Untersuchungen zum toxikologischen Potential Uranabbau-assoziierter Verbindungen und kontaminierter Gewässerproben aus dem Ronneburger Wismut-Gebiet in humanen Kolonzellen

DISSERTATION

zur Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller-Universität Jena

von

Dipl. Ernährungswissenschaftlerin

Yvonne Knöbel

geboren am 02. Dezember 1977 in Gera

Gutachter:

1. Gutachter: Prof. Dr. Beatrice L. Pool-Zobel (FSU Jena)

2. Gutachter: Prof. Dr. Hans Bergmann (FSU Jena)

3. Gutachter: Prof. Dr. Andrea Hartwig (FSU Jena)

INHALTSVERZEICHNIS

ABBILDUNGSVERZEICHNIS

ABKÜRZUNGSVERZEICHNIS

AEO2	Apfelextrakt O2
APC	Adenomatous Polyposis Coli
ATSDR	Agency for Toxic Substances and Disease Registry
BER	Basen-Exzisions-Reparatur
BPDE	Benzo[a]pyrendiolepoxid
Cum-OOH	Cumenhydroperoxid
DAPI	4´,6 Diamiono-2-phenylindoldihydrochlorid
DCC	Deleted in Colon Cancer
dd H_2O	doppelt destilliertes Wasser
2dDCB	2-Dodecylcyclobutanon
DMEM	Dulbeco´s Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
DNA	deoxyribonucleic acid, die englische und international gebräuchliche Abkürzung für Desoxyribonukleinsäure (DNS)
EC_{50}	Effecticve Concentration 50%
EPIC	European Prospective Investigation into Cancer and Nutrition
EtOH	Ethanol
FAP	Familiäre Adenomatöse Polyposis
FCS	Foetal Calf Serum
Fe	Eisen
Fe-NTA	Eisen-Nitrilotriazetat
FISH	Fluoreszenz *in situ* Hybridisierung
GAP	GTPase aktivierendes Protein
γ-GCS	gamma glutamyl cysteine synthetase
GSH	reduziertes Glutathion
GST	Glutathion-S-Transferase
GSTT2	Glutathion-S-Transferase Theta 2
GSTP1	Glutathion-S-Transferase Pi 1
GTP	Guanosintriphosphat
H_2O_2	Wasserstoffperoxid
HNPCC	hereditary non-polyposis colorectal cancer

HNE	4-Hydroxy-2-nonenal
HT29	humane Kolonkarzinomzelllinie
HT29 clone 19	aus HT29 rückdifferenzierte Kolonkarzinomzelllinie
kb	Kilobasen
kDa	Kilodalton
KG	Körpergewicht
K-RAS	Kirsten Rat Sarcoma
LT97	humane Kolonadenomzelllinie
MCC	Mutated in Colon Cancer
MCDB	Zellkulturmedium
M	Metalle
NER	Nukleotid-Exzisions-Reparatur
PZ	Primäre Kolonepithelzellen (Primärzellen)
ROS	reaktive Sauerstoffspezies
RPMI	Zellkulturmedium
RT	Raumtemperatur
SD	Standardabweichung
SDAG Wismut	Sowjetisch-Deutsche Aktiengesellschaft Wismut
SMAD2/4	mothers against decapentaplegic homolog 2/4
TDI	tolerable daily intake (tolerierbare tägliche Aufnahme)
TI	Tailintensität
TP53	Tumorprotein 53
U	Uran
UNSCEAR	United Nations Scientific Committee on the effects of Atomic Radiation
U-NTA	Uranyl-Nitrilotriazetat
WCRF	World Cancer Research Fund

1 EINLEITUNG

1.1 Wirkungspotentiale Uranbergbau

Durch die ehemaligen Bergbaugebiete der SDAG-Wismut hat sich für die dortige Biosphäre eine veränderte Umweltsituation ergeben, die es erforderlich macht, Wirkungspotentiale komplexer Umweltproben zu erforschen. Bisherige Untersuchungen bezogen sich vor allem auf die Kanzerogen-Exposition von Minenarbeitern [HVBG, 1997]. Großflächige Untersuchungen zu Stoffflüssen und der Exposition der umliegenden Bevölkerung liegen nur sehr lückenhaft vor. Dies erscheint aber notwendig, da z. B. Au et al. in einem Umkreis von 0,5-1 Meile um eine Mine in den Vereinigten Staaten von Amerika erhöhte Uranbelastungen nachweisen konnten [Au *et al.*, 1998].

Die SDAG-Wismut betrieb eines der größten Uranbergbaugebiete der Welt. Das Unternehmen produzierte in den 45 Jahren seiner Existenz bis 1990 ca. 231000 Tonnen Uran. Es ist deshalb davon auszugehen, dass auch hier erhöhte Belastungen vorliegen und dass die mit dem Uranabbau verbundenen Stoffe auf die Umwelt und somit auf die Organismen einwirken. Die komplexen Expositionsgemische mit unterschiedlichen Stoffen in variierenden Konzentrationen bergen unterschiedliche Wirkungspotentiale. Uranerze wirken aufgrund ihrer radioaktiven Eigenschaften und durch die Freisetzung von Radongas genotoxisch [Jostes, 1996]. Bei den gesundheitlichen Risiken durch die chemische Toxizität steht die Schadwirkung auf die Nieren im Vordergrund [Meinrath *et al.*, 2003]. Neben dem Uran vorkommende Schwermetalle wie Cadmium, Arsen, Nickel und Eisen sind ebenfalls genotoxisch und zum Teil auch karzinogen [IARC, 1994].

Im Rahmen eines DFG-Projektes der Friedrich-Schiller-Universität Jena wurden im Ronneburger Wismutgebiet im Jahr 2001 zu Beginn der Sanierung des Areals um die Gessenhalde, Wasserproben gezogen (Abbildung 1) und am Institut für Geowissenschaften hinsichtlich ihrer Gehalte an Mengen- und Spurenelementen sowie Radionukliden charakterisiert (siehe Tabelle 1 **Publikationsmanuskript IV**). Drei der Proben wurden nach Kontaminationsgraden (wenig belastet, mittelstark belastet, stark belastet) für toxikologische Untersuchungen im Rahmen dieser Arbeit ausgewählt. Dabei handelt es sich bei Probe A um die wenig belastete, bei Probe B

um die mittelstark belastete und bei Probe C um die stark kontaminierte Gewässerprobe.

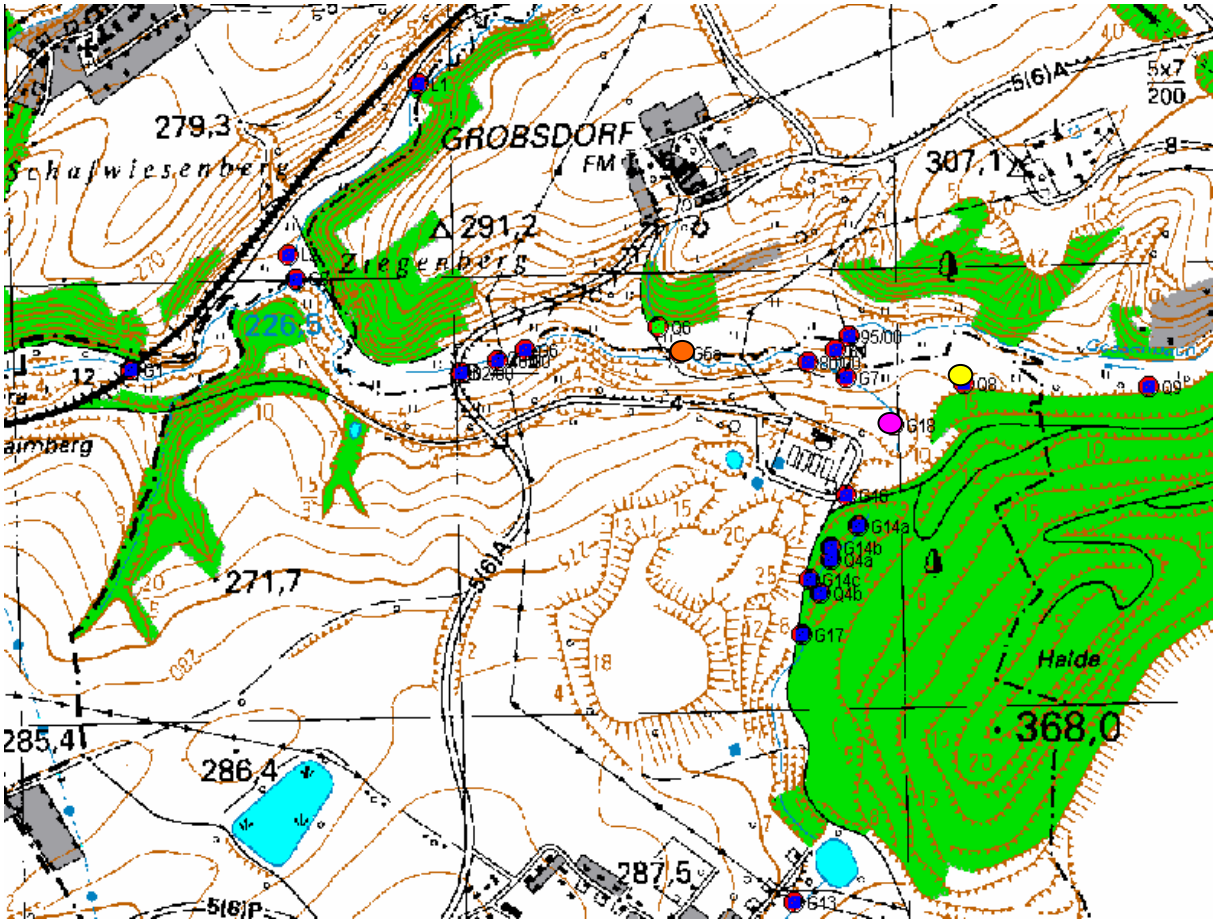


Abbildung 1: Probenentnahmestellen im Einzugsgebiet des Gessenbaches im ehemaligen Ronneburger Wismutgebiet im Jahre 2001, zu Beginn der Sanierung. Die Probenentnahmestellen für die toxikologischen Untersuchungen sind in der Abbildung wie folgt gekennzeichnet: orange = G6 = Probe A, pink = G18 = Probe B, gelb = Q8 = Probe C.

1.2 Uran

1.2.1 Eintrag in die Nahrungskette

Normalerweise besteht die Gefahr einer Urankontamination der Umwelt weniger in der Nachbarschaft von Kernreaktoren, sondern hauptsächlich in Uranbergbaugebieten und in der Umgebung von Uranaufbereitungs- und Anreicherungsanlagen [Wiechen, 1970]. Darüber hinaus kann neben dem Eintrag von Uran in die Nahrungskette durch die Verarbeitung und Anwendung von Phosphaten [Bouwer *et al.*, 1978; Izak-Biran *et al.*, 1989; Martinez-Aguirre *et al.*,

1994] eine Uranemissionen auch über die Flugasche von Kohlekraftwerken erfolgen [Jaworowski & Grzybowska, 1996].

Mikroorganismen und Pflanzen können sich den toxischen Bedingungen in und auf kontaminierten Böden über verschiedene Wege anpassen: (i) dem spezifischen Ausschluss der Schadstoffe, (ii) ihrer Verlagerung in Zellkompartimente und akkumulierenden Geweben, (iii) durch biotische Assoziation und insbesondere durch mykorrhizale Symbiosen, wobei einer der Partner zu (i) oder (ii) befähigt ist [Leyval *et al.*, 1997]. Eine wesentliche Rolle hinsichtlich der Mobilisierung und Immobilisierung von Radionukliden und Schwermetallen beim Transfer in die Nahrungskette spielt das System Mykorrhiza/Pflanze. Bei Überschreitung eines bestimmten Schwellenwertes bei der Aufnahme von Schwermetallen und Radionukliden in die Pflanze werden in dieser Veränderungen des Stoffwechsels induziert, die darauf ausgerichtet sind, die negativen Auswirkungen der Stressoren zu kompensieren [Bergmann *et al.*, 1996; Bergmann *et al.*, 1999; Bergmann *et al.*, 2001; Leinhos & Bergmann, 1995; Machelett *et al.*, 1996].

In Regionen mit Uranbergbau liegt aufgrund der Urankontamination der Grund- und Oberflächenwässer oftmals eine erhöhte Uranbelastung vor, die auch in den Pflanzen nachweisbar ist [Rumble & Bjugstad, 1986; Seeber, 1998; Tracy *et al.*, 1983]. So konnten zum Beispiel im Uranbergbaugebiet um Ronneburg zwei- bis achtfach höhere Urangehalte in verschiedenen Nutz- und Futterpflanzen im Vergleich zu einem Kontrollgebiet gemessen werden [Seeber, 1998]. Seeber kalkulierte ausgehend von den analysierten Urangehalten in Lebensmitteln und denen von Thiel et al. für die neuen Bundesländer gewonnenen Verzehrsdaten die Uranaufnahme Erwachsener [Thiel *et al.*, 1996]. Danach nahmen Frauen in den neuen Bundesländern 2,05 µg Uran pro Tag und Männer 2,40 µg Uran pro Tag über verschiedene Lebensmittel zu sich. Fisenne *et al*. ermittelten für die New Yorker Bevölkerung eine mittlere Uranaufnahme von 1,30 µg/d [Fisenne *et al.*, 1987]. Nach Dang und Pullat galt für die indische Landbevölkerung ein Wert von 2,2 µg/d [Dang & Pullat, 1993]. In Japan lag die durchschnittliche Uranaufnahme mit 1,25 µg/d unterhalb der von Seeber ermittelten Werte [Shiraishi & Yamamoto, 1995]. In der Studie von Shiraishi und Yamamoto wurde jedoch die Aufnahme über Trinkwasser und Getränke nicht berücksichtigt. Geht man davon aus, dass ein Erwachsener (70 kg KG) ca. 1,5 Liter Trinkwasser pro Tag konsumiert, spiegelt der von Shiraishi und Yamamoto ermittelte Wert nicht die reale Uranaufnahme wider, denn ca. 20-30 %

der täglichen Uranaufnahme erfolgt über Trinkwasser [Weir, 2004]. Laut Agency for Toxic Substances and Disease Registry (ATSDR) beträgt die tägliche Uranaufnahme ca. 1,5 µg über Wasser und 1-2 µg über Nahrungsmittel [ATSDR), 1999]. Hauptquellen für die orale Aufnahme natürlichen Urans sind damit Wasser und Nahrungsmittel [Fisenne *et al.*, 1987; UNSCEAR, 2000]. Als tolerierbare tägliche Aufnahme (TDI) wurde von der ATSDR ein Wert von 1 µg/kg KG und d festgesetzt [ATSDR, 1999]. Kalkulationen von Jacob et al. (0,7 µg/kg KG und d) und der WHO (0,6 µg/kg KG und d) liegen noch unter den von der ATSDR empfohlenen Werten [Jacob *et al.*, 1997; WHO, 1998].

In neueren Studien zur Uranaufnahme zeigte sich in Vietnam keine erhöhte Exposition. Giang et al. ermittelten eine mittlere Uranaufnahme von 0,66 µg/d (Vgl. Seeber 2,1 µg/d) [Giang *et al.*, 2001]. Über die toxikologischen Folgen einer oralen Uran-Exposition ist bisher wenig bekannt. Im Gegensatz dazu wurde für die Inhalation von Uran eine positive Assoziation zum Lungenkrebsrisiko postuliert [Kathren, 2001].

Die ehemalige DDR gehörte neben den Vereinigten Staaten von Amerika und Kanada zu den größten Uranproduzenten. Die Strahlenexposition in den Abbaugebieten bedeutete für die Bevölkerung und vor allem für die Selbstversorger im Gebiet um Ronneburg ein erhöhtes Risiko. Die radioaktive Belastung über kontaminierte Lebensmittel betrug 0,36 ms, zusätzlich zur effektiven Äquivalenzdosis von 4,25 ms über die Inhalation von Radon und dessen Zerfallsprodukten. Damit wurden die erlaubten Maximalwerte überschritten [Küppers & Schmidt, 1994].

Die hohe Arbeitsplatzexposition der Minenarbeiter war mit einer Verminderung der Lebenszeit, einer erhöhten Lungenkrebsinzidenz und chromosomalen Aberrationen in peripheren Lymphozyten verbunden. In Untersuchungen von 225 Minenarbeitern ergab sich eine signifikante Korrelation zwischen Chromatidbrüchen und aberranten Zellen und der Radonexposition [Smerhovsky *et al.*, 2002]. Es ist bisher jedoch nicht geklärt, inwieweit andere, gemeinsam mit Uran vorkommende Metalle wie Cadmium, Eisen oder Arsen zu den oben genannten Gesundheitsrisiken beitragen.

1.2.2 Toxikologie Uran

Viele der mit dem Uranbergbau verbundenen Kontaminanten sind toxisch. Verschiedene Mechanismen spielen dabei eine Rolle, z. B. die Bildung reaktiver Sauerstoffspezies (ROS) [Hei *et al.*, 1998], Strahlung (Radongas) [Jostes, 1996] oder eine direkte Interaktionen mit Makromolekülen (Hemmung der DNA-Reparatur) [Hartmann & Speit, 1996; Hartwig *et al.*, 1996; Hartwig, 1998; Hartwig & Beyersmann, 1989a].

Für natürliches Uran spielt die Radioaktivität aufgrund der langen Halbwertszeit nur eine untergeordnete Rolle. Das toxische Wirkungspotential entfaltet sich vorwiegend über die chemische Toxizität [Burkart, 1991].

Für die chemische Toxizität des Urans sind verschiedene Mechanismen denkbar. Yazzie et al. konnten zeigen, dass U(VI) Einzelstrangbrüche in Plasmid-DNA nach Reduktion von U(VI) mit Ascorbat induziert und vermuteten hierfür ursächlich die Generation von Radikalen über die Fenton Reaktion (Abbildung 2A) [Yazzie *et al.*, 2003]. Die Autoren konnten den Mechanismus der Radikalbildung jedoch nicht untermauern. ROS beeinflussen die Regulation der Expression von Genen des Zellwachstums und der Differenzierung und sind über diese Mechanismen an der Tumorprogression beteiligt [Crawford *et al.*, 1988]. In Untersuchungen an Ratten konnte nachgewiesen werden, dass ROS auch mit der Proliferation von Kryptzellen in Zusammenhang stehen [Lund *et al.*, 1998]. Für die genotoxische Wirkung des Urans wird auch eine direkte Interaktion des Uranylkations mit der DNA diskutiert [Franklin, 2001; Yazzie *et al.*, 2003]. In Abbildung 2B ist dieser Mechanismus schematisch dargestellt. Ein Uranyl-Ascorbat-Komplex interagiert mit dem negativ geladenen DNA-Phosphat-Rückgrad. Der Entzug von Elektronen stabilisiert die Phosphodiesterreste gegenüber nukleophilen Angriffen von Hydroxylradikalen und resultiert in der Hydrolyse der DNA.

A

$$U^{6+} + Asc \rightarrow U^{4+} + DHA$$
$$U^{4+} + O_2 + 2H^+ \rightarrow U^{6+} + H_2O_2$$

1) $Asc + O_2 + 2H^+ \rightarrow DHA + H_2O_2$

2) $\frac{1}{2}\, U^{4+} + H_2O_2 \rightarrow \frac{1}{2}\, U^{6+} + HO^{\bullet} + HO$

B

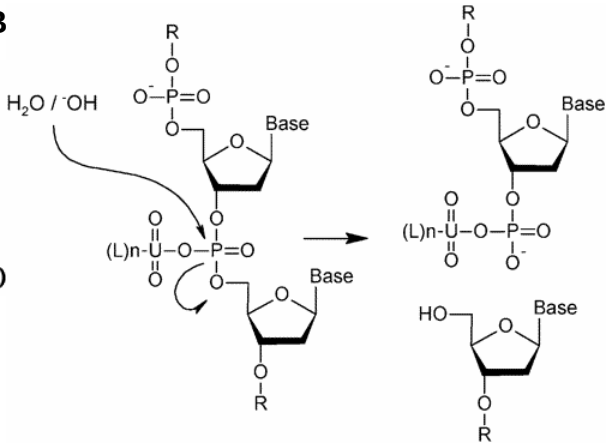


Abbildung 2: (A) Indirekte Mechanismen für uraninduzierte Strangbrüche: Bildung von radikalischen Verbindungen über die Reduktion von U^{6+} durch Ascorbat (Asc), als Nebenprodukt entsteht Dihydroascorbat (DHA). **(B)** Direkte Mechanismen für uraninduzierte Strangbrüche: Hydrolyse der DNA durch Interaktion eines Uranyl-Ascorbat-Komplexes mit dem negativ geladenem DNA-Phosphat-Rückgrad.

Immer mehr Studien befassen sich mit dem genotoxischen Potential von Uran, oftmals wird dabei jedoch nicht zwischen chemischer und radiologischer Toxizität unterschieden [Hu & Shoupeng, 1990; Prabhavathi *et al.*, 2000]. Lin et al. schreiben die von ihnen gefundenen zytogenetischen Schäden eher einer direkten Wirkung von Uran zu [Lin *et al.*, 1993]. Auch Miller und Kollegen schließen Strahlung als Ursache für die durch Uranylchlorid verursachten zellulären Schäden aus, da sie nachweisen konnten, dass nur ca. 0,0014 % der Zellen von α-Partikeln getroffen wurden [Miller *et al.*, 1998; Miller *et al.*, 2002b].

Viele Untersuchungen analysierten die Wirkungen von Uranverbindungen auf chromosomaler Ebene. Eine Inkubation von CHO-Zellen mit Uranylnitrat verursachte eine erhöhte Frequenz von Mikrokernen, Schwester-Chromatid-Austauschen und chromosomalen Aberrationen [Lin *et al.*, 1993]. In Arbeitern der WISMUT-Minen konnte in Lungenmakrophagen ebenfalls eine erhöhte Mikrokernrate nachgewiesen werden [Popp *et al.*, 2000]. Andere Studien hingegen zeigten keinen Zusammenhang zwischen der Exposition mit Uranverbindungen und chromosomalen Veränderungen. So konnten Lloyd et al. keine expositionsbedingten chromosomalen Aberrationen in Lymphozyten von Bergarbeitern nachweisen [Lloyd *et al.*, 2002]. Golfkrieg-Soldaten, die mit abgereicherten Uran in Kontakt waren, zeigten keine Veränderungen in Chromosomen peripherer Lymphozyten [McDiarmid *et al.*, 2000]. Beim Vergleich dieser Studien müssen aber die unterschiedlichen Expositionsarten und Zielzellen berücksichtigt werden. Sie erschweren eine vergleichende Bewertung des Risikos. Minenarbeiter sind vor allem über die Inhalation des hochradioaktiven Radons

exponiert, wohingegen für Soldaten ein Risiko von Waffen mit abgereichertem Uran ausgeht.

Über die Kontamination regionaler Lebensmittel in der humanen Nahrungskette könnte für die Bevölkerung ein erhöhtes Risiko für gastrointestinale Krebserkrankungen bestehen [Küppers & Schmidt, 1994]. Bisher ist wenig bekannt über das genotoxische Gefährdungspotential von Uran und anderer mit dem Uranbergbau verbundener Substanzen nach oraler Exposition. Untersuchungen zur Genotoxizität in humanen Kolonzellen fehlen. Bedenkt man, dass diese Zellen Hauptangriffspunkte von Nahrungsmittelkontaminanten sind, ist die orale Toxikologie der mit dem Uranbergbau assoziierten Verbindungen von großer Bedeutung und sollte neben der inhalativen Exposition in Studien einbezogen werden.

Im Rahmen der Studien, die dieser Arbeit zugrunde liegen, wurde unter anderem die Uranylverbindung Uranylazetat als Einzelnoxe im Vergleich zu den komplexen Umweltproben ausgewählt, da die Anwendung in Genotoxizitätsprüfungen bereits beschrieben wurde [Yazzie *et al.*, 2003]. Mittels Nitrilotriazetat (siehe auch Abschnitt 1.4.2) wurde die wasserlösliche Verbindung Uranyl-Nitrilotriazetat (U-NTA) für die Untersuchungen hergestellt. Die wesentlichen Ergebnisse der toxikologischen Arbeiten mit U-NTA wurden im **Publikationsmanuskript IV** publiziert.

1.3 Eisen

1.3.1 Nahrungseisen und Kolorektalkrebs

Ein Zusammenhang zwischen dem Verzehr von rotem Fleisch und Dickdarmkrebs gilt heute als wahrscheinlich. Potter fasste die epidemiologische Datenlage beim Menschen zusammen und schlussfolgerte, dass die Assoziation zwischen Fleischverzehr und dem Risiko für Dickdarmkrebs stärker zu bewerten ist, als für andere Nährstoffe [Potter, 1999]. Willet et al. reduzierten diesen Zusammenhang nur auf rotes Fleisch [Willet, 2001]. Der Verzehr von rotem Fleisch steht im Verdacht über Hämoglobin die Eisenkonzentrationen im Darmlumen zu erhöhen, dies ist mit der Bildung reaktiver Sauerstoffspezies (ROS) via Fenton Reaktion verbunden [Hata *et al.*, 1997; Imlay *et al.*, 1988]. ROS sind in der Lage DNA-Schäden in epithelialen Zellen der Kolonmukosa zu induzieren [Potter, 1999]. Hämoglobin stellt eine Quelle für das genotoxische Häm dar [Glei *et al.*, 2006]. Die Porphorinstruktur enthält Eisen, für das ebenfalls genotoxische Wirkungen nachgewiesen wurden [Glei *et al.*, 2002].

In der EPIC-Studie ergab sich für einen hohen Verzehr von rotem und verarbeitetem Fleisch eine positive Korrelation zum Kolorektalkrebs [Norat *et al.*, 2005].
Drei große Kohorten-Studien befassten sich mit dem Zusammenhang von Eisen und Kolorektalkrebs [Herrinton *et al.*, 1995; Knekt *et al.*, 1994; Wurzelmann *et al.*, 1996], wobei nur eine der Studien eine Korrelation zwischen Eisenaufnahme und der Krebsinzidenz im proximalen Kolon nachwies [Wurzelmann *et al.*, 1996].

1.3.2 Toxikologie Eisen

Als essentielles Element spielt Eisen eine wesentliche Rolle im Zellwachstum und der DNA-Replikation. Es hat eine zentrale Bedeutung für Schlüsselenzyme der Atmung (Hämoglobin und Myoglobin), des Energiestoffwechsels (Eisen-Schwefelproteine der Atmungskette, Zytochrome) und der DNA-Synthese (Ribonukleotid-Reduktase) [Richardson & Ponka, 1997].
Eisen und andere zu den Transitionselementen zählende Metalle wirken als Katalysatoren der Aktivierung von Sauerstoff in der Fenton- und Haber-Weiss-Reaktion (Abbildung 3), was zu deren toxischem Potential beiträgt.

(1)	$M^{n+} + H_2O_2$	→	$M^{(n+1)+} + OH^- + OH^{\theta}$	Fenton-Reaktion
(2)	$M^{(n+1)+} + O_2^{-\theta}$	→	$M^{n+} + O_2$	Rezyklierung des Metalls
(3)	$H_2O_2 + O_2^{-\theta}$	→	$O_2 + OH^- + OH^{\theta}$	Haber-Weiss-Reaktion (netto)

Abbildung 3: Rolle der Schwermetalle als Katalysatoren in der Fenton-Reaktion und Haber-Weiss-Reaktion (M: Metalle)

Die essentielle Bedeutung des Eisens für den Zellstoffwechsel wird von klinischen Untersuchungen unterstrichen, die ergaben, dass Eisenchelatoren die Proliferation von Krebszellen hemmen können [Richardson & Ponka, 1997].

Weinberg (1996) diskutiert drei mögliche Mechanismen über die Eisen an der Karzinogenese beteiligt sein könnte [Weinberg, 1996]:

1) Eisen katalysiert über die Fenton-Reaktion die Bildung reaktiver Hydroxylradikale, die wiederum Punktmutationen, Strangbrüche und Crosslinks

in der DNA induzieren. Über ROS-induzierte Lipidperoxidation in Membranen kann die Signaltransduktion beeinflusst werden.

2) Neben der Initiation des neoplastischen Wachstums unterdrückt Eisen die zelleigene Immunabwehr. In diesem Zusammenhang wird die Funktion von Makrophagen durch Phagozytose von Erythrozyten, Hämoglobin, Eisendextran oder Eisensalzen beeinflusst.
3) Eisen ist auch für Tumorzellen ein essentieller Nährstoff. Die unbegrenzte Proliferation von Tumorzellen erfordert eine erhöhte Eisenversorgung.

Meneghini (1997) konnte nachweisen, dass niedermolekulare Substanzen wie Metallionen frei durch die Kernporen diffundieren können und Eisen an das Chromatin binden kann [Meneghini, 1997]. Dies ist Voraussetzung dafür, dass Eisen über Hydroxylradikale die DNA im Zellkern schädigen kann.
Verschiedene Studien belegen, dass Schwermetalle wie Cadmium, Nickel oder Arsen in der Lage sind die DNA-Reparatur zu modifizieren [Hartmann & Speit, 1996; Hartwig A., 2002; Hartwig, 1994; Hartwig *et al.*, 1996; Hartwig, 1998; Hartwig & Beyersmann, 1989a; Hartwig & Beyersmann, 1989b; Hartwig & Schwerdtle, 2002]. Für Eisen konnten Abalea et al. (1998) eine eisenvermittelte, zeitabhängige Aktivierung der Reparatur beobachteten, die vermutlich auf der Induktion spezifischer Reparaturenzyme für oxidierte Pyrimidinbasen basiert [Abalea *et al.*, 1998].
Neben der in Abschnitt 1.2.2 beschriebenen Einzelnoxe U-NTA diente für die vorliegende Arbeit auch Eisen in Form von Eisen-Nitrilotriazetat (Fe-NTA) als Modellsubstanz. Der niedermolekulare Komplex Fe-NTA entsteht durch die Komplexierung von Eisen durch den Chelator Nitrilotriazetat (NTA). NTA ist ein synthetischer stickstofforganischer Komplexbildner mit der Summenformel $C_6H_9NO_6$. Mit steigendem pH-Wert dissoziiert die Azetylgruppe, so dass Metallionen gebunden werden können und bei neutralen pH-Werten wasserlösliche Komplexe gebildet werden [Kawabata *et al.*, 1986]. NTA allein wirkt nicht genotoxisch. In Verbindung mit Metallen konnte die Genotoxizität nur für den Komplex Fe-NTA *in vitro* nachgewiesen werden [Cai *et al.*, 1998; Glei *et al.*, 2002; Hartwig & Schlepegrell, 1995]. Fe-NTA ist ein anerkanntes Modell in Untersuchungen zur Kanzerogenese [Okada, 2003]. Als Zielorgane für die Toxizität bzw. kanzerogene Wirkung gelten vor allem die Niere [Zhang *et al.*, 1997] und die Leber [Abalea *et al.*, 1998; Iqbal *et al.*, 1995]. Die im Rahmen dieser Arbeit durchgeführten Untersuchungen mit der Einzelnoxe Fe-NTA

erfolgten mit Primären Kolonepithelzellen, Kolonadenomzellen (LT97) und Kolonkarzinomzellen (HT29 clone 19A) und wurden in 3 Publikationen zusammengestellt **(Manuskripte I, II und V)**.

1.4 Kolorektalkrebs

Kolorektalkrebs war im Jahr 2004 die mit über 376000 Fällen am zweithäufigsten diagnostizierte Krebserkrankung in Europa [Boyle & Ferlay, 2005]. Auch bei den krebsbedingten Todesursachen belegte der Kolorektalkrebs den 2. Platz. Betrachtet man jedoch nur die 25 Mitgliedsstaaten der Europäischen Union (EU), so zeigt sich, dass bei den Krebsneuerkrankungen der Lungenkrebs inzwischen durch den Kolorektalkrebs von der 1. Position verdrängt wurde. 2004 erkrankten in der EU etwa 279000 Menschen an Darmkrebs (13,6 % der Krebsneuerkrankungen), gefolgt von Brustkrebs (13,3 %) und Lungenkrebs (12,5 %). Im selben Jahr starben in der EU etwa 234000 Menschen an Lungenkrebs und 139000 Menschen an Kolorektalkrebs. Bei Männern ist die häufigste Krebstodesursache Lungenkrebs, gefolgt von Darm- und Prostatakrebs. Die häufigste Krebstodesursache bei Frauen ist Brustkrebs vor Darm- und Lungenkrebs. Die altersstandardisierte Inzidenzrate für Deutschland bewegt sich im europaweiten Durchschnitt [Robert Koch Institut, 2003; Robert Koch Institut, 2006]. Etwa 6 % der Männer und Frauen im Alter von 65 Jahren sind davon betroffen, wobei das Risiko exponentiell mit dem Alter steigt. Es gibt Individuen, die aufgrund bestimmter Erkrankungen oder einer familiären Prädisposition ein erhöhtes Risiko für die Entstehung kolorektaler Tumore aufweisen. Zu den erblich bedingten Formen des Dickdarmkrebses werden die Familiäre Adenomatöse Poliposis (FAP) und das nichtpolipöse kolorektale Krebssyndrom (HNPCC) gezählt, die zusammen allerdings nur 4-6 % aller auftretenden Kolontumore ausmachen. Weiterhin müssen die aufgrund chronisch-entzündlicher Darmerkrankungen oder aufgrund von Enzympolymorphismen entstandenen Tumore zu den hereditären Kolonkarzinomen gezählt werden [Potter, 1999]. Etwa 85 % der gesamten kolorektalen Krebserkrankungen entwickeln sich jedoch sporadisch, indem sich ohne genetische Prädisposition über einen langen Zeitraum hinweg gesunde Körperzellen in Krebszellen verwandeln [Potter, 1999].

1.4.1 Ernährung und Kolorektalkrebs

Kolorektale Tumore sind besonders stark mit Ernährungsfaktoren assoziiert. Zu diesen gehören Risikosubstanzen, die in den Stammzellen des Kolons Mutationen in krebsrelevanten Genen induzieren, sowie protektive Stoffe, die vor den Risikosubstanzen schützen [Beyer-Sehlmeyer *et al.*, 2003; Schäferhenrich *et al.*, 2003a]. Zu den Schutzmechanismen zählt die Induktion von Phase II Enzymen, die zur Entgiftung beitragen. Außerdem erfolgt über Tumorsuppressorgene eine Hemmung der Tumorprogression [Ebert *et al.*, 2001; Ebert *et al.*, 2003; Klinder *et al.*, 2004]. Möglicherweise können pflanzliche Lebensmittelinhaltsstoffe durch Genaktivierung in Darmzellen und Mutationen in bestimmten Genen einen Beitrag zur Reduktion des Kolonkrebsrisikos leisten. Für diese Schutzwirkungen kommen lösliche Inhaltsstoffe oder Produkte, die bei der Fermentation im Darm entstehen, in Betracht [Duthie *et al.*, 1997b; Pool-Zobel *et al.*, 2005a].

Der World Cancer Research Fund (WCRF) geht davon aus, dass bis zu 75 % der durch Dickdarmkrebs verursachten Todesfälle durch eine Ernährungsumstellung vermieden werden könnte [WCFR, 1997]. Grundlage dieser Aussage bilden verschiedene epidemiologische Studien, die eine „Western Style Diet“ mit einer erhöhten Krebsinzidenz assoziieren [Joossens & Kesteloot, 2001; La Vecchia *et al.*, 2003]. Die „Western Style Diet“ zeichnet sich durch einen hohen Anteil an Fleisch, tierischen Fetten und raffinierten Kohlenhydraten und einen geringen Anteil an Obst, Gemüse und Ballaststoffen aus [Slattery, 2000].

Epidemiologische Untersuchungen belegen, dass ein hoher Verzehr von Fleisch , insbesondere rotem und verarbeitetem Fleisch, positiv mit dem kolorektalen Risiko verknüpft ist [Breuer-Katschinski *et al.*, 2001; Norat *et al.*, 2005; Norat & Riboli, 2001; Sandhu *et al.*, 2001].

Neben der in Abschnitt 1.3.1 geschilderten Rolle von Hämeisen werden verschiedene andere Mechanismen für die positive Korrelation zwischen Fleischaufnahme und Kolonkrebs diskutiert. Hierzu zählen (i) die Bildung potentieller krebserregender N-Nitroso-Verbindungen durch die Darmflora [Hughes *et al.*, 2001], (ii) die Bildung heterozyklischer Amine beim Grillen von Fleisch [Sinha *et al.*, 2001], und (iii) die erhöhte Ausscheidung von Fett- und Gallensäuren durch den hohen Gehalt an gesättigten Fettsäuren im Fleisch (Newmark et al., 1984).

Eine hohe Gesamtfettaufnahme ist nur in einigen epidemiologischen Studien mit einem höheren Darmkrebsrisiko verbunden [Slattery, 2000], wobei vor allem tierische Fette das Risiko steigern [Slattery *et al.*, 1997]. Darüber hinaus spielen auch endogene Lipidperoxidationsprodukte, wie Malondialdehyd oder 4-Hydroxynonenal (HNE) eine wichtige Rolle bei der Krebsentstehung. HNE und seine Epoxide bilden durch elektrophile Addition an die DNA Propano-DNA-Addukte und Etheno-DNA-Addukte [Esterbauer *et al.*, 1990; Toyokuni *et al.*, 1997].
Alkohol steht ebenfalls im Zusammenhang mit einem erhöhten Risiko an Kolonkrebs zu erkranken, wobei hier vor allem die Induktion einer Mikrosatelliteninstabilität durch inhibierte DNA-Reparatur, die Bildung von DNA-Addukten über Acetaldehyd sowie der Einfluss auf die Zellproliferation als mögliche Ursachen zu nennen sind [Kune & Vitetta, 1992; Slattery *et al.*, 2001]. Neben dem Alkoholkonsum sind auch andere individuelle “Life Style“ Faktoren wie Rauchen [Sharpe *et al.*, 2002], Übergewicht und mangelnde Bewegung [Boutron-Ruault *et al.*, 2001; Slattery & Potter, 2002] als Risikofaktoren zu nennen.
Den potentiell Risiko-erhöhenden Faktoren stehen solche mit protektiven Wirkungen gegenüber. So werden einigen Nahrungsinhaltsstoffen chemoprotektive Eigenschaften zugesprochen. Epidemiologische Untersuchungen zeigten eine inverse Korrelation zwischen Obst- und Gemüse-, sowie Ballaststoffzufuhr und dem Risiko für Kolonkrebs [Bingham *et al.*, 2003; Norat & Riboli, 2002; Peters *et al.*, 2003]. Der Aspekt der Chemoprävention durch Ernährungsfaktoren wird unter Punkt 1.6.2 vertieft.

1.4.2 Die Adenom-Karzinom-Sequenz

Die Tumorentstehung ist ein Mehrstufenprozess, der in Initiation, Promotion und Progression unterteilt wird. In der Initiation führen durch exogene und/oder endogene Faktoren verursachte irreversible DNA-Veränderungen in der Zelle zu manifesten Mutationen in den nachfolgenden Zellgenerationen. An diese ersten initiierenden Mutationen schließen sich verschiedene molekulare und histologische Veränderungen an [Mendelsohn, 2001]. Promotoren bewirken dann eine beschleunigte Proliferation initiierter Zellen (Promotionsphase). Dies führt meist zur Bildung gutartiger Läsionen oder Präneoplasien. Die Progression führt im Weiteren zu einer malignen Entartung benigner Tumoren. Die Malignität ist mit zunehmender

Heterogenität und Autonomie des Tumors, sowie mit infiltrierendem Wachstum und Metastasierung verbunden [Mendelsohn, 2001].

Das Mehrstufenmodell von Fearon und Vogelstein (1990), auch als Adenom-Karzinom-Sequenz bezeichnet, beschreibt die Zusammenhänge der beim Kolonkrebs auftretenden histologisch/morphologischen Veränderungen unter Berücksichtigung der entsprechenden genetischen Modifikationen [Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996; Lengauer *et al.*, 1997]. In Abbildung 4 sind die in der Kolonkarzinogenese beteiligten Mutationen und deren Konsequenzen veranschaulicht. Die genetischen Veränderungen betreffen vor allem Gene des Zellwachtums, der Zelldifferenzierung, der DNA-Reparatur und Gene, die in die Interaktion zwischen zellulärer und extrazellulärer Matrix involviert sind. Fearon und Vogelstein nennen Mutationen im *APC*-Gen (adenomatous poliposis coli, 5q21-q22), im *K-RAS*-Gen (kirsten rat sarcoma, 12p12.1), im *MCC*-Gen (mutated in colon cancer, 5q21-q22) und im *TP53*-Gen (tumor protein 53, 17p13.1). Ausserdem spielen Veränderungen im *SMAD2/SMAD4*-Gen (mothers against decapentaplegic homolog 2/4, 18q21.1) bzw. *DCC*-Gen (deleted in colon cancer, 18q21.3) eine Rolle [Fearon & Vogelstein, 1990; Hahn *et al.*, 1996; Takagi *et al.*, 1998].

Mutationen des *APC*-Gens stellen die initiierenden Ereignisse der kolorektalen Karzinogenese dar, weshalb das *APC*-Gen auch als “Pförtner“ (gatekeeper) der Kolonkarzinogenese bezeichnet wird. Eine Mutation des auf Chromosom 5 lokalisierten Gens ist in etwa 60-80 % der sporadischen kolorektalen Adenome und Karzinome nachweisbar [Groden *et al.*, 1991; Miyaki *et al.*, 1994; Vogelstein *et al.*, 1988]. Bei den Mutationen zwischen Kodon 1280 und 1500 handelt es sich hauptsächlich um Frameshift- und Nonsens-Mutationen. Während der Entwicklung vom Adenom zum invasiven Karzinom kommt es auch häufig zum vollständigen Verlust des *APC*-Allels [Miyaki *et al.*, 1994]. Das Produkt des *APC*-Gens ist ein 312 kDa großes Protein, das aus 2843 Aminosäuren besteht. Im oberen Teil der Kolonkrypten wird *APC* verstärkt exprimiert und ist über den Wnt-ß-Catenin-Signalweg mit der Adhäsion, Migration, Differenzierung und Apoptose der Zellen verknüpft [Fodde *et al.*, 2001].

Das *K-RAS*-Gen gehört zur *RAS*-Genfamilie und ist auf Chromosom 12 lokalisiert. Es kodiert für ein 21 kDa Protein aus 189 Aminosäuren [Bos, 1989]. Mutationen im *K-RAS*-Gen sind in vielen humanen Tumoren zu finden. In der kolorektalen Karzinogenese sind *K-RAS*-Mutationen in 30-50 % der Adenome und Karzinome

nachweisbar [Andreyev *et al.*, 2001; Jen *et al.*, 1994; Rashid *et al.*, 1999; Scott *et al.*, 1993]. In 90 % der Fälle sind die Kodons 12 und 13 von Punktmutationen betroffen [Bos, 1989; Fearon, 1994]. Das *K-RAS*-Gen ist über den RAS-RAF-MEK-ERK-Reaktionsweg in die Signaltransduktion von Wachstumsfaktoren integriert und somit für Zellproliferation und Differenzierung von Bedeutung [Bos, 1989]. Die RAS-Aktivität wird durch die Hydrolyse des RAS-GTPs zum inaktiven RAS-GDP mittels eines GTPase aktivierenden Proteins (GAP) begrenzt. Durch *RAS*-Mutationen können RAS-Proteine entstehen, die ohne gebundenes Nukleotid aktiv sind oder durch ein hydrolysestabiles GTP des aktiven RAS-GTPs aktiv bleiben. Die Folge ist eine von äußeren Faktoren unabhängige ständige Weiterleitung von Wachstumssignalen [Chang *et al.*, 2003; Leslie *et al.*, 2002; McCormick, 1995].

Als spätes Ereignis der Karzinogenese kommen *TP53*-Mutationen in etwa 40-50 % der kolorektalen Tumore vor [Rashid *et al.*, 1999; Scott *et al.*, 1993; Vogelstein *et al.*, 1988]. Über 80 % der *TP53*-Mutationen treten in den Exons 5-8 auf. Hier sind vor allem die Aminosäuren 175, 245, 248, 249, 273 und 282 (Hot Spots) betroffen [May & May, 1999; Oren & Rotter, 1999]. Das Tumorsuppressorgen auf dem kurzen Arm von Chromosom 17 (17p13.1) kodiert für ein 53 kDa großes Phosphoprotein aus 393 Aminosäuren und ist für den Erhalt der genomischen Stabilität von großer Bedeutung. Es wird deshalb auch als „Wächter“ des Genoms bezeichnet. Mutationen können dem *TP53*-Gen einen onkogenen Charakter verleihen und die Zellteilung stimulieren. Zum einen kann so die Tumorentstehung induziert werden, zum anderen wird durch die Ausschaltung der Tumorsuppressorfunktion ein Wachstum geschädigter oder entarteter Zellen begünstigt. Beim Auftreten von DNA-Schäden stimuliert es normalerweise die Reparatur der Schäden, blockiert die Proliferation und induziert bei mangelhafter Reparatur die Apoptose [Lane, 1992; May & May, 1999; McKay *et al.*, 2000; Oren & Rotter, 1999]. Das *TP53*-Genprodukt ist ein Zellzyklusregulator mit wachstumshemmender Aktivität, weshalb der Allelverlust mit ungehemmtem Tumorwachstum und Metastasierung assoziiert ist [Ko & Prives, 1996]. Der Verlust von *TP53* kann auch ohne weitere Exposition gegenüber externen Noxen zu einer genomischen Instabilität führen [Leslie *et al.*, 2002; May & May, 1999; Oren & Rotter, 1999; Vogelstein *et al.*, 2000].

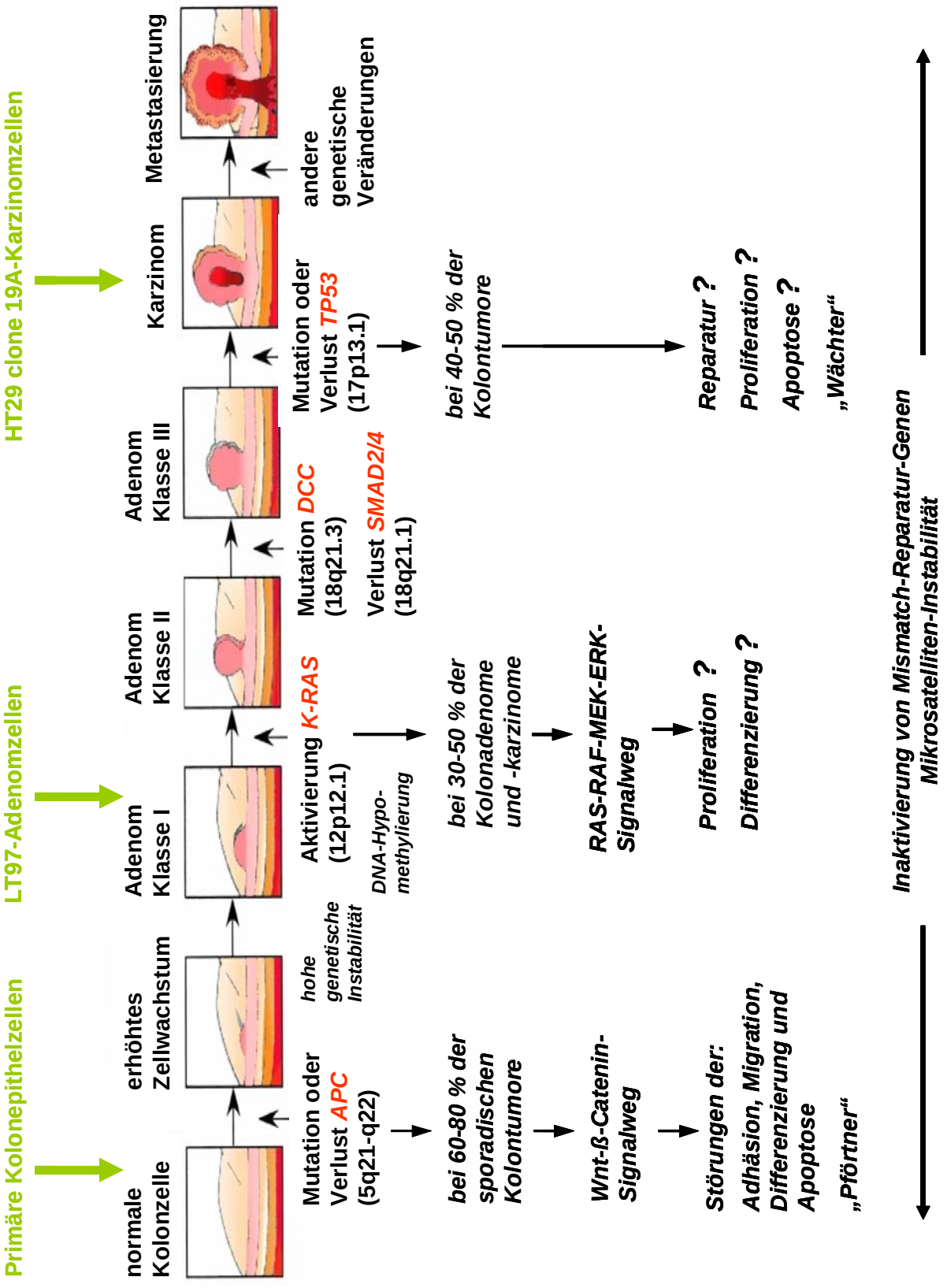


Abbildung 4: Modell der Kolonkarzinogenese nach Fearon und Vogelstein modifiziert nach [Davies *et al.*, 2005; Fearon & Vogelstein, 1990; Pan & Godwin, 2005]. In grün ist dargestellt, in welche Stufe des Karzinogeneseprozesses die in dieser Arbeit verwendeten Kolonzellen einzuordnen sind (siehe Kapitel 1.7).

1.5 Zelluläre Schutzmechanismen gegenüber oxidativen und genotoxischen Verbindugen

Die Zelle verfügt über eine Vielzahl von Schutzmechanismen gegenüber oxidativen und genotoxischen Angriffen. Hierzu zählen (i) antioxidative Abwehrmechanismen zur Minimierung von oxidativen Stress, (ii) enzymatische Systeme des Fremdstoffmetabolismus zur Entgiftung von Noxen und (iii) die Reparatur von DNA-Schäden. Über eine geeignete Ernährung können diese Schutzsyteme positiv beeinflusst werden und somit zur Senkung des Darmkrebsrisikos beitragen [Pool-Zobel *et al.*, 1999].

***Antioxidative Schutzsysteme*:** [Younes, 2004]

- enzymatische Schutzsysteme (Superoxid-Dismutasen, Katalase, Glutathion Peroxidase)
- nichtenzymatische Systeme (Ascobinsäure, Glutathion, Carotinoide, α-Tocopherol, Harnsäure)
- Mechanismus: Abfangen freier Radikale und nichtradikalischer Spezies

Enzyme des Fremdstoffmetabolismus: [Arand & Oesch, 2004; Sheehan *et al.*, 2001]

- Phase II-Enzyme
- Mechanismus: elektrophile Substrate werden von Glutation-S-Transferasen (GST) mit Gluathion (GSH) konjugiert, nukleophile Substrate werden von UDP-Glucuronosyltransferasen, Sulfotransferasen, Acetyltransferasen, Acyl-CoA-Aminosäure-N-Acetyltransferasen und Methyltransferasen metabolisiert

DNA-Reparatur:

- Reparatur von Basenläsionen durch Basen-Exzisions-Reparatur (BER) und Nukleotid-Exzisions-Reparatur (NER) [Cooke *et al.*, 2003]
- Reparatur von Einzel- und Doppelstrangbrüchen durch nichthomologe End-zu-End-Verknüpfung und homologe Rekombination [Christmann *et al.*, 2003]

1.6 Chemoprävention

1.6.1 Mechanismen der Chemoprävention

Unter Chemoprävention versteht man die Nutzung natürlicher oder pharmakologischer Stoffe zur Hemmung der Entwicklung invasiver Tumoren über die Verhinderung oder Reparatur von DNA-Schäden, welche die Karzinogenese initiieren bzw. über die Inhibierung bzw. Umkehrung der Progression premaligner Zellen, in denen bereits Schäden vorhanden sind [Hong & Sporn, 1997]. Durch die Aufnahme natürlich vorkommender Stoffe (sekundäre Pflanzenstoffe) und pharmazeutischer Präparate kann die Entwicklung von Tumoren verhindert, verlangsamt oder umgekehrt (sofern bereits Vorstufen bestehen) werden [Sporn & Newton, 1979]. Hierbei unterscheidet man zwischen Blockierung („Blocking Agent Activity“) und Suppression („Suppressing Agent Activity“). Blocking Agents verhindern den ersten Schritt der Krebsentstehung, die Initiation. Suppressing Agents hemmen den Übergang initiierter Zellen in stärker transformierte Zellen, die Progression. Während in der Primärprävention Maßnahmen zur Vermeidung von toxifizierenden Mechanismen und deren unmittelbaren Folgen, im Fall der Kanzerogenese speziell die Reduktion von Mutationen, im Vordergrund stehen, liegt das Hauptaugenmerk bei der Sekundärprävention auf der Modulierung der Proliferation von initiierten Zellen und von Signaltransduktionswegen. In der Therapie, auch als Tertiärprävention bezeichnet, spielt die Hemmung der für späte Prozesse der Karzinogenese charakteristischen Zellvermehrung und die Entdifferenzierung eine entscheidende Rolle. Chemopräventive Stoffe können sein: (i) körpereigene Stoffe bzw. deren Analoga (z. B. Hormone), (ii) essentielle oder nicht essentiellen Nahrungsbestandteile (z.B. Vitamine, Polyphenole), (iii) Xenobiotika bzw. Arzneimittel (z. B. Aspirin). Unsere tägliche Ernährung enthält eine Vielzahl von chemoprotektiven Verbindungen. Neben Vitaminen, Ballaststoffen und Mineralstoffen besitzen vor allem sekundäre Pflanzenstoffe ein möglicherweise chemopräventives Potential.

