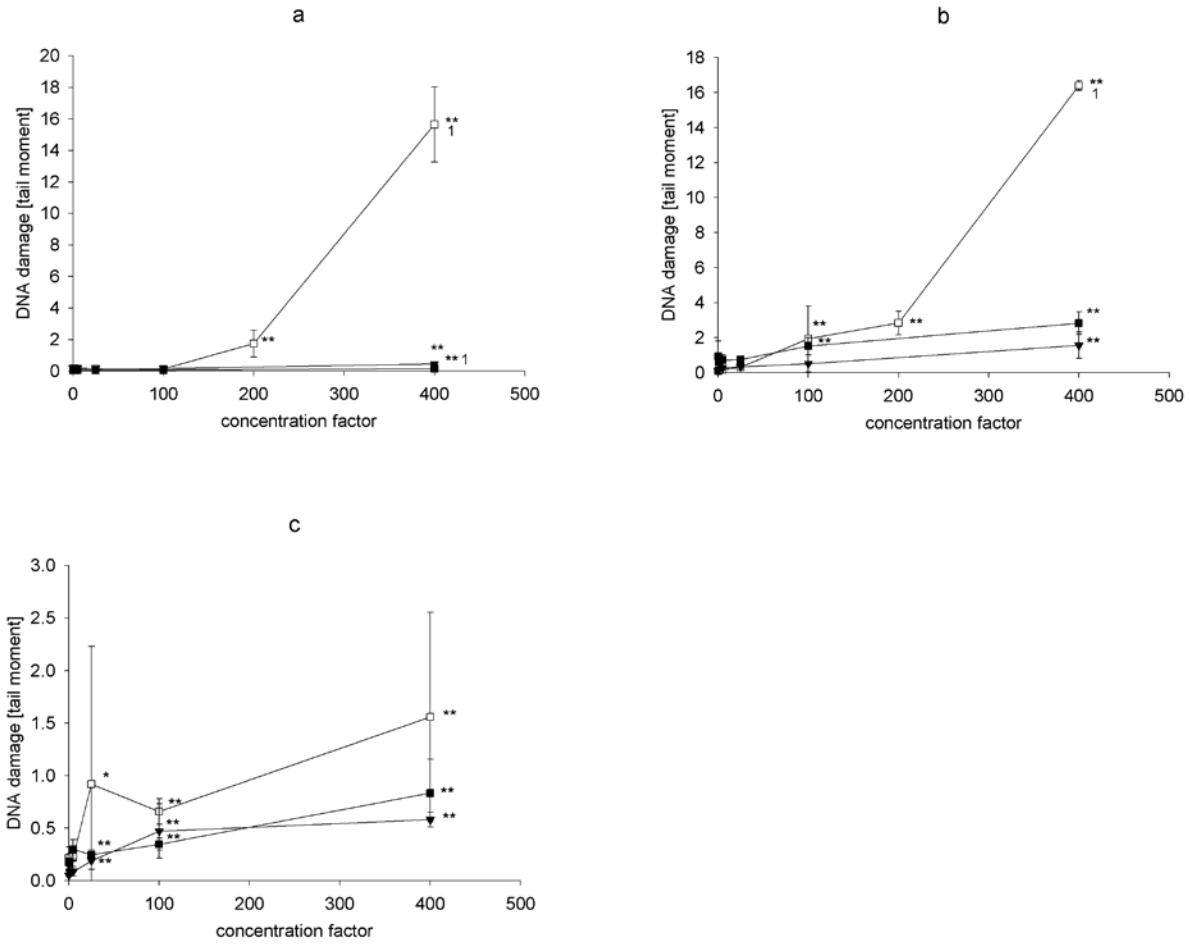

Fig. 4: Concentration dependent DNA damage induced by environmental samples determined with the comet assay (a) TWW STP, (b) surface water 3 and (c) groundwater 4 (□human fibroblast -S9, ■human fibroblast +S9, ▼Hep G2 cells); significance level compared to the control: ** p≤0.01, * p≤0.05; 1: cytotoxicity was ≥30 %

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Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbständig verfasst, keine anderen als die angegebenen Hilfsmittel und Quellen benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Ferner versichere ich, dass die vorliegende Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat

Oldenburg, den 08.07.09

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Curiculum vitea

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Publikationen

2008	Lam C, Stang A, Harder T: *Planktonic epiphytic and fungi are selectively eliminated by marine macroalgae* FEMS microbiology ecology. Vol. 63, 283-291