1.6.2 Chemoprävention durch Ernährungsfaktoren

Welche Rolle Ballaststoffe bei der ernährungbedingten Verminderung von Darmkrebserkrankungen spielen, wird seit langem kontrovers diskutiert. Hinsichtlich

der Ballaststoffzufuhr weisen sowohl die prospektive Studie von Bingham *et al*. (2003) als auch die Fall-Kontroll-Studie von Peters (2003) eine inverse Beziehung zwischen Ballaststoffaufnahme und dem Risiko Kolonadenome bzw. -karzinome zu entwickeln auf [Bingham *et al.*, 2003; Peters *et al.*, 2003]. In Untersuchungen mit Weizenkleie, Fructooligosacchariden und resistenter Stärke im Rahmen einer gesunden Ernährung zeigte sich eine Protektion hinsichtlich der kolorektalen Karzinogenese [Pool-Zobel, 2005]. Allerdings gibt es auch Studien, die das kolonkrebssenkende Potential von Ballaststoffen nicht konsistent bestätigen [Michels *et al.*, 2005]. Ballaststoffe wirken vor allem über die Verdünnung karzinogen wirksamer Substanzen im Fäzes und die Verkürzung der Transitzeit im Kolon durch Zunahme des Stuhlvolumens protektiv. Zudem werden Gallensäuren und andere potentiell toxische Stoffe gebunden, wodurch ebenfalls die Exposition der Kolonmukosa mit Karzinogenen vermindert wird [Kim & Mason, 1996]. Ferner werden Ballaststoffe von der intestinalen Bakterienflora zu kurzkettigen Fettsäuren fermentiert, von denen vor allem Butyrat eine chemoprotektive Wirkung besitzt [Pool-Zobel *et al.*, 2002; Pool-Zobel *et al.*, 2003; Pool-Zobel *et al.*, 2005b].

Epidemiologische Studien zeigten, dass die Inzidenz für kolorektale Tumore über den Verzehr von flavonoidreichen Obst- und Gemüsesorten beeinflusst werden kann [Knekt *et al.*, 1997; Witte *et al.*, 1996]. Hierbei tragen neben Ballaststoffen auch ihre antioxidativen Inhaltsstoffe zur Prävention kolorektaler Erkrankungen bei [Block *et al.*, 1992]. Obst und Gemüse versorgen den Organismus mit Ballaststoffen, den antioxidativ wirksamen Vitaminen A, C und E sowie mit sekundären Pflanzenstoffen (Carotinoide, Isothiocyanate, Polyphenole, Phytoestrogene u.a.) und essentiellen Mikronährstoffen (z. B. Selen und Calcium). Diese Inhaltsstoffe haben eine bedeutende Rolle für die chemoprotektive Wirkung von Obst und Gemüse [Breuer-Katschinski *et al.*, 2001; Levi *et al.*, 2000; Mobarhan, 1999; Voorrips *et al.*, 2000].

1.6.2.1 Chemoprävention durch Polyphenole

Polyphenole sind ubiquitär in Nahrungsmitteln pflanzlichen Ursprungs zu finden. Ihnen werden antikanzerogene, antimikrobielle, immunmodulierende, antioxidative und antiinflammatorische Wirkungen zugeschrieben [Dryden *et al.*, 2006; Yoon & Baek, 2005]. Aufgrund dieser Wirkungen können Polyphenole präventiv bezüglich

Krankheiten wirken, die wie Krebs, kardiovaskuläre oder neurodegenerative Erkrankungen mit oxidativem Stress assoziiert sind.

Zu den Hauptquellen für Flavonoide in der westlichen Ernährung zählen neben Tee und Zwiebeln auch Äpfel [Hermann-Kunz & Thamm, 1999]. Aus 100 g frischen Golden Delicious Äpfeln konnten Chinnici und Kollegen bis zu 357 mg Polyphenole extrahieren [Chinnici *et al.*, 2004]. In Abhängigkeit von der Apfelsorte, dem Reifegrad und Lagerbedingungen enthalten Äpfel pro kg Frischfrucht 0,1 bis 5 g Polyphenole [Manach *et al.*, 2004]. Eine tägliche Aufnahme von Polyphenolen über die Nahrung ist notwendig und empfehlenswert, um eine hohe Plasmakonzentration aufrecht zu erhalten, da aufgrund der schnellen Elimination eine maximale physiologische Konzentration von 10 µM an Polyphenolen und deren Metaboliten im Blutplasma nicht überschritten wird [Williamson & Manach, 2005]. Im Duodenum werden nur ca. 0,2 bis 0,5 % der aufgenommenen Menge absorbiert, demnach erreichen mehr als 95 % aller Polyphenole den Dickdarm, wo sie durch die mikrobielle Darmflora fermentiert werden [Clifford, 2004; Scalbert & Williamson, 2000].

Epidemiologische Studien konnten zeigen, dass die Kolonkrebsinzidenz durch die Aufnahme von Flavonoiden über Obst und Gemüse deutlich beeinflusst werden kann [Knekt *et al.*, 1997; Witte *et al.*, 1996]. Hierbei kommen neben den antioxidativen Eigenschaften auch die Modulation von Signaltransduktionswegen, die in einer Stressantwort münden und die Inhibierung der Proliferation von entarteten Zellen zum Tragen [Block *et al.*, 1992; Briviba *et al.*, 2002; Kuntz *et al.*, 1999; Lin, 2002; Silberberg *et al.*, 2006; Tanaka *et al.*, 2001; Yanagihara *et al.*, 1993]. Polyphenole können aufgrund ihrer chemischen Struktur Wasserstoff oder Elektronen übertragen. Über die Bildung von stabilen Phenoxylradikalen können so ROS abgefangen werden [Rice-Evans *et al.*, 1997]. Außerdem können Polyphenole in ihrer Funktion als Metallchelatoren die durch die Fenton-Reaktion vermittelte Bildung von Radikalen verhindern [Rice-Evans *et al.*, 1996].

In der vorliegenden Arbeit wurde für Untersuchungen zum protektiven Potential von Apfelpolyphenolen ein Apfelextrakt verwendet, der aus einem klaren Apfelsaft (20 % Jonagold; 25 % Topaz; 20 % Bohnapfel; 22,5 % Winteramber und 15 % Bittenfelder) gewonnen wurde [Veeriah *et al.*, 2006]. Der Polyphenolgehalt dieses Apfelextraktes (im Folgenden als AEO2 bezeichnet) beträgt 533,9 mg/g, wobei Phloretinxyloglycosid und Chlorogensäure die Hauptmetaboliten darstellen.

1.7 Verwendete Zellen

Für die Untersuchungen wurden humane Kolonzellen unterschiedlicher Transformationsgrade verwendet. Hierbei handelt es sich um primäre Kolonepithelzellen und jeweils eine Kolonadenomzelllinie (LT97) und eine Kolonkarzinomzelllinie (HT29 clone 19A, für Untersuchungen zum chemopräventiven Potential von Apfelpolyphenolen HT29). Die Einstufung der verwendeten Zellmodelle innerhalb des Kolonkarzinogenese-Prozesses ist in Abbildung 4 zusammenfassend dargestellt. Außerdem wurden humane Leukozyten in die Genotoxizitäts-Untersuchungen mit Eisen einbezogen.

1.7.1 Primäre Kolonepithelzellen

Primäre Kolonepithelzellen wurden aus gesundem, histologisch unauffälligem Kolongewebe, das aus medizinischen Gründen bei einer Operation mit entfernt wurde, gewonnen. Die Gewebeproben wurden vom Klinikum für Allgemeine und Viszerale Chirurgie der Friedrich-Schiller-Universität Jena mit dem Einverständnis der Patienten und der Ethikkommission der Friedrich-Schiller-Universität Jena zur Verfügung gestellt. Aus diesen Gewebeproben wurden Streifen des Kolonepithels gewonnen und Epithelzellen isoliert [Schäferhenrich *et al.*, 2003a; Schäferhenrich *et al.*, 2003b].

1.7.2 Humane Kolonadenomzellen - LT97

Die humane Kolonadenomzelllinie LT97 wurde 1997 von Frau B. Marian (Institut für Krebsforschung der Universitätsklinik Wien) aus Mikroadenomen einer Patientin mit Familiärer Adenomatöser Poliposis etabliert. Die differenzierten Epithelzellen entsprechen dem Adenomstadium der kolorektalen Karzinogenese. Sie wachsen unter standardisierten Bedingungen (LT97-Komplettmedium, 37°C, 5 % CO_2 und 95 % Luftfeuchte) adhärent in dreidimensionalen Strukturen. Die Verdopplungszeit beträgt 72-96 Stunden [Richter *et al.*, 2002]. Zu Beginn der eigenen Arbeiten mit dieser Zelllinie bestand diese aus 2 Klonen (Verhältnis 1:1). Beide Klone wiesen eine Translokation zwischen den Chromosomen 7 und 17 und ein verändertes Chromosom 1 auf. Klon 1 besaß außerdem ein Isochromosom 1q, Klon 2 eine dizentrische Translokation des langen Armes von Chromosom 1 und 18. Der

Karyotyp mit 46-50 Chromosomen je Zelle erwies sich in Routinekaryotypisierungen bis zum heutigen Kenntnisstand als relativ stabil [Schäferhenrich *et al.*, 2003a].

1.7.3 Humane Kolonkarzinomzellen - HT29 und HT29 clone 19A

Die HT29 clone 19A-Zellen sind ein Klon der humanen Kolonkarzinomzelllinie HT29, die 1964 von Fogh und Trempe (1975) aus dem Kolontumor einer 44-jährigen Patientin isoliert und etabliert wurde [Fogh & Trempe, 1975]. Die undifferenzierten Stammzellen entsprechen einem sehr späten Stadium der kolorektalen Karzinogenese. Durch Behandlung der Stammzellen mit 5 mM Natriumbutyrat wurde der Klon HT29 clone 19A rückdifferenziert [Augeron & Laboisse, 1984]. Unter standardisierten Kulturbedingungen (DMEM-Medium, 37 °C, 5 % CO_2 und 95 % Luftfeuchte) wachsen diese adhärent mit einer Verdopplungszeit von etwa 24 Stunden. HT29 clone 19A-Zellen weisen einen heterogenen hyperploiden Karyotyp mit zahlreichen Trisomien auf. Die Anzahl der Chomosomen liegt zwischen 64 und 69, wobei eine große Variation zwischen den einzelnen Metaphasenkernen besteht [Kuechler *et al.*, 2003]. Die in der Entwicklung von Kolonkrebs eine wichtige Rolle spielenden Gene, wie *APC*, *K-RAS* und *TP53* liegen in unterschiedlicher Anzahl vor, weshalb sich diese Zelllinie nicht für Untersuchungen mit der Methode des Comet FISH eignet.

1.7.4 Humane Leukozyten

Humane periphere Leukozyten sind Primärzellen, die durch relativ nicht-invasive Methoden von gesunden oder kranken Menschen isoliert werden können. Innerhalb von *ex vivo* und *in vitro* Studien können nicht nur direkte Einflüsse auf Leukozyten als Zielzellen, sondern auch systemische Effekte im gesamten Organismus nachgewiesen werden. Die in dieser Arbeit für *in vitro*-Untersuchungen verwendeten humanen Leukozyten wurden aus so genannten „Buffy Coats“ (mit Leukozyten angereicherte Blutproben) mittels Dichtegradientenzentrifugation unter Verwendung von Histopaque® -1077 isoliert. Diese können entweder frisch verwendet werden oder in Einfriermedium (90 % FCS und 10 % DMSO) bis zur Nutzung eingefroren werden.

2 PROBLEM- UND ZIELSTELLUNGEN

Durch die ehemaligen Bergbaubetriebe der Wismut AG hat sich in der dortigen Umgebung eine besondere Umweltsituation ergeben, die es in herausragender Weise ermöglichte, unter natürlichen, wenn auch anthropogen modifizierten Bedingungen, die Auswirkung der Stoffflüsse auf die Biosphäre zu prüfen. Die Wirkungspotentiale der in den geologischen Kompartimenten enthaltenen Radionuklide und Schwermetallsalze können nach Überwindung von Transportprozessen tierische und menschliche Zielzellen erreichen und dort über verschiedene Mechanismen zu besonderen Gen-Umwelt-Interaktionen führen. Ziel der vorliegenden Arbeit war es, ausgewählte Wasserproben aus dem ehemaligen Ronneburger Uranbergbaugebiet bzw. die Leitsubstanzen Eisen und Uran hinsichtlich ihrer Wirkungspotentiale zu charakterisieren und grundlegende Erkenntnisse über die beteiligten Wirkungsmechanismen zu erhalten. Für die Untersuchungen wichtiger toxikologischer Eigenschaften und Mechanismen der Testsubstanzen sollte hauptsächlich die Kolonkarzinomzelllinie HT29 clone 19A herangezogen werden. Darauf basierend erfolgte in weiteren Versuchen die Charakterisierung von Kolonzellen unterschiedlicher Transformationsgrade im Karzinogeneseprozess (Primäre Kolonepithelzellen, Kolonadenomzellen – LT97, Kolonkarzinomzellen – HT29 und HT29 clone 19A) und zum Teil auch humaner Leukozyten hinsichtlich ihrer Sensitivität gegenüber den Noxen. Des Weiteren sollte geprüft werden, ob Apfelpolyphenole chemoprotektiv gegenüber Eisen und anderen Genotoxinen wirken. Im Einzelnen wurden folgende Fragestellungen bearbeitet:

1) Unter welchen Bedingungen (Zeit-, Konzentrations-, Effektbeziehungen) beeinflussen Eisen, Uran und die komplexen Umweltproben das Wachstum von Kolonkarzinomzellen?

   Unter Verwendung aller Testsubstanzen sollte in der stark proliferierenden Kolonkarzinomzelllinie HT29 clone 19A mittels des DAPI-Assays das Wachstum und die Überlebensrate beurteilt werden, ein Konzentrations-Wirkungs-Zusammenhang erstellt werden und geeignete Konzentrationen für die weiterführenden Fragestellungen abgeleitet werden.

2) Welchen Einfluss haben Eisen, Uran und die komplexen Umweltproben auf die Bildung reaktiver Sauerstoffspezies (ROS) und den Glutathion-Status?

   Transitionsmetalle sind durch ihre Fähigkeit Elektronen aufzunehmen oder abzugeben in der Lage ROS zu induzieren. In der Karzinomzelllinie sollte das ROS-bildende Potential der Testsubstanzen mittels ROS-Assay untersucht werden.

Glutathion kann diesem Prozess entgegenwirken. Mit einem fluoreszenz-photometrischen Assay sollte deshalb in HT29 clone 19A-Zellen überprüft werden, ob die Testsubstanzen den GSH-Gehalt der Zellen modulieren.

3) Wirken Eisen, Uran und die komplexen Umweltproben genotoxisch in Kolonzellen unterschiedlicher Transformationsgrade?

Die genotoxischen Wirkungen der Testsubstanzen in primären Kolonzellen, LT97-Kolonadenomzellen bzw. HT29clone19a-Kolonkarzinomzellen wurden mittels Comet Assay quantifiziert. Die verschiedenen Zellmodelle sollten so hinsichtlich ihrer Sensitivität und die Testsubstanzen hinsichtlich ihres genotoxischen Potentials charakterisiert werden.

4) Führen die Umweltproben und die Leitsubstanzen zu einer veränderten Sensitivität der Zellen gegenüber weiteren Noxen bzw. wird die Reparatur induzierter Schäden beeinflusst?

Für viele der in den Wismut-Proben enthaltenen Schwermetalle ist eine Hemmung der DNA-Reparatur nachgewiesen. Dies sollte deshalb auch für die beiden Leitelemente Eisen und Uran sowie für die am stärksten kontaminierte Gewässerprobe untersucht werden. Hierfür wurden HT29 clone 19A-Zellen mit nicht-genotoxischen Konzentrationen der Testsubstanzen 24 Stunden vorinkubiert und anschließend mit verschiedenen Genotoxinen geschädigt. Der Einfluss der Testsubstanzen auf die Höhe der induzierten Schäden und deren Reparatur sollte anschließend im Comet Assay ausgewertet werden.

5) Induzieren Eisen, Uran und die komplexen Umweltproben DNA-Schäden in spezifischen Genen bzw. in spezifischen Chromosomen humaner Kolonzellen?

In diesem Teil der Arbeit sollte das Augenmerk auf die Interaktion der Testsubstanzen mit einem für die kolorektale Karzinogenese relevanten Gen, dem Tumorsuppressorgen TP53, gelegt werden. Dazu sollte der Comet-FISH genutzt werden. Hierbei handelt es sich um eine Kombination des Comet Assays und einer Fluoreszenz-in-situ-Hybridisierung, die eine Einschätzung der Sensitivität spezifischer Gene bzw. Genomabschnitte gegenüber genotoxischen Substanzen ermöglicht.
Zum Nachweis substanzinduzierter chromosomaler Veränderungen sollte erstmals eine neue innovative Methode, der 24-Farben-FISH, in vitro an humanen Kolonzellen eingesetzt werden.

6) Sind Leukozyten als Biomarker zur Erfassung von hohen Eisen-Expositionen geeignet?

Humane Leukozyten sollten in vitro mit in humanen Kolonzellen als effektiv erwiesenen Eisen-Konzentrationen behandelt und die DNA-Schädigung im Comet Assay und Comet FISH untersucht werden. Für in vivo-Untersuchungen werden Ratten-Leukozyten herangezogen.

7) Kann ein polyphenolreicher Apfelextrakt chemopräventiv wirken und die durch Eisen und andere Genotoxine induzierten DNA-Schäden vermindern?

Kolonadenomzellen und Kolonkarzinomzellen sollten mit effektiven Konzentrationen eines polyphenolreichen Apfelextraktes vorinkubiert und anschließend mit Eisen und anderen Genotoxinen geschädigt werden. Im Comet Assay kann dann ein möglicher modulierender Effekt nachgewiesen werden.

3 MANUSKRIPTE

Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells

Y. Knöbel, M. Glei, K. Osswald, B.L. Pool-Zobel
Toxicology In Vitro, 2006, Vol. 20(6), 793-800

Ferric iron is genotoxic in non-transformed and preneo-plastic human colon cells

Y. Knöbel, A. Weise, M. Glei, W. Sendt, U. Claussen, B. L. Pool-Zobel
zur Publikation angenommen bei Food and Chemical Toxicology am 30. Oktober 2006, online publiziert doi:10.1016/j.fct.2006.10028

Uranyl nitrilotriacetate (U-NTA), a stabilized salt of uranium, is genotoxic in nontransformed human colon cells and in the human colon adenoma cell line LT97

Y. Knöbel, M. Glei, A. Weise, K. Osswald, A. Schäferhenrich, K. K. Richter, U. Claussen, B. L. Pool-Zobel
Toxicological Sciences, 2006, Vol. 93(2), 286-297

Heavy metal- and radionuclid-contaminated water samples have genotoxic potential to human colon cells

Y. Knöbel, M. Glei, D. Merten, A. Weise, K. K. Richter, U. Claussen, B. L. Pool-Zobel
zur Publikation vorbereitet für Toxicological Sciences

Blood mononucleocytes are sensitive to the DNA damaging effects of iron overload – in vitro and ex vivo results with Fe-NTA

E. Park, M. Glei, **Y. Knöbel**, B. L. Pool-Zobel
zur Publikation angenommen bei Mutation Research am 26. Januar 2007

Apple flavonoids modulate the genotoxic effects of different DNA damaging compounds in human colon cells

S. Klenow*, S. Veeriah*, Y. Knöbel*, B. L. Pool-Zobel (* 3 Erstautoren)
zur Publikation vorbereitet für Mutation Research

3.1 Manuskript I

Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells

Y. Knöbel, M. Glei, K. Osswald, B.L. Pool-Zobel

Toxicology In Vitro, 2006, Vol. 20(6), 793-800

Zusammenfassung

Durch sein genotoxisches Potential ist Eisen ein für Kolorektalkrebs relevanter Risikofaktor. In dieser Arbeit sollten beteiligte Mechanismen, wie die Bildung reaktiver Sauerstoffspezies (ROS), die DNA-Reparatur, das Zellwachstum und der Glutathiongehalt (GSH) untersucht werden.

Fe-NTA verursachte zeitabhängig eine signifikante Bildung von ROS. Fe-NTA zeigte aber keinen Einfluss auf die Reparatur H_2O_2-, HNE- und NOHPhiP-induzierter DNA-Schäden. Interessant war vor allem der synergistische Effekt nicht genotoxischer Eisendosen im Hinblick auf HNE-induzierte DNA-Schäden. Diese wurden durch eine 24-stündige Vorinkubation mit Fe-NTA signifikant verstärkt. Außerdem kann zusammengefasst werden, dass Fe-NTA in hohen, eher unphysiologischen Konzentrationen die Bildung von ROS vermittelt und zelluläre Effekte hervorruft, die eine Antwort auf zellulären Stress widerspiegeln. Ein karzinogenes Risiko kann jedoch auch aus niedrigen, physiologischen Konzentrationen über pro-genotoxische Effekte und ein erhöhtes Zellwachstum resultieren.

Darstellung des Eigenanteils

- Planung, Durchführung und Auswertung aller praktischer Arbeiten, außer Durchführung und Auswertung der ROS-Bestimmung und Versuche zur Schadenspersistenz
- Erstellen und Korrektur des Publikationsmanuskriptes

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Toxicology in Vitro 20 (2006) 793–800
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells

Y. Knöbel, M. Glei, K. Osswald, B.L. Pool-Zobel *

Institute for Nutrition, Department of Nutritional Toxicology, Biological-Pharmaceutical Faculty, Friedrich-Schiller-University, Dornburger Str. 25, 07743 Jena, Germany

Received 23 June 2005; accepted 25 November 2005
Available online 18 January 2006

Abstract

Iron is a relevant risk factor for colorectal cancer due to its genotoxic properties. Here we hypothesised that iron-overload causes other toxic effects, which contribute to carcinogenesis. For this, we investigated formation of reactive oxygen species (ROS), DNA repair, cell growth and glutathione (GSH) in human colon tumor cells (HT29 clone 19A) treated with ferric nitrilotriacetate (Fe-NTA, 0–2000 μM). Intracellular formation of ROS was analysed with the peroxide-labile fluorescent dye carboxy-dichlorodihydrofluorescine-diacetate. DNA repair, reflected as the persistency of DNA damage induced by selected genotoxins, was determined with the Comet assay. Cell growth and GSH were measured by fluorimetrical analysis. Key findings were that ROS formation increased with time (1000 μM Fe-NTA, $p < 0.001$). DNA damage was largely repaired after 120 min, but was not affected by 10 μM Fe-NTA. In contrast, 10 μM Fe-NTA significantly increased DNA damage induced by 4-hydroxynonenal. Doses of 25 μM Fe-NTA increased cell growth ($p < 0.05$), whereas high concentrations (2000 μM) resulted in growth arrest ($p < 0.05$), that was accompanied by increased GSH levels ($p < 0.01$). In conclusion, high concentrations of Fe-NTA caused cellular effects, which reflect a stress response, and resulted in formation of ROS. Carcinogenic risks from ferric iron could be derived also from lower concentrations, which enhance tumor cell growth and cause progenotoxic effects.

Keywords: Colon cells; Ferric nitrilotriacetate (Fe-NTA); Glutathione (GSH); Reactive oxygen species (ROS); DNA repair

1. Introduction

Colorectal cancers are strongly associated with dietary factors. Epidemiological evidence suggests that high consumption of red meat, fat and alcohol increase risks, whereas physical activity and high vegetable intake decrease risks (Bingham, 1996; Giovannucci and Willett, 1994; Norat et al., 2005; Riboli, 2001; World Cancer Research Fund, 1997). Of these, red meat consumption is considered to increase gut lumen concentrations of iron, which could lead to reactive oxygen species (ROS) via the Fenton reaction (Hata et al., 1997; Imlay et al., 1988). The consequences of this are probably damage in epithelial cells of the colon mucosa (Potter, 1999).

Arrest of cell growth is a reliable indicator of loss of cell viability, which may be used to assess the toxicity of iron in relevant target cells. Opposed to this, iron is an essential growth factor and should promote cell growth at lower concentrations (Kicic and Baker, 1998). Therefore, it was of interest to determine in which concentration ranges iron promotes or inhibits growth of human colon cells. The assumption was that enhancement or suppression of colorectal tumor cell growth could possibly increase or diminish cancer progression.

We have recently demonstrated that the carcinoma cell line HT29 clone 19A, treated with 250 μM Fe-NTA for up

* Corresponding author. Tel.: +49 3641 949670; fax: +49 3641 949672.
E-mail address: b8pobe@uni-jena.de (B.L. Pool-Zobel).
URL: http://www.uni-jena.de/biologie/ieu/et (B.L. Pool-Zobel).

0887-2333/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2005.11.009

to 24 h, rapidly absorbed 50–70% of the dose (Glei et al., 2002). Moreover, Fe-NTA (250–1000 μM) induced DNA strand breaks and oxidized bases, which were enhanced by subsequent exposure to H_2O_2. This led to the suggestion that iron, or its putative capacity to catalyse ROS formation, is an important colon cancer risk factor. In this study, we now aimed to further investigate the hypothesis of possible ROS formation by direct measurements.

Furthermore we also determined possible co-genotoxic effects of Fe-NTA. In particular, we studied the effects of ferric iron on the persistency of DNA damage caused by chemical carcinogens. This was a parameter of interest since different studies have reported that there is a marked interference of carcinogenic metal compounds, such as Ni(II) and Cd(II), with DNA repair processes. Nickel, cadmium, cobalt and arsenic compounds are well-known carcinogens, even though their DNA-damaging potentials are rather weak. It has been hypothesised that low and non-cytotoxic concentrations of these metals can contribute to carcinogenesis by interfering with repair of DNA damage induced by other exposures (Hartwig et al., 2002; Hartwig, 1998; Hartwig and Beyersmann, 1989a; Hartwig and Schwerdtle, 2002; Hartwig et al., 1996). Ni(II), for example, inhibits the removal of UV-light induced DNA damage by interfering with the incision and ligation of the nucleotide excision repair process (Hartwig and Beyersmann, 1989b; Hartwig et al., 1994; Snyder et al., 1989). Cd(II) inhibits the repair of UV-light, benzo[*a*]pyrene- and methyl methanesulfonate-induced DNA damage (Hartman and Speit, 1996; Hartwig et al., 1996; Nocentini, 1987). Opposed to this, however, Abalea and colleagues found that DNA repair pathways were not inhibited, but were actually activated by the iron chelate, ferric nitrilotriacetate (Fe-NTA, 10 and 100 μM), in hepatocyte cultures (Abalea et al., 1998). Thus, these authors reported that DNA repair activity was increased in the presence of Fe-NTA in a time- and dose-dependent manner, although the repair stimulation was not enough to prevent an accumulation of highly mutagenic oxidative products in genomic DNA. In this present study we therefore aimed to assess whether and how Fe-NTA modulates repair of DNA damage in human colon cells, which can presumably be more exposed to an iron-overload (e.g. via the colon lumen) than would be expected for hepatocytes, which are only exposed systemically. As inducers of DNA damage we used model compounds that are suggested to be putative risk factors of colorectal carcinogenesis, namely hydrogen peroxide (H_2O_2), 4-hydroxynonenal (HNE) and 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (NOH-PhIP) (Berlau et al., 2004; Pool-Zobel and Leucht, 1997).

The tripeptid glutathione (GSH) is essential for the detoxification of hydrogen peroxide and lipid peroxides. As cosubstrat of glutathione-*S*-transferases (GSTs) GSH not only protects the cell against reactive electrophiles but also against oxidative and nitrosative stress (Bray and Taylor, 1995; Griffith, 1999; Marquardt and Schäfer, 1997). Seymen and colleagues studied the effect of iron supplementation on erythrocyte metabolism in experimental hyperthyroidism and determined the modulation of GSH levels, as well as of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities (Seymen et al., 1997). They showed that iron supplementation increases GSH, GSH-Px and SOD levels of erythrocytes. Since iron may thus lead to specific types of stress responses and GSH induction may counteract the effects related to ROS toxicity, we here also investigated these associations in the human colon tumor cells.

The objectives of our present study were therefore to investigate, in human colon carcinoma cells, how Fe-NTA influences ROS formation, whether Fe-NTA influences DNA damage induced by putative colon carcinogens, at which concentrations growth of colon cells is differently affected by Fe-NTA, and whether this is associated with a modulated GSH homeostasis. Together these types of molecular and cellular toxic effects during iron-overload in the colonic lumen may enhance processes of initiation and progression of carcinogenesis. In addition to our recently reported findings on genotoxic effects of Fe-NTA in human colon cells, the complementary parameters that were determined in this study, will improve the data basis for risk characterisation of iron.

2. Materials and methods

2.1. Compounds

Ferric nitrate (40 mg) [$Fe(NO_3)_3$] (Sigma–Aldrich, Deisenhofen, Germany) was dissolved in 10 ml double destilled water (ddH_2O) and the chelating agent disodium nitrilotriacetate (NTA) (Sigma–Aldrich, Deisenhofen, Germany) was added (23.5 mg). The pH of the solution was adjusted to 7.4 by sodium hydrogen carbonate ($NaHCO_3$). The final iron concentration of the stock solution was 10 mM.

2.2. Cells and culture conditions

The human colon cell line HT29 was established in 1964 by J. Fogh (Memorial Sloan Kettering Cyncer Cetre, New York) (Rousset, 1986). The clone 19A was terminally differentiated with 5 mM sodium butyrate, and characterised by Augeron and Laboisse (1984). The origin of our cells was a kind gift of Laboisse to G. Rechkemmer (Federal Research Centre, Karlsruhe), from whom we obtained them in passage 12. The cells were grown in tissue culture flasks with Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Eggenstein, Germany) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin at 37 °C in a (95%) humidified incubator (5% CO_2). For this study we used passages 20–50.

2.3. Determination of cell growth—DAPI-assay

The determination of cell growth was performed to define non-toxic concentration ranges for the other investi-

gations described below. Growth of HT29 clone 19A cells was determined in 96-well-microtiter plates. 8000 cells were seeded per well and after 48 h they were treated with Fe-NTA (0–2000 μM) dissolved in Dulbecco's Modified Eagle Medium (DMEM), NTA was used as control, and was also dissolved in the culture medium. Residual cells were determined after 24 h, 48 h, and 72 h of treatment. For this, DNA was quantified by fixing and permeating cells with methanol for 5 min and then adding 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma–Aldrich, Deisenhofen, Germany). DNA content was detected by fluorimetrical analysis with E_x/E_m at 360/465 nm, 30 min after application of DAPI using a Microplate Reader (Spectra Flour Plus, Tecan, Austria; Software: X-Flour). Mean values of at least three independent experiments are shown in the figures.

2.4. Detection of intracellular oxidative stress—ROS-assay

This wide spread method used to detect ROS formation is based on the oxidation of the fluorescent dye 2′,7′-dichlorodihydrofluorescein-diacetate (Batandier et al., 2002). The application of the ROS-assay was based on the method of Srinivasan et al. (2001). 4×10^6 cells were seeded in small culture flasks and harvested after 24 h. Cell number and viability were determined with the trypan blue exclusion test and the cell number was adjusted to 1.2×10^6 cells/ml. The cells were stored on ice until use. H_2O_2 was diluted with PBS (50 and 200 μM) for calibration. The colouring agent carboxy-dichlorodihydrofluorescin-diacetate (C-DCFD) was diluted with PBS (40 μl C-DCFD and 4960 μl PBS). The cells were than placed in a 96-well-microtiter plate, followed by addition of 50 μl C-DCFD and 100 μl of the test compound (0–1000 μM Fe-NTA). In the presence of oxidants, C-DCFD is metabolized to the fluorescent carboxy-dichlorodihydroflurescein (C-HDCF). The fluorescence was detected by fluorometric analysis with E_x/E_m at 485/535 nm, at 37 °C in intervals of 10 min (13 cycles).

2.5. Determination of DNA damage persistency—Comet assay

To investigate the influence of a low iron dose on the persistency of induced DNA damage, HT29 clone 19A cells were incubated for 24 h with a non-genotoxic concentration of Fe-NTA (10 μM). After centrifugation of the harvested cells, the cell pellets were incubated with 75 μM H_2O_2 (5 min, 4 °C), 200 μM HNE or 25 μM NOH-PhIP (30 min, 37 °C). We selected the concentrations of H_2O_2, HNE and NOH-PhIP because they were previously shown to induce DNA damage in HT29 clone 19A cells (unpublished results). The cells were distributed onto slides directly and after 30, 60 and 120 min of incubation (to allow for repair of damage) and the Comet assay was carried out according to the conditions described by Glei et al. (2005). By incubating the lysed cells with repair-specific enzymes directly on the slides, we measured the induction and repair of oxidized DNA bases. For measurement of oxidized pyrimidine bases we used endonuclease III (ENDO) and for measurement of oxidized purine bases we used formamidopyrimidine-glycosylase (Fpg). DNA damage was microscopically quantified using a ZEISS Axiovert 25 microscope (Carl Zeiss Jena GmbH, Jena, Germany) and an image analysis system (Komet 4.0, Kinetic Imaging Corp., Liverpool, UK). Fifty cells were evaluated per slide and the percentage of fluorescence in the tail (TI, "tail intensity") was scored. The values of 3 replications from one experiment were used to calculate the overall means of at least three independently reproduced experiments.

2.6. Quantification of GSH

The measurement of GSH was performed according to the protocol of Habig et al. (1974). HT29 clone 19A cells were seeded in 6-well-plates (0.4×10^6 cells per well). After a preincubation period of 24 h, the cells were incubated with Fe-NTA (0–2000 μM, 24 h). Cell numbers and viabilities of the harvested cells were determined with the trypan blue exclusion test. The cell suspensions were centrifuged (13000 rpm, 10 min, 4 °C). For the GSH measurement, the pellets were resuspended in 5% metaphosphor acid (MPA, 2.5 g in 50 ml) and were placed in ultrasound for 1 min. Fluorescence, which is proportional to the GSH content, was quantified at a wavelength of 400 nm in a microtiter-plate-fluorescence photometer against MPA.

2.7. Statistical evaluation

The GraphPad Prism software Version 4.0 (GraphPad Software Inc., San Diego, USA) was used to establish significance levels. Statistics were carried out on the mean values of independently reproduced experiments, as is also specified in the diagrams and tables. One-way ANOVA with Dunnett's post-test, Bonferroni post-test or two-way ANOVA with Bonferroni post-test were used whenever appropriate.

3. Results

3.1. Effects of iron on cell growth

The results from studying the effects of iron on cell growth helped us to define non-toxic concentrations for other investigations. 25 μM Fe-NTA moderately increased growth of HT29 clone 19A cells after 72 h of incubation (Fig. 1, +14%), whereas very high amounts of Fe-NTA (2000 μM, 24 and 72 h) were needed to cause detectable growth arrest (−15%). All together the effects on cell growth were only marginal. For our investigations we therefore used concentrations ranging from 10 to 1000 μM Fe-NTA, which were considered to be non-cytotoxic.

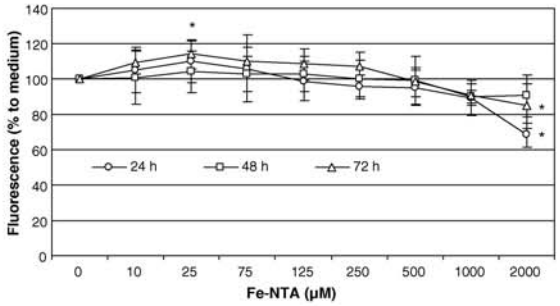


Fig. 1. Growth of HT29 clone 19A cells after 24, 48 and 72 h of treatment with Fe-NTA (0–2000 µM). Shown are mean values and standard deviation from independent experiments (24 h, 48 h, $n = 4$; 72 h, $n = 8$). Statistical evaluation was performed applying one-way ANOVA combined with Dunnett's multiple comparison test ($^{*}p < 0.05$) against medium control.

3.2. Effects of iron on ROS formation

Under in vivo conditions iron overload in the gut lumen and in the colonocytes may result in Haber–Weiss and Fenton-type reactions thus yielding radicals from peroxides. These radicals in turn may be toxic and increase the risks of colorectal cancers (Everse and Hsia, 1997; Imlay et al., 1988; Tseng et al., 1997). To find experimental support for this hypothesis, we determined the intracellular ROS formation in cells treated with increasing concentrations of Fe-NTA (10–1000 µM). We observed a time- and dose-related increase of intracellular fluorescence. Table 1 shows kinetics of ROS formation in HT29 clone 19A cells treated with 1000 µM Fe-NTA. The treatment of cells for 80–120 min with Fe-NTA resulted in a significantly higher ROS formation than NTA, (solvent control) or PBS, (negative control). In comparison to our negative control PBS, the chelating agent NTA also induced a significantly higher formation of ROS after 100–120 min, but lesser than Fe-NTA.

3.3. Effects of iron on DNA damage and its persistency (repair)

H_2O_2 (75 µM) induced significant damage (Table 2, TI 48.6%) which, however, was partially repaired after 120 min postincubation (TI 13.5%). Fe-NTA (10 µM) did not affect H_2O_2-induced DNA damage nor its persistency. In addition to single strand breaks H_2O_2 also induced oxidized bases. Oxidized purine bases were also repaired significantly during the postincubation period, as were pyrimidine bases as well (data not shown).

NOH-PhIP (25 µM) also induced DNA damage, which was repaired by about 30–40% after 30–120 min of postincubation (Table 2). Preincubation with the non-genotoxic Fe-NTA concentration of 10 µM did not affect NOH-PhIP-induced damage or its repair.

Furthermore, Table 2 shows that cells preincubated with 10 µM Fe-NTA had significantly higher levels of HNE-induced DNA damage (30% higher), a difference which persisted after 120 min of repair.

3.4. Effects of iron on GSH level

Reduced GSH catalyses the detoxification of ROS and free radicals. We therefore investigated the influence of iron incubation on the GSH levels of the colon tumor cells. High Fe-NTA (1000, 2000 µM) concentrations significantly increased the GSH content in HT29 clone 19A cells (Fig. 2, 2-fold).

Table 1
Absolute fluorescence as a sign of ROS formation, respectively, intracellular oxidative stress after incubation of HT29 clone 19A cells with 1000 µM Fe-NTA, NTA and PBS ($n = 3$)

Point of measurement after incubation (min)	Fe-NTA		NTA		PBS	
	Mean	SD	Mean	SD	Mean	SD
0	−1.4	2.2	−4.2	2.3	−0.4	3.3
10	−2.3	3.4	1.1	0.5	−1.1	0.8
20	3.4	4.0	2.4	2.3	−1.1	3.2
30	8.5	6.5	6.2	3.0	−0.6	1.6
40	19.6	9.3	14.4	3.9	−0.2	2.7
50	34.4	18.4	18.3	2.0	2.5	4.2
60	55.2	30.5	29.9	2.7	5.5	6.8
70	77.7	40.2	46.4	1.0	8.9	11.8
80	107.4 a*	53.7	59.2	3.0	12.2	15.8
90	138.7 a**b**	64.4	76.0	3.2	18.7	25.3
100	175.4 a***b***	84.0	93.8	1.0	23.1	28.2
110	217.2 a***b***	104.6	116.0	4.7	30.8	41.5
120	258.2 a***b***	122.9	139.6	7.7	39.6	54.6

Statistical significance was tested by two-way ANOVA ($p < 0.001$), including Bonferroni's post-test (a: versus NTA, b: versus PBS).
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 2
Persistency of H_2O_2-, HNE- and NOH-PhIP-induced DNA damage after preincubation with 10 μM Fe-NTA (*t* 0 min: time point after 30 min of incubation with H_2O_2-, HNE- or NOH-PhIP, starting point of repair; *t* 30 min: after 30 min of repair; *t* 60 min: after 60 min of repair; *t* 120 min: after 120 min of repair)

Preincubation (μM)	Damaging agent (μM)	Tail intensity (%)							
		t 0 min		*t* 30 min		*t* 60 min		*t* 120 min	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fe	H_2O_2 ($n = 3$)								
0	0	2.4	0.3	2.8	0.8	3.4	1.2	3.7	1.0
10	0	2.9	0.5	2.7	0.5	2.6	0.9	2.9	0.8
0	75	48.6	11.3	21.2**	1.7	15.3**	2.4	13.5**	6.1
10	75	43.3	11.4	21.3**	7.5	16.3**	3.6	11.8**	4.0
Fe	NOH-PhIP ($n = 5$)								
0	0	2.2	0.5	2.2	0.4	2.5	0.5	2.2	0.4
10	0	1.7	0.3	2.1	0.4	1.9	0.3	1.9	0.8
0	25	17.5	12.8	12.6	9.0	13.0	12.0	11.3	11.6
10	25	20.1	10.4	13.4	9.2	11.6	6.8	10.3	7.7
Fe	HNE ($n = 3$)								
0	0	1.7	0.2	1.9	0.6	1.8	0.5	2.1	0.7
10	0	1.4	0.5	1.7	0.4	1.7	0.3	1.4	1.2
0	200	14.8	3.0	10.4*	2.3	8.2**	1.7	7.6**	0.2
10	200	21.9###	1.7	14.0**,#	2.8	12.1**,#	2.4	11.8**,#	2.1

The significance of the time-dependent repair was calculated by one-way ANOVA (H_2O_2, $p < 0.001$; HNE, $p < 0.001$; NOH-PhIP, ns.), including Dunnett's multiple comparison post-test; $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from timepoint *t* 0. The differences between Fe-NTA preincubation versus non-preincubation were tested by two-way ANOVA, including Bonferroni's post-test; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$. Persistency of H_2O_2-, HNE- and NOH-PhIP-induced DNA damage after preincubation with 10 μM Fe-NTA. The significance of the time-dependent repair was calculated by one-way ANOVA (H_2O_2, $p < 0.001$; HNE, $p < 0.001$; NOH-PhIP, ns.), including Dunnett's multiple comparison post-test; $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from timepoint *t* 0. The differences between Fe-NTA preincubation versus non-preincubation were tested by two-way ANOVA, including Bonferroni's post-test; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$.

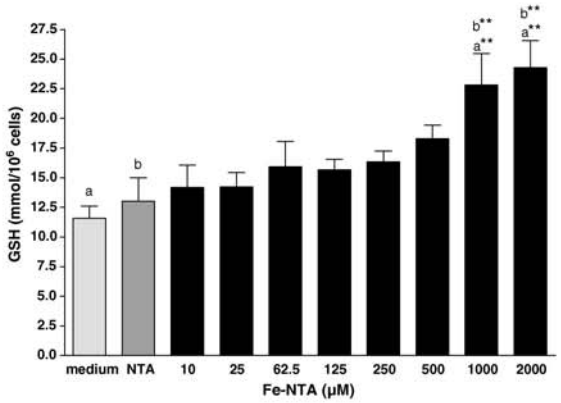


Fig. 2. GSH levels of HT29 clone 19A cells after incubation with 0–2000 μM Fe-NTA (24 h at 37 °C). Shown are mean values and standard derivation from independent experiments by reproduced values ($n = 3$). The significance of individual compound-induced effects was calculated by one-way ANOVA (a: versus medium, $p < 0.001$; b: versus NTA, $p < 0.001$), including Bonferroni's multiple comparison test; $^{**}p < 0.01$.

4. Discussion

Different compounds from dietary or microbial origin or from digestive and excretory processes may be risk factors for colon cancer. The genotoxic potency of these compounds, the ability of the mucosal cells to deactivate the compounds, and the repair of induced damage may influence the impact of risk factors on the epithelial colon cells.

For HT29 clone 19A cells we could show that low levels of Fe-NTA (25 μM) resulted in a slight but significant increase in cell growth (+14%). In contrast, very high amounts of Fe-NTA (2000 μM) caused a growth arrest (−15%) and increased GSH levels, which possibly reflect a stress response to iron-overload. Since most heavy metals have a high affinity to SH groups, two different mechanisms of defensive action of SH compounds are imaginable. One mechanism is the direct action as a chelating agent, and the second is the indirect action as an antioxidant (Hirayama and Yasutake, 1998). Yasutake and Hirayama observed increased GSH levels in kidney and plasma but decreased hepatic levels after 24 h administration of mice with a sublethal MeHg dose (Yasutake and Hirayama, 1994). The decreased GSH levels in the liver could be a result of the enhanced GSH turnover for the elimination of accumulated MeHg in the other tissues. The kidney, the final excretion organ for MeHg, showed increased GSH levels as a result of gamma-glutamylcysteine synthetase (γ-GCS)-stimulation. A similar mechanism could be the reason for the increased GSH level after Fe-NTA treatment of our HT29 clone 19A cells, because compounds, which induce oxidative stress increase the transcription of the γ-GCS genes and induce GSH synthesis (Dickinson and Forman, 2002). Heavy metal-induced elevation of GSH levels have also been reported in other organs and cultured cells.

Standeven and Wetterhahn measured increased hepatic GSH levels in rats treated with a moderately toxic dose of hexavalent chromium (Standeven and Wetterhahn, 1991). In vitro investigations showed a significant increase of intracellular GSH in cadmium-treated cultured mesangial cells and arsenite-treated Swiss 3T3 cells (Chin and Templeton, 1993; Li and Chou, 1992). These results suggest that the GSH elevation might reflect a self-protective mechanism against cellular injury and may be interpreted as a defensive response of the cells.

Iron or the formation of reactive oxygen species (ROS) via the Fenton reaction, is a very potent risk factor for colon cancer which possibly predisposes to colon diseases by some of these mechanisms (Aruoma et al., 1989; Babbs, 1992; Imlay et al., 1988; Kitahora et al., 1988). For instance, ROS play an important role in the promotion phase of tumor generation by contributing to the regulation of gene expression during cell growth and differentiation (Crawford et al., 1988). Lund et al. showed that iron or ROS is associated with changes in crypt cell proliferation in rat large intestine (Lund et al., 1998). In a former study we investigated the iron uptake by the colon cells and could show, that after 15 min 48% of the iron was already detectable in the cells (Glei et al., 2002). This increased intracellular iron concentration may be expected to facilitate the formation of ROS via ferrous iron Fe(II). Iron uptake by colonic cells, iron metabolism, transport and secretion are complex and tightly regulated mechanisms (Richardson and Ponka, 1997). In spite of that, an iron-overload in the gut is possible and this may result in an increased oxidative stress (Erhardt et al., 1998; Lund et al., 1999). We observed a time- and dose-related increase of intracellular fluorescence and consequently of ROS formation, which indicates that these associations do occur during extreme exposure situations. The exposure, however, was only 3 times higher than that previously reported to occur in the gut lumen. Thus, Lund et al. reported that the concentration of water-soluble iron in the gut lumen is normally around 25 μM, but could rise to >100 μM in human feces, with a total iron concentration reaching 350 μM in the intraluminal pool after oral supplementation of ferrous sulfate (Lund et al., 1998, 1999).

Another question was whether low doses of Fe-NTA affected the repair of DNA damage. The present study reported that low doses of Fe-NTA did not influence the persistency of H_2O_2-, HNE- and NOH-PhIP-induced DNA damage. Thus, we neither observed an inhibition of DNA repair, as has been reported for Ni or Cd, nor did we observe the enhanced DNA repair activity as has been reported for Fe-NTA in hepatocytes. We did, however, see an important co-genotoxic effect of Fe-NTA on the genotoxic action of HNE. This possibly demonstrates over-additive actions of HNE and very low Fe-NTA doses (10 μM). HNE is a major reactive product of lipid peroxidation caused by oxidative stress. It is formed by radical-initiated degradation of ϖ-6-polyunsaturated fatty acids such as linoleic acid and arachidonic acid, two relatively abundant fatty acids in human cells. Products of lipid peroxidation cause cell damage in model systems (Abrahamse et al., 1999; Gölzer et al., 1996) and global DNA damage in human colon cells (Schäferhenrich et al., 2003a,b). Possible mechanisms involved in the enhancement of HNE-induced damage are speculative, but could involve similar reactions of HNE with metal ions as have been reported for H_2O_2 (Termini, 2000). Thus, in analogy to the Fenton-catalysed decomposition of H_2O_2, alkyl hydroperoxides (from HNE) could be reduced to alkoxyl radicals by iron complexes (Winston et al., 1983). Hammer et al. e.g. found an effect of oxidative stress by iron on HNE formation in hepatoma cells (Hammer et al., 1997). In addition, an iron-mediated increase of lipid peroxidation is conceivable. Transition metals such as iron are powerful catalysts for hydroxyl radical formation and lipid peroxidation (Goddard and Sweeney, 1983; Halliwell and Gutteridge, 1984; Sakurai and Cederbaum, 1998). Lipid peroxidation, and therefore formation of HNE, was observed in kidney and liver homogenates of rats treated with Fe-NTA (Lamb et al., 1999; Toyokuni et al., 1994). Toyokuni et al. observed generation of HNE after the treatment with Fe-NTA (15 mg/kg) with a maximum occurring after 1 h. However, it would be conceivable that iron in vitro also enhances the oxidation of lipids in the cells or in the medium to yield reactive products like HNE. The iron-catalysed lipid peroxidation and an increased content of HNE and other products could explain our observations.

In conclusion, we could show that high concentrations (1000–2000 μM) of Fe-NTA inhibited cell growth, enhanced GSH and resulted in formation of ROS. These are considered to be cellular effects which reflect a stress response. Carcinogenic risks from ferric iron, however, could be derived also from lower concentrations about 25 μM. These could promote tumor cell growth. Additional risks may be encountered at 10 μM, a concentration which markedly enhanced the genotoxic effects of HNE, a relevant genotoxic product of lipid peroxidation.

Acknowledgments

This work has been carried out with financial support by Deutsche Forschungsgemeinschaft Project Number PO 284/6-1 and PO 284/6-2. Furthermore, we are thankful to Dr. William M. Pool for editorial assistance.

References

Abalea, V., Cilliard, J., Dubos, M.P., Anger, J.P., Cilliard, P., Morel, I., 1998. Iron-induced oxidative DNA damage and its repair in primary rat hepatocyte culture. Carcinogenesis 19, 1053–1059.

Abrahamse, S.L., Pool-Zobel, B.L., Rechkemmer, G., 1999. Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration in isolated rat colon cells. Carcinogenesis 20, 629–634.

Aruoma, O.I., Halliwell, B., Gajewski, E., Dizdaroglu, M., 1989. Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. The Journal of Biological Chemistry 264, 20509–20512.

Augeron, C., Laboisse, C.L., 1984. Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. Cancer Researches 44, 3961–3969.

Babbs, C.F., 1992. Oxygen radicals in ulcerative colitis. Free Radical Biology and Medicine 13, 169–181.

Batandier, C., Fontaine, E., Keril, C., Levere, X.M., 2002. Determination of mitochondrial reactive oxygen species: methodological aspects. Journal of Cellular and Molecular Medicine 6, 175–187.

Berlau, J., Glei, M., Pool-Zobel, B.L., 2004. Colon cancer risk factors from nutrition. Analytical Bioanalytical Chemistry 30, 737–743.

Bingham, S.A., 1996. Epidemiology and mechanisms relating diet to risk of colorectal cancer. Nutrition Research Reviews 9, 197–239.

Bray, T.M., Taylor, C.G., 1995. Tissue glutathione, nutrition and oxidative stress. Canadian Journal of Physiology and Pharmacology 71, 746–751.

Chin, T.A., Templeton, D.M., 1993. Protective elevation of glutathione and metallothionein in cadmium-exposed mesangial cells. Toxicology 77, 145–156.

Crawford, D., Zbinden, I., Amstad, P., Cerutti, P.A., 1988. Oxidant stress induces the proto-oncogenes c-*fos* and c-*myc* in mouse epidermal cells. Oncogene 3, 27–32.

Dickinson, D.A., Forman, H.J., 2002. Cellular glutathione and thiols metabolism. Biochemical Pharmacology 64, 1019–1026.

Erhardt, J.G., Lim, S.S., Bode, J.C., Bode, C., 1998. A diet rich in fat and poor in dietary fiber increases the in vitro formation of reactive oxygen species in human feces. Journal of Nutrition 127, 706–709.

Everse, J., Hsia, N., 1997. The toxicities of native and modified hemoglobins. Free Radical Biology and Medicine 22, 1075–1099.

Giovannucci, E., Willett, W.C., 1994. Dietary factors and risk of colon cancer. Annals of Medicine 26, 443–452.

Glei, M., Klinder, A., Latunde-Dada, G.O., Becker, T.W., Hermann, U., Voigt, K., Pool-Zobel, B.L., 2002. Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A. Mutation Research 519, 151–161.

Glei, M., Habermann, N., Osswald, K., Seidel, C., Persin, C., Jahreis, G., Pool-Zobel, B.L., 2005. Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the Comet assay: a biomarker model. Biomarkers 10, 203–217.

Goddard, J.G., Sweeney, G.D., 1983. Fe-NTA: a potent stimulant of in vivo lipid peroxidation in mice. Biochemical Pharmacology 32, 3879–3882.

Gölzer, P., Janzowski, C., Pool-Zobel, B.L., Eisenbrand, G., 1996. Hexenal-induced DNA damage and formation of cyclic 1,N^2-(1,3-propano)-2′-deoxygunanosine adducts in mammalian cells. Chemical Research in Toxicology 9, 1207–1213.

Griffith, O.W., 1999. Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radical Biology and Medicine 27, 922–935.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferase. The first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249, 7130–7139.

Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radicals, transition metals, and disease. Biochemical Journal 219, 1–14.

Hammer, A., Ferro, M., Tillian, H.M., Tatzbar, F., Zollner, H., Schauenstein, E., Schaur, R.J., 1997. Effect of oxidative stress by iron on 4-hydroxynonenal formaton and proliferative activity in hepatomas of different degrees of differentiation. Free Radical Biology and Medicine 23, 26–33.

Hartman, A.M., Speit, G., 1996. Effect of arsenic and cadmium on the persistence of mutagen-induced DNA lesions in human cells. Environmental and Molecular Mutagenesis 27, 98–104.

Hartwig, A., 1998. Carcinogenity of metal compounds: possible role of DNA repair inhibition. Toxicological Letter 28, 235–239.

Hartwig, A., Beyersmann, D., 1989a. Comutagenicity and inhibition of DNA repair by metal ions in mammalian cells. Biological Trace Elements Research 21, 359–365.

Hartwig, A., Beyersmann, D., 1989b. Enhancement of UV-induced mutagenesis and sister-chromatid exchanges by nickel ions in V79 cells: evidence for inhibition of DNA repair. Mutation Research 217, 65–73.

Hartwig, A., Schwerdtle, T., 2002. Interactions by carcinogenic metal compounds with DNA repair processes: toxicological implications. Toxicological Letter 127, 47–54.

Hartwig, A., Mullender, L.H.F., Schlepergrell, R., Kasten, U., 1994. Nickel(II) interferes with the incision step in nucleotide excision repair. Cancer Research 54, 4045–4051.

Hartwig, A., Schlepergrell, R., Dally, H., Hartmann, M., 1996. Interaction of carcinogenic metal compounds with deoxyribonucleic acid repair process. Annals of Clinical and Laboratory Sciences 26, 31–38.

Hartwig, A., Asmuss, M., Ehleben, I., Hetzer, U., Kastelac, D., Pelzer, A., Schwerdtle, T., Burkle, A., 2002. Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanism. Environmental Health Perspectives 110, 797–799.

Hata, Y., Kawabe, T., Hiraishi, H., Ota, S., Terano, A., Ivey, K.J., 1997. Hydrogen peroxide-mediated cytotoxicity to cultured colonic epithelial cells. Life Sciences 60, 2221–2230.

Hirayama, K., Yasutake, A., 1998. Free radicals and trace elements. Journal of Trace Elements and Experimental Medicine 11, 209–217.

Imlay, J., Chin, S.M., Linn, S., 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science, 640–642.

Kicic, A., Baker, E., 1998. The effects of iron chelators Upon cellular proliferation and ion transport in hepatoma cells. INABIS'98—5th Internet World Congress on Biomedical Sciences, Toronto, Canada, URL <http://www.mcmaster.ca/inabis98/>.

Kitahora, T., Suzuki, K., Asakura, H., Yoshida, T., Suematsu, M., Watanabe, M., 1988. Active oxygen species generated by monocytes and polymorphonuclear cells in Crohn's disease. Digestive Diseases and Sciences 33, 951–955.

Lamb, N., Quinlan, G.J., Mumby, S., Evans, T.W., Gutteridge, M.C., 1999. Haem oxygenase shows pro-oxidant activity in microsomal and cellular systems: implications for the release of low-molecular-mass iron. Biochemical Journal 344, 153–158.

Li, W., Chou, I.-N., 1992. Effects of arsenite on the cytoskeleton and cellular glutathione levels in cultured cells. Toxicology and Applied Pharmacology 114, 132–139.

Lund, E.K., Wharf, S.G., Fairweather-Tait, S.J., Johnson, I.T., 1998. Increases in the concentrations of available iron in response to dietary iron supplementation are associated with changes in crypt cell proliferation in rat large intestine. Journal of Nutrition 128, 175–179.

Lund, E.K., Wharf, S.G., Fairweather-Tait, S.J., Johnson, I.T., 1999. Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. American Journal of Clinical Nutrition 69, 250–255.

Marquardt, H., Schäfer, S.G., 1997. Lehrbuch der Toxikologie. Spektrum Akademischer Verlag, Berlin.

Nocentini, S., 1987. Inhibition of DNA replication and repair by cadmium in mammalian cells. Protective interaction of zinc. Nucleic Acids Research 15, 4211–4225.

Norat, T., Bingham, S., Ferrari, P., Slimani, N., Jenab, M., Mazuir, M., Overvad, K., Olsen, A., Tjønneland, A., Clavel, F., Boutron-Ruault, M.-C., Kesse, E., Bergmann, M.M., Nieters, A., Linseisen, J., Trichopulou, A., Trichopoulou, D., Tountas, Y., Berrino, F., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., Peeters, P.H.M., Engeset, D., Lund, E., Skeie, G., Ardanaz, E., González, C., Navarro, C., Quirós, J.R., Sanchez, M.-J., Berglund, G., Mattisson, I., Hallmans, G., Palmquist, R., Day, N.E., Khaw, K.-T., Key, T.J., San Joaquin, M., Hémon, B., Saracci, R., Kaaks, R., Riboli, E., 2005. Meat, fish, and colorectal cancer risk: the European prospective investigation into cancer and nutrition. Journal of the National Cancer Institute 97, 906–916.

Pool-Zobel, B.L., Leucht, U., 1997. Induction of DNA damage in human colon cells derived from biopsies by suggested risk factors of colon cancer. Mutation Research 375, 105–116.

Potter, J.D., 1999. Colorectal cancer: molecules and populations. Journal of the National Cancer Institute 91, 916–932.

Riboli, E., 2001. The European prospective investigation into cancer and nutrition (EPIC): plans and progress. Journal of Nutrition 131, 170S–175S.

Richardson, D.R., Ponka, P., 1997. The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. Biochimica et Biophysica Acta 1331, 1–40.

Rousset, M., 1986. The human colon carcinoma cell lines HT-29 and Caco-2: two in vitro models for the study of intestinal differentiation. Biochimie 68, 1035–1040.

Sakurai, K., Cederbaum, A., 1998. Oxidative stress and cytotoxicity induced by ferric-nitrilotriacetate in HepG2 cells that express cytochrome P450 2E1. Molecular Pharmacology 54, 1024–1035.

Schäferhenrich, A., Beyer-Sehlmeyer, G., Festag, G., Kuechler, A., Haag, N., Weise, A., Liehr, T., Claussen, U., Marian, B., Sendt, W., Scheele, J., Pool-Zobel, B.L., 2003a. Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. Mutation Research 9496, 1–14.

Schäferhenrich, A., Sendt, W., Scheele, J., Kuechler, A., Liehr, T., Claussen, U., Rapp, A., Greulich, K.O., Pool-Zobel, B.L., 2003b. Endogenously formed cancer risk factors induce damage-of p53 in human colon cells obtained from surgical samples. Food and Chemical Toxicology 41, 655–664.

Seymen, O., Seven, A., Candan, G., Yigit, G., Hatemi, S., Hatemi, H., 1997. The effect of iron supplementation on GSH levels, GSH-Px, and SOD activities in L-thyroxine administration. Acta Medicinae Okayama 51, 129–133.

Snyder, R.D., Davis, G.F., Lachmann, P.J., 1989. Inhibition by metals of X-ray and ultraviolet-induced DNA repair in human cells. Biological Trace Elements Research 21, 389–398.

Srinivasan, A., Lehmler, H.-J., Robertson, L.W., Ludewig, G., 2001. Production of DNA strand breaks in vitro and reactive oxygen species in vitro and in HL-60 cells by PCB metabolites. Toxicological Sciences 60, 92–102.

Standeven, A.M., Wetterhahn, K.E., 1991. Possible role of glutathione in chromium(VI) metabolism and toxicity in rats. Pharmacology and Toxicology 69, 469–476.

Termini, J., 2000. Hydroperoxide-induced DNA damage and mutations. Mutation Research 450, 107–124.

Toyokuni, S., Uchida, K., Okamoto, K., Hattori-Nakakuki, Y., Hiai, H., Stadtman, E.R., 1994. Formation of 4-hydroxy-2-nonenal-modified proteins in the renal proximal tubules of rats treated with a renal carcinogen, ferric nitrilotriacetate. Proceedings of the National Academy of Sciences of the United States of America 91, 2616–2620.

Tseng, M., Sandler, R.S., Greenberg, E.R., Mandel, J.S., Haile, R.W., Baron, J.A., 1997. Dietary iron and recurrence of colorectal adenomas. Cancer Epidemiology, Biomarkers and Prevention 6, 1029–1032.

Winston, G.W., Harvey, W., Berl, L., Cederbaum, A.L., 1983. The generation of hydroxy and alkoxyl radicals from the interaction of ferrous bipyridyl with peroxides. Biochemical Journal 216, 421.

World Cancer Research Fund and American Institute for Cancer Research, 1997. Food, Nutrition and the Prevention of Cancer: A Global Perspective. American Institute for Cancer Research, Washington, DC. p p. 1–670.

Yasutake, A., Hirayama, K., 1994. Acute effects of methylmercury on hepatic and renal glutathione metabolism in mice. Archives of Toxicology 68, 512–516.

3.2 Manuskript II

Ferric iron is genotoxic in non-transformed and preneoplastic human colon cells

Y. Knöbel, A. Weise, M. Glei, W. Sendt, U. Claussen, B. L. Pool-Zobel

zur Publikation angenommen bei Food and Chemical Toxicology am 30. Oktober 2006, online publiziert doi:10.1016/j.fct.2006.10028

Zusammenfassung

Eisen ist ein relevanter Krebsrisikofaktor, da es die Bildung von ROS katalysiert, welche wiederum die DNA schädigen. In der vorliegenden Arbeit wurde Eisen in primären, nicht transformierten Kolonepithelzellen und in der preneoplastischen Kolonadenomzelllinie LT97 hinsichtlich seines genotoxischen Potentials untersucht. Ausserdem wurde die Sensitivität des *TP53*-Gens gegenüber Eisen geprüft.

Fe-NTA induzierte signifikant Einzelstrangbrüche in primären Kolonzellen und in LT97-Zellen. Bereits für geringe Konzentrationen (100 und 250 µM Fe-NTA) konnten mittels Comet FISH signifikant erhöhte genspezifische *TP53*-Schadensraten ermittelt werden. Dies spricht für eine hohe Sensitivität dieses tumorrelevanten Gens gegenüber Fe-NTA.

Überträgt man diese Ergebnisse auf die *in vivo*-Situation, birgt Eisenüberschuss aufgrund des genotoxischen Potentials in humanen Kolonzellen ein potentielles kanzerogenes Risiko.

Darstellung des Eigenanteils

- Planung, Durchführung und Auswertung aller praktischer Arbeiten
- Erstellen und Korrektur des Publikationsmanuskriptes

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

Available online at www.sciencedirect.com
ScienceDirect

Food and Chemical Toxicology xxx (2006) xxx–xxx

www.elsevier.com/locate/foodchemtox

Ferric iron is genotoxic in non-transformed and preneoplastic human colon cells

Y. Knöbel [a], A. Weise [b], M. Glei [a], W. Sendt [c], U. Claussen [b], B.L. Pool-Zobel [a,*]

[a] *Institute of Nutrition, Department of Nutritional Toxicology, Friedrich-Schiller-University, Dornburger Str. 25, 07743 Jena, Germany*
[b] *Institute for Human Genetics and Antropology, Friedrich-Schiller-University, Kollegiengasse 10, 07743 Jena, Germany*
[c] *Department of Surgery, Friedrich-Schiller-University, Erlanger Allee 101, 07743 Jena, Germany*

Received 15 November 2005; accepted 30 October 2006

Abstract

Iron could be a relevant risk factor for carcinogenesis since it catalyses the formation of reactive oxygen species (ROS), which damage DNA. We previously demonstrated genotoxic effects by ferric iron using the human colon cancer cell line HT29. Here we investigated ferric iron in primary non-transformed colon cells and in a preneoplastic colon adenoma cell line (LT97), which both are suitable models to study effects of carcinogens during early stages of cell transformation. Genetic damage was determined using the Comet assay. Comet FISH (fluorescence *in situ* hybridization) was used to assess specific effects on *TP53*. Fe-NTA (0–1000 μM, 30 min, 37 °C) significantly induced single strand breaks in primary colon cells (500 μM Fe-NTA: Tail intensity [TI] 22.6% ± 5.0% versus RPMI control: TI 10.6% ± 3.9%, $p < 0.01$) and in LT97 cells (1000 μM Fe-NTA: TI 26.8% ± 7.3% versus RPMI control: TI 11.1% ± 3.7%, $p < 0.01$). With the Comet FISH protocol lower concentrations of Fe-NTA significantly increased DNA damage already at 100 and 250 μM Fe-NTA in primary colon and LT97 adenoma cells, respectively. This damage was detected as an enhanced migration of *TP53* signals into the comet tail in both cell types, which indicates a high susceptibility of this tumor relevant gene towards Fe-NTA. In conclusion, Fe-NTA acts genotoxic in non-transformed and in preneoplastic human colon cells, in which it also enhances migration of *TP53* at relatively low concentrations. Translated to the *in vivo* situation these results suggest that iron overload putatively contributes to a genotoxic risk during early stages of colorectal carcinogenesis on account of its genotoxic potential in non-tumorigenic human colon cells.

Keywords: Primary human colon cells; Human colon adenoma cells; Comet assay; Comet FISH; Fe-NTA

1. Introduction

Nutritional factors are strongly associated with colorectal carcinogenesis as has been hypothesized in numerous observational and experimental studies. Evidence from epidemiological studies suggests that there is a higher risk for development of this disease after high consumption of red and processed meat, animal fat and alcohol. Opposed to this adequate levels of physical activity and high vegetable intake decrease risks (Bingham, 1996; Giovannucci and Willett, 1994; Boutron-Ruault et al., 2001). The immediate mechanisms involved are probably increases of genotoxic carcinogens and toxic compounds in the gut lumen. These compounds may be directly available from the ingested foods or may be metabolites which are produced endogenously (Berlau et al., 2004). Their contribution is to enhance cancer progression via different mechanisms (World Cancer Research Fund, 1999). Risk factors derived from red meat, for example, include heterocyclic amines,

Abbreviations: DNA, desoxy ribonucleic acid; *APC*, adenomatous polyposis coli; ddH_2O, double distilled water; FCS, fetal calf serum; Fe-NTA, ferric nitrilotriacetate; FISH, fluorescence in situ hybridization; *K-RAS*, Kirsten rat sarcoma; HNE, 4-hydroxynonenal; H_2O_2, hydrogen peroxide; NTA, nitrilotriactetate; ROS, reactive oxygen species; SD, standard derivation; TI, tail intensity; *TP53*, tumor protein p53.

* Corresponding author. Tel.: +49 3641 949670; fax: +49 3641 949672.
E-mail address: b8pobe@uni-jena.de (B.L. Pool-Zobel).
URL: www.uni-jena.de/biologie/ieu/et (B.L. Pool-Zobel).

0278-6915/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2006.10.028

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

polycyclic aromatic hydrocarbons and hemoglobin (Sinha et al., 2001; Muscat and Wynder, 1994; Pierre et al., 2003). The data of Norat et al. confirm that colorectal cancer is positively associated with high consumption of red and processed meat as a relevant source of hemoglobin (Norat et al., 2005). Hemoglobin is a source of heme, which acts toxic and genotoxic (Glei et al., 2006). The porphyrine structure of heme contains iron, which also acts genotoxic in human colon cells (Glei et al., 2002). Moreover, there are also epidemiological studies available showing that an iron overload is related to an increase of colorectal cancer as well (Senesse et al., 2004; Wurzelmann et al., 1996). Shaheen et al. described that there is an association between iron exposure and colorectal carcinogenesis in highly susceptible individuals with genetic based iron storage deficiencies (Shaheen et al., 2003), although in another cohort no association between iron stores and the risk of colorectal cancer was observed (Herrinton et al., 1995). Next to mutations in the hemochromatosis (HFE) gene, mutations in the recently identified heme transporter HCP1 could also be associated with increased total body iron stores and hence with increased cancer risk in some individuals (Shayeghi et al., 2005). Cross et al. however, published data of a nested case-cohort study which was, however, did not support previous data on risk enhancing properties of iron, namely an inverse association between several serum iron indices and colon cancer risk (Cross et al., 2006). These findings exemplify why the role of ferric iron and associated compounds in the process of carcinogenesis is still unclear. More basic knowledge is needed among others on concentration effects and on the sensitivity of human colon cells to iron in order to understand more on mechanisms and possibly resolve some of the discrepancies.

Fe-NTA is a suited model compound to cause iron overload, and moreover has a similar pattern of genotoxic activity as an important physiological source of iron which is hemoglobin (Glei et al., 2006). Concentrations used in this recently published study covered a broad range from 1 to 1000 μM and the tested amounts included concentrations that had been reported to occur in the colon after dietary iron supplementation *in vivo*. Lund et al. for instance reported that the concentration of iron in the active intraluminal pool during the intervention with ferrous sulphate (100 mg/d) increased from 60 μmol/l up to 350 μmol/l (Lund et al., 1999). Ferrous iron is primarily used for the enrichment of food, but also ferric iron. The bioavailability of ferrous salts are higher than that of ferric salts, however, ferrous salts are considerably more reactive in foods, and oxidize faster and convert to ferric iron (Cook and Reuser, 1983).

The most important target cells of colon carcinogenesis are epithelial stem cells or the migrating daughter cells, both located at the bottom of the colon crypts (Bach et al., 2000; Preston et al., 2003). In these cells, mutations in tumor relevant genes, namely *APC*, *K-RAS* and *TP53*, are the key molecular mechanism which drive transformation of normal cells into tumor cells (Fearon and Vogelstein, 1990; Renehan et al., 2002; Leslie et al., 2002). The first of these genetic lesions probably often occur in *APC* and *K-RAS* (Fodde et al., 2001). The resulting downstream alterations (lack of *APC* protein, activated *K-RAS* protein) then transform normal cells into more rapidly proliferating cells to yield lesions detectable as dysplasia and adenoma. These types of preneoplastic cells conceivably may be further targeted by genotoxic risk compounds (Berlau et al., 2004). In particular subsequent genetic alterations in *TP53* may be the cause for the transition of adenoma cells into carcinoma cells (Kinzler and Vogelstein, 1996; Lane, 1992). Since adenomatous preneoplastic lesions occur frequently with increasing age and are related to diet (Heavey et al., 2004), they may be just as important target cells for cancer progression as normal cells are for cancer initiation. It is thus self evidently important to study the sensitivities of normal epithelial cells and of adenoma cells to genotoxic risk factors to learn more about the associations between exposure and colorectal carcinogenesis (Pool-Zobel and Leucht, 1997).

For the example of the aforementioned risk factor iron, we have recently demonstrated that this is genotoxic in the carcinoma cell line HT29 clone 19A, which were treated up to 24 h with 250 μM Fe-NTA, rapidly absorbed 50–70% of the given dose. Moreover we were able to show that Fe-NTA (250–1000 μM) caused DNA breaks and oxidised bases, which were enhanced by subsequent H_2O_2- or HNE-exposure (Glei et al., 2002; Knöbel et al., 2006). These results allowed us to conclude that iron or its capacity to catalyse ROS formation, is an important colon cancer risk factor. The HT29 clone 19A cells, used for these investigations, however, were a differentiated, but still highly transformed cell line that had originally been derived from the parent HT29 tumor cell line. An unanswered question was therefore, how do iron-derived compounds affect non-transformed cells that occur in the normal colon mucosa. Thus, here we used primary human colon cells and human colon adenoma cells which both express different levels of genes related to drug metabolism and are thus expected to be different susceptible to genotoxic risk factors, including Fe-NTA, than previously studied colon tumor cells (Schäferhenrich et al., 2003; Pool-Zobel et al., 2005; Knöbel et al., 2006). We investigated global DNA damage using the alkaline version of the Comet assay. Our adenoma cells carry mutations in *APC* and *KRAS*, but have an apparently normal wild type for *TP53* (Schäferhenrich et al., 2003). For this we also determined gene specific damage in *TP53* of LT97 adenoma cells. We used a recently developed new modification of the Comet FISH technique (Liehr et al., 2002; Schäferhenrich et al., 2003).

2. Materials and methods

2.1. Compounds

Ferric nitrate (40 mg) [$Fe(NO_3)_3$] (Sigma-Aldrich, Deisenhofen, Germany, CAS-Nr. 7782-61-8) was dissolved in 10 ml double distilled water and the chelating agent disodium nitrilotriacetate (NTA) (Sigma-Aldrich,

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

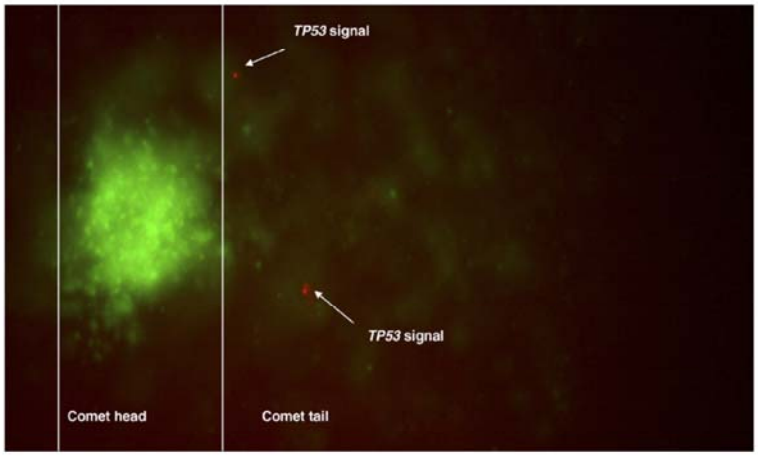


Fig. 1. Comet image of a cell after treatment with Fe-NTA with focus on the comet head and comet tail. Total DNA is stained with Sybr Green, *TP53* signal is stained with Texas Red. The picture shows the localisation of two *TP53* signals in the comet tail.

Deisenhofen, Germany, CAS-Nr. 15467-20-6) was added (23.5 mg). The pH of the solution was adjusted to 7.4 by addition of sodium hydrogencarbonate ($NaHCO_3$) (Sigma-Aldrich, Deisenhofen, CAS-Nr. 144-55-8). The final iron concentration of the stock solution was 10 mM.

2.2. Primary cells

Primary human colon cells were isolated from surgical tissue (non-tumorous material) as recently described (Schäferhenrich et al., 2003). The Ethical Committee of the Friedrich-Schiller-University Jena approved the study and the tissue was made available from 6 patients who had given their informed consent [3 patients for the normal Comet assay (Fig. 1) and another 3 patients for the Comet FISH method (Figs. 2 and 3)]. The average age of the donors was 55 ± 14 years ($n = 5$), 2 of 5 donors were female and 3 were male. For one donor the data are missing.

The human colonic epithelium was separated from the underlying layers of tissue by perfusion supported mechanical disaggregation. The epithelial stripes were cut into small pieces and incubated with 2 mg/ml proteinase K (Sigma, Steinheim, Germany) and 1 mg/ml collagenase P (Boehringer, Mannheim, Germany) for 60 min in a shaking water-bath at 37 °C. Pellets containing erythrocytes were treated with erythrocyte lysis buffer (hypertonic ammonium chloride solution: 155 μM NH_4Cl; 5 μM KCl; 0.06 μM EDTA; pH 7.0), centrifuged, and resuspended in RPMI 1640 (Life Technologies, Karlsruhe, Germany) to exclude erythrocytes in our suspension and to enhance the purity of the epithelial cells. Yield of the cells and viability were determined using trypan blue.

2.3. LT97 adenoma cells

The human colon adenoma cell line LT97 was originally established from a colon microadenoma of a patient with familial adenoma polyposis

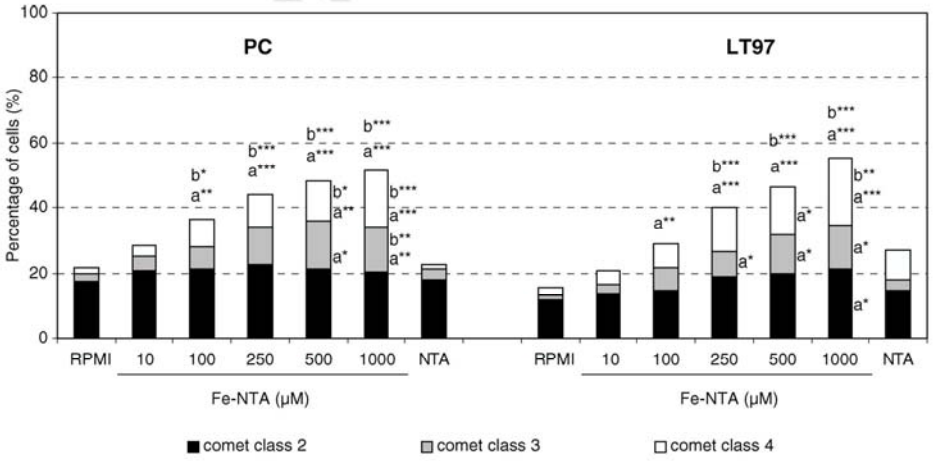


Fig. 2. Percentage of damaged cells belonging to comet classes 2–4 in primary colon cells (PC) and LT97 cells after 30 min treatment with Fe-NTA (10–1000 μM, 37 °C), ($n = 3$). The significant differences to the untreated medium control (RPMI) (a) or NTA control (b) were calculated by One way ANOVA, including Dunnett's multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (The percentage of cells in comet class 1 is not outlined in this figure, but is equal to the difference 100% minus sum of percentage of cells in comet classes 2–4. The significance levels for the changes in comet class 1 are outlined above the total bars.)

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

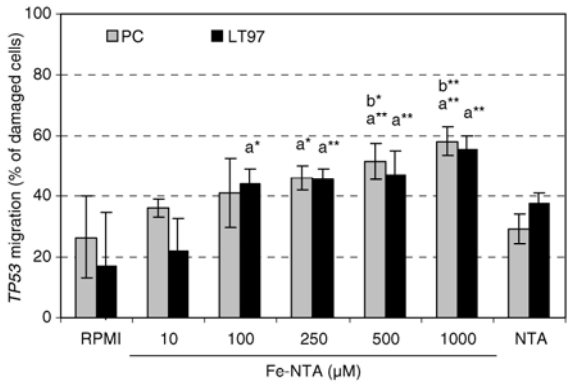


Fig. 3. Percentage of damaged cells (comet classes 2–4) with *TP53* signals in the comet tail after a 30 min incubation of primary human colon cells (PC) or LT97 cells with Fe-NTA (10–1000 μM, 37 °C), ($n = 3$). The significant differences to the untreated medium control (RPMI) (a) or NTA control (b) were calculated by One way ANOVA, including Dunnett's multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.01$).

coli (Richter et al., 2002) and was kindly provided by B. Marian (University of Vienna). LT97 cells were maintained in a culture medium (MCDB 302) containing 20% of L15 Leibovitz medium, 2% FCS, 1% penicillin/streptomycin, 0.2 nM triiodo-L-thyronine, 1 μg/ml hydrocortisone (302 basic medium), 10μg/ml insulin, 2μg/ml transferrin, 5 nM sodium selenite and 30 ng/ml epidermal growth factor (EGF). Cells were grown in a humidified incubator under standardized culture conditions (5% CO_2, 95% humidity, 37 °C). Mycoplasm free passages from 15 to 30 were used to perform the experiments presented here.

2.4. Determination of genetic damage

Cell numbers and viabilities of the harvested cells were determined with the trypan blue exclusion test and the cell number was adjusted to 2×10^6 cells per ml. Cells were incubated with Fe-NTA (1–1000 μM) at 37 °C for 30 min on a thermomixer. The remaining washed cells were mixed with 0.7% low melting point agarose and distributed onto microscope slides, 3 slides per data point. The Comet assay was performed as described before (Ebert et al., 2001). After the agarose solidified, cells on slides were lysed (10 mM Tris–HCl, 100 mM Na_2 EDTA, 2.5 M NaCl, 10% dimethylsulfoxide, 1% Triton X-100, pH 10) for at least 60 min. After 20 min of DNA unwinding (1 mM Na_2EDTA, 300 mM NaOH, pH 13) electrophoresis was carried out at 1.25 V/cm, 300 mA for another 20 min. The slides were treated with neutralising buffer (4.2 M Tris–HCl, 0.08 M Tris-base, pH 7.2), washed three times, and were stained with SYBR-green (1 μl/ml; 30 μl/slide) (Sigma-Aldrich, Deisenhofen, Germany, CAS-Nr. 163795-75-3).

All steps of the Comet assay were conducted under red light. Each experiment was reproduced independently at least three times. The viability of the treated cells was determined with the trypan blue exclusion test.

Global DNA damage was microscopically quantified using a ZEISS Axiovert 25 microscope (Carl Zeiss Jena GmbH, Jena, Germany) and the Komet 4.0 image analysis system (Kinetic Imaging Corp., Liverpool, UK). Fifty cells were evaluated per slide and the percentage of fluorescence in the comet tail (TI, "tail intensity") was scored. The mean values of 3 slides per data point and experiment were used to calculate the mean values of at least three reproduced experiments.

2.5. Determination of gene specific damage in TP53 (Comet FISH)

The Comet assay was performed as described above and the slides were additionally treated with a Texas Red labelled *TP53* probe. This region-specific probe were generated by glass-needle based microdissection and the isolated DNA was amplified by DOP-PCR (Liehr et al., 2002; Lüdecke et al., 1989; Telenius et al., 1992). The probe was a mixture of directly labelled probes that are specific for genomic sequences including the *TP53* locus (17pter-p12).

FISH experiments were performed following the method described by Schäferhenrich et al. (2003). We had to modify and adapt the hybridisation conditions to meet the requirements of our Texas Red labelled *TP53* probes. Hybrisol VI (Oncor, Gaithersburg, UK) was dropped on the Comet assay slides, spread, and covered with a plastic cover slip. The hybridisation mixture [5 μl *TP53* probe per slide +7 μl Hybrisol VI per slide] was denatured at 75 °C for 5 min, at 4 °C for 2 min, and at 37 °C for 30 min. Twelve μl of this mixture were added onto one half of the slides and 12 μl Hybrisol VI as control onto the other half of the slides. The slides were covered with plastic cover slips (24 × 24 mm) and incubated in hybridisation chambers for 72 h at 37 °C. After hybridization, the slides were washed for 5 min in 2× saline sodium citrate solution without formamid (SSC: 0.3 M NaCl; 0.03 M NaCitrate; pH 7.2) at 65 °C. This was followed by washing for 5 min in 1× phosphate buffered detergent (PBD: 15.6 g/l NaH_2PO_4; 17.8 g/l Na_2HPO_4; 1 g/l Nonidet-P40; pH 8.0) at room temperature. Texas Red labelled probes were counter-stained with SYBR-Green (1 μl/ml, 30 μl per slide).

To evaluate the Comet FISH experiments, the comets were categorised into four degrees of damage ranging from non-damaged images (class 1) to severely damaged images (class 4) (Wollowski et al., 1999). For a first evaluation, we determined the total number of *TP53* signals per cell and the hybridization efficiency. For the further Comet FISH evaluation, only cells with two fluorescent spots were used and the position of the *TP53* signals in the comet head or comet tail was recorded (Fig. 1). The percentage of damaged cells (belonging to comet classes 2–4), in which we could find a migration of at least one *TP53* signal into the comet tail, was recorded as the parameter of *TP53* migration. About 100 cells per slide were scored.

Fluorescence microscopy was performed using a ZEISS Axiovert M100 (Carl Zeiss Jena GmbH, Jena, Germany), equipped with filters for observation of Texas Red (red, ZEISS filter No. 15) and SYBR Green (green, ZEISS filter No. 09). Images were captured using a MicroMAX Digital CCD camera (BFI OPTILAS GmbH, Puchheim, Germany), Meta View Imaging Software (Visitron Systems GmbH, Puchheim, Germany) was used to process the images.

2.6. Statistical evaluation

The GraphPad Prism software Version 4.0 (GraphPad Software Inc., San Diego, USA) was used to calculate statistically significant differences. Statistical evaluations were carried out on the mean values of the data and these values are also presented (mean and SD) in the figures and tables. As specified in the legends of the figures and tables, One-way ANOVA with Dunnett's post-test, Bonferroni's post-test, or two-way ANOVA with Bonferroni's post-test were utilized when appropriate.

3. Results

3.1. Effects of Fe-NTA on DNA damage

Table 1 shows that the treatment with Fe-NTA resulted in enhanced DNA strand breaks in primary colon cells. The effects were statistically significant for 500 and 1000 μM Fe-NTA. The chelating agent NTA alone was not genotoxic. Moreover, the investigated Fe-NTA concentrations were not cytotoxic. Cell viability was between 80% and 100% after 30 min of treatment.

For LT97 cells, we observed a comparable damage profile (Table 1) and significant DNA damage was induced at a concentration of 1000 μM.

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

Table 1
Levels of DNA damage induced by 0–1000 μM Fe-NTA (30 min at 37 °C) in primary human colon cells and LT97 cells measured with the Comet assay[a]

Treatment	Primary colon cells ($n = 3$)				LT97 ($n = 4$)			
	Tail intensity [%]		Viability [%]		Tail intensity [%]		Viability [%]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
RPMI	10.6	3.9	87.7	2.8	11.1	3.7	89.5	2.7
1000 μM NTA	12.1	3.0	89.4	10.1	12.7	3.7	88.7	3.7
10 μM Fe-NTA	10.0	1.4	91.6	8.4	15.5	5.9	89.4	7.2
100 μM Fe-NTA	11.3	0.6	91.5	4.2	14.9	3.7	90.5	2.1
250 μM Fe-NTA	14.9	1.5	89.9	5.1	14.5	4.0	86.0	4.8
500 μM Fe-NTA	20.1**	1.7	86.4	0.9	20.4	8.5	87.6	3.1
1000 μM Fe-NTA	30.0**	3.5	83.2	6.9	26.8**	7.3	83.7	3.0

[a] The data show mean values from three to four independent experiments. The significance of individual compound induced effects was calculated by one way ANOVA, including Dunnett's post-test.
** $p < 0.01$ significantly different from RPMI control.

3.2. Effects of Fe-NTA on TP53 damage

A normal metaphase or an interphase nucleus stained with the *TP53* specific probe usually has two signals. Cells without signals and cells with only one or two signals were counted, and the resulting hybridisation efficiency was estimated to be 79% for primary human colon cells and 87% for LT97 cells. Only very few comets had 3 signals, an observation which reflects the actual breakage and isolated migration of a *TP53*-gene fragment.

Fig. 2 shows the frequencies of comet classes 2–4 (damaged cells) resulting from treatment of primary colon cells and LT97 cells with Fe-NTA for 30 min. It is apparent that the total score of damaged cells increased with increasing concentrations in Fe-NTA. A significant shift was observed primarily in the direction of the relative strongly damaged cells. Fe-NTA was thus significantly causing DNA damage in comparison to the medium control already at a concentration of 100 μM in primary colon cells and in LT97 adenoma cells. There were no significant differences in the response of primary colon cells and LT97 cells.

Fig. 3 shows the proportion of damaged cells with the *TP53* signals in the comet tail. There is a concentration dependent migration of *TP53* into the comet tail, which is significant compared to medium control at 100–1000 μM for LT97 cells and at 250–1000 μM for primary colon cells. There were no significant differences between responses of primary cells and LT97 cells detectable.

4. Discussion

Compounds from dietary or microbial origin or from digestive and excretory processes may have genotoxic properties and thus pose risks during colorectal carcinogenesis (Bingham, 1996; Giovannucci and Willett, 1994). The genotoxic potency of these compounds, the ability of the mucosal cells or of the intestinal flora to deactivate the compounds, the repair capacity of the cells to remove damage and the apoptosis rate of damaged cells together influence the action of such compounds on the epithelial colon cells (Takayama et al., 1998). One of these compounds, iron, is a particular risk factor for colon cancer and related diseases through by its ability to form reactive oxygen species via the Fenton reaction (Imlay et al., 1988; Aruoma et al., 1989; Babbs, 1992). The alkaline version of the Comet assay used in this study is a suitable tool to investigate the genotoxic potential of risk compounds, like ROS or iron (Tice et al., 2000). The resulting lesions represent the very first steps of the mutations in the carcinogenesis, since they indicate the probability of occurrence of promutagenic lesions (Fearon and Vogelstein, 1990).

ROS play an important role during tumor progression and contribute to regulation of the expression of genes during cell growth and differentiation (Crawford et al., 1988). Lund et al. could show that there is an association between increased concentrations of biologically available iron and changes in crypt cell proliferation in the rat large intestine (Lund et al., 1998). We demonstrated that Fe-NTA induces DNA strand breaks in primary colon cells and in LT97 cells. This confirms similar effects of a recent study with HT29 clone 19A cells also showing that DNA damage was induced at 500–1000 μM (Glei et al., 2002). Hemin and hemoglobin, as other sources of iron also induced DNA damage in human colon cells in a similar order of magnitude (Glei et al., 2006). Hartwig et al. were the first who described DNA strand breaks induced by ferric iron in V79 cells and oxidative DNA damage in mammalian cells (Hartwig et al., 1993; Hartwig and Schlepegrell, 1995). Abelea et al. reported on iron-induced oxidative damage in primary rat hepatocyte culture (Abalea et al., 1998). In our study NTA was used as one of our control treatments to exclude the possibility that this chelator contributes to the genotoxic effect of Fe-NTA. Thus all available information points to a considerable genotoxic action by ferric iron. The data also indicates that colon cells of three different stages of cell transformation are of equal sensitivity, and supports the hypothesis that iron overload can be a risk factor throughout the process of colorectal carcinogenesis. To our knowledge, this is the first report on the genotoxic potential of ferric iron in non-transformed human colon cells and in cells derived from preneoplastic colon lesions.

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

We were not able to find significant differences in the response of primary colon cells and LT97 cells in these studies. There are only marginal differences towards the genotoxic effect of Fe-NTA between the two cell types used in this study and a previously used colon cancer line possible due to differences in the expression of antioxidaitve enzymes (Glei et al., 2002). This could lead to differences in protective mechanisms to initial DNA damage. We have recently also characterised the cells for expression of genes related to drug metabolism and stress response. Catalase and superoxide dismutase 2 are more expressed in the adenoma cell line than in primary cells isolated from surgical donors, whereas superoxide dismutase 1 and glutathione peroxidase are expressed at approximately equal levels in both cells lines (unpublished data). Together the data imply that primary colon cells are more susceptible to ROS due to their lower levels of antioxidative systems. When comparing the basal DNA damage of primary human colon cells and LT97 adenoma cells we may assume that the isolation of the primary cells does not cause any additional isolation stress because both cell types have comparable levels of basal damage. In a recent study we could also show that non-physiological high doses of iron modulate the growth and GSH content of HT29 clone 19A colon carcinoma cells. High doses (1000, 2000 μM) inhibited cell growth and significantly increased the GSH content in these cells (Knöbel et al., 2006).

After loss of heterozygosity in *APC* (adenomatous polyposis coli), alterations of *K-RAS* (Kirsten rat sarcoma) and mutations in *SMAD4* (mothers against DPP homolog 4), mutations in *TP53* are the final steps in colon carcinogenesis (Fodde et al., 2001). These are responsible for converting adenoma to invasive carcinoma cells. Hussain et al. found a higher frequency of *TP53* mutated alleles in non-tumorous liver tissue of patients with Wilson disease and hemochromatosis compared with liver samples from normal controls (Hussain et al., 2000). Furthermore they exposed a wild-type *TP53* TK-6 lymphoblastoid cell line to 4-hydroxynonenal and observed an increase in G to T transversions at *TP53* codon 249 (AGG to AGT). These results are consistent with the hypothesis that iron overload in Wilson disease and hemochromatosis cause mutations in the *TP53* tumor suppressor gene via generation of ROS. Therefore we investigated whether a simulated iron overload induces gene specific damage in *TP53* of primary colon cells and LT97 adenoma cells using Comet FISH. While the results of the Comet assay provide information on the overall DNA damage-induction, the Comet FISH-modification allows the identification of a specific gene sequence within the damaged or un-damaged part of the comet. It therefore provides additional information on damage to specific gene sequences or genome regions. In this study, there was a concentration-related higher proportion of damaged cells with *TP53* migration into the comet tail. Thus treatment of the cells with 500 μM Fe-NTA resulted in a *TP53* migration in about 50% of the damaged cells. Thus we can conclude that the tumor suppressor gene *TP53* was sensitive toward Fe-NTA. The observed dose-dependent migration of *TP53* into the comet tail indicates an interaction of Fe-NTA, probably via ROS, with the gene. In support to this mechanism Schäferhenrich et al. demonstrated that H_2O_2 also caused a migration of the *TP53* into the comet tail which was comparable to the global DNA damage in LT97 cells, but higher than the global damage in primary colon cells (Schäferhenrich et al., 2003). *TP53* in primary human colon cells was significantly more sensitive towards H_2O_2 than *TP53* in LT97 cells. In contrast there were not any significant differences between the global genotoxic effects in the two cell types, which is in support of our data with Fe-NTA. In the same study HNE was selectively more active in *TP53* than in global DNA of both cell types, but LT97 cells were significantly more sensitive than primary colon cells, which is not expected on account of the relatively lower activity and expression levels of the GSH/GST system in primary colonocytes compared to the adenoma cells. Our previous studies have shown that GST activity and GSTP1 expression is twice as high in LT97 cells compared to primary cells (Schäferhenrich et al., 2003; Pool-Zobel et al., 2005).

In regard to the relative high susceptibility of *TP53* in both cell types our results are of significance since alterations in this tumor suppressor gene are frequently encountered genetic events in colorectal cancer and they are late events occurring during the transition of adenoma to malignant carcinoma (Fodde et al., 2001). Now it is important to determine whether an altered migration of *TP53* is actually associated with enhanced mutations in this gene and how Fe-NTA modulates the spectrum of mutations. This could be possible with the 24-colour-FISH an innovative method to detect compound-induced chromosomal aberrations. In conclusion, we have clearly demonstrated that ferric iron was significantly genotoxic in primary human colon cells and LT97 adenoma cells. The results imply that ferric iron may play a role in initiation and early progression by acting genotoxic in non-transformed and adenoma-derived cells, respectively. Both processes are driven by genotoxic burdens in the gut lumen, including molecules involved in oxidative stress (Bartsch et al., 2002). Our observations contribute to the current understanding of the genotoxic and putatively carcinogenic mechanism of Fe-NTA in which the tumor suppressor gene *TP53* seems to play a role as an important target gene. Thus we have provided new evidence showing that primary colon cells are of comparable sensitivity towards genotoxic actions of ferric iron as HT29 tumor cells are, and that these effects are visible at concentrations which can be detected in the colon lumen during conditions of iron overload. Translated to the *in vivo* situation these results imply that iron overload could pose a carcinogenic risk factor during early stages of colorectal carcinogenesis.

5. Uncited reference

Potter (1999).

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

Acknowledgement

This work was carried out with financial support by Deutsche Forschungsgemeinschaft project number PO 284/6-1 and PO 284/6-2. We are grateful to Dr. W.M. Pool for editorial assistance and to Dr. A. Schäferhenrich for valuable discussions and for contribution in establishing the Comet FISH method.

References

Abalea, V., Cilliard, J., Dubos, M.P., Anger, J.P., Cilliard, P., Morel, I., 1998. Iron-induced oxidative DNA damage and its repair in primary rat hepatocyte culture. Carcinogenesis 19, 1053–1059.

Aruoma, O.I., Halliwell, B., Gajewski, E., Dizdaroglu, M., 1989. Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. J. Biol. Chem. 264, 20509–20512.

Babbs, C.F., 1992. Oxygen radicals in ulcerative colitis. Free Radical Biol. Med. 13, 169–181.

Bach, S.P., Renehan, A.G., Potten, C.S., 2000. Stem cells: the intestinal stem cell as a paradigm. Carcinogenesis 21, 469–476.

Bartsch, H., Nair, J., Owen, R.W., 2002. Exocyclic DNA adducts as oxidative stress markers in colon carcinogenesis: Potential role of lipid peroxidation, dietary fat and antioxidants. Biol. Chem. 383, 915–921.

Berlau, J., Glei, M., Pool-Zobel, B.L., 2004. Colon cancer risk factors from nutrition. Anal. Bioanal. Chem. 30, 737–743.

Bingham, S.A., 1996. Epidemiology and mechanisms relating diet to risk of colorectal cancer. Nutr. Res. Rev. 9, 197–239.

Boutron-Ruault, M.-C., Senesse, P., Méance, S., Belghiti, C., Faivre, J., 2001. Energy intake, body mass index, physical activity and the colorectal adenoma–carcinoma sequence. Nutr. Cancer 39, 50–57.

Cook, J.D., Reuser, M.E., 1983. Iron fortification: an update. Am. J. Clin. Nutr. 38, 648–649.

Crawford, D., Zbinden, I., Amstad, P., Cerutti, P.A., 1988. Oxidant stress induces the proto-oncogenes c-*fos* and c-*myc* in mouse epidermal cells. Oncogene 3, 27–32.

Cross, A.J., Gunter, M.J., Wood, R.J., Pietinen, P., Taylor, P.R., Virtamo, J., Albanes, D., Sinha, R., 2006. Iron and colorectal cancer risk in the a-tocopherol, b-carotene cancer prevention study. Int. J. Cancer 118, 3152.

Ebert, M.N., Beyer-Sehlmeyer, G., Liegibel, U.M., Kautenburger, T., Becker, T.W., Pool-Zobel, B.L., 2001. Butyrate-induces glutathione *S*-transferase in human colon cells and protects from genetic damage by 4-hydroxynonenal. Nutr. Cancer 41, 156–164.

Fearon, E.R., Vogelstein, B., 1990. A genetic model for colorectal tumorigenesis. Cell 61, 759–767.

Fodde, R., Smits, R., Clevers, H., 2001. *APC* signal transduction and genetic instability in colorectal cancer. Nat. Rev. Cancer 1, 55–67.

Giovannucci, E., Willett, W.C., 1994. Dietary factors and risk of colon cancer. Ann. Med. 26, 443–452.

Glei, M., Klinder, A., Latunde-Dada, G.O., Becker, T.W., Hermann, U., Voigt, K., Pool-Zobel, B.L., 2002. Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A. Mutat. Res. 519, 151–161.

Glei, M., Klenow, S., Sauer, J., Wegewitz, U., Richter, K., Pool-Zobel, B.L., 2006. Hemoglobin and hemin induce DNA damage in human colon tumor cells HT29 clone 19A and in primary human colonocytes. Mutat. Res. 594, 162–171.

Hartwig, A., Schlepegrell, R., 1995. Induction of oxidative DNA damage by ferric iron in mammalian cells. Carcinogenesis 16, 3009–3013.

Hartwig, A., Klyszcz-Nasko, H., Schlepegrell, R., Beyersmann, D., 1993. Cellular damage by ferric nitrilotriactate and ferric citrate in V79 cells: interrelationship between lipid peroxidation, DNA strand breaks and sister chromatid exchanges. Carcinogenesis 14, 107–112.

Heavey, P.M., McKenna, D., Rowland, I.R., 2004. Colorectal cancer and the relationship between genes and the environment. Nutr. Cancer 48, 124–141.

Herrinton, L.J., Friedman, G.D., Baer, D., Selby, J.V., 1995. Transferrin saturation and risk of cancer. Am. J. Epidemiol. 142, 692–698.

Hussain, S.P., Raja, K., Amstad, P.A., Sawyer, M., Trudel, L.J., Wogan, G.N., Hofseth, L.J., Shields, P.G., Billiar, T.R., Trautwein, C., Höhler, T., Galle, P.R., Phillips, D.H., Markin, R., Marrogi, A.J., Harris, C.C., 2000. Increased p53 mutation load in nontumorous human liver of Wilson disease and hemochromatosis: oxyradical overload diseases. Proc. Natl. Acad. Sci. USA 97, 12770–12775.

Imlay, J., Chin, S.M., Linn, S., 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science 240, 640–642.

Kinzler, K.W., Vogelstein, B., 1996. Lessons from hereditary colorectal cancer. Cell 87, 159–170.

Knöbel, Y., Glei, M., Osswald, K., Pool-Zobel, B.L., 2006. Ferric iron increases formation of reactive oxygen species, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells. Toxicol. In Vitro 20, 793–800.

Lane, D.P., 1992. Cancer. *TP53*, the guardian of the genome. Nature 358, 15–16.

Leslie, A., Carey, F.A., Pratt, N.R., Steele, R.J., 2002. The colorectal adenoma–carcinoma sequence. Br. J. Surg. 89, 845–860.

Liehr, T., Heller, A., Starke, H., Rubtsov, N., Trifonov, V., Mrasek, K., Weise, A., Kuechler, A., Claussen, U., 2002. Microdissection based high resolution multicolor banding for all 24 human chromosomes. Int. J. Mol. Med. 9, 335–339.

Lüdecke, H.J., Senger, G., Claussen, U., Horsthemke, B., 1989. Cloning defined regions of the human genome by microdissection of banded chromosomes and enzymatic amplification. Nature 338, 348–350.

Lund, E.K., Wharf, S.G., Fairweather-Tait, S.J., Johnson, I.T., 1998. Increases in the concentrations of available iron in response to dietary iron supplementation are associated with changes in crypt cell proliferation in rat large intestine. J. Nutr. 128, 175–179.

Lund, E.K., Wharf, S.G., Fairweather-Tait, S.J., Johnson, I.T., 1999. Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. Am. J. Clin. Nutr. 69, 250–255.

Muscat, J.E., Wynder, E.L., 1994. The consumption of well-done red meat and the risk of colorectal cancer. Am. J. Public Health 84, 856–858.

Norat, T., Bingham, S., Ferrari, P., Slimani, N., Jenab, M., Mazuir, M., Overvad, K., Olsen, A., Tjønneland, A., Clavel, F., Boutron-Ruault, M.-C., Kesse, E., Bergmann, M.M., Nieters, A., Linseisen, J., Trichopulou, A., Trichopoulou, D., Tountas, Y., Berrino, F., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., Peeters, P.H.M., Engeset, D., Lund, E., Skeie, G., Ardanaz, E., González, C., Navarro, C., Quirós, J.R., Sanchez, M.-J., Berglund, G., Mattisson, I., Hallmans, G., Palmquist, R., Day, N.E., Khaw, K.-T., Key, T.J., San Joaquin, M., Hémon, B., Saracci, R., Kaaks, R., Riboli, E., 2005. Meat, fish, and colorectal cancer risk: the European prospective investigation into cancer and nutrition. J. Natl. Cancer Inst. 97, 906–916.

Pierre, F., Tache, S., Petit, C.R., Van der Meer, R., Corpet, D.E., 2003. Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. Carcinogenesis 24, 1683–1690.

Pool-Zobel, B.L., Leucht, U., 1997. Induction of DNA damage in human colon cells derived from biopsies by suggested risk factors of colon cancer. Mutat. Res. 375, 105–116.

Pool-Zobel, B.L., Veeriah, S., Kautenburger, T., Kiefer, J., Richter, K.K., Sauer, J., Soom, M., Wölfl, S., 2005. Butyrate may enhance toxicological defence in primary, adenoma and tumour human colon cells by favourably modulating expression of glutathione *S*-transferases genes. Carcinogenesis 26, 1064–1076.

Potter, J.D., 1999. Colorectal cancer: molecules and populations. J. Natl. Cancer Inst. 91, 916–932.

Preston, S.L., Wong, W.M., Chan, A.O., Poulsom, R., Jeffery, R., Goodlad, R.A., Mandir, N., Elia, G., Novelli, M., Bodmer, W.F.,

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

FCT 3716
22 November 2006 Disk Used
ARTICLE IN PRESS
No. of Pages 8, Model 5+

Tomlinson, R.B., Wright, N.A., 1996. Bottom-up histogenesis of colorectal adenomas: origin in the monocryptal adenoma and initial expansion by crypt fission. Cancer Res. 63, 3819–3825.

Renehan, A.G., O'Dwyer, S.T., Haboubi, N.J., Potten, C.S., 2002. Early cellular events in colorectal carcinogenesis. Colorectal Dis. 4, 76–89.

Richter, M., Jurek, D., Wrba, F., Kaserer, K., Wurzer, G., Karner-Hanusch, J., Marian, B., 2002. Cells obtained from colorectal microadenomas mirror early premalignant growth patterns in vitro. Eur. J. Cancer 38, 1937–1945.

Schäferhenrich, A., Beyer-Sehlmeyer, G., Festag, G., Kuechler, A., Haag, N., Weise, A., Liehr, T., Claussen, U., Marian, B., Sendt, W., Scheele, J., Pool-Zobel, B.L., 2003. Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. Mutat. Res. 9496, 1–14.

Senesse, P., Méance, S., Cotter, V., Faivre, J., Boutron-Ruault, M.-C., 2004. High dietary iron and copper and risk of colerectal cancer: a case-control study in Burgundy, France. Nutr. Cancer 49, 66–71.

Shaheen, N.J., Silverman, L.M., Keku, T., Lawrence, L.B., Rohlfs, E.M., Martin, C.F., Galanko, J., Sandler, R.S., 2003. Association between hemochromatosis (HFE) gene mutation carrier status and the risk of colon cancer. J. Natl. Cancer Inst. 95, 154–159.

Shayeghi, M., Latunde-Dada, G.O., Oakhill, S., Laftah, A.H., Takeuchi, K., Halliday, N., Khan, Y., Warley, A., McCann, F.E., Hider, R.C., Frazer, D.M., Anderson, G.J., Vulpe, C.D., Simpson, R.J., McKie, A.T., 2005. Identification of an intestinal heme transporter. Cell 122, 789–801.

Sinha, R., Kulldorff, M., Chow, W.H., Denobile, J., Rothman, N., 2001. Dietary intake of heterocyclic amines, meat-derived mutagenic activity, and risk of colorectal adenomas. Cancer Epidemiol. Biomarkers Prev. 10, 559–562.

Takayama, T., Katsuki, S., Takahashi, Y., Ohti, M., Nojiri, S., Sakamaki, S., Kato, J., Kogawa, K., Miyake, H., Nitsu, Y., 1998. Aberrant crypt foci of the colon as precursors of adenoma and cancer. N. Engl. J. Med. 339, 1277–1284.

Telenius, H., Carter, N.P., Bebb, C.E., Nordenskjold, M., Ponder, B.A., Tunnacliffe, A., 1992. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 13, 718–725.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Wollowski, I., Ji, S., Bakalinsky, A.T., Neudecker, C., Pool-Zobel, B.L., 1999. Bacteria used for the production of yogurt inactivate carcinogens and prevent DNA damage in the colon of rats. J. Nutr. 129, 77–82.

World Cancer Research Fund, American Institute for Cancer Research, and Deutsches Institut für Ernährungsforschung. Krebsprävention durch die Ernährung: Forschung, Daten, Begründungen, Empfehlungen, 1999. Deutsches Institut für Ernährungsforschung, Potsdamm-Rehbrücke.

Wurzelmann, J.I., Silver, A., Schreinemachers, D.M., Sandler, R.S., Everson, R.B., 1996. Iron intake and risk of colon cancer. Cancer Epidemiol. Biomarkers Prev. 5, 507.

Please cite this article in press as: Knöbel, Y. et al., Ferric iron is genotoxic in non-transformed and ..., Food Chem. Toxicol. (2006), doi:10.1016/j.fct.2006.10.028

3.3 Manuskript III

Uranyl nitrilotriacetate (U-NTA), a stabilized salt of uranium, is genotoxic in nontransformed human colon cells and in the human colon adenoma cell line LT97

Y. Knöbel, M. Glei, A. Weise, K. Osswald, A. Schäferhenrich, K. K. Richter, U. Claussen, B. L. Pool-Zobel

Toxicological Sciences, 2006, Vol. 93(2), 286-297

Zusammenfassung

Substanzen, die mit dem Uranbergbau assoziiert sind, können über ihre Radioaktivität oder durch direkte Interaktion mit zellulären Makromolekülen genotoxisch wirken. Um ein mögliches Risikopotential zu beurteilen, untersuchten wir die genotoxischen Effekte von Uranyl-Nitriolotriazetat (U-NTA) in humanen Kolonzellen unterschiedlichen Transformationsgrades.

U-NTA inhibierte das Wachstum von HT29 clone 19A-Zellen und erhöhte den Gehalt an zellulärem GSH. In hohen Konzentrationen wirkte U-NTA genotoxisch. In LT97-Zellen und primären Kolonzellen reagierte das *TP53*-Gen besonders sensitiv. In den LT97-Zellen induzierte U-NTA chromosomale Veränderungen in den Chromosomen 5, 12 und 17, die die tumorrelevanten Gene *APC*, *K-RAS* und *TP53* tragen.

Es kann geschlussfolgert werden, dass Uranverbindungen über die orale Exposition das genotoxische Gefährdungspotential in Menschen erhöhen können.

Darstellung des Eigenanteils

- Planung, Durchführung und Auswertung aller praktischer Arbeiten, außer Durchführung und Auswertung der GSH-Bestimmung, Versuche zur Schadenspersistenz und 24-Farben-FISH-Versuche
- Erstellen und Korrektur des Publikationsmanuskriptes

TOXICOLOGICAL SCIENCES **93(2)**, 286–297 (2006)
doi:10.1093/toxsci/kfl060
Advance Access publication July 13, 2006

Uranyl Nitrilotriacetate, a Stabilized Salt of Uranium, is Genotoxic in Nontransformed Human Colon Cells and in the Human Colon Adenoma Cell Line LT97

Yuonne Knöbel,* Michael Glei,* Anja Weise,† Kerstin Osswald,* Anja Schäferhenrich,* Konrad Klaus Richter,‡ Uwe Claussen,† and Beatrice Louise Pool-Zobel*,[1]

Department of Nutritional Toxicology, Institute for Nutrition, †Institute for Human Genetics and Anthropology, and ‡Department of General and Visceral Surgery, Friedrich-Schiller-University Jena, 07743 Jena, Germany

Received March 24, 2006; accepted June 28, 2006

Previous uranium mining in the "Wismut" region in Germany enhanced environmental distribution of heavy metals and radionuclides. Carryover effects may now lead to contamination of locally produced foods. Compounds of "Wismut" origin are probably genotoxic via their irradiating components (radon) or by interacting directly with cellular macromolecules. To assess possible hazards, we investigated the genotoxic effects of uranyl nitrilotriacetate (U-NTA) in human colon tumor cells (HT29 clone 19A), adenoma cells (LT97), and nontransformed primary colon cells. These are target cells of oral exposure to environmentally contaminated foods and represent different cellular stages during colorectal carcinogenesis. Colon cells were incubated with U-NTA. Cell survival, cytotoxicity, cellular glutathione (GSH) levels, genotoxicity, and DNA repair capacity (comet assay), as well as gene- and chromosome-specific damage combination of comet assay and fluorescence *in situ* hybridization [FISH], 24-color FISH) were determined. U-NTA inhibited growth of HT29 clone 19A cells (75–2000μM, 72 h) and increased GSH (125–2000μM, 24 h). U-NTA was genotoxic (1000μM, 30 min) but did not inhibit the repair of DNA damage caused by hydrogen peroxide (H_2O_2), 4-hydroxy-nonenal, and 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine. U-NTA was also genotoxic in LT97 cells and primary colon cells, where it additionally increased migration of *TP53* into the comet tail. In LT97 cells, 0.5–2mM U-NTA increased chromosomal aberrations in chromosomes 5, 12, and 17, which harbor the tumor-related genes *APC*, *KRAS*, and *TP53*. It may be concluded that uranium compounds could increase alimentary genotoxic exposure in humans if they reach the food chain in sufficient amounts.

Key Words: **colon cells; U-NTA; GSH; comet assay; *TP53*; chromosomal aberrations.**

Previous extensive uranium mining in the "Wismut" region near Ronneburg (Thuringia, Germany) has enhanced environmental distribution of heavy metals and radionuclides. The compounds are considered to be hazardous based on what we know about their toxicity, but it is not known how environmental exposure to these compounds will affect human health. Several of the "Wismut"-related compounds probably are toxic through formation of free radical oxygen species (ROS) (Hei *et al.*, 1998), via irradiating mechanisms (radon) (Jostes, 1996), or by interacting directly with cellular macromolecules (e.g., inhibition of DNA repair) (Hartmann and Speit, 1996; Hartwig and Beyersmann 1989; Hartwig *et al.*, 1996). The release of radionuclides and uranyl compounds into the environment during uranium mining is associated with the transfer of these compounds into the food chain (Fisenne *et al.*, 1987; Thomas, 2000). The ingestion of contaminated soil or dust, moreover, increases the burden on the gastrointestinal system as well (Thomas, 2000).

Studies to determine the nutritional intake of radionuclides in Vietnam did not indicate an increased exposure to the noxious agents (Giang *et al.*, 2001). The estimated uranium intake in these studies was 0.66 μg U^{238}/day. This is lower than the estimated intake of 2.1 μg U/day at Ronneburg (Seeber, 1998). Kathren (2001) reported on the association between U^{238} inhalation and the risk of lung cancer. In contrast, there are hardly any investigations available on the importance of oral uranium intake.

The genotoxicity of uranium has not been investigated extensively in human cells, especially in cells of the colon, which is the major site of cancer related to nutrition. This is probably due to the lack of appropriate experimental methods. Since contamination of regional foods could increase the risk of gastrointestinal cancer, it is important that we assess the genotoxicity of "Wismut"-related compounds in human colon cells. We used normal primary colon cells, preneoplastic LT97 adenoma cells, and highly transformed tumor cells (HT29 clone 19A) to study the impacts of uranyl nitrilotriacetate (U-NTA). For this we determined a number of parameters related to the process of colorectal carcinogenesis, such as cell

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

[1] To whom correspondence should be addressed at Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller-University Jena, Dornburger Str. 25, 07743 Jena, Germany. Fax: +49 3641-949672. E-mail: b8pobe@uni-jena.de.

growth and DNA damage. The effects on DNA repair were studied as well since previous investigations had shown that heavy metals interfere with this important process of maintaining DNA integrity (Hartmann and Speit, 1996; Hartwig and Beyersmann, 1989; Hartwig *et al.*, 1996, 2002). Furthermore, we used a new technique developed in our laboratory to determine specific damage in *TP53*, an important gene which is altered during colorectal carcinogenesis (Fearon and Vogelstein, 1990). For this we combined the techniques of single-cell gel electrophoresis (comet assay) and fluorescence *in situ* hybridization (FISH) (Schäferhenrich *et al.*, 2003a). Last but not least, chromosomal aberrations in LT97 adenoma cells were detected with 24-color FISH. This is to our knowledge one of the first reports on the detection of cytogenetic lesions in human colon cells *in vitro* (Knoll *et al.*, 2006a).

MATERIALS AND METHODS

Compounds. Uranyl acetate [$UO_2(OCOCH_3)_2{\cdot}2H_2O$] from our laboratory stock (42.4 mg) was dissolved in 10 ml of double-distilled water (ddH_2O). The solution was supplemented with the chelating agent disodium NTA (23.5 mg) (Sigma-Aldrich, Deisenhofen, Germany). The pH of the solution was adjusted to 7.4 by adding sodium hydrogen carbonate ($NaHCO_3$). The final U-NTA concentration of this stock solution was 10mM.

Cells and culture conditions. HT29 clone 19A is a permanent subclone derived from the carcinoma cell line HT29 treated with sodium butyrate (Augeron and Laboisse, 1984). The cells were grown in tissue culture flasks with Dulbecco modified Eaglemedium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C in a humidified incubator (5% CO_2/95% air). For this study we used passages 20–50.

The human colon adenoma cell line LT97 was established from a colon microadenoma of a patient with familial adenoma polyposis coli (Richter *et al.*, 2002). LT97 was maintained in a culture medium containing MCDB 302 medium (Biochrom, Berlin, Germany) plus 20% of L15 Leibovitz medium, 2% FCS, and 1% penicillin/streptomycin. This medium was supplemented with 0.2nM triiodo-L-thyronine, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 2 μg/ml transferrin, 5nM sodium selenite, and 30 ng/ml epidermal growth factor. Cells were grown in a humidified incubator under standardized culture conditions (5% CO_2, 95% humidity, 37°C). Passages 17–33 were used for the experiments.

Primary human colon cells were isolated from surgical tissues (nontumor tissue) (Schäferhenrich *et al.*, 2003b). The Ethical Committee of the Friedrich-Schiller-University Jena approved the study, and the tissues were made available from patients who had given their informed consent. The human colon epithelium was separated from the underlying layers of tissue by perfusion-supported mechanical disaggregation. The epithelium stripes were cut into small pieces and incubated with 2 mg/ml proteinase K (Sigma, Steinheim, Germany) and 1 mg/ml collagenase P (Boehringer, Mannheim, Germany) for 60–120 min in a shaking water bath at 37°C. After washing and centrifugation (5 min at 400 × g), the pellets were resuspended in phosphate-buffered saline (PBS: 8.0 g/l NaCl, 1.44 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.3). Pellets containing erythrocytes were treated with erythrocyte lysis buffer (hypertonic ammonium chloride solution: 155μM NH_4Cl, 5μM KCl, 0.06μM EDTA, pH 7.0), centrifuged, and resuspended in RPMI 1640 (Gibco BRL). Yield of the cells and their viability were determined using the trypan blue exclusion test. The average age of the donors was 54 ± 15 years ($n = 3$, male). For the fourth donor, the data are missing.

The investigations with long-time exposure (24–72 h; cell survival, glutathione [GSH] modulation, damage persistence) were done in the rapidly growing cancer cell line HT29 clone 19A to obtain just basic information on effective concentrations and basic biological activities. The comet assay was performed in all three cell types to characterize the genotoxic potential of U-NTA and the relative sensitivities of the cells in different stages of tumor development. The detection of gene-specific damage (Comet FISH) could only be performed with LT97 cells and primary colon cells since HT29 clone 19A cells have a polyploid karyotype, which prevents their usefulness in this type of assay (Schäferhenrich *et al.*, 2003a). LT97 cells were also used to assess chromosomal aberrations because they are both capable of proliferating and have a diploid and relative stable karyotype.

Determination of cell survival. This basal investigation was done in the fast-growing cancer cell line HT29 clone 19A to define nontoxic concentration ranges for the genotoxicity assays. Survival of HT29 clone 19A cells was determined in 96-well microtiter plates (Beyer-Sehlmeyer *et al.*, 2003). Eight thousand cells per well were seeded, and after 24 h they were treated with 0–2000μM U-NTA dissolved in culture medium. Proliferation rates were determined after 24, 48, and 72 h of treatment. Quantification of DNA was achieved by first fixing and permeabilizing cells with methanol for 5 min, and then adding 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) to stain the residual cellular DNA. DNA content was detected by fluorometric analysis at Ex/Em 360/465 nm, 30 min after application of DAPI in a Microplate Reader (Spectra Fluor Plus, Tecan, Austria; software: XFluor). Mean values of at least three independent experiments are shown in the figures. DNA content directly reflected the number of remaining cells.

Quantification of GSH. For the GSH measurement, HT29 clone 19A cells were seeded in 96-well microtiter plates at 0.4×10^6 cells per well. After preincubating the cells for 24 h, U-NTA (0–2000μM) was added for another 24 h. Cell morphology was judged and the cells were harvested. Cell numbers and viability of the cells were determined using the trypan blue exclusion test. The cell suspensions were centrifuged (855 × g, 10 min, 4°C). GSH was measured in the cytosols. Therefore, the pellets were resuspended in 5% metaphosphoric acid (MPA, 2.5 g in 50 ml) and disrupted by ultrasound for 1 min. Fluorescence, which is proportional to the GSH content, was quantified at a wavelength of 400 nm in a microtiter plate fluorescence photometer against MPA.

Determination of genetic damage. The alkaline version of the comet assay was performed according to the guidelines published by Tice *et al.* (2000). This method allows the detection of compound-induced DNA damage, including DNA single-strand breaks (SSBs), alkali-labile sites, DNA-DNA/DNA-protein cross-links, and SSB resulting from incomplete excision repair (Tice *et al.*, 2000). LT97 cells grown in 25-cm^2 tissue flasks were washed with PBS containing EDTA (5mM) and subsequently removed from the surface of the flasks with trypsin/versene (10% [vol/vol], Invitrogen, Karlsruhe, Germany) at 37°C for 2–5 min. The cells were then taken up in RPMI to yield suspension cultures. HT29 clone 19A cells were processed in the same manner. Primary human colon cells were isolated as described above. Cell numbers and viabilities were determined with the trypan blue exclusion test, and the cell number was adjusted to 2×10^6 cells/ml. Aliquots of these cell suspensions were centrifuged (380 × g, 5 min), and the cell pellets were incubated with 0–1000μM U-NTA at 37°C for 30 min on a thermomixer. We used Fe-NTA as positive or reference control in the comet assay investigations of this study. NTA treatment alone was not performed since our previous studies had not shown an effect of NTA using comet assay and Comet FISH (Knöbel *et al.*, 2006). The suspensions were then centrifuged (380 × g, 5 min), and the cell pellets were washed in PBS. Twenty microliters of the cell suspensions was stored on ice for determination of cell numbers and viability. The remaining cell suspensions were centrifuged (380 × g, 5 min), and the pellets were mixed with agarose and distributed onto microscopic slides. After the agarose solidified, cells on slides were lysed for at least 60 min (pH 10). For DNA unwinding (pH > 13), the slides were placed in an electrophoresis chamber. After 20 min the electrophoresis was carried out at 1.25 V/cm and at 300 mA for another 20 min. The slides were neutralized by washing three times, each for 5 min, and afterward they were stained with SYBR-Green (1 μl/ml, 30 μl per slide). All steps of the comet assay were conducted under red light.

The images were quantified by microscopic evaluation using the image analysis system of Kinetic Imaging (Liverpool, UK). The percentage of fluorescence in the tail (tail intensity) was measured for 50 cells per slide. The means of three replicate slides were used to calculate the means of at least three independently reproduced experiments.

Persistence of DNA damage (DNA repair). To investigate the influence of a low uranium dose on the persistence of induced DNA damage, HT29 clone 19A cells were incubated for 24 h with 10µM U-NTA or RPMI and then were harvested as described above. After centrifugation, the cell pellets were resuspended in PBS or RPMI containing 75µM hydrogen peroxide (H_2O_2), 200µM 4-hydroxy-2-nonenal (HNE), or 25µM 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (NOH-PhIP) for 5 min at 4°C (H_2O_2) or for 30 min at 37°C (HNE and NOH-PhIP). One quarter of the cells were applied to the slides, and the remaining cells were incubated at 37°C to allow DNA repair processes to begin (postincubation period). These cells were distributed onto slides after 30, 60, and 120 min of postincubation. All slides were then incubated in lysis solution for at least 60 min. The H_2O_2-damaged DNA was also digested with endonuclease III (Endo III) for 45 min to determine oxidized pyrimidine bases along with other endonuclease-specific lesions. Alternatively, the slides were treated with formamidopyrimidine-glycosylase (Fpg) for 30 min to determine oxidized purine bases, among other Fpg-specific lesions (Collins *et al.*, 1995). Subsequently, all steps of the comet assay were carried out as described above.

Determination of gene-specific damage in TP53 (Comet FISH). For the Comet FISH experiments, we used LT97 adenoma cells and primary colon cells. Direct Texas Red–labeled *TP53* probe was a mixture of directly labeled probes that were specific for genomic sequences, which included the *TP53* locus (microdissection derived probe spanning 17pter-p12) (Liehr *et al.*, 2002). FISH experiments were performed according to Schäferhenrich *et al.* (2003a) but were adapted to the hybridization conditions needed for the Texas Red–labeled *TP53* probes used here. The dehydrated slides from the comet assay were rehydrated in ddH_2O for 10 min. Afterward, the target DNA was denatured with 0.5M NaOH for 30 min and then neutralized in 0.01M PBS for 1 min. The slides were dehydrated through an ethanol series (70, 80, and 95%, each for 5 min) and dried at room temperature. Thirty microliters of Hybrisol VI (Oncor, Gaithersburg, UK) was dropped on each slide gel and spread with a plastic coverslip. The *TP53* probe (5 µl for each slide) was diluted with Hybrisol VI (7 µl for each slide) and denatured at 75°C for 5 min, 4°C for 2 min, and 37°C for 30 min. This hybridization mixture (12 µl per slide) was added to the slides, covered with a plastic coverslip (24 × 24 mm), and incubated in hybridization chambers for 72 h at 37°C. The coverslips were then removed, and the slides were washed for 5 min in 2× saline sodium citrate (0.3M NaCl, 0.03M Na citrate, pH 7.2) without formamide at 65°C. This step was followed by a 5-min washing in 1× phosphate-buffered detergent (Oncor) at room temperature. SYBR-Green (1 µl/ml, 30 µl per slide) was used to counterstain the Texas Red–labeled probes. Negative controls were included for each concentration on the same slide using Hybrisol VI without the DNA probe (24 × 24-mm coverslip).

For evaluating Comet FISH experiments, the comets were categorized into four classes representing different degrees of DNA damage ranging from nondamaged to severely damaged images (classes 1–4) (Wollowski *et al.*, 1999). Also, the total numbers of *TP53* signals per cell were scored. The expected number of spots in a normal interphase nucleus is two. The hybridization efficiency was calculated on the basis of the number of cells without signals, with only one signal, and with two and more signals. Moreover, cells with broken fluorescent signals were counted as an indication of breaks which occur directly in the gene. For the further Comet FISH evaluation, only cells with two fluorescent spots were used and the localization of the *TP53* signals in the comet head or comet tail was recorded. The parameter of *TP53* migration was based on the percentage of damaged cells (belonging to comet classes 2–4), in which we could find a migration of at least one *TP53* signal into the comet tail. One hundred to 120 images per slide were measured during the Comet FISH evaluation.

Fluorescence microscopy was performed using a ZEISS Axiovert M100 (Carl Zeiss Jena GmbH, Jena, Germany) equipped with filters to detect Texas Red (red, ZEISS filter No. 15) and SYBR-Green (green, ZEISS filter No. 09). Images were captured using a MicroMAX Digital CCD camera (BFI OPTILAS GmbH, Puchheim, Germany). Meta View Imaging Software (Visitron Systems GmbH, Puchheim, Germany) was used to capture and process the images.

Determination of chromosomal aberrations (24-color FISH). The protocol was based on the guidelines of the OECD (1997). To detect U-NTA–induced chromosomal aberrations, subconfluent grown LT97 cells of the same passage were treated with medium, U-NTA (0.5, 1, and 2mM), the solvent control NTA, or the positive control compound ethylmethanesulfonate (EMS, 1mM) for 6 h without gas exchange. Cell culture medium served as a negative control. LT97 cells were grown under standard conditions. After the medium was changed, the culture was further incubated for 46 h to allow the minimum one reproduction cycle.

Thereafter, metaphase chromosomes were prepared according to standard protocols for 24-color FISH (Claussen *et al.*, 2002). Further steps were performed as described by Schäferhenrich *et al.* (2003a). In short, prepared slides for cytogenetic analyses were pretreated to perform multicolor FISH (M-FISH) hybridization according to previously described techniques (Senger *et al.*, 1998). After incubating the samples for 48 h (37°C) with the probe mixture, they were subsequently washed and the detection was carried out. Metaphase images were captured using a fluorescence microscope (Axioplan 2, Zeiss, Germany) with a PCO VC45 CCD camera (PCO, Kehl, Germany) and suitable filter combinations (DAPI/fluorescein isothiocyanate/Spectrum Orange/Texas Red/Cyanine 5/Diethylamino-coumarine). Evaluation was done with the ISIS3-software (MetaSystems, Altlussheim, Germany). Using this method, each of the 24 different chromosomes (22 autosomes, X, Y) was labeled with a specific combination of fluorochromes that allowed an unambiguous identification of the origin of the chromosomal material (Speicher *et al.*, 1996). Fifty metaphases were analyzed per concentration.

Statistical evaluation. Each experiment was independently reproduced three to four times, as is specified in the legends to the figures. There were two to three replicates for each exposure level within each experiment. The GraphPad Prism software Version 4.0 (GraphPad Software, Inc., San Diego, CA) was used for establishing significance levels. Statistics were carried out on the mean values of the data, and these are also presented (mean and standard deviation) in the figures and tables. As is specified in the figure legends, one-way analysis of variance (ANOVA) with Dunnett posttest or two-way ANOVA with Bonferroni posttest was used whenever appropriate.

RESULTS

Cell Survival

U-NTA was analyzed for its effects on cell growth (or rather cell survival) to determine cytotoxic dose, in relation to treatment time and concentration. These studies were performed with the most highly proliferating cells, namely, with the HT29 clone 19A tumor cell line. Figure 1 shows that at 24- and 48-h treatments with U-NTA, there was a significant growth arrest only at the highest tested U-NTA concentration, which was 2000µM ($p < 0.01$). After 72 h incubation, however, U-NTA was cytotoxic in a wide concentration range. A significant decrease in cell number was observed from 75 to 2000µM U-NTA ($p < 0.01$, $p < 0.001$). The 50% effective concentration (EC_{50}) at 72 h was reached already at 215.2 ± 19.2µM U-NTA.

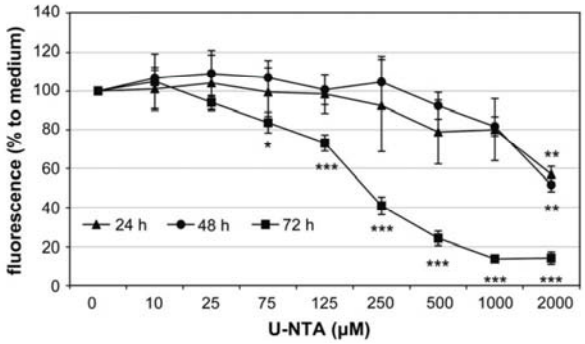


FIG. 1. Growth of HT29 clone 19A cells after 24, 48, and 72 h of treatment with U-NTA. Growth rates are presented as mean values ± SD ($n = 4$). Statistical evaluation was performed with one-way ANOVA versus medium control combined with the Dunnett multiple comparison posttest (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

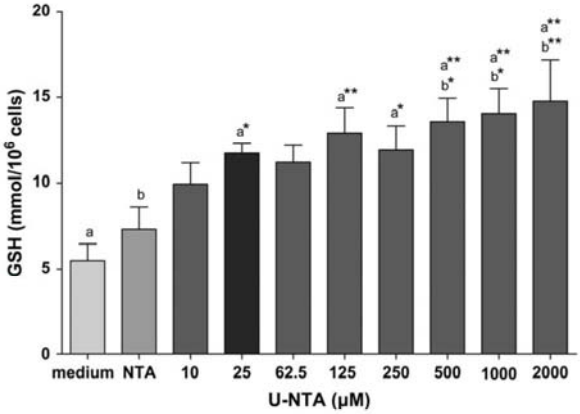


FIG. 2. GSH levels of HT29 clone 19A cells after incubation with 0–2000µM U-NTA (24 h at 37°C). Shown are mean values and SD from independent experiments ($n = 3$). The significance of individual compound-induced effects was calculated by one-way ANOVA, including the Dunnett multiple comparison posttest versus medium control (a) and versus NTA (b); *$p < 0.05$, **$p < 0.01$.

GSH Content in the Cells

Reduced GSH catalyzes the detoxification of electrophilic compounds, such as reactive oxygen species and free radicals. Moreover, intracellular GSH levels are directly related to the cell density and thus to growth properties. Therefore, we also investigated the influence of a uranium incubation on the GSH level in HT29 clone 19A cells. HT29 clone 19A cells were treated for 24 h with U-NTA. Figure 2 shows that there was a significant increase of the GSH content in the U-NTA–treated cells in comparison with control cells (a, vs. medium 125–2000µM U-NTA; b, vs. NTA 500–2000µM U-NTA).

Effects on DNA Damage

The key aim of this work was to determine whether U-NTA is genotoxic in human colon cells. For this we first analyzed DNA strand breaks at concentrations which did not affect cell growth (30 min, 0–1000µM). We also did not observe cytotoxic effects of our substances at these tested concentrations when using other methods to determine viability. Thus, with the trypan blue exclusion assay, the viability was between 70 and 100%. Table 1 shows these data as well as the impact of U-NTA on induction of strand breaks in HT29 clone 19A cells, LT97 cells, and primary colon cells after 30 min of treatment. At these doses, U-NTA was genotoxic in the different cell types. These effects were significant in HT29 clone 19A cells at 1000µM U-NTA ($p < 0.01$), in LT97 cells at 500µM ($p < 0.05$)

TABLE 1
Comparison of Genotoxic Effects of U-NTA (30 min, 37°C) in Primary Human Colon Cells, LT97 Cells, and HT29 Clone 19A Cells[a]

	Primary colon cells ($n = 4$)				LT97 ($n = 3$)				HT29 clone 19A ($n = 3$)			
	Tail intensity (%)		Viability (%)		Tail intensity (%)		Viability (%)		Tail intensity (%)		Viability (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
RPMI	8.9	1.8	87.5	1.9	10.1	1.7	86.7	1.5	4.1 b*	1.0	86.6	5.8
10µM U-NTA	11.2	2.8	85.7	2.3	7.1	0.8	85.1	2.4	4.3 a**	1.2	81.6	16.0
100µM U-NTA	12.9	2.2	86.9	6.2	10.7	2.3	84.4	4.3	4.0 a*** b*	1.2	85.9	3.1
250µM U-NTA	13.9	3.5	85.8	3.2	12.8	2.1	84.3	1.0	5.5 a*** b*	0.7	79.4	10.0
500µM U-NTA	14.6	4.0	76.2	13.2	15.5*	1.6	74.2	15.5	6.6 a*** b**	1.5	76.0	8.5
1000µM U-NTA	18.3**	3.9	79.3	4.8	20.9**	3.1	80.6	4.9	8.7** a*** b***	3.2	72.1	21.9
1000µM Fe-NTA	25.1**	4.3	79.2	7.2	19.5**	2.4	80.9	7.7	23.5	X	85.8	X

[a]The data show mean values from three or four independent experiments. The significance of individual compound-induced effects was calculated by one-way ANOVA, including the Dunnett posttest; *$p < 0.05$; **$p < 0.01$ significantly different from control. Differences between the cells were calculated by two-way ANOVA, including Bonferroni posttest (a, significant different from primary colon cells; b, significant different from LT97 cells; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; X, only measured in one experiment).

TABLE 2
Persistence of H_2O_2-, HNE-, and NOH-PhiP–Induced DNA Damage in HT29 Clone 19A Cells after a 24-h Preincubation with 10μM U-NTA[a]

		Tail intensity (%)							
		t 0 min		*t* 30 min		*t* 60 min		*t* 120 min	
Preincubation (μM)	Damaging agent (μM)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
U-NTA	H_2O_2								
0	0	2.81	0.62	2.88	0.67	2.06	0.26	2.07	0.89
10	0	3.49	1.00	2.47	0.57	1.98	0.64	2.13	0.53
0	75	25.70	7.38	9.62**	2.72	6.82**	3.23	5.98**	3.75
10	75	29.13	9.25	12.43**	2.99	8.72**	2.97	7.09**	6.11
U-NTA	NOH-PhiP								
0	0	2.92	0.56	2.94	0.97	2.45	0.40	3.10	0.44
10	0	2.20	0.68	3.32	0.69	2.92	1.19	2.12	0.25
0	75	28.45	5.14	20.05	6.00	15.37**	5.17	12.15**	7.17
10	75	28.88	4.87	18.84	5.59	14.90**	7.13	10.39**	6.16
U-NTA	HNE								
0	0	2.72	1.05	2.17	0.31	2.44	0.51	2.16	0.78
10	0	1.93	0.44	2.20	0.53	1.97	0.28	1.67	6.25
0	75	11.96	6.25	7.53	3.87	8.21	3.28	6.16	7.17
10	75	9.38	5.82	6.95	5.87	7.72	5.18	5.99	6.16

Note. *t* 0 min, time point after incubation with H_2O_2 (5 min, 4°C) or HNE or NOH-PhIP (30 min, 37°C), starting point of repair; *t* 30 min, after 30 min of repair; *t* 60 min, after 60 min of repair; *t* 120 min, after 120 min of repair.

[a]The significance of the time-dependent repair was calculated by one-way ANOVA, including the Dunnett multiple comparison posttest; $^{**}p < 0.01$ significantly different from time point *t* 0. The differences between U-NTA preincubation and non-preincubation were tested by two-way ANOVA, including Bonferroni posttest; they were not significant.

and at 1000μM ($p < 0.01$), and in primary colon cells at 1000μM U-NTA ($p < 0.05$). HT29 clone 19A cells reacted differently than the other two cell types and were significantly less sensitive.

Persistence of DNA Damage (DNA Repair)

The effects of U-NTA on repair of damage by putative colon carcinogens were investigated at 10μM for 30, 60, and 120 min. These were conditions that had been found to be nongenotoxic and were also previously reported to be effective for other metals. We did not observe an influence of 10μM U-NTA (24 h, 37°C) on the repair of damage induced by our genotoxic agents. For instance, H_2O_2-induced damage (75μM) was removed after 30 to 120 min postincubation ($p < 0.01$), without additional effects of U-NTA (Table 2). In addition to SSBs, H_2O_2 induces oxidized bases. H_2O_2-induced oxidized pyrimidine bases plus strand breaks were also repaired significantly ($p < 0.01$) after 30 min, but U-NTA again did not have an additional effect on the persistence of this type of damage (Table 3).

NOH-PhiP–induced DNA damage was significantly repaired after 60 min postincubation ($p < 0.01$). Preincubation with the nongenotoxic U-NTA concentration of 10μM, however, did not affect damage persistence (Table 2).

Finally, HNE-induced DNA damage was also repaired, but without statistical significance. Again, there was no detectable influence of U-NTA on the persistence of induced DNA damage (Table 2).

Effects on TP53 (Comet FISH)

This set of experiments addresses the possibility that U-NTA specifically damages genes, which are known to be altered during the carcinogenesis process. For Comet FISH experiments, we used LT97 and primary colon cells. Damage induced in *TP53* was evaluated by first grouping the images into comet classes. We determined the total number of signals per slide and per cell. A normal metaphase or interphase nucleus has two spots. The initial evaluation was done to exclude cells without and with only one signal and to determine the hybridization efficiency. This was found to be 86.8 ± 2.2% for primary cells and 83.4 ± 1.2% for LT97 cells. Only very few comets had three signals, which would indicate an actual break of the *TP53* gene. For further Comet FISH evaluation, only cells with two spots were used.

Figure 3 shows the frequency of comet classes 2–4 (damaged cells) resulting from the treatment of primary colon cells and LT97 with U-NTA (30 min, 37°C). It is apparent that the total score of damaged cells increased with increasing concentrations of the test compound. A significant shift was observed primarily in the direction of the stronger damaged cells. Thus, U-NTA significantly caused DNA damage, in comparison to the medium control, already at 100μM U-NTA in primary

TABLE 3
Persistence of H_2O_2-Induced Strand Breaks and Oxidized Bases in HT29 Clone 19A Cells after a 24-h Preincubation with 10μM U-NTA[a]

		Tail intensity (%)							
		t 0 min		*t* 30 min		*t* 60 min		*t* 120 min	
Preincubation (μM)	Damaging agent (μM)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
U-NTA	H_2O_2								
0	0	5.05	0.91	4.79	0.75	5.46	0.58	8.83	5.25
10	0	5.89	1.91	5.76	0.29	4.84	2.14	5.43	1.21
0	75	23.09	1.73	11.40**	4.32	10.05**	2.75	7.24**	2.64
10	75	28.60	6.80	6.82**	2.00	8.71**	1.49	6.90**	3.66

Note. *t* 0 min, time point after incubation with H_2O_2 (5 min, 4°C), starting point of repair; *t* 30 min, after 30 min of repair; *t* 60 min, after 60 min of repair; *t* 120 min, after 120 min of repair.

[a]The significance of the time-dependent repair was calculated by one-way ANOVA, including the Dunnett multiple comparison posttest; $^{**}p < 0.01$ significantly different from time point *t* 0. The differences between U-NTA preincubation and non-preincubation were tested by two-way ANOVA, including Bonferroni posttest; they were not significant.

human colon cells and in LT97 adenoma cells. The differences in the response between primary colon cells and LT97 adenoma cells were not significant.

Figure 4 shows the proportion of damaged cells with the *TP53* signals in the comet tail. We observed a concentration-dependent *TP53* migration into the comet tail, which was significant compared to medium control at 500 and 1000μM for primary colon cells and at 1000μM for LT97 cells. Significant differences between responses of the two cell types were not apparent.

Effects on Chromosome-Specific Damage (24-color FISH)

Figure 5 exemplifies some cytogenetic alterations in representative 24-color-FISH karyograms of each data point. The depicted alterations in the U-NTA–treated cells are compound related and have not been observed in the medium control and during previous routine analysis of LT97 karyotype (Schäferhenrich *et al.*, 2003a).

Figure 6 describes the range of observed chromosomal abnormalities in LT97 adenoma cells, which were translocations, deletions, and isochromosomes. It is apparent that EMS

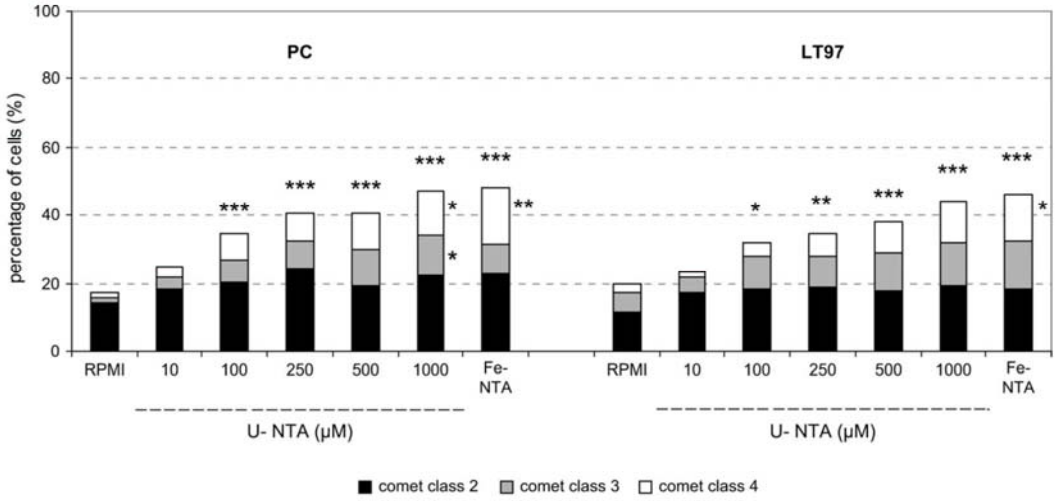


FIG. 3. Proportional distribution of primary colon cells (PC) and LT97 cells in the comet classes 2–4 (damaged cells) after 30 min treatment with U-NTA (10–1000μM, 37°C) ($n = 3$). The significant differences in the comet classes to the untreated medium control (RPMI) were calculated by one-way ANOVA, including the Dunnett multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (The percentage of cells in comet class 1 is not outlined in this figure but can be calculated as followed: 100% minus the percentage of cells in comet classes 2–4. The significance levels for the changes in comet class 1 are outlined above the total bars.)

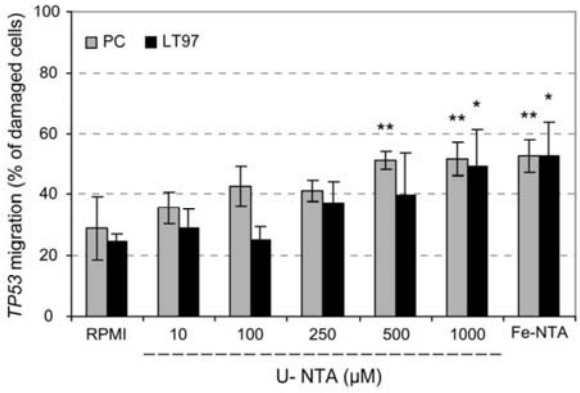


FIG. 4. Percentage *TP53* migration in the comet tail of damaged cells (comet classes 2–4) after a 30-min incubation of primary human colon cells (PC) or LT97 cells with U-NTA (10–1000µM, 37°C) ($n = 3$). The significant differences to the untreated medium control (RPMI) were calculated by one-way ANOVA, including the Dunnett multiple comparison test (*$p < 0.05$, **$p < 0.01$).

mainly caused translocations, whereas uranium caused more deletions. The proportion of translocations, however, increased with increasing U-NTA concentrations as well. The number of aberrant metaphases is shown in Figure 7. The total score of aberrant metaphases increased with increasing concentrations of U-NTA. Interestingly, when regarding only chromosomes 5, 12, and 17, which harbor the tumor-related target genes *APC* (adenomatous polyposis coli), *KRAS* (Kirsten rat sarcoma), and *TP53*, 2mM U-NTA caused a relatively higher proportion of damage than was expected for EMS. The data clearly point to a cytogenetic potential of U-NTA in human colon adenoma cells.

DISCUSSION

Natural uranium is weakly radioactive and potentially chemotoxic (Burkart, 1991). Target organs for uranium toxicity are the kidneys, as has also been shown for cadmium, lead, and mercury (Meinrath *et al.*, 2003). Drinking water and food are the primary sources of natural uranium (Fisenne *et al.*, 1987; UNSCEAR, 2000). The daily intake is estimated to be 1.5 µg from water and 1–2 µg from diet (ATSDR, 1999). The Agency for Toxic Substances and Disease Research sets the tolerable daily intake (TDI) level for uranium to 1 µg/kg/day (ATSDR, 1999). Jacob *et al.* (1997) suggests a TDI of 0.7 µg/kg/day, whereas the World Health Organization (WHO, 1998) recommends a TDI of less than 0.6 µg/kg/day. In recent years, several groups have published articles related to the toxicological assessment of uranium and other radionuclides (Cardis and Richardson, 2002; Priest, 2001). Some of this work was related to studies of exposure to depleted uranium (DU) used in the production of artillery (Durakovic, 2001; Giannardi and Domininci, 2003; McDiarmid *et al.*, 2000). The radiological toxicity of uranium results from its alpha-, beta-, and gamma-emissions (Meinrath *et al.*, 2003). Because of the long half-life of U-NTA and the relatively short incubation times in our experiments (0.5–72 h), the results of our studies probably only reflect the chemical toxicity of the compound.

One important mechanism of chemical toxicity could be the generation of oxidative stress. Uranium has been shown to catalytically oxidize ascorbate to dehydroascorbate at low pH (0.99–2.00) in the presence of dioxygen (Taqui Khan and Martell, 1969). Hamilton *et al.* (1997) could show that uranyl nitrate produces hydroxyl radicals in the presence of H_2O_2 at pH < 4. In *in vitro* experiments with plasmid DNA, Yazzie *et al.* (2003) showed that uranyl-acetate induced SSBs after reaction with ascorbate. Their data suggest that uranium may be directly genotoxic and may, like chromium, react with DNA by more than one pathway. Lund *et al.* (1998) showed an association of ROS with toxicity related to crypt cell proliferation in the large intestine of rats. Finally, another response to oxidative stress could be an enhanced cellular level of the tripeptide GSH, which is essential for detoxification. Metals can inhibit antioxidative enzymes and decrease intracellular GSH (Quig, 1998). Hg and Cd have high affinities for GSH, so they lead to a GSH depletion in the cells. In contrast, Seymen *et al.* (1997) showed that iron supplementation increases GSH, GSH-Px, and superoxide dismutase (SOD) levels in erythrocytes. This finding corresponds to our results with Fe-NTA (Knöbel *et al.*, 2006). In this study, U-NTA also significantly increased intracellular GSH.

Next to cytotoxicity, ROS are also genotoxic, and uranium has also been shown to have DNA-damaging effects. Reactions of H_2O_2 with U(IV) may generate the DNA-damaging hydroxyl radical (Taqui Khan and Martell, 1969). Yazzie *et al.* (2003) reported genotoxic effects of U(VI) in plasmid DNA after reduction with ascorbate, but the authors could not confirm the Fenton-type chemistry. Oxidative damage was recently found in DU-treated calf thymus DNA in the presence of H_2O_2 and ascorbate (Miller *et al.*, 2002). Another possible mechanism of uranium genotoxicity could be the direct interaction of uranyl cation with the DNA, as has been suggested previously (Franklin, 2001).

HNE and H_2O_2 are genotoxic products of oxidative stress (Abrahamse *et al.*, 1999; Knoll *et al.*, 2006a). In a recent study we have been able to show that both compounds are genotoxic in human colon cells and that their genotoxicity can be reduced by GSH/glutathione-*S*-transferases (Pool-Zobel *et al.*, 1996). To study more interactions, we therefore assessed how low doses of U-NTA affect the genotoxicity of these compounds and the repair of the DNA damage that they cause. It had, for instance, been shown that very low doses of Fe-NTA clearly increased the DNA-damaging effects of HNE, indicating synergistic potentials of the combination HNE + Fe-NTA (Knöbel *et al.*, 2006). Here, using the same approach with uranium, we did not see similar effects. We also did not observe that U-NTA affected the DNA repair of H_2O_2-mediated

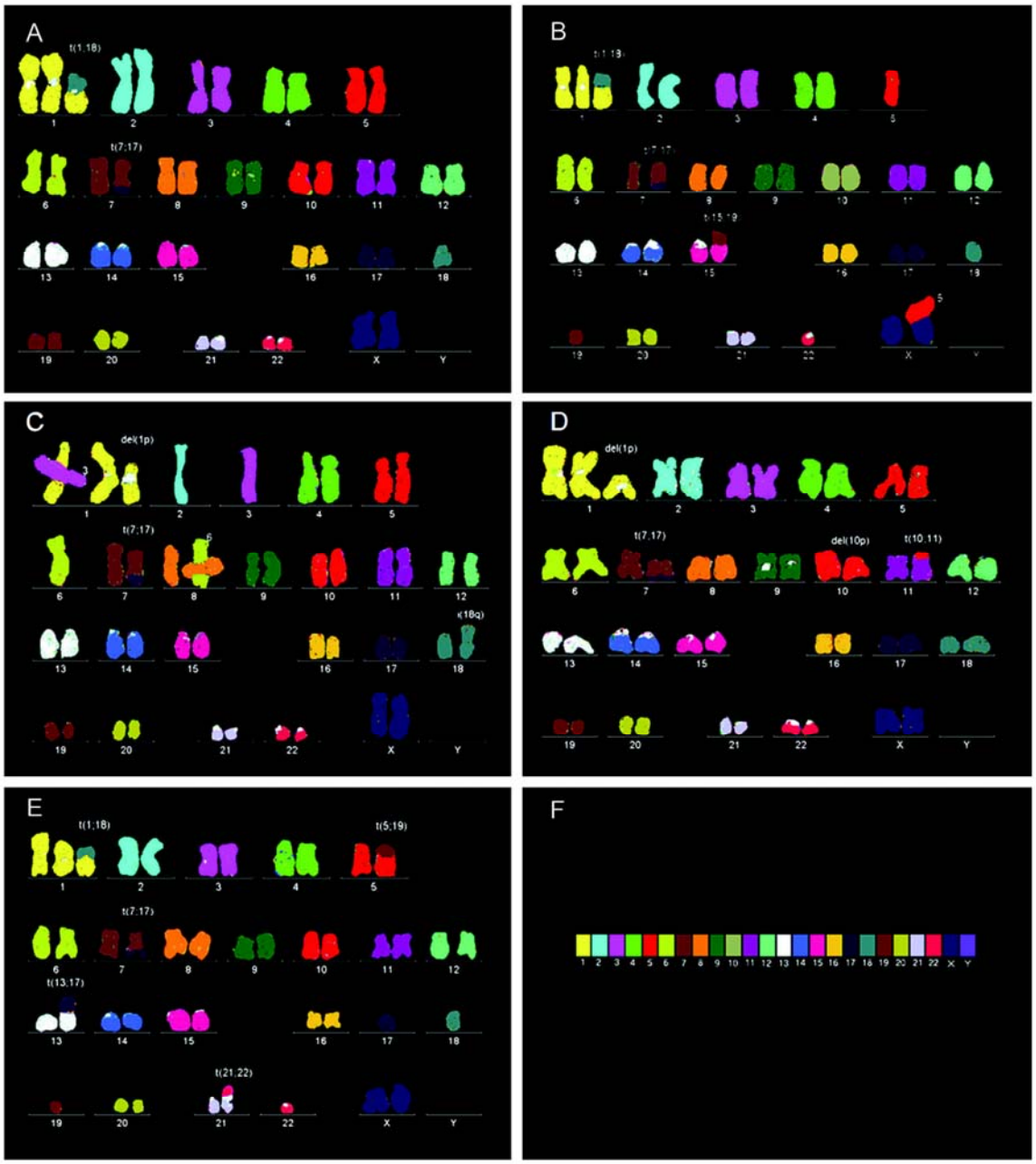


FIG. 5. 24-color-FISH examples of the same passage of the cell line LT97 treated with solvent control (A), 1mM EMS (B), 0.5mM U-NTA (C), 1mM U-NTA (D), and 2mM U-NTA (E) for 6 h at 37°C. The details of the pseudocolor code are shown in (F). Chromosomal aberrations are summarized in the karyotype formula for each example. (A) 46,XX,+dic(1;18)(q10;q10),der(7)t(7;17)(q31.3;q21.3),−18; (B) 44,XX,+dic(1;18)(q10;q10),der(7)t(7;17)(q31.3;q21.3),der(15)t(15;19)(p11.1?;q11?),−18,−19,−22; (C) 46,XX,del(1)(p21),−2,der(7)t(7;17)(q31.3;q21.3),i(18)(q11.1); (D) 47,XX,del(1)(p21),der(7)t(7;17)(q31.3;q21.3),del(10)(p11.2?),der(11)t(10;11)(p11.2?;p15?); (E) 44,XX,+dic(1;18)(q10;q10),der(5)t(5;19)(p11?;q12?),der(7)t(7;17)(q31.3;q21.3),der(13)t(13;17)(p11.1?;11.2?),17,18,19,der(21)t(21;22)(p11.1?;q11.2?),−22.

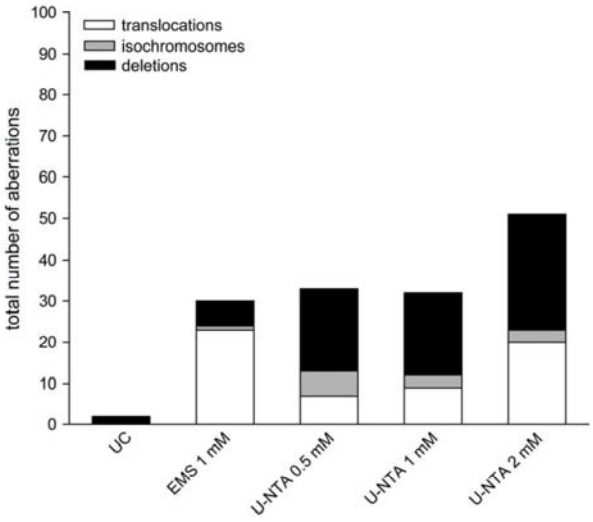


FIG. 6. Chromosomal breaks observed in 50 evaluated metaphases in LT97 adenoma cells after incubation with U-NTA, EMS, and untreated control (UC) medium (6 h, 37°C). Plotted is the total number of translocations, deletions, and isochromosomes induced by the treatment.

damage or of HNE- and NOH-PhiP–induced DNA damage. In contrast, similar types of interactions have been detected with other carcinogenic metal compounds, such as nickel, cadmium, arsenic, and cobalt. Nickel, cadmium, cobalt, and arsenic interfere with the nucleotide and base excision repair at low, nongenotoxic concentrations (Hartwig and Schwerdtle, 2002). As a possible mechanism of repair inhibition, it was suggested

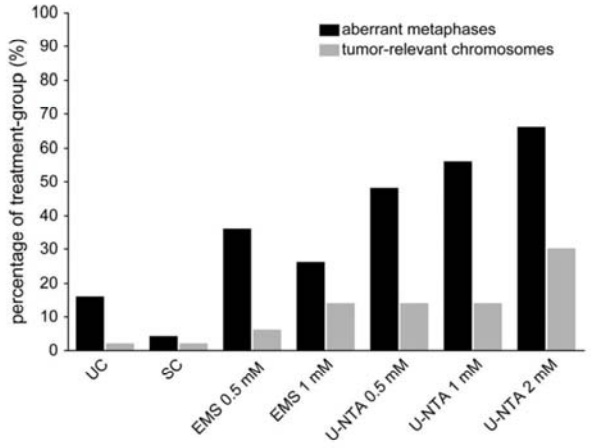


FIG. 7. Percentage of the tumor-relevant chromosomes 5, 12, and 17 (genes: *APC*, *KRAS*, *TP53*) in total aberrant metaphases of LT97 adenoma cells. UC, untreated medium control, which was different from the SC (solvent control) containing only NTA. Percentage of affected metaphases with at least one damaged chromosome of the tumor-relevant chromosomes was calculated for 50 evaluated metaphases.

that Ni(II) replaced other divalent ions in repair enzymes, which are essential for enzyme activity (Hartwig and Beyersmann, 1989). Here, we saw no indication that U-NTA modulates the DNA repair of damage caused by different genotoxins, and thus U-NTA probably does not inhibit repair by this mechanism.

Putative carcinogens initiate colon cancer, presumably by first damaging the *APC* gene. This step is followed by alterations of *KRAS* and *SMAD*4 (mothers against decapentaplegic homolog 4) (Potter, 1999). These genetic alterations may cause cells to proliferate more rapidly and thus form aberrant crypts, polyps, and microadenomas. Mutations in the tumor suppressor gene *TP53* are the final genetic changes in colon carcinogenesis and they are responsible for converting adenoma cells into invasive carcinoma cells. In order to study whether U-NTA damages human colon cells in tumor-relevant genes, such as in *TP53*, we stained comet images of the comet assay slides using FISH technique. U-NTA caused a dose-dependent migration of *TP53* into the comet tail in both LT97 and primary colon cells. Treatment of cells with high U-NTA concentrations was associated with a detectable migration of *TP53* in about 40–50% of the damaged cells. Thus, we can conclude that the tumor suppressor gene *TP53* is sensitive toward U-NTA at high concentrations, especially in primary human colon cells. Comparable results were obtained by Schäferhenrich *et al.* (2003a) with HNE- and H_2O_2-mediated genotoxic damage in LT97 cells. They concluded that these compounds were more effective in damaging *TP53* than total genomic DNA.

Our LT97 adenoma cells were derived from preneoplastic stages of tumorgenesis and were thus interesting target cells to study clastogenic effects of U-NTA (Knoll *et al.*, 2006a). EMS was used as the positive control compound. It is an alkylating agent which induces apurinic and apyrimidine sites (AP sites) and forms SSBs in the next S-phase. These SSBs are converted into double-strand breaks in the second S-phase (Kaufmann and Paules, 1996). We incubated our cells for 6 h, after which a medium change was performed. The culture was further incubated for 46 h to allow the minimum one reproduction cycle. Since the cells are not all in the same phase of the cycle when the incubation starts, the cells pass different numbers of S-phases. In our study we could also demonstrated that EMS induce translocations or chromosome deletions (Knoll *et al.*, 2006b). Here, uranyl acetate was shown to be clastogenic in LT97 cells, where it also caused deletions and translocations. The proportion of tumor-related chromosomes in the total score of damaged chromosomes was about 30% (2mM U-NTA). These results support the hypothesis that chromosomal rearrangements might be causally involved in early stages of carcinogenesis. Lin *et al.* (1993) found increased frequencies of micronuclei, sister-chromatid exchanges, and chromosomal aberrations in CHO cells after incubation with uranyl nitrate (10–300μM). Opposed to this, McDiarmid *et al.* (2000) did not observe chromosomal alterations in peripheral blood

lymphocytes of Gulf War veterans, who were exposed to DU. The same was true of uranium-mining workers experiencing higher and longer exposures to uranium-associated compounds, who did not show an increased incidence of chromosomal aberrations in the white blood cells (Lloyd *et al.*, 2002). In contrast, a group of workers from the "Wismut" mines exhibited a significantly increased incidence of micronuclei in their lung macrophages (Popp *et al.*, 2000). These differences could possibly be due to varying types and the extents of exposures or to differences in the target cells that were examined.

We were able to detect significant genotoxic and gene-specific effects at higher concentrations (at least 100μM) that normally do not occur in our environment. There are, however, geographical regions with high concentrations of uranium and other radionuclides in the groundwater resulting from uranium mining or processing or from special events like reactor accidents or use of atomic bombs. This is associated with a higher transfer of these compounds into the water cycle and food chain (Fisenne *et al.*, 1987; Priest, 2001). The burden of the gastrointestinal system by consuming contaminated soil, dust, food, or drinking water has been shown to increase (Thomas, 2000). Kurttio *et al.* (2002) measured 131 μg U/l drinking water in South Finland, which exceeded the WHO (2004) guideline level of 15 μg/l. The authors postulated that an increase in the daily intake of uranium from drinking water by 1 μg was associated with an increase of 0.21 ng U/mmol creatinine in urine. They detected a mean urine concentration of 424 μg U/l. This concentration (424 μg U/l = 1.8μM) is only about 50-fold lower than the concentration, which was effective in our study. Considering the longer term exposure durations in humans as compared to the treatment periods in our cell culture experiments, the respective doses may be even more similar to each other. A number of other investigations have also reported on detectable urinary uranium concentrations after environmental uranium exposure, which, however, did not reach the levels reported by Kurttio *et al.* (2002; Hooper *et al.*, 1999; Orloff *et al.*, 2004). In any case, the available data do suggest that the contamination of regional foods in the human food chain could increase genotoxic exposure and thus the risk of gastrointestinal diseases for exposed populations (Küppers and Schmidt, 1994).

In summary, U-NTA was cytotoxic in HT29 clone 19A tumor cells. U-NTA was also genotoxic in nontransformed human colon cells and in a cell line derived from a human colon adenoma. Health effects from environmental uranium exposures have been well documented in the past, but the potential genotoxicity to the gastrointestinal tract via dietary exposure was not previously considered. The results of our study, therefore, add support to the assumption that this contaminant may increase alimentary genotoxicity in humans if it reaches the food chain in sufficient amounts. It is therefore necessary, in the future, that these types of exposures be included in environmental studies.

ACKNOWLEDGMENT

This work has been carried out with financial support by Deutsche Forschungsgemeinchaft project number PO 284/6-1 and PO 284/6-2. Furthermore, we are thankful to Dr. William M. Pool for editorial assistance and to Prof. B. Marian, Institute of Cancer Research, University of Vienna, Austria for the generous gift of LT97 adenoma cells.

REFERENCES

Abrahamse, S. L., Pool-Zobel, B. L., and Rechkemmer, G. (1999). Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration in isolated rat colon cells. *Carcinogenesis* **20,** 629–634.

Agency for Toxic Substances and Disease Research. (ATSDR). (1999). *Toxicological Profile for Uranium*. U.S. Public Health Service, Department of Health & Human Services, Atlanta, GA.

Augeron, C., and Laboisse, C. L. (1984). Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. *Cancer Res.* **44,** 3961–3969.

Beyer-Sehlmeyer, G., Glei, M., Hartmann, F., Hughes, R., Persin, C., Böhm, V., Rowland, I. R., Schubert, R., Jahreis, G., and Pool-Zobel, B. L. (2003). Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources. *Br. J. Nutr.* **90,** 1057–1070.

Burkart, W. (1991). Uranium, thorium and decay products. In *Metals and Their Compounds in the Environment, Occurrence, Analysis and Biological Relevance*. E. Merian (editor), VCH Verlagsgesellschaft, Waldheim, Germany.

Cardis, E., and Richardson, D. (2002). Invited editorial: Health effects of radiation exposure at uranium processing facilities. *J. Radiol. Prot.* **20,** 95–97.

Claussen, U., Michel, C., Mühlig, P., Westermann, M., Grummt, U.-W., Kromeyer-Hauschild, K., and Liehr, T. (2002). Demystifying chromosome preparation and the implications for the concept of chromosome condensation during mitosis. *Cytogenet. Genome Res.* **98,** 136–146.

Collins, A. R., Duthie, S. J., and Dobson, V. L. (1995). Direct enzymic detection of endogenous oxidative base damage in human lymphocyted DNA. *Carcinogenesis* **14,** 1733–1735.

Durakovic, A. (2001). On depleted uranium: Gulf War and Balkan Syndrome. *Croat. Med. J.* **42,** 130–134.

Fearon, E. R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* **61,** 759–767.

Fisenne, I. M., Perry, P. M., Decker, K. M., and Keller, H. W. (1987). The daily intake of 234, 235, 238U, 228, 230, 232Th and 226, 228Ra by New York city residents. *Health Phys.* **53,** 357–363.

Franklin, S. J. (2001). Lanthanide-mediated DNA hydrolysis. *Curr. Opin. Chem. Biol.* **5,** 201–208.

Giang, N., Shiraishi, K., Sinh, N. M., Kimura, S., Tuan, N. N., and Arae, H. (2001). Estimation of dietary ^{232}Th, ^{238}U, cesium, and strontium intakes in Vietnamese peoples from different geographical regions. *Health Phys.* **80,** 605–611.

Giannardi, C., and Dominini, D. (2003). Military use of depleted uranium: Assessment of prolonged population exposure. *J. Environ. Radioact.* **64,** 227–236.

Hamilton, M. M., Ejnik, J. W., and Carmichael, A. J. (1997). Uranium reactions with hydrogen peroxide studies by EPR-spin trapping with DMPO. *J. Chem. Soc. Perkin Trans.* **2,** 2491–2494.

Hartmann, A. M., and Speit, G. (1996). Effect of arsenic and cadmium on the persistence of mutagen-induced DNA lesions in human cells. *Environ. Mol. Mutagen.* **27,** 98–104.

Hartwig, A., and Beyersmann, D. (1989). Comutagenicity and inhibition of DNA repair by metal ions in mammalian cells. *Biol. Trace Elem. Res.* **21,** 359–365.

Hartwig, A., Schlepegrell, R., Dally, H., and Hartmann, M. (1996). Interaction of carcinogenic metal compounds with deoxyribonucleic acid repair process. *Ann. Clin. Lab. Sci.* **26,** 31–38.

Hartwig, A., and Schwerdtle, T. (2002). Interactions by carcinogenic metal compounds with DNA repair processes: Toxicological implications. *Toxicol. Lett.* **127,** 47–54.

Hei, T. K., Liu, S. X., and Waldren, C. (1998). Mutagenicity of arsenic in mammalian cells: Role of reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8103–8107.

Hooper, F. J., Squibb, K. S., Siegel, E. L., McPhaul, K., and Koegh, J. P. (1999). Elevated urine uranium excretion by soldiers with retained uranium shrapnel. *Health Phys.* **77,** 512–519.

Jacob, P., Pröhl, G., Schneider, K., and Voß, J.-U. (1997). *Machbarkeitsstudie zur Verknüpfung der Bewertung radiologischer und chemisch-toxischer Wirkungen von Altlasten.* texts 43/97. Umweltbundesamt, Berlin.

Jostes, R. E. (1996). Genetic, cytogenetic, and carcinogenic effects of radon: A review. *Mutat. Res.* **340,** 125–139.

Kathren, R. L. (2001). Tissue studies of persons with intakes of the actinide elements: The U.S. transuranium and uranium registries. *Occup. Med.* **16,** 317–329.

Kaufmann, W. K., and Paules, R. S. (1996). DNA damage and cell cycle checkpoints. *FASEB J.* **10,** 238–247.

Knöbel, Y., Glei, M., Osswald, K., and Pool-Zobel, B. L. (2006). Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells. *Toxicol. In Vitro* **20,** 793–800.

Knoll, N., Weise, A., Claussen, U., Sendt, W., Marian, B., Glei, M., and Pool-Zobel, B. L. (2006a). 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary human colon cells and in cells from preneoplastic lesions. *Mutat. Res.* **594,** 10–19.

Knoll, N., Weise, A., Liehr, T., Pool-Zobel, B. L., Glei, M., and Claussen, U. (2006b). Application of fluorescence in-situ hybridisation (24-color-fish) for testing mutagenicity of ethylmethanesulfonate in preneoplastic human colon cells using the OECD guideline 473. Medizinische Genetik. *Proceedings of the 17th Conference of the German Society of Human Genetics*, Vol. 1, p. 46 (Abstract).

Küppers, C., and Schmidt, G. (1994). Strahlenschutzaspekte bei Altlasten des Uranbergbaus in Thüringen und Sachsen. *Öko-Institut e. V. Werkstattreihe* 86.

Kurttio, P., Auvinen, A., Salonen, L., Saha, H., Pekkanen, J., Mäkeläinen, I., Välsänen, S. B., Pentillä, I. M., and Komulainen, H. (2002). Renal effects of uranium in drinking water. *Environ. Health Perspect.* **110,** 337–342.

Liehr, T., Heller, A., Starke, H., Rubtsov, N., Trifonov, V., Mrasek, K., Weise, A., Kuechler, A., and Claussen, U. (2002). Microdissection based high resolution multicolour banding (MCB) for all 24 human chromosomes. *Int. J. Mol. Med.* **9,** 335–339.

Lin, R. H., Wu, L. J., Lee, C. H., and Lin-Shiau, S. Y. (1993). Cytogenetic toxicity of uranyl nitrate in Chinese hamster ovary cells. *Mutat. Res.* **319,** 197–203.

Lloyd, D. C., Lucas, J. N., Edwards, A. A., Deng, W., Valente, E., Hone, P. A., and Moquet, J. E. (2002). A study to verify a reported excess of chromosomal aberrations in blood lymphocytes of Namibian uranium miners. *Radiat. Res.* **155,** 809–817.

Lund, E. K., Wharf, S. G., Fairweather-Tait, S. J., and Johnson, I. T. (1998). Increases in the concentrations of available iron in response to dietary iron supplementation are associated with changes in crypt cell proliferation in rat large intestine. *J. Nutr.* **128,** 175–179.

McDiarmid, M. A., Keogh, J. P., Hooper, F. J., McPhaul, K., Squibb, K., Kane, R., DiPino, R., Kabat, M., Kaup, B., Anderson, L., *et al.* (2000). Health effects of depleted uranium on exposed Gulf War veterans. *Environ. Res.* **82,** 168–180.

Meinrath, A., Schneider, P., and Meinrath, G. (2003). Uranium ores and depleted uranium in the environment, with a reference to uranium in the biosphere from Erzgebirge/Sachsen, Germany. *J. Environ. Radioact.* **64,** 175–193.

Miller, A. C., Stewart, M., Brooks, K., Shi, L., and Page, N. (2002). Depleted uranium-catalyzed oxidative DNA damage: Absence of significant alpha particle decay. *J. Inorg. Biochem.* **91,** 246–252.

OECD (1997). *Text Guidance 473, In Vitro* Mammalian Chromosome Aberration Test. In *OECD Guidance for Testing of Chemicals.* Paris, Organization for Economic Cooperation and Development.

Orloff, K. G., Mistry, K., Charp, P., Metcalf, S., Marino, R., Shelly, T., Melaro, E., Donohoe, A. M., and Jones, R. L. (2004). Human exposure to uranium in groundwater. *Environ. Res.* **94,** 319–326.

Pool-Zobel, B. L., Abrahamse, S. L., and Rechkemmer, G. (1996). Protective effects of short-chain fatty acids on early events of carcinogenesis: Antigenotoxic effects of butyrate in rat and human colon cells. In *Cost Action 92: Dietary Fibre and Fermentation in the Colon, Proceedings of the COST Action 92 Workshop, Espoo, Finland, 15–17 June 1995*, pp. 350–357. European Commission, DG XII, Science, Research and Development, Brussels.

Popp, W., Plappert, U., Müller, W. U., Rehn, B., Schneider, J., Braun, A., Bauer, P. C., Vahrenholz, C., Presek, P., Brauksiepe, A., *et al.* (2000). Biomarkers of genetic damage and inflammation in blood and bronchoalveolar lavage fluid among former German uranium miners: A pilot study. *Radiat. Environ. Biophys.* **39,** 275–282.

Potter, J. D. (1999). Colorectal cancer: Molecules and populations. *J. Natl. Cancer Inst.* **91,** 916–932.

Priest, N. D. (2001). Toxicity of depleted uranium. *Lancet* **357,** 244–246.

Quig, D. (1998). Cysteine metabolism and metal toxicity. *Altern. Med. Rev.* **3,** 262–270.

Richter, M., Jurek, D., Wrba, F., Kaserer, K., Wurzer, G., Karner-Hanusch, J., and Marian, B. (2002). Cells obtained from colorectal microadenomas mirror early premalignant growth patterns *in vitro*. *Eur. J. Cancer* **38,** 1937–1945.

Schäferhenrich, A., Beyer-Sehlmeyer, G., Festag, G., Kuechler, A., Haag, N., Weise, A., Liehr, T., Claussen, U., Marian, B., Sendt, W., *et al.* (2003a). Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. *Mutat. Res.* **9496,** 1–14.

Schäferhenrich, A., Sendt, W., Scheele, J., Kuechler, A., Liehr, T., Claussen, U., Rapp, A., Greulich, K.-O., and Pool-Zobel, B. L. (2003b). Putative colon cancer risk factors damage global DNA and *TP53* in primary human colon cells isolated from surgical samples. *Food Chem. Toxicol.* **41,** 655–664.

Seeber, O. (1998). Das Angebot, die Aufnahme und die Bilanz des toxischen Schwermetalls Uran bei erwachsenen Mischköstlern in Deutschland. PhD Thesis, Friedrich-Schiller-University Jena, Germany.

Senger, G., Chudoba, I., and Plesch, A. (1998). Multicolour-FISH—The identification of chromosome aberrations by 24 colours. *Bioforum* **9,** 499–503.

Seymen, O., Seven, A., Candan, G., Yigit, G., Hatemi, S., and Hatemi, H. (1997). The effect of iron supplementation on GSH levels, GSH-Px, and SOD activities of erythrocytes in L-thyroxine administration. *Acta Med. Okayama* **51,** 129–133.

Speicher, M. R., Ballard, S. G., and Ward, D. S. (1996). Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat. Genet.* **12,** 368–375.

Taqui Khan, M. M., and Martell, A. E. (1969). Kinetics of metal ion and metal chelate catalyzed oxidation of ascorbic acid. IV. Uranyl ion catalyzed oxidation. *J. Am. Chem. Soc.* **91,** 4668–4672.

Thomas, P. A. (2000). Radionuclides in the terrestrial ecosystem near a Canadian uranium mill—Part II: Small mammal food chains and bioavailability. *Health Phys.* **78,** 625–632.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. C., and Sasaki, Y. F. (2000). Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **35,** 206–221.

UNSCEAR (2000). *Sources and Effects of Ionizing Radiation*. United Nations, UNSCEAR, New York. United Nations Sales publication E.00.IX.3.

WHO (1998). *Guidelines for Drinking Water Quality. Health Criteria and Other Supporting Information*, 2nd ed. WHO, Geneva, Switzerland.

WHO (2004). *Guidelines for Drinking-Water Quality. Uranium in Drinking-Water*, 3rd ed. Background document. WHO, Geneva, Switzerland.

Wollowski, I., Ji, S., Bakalinsky, A. T., Neudecker, C., and Pool-Zobel, B. L. (1999). Bacteria used for the production of yogurt inactivate carcinogens and prevent DNA damage in the colon of rats. *J. Nutr.* **129,** 77–82.

Yazzie, M., Gamble, S. L., Civitello, E. R., and Stearns, D. M. (2003). Uranyl acetate causes DNA single strand breaks in vitro in the presence of ascorbate (vitamin C). *Chem. Res. Toxicol.* **16,** 524–530.

3.4 Manuskript IV

Heavy metal- and radionuclid-contaminated water samples have genotoxic potential to human colon cells

Y. Knöbel, M. Glei, D. Merten, A. Weise, K. K. Richter, U. Claussen, B. L. Pool-Zobel

zur Publikation vorbereitet für Toxicological Sciences

Zusammenfassung

Über den Wasserkreislauf ist der Transfer von mit dem Uranbergbau verbundenen Kontaminanten in die menschliche Nahrungskette möglich. Ziel dieser Studie war es zu untersuchen, ob kontaminierte Wasserproben genotoxisch in humanen Kolonzellen wirken, welche über die orale Aufnahme kontaminierter Nahrungsmittel oder Wässer mit den Wismut-relevanten Verbindungen exponiert werden können.
Im Zellkulturmedium erfolgte die Bildung von H_2O_2 durch die am stärksten kontaminierte Wismut-Probe. Diese hemmte außerdem das Zellwachstum signifikant. Zwei der drei Proben waren in nicht-zytotoxischen Konzentrationen genotoxisch in allen Kolonzellmodellen.
Die kontaminierten Wasserproben und somit die wismutrelevanten Verbindungen haben das Potential zur Bildung von ROS und schädigen die DNA humaner Kolonzellen. Erreichen diese Verbindungen die humane Nahrungskette können sie das genotoxische Belastungspotential erhöhen.

Darstellung des Eigenanteils

- Planung, Durchführung und Auswertung aller praktischer Arbeiten, außer Versuche zur Schadenspersistenz und Bestimmung der Element-Gehalte in den Wismut-Proben
- Erstellen und Korrektur des Publikationsmanuskripte

Heavy metal- and radionuclid-contaminated water samples have genotoxic impact on human colon cells

Y. Knöbel[1], M. Glei[1], K. Osswald[1], K. Richter[2] , D. Merten[3] B.L. Pool-Zobel[1]

[1]Institute for Nutrition, Department of Nutritional Toxicology, Friedrich-Schiller-University, Dornburger Str. 25, 07743 Jena, Germany

[2]Department of General and Visceral Surgery, Friedrich-Schiller-University, Bachstrasse 18, 07743 Jena, Germany

[3]Institute of Geosciences, Department of Applied Geology, Friedrich-Schiller-University, Burgweg 11, 07749 Jena, Germany

Last update 04.01.2007

Address of corresponding author

Prof. Dr. rer. nat. Beatrice L. Pool-Zobel
Friedrich-Schiller-University Jena
Biologisch-Pharmazeutische Fakultät
Department of Nutritional Toxicology
Dornburger Str. 25
07743 Jena
Germany

Tel: + 49 3641 949670
Fax: + 49 3641 949672
E-mail: b8pobe@uni-jena.de
Homepage: www.uni-jena.de/biologie/ieu/et

Running title: Genotoxicity of contaminated water samples

Abstract:

Introduction: Previous extensive uranium mining in the „Wismut“ region of Ronneburg has led to environmental contamination of water with heavy metals and radionucleids. It now appears possible that „Wismut“-related compounds could result in the contamination also of locally produced foods. This could be a hazard if the contaminants have genotoxic potential. This study addresses the question of whether “Wismut” related water samples are genotoxic in human colon cells. Methods: The human colon tumor cells (HT29 clone 19A), adenoma cells (LT97) and primary cells (freshly isolated from colon tissue) were incubated with water samples of the “Wismut” region (A, B, C: 0.5-20 %). We investigated cell growth, formation peroxides, cytotoxic effects and genotoxicity. Results: In aqueous solution water sample C lead to marked production of ROS, determined as H_2O_2. Treatment of HT29 clone 19A cells with water sample C resulted in a significant growth arrest of the cells after 72 h of incubation. Both water samples B and C were genotoxic at non-cytotoxic concentrations in all three cell types. We also detected an enhanced migration of *TP53* signals into the comet tail of LT97 adenoma cells, which indictes a high susceptibility of this gene region towards the exposure with the complecx contaminated sample B. Conclusion: The highly contaminated water samples C gave rise to ROS formation, induced DNA damage in human colon cells and inhibited their cell growth. Thus, if compounds like uranium, iron, nickel or cadmium reach the nutritional cycle via contaminated water they can increase the risk of alimentary genotoxic exposure in humans.

Keywords: colon cells, Comet Assay, Comet FISH, Wismut, heavy metals, uranium

Introduction

Previous extensive uranium mining in the „Wismut“ region of Ronneburg (Thuringia, Germany) has led to an enhanced environmental distribution of heavy metals and radionuclids. The compounds are considered to be hazardous on account of several mechanisms and little is known of potential human health effects due to environmental exposure. Several of the „Wismut“-relevant compounds probably act through formation of free radical oxygen species (ROS) [1-5], through irradiation (radon) [6] or through direct interaction with cellular macromolecules (e.g. inhibition of DNA repair) [7].

The migration of radionuclids and uranyl compounds from uranium mining is associated with the transfer of these compounds into the food chain and water cycle [8-11]. Contamination of surface water and groundwater by effluents from uranium mining, milling and production operations has been documented [12-15]. The transport and dispersion of uranium in water are affected by the presence of aquatic sediments [16]. Uranium in surface water can be dispersed over large distances to rivers and oceans. Ocean water contains about 3.3 µg/l and rivers contain between 0.03 and 3 µg/l (Amazonas 0.03 µg/l, Ganges 3.9 µg/l, german rivers 1-3 µg/l) [17]. In Canada (Ontario) concentrations from 0.05 to 4.21 µg/l were measured in drinking water [18]. In the United States of America drinking water has been reported to contain a mean uranium concentration of 2.55 µg/l [19]. In 18 % of Norwegian groundwater samples, uranium concentrations exceeded 20 µg/l [20]. A study in Finland examined a population receiving drinking water that contained 28 µg/l uranium. Drinking waters in Germany were reported to contain from <1 up to 100 µg/l uranium. The intake of drinking water with high uranium concentrations increases the risk of kidney cancer. Studies in rats indicated a lowest observed adverse effect level (LOAEL) of 60 µg/kg body weight per day. With an uncertainty factor of 100 for intra-

and interspecies variation, the World Health Organisation (WHO) estimated that the tolerable daily intake (TDI) level for uranium is 0.6 µg/kg bodyweight per day. For drinking water, the WHO recommended a limit of 15 µg/l [21;22]. The Environmental Protection Agency (EPA) reported risks for human health above a limiting value of 30 µg/l [23]. In Germany no value for uranium in drinking water exists. The German Environmental Office proposes a value of 10µg/l as perpetual regards health dose in drinking water [24]. Our water samples exceed these recommended values partly obviously. Thus this water samples hold a health risk for animals and humans. The burden of the gastrointestinal system by consuming contaminated soil, dust, food or drinking water has been shown to increase [25]. The contamination of the water cycle is associated with the transfer of these compounds into the food chain. In plants, uptake of uranium may be restricted to the root systeme [26]. In uranium mining areas there is often a higher uranium contamination of the soil. These increased uranium concentrations are mostly detectable in the plants [27-29]. In the uranium mining area around Ronneburg (Germany) different food plants had 2 to 8-fold higher uranium levels than in control areas [30]. An accumulation in the food chain via feeding stuff would be imaginable. In Ocean water uranium exists as tricarbonatouranylation which has a low bioavailability [31]. Therefore, there is no accumulation of uranium in marine ecosytems and sea food [32]. Due to the relative high consumption of beverages, drinking water significantly contribute to the uranium intake of the population.

Beside the risk, connecting with the inhalation of wismut related compounds and the damage of the lung, there is an increased risk for the gastrointestinal section by the entry of these compounds into the food chain and water cycle. Thus, the objectives of our present study were to investigate in cells representing different stages of transformation of human colon cells how heavy metal- and radionuclid-

contaminated water samples influences cell proliferation, peroxide formation, DNA damage and their repair. Furthermore we are interested in *TP53* gene-specific interactions.

Materials and methods

Samples: Water samples (50 mL each) were collected at the northeastern boundary of the former waste rock dump “Gessenhalde” (sample C) and along the Gessenbach creek (sample A and B). pH, electrical conductivity, redox potential and temperature of the unfiltered samples were directly measured in the field using portable instruments pH320, LF320 and an external thermocouple (WTW Wissenschaftlich Technische Werkstätten, Germany). The water samples were filtered with a pore size of 0.20 µm. 100 µl of nitric acid (65 %, Baker Ultrex, USA) were added to the water samples for stabilization. Fe was analyzed by flame atomic absorption spectrometry (F-AAS 5, Zeiss, Germany), Al, Ba, Cd, Co, Cu, Mn, Sr, Pb, Th, U and Zn were determined by inductively coupled plasma mass spectrometry (PQ3, ThermoElemental, U.K.).

In F-AAS a liquid sample is aspirated and mixed as an aerosol with combustible gasses (acetylene and air). A flame with a temperature of about 2500 K is used to transfer the analytes to the atomic state. A light beam from a lamp whose cathode is made of the element being determined is passed through the flame into a monochromator and detector. Free, unexcited ground state atoms of the element absorb light at characteristic wavelengths; this reduction of the light energy at the analytical wavelength is a measure of the amount of the element in the sample.

In ICP-MS a plasma, consisting of ionized Ar, is used to generate temperatures in the range of 5000 K. Due to this high temperature the analytes are quantitatively transformed into ions. These are extracted by means of electrical potentials in to the mass spectrometer being kept under vacuum. A quadrupole selects ions according to their mass to charge ratio. The specific analytes are registrated as an electrical current at the detector allowing quantification.

The samples were stored at 4 °C, the chelating agent disodium nitrilotriacetae was added to prevent oxidation of iron.

Cells and culture conditions: The target cells were HT29 clone 19A cells which are derived from the carcinoma cell line HT29 by treatment with sodium butyrate [33]. The cells were grown in tissue culture flasks with Dulbecco´s Modified Eagle Medium (DMEM, Gibco BRL, Eggenstein, Germany), supplemented with 10 % foetal calf serum and 1 % penicillin/streptomycin, at 37°C in a humidified incubator (5 % CO_2/95 % air). Passages 20-50 were used for the experiments described here.

The human colon adenoma cell line LT97 was established from colon microadenoma of a patient with familial adenoma polyposis coli [34]. LT97 cells were maintained in a culture medium (MCDB 302 basis medium) containing 20% of L15 Leibovitz medium, 2% FCS (foetal calf serum), 1% penicillin/streptomycin, 0.2 nM triiodo-L-thyronine, 1 µg/ml hydrocortisone, supplemented with 10 µg/ml insulin, 2 µg/ml transferrin, 5 nM sodium selenite and 30 ng/ml epidermal growth factor (EGF). Cells were grown in a humidified incubator under standardized culture conditions (5 % CO_2, 95 % humidity, 37°C). Passages 17-33 were used for the experiments in this study.

Primary human colon cells were isolated from surgical tissue (non-tumor tissue [35]. The Ethical Committee of the Friedrich-Schiller-University Jena approved the study and the tissue was obtained from patients who gave their informed consent. First, the human colon epithelium was separated from the underlying layers of tissue by perfusion-supported mechanical disaggregation. The epithelium stripes were cut into small pieces and incubated with 2 mg/ml proteinase K (Sigma, Steinheim, Germany) and 1 mg/ml collagenase P (Boehringer, Mannheim, Germany) for 60 min in a shaking water-bath at 37°C. After washing and centrifugation (5 min at 400 x g), the pellets were resuspended in phosphate buffered saline (PBS; 8.0 g/l NaCl; 1.44 g/l Na_2HPO_4; 0.2 g/l KH_2PO_4; pH 7.3). Pellets containing erythrocytes were treated with erythrocyte-lysis buffer (hypertonic ammonium chlorid solution: 155 µM NH_4Cl; 5 µM KCl; 0.06 µM EDTA; pH 7.0), centrifugated, and resuspended in RPMI 1640 (GibcoBRL, Eggenstein, Germany). Yields of the cells and viabilities were determined using the trypan blue exclusion assay.

Determination of cell growth: Growth of HT29 clone 19A cells was determined in 96-well microtiter plates [36]. 8000 cells/well were seeded and after 24 h they were treated with 0-20 % of the Wismut samples dissolved in culture medium. Proliferation rates were detected after 24 h, 48 h and 72 h of treatment. Quantification of DNA was achieved by fixation and permeabilisation of cells using methanol for 5 minutes, followed by the addition of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Deisenhofen, Germany). DNA content was detected by fluorimetric analysis with Ex/Em 360/465 nm, 30 min after application of DAPI in a Microplate Reader (Spectra Fluor Plus, Tecan, Austria; Software: XFlour). Mean values of at least three independent experiments are shown in the figures.

Determination of H_2O_2 formation in culture medium – FOX Assay: Peroxide production, or rather hydrogen peroxide formation, in the cell culture medium DMEM in the presence of wismut related compounds was analysed using the FOX method [37]. Different concentrations of water samples B or C (1-10 % in culture medium DMEM) were incubated for 30 minutes at room temperature to allow for the formation of H_2O_2. Then, 40 µl of the test suspensions were mixed with 360 µl of the FOX solution and incubated for 30 minutes at room temperature, during which time ferrous ions were oxidized to ferric ions by the formed hydroperoxides. Serial concentrations of H_2O_2 (0-75 µM) were processed in parallel for calibration. Ferric ions are detected by the formation of a coloured complex with xylenol orange. After centrifugation (1900 x g, 10 minutes) 3 x 100 µl of the supernatant were transferred into wells of a 96-well plate and the absorption at 560 nm was measured. Concentrations were determined from the hydrogen peroxide standard curve. The results were expressed as hydrogen peroxide-equivalents.

Determination of genetic damage: LT97 cells were washed with PBS containing ethylenediaminetetraacetic acid (EDTA, 5 mM) and subsequently trypsinized with trypsin/versene (10% (v/v), Invitrogen, Karlsruhe, Germany) at 37°C for 2-5 min and then suspended in RPMI. HT29 clone 19A cells were also trypsinized and resuspended in RPMI. Primary human colon cells were isolated as described above. Cell numbers and viabilities were determined with trypan blue exclusion and the cell numbers were adjusted to 2 x 10^6 cells per ml. 200 µl of this cell suspension were incubated with 0-20 % of the Wismut water sample at 37°C for 30 minutes on a thermomixer. Then the suspensions were centrifugated at 380 x g for 5 minutes and the cell pellets were washed with 200 µl PBS. 20 µl were stored on ice for determination of cell number and viability. The remaining cell suspensions were

centrifuged at 380 x g for 5 minutes. The cell pellets were mixed with agarose and distributed onto microscopical slides. The Comet Assay was performed as described before [36].

Microscopic evaluation of the images was quantified using the image analysis system of Kinetic Imaging (Liverpool, UK). 50 cells were evaluated per slide and the percentage of fluorescence in the tail (TI, "tail intensity") was scored. The means of 3 replicates were used to calculate the means of at least three independently reproduced experiments.

Determination of gene-specific damage in *TP53* (Comet FISH): LT97 microadenoma cells were used to determine the sensitivity of the gene *TP53* towards the the complex water sample B with the technique of “Comet FISH”. Direct Texas Red labelled *TP53* probes, kind of Prof. Claussen, Jena, were mixtures of directly labelled probes specific for a number of genomic sequences including the *TP53* locus (17pter-p12). These probes yielded a signal that was clearly outlined in interphase nucleus.

FISH experiments were performed as described by Schäferhenrich et al. [38;39], but modifications of the hybridisation conditions were adapted to detect the texas red labelled *TP53* probes. The dehydrated slides from the Comet Assay were rehydrated in ddH_2O for 10 min. Thereafter, the target DNA was denatured with 0.5 M NaOH for 30 minutes and neutralised in 0.01 M PBS for 1 minute. The slides were dehydrated using ethanol (70 %, 80 % and 95 % ethanol solutions 5 minutes each) and dried at room temperature. 30 µl Hybrisol VI (Oncor, Gaithersburg, UK) were dropped onto each gel and spread with a plastic cover slip. The *TP53* probe (5 µl for each slide) was diluted with Hybrisol VI (7 µl for each slide) and denatured at 75°C for 5 minutes, 4°C for 2 minutes and 37°C for 30 minutes. This hybridisation mixture (12 µl/slide) was added to the slides, covered with a plastic cover slip (24x24 mm) and incubated in hybridisation

chambers for 72h at 37°C. The cover slips were removed and the slides were first washed for 5 minutes in 2xSSC without formamid (saline sodium citrate; 0.3 M NaCl; 0.03 M NaCitrate, pH 7.2) at 65°C and then washed for further 5 minutes with 1xPBD (phosphate buffered detergent; Oncor, Gaithersburg, UK) at room temperature. SYBR-Green (1 µl/ml, 30 µl per slide) was used to counterstain the texas red labelled probes. Negative controls were performed for each concentration on the same slide using Hybrisol VI without the DNA probe during hybridisation (24x24 mm cover slip).

To evaluate results from the Comet FISH experiments, the comets were categorised into four degrees of damage ranging from non-damaged to severely damaged images (classes 1-4) [40]. First, the total number of *TP53* signals per cell was determined. The expected number of spots in a normal interphase nucleus is two. On the slides hybridised with the fluorescent labelled probes, cells without signals, with only one signal and with two signals were counted and the hybridization efficiency was determined. Moreover, cells with broken fluorescent signals were counted so that breaks occurring directly in the analysed genes could be quantified. For the further Comet FISH evaluation only cells with two fluorescent spots were used and the localization of the *TP53* signals in the comet head or comet tail was recorded. The parameter of *TP53* migration was based on the percentage of damaged cells (belonging to comet classes 2-4), in which we could find a migration of at least one *TP53* signal into the comet tail. 100 to 120 images per slide were measured during the Comet FISH evaluation.

Fluorescence microscopy was performed using a ZEISS Axiovert M100 (Carl Zeiss Jena GmbH, Jena, Germany) equipped with filters for detection of Texas Red (red, ZEISS filter No 15) and SYBR Green (green, ZEISS filter No 09). Images were captured using a MicroMAX Digital CCD camera (BFI OPTILAS GmbH, Puchheim,

Germany) and Meta View Imaging Software (Visitron Systems GmbH, Puchheim, Germany) was used to process the images.

Statistical Evaluation: The GraphPad Prism software Version 4.0 (GraphPad Software Inc., San Diego, USA) was used for establishing significance of effects. Statistics were carried out on the mean values of the data and these are also presented (mean and SD) in the diagrams and tables. As is specified in the legends to the figure, one-way ANOVA with Dunnett's post test or Bonfferoni´s post test and two-way ANOVA with Bonfferoni´s post test were used as appropriate.

Results

Chemical Analysis of the water samples

Table 1 outlined the most important macro- and micro elements. Related to the amounts of iron and uranium we can characterize sample A as not contaminated, sample B as moderately contaminated and sample C as highly contaminated.

Effects of complex water samples on cell growth

The influence of heavy metal-contaminated water samples on cell growth was studied to determine the sub-toxic concentrations needed to perform subsequent investigations. The growth of HT29 clone 19A cells was not influenced by a 72-hour treatment with the wismut samples A or B. In contrast, a significant growth arrest resulted from the treatment of the HT29 clone 19A cells with sample C (Figure 1, 10 and 20 % of the sample: $p< 0.01$).

Effects of complex water samples on the formation of H_2O_2

We measured significant concentrations of H_2O_2 after adding of increasing concentrations of samples C to the cell free culture medium (Figure 2). Sample C significantly increased H_2O_2 equivalents in a dose-dependent manner. For sample B no H_2O_2-formation was detectable.

Effects of complex water samples on DNA damage

Tables 2 and 3 summarizes the data on strand breaks in HT29 clone 19A cells, LT97 cells and primary colon cells after 30 minutes of incubation with the water samples B (Table 2) and C (Table 3). Both samples were genotoxic in all three cell types in a dose-related manner. For LT97 cells significant results were observed only

at 10 % of sample B and the positive control Fe-NTA. At 7.5-10 % of sample B, 2.5-10 % of sample C and for Fe-NTA we could show significant genotoxic effects in primary human colon cells. In HT29 clone 19A cells these effects were significant for 5-10 % of sample B, respectively 2-10 % of sample C and for the positive control Fe-NTA. In HT29 clone 19A cells and in LT97 cells higher levels of DNA damage were observed at the highest tested concentration of sample C (10 %, 29 µM Fe and 27 nM U) than for 500 µM Fe-NTA. We observed no cytotoxic effects of our substances at the tested concentrations. The viability after 30 minutes of incubation was between 70 and 100 %.

Effects of complex water samples on gene specific damage in *TP53*

For Comet FISH experiments we used LT97 and primary colon cells. HT29 clone 19A cells are unsuitable for these experiments since they have an unstable karyotype. Damage induced in *TP53* was evaluated by first categorizing the images according to comet classes. We determined the total number of signals per slide and per cell. A normal metaphase or interphase nucleulus has two spots. Cells without and with only one signal were counted and the hybridisation efficiency was determined to be …….for primary colon cells and 85.0 ± 2.6 % for LT97 cells. Only very few comets had 3 signals which would represent the actual breakage and isolated migration of a *TP53*-gene fragment. Also there was no compound- or comet class-related effect on the yield of 3 distinct *TP53* signals (results not shown). For further Comet FISH evaluation, only cells with two spots were used.

Figure 4A summarizes the frequency of damaged cells (comet classes 2-4) resulting from the treatment of LT97 with the water sample B (30 minutes, 37 °C). The total score of damaged cells increased with increasing concentrations of the sample. A significant shift was observed primarily in the direction of the stronger

damaged cells. This water sample significantly induced DNA damage, already at 2.5 % of the sample in LT97 adenoma cells.

The proportion of damaged cells with the *TP53* signals in the comet tail is outlined in Figure 4B. We observed a significant *TP53*-migration into the comet tail comparing to the RPMI control also already at 2.5 % for LT97.

Discussion

An investigation on the nutritional intake of uranium in the former GDR demonstrated that an elevated consumption had occurred in the population of these region resulted in a daily intake of 2.05 µg for women and 2.40 µg for men (Seeber arbeit). In Vietnam Giang et al. found an uranium intake of 0.66 µg U^{238}/d [41], whereas Shiraishi and Yamamoto described a daily intake of 1.25 µg/d. Dang et al. reported a daily uranium intake of 2.2 µg for India which correlates with the data of Seber for the region of the former GDR. Seber et al. also compared the mean intake of uranium of Germany with this of Mexico, in both countries it was about 2.0 µg/d. Interestingly when comparing regions with higher occurrence of natural uranium in Germany (Thuringia, Saxonia) with regions with normal uranium occurrence (for example Brandenburg, Mecklenburg-Vorpommern) they observed significant differences of the daily uranium intake. For the former uranium mining area around Ronneburg an uranium intake of 35 µg/ d for women and 24 ng/kg body weight for men was estimated in the year 1996.

The contamination level of the water samples correlates with the near of the sampling points to the former waste rock dump “Gessenhalde”. Sample C with the highest iron and uranium concentrations was collected directly at the northern boundary of this waste rock dump. Samples B and C were collected in different distances to the waste rock dump along the Gessenbach.

Long time incubation (72 hours) of the carcinoma cell line HT29 clone 19A with the highly contaminated water sample C resulted in a significant decreased cell survival. In recent studies the two single noxes iron and uranium which are main contaminants in our samples also decreased cell survival after 72 hours [36;42]. The effective iron concentration (2000 µM) is comparable with the concentration of iron in 20 % of the water sample C (about 1620 µM iron) which resulted in a significant reduction of cell

survival in our present study. The uranium concentration in sample are about 1000-fold lower (20 % of sample C contains about 60 nm uranium) than the first effective concentration in the investigations with uranium alone (75 µM). These results argue for the possibility that other contaminants in our environmental samples, like nickel or copper contribute to the decreased cell survival after the long time incubation of the carcinoma cells with the complex water sample C.

Because of the high heavy metal concentrations in our samples, especially high iron concentrations we assumed that our water samples are able to produce hydrogen peroxide. The formation of reactive oxygen species (ROS) via the Fenton reaction, is a very potent risk factor for colon cancer which possibly predisposes to colon diseases by some of these mechanisms [43-46]. ROS are involved in the promotion phase of tumor generation by contributing to the regulation of gene expression during cell growth and differentiation [47]. In a recent study we could demonstrate that the main contaminant in our water sample, namely iron induces ROS [36]. Thus we wanted to know, if our complex environmental samples are able to induce oxidative stress, too. The FOX assay was used to investigate the H_2O_2 generation by our samples. For the highly contaminated probe we measured a significant dose-dependent generation of H_2O_2 up to 100 µM.

The consequence of ROS could be a damaging of DNA. Here we could show that the two contaminated samples are genotoxic in colon cells of different transformation state. Recently we verified the genotoxic potential of the two single noxes iron and uranium [36;42]. But it is probably that other metals like nickel, arsenic or copper also plays a role in this genotoxic potential of our complex samples. Nickel, cadmium, cobalt and arsenic compounds are well-known carcinogens by interfering with DNA repair processes, even though their direct DNA-damaging potentials are rather weak [48-51].

Acknowlegements

This work has been carried out with financial support by Deutsche Forschungsgemeinchaft project number PO 284/6-1 and PO 284/6-2. Furthermore, we are thankful to Dr. William M. Pool for editorial assistance and to Prof. B. Marian, Institute of Cancer Research, University of Vienna, Austria for the generous gift of LT97 adenoma cells.

Legends to the figures

Figure 1: Growth of HT29 clone 19A cells after 72 h of treatment with the wismut samples A, B and C. Growth rates are depicted as means ± SD (n=8). Statistical evaluation was performed applying One-way-ANOVA versus medium control combined with Dunnett's Multiple Comparison Post Test (**p<0.01).

Figure 2: Formation of H_2O_2 after 30 minutes incubation of the cell culture medium DMEM with the wismut samples B and C. Statistical evaluation was performed applying One-way-ANOVA combined with Dunnett's Multiple Comparison Test (*p<0.05, **p<0.01) against the blank.

Figure 3: Proportional distribution of LT97 cells in the comet classes2-4 (damaged cells) after 30 min treatment with the water sample B (1-10 %, 37°C, n=3). The significant differences in the comet classes to the untreated medium control (RPMI) were calculated by one-way ANOVA, including the Dunnett multiple comparison test (*p<0.05, **p<0.01, ***p<0.001). The percentage of cells in comet class 1 is not outlined in this figure but can be calculated as followed: 100 % minus the percentage of cells in comet classes 2-4. The significance levels for the changes in comet class 1 are outlined above the total bars.

Figure 4: Percentage *TP53* migration in the comet tail of damaged cells (comet classes2-4) after 30 min treatment with the water sample B (1-10 %, 37°C, n=3). The significant differences to the untreated medium control (RPMI) were calculated by one-way ANOVA, including the Dunnett multiple comparison test (*p<0.05, **p<0.01)

Reference List

1. Chen,Y.C., Lin-Shiau,S.Y., and Lin,J.K. (1998) Involvement of reactive oxygen species and caspase 3 activation in arsenite-induced apoptosis. *J Cell Physiol*, **177**, 324-333.

2. Cimino,F., Esposito,F., Ammendola,R., and Russo,T. (1997) Gene regulation by reactive oxygen species. *Current Topics in Cellular Regulation*, **35**, 123-148.

3. Hei,T.K., Liu,S.X., and Waldren,C. (1998) Mutagenicity of arsenic in mammalian cells: role of ractive oxygen species. *Proc Natl Acad Sci USA*, **95**, 8103-8107.

4. Kasprzak,S. (1992) Animal studies: An overview. In Nieboer,E. and Nriagu,J.O. (eds.) *advances in Environmental Science and Technology, Nickel and Human Helath: Crurrent Perspectives Volume 25*. John Wiley & Sons, Inc., New York, Chichester, Brisbane, Toronto, Singapore, pp 387-420.

5. Wang,T.S., Kuo,c.F., Jan,K.Y., and Huang,H. (1996) Arsenite induces apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species. *J Cell Physiol*, **169**, 256-268.

6. Jostes,R.E. (1996) Genetic, cytogenetic, and carcinogenic effects of radon: a review. *Mutation Res*, **340**, 125-139.

7. Dally,H. and Hartwig,A. (1997) Induction and repair inhibition of oxidative DNA damage by nickel (II) and cadmium (II) in mammalian cells. *Carcinogenesis*, **18**, 1021-1026.

8. Priest,N.D. (2001) Toxicity of depleted uranium. *The Lancet*, **357**, 244-246.

9. Fisenne.I.M.,Perry, P.M., Decker, K.M. and Keller, H.W. (1987) The daily intake of 234, 235, 238U, 228, 230, 232Th and 226, 228Ra by New York city residents. *Health Phys.*, **53**, 357-363.

10. UNSCEAR (United Nations Scientific Committee on the effects of Atomic Radiation). Genetic and somatic effets of ionizing radiation. Report to the General Assembly New York. 1986. NY, UNSCEAR.

11. UNSCEAR (United Nations Scientific Committee on the effects of Atomic Radiation). Sources and effects of ionizing radiation. 2000. New York:United Nations, UNSCEAR. United Nations Sales Publikation E.00.IX.3.

12. Eadie,G.G. and Kaufmann,R.F. (1977) Radiological evaluation of the effects of uranium mining and milling operations of selected ground water supplies in the Grants Mineral Belt, New Mexico. *Health Phys.*, **32**, 231-241.

13. Brandvold,L.L., Brandvold,D.K., and Popp,C.J. (1981) Effects of uranium mining and milling on surface water in New Mexico. 7th National Conference. *Energy Environ*,467-476.

14. Swanson,S.M. (1985) Food chain transfer of U-series radionucleids in Northern Saskatchewan aquatic system. *Health Phys.*, **49**, 747-770.

15. Hart,D.R., McKee,P.M., and Burt,A.J. (1986) Benthic community and sediment quality assessment of Port Hope Harbour, Lake Ontario. *J Great Lakes Res.*, **12**, 206-220.

16. Brunskill,G.J. and Wilkinson,P. (1987) Annual supply of ^{238}U, ^{234}U, ^{230}Th, ^{226}Ra, ^{210}Pb, ^{210}Po and ^{232}Th to Lake 239 (Experimental Lakes Area, Ontario) from terrestrial and atmospheric sources. *Can J Fish Aquat Sci.*, **44**, 215-230.

17. Uran im Trinkwasser. http://www.geo.tu-freiberg.de/~merkel/uran_index.htm . 2006.

18. OMEE. Monitoring data uranium - 1990-1995. Toronto, Ontario. 1996. OMEE (Ontario Ministry of Environment and Energy), Ontario Drinking Water Surveillance Programm.

19. US EPA. Occurence and exposure assessment for uranium in public drinking water supplies. Report prepared by Wade Miller Associates, Inc. for the Office of Drinking Water, US Environmental Protection Agency. EPA Contact No. 68-03-3514. 1990. US Environmetal Protection Agency.

20. Frengstad,B., Skrede,A.K., Banks,D., Krog,J.R., and Siewers,U. (2000) The chemistry of Norwegian groundwaters: III. The distribution of trace elements in 476 crystalline bedrock groundwaters, as analysed by ICP-MS techniques. *The Science of the Total Environment*, **31**, 21-40.

21. WHO. WHO Guidelines for drinking-water quality. Uranium in Drinking-water, Background document. Third Edition. 2004. WHO. Ref Type: Pamphlet

22. Uranium Toxicity. http://www.wise-uranium.org/utox.html . 2005.

23. Uran im Trinkwasser. http://www.geo.tu-freiberg.de/~merkel/uran_index.htm . 2006.

24. Uran im Trinkwasser. http://www.geo.tu-freiberg.de/~merkel/uran_index.htm . 2006.

25. Thomas,P.A. (2000) Radionuclides in the terrestrial ecosystem near a Canadian uranium mill- Part II: small mammal food chains and bioavailability. *Health Phys*, **78**, 625-632.

26. Sheppard,M.I., Vandergraaf,T.T., and Thibault,D.H. (1983) Technetium and uranium: sorption by plant uptake peat and sand. *Health Phys.*, **44**, 644.

27. Yamamoto,T., Masuda,K., and Onishi,N. (1968) Studies on environmental contamination by uranium. 1. Environmental Survey of uranium in Kamisaibara Village, Okayama Prefecture. *Journal of Radiation Research*, **9**, 92-99.

28. Tracy,B.L., Prantl,F.A., and Quinn,J.M. (1983) Transfer of ^{226}Ra, ^{210}Pb and uranium from soil to garden products: assessment of risk. *Health Phys.*, **44**, 469-477.

29. Rumble,M.A. and Bjugstad,A.J. (1986) Uranium and radium concentrations in plants growing on uranium mill tailings in South Dakota, USA. *Reclamation and Revegetation Research*, **4**, 271-278.

30. Seeber, O. Das Angebot, die Aufnahme und die Bilanz des toxischen Schwermetalls Uran bei erwachsenen Mischköstlern in Deutschland. Doctoral Thesis. 1998.

31. Fautz,R., Forster,R., Hechenberger,C.M.A., Hertner,T., von der Hude,W., Kaufmann,G., Madle,H., Madle,S., Miltenburger,H.G., Müller,L., Pool,B.L., Puri,E.C., Schmezer,P., Seeberger,A.H., Strobel,R., Suter,W., and Baumeister,M. (1991) Report of a comparative study of DNA damage and repair assays in primary rat hapatocytes with coded chemicals. *Mutation Res*, **260**, 281-294.

32. Szefer,P. (1991) Interphase and trophic relationship of metals in Southern Baltic ecosystem. *The Sciences of the Total Environment*, **101**, 201-215.

33. Augeron,C. and Laboisse,C.L. (1984) Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. *Cancer Res*, **44**, 3961-3969.

34. Richter,M., Jurek,D., Wrba,F., Kaserer,K., Wurzer,G., Karner-Hanusch,J., and Marian,B. (2002) Cells obtained from colorectal microadenamas mirror early premalignant growth patterns *in vitro*. *European Journal of Cancer*, **38**, 1937-1945.

35. Schäferhenrich,A., Sendt,W., Scheele,J., Kuechler,A., Liehr,T., Claussen,U., Rapp,A., Greulich,K.-O., and Pool-Zobel,B.L. (2003) Putative colon cancer risk factors damage global DNA and *TP53* in primary human colon cells isolated from surgical samples. *Food Chem Toxicol.*, **41**, 655-664.

36. Knöbel,Y., Glei,M., Osswald,K., and Pool-Zobel,B.L. (2006) Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells. *Toxicology In Vitro*, **20**, 793-800.

37. Jaeger,J., Sorensen,K., and Wolff,S.P. (1994) Peroxide accumulation in detergents. *J Biochem Biophys Metho*, **29**, 77-81.

38. Schäferhenrich, A., Sendt, W., Scheele, J, and Pool-Zobel, B. L. Putative colon cancer risk factors specifically induce damage of *p53* in primary human colon cells. Toxicological Sciences 66 1-S, 307. 2002. Ref Type: Abstract

39. Schäferhenrich,A., Beyer-Sehlmeyer,G., Festag,G., Kuechler,A., Haag,N., Weise,A., Liehr,T., Claussen,U., Marian,B., Sendt,W., Scheele,J., and Pool-Zobel,B.L. (2003) Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. *Mutation Research*, **9496**, 1-14.

40. Wollowski,I., Ji,S., Bakalinsky,A.T., Neudecker,C., and Pool-Zobel,B.L. (1999) Bacteria used for the production of yogurt inactivate carcinogens and prevent DNA damage in the colon of rats. *J Nutr*, **129**, 77-82.

41. Giang,N., Shiraishi,K., Sinh,N.M., Kimura,S., Tuan,N.N., and Arae,H. (2001) Estimation of dietary ^{232}Th, ^{238}U, cesium, and strontium intakes in Vietnamese peoples from different geographical regions. *Health Phys*, **80**, 605-611.

42. Knöbel,Y., Glei,M., Weise,A., Osswald,K., Schäferhenrich,A., Richter,K.K., Claussen,U., and Pool-Zobel,B.L. (2006) Uranyl nitrilotriacetate (U-NTA), a stabilized salt of uranium, is genotoxic in non-transformed human colon cells and in the human colon adenoma cell line LT97. *Toxicological Sciences,* **93**, 286-297

43. Aruoma,O.I., Halliwell,B., Gajewski,E., and Dizdaroglu,M. (1989) Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. *The Journal of Biological Chemistry*, **264**, 20509-20512.

44. Babbs,C.F. (1992) Oxygen radicals in ulcerative colitis. *Free Radical Biology and Medicine*, **13**, 169-181.

45. Imlay,J., Chin,S.M., and Linn,S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science*,640-642.

46. Kitahora,T., Suzuki,K., Asakura,H., Yoshida,T., Suematsu,M., and Watanabe,M. (1988) Active oxygen species generated by monocytes and polymorphonuclear cells in Crohn's disease. *Digestive Diseases and Sciences*, **33**, 951-955.

47. Crawford,D., Zbinden,I., Amstad,P., and Cerutti,P.A. (1988) Oxidant stress induces the proto-oncogenes c-*fos* and c-*myc* in mouse epidermal cells. *Oncogene*, **3**, 27-32.

48. Hartwig,A. (1998) Carcinogenity of metal compounds: possible role of DNA repair inhibition. *toxicol Lett.*, **28**, 235-239.

49. Hartwig,A. and Beyersmann,D. (1989) Comutagenicity and inhibition of DNA repair by metal ions in mammalian cells. *Biol Trace Elem Res.*, **21**, 359-365.

50. Hartwig,A. and Schwerdtle,T. (2002) Interactions by carcinogenic metal compounds with DNA repair processes: toxicological implications. *toxicol Lett.*, **127**, 47-54.

51. Hartwig,A., Asmuss,M., Ehleben,I., Herzer,U., Kostelac,D., Pelzer,A., Schwerdtle,T., and Burkle,A. (2002) Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanism. *Environ Health Perspect.*, **110**, 797-799.

Table 1: Contents of different elements in the wismut samples

	water samples					
elements	***A***		***B***		***C***	
Fe (mg/l)/µM	***0.740***	***13.3***	***16.1***	***288.0***	***454.0***	***8130***
Zn (µg/l)µM	n.d.		408	6.2	287	4.4
Al (µg/l)µM	94.51	3.5	7053	261.4	2349	87
Mn (µg/l)µM	1037	18.9	8010	145.8	50970	927.9
Co (µg/l)µM	29.0	0.5	360.2	6.1	239.9	4.1
Ni (µg/l)µM	***205.4***	***3.5***	***1149***	***19.6***	***1059***	***18.0***
Cu (µg/l)µM	***143.1***	***2.3***	***251.3***	***4.0***	***510.2***	***8.0***
Sr (µg/l)µM	197	2.2	283.8	3.2	348.7	4.0
Cd (µg/l)nM	0.4	3.7	5.6	50.1	4.6	40.7
Ba (µg/l)nM	7.5	54.7	4.99	36.3	4.8	35.0
Pb (µg/l)nM	4.2	21.8	2.4	11.8	3.4	16.3
Th (µg/l)nM	n.d.		0.1	0.3	0.2	0.9
U (µg/l)nM	***23.0***	***96.8***	***64.1***	***296.2***	***73.0***	***306.6***

n.d. not detectable

Table 2: Levels of DNA damage induced by 2.5-10% of the wismut sample B (30 min at 37°C) in primary human colon cells, LT97 cells and HT29 clone 19A cells. The data show mean values from three to four independent experiments. The significance of individual compound induced effects was calculated by one way ANOVA, including Dunnett´s Post Test; *p<0.05, **p<0.01 significantly different from RPMI control.

	Primary colon cells (n=4)				**LT97 (n=3)**				**HT29 clone 19A (n=4)**			
	tail intensity (%)		viability (%)		tail intensity (%)		viability (%)		tail intensity (%)		viability (%)	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
RPMI	10.2	1.3	93.0	4.1	9.0	0.2	86.5	1.4	5.4	0.8	85.5	2.2
10 % NTA	10.3	2.9	87.4	0.8	9.6	0.8	79.8	5.7	7.0	2.2	84.3	4.4
0.5 % B *(1.5 µM Fe, 1.4 nm U^{238})*	_	_	_	_	_	_	_	_	5.1	0.5	86.4	5.4
1 % B *(2.9 µM Fe, 2.7 nm U^{238})*	_	_	_	_	_	_	_	_	6.4	0.5	85.5	3.9
2 % B *(5.8 µM Fe, 5.4 nm U^{238})*	_	_	_	_	_	_	_	_	8.8	1.7	84.8	4.7
2.5 % B *(7.3 µM Fe, 6.8 nM U^{238})*	10.2	1.5	90.2	5.3	13.5	3.9	83.2	6.3	9.3	1.3	83.2	5.0
5 % B *(14.5 µM Fe, 13.5 nm U^{238})*	12.9	2.4	88.6	6.3	14.3	5.4	79.8	4.1	11.7 **	3.1	81.3	4.5
7.5 % B *(21.8 µM Fe, 20.3 nM U^{238})*	16.7 *	3.3	91.4	3.0	14.9	2.1	75.1	1.9	14.7 **	3.5	81.2	7.2
10 % B *(29 µM Fe, 27 nm U^{238})*	19.1 **	5.2	92.1	4.7	18.1 *	3.6	81.6	4.7	19.2 **	3.6	80.1	3.9
Fe-NTA (500 µM)	24.4 **	3.7	90.9	0.5	26.2 **	4.6	80.6	4.3	_	_	_	_

Table 3: Levels of DNA damage induced by 2.5-10% of the wismut sample C (30 min at 37°C) in primary human colon cells, LT97 cells and HT29 clone 19A cells. The data show mean values from three to four independent experiments. The significance of individual compound induced effects was calculated by one way ANOVA, including Dunnett´s Post Test; *p<0.05, **p<0.01 significantly different from RPMI control.

	Primary colon cells (n=3)				**LT97 (n=3)**				**HT29 clone 19A (n=4)**			
	tail intensity (%)		viability (%)		tail intensity (%)		viability (%)		tail intensity (%)		viability (%)	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
RPMI	9.0	1.5	87.8	4.7	7.9	0.7	81.9	4.3	5.2	1.6	89.7	2.4
10 % NTA	9.3	1.0	92.5	6.2	9.7	3.5	82.4	1.3	6.8	2.1	86.0	4.4
0.5 % C *(40.5 µM Fe, 1.5 nm U^{238})*	_	_	_	_	_	_	_	_	7.1	2.0	83.4	6.5
1 % C *(81 µM Fe, 3.1 nm U^{238})*	_	_	_	_	_	_	_	_	9.1	3.1	83.1	6.9
2 % C *(162 µM Fe, 6.1 nm U^{238})*	_	_	_	_	_	_	_	_	11.8 *	4.9	82.8	6.5
2.5 % C *(202.5 µM Fe, 7.7 nM U^{238})*	18.3 *	4.4	87.7	7.3	11.5	1.9	76.5	0.5	13.6 **	3.7	81.0	7.7
5 % C *(405 µM Fe, 15.4 nm U^{238})*	21.3 **	2.2	87.3	3.2	13.7	2.4	79.6	4.8	16.0 **	4.9	81.7	4.5
7.5 % C *(6078 µM Fe, 23 nM U^{238})*	28.7 **	5.7	82.1	3.5	19.6 ** ##	3.9	84.3	3.5	19.8 ** ##	1.8	76.4	10.4
10 % C *(810 µM Fe, 30.7 nm U^{238})*	29.9 **	3.9	86.1	1.1	22.2 **	5.6	83.8	1.8	22.5 **	1.8	77.4	6.1
Fe-NTA *(500 µM)*	24.6 **	4.5	81.3	4.0	19.2 **	1.8	80.1	7.7	_	_	_	_

Figure 1

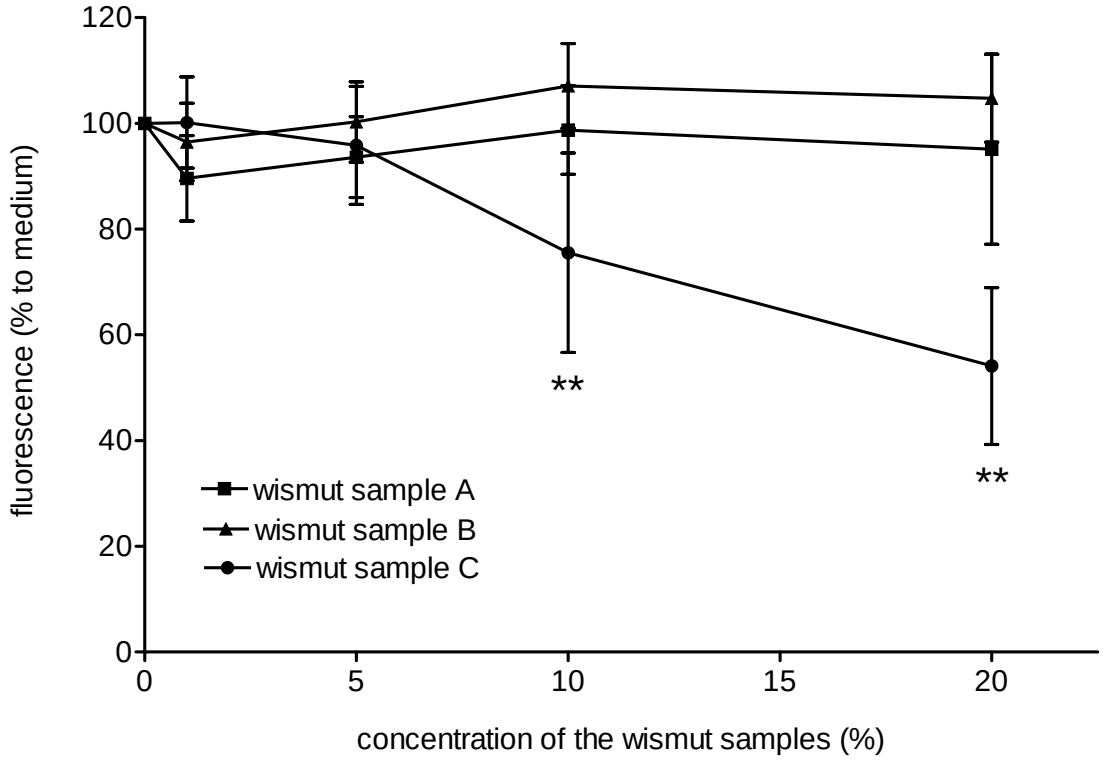

120
100
80
60
40
20
0
fluorescence (% to medium)
0
5
10
15
20
concentration of the wismut samples (%)
wismut sample A
wismut sample B
wismut sample C
**
**

Figure 2

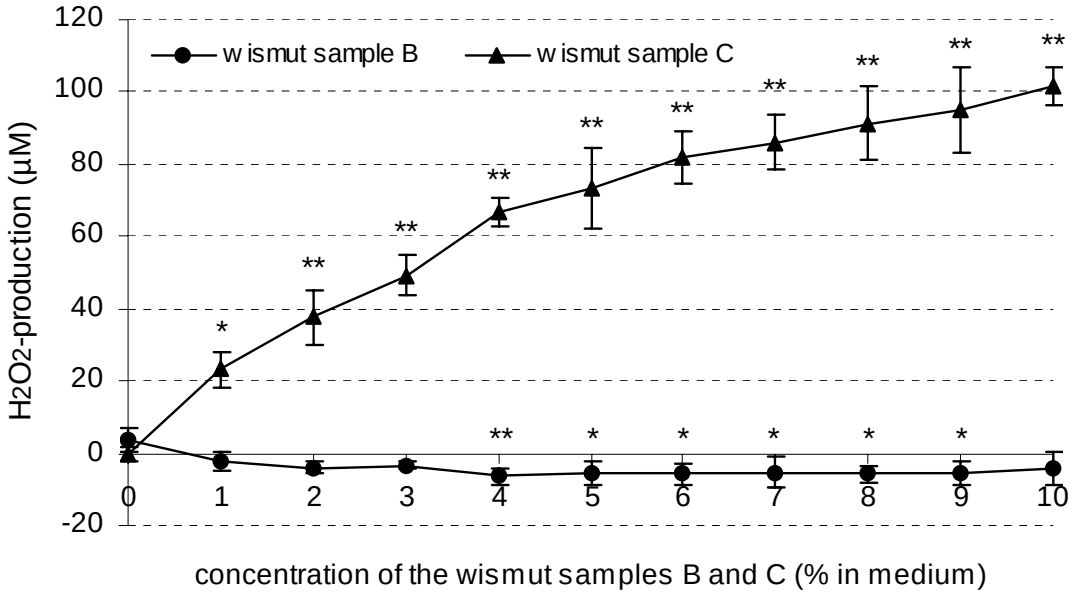

120
100
80
60
40
20
0
-20
H2O2-production (µM)
wismut sample B
wismut sample C
0
1
2
3
4
5
6
7
8
9
10
concentration of the wismut samples B and C (% in medium)

Figure 3

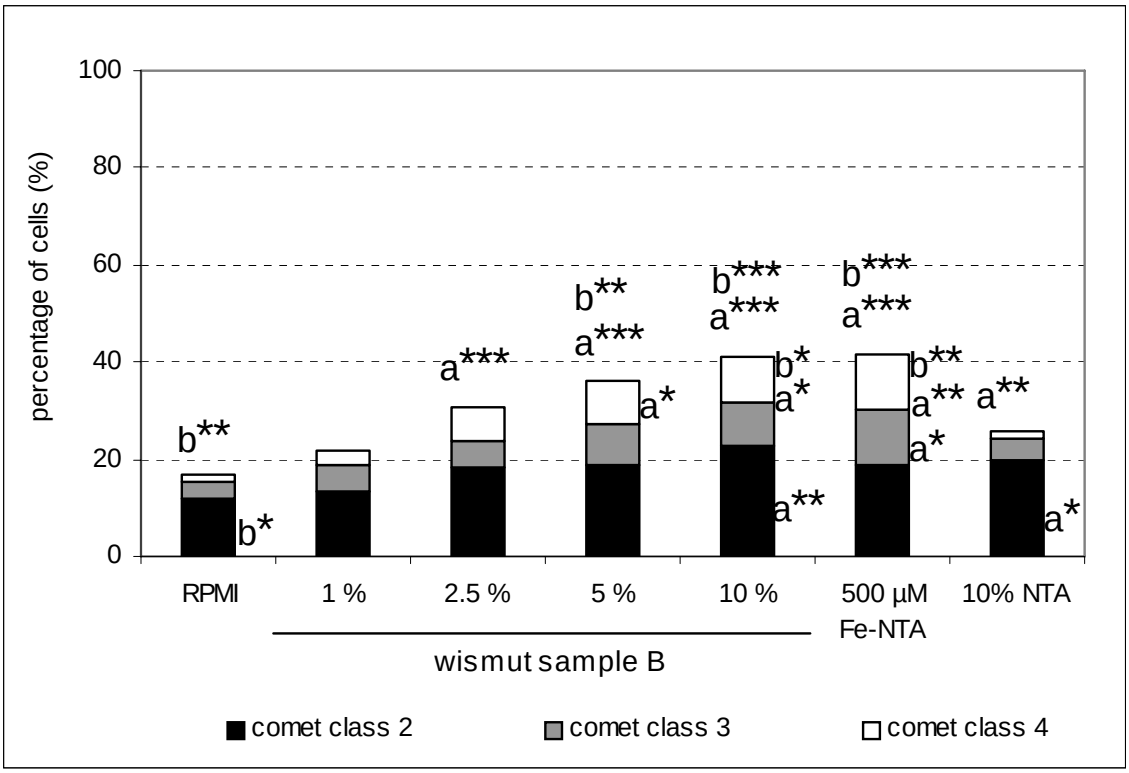
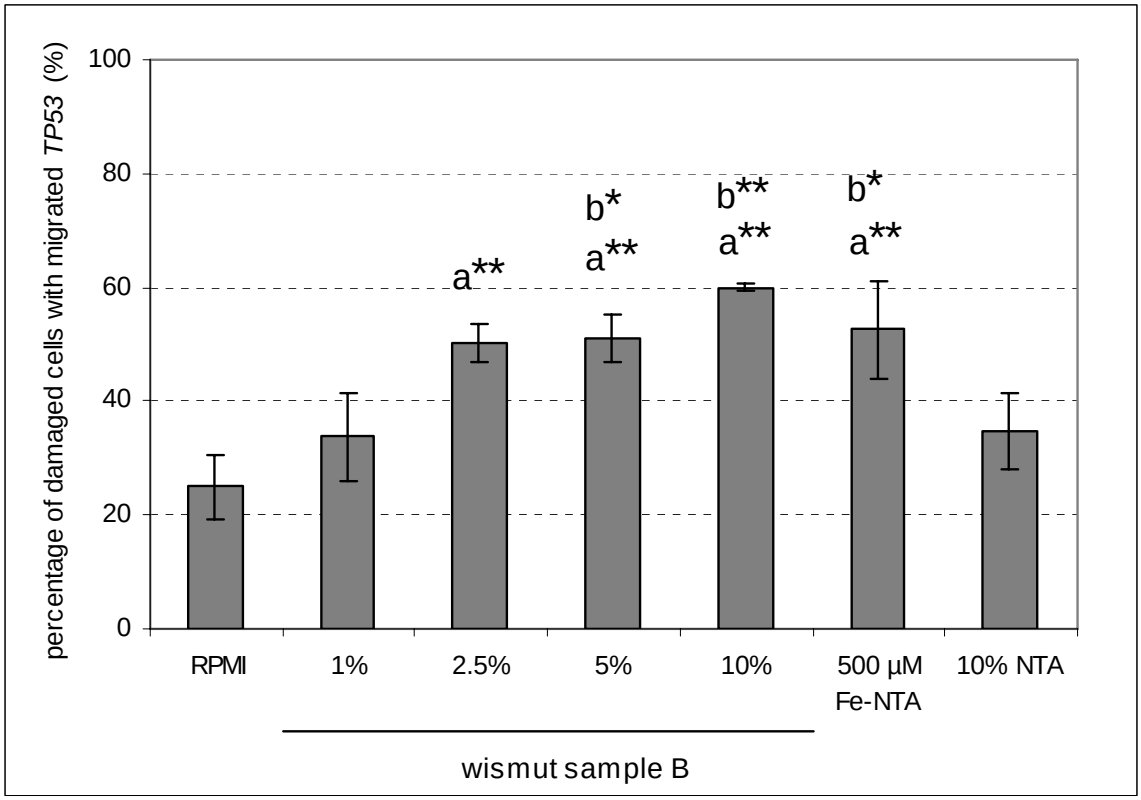

100
80
60
40
20
0
percentage of cells (%)
b**
b*
a***
b**
a***
a*
b***
a***
b*
a*
a**
b***
a***
b**
a**
a*
a**
a*
RPMI
1 %
2.5 %
5 %
10 %
500 µM Fe-NTA
10% NTA
wismut sample B
comet class 2
comet class 3
comet class 4

Figure 4


percentage of damaged cells with migrated TP53 (%)
100
80
60
40
20
0
a**
b*
a**
b**
a**
b*
a**
RPMI
1%
2.5%
5%
10%
500 µM Fe-NTA
10% NTA
wismut sample B

3.5 Manuskript V

Blood mononucleocytes are sensitive to the DNA damaging effects of iron overload – in vitro and ex vivo results with Fe-NTA

E. Park, M. Glei, **Y. Knöbel**, B. L. Pool-Zobel

zur Publikation angenommen bei Mutation Research am 26. Januar 2007

Zusammenfassung

Die Exposition gegenüber Eisen ist direkt mit einem erhöhten kolorektalen Risiko assoziiert. Ziel dieser Studie war es, periphere Leukozyten als Zielzellen systemischer Expositionen einzusetzen, um zu prüfen, ob Fe-NTA auch in diesen Zellen globale oder spezifische DNA-Schäden induziert.

Fe-NTA war *in vitro* in humanen Leukozyten und *ex vivo* in Rattenleukozyten genotoxisch. Die Migration des tumorrelevanten Gens *TP53* in den Kometenschweif humaner Leukozyten wurde durch Fe-NTA verstärkt.

Diese Ergebnisse zeigen, dass Leukozyten sensitiv gegenüber einer systemischen Eisen-Exposition reagieren. Die Messung der DNA-Schädigung in humanen Leukozyten durch Eisenüberschuss könnte als sensitiver Biomarker in kontrollierten humanen Interventionsstudien genutzt werden, um zu prüfen, ob eine zu verabreichende Supplementierung mit einem genotoxischen Risiko verbunden ist.

Darstellung des Eigenanteils

- Einweisung in die Comet FISH Methode und Unterstützung bei der Auswertung
- Zuarbeit zum Publikationsmanuskript

Blood mononucleocytes are sensitive to the DNA damaging effects of iron overload – *in vitro* and *ex vivo* results with human and rat cells

Eunju Park[a], Michael Glei[b], Yvonne Knöbel[b], Beatrice L. Pool-Zobel[b,*]

[a]*Department of Food and Nutrition, Kyungnam University, 449 Wolyoung-dong, Masan, 631-701 South Korea*
[b]*Department of Nutritional Toxicology, Friedrich-Schiller-University Jena, Dornburger Street 25, 07743 Jena, Germany*

*Address of corresponding author

Prof. Dr. rer. nat. Beatrice L. Pool-Zobel
Friedrich-Schiller-University Jena
Biological-Pharmaceutical Faculty
Department of Nutritional Toxicology
Dornburger Str. 25
07743 Jena
Germany

Tel: + 49 3641 949670
Fax: + 49 3641 949672
E-mail: b8pobe@uni-jena.de
Homepage: www.uni-jena.de/biologie/ieu/et

Abstract

Iron exposure enhances colorectal carcinogeneis, by producing reactive oxygen species, which damage lipids, proteins and DNA. We recently demonstrated that ferric-nitrilotriacetate (Fe-NTA) damages DNA of human colon cells in different stages of malignant transformation. Opposed to this, little is known on systemic effects of iron and it is still difficult to determine the border between essential iron supplementation and iron overload in humans. The aim of this study was to determine whether Fe-NTA causes global and specific DNA damage in peripheral leucocytes. Human leucocytes were treated *in vitro* with Fe-NTA for 30 min at 37^{o}C. Male Sprague Dawley rats were fed (6 weeks) with an iron-overload diet (9.9 g Fe/kg DM) and whole blood was collected. DNA damage was measured in human and rat blood cells using the alkaline version of the Comet Assay with repair specific enzymes. In human cells the distribution of *TP53* in the comet images was detected using fluorescence *in situ* hybridization (Comet FISH) to measure DNA damage in the region of the *TP53* gene. Fe-NTA (10-500 µM) was clearly genotoxic in human leucocytes *in vitro*, and also in leucocytes of rats fed the iron overload diet. The induced damage in human leucocytes was approximately two-fold that observed previously in human colon cells. Oxidised bases were induced by iron in rat leucocytes *in vivo*, while they were not induced in human leucocytes *in vitro*. Fe-NTA enhanced the migration of *TP53* signals into the comet tail of human leucocytes, indicating a high susceptibility of this tumor-relevant gene towards DNA damage induced by iron overload. In conclusion, iron markedly induced DNA damage in human and rat leucocytes, which shows that these white blood cells are sufficiently sensitive to assess exposure to iron. The measurement of DNA damage in human leucocytes could be used as a

sensitive biomarker to study iron overload *in vivo* in humans and thus to determine whether supplementation results in genotoxic risk.

Keywords : Iron overload, human leucocytes, rat leucocytes, Comet Assay, Comet FISH, *TP53*

1. Introduction:

Most human tumors are likely the result of lifelong exposure to environmental carcinogens that cause initiation and enhance progression. A feasible strategy for avoiding risks is to find interventions that reduce genotoxic damage and induce toxicological deactivation systems. Cells from tumor target tissues are appropriate models for studying these associations, since they reflect local effects occurring at the target sites of carcinogenesis. This model system has been developed for the example of elucidating possible mechanisms leading to initiation of colorectal carcinogenesis by studying genotoxicity in human colon tissue specimens [1, 2]. However, blood cells are preferable to study aspects of the associations since they are obtained less invasively and are of value to measure systemic genotoxicity [3-7]. Using the Comet Assay, a previous human study has revealed that there is a good correlation between genetic damage in rectal cells and in leucocytes [8]. On the basis of current knowledge, blood cells are probably also suitable systems to analyse chemoprotective effects by dietary intervention with antigenotoxic nutrients [9-12]. In other words, circulating leucocytes are surrogate cells which continuously maintain a surveillance of the body for signs of toxic and antitoxic exposures, that are of importance for diseases, including cancer [13]. In this case, however, it must be recognised that studies based on surrogate end points and tissues, are much less reliable than studies with the ‘true’ end point using the target cells where cancer arises (for example, colon tumors arising from stem cells). For the purpose of using peripheral blood cells as surrogate cells and as biomarkers in human studies [14], it is therefore first necessary to evaluate them as markers for exposure and susceptibility and to compare these two properties to corresponding effects in the specific target cells

(e.g. in colonocytes). This corresponds to the parallelogram type of approach that combines *in vitro/ex vivo* animal and human studies [15].

In the following, we were interested in studying the question of whether peripheral blood cells, when exposed to a genotoxic risk substance related to diet, respond in similar fashions as colon cells did in previous studies [16]. These investigations had shown that iron induces DNA strand breaks in colon cells with different stages of cell transformation (primary colon cells; colon adenoma cells, LT 97; colon carcinoma cells, HT29 clone 19A). In other words, we would like to investigate how do susceptibilities of blood cells compare to colonocytes when exposed *in vivo* (rats) or *in vitro* (human cells) to DNA damaging doses of iron. This is a new approach to develop a biomarker reflecting the iron status and to define the threshold of iron overload which is associated with different disorders, like hemochromatosis with severe organ damage [17], or cancer [18].

Previously, we have reported that iron overload enhanced human colon tumor cell growth and caused progenotoxic effects [16]. Iron overload (resulting possibly from high red meat consumption or intake of iron supplements) is expected to increase the production of reactive oxygen species (ROS) from peroxides via Fenton reaction in the gut lumen, which may be contribute to the aetiology of cancer in the human colon [19-21].

In this study human leucocytes were isolated by gradient centrifugation from anonymous buffy coat preserves and rat leucocytes were from blood collected from the animals [22]. The human cells were treated *in vitro* with iron nitrilotriacetate (Fe-NTA), a stabilized form of ferric iron [16]. The rats were fed an iron-overload diet that was supplemented with $FeSO_4$ (Table 1), used on account of its high bioavailability [23]. The used dose of iron was quite high and probably can therefore only model the

effects of iron supplements in humans, which e. g. are recommended during the second half of pregnancy [24].

Different types of DNA damage were studied using various modifications of the Comet Assay, a gel electrophoresis method with individual cells. Global DNA damage was determined with the standard alkaline version of the assay [25], oxidised pyrimidine and purine bases were detected using repair specific enzymes [26, 27] and gene specific damage was assessed by staining *TP53* in the comet images using fluorescence *in situ* hybridization, a method also called Comet FISH [2, 28-30]. A high number of mutations were identified in *TP53* gene of colon tumor [31] some of which were probably crucial for the transition of adenoma to invasive carcinoma. However, little is well known about *TP53* mutation in tumors related with human lymphocytes.

2. Materials and methods:

2.1. Compounds

Ferric nitrate (40 mg) [$Fe(NO_3)_3$] (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in 10 ml double distilled water (ddH_2O) and the chelating agent disodium nitrilotriacetate (NTA) (Sigma-Aldrich, Deisenhofen, Germany) was added (23.5 mg). The pH of the solution was adjusted to 7.4 by sodium hydrogencarbonate ($NaHCO_3$) (Sigma-Aldrich, Deisenhofen, Germany). The final iron concentration of the stock solution was 10 mM. The tested concentration range (1-500 µM) considers physiological conditions. Levels of relevant non-transferrin-bound iron (NTBI) in blood serum vary between almost zero in healthy controls, about 1 µM in patients with diabetes and up to 10 µM in patients with hemochromatosis [32-34].

2.2. Animals, diets

Eight-week-old male Sprague Dawley rats (n = 20) were purchased from Semtako Inc. (Osan, Korea) and housed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* [35]. The study was approved by the ethical committee of Kyungnam University.

The rats were acclimatized to the animal facility room in the Kyungam University for one week, being housed individually (20–22°C; 12:12-h light-dark cycle) with free access to a commercially prepared pelleted diet and water. The rats were then randomly divided into two groups of 10 animals each and fed either a standard diet (control group), or a standard diet supplemented with 0.99 % ferrous iron (iron overload group) for 6 weeks (Table 1). So we fed 0.25 g Fe/kg dry matter (DM) in the control group and 9.9 g Fe/kg DM in the iron-overload group according to Lafay *et al*. [36]. Animals were monitored daily for general health and body weights were re-

corded every week for the duration of the study. At the end of the experimental period, the rats were anesthetized with ethyl ether and blood was collected from the abdominal artery in a heparinised sterile tube. This was then used to analyze the effect of iron overload on global DNA damage in leucocytes *in vivo*.

2.3. Preparation of human leucocytes

Leucocytes were isolated as a fraction of mononuclear cells (containing lymphocytes and monocytes) from anonymous buffy coat preserves by gradient centrifugation with HISTOPAQUE®-1077 (Sigma, Deisenhofen, Germany), as described earlier [22, 37]. The cells were frozen and stored at -140 °C (deep freezer QC 10140, Nunc, Wiesbaden, Germany). They were thawed rapidly prior to each experiment in a water bath at 37 °C. Leucocytes from different donors were used for the triplicate experiments.

2.4. Determination of genetic damage

To measure global DNA damage, we used our protocol of the alkaline version of the Comet Assay, according to recommendations published previously [38, 39]. 10 µl of the cell suspensions (containing 2 x 10^5 peripheral leucocytes) for the *in vitro* study or 5 µl of rat whole blood for the *ex vivo* study were mixed with 75 µl 0.7 % low melting point agarose (Biozym, Oldendorf, Germany) and distributed onto microscope slides coated with 0.5 % normal melting agarose. After solidification of the agarose, slides were covered with another 75 µl 0.7 % low melting point agarose and then immersed in a lysis solution (100 mM Na_2EDTA, 1 % Triton X 100, 2.5 M NaCl; 1 % N-lauroyl sarcosin sodium salt, 10 % DMSO, 10 mM Tris, pH 10) for at least 60 min at 4 °C. All slides were placed in an electrophoresis chamber containing alkaline

solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) for DNA unwinding. After 20 min, the current was switched on and electrophoresis was carried out at 1.25 V/cm and 300 mA for 20 min. The slides were removed from the alkaline solution and washed 3 times for 5 min with neutralisation buffer (0.4 M TRIS, pH 7.5). Slides were stained with SYBR Green (Sigma, Deisenhofen, Germany,1 µl/ml antifade buffer, 30 µl/slide). All steps with the isolated cells were conducted under red light. All experiments were carried out at least three times [40], usually each with three parallel slides per data point. Microscopical evaluation was performed with the image analysis system of Perceptive Instruments (Halstead, UK). Fifty images were evaluated per slide and the percentage of fluorescence in the tail (TI) was scored as a reflection of DNA damage [10].

Determination of oxidised DNA bases was performed by incubating the slides after the lysis step with repair specific enzymes [26, 27]. The enzymes used in the experiments with human leucocytes were kindly provided by Prof. Andrew Collins (Rowett Research Institute, Aberdeen, UK), those used in the rat cell study were purchased from Trevigen, Inc., Gaithersburg, USA. For this, DNA was digested for 45 min with endonuclease III (Endo III from Trevigen: 0.1 units/slide; Endo III from Prof. Collins: 1 µl/ml buffer, 50 µl/slide) or for 30 min with formamidopyrimidine DNA glycosylase (Fpg from Trevigen: 4 units/slide; Fpg from Prof. Collins: 1 µl/3 ml buffer, 50 µl/slide) and the corresponding buffers. The different enzymes were needed to detect oxidised pyrimidine bases and purine bases, respectively.

Furthermore, we hybridized comet slides with a Texas Red labelled *TP53* containing FISH probe to determine “gene specific” effects of iron. The probe which was kindly provided by Prof. Uwe Claussen (Institute for Human Genetics and Anthropology, Friedrich-Schiller-University Jena, Germany) was derived from microdissection

and was a mixture of directly labelled probes that are specific for genomic sequences including the *TP53* locus (17pter-p12) [41]. The size of the probe was 10.6 Mb including the *TP53* gene (about 7.50 Mb) (unpublished data). “Gene specific” damage (*TP 53* plus surrounding regions) was reflected by the extent of migration of *TP53* signals into the comet tail. The Comet FISH experiments were performed according to the method described by Schäferhenrich et al. [2, 30], using modifications needed to adapt the hybridization conditions to Texas Red labelled *TP53* probes [42]. The dehydrated slides from the Comet Assay were rehydrated in ddH_2O for 10 min. Afterward, the target DNA was denatured with 0.5 M NaOH for 30 min and then neutralized in 0.01 M PBS for 1 min. The slides were dehydrated through an ethanol series (70, 80, and 95 %, each for 5 min) and dried at room temperature. Thirty microliters of Hybrisol VI (Oncor, Gaithersburg, UK) was dropped on each slide gel and spread with a plastic coverslip. The *TP53* probe (5 µl for each slide) was diluted with Hybrisol VI (7 µl for each slide) and denatured at 75°C for 5 min, 4°C for 2 min, and 37°C for 30 min. This hybridization mixture (12 µl per slide) was added to the slides, covered with a plastic coverslip (24 x 24 mm), and incubated in hybridization chambers for 72 h at 37°C. The coverslips were then removed, and the slides were washed for 5 min in 2x saline sodium citrate (0.3M NaCl, 0.03M Na citrate, pH 7.2) without formamide at 65°C. This step was followed by a 5 min washing in 1x phosphate-buffered detergent (Oncor, Gaithersburg, UK) at room temperature. SYBR-Green (1 µl/ml, 30 µl per slide) was used to counterstain the Texas Red labelled probes. Negative controls were included for each concentration on the same slide using Hybrisol VI without the DNA probe.

To evaluate the Comet FISH experiments, the comets were categorised into four degrees of damage ranging from non-damaged images to severely damaged

images (classes 1-4). After determining the hybridization efficiency, 100 cells per slide with two fluorescent spots were used for the final evaluation by recording the positions of the *TP53* signals in the comet head or comet tail (Figure 1). The parameter of *TP53* migration was based on the percentage of damaged cells (belonging to comet classes 2-4), in which we could find a migration of at least one *TP53* signal into the comet tail. Evaluation proceeded with a ZEISS Axiovert M100 (Carl Zeiss Jena GmbH, Jena, Germany), equipped with filters to score images stained with Texas Red (red, ZEISS filter No 15) and SYBR Green (green, ZEISS filter No 09). Images were captured using a MicroMAX Digital CCD camera (BFI OPTILAS GmbH, Puchheim, Germany) and processed with the Meta View Imaging Software (Visitron Systems GmbH, Puchheim, Germany).

To minimize systemic errors the persons measuring the slides used a numeric codification, but the study was not fully blinded.

2.5. Statistical analysis

Data were analyzed using the SPSS package for Windows (Version 10) and GraphPad Prism for Windows (Version 4.0). Values are expressed as mean±standard deviation (SD). The mean values of each treatment were compared using One-way analysis of variance (ANOVA) followed by Duncan's multiple range test or Dunnett's Multiple Comparison Test. P-values of less than 0.05 were considered significant. The significance of differences between global DNA damage (TI %) and *TP53* signals in the tail were calculated by Two-way-ANOVA, including Bonferroni Post-Test for comparing selected pairs of columns ($p<0.05$). Statistical differences between the control and iron overload group (animal study) were considered significant at $p<0.05$ by Student's t-test.

3. Results:

The effect of Fe-NTA on DNA damage in human leucocytes is shown in Fig. 2, indicating that Fe-NTA was clearly genotoxic. The effect was dependent on dose, but there was no concomitant cytotoxicity (data not shown). 500 µM Fe-NTA induced tail intensity (34.1 ± 7.4 %) was about 3 times higher than those of RPMI control (9.9 ± 3.1 %) or 500 µM NTA control (9.6 ± 2.1 %). This value was considerably higher than the corresponding values previously observed in HT29 clone 19A cells (8.5 ± 1.9 %, [16]), LT97 cells (20.4 ± 8.5 %) or primary colon cells (20.1 ± 1.7 %) [42].

In a set of experiments we investigated the level of oxidised bases using repair specific enzymes. Here we found that human leucocytes (Fig. 3) had considerable levels of Endo III- and Fpg-sensitive lesions, reflecting oxidised pyrimidine and purine bases, respectively. The oxidised bases were mainly evident in the two negative control samples (RPMI, NTA). The additional treatment with Fe-NTA, however, did not seem to enhance these effects. Instead, the increasing concentrations of iron which clearly led to an increase in the strand break rates seemed to suppress the occurrence of base-specific damage. This was particularly apparent for 250 µM Fe-NTA, a concentration at which there were no additional effects by the repair enzymes (SBs 31.7 ± 2.6 %, Endo 32.3 ± 7.2 %, Fpg 32.2 ± 5.6 %, respectively).

The leucocytes of rats fed with an iron-overload diet (Fig. 4) showed a significantly increased DNA strand break level (8.5±0.6 % vs. 18.4±1.4 %, $p<0.001$) in comparison with the cells of control animals. Thus the results of the rat study confirmed the *in vitro* findings on effects of iron using human leucocytes. Unlike the results from *in vitro* studies with human leucocytes, however, iron overload additionally resulted in oxidised pyrimidine and purine bases (Endo III- and Fpg-sensitive sites) in the rat peripheral white blood cells ($p<0.05$).

Iron overload did not affect body weight gain, food efficiency ratio or organ weight during the experiment, although the food intake in the iron overload group was significantly higher than in the control group (Table 2).

Fig. 5 shows the effect of Fe-NTA in human leucocytes *in vitro* using a different mode of evaluation as is needed for evaluating the Comet FISH results. This was to score comet classes with different damage levels (from 1 to 4 meaning undamaged to highly damaged). Increasing Fe-amounts resulted in an increased proportion of highly damaged cells (class 4), whereas the undamaged cells (class 1) were reduced. The evaluation mode disclosed a high sensitivity of peripheral leucocytes to the damaging activity of even low Fe-NTA concentrations. Fig. 6 shows the proportion of all damaged cells (comet classes 2-4) which revealed *TP53* signals in the comet tail. There was a concentration dependent *TP53*-migration into the comet tail, which was significantly different from the medium control and from the NTA control beginning already at 100 µM Fe-NTA.

4. Discussion:

Transition metals, like iron and copper, with their multiple oxidation states are vital for the cascades of electron transfer reactions. These are characteristic of cellular processes such as oxygen transport, photosynthesis, nitrogen fixation, and respiration in most organisms [43-45]. Iron deficiency leads to anemia. Worldwide, up to a billion individuals suffer adverse effects from insufficient iron supply, making iron deficiency anemia the most common nutritional anemia [44]. Opposed to this it is likely that there is an association between high amounts of iron in the body storage and the risk of chronic diseases (e.g. diabetes mellitus, cancer, or cardiac failure in patients with hemochromatosis) [46]. One reason for these risks could be that iron induces DNA damage and increases lipid peroxidation, both damaging effects which can be measured after iron infusion during hemodialysis [47]. This also implies that by modifying dietary patterns and avoiding excessive amounts of iron-containing dietary supplements, it could be possible to lower the risk of developing high iron body loads and thus, the risk of developing diseases [48]. Alternatively, appropriate dietary ingredients could also counteract ill effects of iron. Thus, using an *in vitro* rat model of oxidative stress, Lafay et al. have shown that caffeic acid fully prevented the pro-oxidant effects (lipid oxidation) of high iron doses [36].

Although there have been numerous direct human trials studying iron supply and metabolism, there is still controversy regarding the identification of risks [49]. The iron absorption is marginal and normally very tight controlled. This minimal absorption of excess iron in the small intestine leads to significant concentrations in the colon. This is the reason why damage to colonocytes is more likely than to lymphocytes. Inborn errors of iron metabolism (e.g. hemochromatosis) or other factors (secondary or acquired iron overload) lead to progressive body iron overload [50]. When plasma

iron content exceeds the iron-binding capacity of transferrin, iron accumulates in the body and causes cell damage [46] with different clinical symptoms, such as inflammation, arrhythmias or diabetes mellitus [44].

Obviously it is necessary to measure potential adverse health effects by iron supplementation and several approaches have been reported. Schumann et al. showed that urinary 8-hydroxy-2-desoxyguanosine (8-oxodGuo) and isoprostane of humans could be used as reliable indicators of oxidative and inflammatory response to oral ferrous sulphate [51] and Gackowski et al. found a significant correlation (r=0.57) between 8-oxodGuo and the labile iron pool in lymphocytes [52]. In this study, we were now able to demonstrate that also the measurement of DNA damage in peripheral leucocytes using the Comet Assay could be a suitable biomarker to study iron overload.

Thus here evidence is presented that iron-treatment *in vitro* resulted in strand breaks in isolated human leucocytes. Also, leucocytes from rats treated *in vivo* with an iron-overload diet were significantly damaged which reflected the sensitivity of this suggested biomarker.

The present study also reveals that there was an effect of iron overload on the occurrence of oxidised bases (detected using DNA repair specific enzymes: Endo III and Fpg) in rats fed a diet with high levels of $FeSO_4$, but not in human leucocytes damaged *in vitro* with Fe-NTA. Possible reasons for the disagreement between the two studies could be species specificity or the type of iron used. Another explanation could be different levels of antioxidant enzymes which, however, were not determined here. Further studies will be needed to elucidate exact mechanisms. In a previous study we have found that 250-1000 µM Fe-NTA did induce oxidised bases (Fpg-sensitive sites) in a clear concentration-response relationship in human colon

cancer cells (HT29 clone 19A cells), whereas oxidized pyrimidine bases (Endo III-sensitive sites) were only marginally detectable [53]. This indicates that Fe-NTA is basically capable of causing oxidized DNA bases in human cells, although with low potency.

Furthermore, we investigated whether a simulated iron overload induces gene specific damage in *TP53* of human leucocytes using the method of Comet FISH. The dose-dependent migration of *TP53* into the comet tail of damaged leucocytes indicates an interaction of Fe-NTA with this gene region. These results are consistent with those obtained in LT97 colon adenoma cells and primary human colon cells [42]. When we compare these *TP53*-specific effects of iron in colon cells with the potential systemic effect in human leucocytes, we can conclude that *TP53* is significantly more sensitive in human colon cells than in human leucocytes even though global DNA damage was higher in leucocytes. Schäferhenrich et al. also observed a *TP53*-specific effect in human colon adenoma cells after the treatment with 4-hydroxy-2-nonenal [30]. Related to this could be the finding of Hussain et al. who found that in patients suffering from Wilson disease and hemochromatosis there was a higher frequency of *TP53* mutated alleles in non-tumorous liver tissue compared with liver samples from normal controls [54].

Using DNA damage in leucocytes as a biomarker, it should presumably be possible to define the threshold between an adequate iron supply and an iron overload which enhances DNA damage. This should be investigated in human intervention studies, using iron supplements, and also could be important for patients with hereditary hemochromatosis (reviewed in [55]) or other kinds of iron overload diseases (reviewed in [44, 56]). Epidemiological studies gave evidence that iron overload is related to an increase of colorectal cancer [57, 58]. Shaheen et al. described

an association of iron exposure with colorectal carcinogenesis in highly susceptible individuals with genetically based iron storage deficiencies [59]. Other groups did not find this association [60, 61]. The biomarker developed here could provide an additional tool to study these types of activities and provide more insight to mechanisms of iron risk.

Iron induces global DNA damage in human colon cancer cells [16], in colon adenoma cells and in primary colon cells [42]. Fe-NTA also increases oxidative DNA damage in rat sperm cells [62]. In this study we were able to demonstrate that this is also true for human leucocytes and for leucocytes of rats fed with 9.9 g Fe/kg DM. Peripheral leucocytes are more sensitive to the genotoxic effects of Fe-NTA than cells derived from human colon tumors, adenomas and primary tissue [42]. Thus, 500 µM Fe-NTA induced higher DNA damage levels in human leucocytes than in human colon cells (leucocytes: about 35 % TI, primary human colon cells and LT97 colon adenoma cells: about 20 % TI).

In conclusion, iron markedly induces DNA damage in human leucocytes *in vitro* and rat leucocytes *in vivo*. This shows that these types of white blood cells are sufficiently sensitive to assess excessive iron exposure. The measurement of DNA damage in human leucocytes could thus be used as a sensitive biomarker to study iron overload *in vivo* during well controlled human studies to determine whether a given supplementation results in genotoxic risk or not.

Acknowledgements

This work was carried out with financial support by Deutsche Forschungsgemeinschaft project number PO 284/6-1 and PO 284/6-2 and promotion of the Scientist Exchange Program by Korean Science and Engineering Foundation (F03-2002-000-00053-0).

Reference List

[1] B.L.Pool-Zobel, S.L.Abrahamse, A.R.Collins, W.Kark, R.Gugler, D.Oberreuther, E.G.Siegel, S.Treptow-van Lishaut, G.Rechkemmer. Analysis of DNA strand breaks, oxidized bases and glutathione S-transferase P1 in human colon cells, Canc Epid Biom Prev 8 (1999) 609-614.

[2] A.Schäferhenrich, W.Sendt, J.Scheele, A.Kuechler, T.Liehr, U.Claussen, A.Rapp, K.O.Greulich, B.L.Pool-Zobel. Endogenously formed cancer risk factors induce damage-of *p53* in human colon cells obtained from surgical samples, Fd Chem Toxicol 41 (2003) 655-664.

[3] C.Betti, T.Davini, L.Giannessi, N.Loprieno, R.Barale. Microgel electrophoresis assay (comet test) and SCE analysis in human lymphocytes from 100 normal subjects, Mutation Res 307 (1994) 323-333.

[4] A.Hartmann, E.Agurell, C.Beevers, S.Brendler-Schwaab, B.Burlinson, P.Clay, A.Collins, A.Smith, G.Speit, V.Thybaud, R.R.Tice. Recommendations for conducting the in vivo alkaline Comet assay, Mutagenesis 18 (2003) 45-51.

[5] M.Glei, N.Habermann, K.Osswald, C.Seidel, V.Böhm, C.Persin, G.Jahreis, B.L.Pool-Zobel. Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the comet assay: a biomarker model, Biomarkers 10 (2-3) (2005) 203-217.

[6] A.R.Collins. The Comet Assay for DNA Damage and Repair, Principles, Applications, and Limitations. Molecular Biotechnology 26 (2004) 249-261.

[7] S.Bonassi, L.Hagmar, U.Stromberg, A.H.Montagud, H.Tinnerberg, A.Forni, P.Heikkila, S.Wanders, P.Wilhardt, I.L.Hansteen, L.E.Knudsen, H.Norppa. Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health., Cancer Res 60 (2000) 619-625.

[8] B.L.Pool-Zobel, I.Dornacher, R.Lambertz, M.Knoll, H.K.Seitz. Genetic damage and repair in human rectal cells for biomonitoring: sex differences, effects of alcohol exposure, and susceptibilities in comparison to peripheral blood lymphocytes, Mutat. Res. 551 (2004) 127-134.

[9] S.J.Duthie, A.Ma, M.A.Ross, A.R.Collins. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes, Cancer Res 56 (1996) 1291-1295.

[10] B.L.Pool-Zobel, A.Bub, H.Müller, I.Wollowski, G.Rechkemmer. Consumption of vegetables reduces genetic damage in humans: first results of an intervention trial with carotenoid-rich foods, Carcinogenesis 18 (1997) 1847-1850.

[11] A.Bub, B.Watzl, M.Blockhaus, K.Briviba, U.M.Liegibel, H.Müller, B.L.Pool-Zobel, G.Rechkemmer. Fruit juice consumption modulates antioxidative status, immune status and DNA damage, J. Nutr. Biochem. 14 (2003) 98 .

[12] M.Fenech, C.Stockley, C.Aitken. Moderate wine consumption protects against hydrogen peroxide-induced DNA damage, Mutagenesis 12 (1997) 289-296.

[13] A.R.Whitney, M.Diehn, S.J.Popper, A.A.Alizadeh, J.C.Boldrick, D.A.Relman, P.O.O'Brown. Individuality and variation in gene expression patterns in human blood. Proceedings of the National Academy of Sciences U.S.A. 100, 1896-1901. 2003.

[14] J.C.Rockett, M.E.Burczynski, A.J.Fornace, P.C.Herrmann, S.A.Krawetz, D.J.Dix. Surrogate tissue analysis: monitoring toxicant exposure and health status of inaccessible tissues through the analysis of accessible tissues and cells., Toxicology and Applied Pharmacology 194 (2004) 189-199.

[15] G.Eisenbrand, B.Pool-Zobel, V.Baker, M.Balls, B.J.Blaauboer, A.Boobis, A.Carere, S.Kevekordes, J.C.Lhuguenot, R.Pieters, J.Kleiner. Methods of in vitro toxicology, Food Chem. Toxicol. 40 (2002) 193-236.

[16] M.Glei, A.Klinder, G.O.Latunde-Dada, T.W.Becker, U.Hermann, K.Voigt, B.L.Pool-Zobel. Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A , Mutation Research 519 (2002) 151-161.

[17] A.Pietrangelo. Haemochromatosis, Gut 52 Suppl 2 (2003) ii23-ii30.

[18] X.Huang. Iron overload and its association with cancer risk in humans: evidence for iron as a carcinogenic metal, Mutat. Res. 533 (2003) 153-171.

[19] C.F.Babbs. Oxygen radicals in ulcerative colitis, Free Radic. Biol. Med. 13 (1992) 169-181.

[20] J.A.Imlay, S.Linn. DNA damage and oxygen radical toxicity, Science 240 (1988) 1302-1309.

[21] M.Tseng, R.S.Sandler, E.R.Greenberg, J.S.Mandel, R.W.Haile, J.A.Baron. Dietary iron and recurrence of colorectal adenomas, Cancer Epidemiol. Biomarkers Prev. 6 (1997) 1029-1032.

[22] A.Boyum. Isolation of mononuclear cells and granulocytes from human blood, Scand. J. Clin. Lab Invest 21 (1968) 77.

[23] J.H.Swain, S.M.Newman, J.R.Hunt. Bioavailability of elemental iron powders to rats is less than bakery-grade ferrous sulfate and predicted by iron solubility and particle surface area, J. Nutr. 133 (2003) 3546-3552.

[24] H.Nordeng, A.Eskild, B.I.Nesheim, I.Aursnes, G.Jacobsen. Guidelines for iron supplementation in pregnancy: compliance among 431 parous Scandinavian women, Eur. J. Clin. Pharmacol. 59 (2003) 163-168.

[25] N.P.Singh, M.T.McCoy, R.R.Tice, E.L.Schneider. A simple technique for quantitation of low levels of DNA damage in individual cells, Exp Cell Res 175 (1988) 184-191.

[26] A.R.Collins, S.J.Duthie, V.L.Dobson. Direct enzymic detection of endogenous oxidative base damage in human lymphocyted DNA, Carcinogenesis 14 (1995) 1733-1735.

[27] M.Dusinská, A.R.Collins. Detection of oxidised purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay, ATLA 24 (1996) 405-411.

[28] V.J.McKelvey-Martin, E.T.Ho, S.R.McKeown, S.R.Johnston, P.J.McCarthy, N.F.Rajab, C.S.Downes. Emerging applications of the single cell gel electrophoresis (Comet) assay. I. Management of invasive transitional cell human bladder carcinoma. II Fluorescent in situ hybridization Comets for the identification of damaged and repaired DNA sequences in individual cells, Mutagenesis 13 (1998) 1-8.

[29] C.Bock, A.Rapp, H.Dittmar, S.Monajembashi, K.O.Greulich. Localisation of specific sequences and DNA single strand breaks in individual UV-A irradiated human lymphocytes by COMET FISH, Progress in Biomedical Optics 3568 (1999) 207-217.

[30] A.Schäferhenrich, G.Beyer-Sehlmeyer, G.Festag, A.Kuechler, N.Haag, A.Weise, T.Liehr, U.Claussen, B.Marian, W.Sendt, J.Scheele, B.L.Pool-Zobel. Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal , Mutation Research 9496 (2003) 1-14.

[31] G.P.Pfeifer. p53 mutational spectra and the role of methylated CpG sequences, Mutat. Res. 450 (2000) 155-166.

[32] D.H.Lee, D.Y.Liu, D.R.Jacobs, Jr., H.R.Shin, K.Song, I.K.Lee, B.Kim, R.C.Hider. Common presence of non-transferrin-bound iron among patients with type 2 diabetes, Diabetes Care 29 (2006) 1090-1095.

[33] L.J.van Tits, E.M.Jacobs, D.W.Swinkels, H.L.Lemmers, d.van, V, G.J.de, A.F.Stalenhoef. Serum non-transferrin-bound iron and low-density lipoprotein oxidation in heterozygous hemochromatosis, Biochem. Biophys. Res. Commun. 345 (2006) 371-376.

[34] M.Grootveld, J.D.Bell, B.Halliwell, O.I.Aruoma, A.Bomford, P.J.Sadler. Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy, J. Biol. Chem. 264 (1989) 4417-4422.

[35] National Research Council. Guide for the care and use of laboratory animals, National Academy Press, Washington, DC, 1996.

[36] S.Lafay, E.Gueux, Y.Rayssiguier, A.Mazur, C.Remesy, A.Scalbert. Caffeic acid inhibits oxidative Stress and reduces hypercholesterolemia induced by iron overload in rats, Int. J. Vitam. Nutr. Res. 75 (2005) 119-125.

[37] M.Glei, U.M.Liegibel, M.N.Ebert, V.Böhm, B.L.Pool-Zobel. ß-Carotene reduces bleomycin-induced genetic damage in human lymphocytes, Toxicology and Applied Pharmacology 179 (2002) 65-73.

[38] R.R.Tice, E.Agurell, D.Anderson, B.Burlinson, A.Hartmann, H.Kobayashi, Y.Miyamae, E.Rojas, J.C.Ryu, Y.F.Sasaki. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206-221.

[39] C.H.Chuang, M.L.Hu. Use of whole blood directly for single-cell gel electrophoresis (comet) assay in vivo and white blood cells for in vitro assay, Mutat. Res. 564 (2004) 75-82.

[40] H.Verhagen, O.I.Aruoma, J.H.M.van Delft, L.O.Dragsted, L.R.Ferguson, S.Knasmüller, B.L.Pool-Zobel, H.E.Poulson, G.Williamson, S.Yannai. The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in *in vitro* experiments and animal studies *in vivo*, Fd Chem Toxicol 41 (2003) 603-610.

[41] T.Liehr, A.Heller, H.Starke, N.Rubstov, V.Trifonov, K.Mrasek, A.Weise, A.Kuechler, U.Claussen. Microdissection based high resolution multicolour banding (MCB) for all 24 chromosomes., Int. J. Mol. Med. 9 (2002) 335-339.

[42] Y.Knöbel, A.Weise, M.Glei, W.Sendt, U.Claussen, B.L.Pool-Zobel. Ferric iron is genotoxic in non transformed and preneoplastic human colon cells., Food Chem. Toxicol. (2006) Nov 7 [E pub ahead of print].

[43] J.Y.Uriu-Adams, C.L.Keen. Copper, oxidative stress, and human health, Mol. Aspects Med. 26 (2005) 268-298.

[44] N.C.Andrews. Iron metabolism: iron deficiency and iron overload, Annu. Rev. Genomics Hum. Genet. 1 (2000) 75-98.

[45] J.Emerit, C.Beaumont, F.Trivin. Iron metabolism, free radicals, and oxidative injury, Biomed. Pharmacother. 55 (2001) 333-339.

[46] G.Papanikolaou, K.Pantopoulos. Iron metabolism and toxicity, Toxicol. Appl. Pharmacol. 202 (2005) 199-211.

[47] C.Müller, G.Eisenbrand, M.Gradinger, T.Rath, F.W.Albert, J.Vienken, R.Singh, P.B.Farmer, J.P.Stockis, C.Janzowski. Effects of hemodialysis, dialyser type and iron infusion on oxidative stress in uremic patients, Free Radic. Res. 38 (2004) 1093-1100.

[48] D.J.Fleming, K.L.Tucker, P.F.Jacques, G.E.Dallal, P.W.F.Wilson, R.J.Wood. Dietary factors associated with the risk of high iron stores in the elderly Framingham Heart Study cohort, Am J Clin Nutr 76 (2002) 1375-1384.

[49] J.Beard. Dietary iron intakes and elevated iron stores in the elderly: is it time to abandon the set-point hypothesis of regulation of iron absorption?, Am J Clin Nutr 76 (2002) 1189-1190.

[50] G.Cairo, F.Bernuzzi, S.Recalcati. A precious metal: iron, an essential nutrient for all cells. Genes & Nutrition 1[1], 25-40. 2006.

[51] K.Schumann, S.Kroll, G.Weiss, J.Frank, H.K.Biesalski, H.Daniel, J.Friel, N.W.Solomons. Monitoring of hematological, inflammatory and oxidative reactions to acute oral iron exposure in human volunteers: preliminary screening for selection of potentially-responsive biomarkers, Toxicology 212 (2005) 10-23.

[52] D.Gackowski, M.Kruszewski, T.Bartlomiejczyk, A.Jawien, M.Ciecierski, R.Olinski. The level of 8-oxo-7,8-dihydro-2'-deoxyguanosine is positively correlated with the size of the labile iron pool in human lymphocytes, J. Biol. Inorg. Chem. 7 (2002) 548-550.

[53] M.Glei, G.O.Latunde-Dada, A.Klinder, T.W.Becker, U.Hermann, K.Voigt, B.L.Pool-Zobel. Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A, Mutat. Res. 519 (2002) 151-161.

[54] S.P.Hussain, K.Raja, P.A.Amstad, M.Sawyer, L.J.Trudel, G.N.Wogan, L.J.Hofseth, P.G.Shields, T.R.Billar, C.Trautwein, T.Höhler, P.R.Galle, D.H.Phillips, R.Markin, A.J.Marrogi, C.C.Harris. Increased *p53* mutation load in nontumorous human liver of Wilson disease and hemochromatosis: Oxyradical overload diseases., Med. Sci. 97 (2000) 12770-12775.

[55] N.C.Andrews, J.E.Levy. Iron is hot: an update on the pathophysiology of hemochromatosis, Blood 92 (1998) 1845-1851.

[56] A.Pietrangelo. Haemochromatosis, Gut 52 Suppl 2 (2003) ii23-ii30.

[57] J.I.Wurzelmann, A.Silver, D.M.Schreinemachers, R.S.Sandler, R.B.Everson. Iron intake and risk of colon cancer., Cancer Epidemiol. Biomarkers Prev. 5 (1996) 503-507.

[58] P.Senesse, S.Meánce, V.Cottet, J.Faivre, M.-C.Boutron-Ruault. High dietary iron and copper and risk of colorectal cancer: a case-control study in Burgundy, France., Nutr. Cancer 49 (2004) 66-71.

[59] N.J.Shaheen, L.M.Silverman, T.Keku, L.B.Lawrence, E.M.Rohlfs, C.F.Martin, J.Galanko, R.S.Sandler. Association Between Hemochromatosis (HFE) Gene Mutation Carrier Status and the Risk of Colon Cancer, JNCI Cancer Spectrum 95 (2003) 154-159.

[60] A.T.Chan, J.Ma, G.J.Tranah, E.L.Giovannucci, N.Rifai, D.J.Hunter, C.S.Fuchs. Hemochromatosis gene mutations, body iron stores, dietary iron, and risk of colorectal adenoma in women., J Natl Cancer Inst 97 (2005) 917-926.

[61] T.Norat, S.Bingham, P.Ferrari, N.Slimani, M.Jenab, M.Mazuir, K.Overvad, A.Olsen, A.Tjonneland, F.Clavel, M.C.Boutron-Ruault, E.Kesse, H.Boeing, M.M.Bergmann, A.Nieters, J.Linseisen, A.Trichopoulou, D.Trichopoulos, Y.Tountas, F.Berrino, D.Palli, S.Panico, R.Tumino, P.Vineis, H.B.Bueno-de-Mesquita, P.H.Peeters, D.Engeset, E.Lund, G.Skeie, E.Ardanaz, C.Gonzalez, C.Navarro, J.R.Quiros, M.J.Sanchez, G.Berglund, I.Mattisson, G.Hallmans, R.Palmqvist, N.E.Day, K.T.Khaw, T.J.Key, J.M.San, B.Hemon, R.Saracci, R.Kaaks, E.Riboli. Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition, J. Natl. Cancer Inst. 97 (2005) 906-916.

[62] A.Wellejus, H.E.Poulsen, S.Loft. Iron-induced oxidative DNA damage in rat sperm cells in vivo and in vitro, Free Radic. Res. 32 (2000) 75-83.

Table 1. Composition of experimental diet (%)

ingredients	control	iron overload
casein	20	20
corn starch	55.949	54.959
sucrose	10	10
corn oil	5	5
cellulose	4	4
vitamin mixture[1)]	1	1
mineral mixture[2)]	3.5	3.5
choline bitartarate	0.25	0.25
DL-methionine	0.3	0.3
butylated hydroxy toluene	0.001	0.001
iron ($FeSO_4 \cdot 7H_2O$)	-	0.99
total	100	100

[1)] AIN 76 vitamin mixture contained (in g/kg of mixture): thiamine HCl 0.6; riboflavin 0.6; pyridoxine HCl 0.7; niacin 3; D-calcium pantothenate 1.6; folic acid 0.2; D-biotin 0.02; cyanocobalamin (vitamin B_{12}) 0.001; dry vitamin A palmitate (500,000 U/d) 0.8; dry vitamin E acetate (500 U/d0 10; vitamin D_3 trituration (400,000 U/g) 0.25; menadione sodium bisulfite complex 0.15; sucrose finely powdered 981.08

[2)] AIN 76 mineral mixture contained (in g/kg of mixture): calcium phosphate, dibasic 500; sodium chloride 74; potassium citrate, monohydrate 220; potassium sulfate 52; magnesium oxide 24; manganous carbonate (43-48 % Mn) 3.5; ferric citrate (16-17 % Fe) 6; zinc carbonate (70 % ZnO) 1.6; cupric carbonate (53-55 % Cu) 0.3; potassium iodate 0.01; sodium selenite 0.01; chromium potassium sulfate 0.55; sucrose, finely powdered 118.03

Table 2. The effect of iron overload on body weight gain, food intake, food efficiency ratio and organ weight

	control	iron overload
body weight gain (g/day)	2.4 ± 0.2[1]	2.7 ± 0.1
food intake (g/day)	17.9 ± 0.2	18.8 ± 0.2*
food efficiency ratio	13.3 ± 0.9	14.3 ± 0.7
organ weight (g/100g BW)		
liver	2.55 ±0.04	2.67 ± 0.04
heart	0.32 ± 0.02	0.35 ± 0.01
kidney	0.60 ± 0.01	0.58 ± 0.01
spleen	0.19 ± 0.01	0.20 ± 0.01

1) Values are means ± SD (n=10), * Significantly different at $p < 0.05$ by Student's t-test.

Legends to the Figures:

Fig. 1. Comet image (comet class 4) in leucocytes incubated with Fe-NTA (500 µM) (A) and its Comet FISH image with *TP53* probes (B). The arrows indicate hybridization signals.

Fig. 2. Effect of Fe-NTA on DNA damage in human leucocytes treated for 30 min. The effect was significant by repeated measurement of ANOVA. Bars with different letters are significantly different at $p < 0.05$ by Duncan's multiple range test (n=6).

Fig. 3. Effect of Fe-NTA on oxidized bases in addition to strand breaks (SBs) in human leucocytes after 30 min exposure. Bars with different letters (small letters for SBs; capital letters for Endo III-sensitive sites; bolded capital letters for Fpg-sensitive sites) are significantly different at $p < 0.05$ by Duncan's multiple range test (n=3).

Fig. 4. The effect of iron overload on oxidized bases in addition to strand breaks (SBs) in rat leucocytes. Values are means ± SD (n=10), significantly different at *$p < 0.05$ and ***$p < 0.001$ by Student's t-test. Bars with different letters are significantly different at $p < 0.05$ by Duncan's multiple range tests.

Fig. 5. Proportional distribution of human leucocytes in the comet classes 2-4 (damaged cells) after 30 min treatment with Fe-NTA (1-500 µM, 37°C), (n=3). The significant differences in the comet classes to the (a) untreated medium control (RPMI) or (b) the NTA control were calculated by One-way ANOVA, including Dunnett´s Multiple Comparison Test (*$p<0.05$,***$p<0.001$, beside the bars). The percentage of cells in comet class 1 is not outlined in this figure, but can be calculated as followed: 100 % minus the percentage of cells in comet classes 2-4. The significance levels for the changes in comet class 1 are outlined above the total bars.

Fig. 6. Percentage *TP53* migration in the comet tail of damaged leucocytes (comet classes 2-4) after a 30 min incubation of the cells with Fe-NTA (1-500 µM, 37°C), (n=3). The significant differences to (a) the untreated medium control (RPMI) or (b) the NTA control were calculated by One-way ANOVA, including Dunnett´s Multiple Comparison Test (*$p<0.05$, **$p<0.01$).

Fig. 1

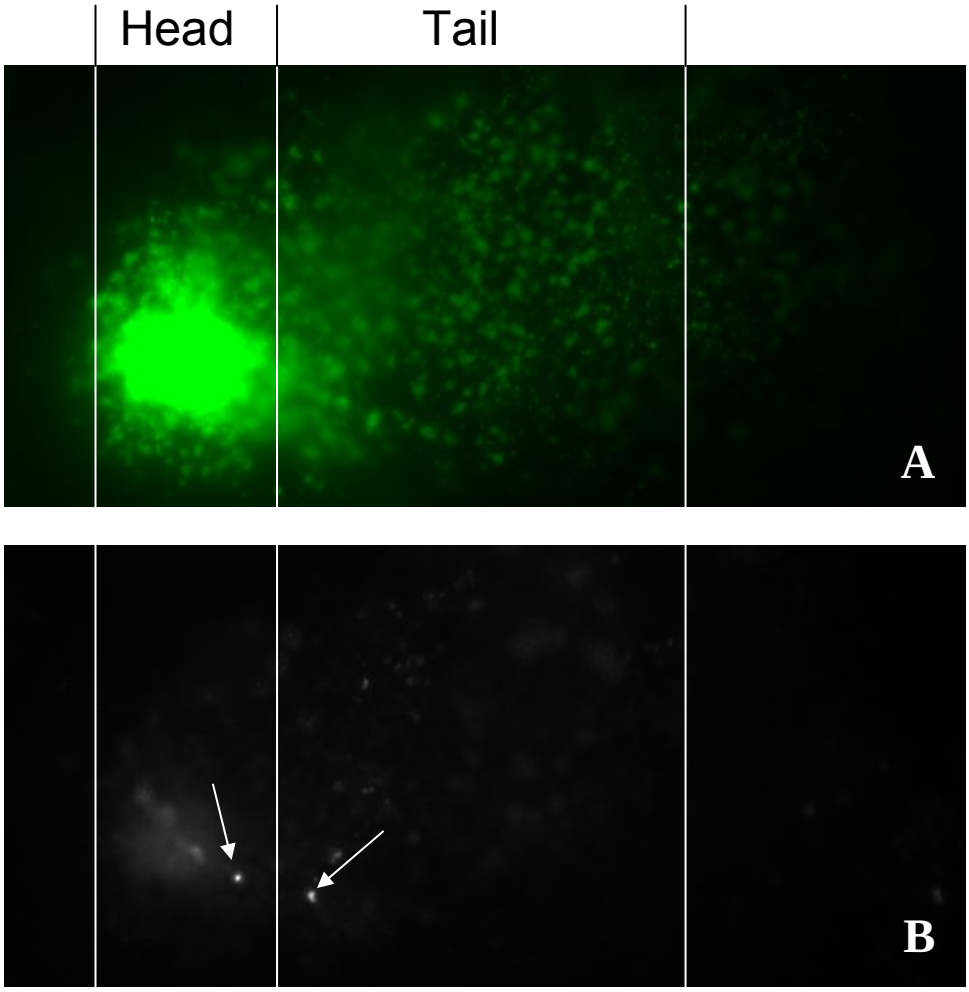


Fig. 2

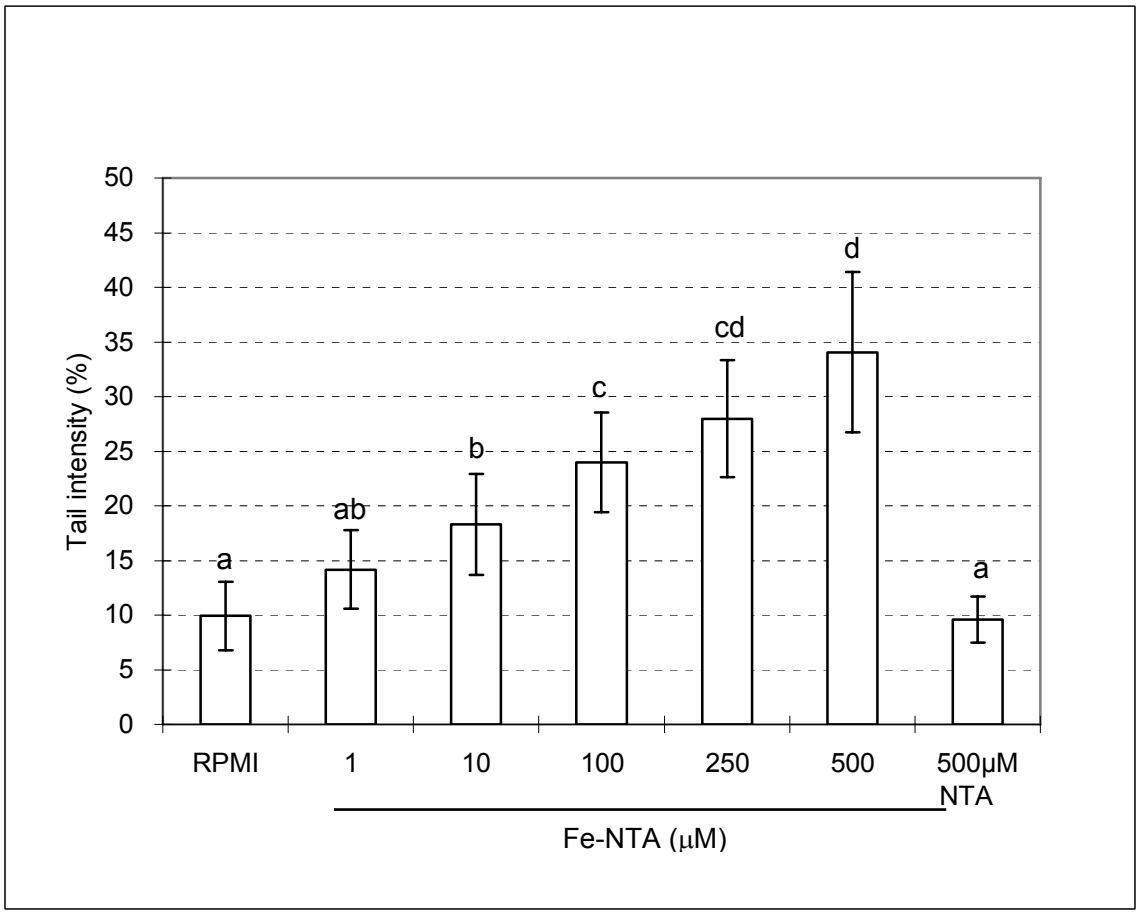

50
45
40
35
30
25
20
15
10
5
0
Tail intensity (%)
a
ab
b
c
cd
d
a
RPMI
1
10
100
250
500
500µM NTA
Fe-NTA (µM)

Fig. 3

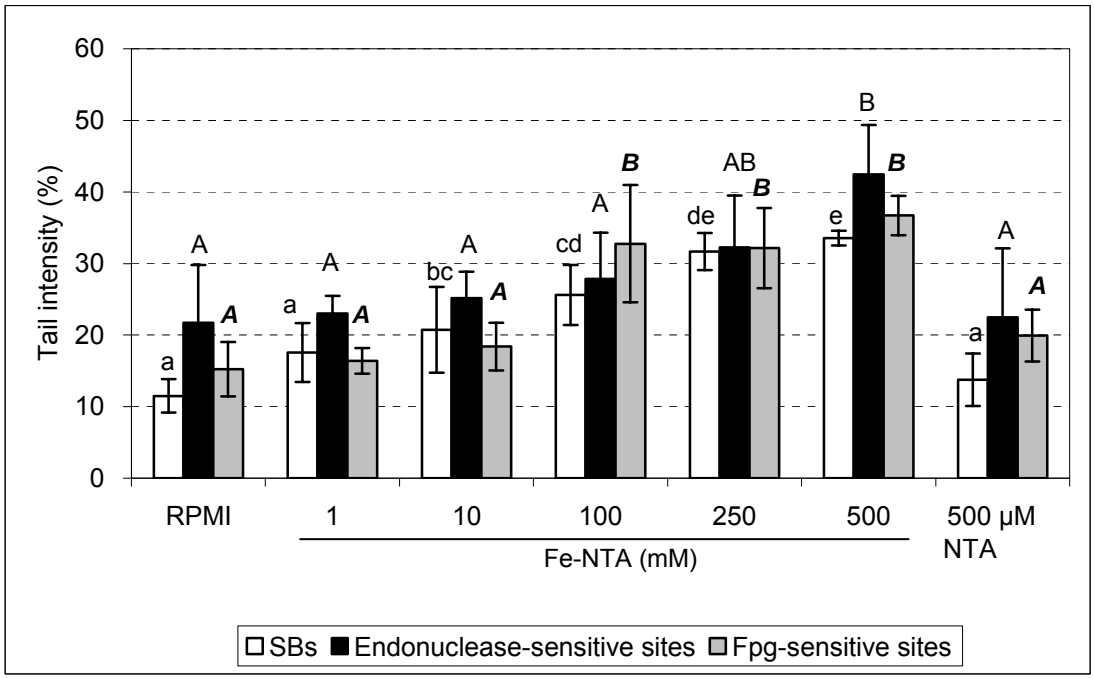

60
50
40
30
20
10
0
Tail intensity (%)
a
A
A
a
A
A
bc
A
A
cd
A
B
de
AB
B
e
B
B
a
A
A
RPMI
1
10
100
250
500
500 µM NTA
Fe-NTA (mM)
SBs
Endonuclease-sensitive sites
Fpg-sensitive sites

Fig. 4

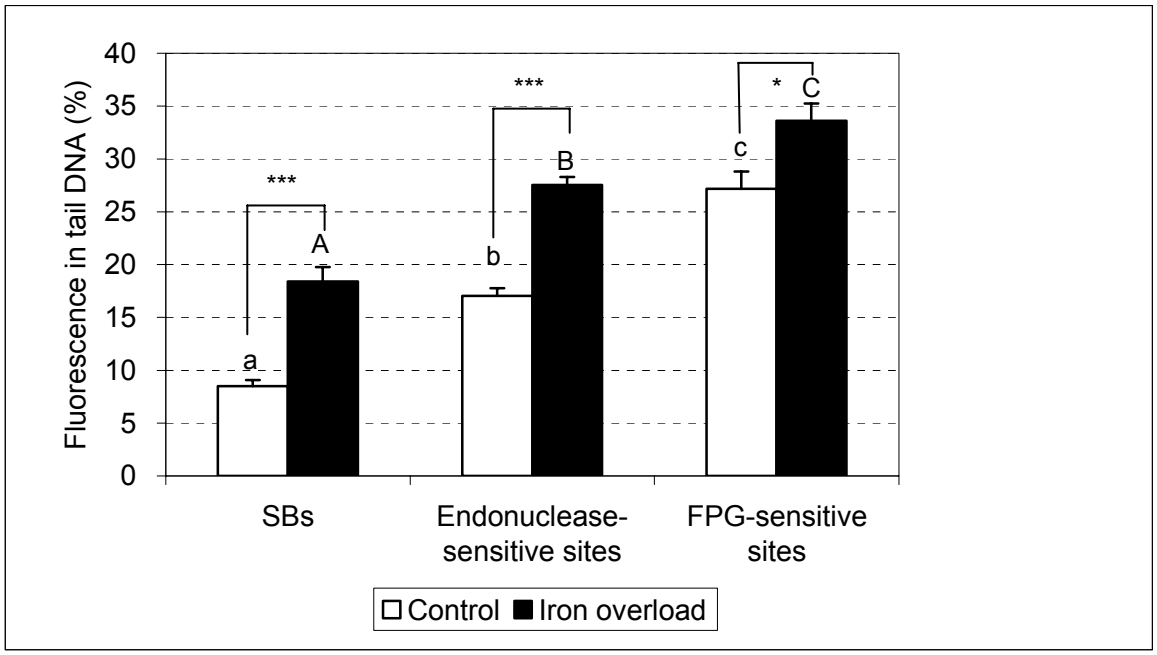

Fluorescence in tail DNA (%)
40
35
30
25
20
15
10
5
0
***
***
*
a
A
b
B
c
C
SBs
Endonuclease-sensitive sites
FPG-sensitive sites
Control
Iron overload

Fig. 5

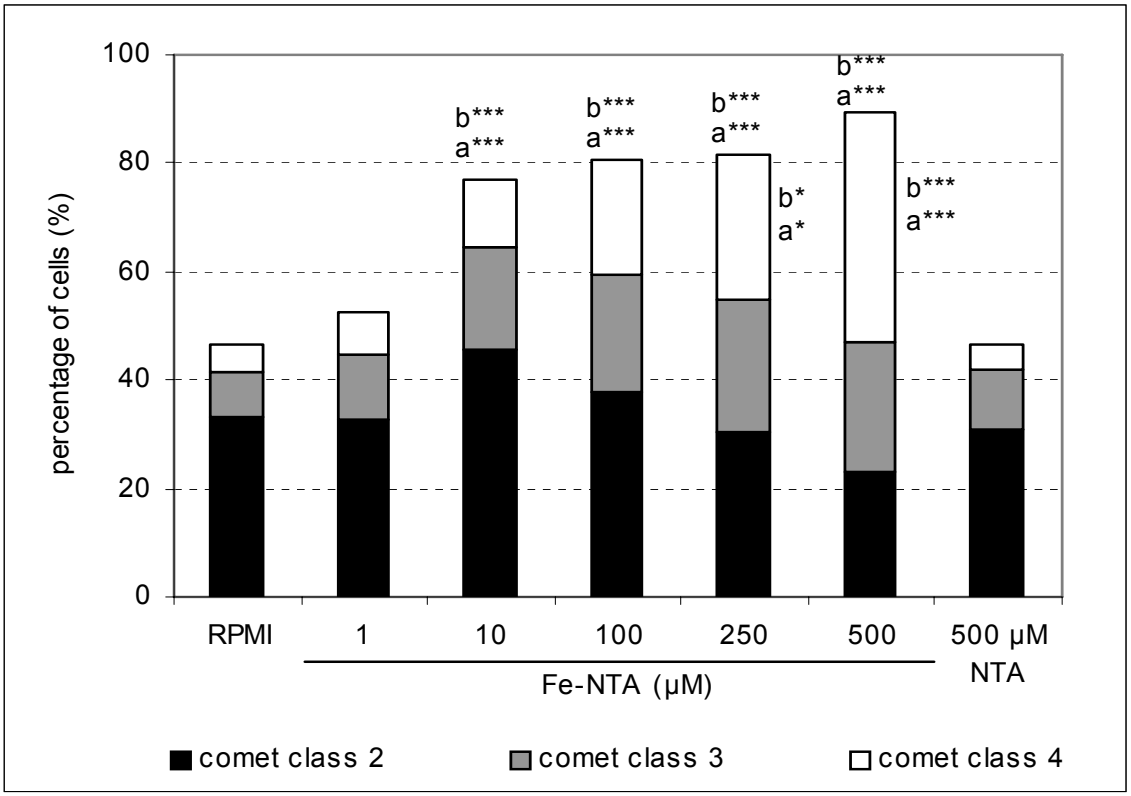
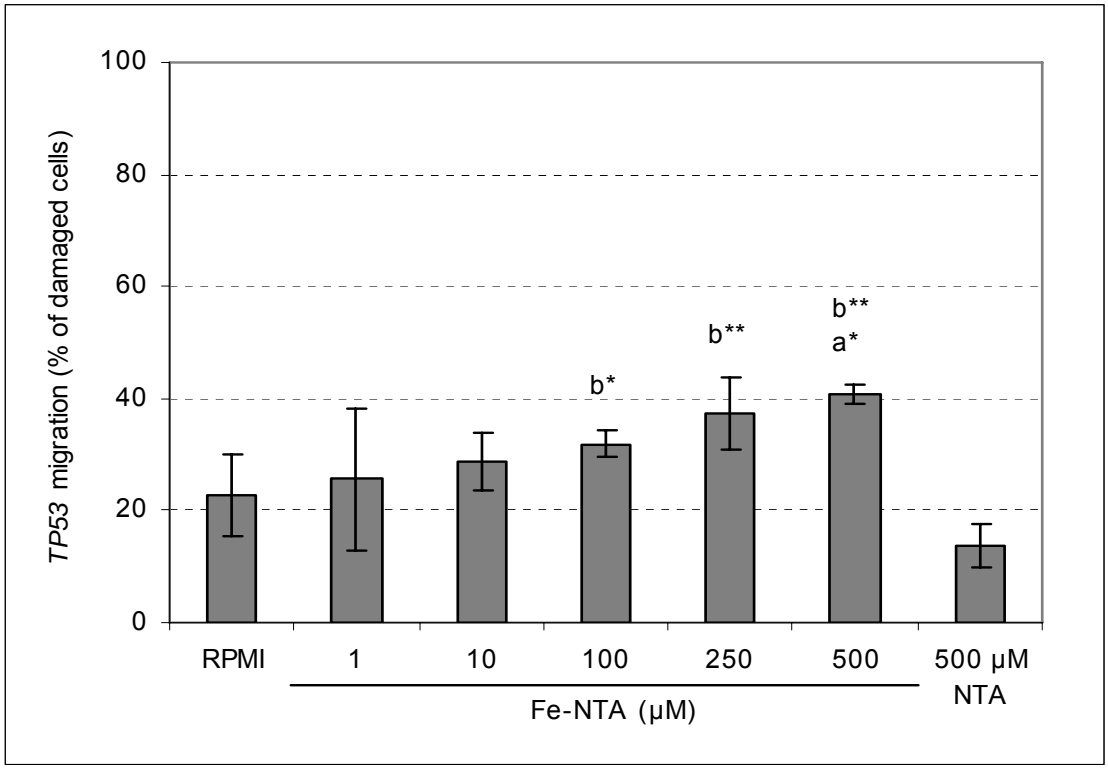

100
80
60
40
20
0
percentage of cells (%)
b***
a***
b***
a***
b***
a***
b*
a*
b***
a***
b***
a***
RPMI
1
10
100
250
500
500 µM
NTA
Fe-NTA (µM)
comet class 2
comet class 3
comet class 4

Fig. 6


100
80
60
40
20
0
TP53 migration (% of damaged cells)
b*
b**
b**
a*
RPMI
1
10
100
250
500
500 µM NTA
Fe-NTA (µM)

3.6 Manuskript VI

Apple flavonoids modulate the genotoxic effects of different DNA damaging compounds in human colon cells

S. Klenow*, S. Veeriah*, **Y. Knöbel***, B. L. Pool-Zobel (* 3 Erstautoren)

zur Publikation vorbereitet für Mutation Research

Zusammenfassung

In der Ernährungstoxikologie spielen Flavonoide wegen ihrer positiven Effekte auf den Fremdstoffmetabolismus, der Radikalfänger-Funktion und der Protektion der Lipidperoxidation eine entscheidende Rolle. Im Rahmen dieser Studie sollte deshalb untersucht werden, ob ein polyphenolreicher Apfelextrakt in der Lage ist, die durch verschiedene Genotoxine induzierten DNA-Schäden zu modulieren, bestenfalls zu vermindern.

Hierfür wurden im Challenge Assay Kolonadenomzellen (LT97) und Kolonkarzinomzellen (HT29 bzw. HT29 clone 19A) mit einem Apfelextrakt für 24 h vorinkubiert und anschließend mit verschiedenen Genotoxinen geschädigt.

Die Vorinkubation mit dem Apfelextrakt verminderte signifikant die genotoxischen Wirkungen des synthetischen Hydroperoxids Cumenhydroperoxid und des auch endogen gebildeten Wasserstoffperoxids. Die durch Benzo[a]pyrendiolepoxid (BPDE) induzierten DNA-Schäden konnten durch den Apfelextrakt nur in den LT97-Adenomzellen vermindert werden. In HT29-Zellen wirkte der Apfelextrakt in Verbindung mit BPDE pro-oxidativ.

Darstellung des Eigenanteils

- Planung, Durchführung und Auswertung der Versuche, außer FOX Assay für ASO2 über 24 h
- Erstellen des Publikationsmanuskriptes

Apple polyphenols modulate the genotoxic effects of different DNA damaging compounds

Stefanie Klenow[¥], Selvaraju Veeriah[¥], Yvonne Knöbel[¥], Beatrice L. Pool-Zobel*

Institute for Nutrition, Department of Nutritional Toxicology, Friedrich-Schiller-University Jena, Dornburger Str. 25, 07743 Jena, Germany

Last update 08.01.2007

*Address of corresponding author

Prof. Dr. rer. nat. Beatrice L. Pool-Zobel

Friedrich-Schiller-University Jena

Institute for Nutrition

Department of Nutritional Toxicology

Dornburger Str. 25

07743 Jena

Germany

Tel: + 49 3641 949670

Fax: + 49 3641 949672

E-mail: b8pobe@uni-jena.de

¥ Authors equilly contributed to this work

Abstract

Background Apple polyphenols are possibly chemoprotective, since they enhance expression of detoxifying glutathione S-transferases (e.g. *GSTT2, GSTP1*) in human colon cells. Aim of this study was to elucidate whether pretreatment of the cells with an apple extract (AE) also reduces DNA-damage caused by compounds that are deactivated by GSTs. **Methods** An AE was prepared and used to treat two human colon cell lines (LT97, HT29) under conditions that induce GSTs. The treated cells were then challenged with genotoxic compounds and DNA damage was determined with the comet assay. Cumene hydroperoxide (Cum-OOH) and hydrogen peroxide (H_2O_2) or benzo(a)pyrene diolepoxide (BPDE) were used as genotoxins, since they may be selectively conjugated and deactivated by *GSTT2* or *GSTP1*, respectively. Production of H_2O_2 by AE was studied to understand additional mechanisms. **Results** The pre-treatment with AE protected HT29 cells against the genotoxic action of Cum-OOH and H_2O_2, whereas BPDE-damage was not modified. In LT97 cells, AE reduced DNA-damage of all three genotoxins used. H_2O_2 was produced when incubating AE in culture medium for HT29 cells but not of LT97 cells. **Conclusions** Apple polyphenols protected against DNA damage induced by Cum-OOH and H_2O_2 in human LT97 and H29 cell lines, which may be due to the enhanced expression of GSTT2 that deactivates the genotoxins. BPDE-mediated DNA damage, however, was only reduced by pre-treated LT97 cells. Differences between cell lines possibly caused by formed H_2O_2 in the HT29 suspension culture, resulting in higher oxidative burden of HT29 cells. The adenoma derived cells (LT97) were more susceptible towards the genotoxic substances than carcinoma derived (HT29) cells, a finding which mirrors a higher sensitivity of preneoplastic cells than tumor cells. The present

study reveals that apple polyphenols have anti-genotoxic and pro-genotoxic activities *in vitro*, the consequences of which need to be resolved for the *in vivo* situation.

Keywords: Apple polyphenols; Chemoprevention; LT97; HT29; Comet Assay; Toxicology

1. Introduction

Apples are widely consumed in the Western diet and they are a major source of flavonoids [1, 2]. Flavonoids are known to possess various beneficial properties that may affect carcinogen metabolism [3], free radical scavenging or lipid peroxidation [4, 5]. Chemopreventive properties of flavonoids may thus be associated with diverse biological activities such as induction of carcinogen-detoxifying systems [6], interaction with cellular signalling pathways, and modulation of gene expression controlling proliferation, differentiation and apoptosis of cancer cells [7]. Recent studies with the apple polyphenol procyanidin have shown that this compound increases the expression of extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and the activity of caspase-3, indicating apoptotic process resulting cell death [8]. In this study the authors also have shown that apple polyphenol procyanidin inhibits G2/M phase cell cycle, activities of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) in human colorectal carcinoma cell line and thus, undermine promotion of colon carcinogenesis [9]. Recently *in vivo* studies properties which are related to anticarcinogenic activities have been reported, showing that treatment of rats with a polyphenol-rich apple extract, resulted in reduced DNA-damage, preneoplastic lesions and number of aberrant crypt foci (ACF) initiated by colon carcinogens dimethylhydrazine (DMH), azoxymethane (AOM) in the colonic tissues [10, 11].

We have recently shown that the pretreatment of HT29 colon cells with non-genotoxic concentration (510 µg/ml) of an apple extract (AE) resulted in an enhanced expression of *GSTT2* mRNA [12]. The *GSTT2* gene has 55 % homology with isoenzyme *GSTT1* [13]. These genes may biologically activated against many industrial

chemicals, such as ethylene oxide, halogenated alkanes and epoxides thus, enhances their toxicity [14, 15]. Alternatively *GSTT2* has peroxidase activity towards t-butyl hydroperoxide in addition to cumene hydroperoxide (Cum-OOH) for which it has pronounced substrate specificity even though GSTA2-2 and GSTK1-1 as well as mu-class GST enzymes have reported activity with Cum-OOH [16-18]. Characterisation of isolated GSTT2-2 enzyme revealed no detectable activity towards hydrogen peroxide [19, 20]. In addition, Cum-OOH is a substrate for glutathione peroxidase (GPX). One of our parallel gene expression study in LT97 cell line showed 2-fold up-regulation of GPX2 in response to AE (128 µg/ml) after 24 h treatment time. *GPX2* is the selenium-dependent glutathione peroxidase and the primary role of *GPX2* seems to scavenge H_2O_2 in the epithelium of the gastrointestinal tract [21]. Mercaptosuccinate is one of the most potent inhibitor of GPX and has been described to reduce detoxification of Cum-OOH via glutathione system [22, 23].

In the following it was therefore one aim to assess whether pretreatment of human colon cells with AE would alter the genotoxic response of Cum-OOH and of H_2O_2. H_2O_2 is a source of endogenous oxidative stress that may decompose to yield reactive oxygen species (ROS). This reaction is catalysed by transition metals like iron, which increase the production of ROS from peroxides (e.g. H_2O_2) via the Fenton reaction [24].

In addition to *GSTT2* (1.7-fold), our previous studies had also shown that *GSTP1* (1.6-fold), was upregulated by AE in HT29 colon cells. A substrate for *GSTP1*, which is deactivated by *GSTP1*, is benzo[a]pyrene diol epoxide (BPDE), the metabolically activated form of benzo(a)pyrene [25, 26]. Benzo(a)pyrene is a well-characterized carcinogenic polycyclic aromatic hydrocarbon produced during food heating [27] and

a substrate for CYP1A-enzymes which biotransform the parent compound to the mutagenic and carcinogenic BPDE. BPDE induces DNA adducts that are repaired by the nucleotide excision repair pathway. The genotoxic effects of BPDE can be prevented by conjugation with GSH catalyzed by GSTs [28]. Thus it was expected that AE pretreatment could protect the cells from genotoxicity of BPDE.

2. Material and Methods

2.1. Compounds

Cum-OOH was obtained from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany. The stock solution (6 M) can be stored at room temperature and working solutions (60-360 µM) diluted with PBS were freshly prepared for each experiment. Stock solution of H_2O_2 (Merck Eurolab GmbH, Darmstadt, Germany) was diluted in PBS to prepare working solutions of 4.7 to 150 µM. H_2O_2 suspensions were incubated for five minutes at 4°C to avoid repair of the induced oxidative DNA damage [Collins et al., 1995]. BPDE (Benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide(±)) was obtained from Sigma Chemie and was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich GmbH, Deisenhofen, Germany). Stock solutions of BPDE (10 - 50 mM) prepared in DMSO were stored at –20 °C, and working solutions (10 - 50 µM) diluted with DMSO were freshly prepared for each experiment.

2.2. Apple polyphenols extract (AE)

Clear apple juice from cultivar year 2002 was produced on an experimental scale. A defined mixture of table apples as has been described before [29]. Polyphenols of 100 l of that juice were retained on 5 l adsorber resin (XAD 16 HP, Rohm & Haas, Frankfurt) packed onto a Pharmacia glass column (BPG 100, 100x10cm). After

washing out water soluble juice ingredients, polyphenols were eluted with 3 bed volumes of ethanol (96%). The ethanolic fraction was concentrated by evaporation, transferred into the water phase and freeze dried until further use [30]. An aliquot was used for analysis of polyphenol contents [31] other aliquots were needed to treat the colon cells prior to determining biological activities. Apple extract concentrations used in the experiment were expressed in “µM” as phloridzin equivalents (Ph.E).

2.3. Cells and culture conditions

The human colon tumor cell line HT29 was established in 1964 by J. Fogh (Memorial Sloan Kettering Cancer Center, New York, NY). The cells were grown in tissue culture flasks with Dulbecco´s Modified Eagle Medium (DMEM, Gibco BRL, Eggenstein, Germany) supplemented with 10 % foetal calf serum (FCS) and 1 % penicillin/streptomycin at 37 °C in a humidified incubator (5 % CO_2/95 % air). For this study we used cells in passages 20-50.

The human colon adenoma cell line LT97 was established from a colon microadenoma of a patient with familial adenoma polyposis coli [32]. LT97 was maintained in a culture medium containing MCDB 302 medium with glutamine (Biochrom, Berlin, Germany) plus 20 % of L15 Leibovitz medium, 2 % FCS and gentamycin (10 µg/ml). This medium was supplemented with 0.2 nM triiodo-L-thyronine, 1 µg/ml hydrocortisone, 10 µg/ml insulin, 2 µg/ml transferrin, 5 nM sodium selenite and 30 ng/ml epidermal growth factor (EGF). Cells were grown in a humidified incubator under standardized culture.

2.4. Challenge Assay

To investigate the influence of an apple extract on the induction of DNA damage, HT29 cells or LT97 cells were incubated with the AE for 24 h. Nongenotoxic concentrations were used that delivered 30 µM, Ph.E (HT29) or 7.5 µM, Ph.E (LT97) to the suspension culture. These concentrations had been previously determined to be equivalent to ~ 38 % of the respective EC_{50} values (cell proliferation) at 48 h in LT97 and 24 h in HT29 cells [33]. They are thus equitoxic and of comparable efficacy in the two cell types. The cells were harvested and damaged with different concentrations of Cum-OOH (60-360 µM, 5 minutes, 4°C), H_2O_2 (4.7-150 µM, 5 minutes, 4°C), or BPDE (10-50 µM, 30 minutes, 37°C). The Comet assay was carried out under the conditions described by Tice et al. [34]. DNA damage was microscopically quantified using a ZEISS Axiovert 25 microscope (Carl Zeiss Jena GmbH, Jena, Germany) and the Komet 4.0 image analysis system (Kinetic Imaging Corp., Liverpool, UK). 50 cells were evaluated per slide and the percentage of fluorescence in the tail (TI, “tail intensity”) was scored. The means of 3 replicates were used to calculate the means of at least three independently reproduced experiments.

2.5. Determination of H_2O_2 formation – Ferric-Xylenol Orange (FOX) Assay

Peroxide production or rather hydrogen peroxide formation in the cell free culture media in the presence of the apple extract or the individual apple compounds chlorogenic acid (CA) and phloridzin (Pl) was analysed using the FOX method [35]. Different concentrations of AE (0 - 50 µM, “Ph.E”, in culture media), CA (20 - 200 µM) or Pl (20 - 200 µM) were incubated for 24 hours at 37°C in a humidified incubator to allow H_2O_2 formation. Then 40 µl of the test concentrations were mixed with 360 µl of the FOX solution and incubated for 30 minutes at room temperature, during which

time ferrous ions were oxidized to ferric ions by the hydroperoxides.. Ferric ions are detected by the formation of a coloured complex with xylenol orange. Serial concentrations of H_2O_2 (0-75 µM) were processed in parallel for calibration purpose After centrifugation (10000 g, 10 minutes) 3 x 100 µl of the supernatant were transferred in to a 96-well plate reading absorptions at 560 nm. Concentrations were determined from the standard curve of hydrogen peroxide. The results were expressed as hydrogen peroxide equivalents.

2.6. Statistical analysis

Statistical analysis was performed using the GraphPad® Prism software Version 4.0 (GraphPad® Software Inc., San Diego, USA). All experiments performed *in vitro* were repeated at least three times independently unless otherwise indicated. Repeated measures (one-way) ANOVA, including Bonferronis multiple comparison tests were used to compare the significance of differences between PBS/DMSO-treated controls and Cum-OOH, H_2O_2, or BPDE-treated cells. Comparisons of medium-treated controls with AE-treatments were calculated using the Repeated measures (mixed model) ANOVA with matched subjects, including pairedwise Bonferroni´s multiple comparison test. Differences with P values ≤ 0.05 were considered significant.

3. Results

3.1. Modulation of genotoxicity

The synthetic hydroperoxide Cum-OOH was significantly genotoxic in both cell lines (grey bars in Figure 1A and 1B). The colon adenoma cell line LT97 was more sensitive towards the DNA damaging action of Cum-OOH than the colon carcinoma cell line HT29 ($p \leq 0.001$, two-way ANOVA). The maximal TI at 360 µM Cum-OOH was about 2-fold higher in LT97 than in HT29 cells. Preincubation of both cell lines with equitoxic concentrations of the apple extract (LT97 7.5 µM, HT29 30 µM) reduced the genotoxic effects of Cum-OOH (black bars in Figure 1A and 1B). Viability of AS02 pre-treated HT29 cells was significantly reduced after the challenge (84±4% in medium control to 56±5% in AS02 treated cells, Cum-OOH had no impact).

H_2O_2 was investigated as a model for endogenous, relevant compounds. H_2O_2 was also significantly genotoxic in both cell lines at 37.5 µM and higher, and LT97 cells were again more sensitive than HT29 cells (grey bars in Figure 2A and 2B; $p \leq 0.001$). Pre-treatment of the cells with the AE resulted in significantly lower levels of H_2O_2 - caused DNA damage in comparison to the cells pre-incubated with cell culture medium (black bars in Figure 2A and 2B; LT97: $p \leq 0.01$; HT29: $p \leq 0.001$, two-way ANOVA). Again, viability was strongly reduced in AS02 treated cells after the challenge (81±5% in medium control to 43±13% in AS02 treated cells, H_2O_2 had no impact).

BPDE induced significant DNA damage (grey bars in Figure 4A and 4B), in LT97 cells at 10 µM ($p \leq 0.01$, one-way ANOVA) and in HT29 cells at 30 µM ($p \leq 0.01$, one-way ANOVA). The difference in the sensitivity of the cells was significant ($p \leq$

0.001, two-way ANOVA). Pre-incubation with the apple extract reduced the amount of DNA damaging effects of higher concentrations of BPDE in LT97 cells (black bars in Figure 4A). In HT29 cells, however, DNA damage was significantly enhanced the cells pre-treated with the apple extract (black bars in Figure 4B). The elevated values of TI in the controls (RPMI, DMSO) in this set of experiments in comparison to the controls (PBS) of the previous experiments is probably due to the different post treatment conditions used to determined the genotoxicity of the BPDE (37°C in suspension culture, 30 min) or of H_2O_2 and Cum-OOH (5 min, 4°C on slides). Normalisation of the medium control of both pre-treatments (medium control and AS02) did not reveal induction or reduction of BPDE induced DNA damage by AS02 (data not shown). Again, viability was reduced in AS02 treated cells after the challenge (91±2% in medium control to 69±4% in AS02 treated cells, BPDE had no impact).

3.2. FOX Assay

Incubation of the AE in HT29 cell culture medium (DMEM with 10% FCS) resulted in a significant production of hydrogen peroxide ($p \leq 0.01$, one-way ANOVA). Significant amounts were already generated at 5 µM AE, Ph.E (Figure 5; $p \leq 0.05$, one-way ANOVA). In LT97 cell culture medium (MCDB with supplements) H_2O_2 generation was not detectable. Amounts of generated H_2O_2 by AE in the two culture media were significantly different from each other ($p \leq 0.001$, two-way ANOVA). Apple extract in DMEM was investigated for its potential to generate H_2O_2 after only short time incubation and it appeared as if AE also produced significant H_2O_2-concentrations after 30 minutes incubation. In a parallel study after addition catalase to the incubation mixture H_2O_2 was not measurable any longer, indicating that H_2O_2 is generated and then decomposed by the enzyme (not shown).

Two major compounds contained in the apple extract namely chlorogenic acid and phloridzin, were also tested for their capacity to lead to the generation of H_2O_2. Chlorogenic acid resulted in a significant dose-dependent H_2O_2 production in DMEM culture medium (Figure 5, $p \leq 0.01$). In contrast high concentrations of chlorogenic acid were needed to resulting H_2O_2 formation in MCDB culture medium (Figure 5). Significantly more H_2O_2 was produced in DMEM compared to MCDB culture medium ($p \leq 0.001$). Phloridzin, did not lead to generation of H_2O_2, incubation in DMEM or in MCDB medium.

4. Discussion

Polyphenolic compounds are implicated in preventing diseases related to oxidative stress. We investigated whether an apple polyphenols extract is chemoprotective in two different human colon cell lines *in vitro*. In particular, the apple extract was tested for its ability to reduce DNA-damage induced by different genotoxic agents or oxidants.

Different studies demonstrated that polyphenols protect against damage of oxidation processes. Roig et al. found that procyanidins, the main polyphenols present in red wine and also in apples, protect rat hepatoma (Fao) cells against hydrogen peroxide-induced oxidative stress [36]. They could also show that a procyanidin mixture had a greater antioxidant effect than each of the individual flavonoids tested alone. Quercetin and rutin, flavonols with high antioxidative capacity, induced favourable changes in the antioxidant defence system of HepG2 cells thus preventing cellular oxidative stress [37]. The results of Graziani et al. indicate that apple polyphenol extracts prevented damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo* by increasing intracellular antioxidant activity four-fold and decreasing xanthine-xanthine oxidase [38]. Chlorogenic acid and catechin, the main polyphenolic compounds of apple extracts, were as effective as complex apple extracts in preventing oxidative injury to gastric cells. A moderate wine consumption, and thus the combined effects of alcohol, glycerol and ascorbate together with specific wine-polyphenols, protect the nuclear material from hydrogen peroxide-derived reactive oxygen metabolites [39].

In the present study we investigated the potential chemoprotective action of the apple polyphenols extract in cells challenged with the synthetic compound Cum-

OOH (substrate for GSTT2-2 enzymes) and the endogenously relevant peroxide H_2O_2. Cum-OOH was significantly genotoxic in both cell lines; LT97 cells were more susceptible towards both compounds than HT29 carcinoma cells. The AE protected both cells from Cum-OOH-induced DNA damage. H_2O_2 was also genotoxic at 37.5 µM up to 150 µM in both LT97 cells and HT29 cells. The DNA damage in LT97 cells was on a higher level than HT29 cells. The apple extract was able to reduce H_2O_2-induced DNA damage in both cells lines. Our results confirm the results of the others studies which demonstrated that polyphenols protects from oxidative damage. <Since chlorogenic acid is the main polyphenolic compound in our apple extract we assume that the protective action is mainly due to this compound. Quercetin and phloridzin are also a high effective polyphenols presented in apples. They protect human lymphocytes and colonocytes (Caco-2) against the oxidative attack of H_2O_2 *in vitro* [40, 41]. Next to quercetin Horvathova and colleagues included the flavonoids rutin, apigenin and luteolin in their investigations. Quercetin was found to possess the highest protective effect in murine leukaemia L1210 cells (45 %) followed by luteolin (40 %) [42].

During the reaction of BPDE with the DNA mainly the (+)-trans-anti-BPDE-N^2-dG adduct is formed. Normally this “bulky adducts” could be repaired via nucleotide excision repair (NER) [43]. B(a)P has tumour-initiating and tumour-promoting properties [44]. An enhanced intake of polycyclic aromatic hydrocarbons (PAHs) leads to an increased expression of CYP1A1 via the binding of PAH at the aryl hydrocarbon receptor (Ah-R). Due to this binding the transcription factor will be activate and migrate into the cell nucleus. After a hetero dimerisation with the Ah-R nuclear translocator this complex binds to the xenobiotic response element (XRE). Thus, the tran-

scription of several genes including genes of the extracellular signal transduction, proliferation genes, apoptosis genes and genes of the cell cycle will be activated [45]. Thus, mechanisms enhance the self-sustaining growth of initiated cells and advance carcinogenesis. Since B(a)P binds directly at the Ah-R already low doses are sufficient to be effective.

The glutathione-*S*-transferase Pi class play an important role in the detoxification of BPDE. The expression of *GSTP1* may be an important factor in susceptibility of cells to the DNA damaging activities of BPDE. Recently we could show that the incubation of the polyphenol-rich apple extract induced the expression of *GSTP1* (1.6 fold) mRNA [46]. A functional consequence of this induction should be an increased detoxification of BPDE and thus reduced BPDE-induced DNA damage. In the present work the apple extract reduced BPDE-induced DNA damage in LT97 adenoma cells. Effects in HT29 cells might be due to significant H_2O_2 production at the used AE concentration (30 µM, Ph.E) for HT29 cells. LT97 have a *GSTM1* null polymorphism, thus BPDE can only detoxified by *GSTP1*. A stable transfection of a rat cell line with GSTP1-1 resulted in a significant reduction of BPDE-DNA adducts about 70 % [47]. Single polyphenolic compounds like quercetin have been shown to protect dose dependently against BPDE-DNA adducts in human lymphocytes *in vitro* [48].

The results of recent researches which found a generation of H_2O_2 in different cell culture media and buffer solutions after addition of catechin are in agreement with our results of the FOX assay [49, 50]. We observed a significant H_2O_2 production in DMEM, but not in MCDB medium. This could be due to the high FCS amount in DMEM connected with a higher amount of transition metal like iron or copper. In the study of Akagawa et al. the metal chelator EDTA was able to inhibit the formation

of a metal-polyphenolic complex and the production of H_2O_2 up to 25-30 % [51]. The incomplete inhibition suggested to a higher chelating ability of polyphenolic compounds or that the autoxidation by O_2 itself is involved in the H_2O_2 generation. The transition elements in the media benefit the autoxidation of the polyphenols of the apple extract and thus the formation of H_2O_2. We could also show that the addition of catalase to the reaction prevent the formation H_2O_2. In our investigation we also included chlorogenic acid and phloridzin, main polyphenols in the apple extract and resulted a significant generation of H_2O_2 in both cell culture media, but in MCDB only at very high concentrations. The individual compound phloridzin did not induce H_2O_2. Akagawa and colleagues determined for numerous polyphenols such a H_2O_2 production, especially for the green tea catechin, gallic acid and caffeic acid. Further investigations are necessary to clarify the H_2O_2-producing property of polyphenols and their prooxidative and on the other hand protective effects *in vitro* and *in vivo*.

In summary, the present data provide evidence that primarily polyphenols-rich apple extract reduced DNA damage in colon adenoma (LT97) and carcinoma (HT29) cell lines initiated by carcinogens with Cum-OOH, H_2O_2 and BPDE. In our previous studies we reported an induction of several phase II enzymes (e.g. *GSTT2, GSTP1*) by treatment with apple extract in HT29 cells and thus, possibly reflecting an interaction of GSTs with genotoxic compounds in a way that detoxify carcinogens. H_2O_2 production by polyphenols is normal however; in the present study it is difficult to evaluate the prooxidative effect of apple extract with Fe-NTA and BPDE for the antigenotoxic effects. The adenoma cell line is more sensitive towards the genotoxins and reduction of DNA damage; it could mirror higher efficiencies in preneoplastic lesions than in tumor tissues. Altogether, the reduction of DNA damage of human

colon cells possibly by apple polyphenols induced phase II enzymes could be new targets for colon cancer chemoprotection.

Acknowledgements

The laboratory acknowledges grant funding from the Bundesministerium für Bildung und Forschung (BMBF), Germany (BMBF FKZ.01EA0103). The authors thank Prof. Brigitte Marian, Institute of Cancer Research, University of Vienna, Austria for the generous gift of LT97 adenoma cells and our collaboration partner Dr. Frank Will and Prof. Dr. Helmut Dietrich for providing the apple extract.

Reference List

[1] I.C.Arts, P.C.Hollman, E.J.Feskens, H.B.Bueno de Mesquita, D.Kromhout. Catechin intake and associated dietary and lifestyle factors in a representative sample of Dutch men and women, Eur. J. Clin. Nutr. 55 (2001) 76-81.

[2] J.A.Vinson, X.Su, L.Zubik, P.Bose. Phenol antioxidant quantity and quality in foods: fruits, J. Agric. Food Chem. 49 (2001) 5315-5321.

[3] J.R.Bacon, G.Williamson, R.C.Garner, G.Lappin, S.Langouet, Y.Bao. Sulforaphane and quercetin modulate PhIP-DNA adduct formation in human HepG2 cells and hepatocytes, Carcinogenesis 24 (2003) 1903-1911.

[4] A.S.Dias, M.Porawski, M.Alonso, N.Marroni, P.S.Collado, J.Gonzalez-Gallego. Quercetin decreases oxidative stress, NF-kappaB activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats, J. Nutr. 135 (2005) 2299-2304.

[5] C.S.Yang, J.M.Landau, M.T.Huang, H.L.Newmark. Inhibition of carcinogenesis by dietary polyphenolic compounds, Annu. Rev. Nutr. 21 (2001) 381-406.

[6] M.C.Canivenc-Lavier, M.F.Vernevaut, M.Totis, M.H.Siess, J.Magdalou, M.Suschetet. Comparative effects of flavonoids and model inducers on drug-metabolizing enzymes in rat liver, Toxicology 114 (1996) 19-27.

[7] U.Wenzel, A.Herzog, S.Kuntz, H.Daniel. Protein expression profiling identifies molecular targets of quercetin as a major dietary flavonoid in human colon cancer cells, Proteomics. 4 (2004) 2160-2174.

[8] F.Gosse, S.Guyot, S.Roussi, A.Lobstein, B.Fischer, N.Seiler, F.Raul. Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis, Carcinogenesis 26 (2005) 1291-1295.

[9] F.Gosse, S.Guyot, S.Roussi, A.Lobstein, B.Fischer, N.Seiler, F.Raul. Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis, Carcinogenesis 26 (2005) 1291-1295.

[10] M.Kern, Z.Tjaden, Y.Ngiewih, N.Puppel, F.Will, H.Dietrich, G.Pahlke, D.Marko. Inhibitors of the epidermal growth factor receptor in apple juice extract, Mol. Nutr. Food Res. 49 (2005) 317-328.

[11] S.W.Barth, C.Fahndrich, A.Bub, H.Dietrich, B.Watzl, F.Will, K.Briviba, G.Rechkemmer. Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats, Carcinogenesis 26 (2005) 1414-1421.

[12] S.Veeriah, T.Kautenburger, J.Saauer, H.Dietrich, F.Will, B.L.Pool-Zobel. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics., Molecular Carcinogenesis 45 (2006) 164-174.

[13] M.Coggan, L.Whitbread, A.Whittington, P.Board. Structure and organization of the human theta-class glutathione S-transferase and D-dopachrome tautomerase gene complex, Biochem. J. 334 (Pt 3) (1998) 617-623.

[14] J.D.Hayes, R.C.Strange. Glutathione S-transferase polymorphisms and their biological consequences, Pharmacology 61 (2000) 154-166.

[15] P.J.Sherratt, D.J.Pulford, D.J.Harrison, T.Green, J.D.Hayes. Evidence that human class Theta glutathione S-transferase T1-1 can catalyse the activation of dichloromethane, a liver and lung carcinogen in the mouse. Comparison of the tissue distribution of GST T1-1 with that of classes Alpha, Mu and Pi GST in human, Biochem. J. 326 (Pt 3) (1997) 837-846.

[16] J.D.Hayes, J.U.Flanagan, I.R.Jowsey. Glutathione transferases, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 51-88.

[17] K.L.Tan, P.G.Board. Purification and characterization of a recombinant human Theta-class glutathione transferase (GSTT2-2), Biochem J 315 (1996) 727-732.

[18] A.J.Hussey, L.A.Kerr, A.D.Cronshaw, D.J.Harrison, J.D.Hayes. Variation in the expression of Mu-class glutathione S-transferase isoenzymes from human skeletal muscle. Evidence for the existence of heterodimers, Biochem. J. 273(Pt 2) (1991) 323-332.

[19] K.L.Tan, P.G.Board. Purification and characterization of a recombinant human Theta-class glutathione transferase (GSTT2-2), Biochem. J. 315 (Pt 3) (1996) 727-732.

[20] A.J.Hussey, J.D.Hayes. Characterization of a human class-Theta glutathione S-transferase with activity towards 1-menaphthyl sulphate, Biochem. J. 286 (Pt 3) (1992) 929-935.

[21] F.F.Chu, J.H.Doroshow, R.S.Esworthy. Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI, J. Biol. Chem. 268 (1993) 2571-2576.

[22] L.Kussmaul, B.Hamprecht, R.Dringen. The detoxification of cumene hydroperoxide by the glutathione system of cultured astroglial cells hinges on hexose availability for the regeneration of NADPH, J. Neurochem. 73 (1999) 1246-1253.

[23] J.Chaudiere, E.C.Wilhelmsen, A.L.Tappel. Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans, J. Biol. Chem. 259 (1984) 1043-1050.

[24] J.Imlay, S.M.Chin, S.Linn. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro, Science (1988) 640-642.

[25] K.Sundberg, A.Seidel, B.Mannervik, B.Jernstrom. Detoxication of carcinogenic fjord-region diol epoxides of polycyclic aromatic hydrocarbons by glutathione transferase P1-1 variants and glutathione, FEBS Lett. 438 (1998) 206-210.

[26] M.Lodovici, C.Luceri, F.Guglielmi, C.Bacci, V.Akpan, M.L.Fonnesu, V.Boddi, P.Dolara. Benzo(a)pyrene diolepoxide (BPDE)-DNA adduct levels in leukocytes of smokers in relation to polymorphism of CYP1A1, GSTM1, GSTP1, GSTT1, and mEH, Cancer Epidemiol. Biomarkers Prev. 13 (2004) 1342-1348.

[27] W.Lijinsky, P.Shubik. Benzo(a)pyrene and other polynuclear hydrocarbons in charcoal-broiled meat, Science 145 (1964) 53-55.

[28] P.Zimniak, B.Nanduri, S.Pikula, J.Bandorowicz-Pikula, S.S.Singhal, S.K.Strivastava, S.Awasthi, Y.C.Awasthi. Naturally occurring human glutathione S-transferase GSTP1-1 isoforms with isoleucine and valine in position 104 differs in enzymic properties, European Journal of Biochemistry 224 (1994) 27566-27573.

[29] S.Veeriah, T.Kautenburger, N.Habermann, J.Sauer, H.Dietrich, F.Will, B.L.Pool-Zobel. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics, Mol. Carcinog. 45 (2006) 164-174.

[30] B.K.D.H.Will F. Apple Pomace Liquefaction with Pectinases and Cellulases - Analytical Data of the corresponding Juices., European Food Research and Technology 211/4:291-297 (2004).

[31] B.K.D.H.Will F. Apple Pomace Liquefaction with Pectinases and Cellulases - Analytical Data of the corresponding Juices., European Food Research and Technology 211/4:291-297 (2004).

[32] M.Richter, D.Jurek, F.Wrba, K.Kaserer, G.Wurzer, J.Karner-Hanusch, B.Marian. Cells obtained from colorectal microadenamas mirror early premalignant growth patterns *in vitro*, European Journal of Cancer 38 (2002) 1937-1945.

[33] S.Veeriah, T.Kautenburger, N.Habermann, J.Sauer, H.Dietrich, F.Will, B.L.Pool-Zobel. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics, Mol. Carcinog. 45 (2006) 164-174.

[34] R.R.Tice, E.Agurell, D.Anderson, B.Burlinson, A.Hartmann, H.Kobayashi, Y.Miyamae, E.Rojas, J.C.Ryu, Y.F.Sasaki. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing., Environ Mol Mutagen. 35 (2000) 206-221.

[35] J.Jaeger, K.Sorensen, S.P.Wolff. Peroxide accumulation in detergents., J Biochem Biophys Metho 29 (1994) 77-81.

[36] R.Roig, E.Cascon, L.Arola, C.Blade, M.J.Salvado. Procyanidins porotect Fao cells against hydrogen peroxide-induced oxidative stress., Biochim. Biophys. Acta 1572 (2002) 25-30.

[37] M.Alia, R.Mateos, S.Ramos, E.Lecumberri, L.Bravo, L.Goya. Influence of quercetin and rutin on growth and antioxidative defense system of a human hepatoma cell line (HepG2)., Eur. J. Nutr. 45 (2006) 19-28.

[38] G.Graziani, G.D´ Argenio, C.Tuccillo, C.Loguercio, A.Ritieni, F.Morisco, C.Del Vecchio Blanco, V.Fogliano, M.Romano. Apple polyphenol extracts prevent damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo.*, Gut 54 (2005) 193-200.

[39] M.Fenech, C.Stockley, C.Aitken. Moderate wine consumption protects against hydrogen peroxide-induced DNA damage, Mutagenesis 12 (1997) 289-296.

[40] S.J.Duthie, A.R.Collins, G.G.Duthie, V.L.Dobson. Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes, Mutat. Res. 393 (1997) 223-231.

[41] S.J.Duthie, V.L.Dobson. Dietary flavonoids protect human colonocyte DNA from oxidative attack *in vitro*., Eur. J. Nutr. 38 (1999) 28-34.

[42] K.Horvathova, L.Novotny, A.Vachalkova. The free radical scavenging activity of four flavonoids determined by the comet assay., Neoplasma 50 (2003) 291-295.

[43] M.T.Hess, D.Gunz, N.Luneva, N.E.Geacintov, H.Naegeli. Base pair conformation-dependent excision of benzo[a]pyrene diol epoxide-guanine adducts by human nucleotide excision repair enzymes., Mol. Cell Biol. 17 (1997) 7069-7076.

[44] A.Luch. Nature and nurture - lessons from chemical carcinogenesis., Nat. Rev. Cancer 5 (2005) 113-125.

[45] W.K.Kaufmann, T.P.Heffernan, L.M.Beaulieu, S.Doherty, A.R.Frank, Y.Zhou, M.F.Bryant, T.Zhou, D.D.Luche, N.Nikolaishvili-Feinberg. Caffeine and human DNA metabolism: the magic and the mystery, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 532 (2003) 85-102.

[46] S.Veeriah, T.Kautenburger, N.Habermann, J.Sauer, H.Dietrich, F.Will, B.L.Pool-Zobel. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics, Mol. Carcinog. 45 (2006) 164-174.

[47] A.J.Townsend, W.R.Fields, R.L.Haynes, A.J.Doss, J.Doehmer, C.S.Morrow. Chemoprotective functions of glutathione S-transferases in cell lines induced to express specific isozymes by stable transfection, Chem -Biol Interac 111-112 (1998) 389-407.

[48] L.C.Wilms, P.C.H.Hollmann, A.W.Boots, J.C.S.Kleinjans. Protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage and formation of BPDE-DNA adducts in human lymphocytes., Mutat. Res. 582 (2005) 155-162.

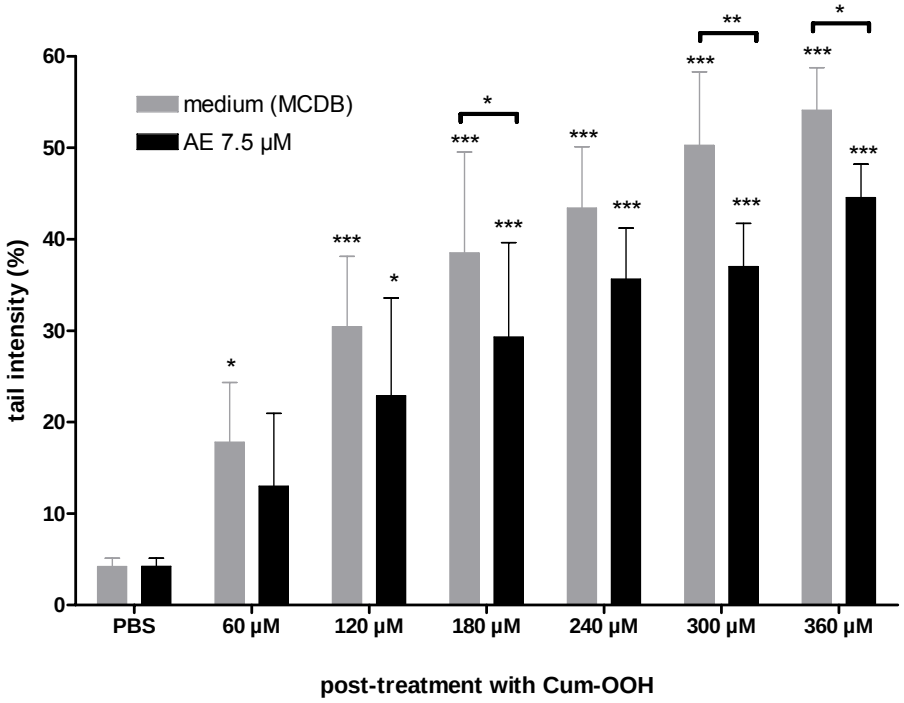
[49] S.C.Roques, N.Landrault, P.-L.Teissédre, C.Laurent, P.Besancon, J.-M.Rouanet, B.Caporiccio. Hydrogen peroxide generation in Caco-2 cell culture medium by addition of phenolic compounds: effect of ascorbic acid., Free Radic. Res. 36 (2002) 593-599.

[50] L.H.Long, M.V.Clement, B.Halliwell. Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (-) epigallocatechin, (-)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media, Biochem Biophys Res Commun 273 (2000) 50-53.

[51] M.Akagawa, T.Shigemitsu, K.Suyama. Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions., Biosci. Biotechnol. Biochem. 67 (2003) 2632-2640.

Figure 1

A


medium (MCDB)
AE 7.5 µM
tail intensity (%)
0
10
20
30
40
50
60
PBS
60 µM
120 µM
180 µM
240 µM
300 µM
360 µM
post-treatment with Cum-OOH

B

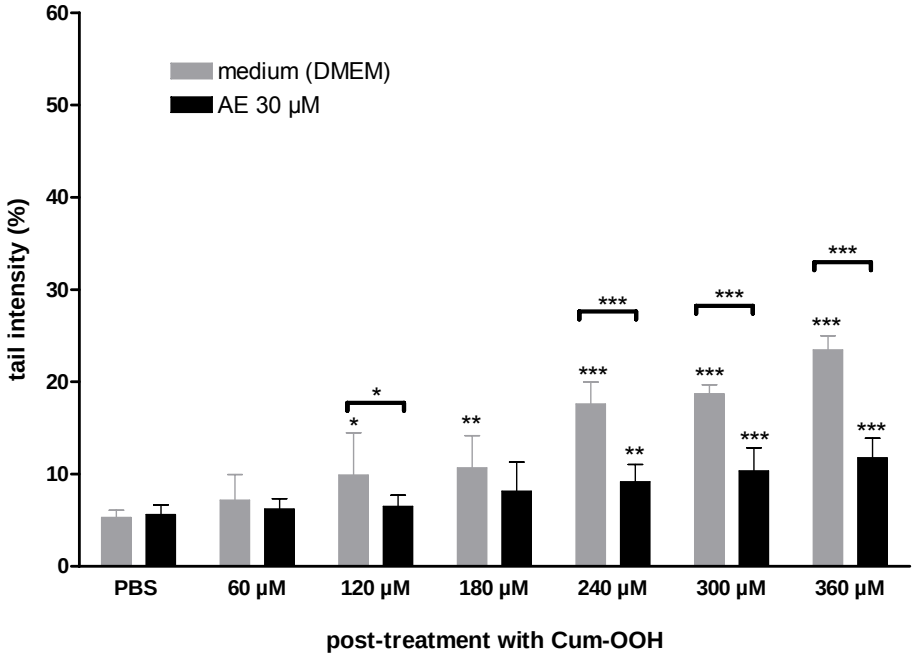
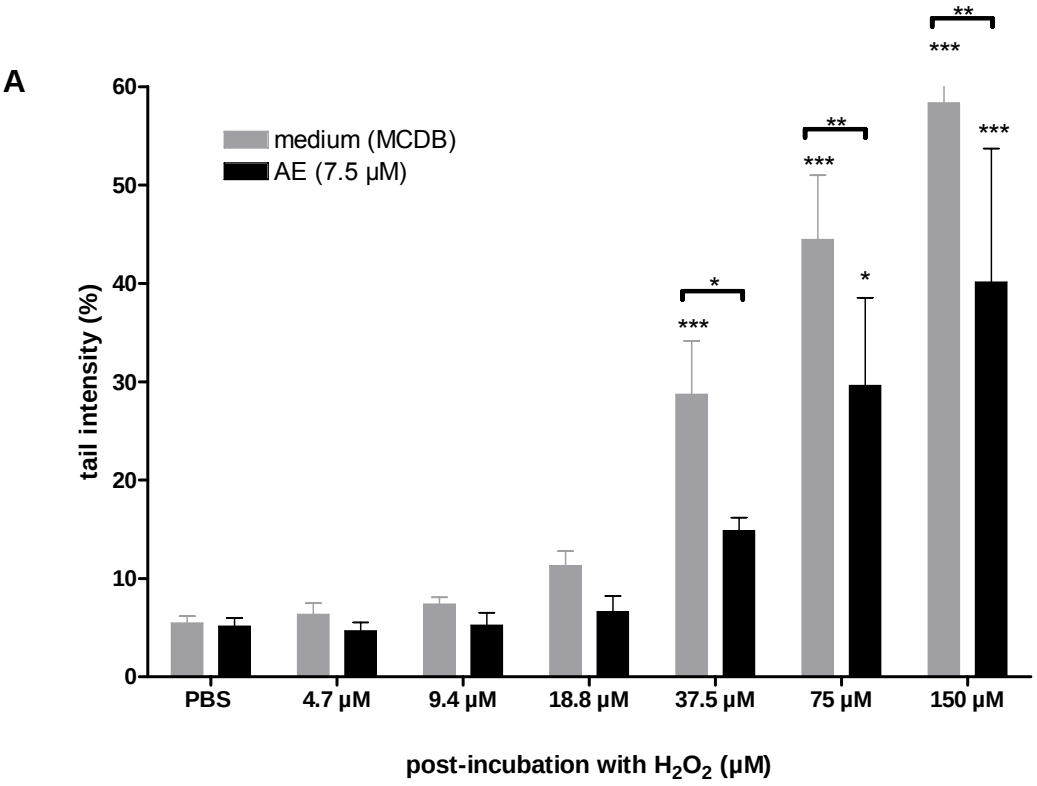

medium (DMEM)
AE 30 µM
tail intensity (%)
0
10
20
30
40
50
60
PBS
60 µM
120 µM
180 µM
240 µM
300 µM
360 µM
post-treatment with Cum-OOH

Figure 2


A
medium (MCDB)
AE (7.5 µM)
tail intensity (%)
0
10
20
30
40
50
60
PBS
4.7 µM
9.4 µM
18.8 µM
37.5 µM
75 µM
150 µM
post-incubation with H2O2 (µM)
*
**
***

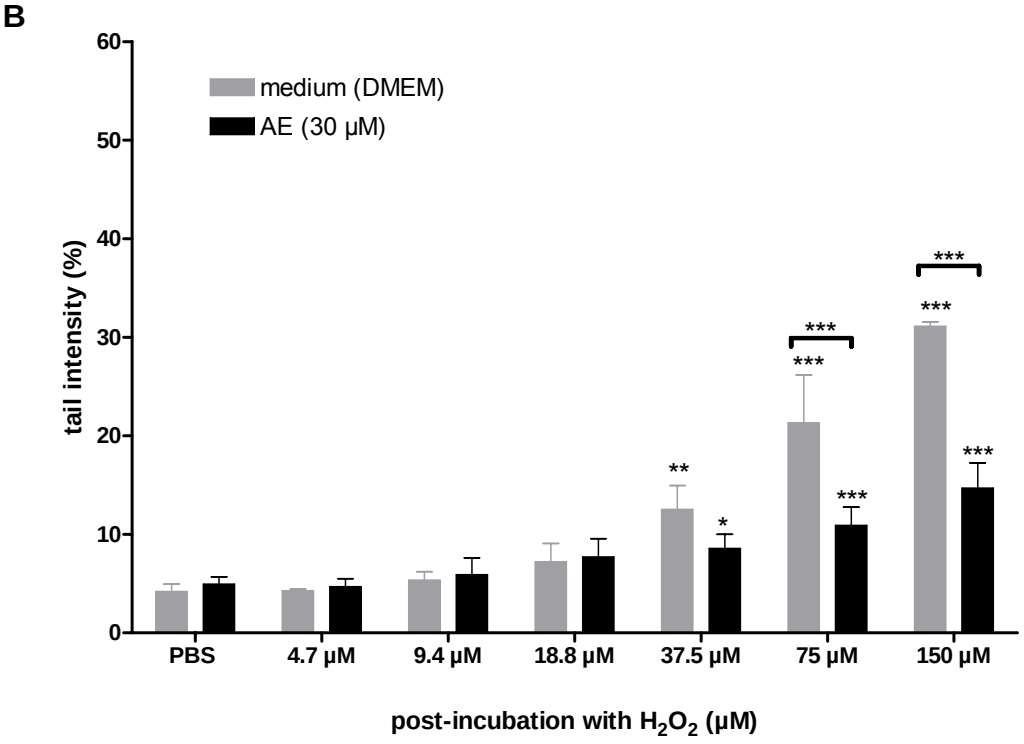
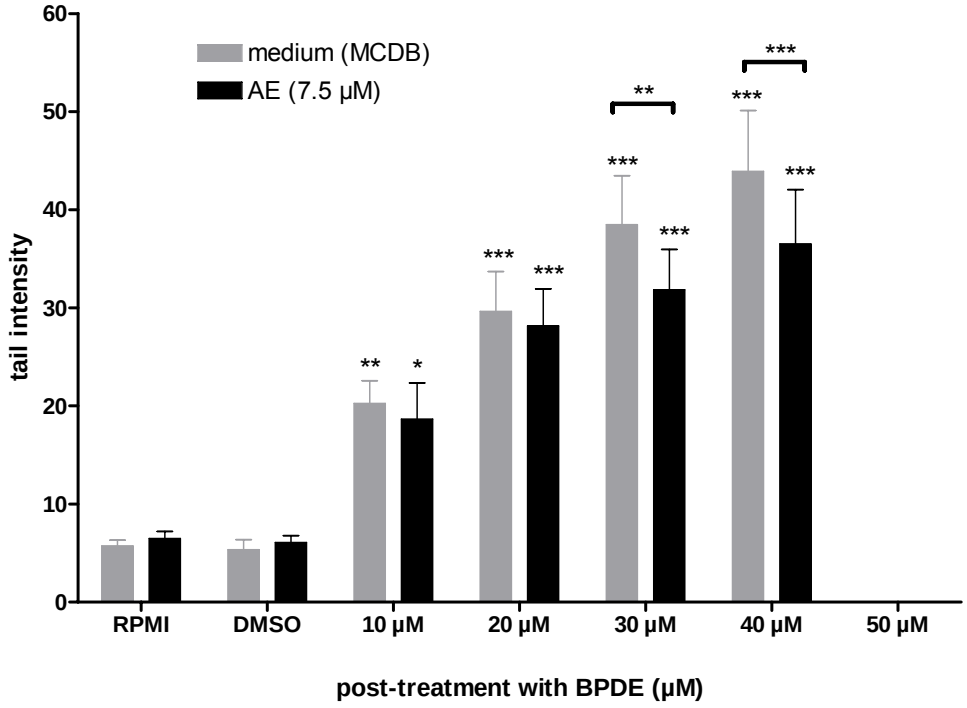

B
medium (DMEM)
AE (30 µM)
tail intensity (%)
0
10
20
30
40
50
60
PBS
4.7 µM
9.4 µM
18.8 µM
37.5 µM
75 µM
150 µM
post-incubation with H2O2 (µM)
*
**
***

Figure 3

A


medium (MCDB)
AE (7.5 µM)
tail intensity
RPMI
DMSO
10 µM
20 µM
30 µM
40 µM
50 µM
post-treatment with BPDE (µM)

B

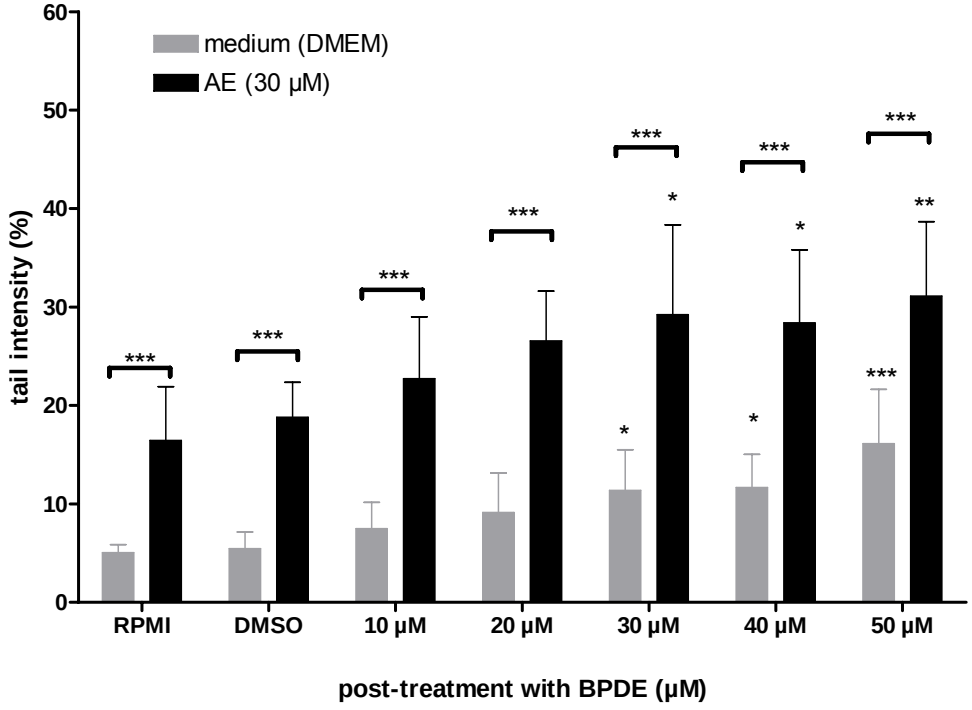
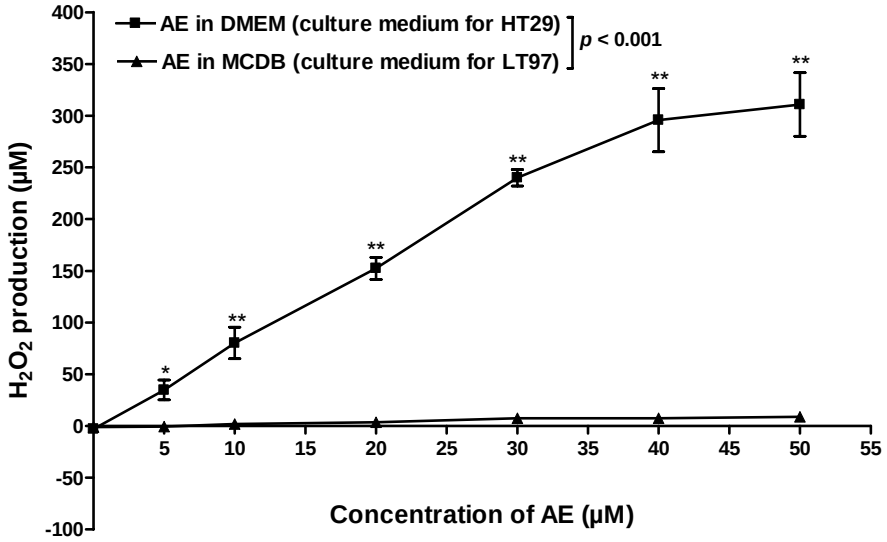

medium (DMEM)
AE (30 µM)
tail intensity (%)
RPMI
DMSO
10 µM
20 µM
30 µM
40 µM
50 µM
post-treatment with BPDE (µM)

Figure 4

A


AE in DMEM (culture medium for HT29)
AE in MCDB (culture medium for LT97)
p < 0.001
H2O2 production (µM)
Concentration of AE (µM)

B

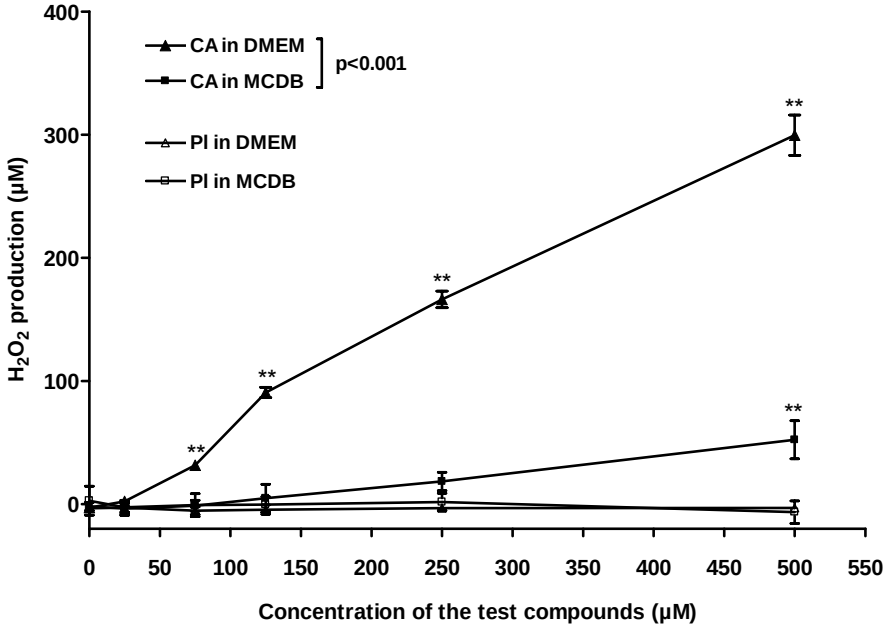

CA in DMEM
CA in MCDB
p<0.001
PI in DMEM
PI in MCDB
H2O2 production (µM)
Concentration of the test compounds (µM)

Figure legends

Figure 1: Levels of DNA damage induced by Cum-OOH (60 - 360 µM, 5 minutes, 4 °C) after preincubation of **(A)** LT97 cells or **(B)** HT29 cells with AE (LT97: 7.5 µM, HT29: 30 µM, black bars) or DMEM (grey bars) measured with the standard version of the alkaline Comet Assay (mean ± SD, n=3). The significant differences to the untreated control (RPMI) were calculated by one-way ANOVA, including Bonferronis Multiple Comparison Test (* $p \leq 0.05$, ** $p \leq 0.01$). The effect of the apple extract preincubation was calculated, using Two-way ANOVA, including Bonferroni´s Multiple Comparison Test (*** $p \leq 0.001$).

Figure 2: Levels of DNA damage induced by H_2O_2 (4.7 - 150 µM, 5 minutes, 4 °C) after preincubation of **(A)** LT97 cells or **(B)** HT29 cells with AE (LT97: 7.5 µM, HT29: 30 µM, black bars) or DMEM (grey bars) measured with the standard version of the alkaline Comet Assay (mean ± SD, n=3). The significant differences to the un-treated control (RPMI) were calculated by One-way ANOVA, including Dunnett´s Multiple Comparison Test (** $p \leq 0.01$). The effect of the apple extract preincubation was calculated, using Two-way ANOVA, including Bonferroni´s Multiple Comparison Test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Figure 3: Levels of DNA damage induced by BPDE (10 - 50 µM, 30 minutes, 37 °C) after preincubation of **(A)** LT97 cells or **(B)** HT29 cells with AE (LT97: 7.5 µM, HT29: 30 µM, black bars) or DMEM (grey bars) measured with the standard version of the alkaline Comet Assay (n=3). The significant differences to the untreated control (RPMI) were calculated by One-way ANOVA, including Dunnett´s Multiple Comparison Test (** $p \leq 0.01$). The effect of the apple extract preincubation was calculated,

using Two-way ANOVA, including Bonferroni´s Multiple Comparison Test (** $p \leq 0.01$).

Figure 4: Hydrogen peroxide formation in the cell free culture media DMEM and MCDB (**A**) in the presence of the apple extract AE (5 - 50 µM, 24 h at 37 °C, n=3). The significant differences to the un-treated medium control were calculated by One-way ANOVA, including Dunnett´s Multiple Comparison Test (* $p \leq 0.05$, ** $p \leq 0.01$). The significant differences between the two media were calculated using Two-way ANOVA, including Bonferroni´s Multiple Comparison Test (*** $p \leq 0.001$). Hydrogen peroxide formation in the cell free culture media DMEM or MCDB (**B**) in the presence of chlorogenic acid (CA) and phloridzin (PI) (25 - 500 µM, 24 h at 37 °C, n=3). The significant differences to the untreated medium control were calculated by One-way ANOVA, including Dunnett´s Multiple Comparison Test (** $p \leq 0.01$). The significant differences between the two media were calculated using Two-way ANOVA, including Bonferroni´s Multiple Comparison Test (*** $p \leq 0.001$).

4 DISKUSSION

4.1 Unter welchen Bedingungen (Zeit-, Konzentrations-, Effektbeziehungen) beeinflussen Eisen, Uran und die komplexen Umweltproben das Wachstum von Kolonkarzinomzellen?

Zunächst sollten anhand des Einflusses der Testsubstanzen und komplexen Proben auf das Zellwachstum nicht-toxische Konzentrationen ermittelt werden. Aufgrund des schnellen Wachstums und der hohen Sensitivität von HT29 clone 19A-Zellen wurden diese Voruntersuchungen nur mit dieser Karzinomzelllinie vorgenommen.
Die beiden Einzelnoxen Fe-NTA und U-NTA wurden hinsichtlich der Modifikation des Zellwachstums in einem relativ breiten Konzentrationsbereich (0-2000 µM) und für drei Inkubationszeiten (24-72 h) getestet. Fe-NTA bewirkte nach 72 h Inkubation in geringen Konzentrationen (25 µM) einen signifikanten Anstieg im Zellwachstum, sehr hohe Konzentrationen (2000 µM) verminderten hingegen das Zellwachstum signifikant **(Publikation I)**. Die Einzelnoxe U-NTA zeigte nach 24 und 48 h bei der höchsten Konzentration einen wachstumshemmenden Effekt **(Publikation III)**. Bei dieser Konzentration war das Wachstum im Vergleich zur unbehandelten Kontrolle um 40-50 % vermindert, was auf zytotoxische Effekte hindeutet. Nach 72 h war diese zytotoxische Wirkung schon ab 75 µM nachweisbar. Eine EC_{50}-Dosis konnte nur nach 72 Stunden ermittelt werden. 215 µM U-NTA verminderten die Zellzahl um die Hälfte. Um die Wirkungen beider Einzelnoxen in den folgenden Untersuchungen vergleichen zu können, wurde deshalb für beide Substanzen der gleiche Konzentrationsbereich gewählt. Die Inkubationszeiten der weiterführenden Experimente reichten von 30 min bis maximal 24 h. Es wurde deshalb ein Konzentrationsbereich von 10-1000 µM festgelegt. In diesem Bereich waren bei den vorgesehenen Inkubationszeiten keine zytotoxischen Wirkungen beobachtet worden.
Um die komplexen Umweltproben mit unterschiedlichen Gehalten an Schwermetallen und Radionukliden (A - wenig, B - mittel und C - stark kontaminiert) unter konstanten Bedingen testen zu können, wurden für alle 3 Proben die gleichen prozentualen Konzentrationen eingesetzt (0-20 % im Medium). Das Wachstum von HT29 clone 19A-Zellen konnte durch eine 72-stündige Inkubation nur durch die stark belastete Probe (C) moduliert werden. Schon ab 10 % wirkte diese Wasserprobe zytotoxisch. Zur Beantwortung weiterführender Fragestellungen wurden deshalb

Konzentrationen von bis zu 10 % gewählt. Die wesentlichen Schwermetalle in den Wismutwässern und deren Gehalte in den Proben sind tabellarisch im **Publikationsmanuskript IV** zusammengefasst.

Die Untersuchungen zeigten, dass auch geringe, durchaus physiologische Eisenkonzentrationen (25 µM) über eine eisenvermittelte Wachstumssteigerung ein karzinogenes Potential bergen **(Publikation I)**. In der Literatur findet man gegensätzliche Aussagen zum Einfluß von Eisen auf die Proliferation gesunder und neoplastischer Zellen. In einer Metaanalyse zeigten nur drei Viertel der Studien eine positive Korrelation zwischen Eisen und einem erhöhten Risiko für kolorektale Neoplasien [Nelson, 2001].

4.2 Welchen Einfluss haben Eisen, Uran und die komplexen Umweltproben auf die Bildung reaktiver Sauerstoffspezies (ROS) und den Glutathion-Status?

Über die Fenton-Reaktion und Haber-Weiss-Reaktion sind Eisen und andere Schwermetalle an der Bildung von Radikalen beteiligt. Auch für Uranverbindungen wurden solche Mechanismen diskutiert [Taqui Khan & Martell, 1969]. Die so entstandenen radikalischen Verbindungen führen zu toxischen Reaktionen in den Zellen, z. B. zu oxidativen Basenveränderungen in der DNA und können somit das Risiko für Kolorektalkrebs erhöhen [Abalea *et al.*, 1998; Hartwig *et al.*, 1993; Hartwig & Schlepegrell, 1995; Imlay *et al.*, 1988]. In eigenen Untersuchungen sollte die mögliche Beteiligung von Eisen und Uran an der Generation reaktiver Verbindungen untersucht werden.

Die Modellsubstanz Fe-NTA war in der Lage ROS zu generieren **(Publikation I)**. Mit dem ROS-Assay, der auf der durch ROS induzierten Oxidation des Fluoreszenzfarbstoffes Dichlorodihydrofluoreszeindiazetat basiert, konnte eine konzentrations- und zeitabhängige Erhöhung der Fluoreszenz und somit die Bildung reaktiver Spezies nachgewiesen werden. Diese Ergebnisse stützen den Mechanismus der eisenvermittelten Fenton-Reaktion. Die gebildeten reaktiven Spezies können oxidative Schäden in der DNA verursachen. Von Glei et al. konnten für Fe-NTA mittels Comet Assay oxidative DNA-Schäden nachgewiesen werden [Glei *et al.*, 2002].

Mit der gleichen Methode (ROS Assay) konnte für die Einzelnoxe U-NTA kein signifikanter Effekt gegenüber der unbehandelten Kontrolle bzw. NTA (eigene unveröffentlichte Daten) nachgewiesen werden.

Die Wismutproben enthalten unterschiedliche Gehalte an Schwermetallen. Eisen ist eine der Hauptkontaminanten in den Wasserproben. Deshalb lag die Vermutung nahe, dass die Wasserproben ebenfalls zur ROS-Bildung befähigt sind. Probe A mit den geringsten Eisen- und Urangehalten zeigte erstaunlicherweise die deutlichste ROS-Bildung im Vergleich zur entsprechenden NTA-Kontrolle. In den Versuchen fiel auf, dass die Ergebnisse großen Schwankungen unterliegen. Die NTA-Kontrolle zeigte eine große Schwankungsbreite, außerdem korrelierte die ROS-Bildung nicht mit der Kontaminationshöhe der unterschiedlichen Proben. Diese widersprüchlichen Ergebnisse legen den Verdacht nahe, dass der von uns verwendete ROS Assay **(Publikation I)** nicht für alle unsere Fragestellungen bzw. Modelle optimal geeignet ist. Um die Ergebnisse zu validieren, wurde zusätzlich der FOX Assay durchgeführt. Dieser basiert auf der durch Peroxide verursachten Oxidation von Fe^{2+} zu Fe^{3+}. Dieses reagiert mit Xylenolorange zu einem Farbkomplex, welcher bei 550 nm gemessen werden kann [Jaeger *et al.*, 1994].

Für Fe-NTA konnte konzentrationsabhängig ein Signal nachgewiesen werden (eigene unveröffentlichte Ergebnisse). Da der Assay aber auf einer Farbreaktion von Fe^{3+} mit Xylenolorange basiert und über Fe-NTA ein zusätzlicher Eintrag von Fe^{3+} in das System erfolgt, ist nicht klar, ob hierfür die Bildung von Peroxiden (H_2O_2) und die damit verbundene Farbreaktion oder zusätzlich eingetragenes Fe^{3+} verantwortlich ist. Zusammenfassend konnte für Fe-NTA im ROS Assay und unter Vorbehalt im FOX Assay die Bildung von ROS nachgewiesen werden.

Auch mit dem FOX Assay konnten keine Hinweise darauf gefunden werden, dass U-NTA ebenfalls über diesen Mechanismus seine Wirkung entfaltet. U-NTA löste keine Reaktion im FOX Assay aus (eigene unveröffentlichte Daten). Aus den Untersuchungen im ROS-Assay und FOX Assay kann zusammenfassend geschlussfolgert werden, dass U-NTA nicht über den Mechanismus der ROS-Bildung seine genotoxische Wirkung entfaltet. Yazzie und Kollegen schlossen ebenfalls aus ihren Untersuchungen, dass bei der Genotoxizität von Uranylazetat die Fenton-vermittelte Bildung reaktiver Sauerstoffspezies vermutlich keine Rolle spielt [Yazzie *et al.*, 2003].

Die beiden mit dem FOX Assay getesteten Wismutwässer (B und C) reagierten sehr unterschiedlich. So war für die stark kontaminierte Wasserprobe eine signifikante,

konzentrationsabhängige Reaktion nachweisbar, für die moderat belastete Probe jedoch nicht. Da auch in den Wismutproben hohe Mengen Eisen enthalten sind, kann nicht ausgeschlossen werden, dass die Reaktion auf in das System eingetragenes Fe^{3+} zurückzuführen ist. Zusammenfassend kann geschlussfolgert werden, dass Fe-NTA und vermutlich auch die stark mit Schwermetallen kontaminierte Wasserprobe reaktive Sauerstoffspezies generieren, was genotoxische Effekte hervorrufen kann. Eine abschließende Beurteilung darüber, welcher der beiden Assays (ROS Assay oder FOX Assay) für unsere Modelle der bessere ist kann anhand der vorliegenden Ergebnisse nicht getroffen werden.

Während für Eisen die ROS-Bildung und die damit verbundene oxidative DNA-Schädigung unbestritten ist, herrscht jedoch keine einheitliche Meinung über die Mechanismen, die hinter der chemischen Toxizität von Uranverbindungen stehen. Das oxidative Potential von Uranylionen konnte bereits 1969 nachgewiesen werden [Taqui Khan & Martell, 1969]. Laut Hamilton und Kollegen ist Uranylnitrat in Anwesenheit von H_2O_2 und Ascorbat in der Lage Hydroxlradikale zu generieren [Hamilton *et al.*, 1997]. Miller et al. wiesen nach, dass abgereichertes Uran oxidative DNA-Schäden verursachen kann und die Bildung von Hydroxylradikalen katalysiert [Miller *et al.*, 2002a]. Die Bildung von ROS konnte mit der Modellsubstanz U-NTA nicht nachgewiesen werden. Die ROS-Bildung bzw. die Reaktion der Wismut-Probe C im FOX Assay ist wahrscheinlich auf den hohen Anteil (8100 µM) an Eisen zurückzuführen. Es ist aber auch nicht auszuschließen, dass andere Schwermetalle wie Nickel und Cadmium, die ebenfalls nennenswert enthalten sind, eine Rolle spielen. Demgegenüber scheint Uran hierbei keine bzw. nur eine untergeordnete Bedeutung zuzukommen.

Reaktiven Sauerstoffspezies werden in der Promotion der Karzinogenese eine bedeutende Rolle zugesprochen. So sind sie an der Regulation der Genexpression wichtiger Zellwachstums- und Differenzierungsgene beteiligt [Crawford *et al.*, 1988]. Lund und Kollegen konnten zeigen, dass ROS mit Veränderungen der Proliferation von Kryptzellen im Dickdarm von Ratten assoziiert sind [Lund *et al.*, 1998]. Das Eisen auch *in vitro* von den Kolonzellen aufgenommen wird, zeigten bereits Glei et al. [Glei *et al.*, 2002]. Erhöhte intrazelluläre Konzentrationen an Eisen bzw. anderen katalytisch wirkenden Verbindungen können dort die Bildung von ROS ermöglichen. Eisenüberschuss im Darm und somit verstärkter oxidativer Stress kann durch fehlerhafte Eisensupplementierung (Überdosierung) entstehen. Dies birgt ein erhöhtes

Kolonkrebsrisiko [Glei *et al.*, 2002; Lund *et al.*, 1998; Lund *et al.*, 1999; Richardson & Ponka, 1997]. Lund et al. berichteten, dass durch Supplementierung mit Eisensulfat im intraluminalen Pool Eisenkonzentrationen (wasserlösliches Eisen) von mehr als 100 µM erreicht werden (Maximalwerte von bis zu 350 µM). Im Vergleich dazu liegt die mittlere Konzentration im Darmlumen bei rund 25 µM [Lund *et al.*, 1998; Lund *et al.*, 1999]. Außerdem wurde eine vermehrte Produktion von freien Radikalen in den Fäzes der Versuchspersonen mit Eisensupplementierung beobachtet, was auf die erhöhte Eisenkonzentration im Darm zurückgeführt werden könnte. Wir konnten für Fe-NTA mittels ROS Assay eine zeit- und dosishängige Bildung von ROS nachweisen, wobei die höchste Dosis (1000 µM) nur etwa eine Zehnerpotenz über den von Lund et al. ermittelten Konzentrationen lag. Auch andere Studien assoziierten erhöhte Eisenkonzentrationen im Kolongewebe oder in den Fäzes mit einem erhöhten Risiko für Kolonkrebs [Babbs, 1992; Toyokuni *et al.*, 1995].

Das Tripeptid Glutathion (GSH) ist an der Entgiftung von ROS und freien Radikalen beteiligt. Als Kosubstrat der Glutathion-*S*-Transferasen schützt es die Zelle nicht nur gegenüber reaktiven Elektrophilen, sondern auch gegen oxidativen und nitrosativen Stress [Bray & Taylor, 1995; Griffith, 1999]. Oxidativer Stress wird unter anderem über die durch Schwermetalle (wie z. B. Eisen) vermittelte Fenton Reaktion und damit verbundnen Bildung von ROS erzeugt [Imlay *et al.*, 1988]. Schwermetalle haben gegenüber SH-Gruppen von schwefelhaltigen Proteinen (z. B. GSH) eine hohe Affinität. Ihre schützenden Wirkungen entfalten diese schwefelhaltigen Verbindungen zum einem direkt als Chelatoren und zum anderen indirekt als Antioxidantien [Hirayama & Yasutake, 1998]. Metalle können antioxidative Enzymsysteme inhibieren und die intrazelluläre GSH-Konzentration senken [Quig, 1998]. Quecksilber und Cadmium haben eine besonders hohe GSH-Affinität. Hirayama und Yasutake beobachteten bei Mäusen 24 Stunden nach Gabe einer sublethalen Dosis (MeHg) erhöhte GSH-Konzentrationen im Nierengewebe und im Blut, in der Leber jedoch sinkende GSH-Gehalte [Yasutake & Hirayama, 1994]. Als Ursache für die gesunkenen GSH-Mengen in der Leber vermuteten sie einen starken Umsatz von GSH durch die Eliminierung von MeHg. Die erhöhten GSH-Level in der Niere, dem entscheidenden Ausscheidungsorgan für MeHg, waren auf eine Stimulierung der γ-Glutamylcystein-Synthetase (γ-GCS) zurückzuführen. Seymen und Kollegen konnten nachweisen, dass eine Supplementierung mit Eisen die GSH-, Glutathionperoxidase-

und Superoxiddismutase-Konzentrationen in Erythrozyten erhöht [Seymen *et al.*, 1997].

Der Nachweis der ROS-Bildung durch unsere Testsubstanzen lies die Frage zu, ob diese ebenfalls in der Lage sind, die GSH-Homeostase zu modulieren und somit auf die Entgiftung von ROS Einfluss zu nehmen. Daher wurde der intrazelluläre GSH-Gehalt von HT29 clone 19A-Zellen nach 24-stündiger Inkubation mit allen Testsubstanzen ermittelt. In Analogie zu Seymen et al. (1997) erhöhte Eisen die intrazelluläre GSH-Konzentration, signifikant jedoch erst bei hohen Dosen (500 und 1000 µM) **(Publikation I)**. U-NTA hingegen bewirkte diesen Effekt schon bei geringeren Konzentrationen (im Vergleich zur unbehandelten Medium-Kontrolle schon ab 25 µM) **(Publikation III)**. Dies stützt die Aussage, dass Substanzen, die oxidativen Stress induzieren, die Transkription von γ-GCS-Genen erhöhen können und die Synthese von GSH stimulieren [Dickinson & Forman, 2002]. Eine schwermetallinduzierte Veränderung der GSH-Homeostase wurde auch für andere Organe und Zellkulturen beschrieben. So führte die Exposition von Ratten gegenüber moderat toxischen Dosen hexavalenten Chroms zu steigenden GSH-Gehalten in der Leber [Standeven & Wetterhahn, 1991]. *In vitro*-Untersuchungen zeigten für Cadmium-behandelte Mesangialzellen und arsenbehandelte Swiss 3T3-Zellen signifikant induzierte intrazelluläre GSH-Gehalte [Chin & Templeton, 1993; Li & Chou, 1992]. Die in der Literatur beschriebene und in dieser Arbeit für Eisen und Uran bestätigte GSH-Erhöhung reflektiert möglicherweise einen Eigenschutzmechanismus der Zellen vor oxidativen Schäden. Obwohl in den Wismut-Proben eine Vielzahl von Schwermetallen enthalten sind, konnte für keine der komplexen Proben eine GSH-Reaktion nachgewiesen werden (eigene unveröffentlichte Daten). Möglicherweise sind die im Inkubationsansatz erreichten Dosen für eine deutliche Reaktion nicht ausreichend oder andere Kontaminanten in dem komplexen Gemisch wirken einer GSH-Erhöhung entgegen.

4.3 Wirken Eisen, Uran und die komplexen Umweltproben genotoxisch in Kolonzellen unterschiedlicher Transformationsgrade?

Ein wesentliches Ziel der vorliegenden Arbeit war es, die verschiedenen Zelltypen hinsichtlich ihrer Empfindlichkeit gegenüber den mit dem Uranbergbau verbundenen Kontaminanten und komplexen Umweltproben zu charakterisieren. Als wichtiger Parameter der Empfindlichkeit wurde die Genotoxizität der Testsubstanzen in den Zellen mittels Einzelzellmikrogelelektrophorese (Comet Assay) untersucht. Dabei sollte unter anderem geklärt werden, ob die ROS-Bildung zu funktionellen Konsequenzen innerhalb der Zellen im Sinne von DNA-Schäden führen. Die Ergebnisse zeigen die genotoxische Wirkung von Fe-NTA in HT29 clone 19A-Kolonkarzinomzellen (eigene unveröffentlichte Daten), was die Untersuchungen von Glei et al. bestätigt [Glei *et al.*, 2002]. Durch Konzentrationen von 500 und 1000 µM Fe-NTA wurde die DNA von HT29 clone 19A signifikant geschädigt. LT97-Adenomzellen reagierten tendenziell weniger sensitiv auf Fe-NTA. Hier zeigte sich nur für die höchste Konzentration (1000 µM) ein signifikanter Effekt **(Publikation II)**. Die Wirkung von Fe-NTA in Primärzellen war mit der in der Karzinomzelllinie vergleichbar. So schädigten 500 und 1000 µM Fe-NTA die DNA signifikant **(Publikation II)**. Die Unterschiede hinsichtlich der Sensitivität der Zellmodelle konnten statistisch gesichert werden.

Bisherige Untersuchungen zur genotoxischen Wirkung von Uranverbindungen beschränkten sich vor allem auf abgereichertes Uran. Nur wenige Studien befassten sich mit der Genotoxizität *in vitro.* So berichteten Yazzie et al. von genotoxischen Effekten von U(VI) in Plasmid-DNA nach Reduktion mit Ascorbat [Yazzie *et al.*, 2003]. In der vorliegenden Arbeit wurden ähnliche Untersuchungen erstmals an humanen Kolonzellen durchgeführt. Hierbei erwiesen sich die Kolonkarzinomzellen signifikant weniger sensitiv gegenüber U-NTA als LT97-Zellen bzw. Primärzellen. In allen 3 Zelltypen konnte eine Dosis-Wirkungsbeziehung nachgewiesen werden, wobei die höchste Konzentration (1000 µM) immer zu signifikanten DNA-Schäden führte **(Publikation III)**. In LT97-Zellen zeigte sich schon ab 500 µM eine signifikant genotoxische Wirkung. Aussagen zum Mechanismus, über den U-NTA seine genotoxische Wirkung entfaltet, sind anhand der Comet Assay-Ergebnisse allein nicht möglich. In den vorangegangenen Untersuchungen konnte für U-NTA über verschiedene Methoden jedoch kein ROS-bildendes Potential nachgewiesen werden. Des-

halb liegt die Vermutung nahe, dass U-NTA, wie von Franklin beschriebenen, direkt mit der DNA interagiert und diese hydrolysiert [Franklin, 2001].

Eine mögliche Ursache für die relativ hohe Sensitivität primärer Kolonepithelzellen ist die im Vergleich zu den beiden Kulturzelllinien geringere Expression antioxidativer Enzymsysteme. So ist z. B. die basale GST-Aktivität in Primärzellen nur halb so hoch wie in LT97-Adenomzellen und Glutathion-*S*-Transferase P1 (GSTP1) ist in LT97-Zellen etwa doppelt so stark exprimiert wie in Primärzellen (425 ng/10^6 Zellen versus 194 ng/10^6 Zellen) [Schäferhenrich *et al.*, 2003a]. Auch das relative Expressionsniveau der Katalase ist in Primärzellen geringer als in LT97-Zellen (unveröffentlichte Ergebnisse der Arbeitsgruppe Pool-Zobel). Die hohe Sensitivität der Primärzellen gegenüber den Noxen Fe-NTA und U-NTA spricht dafür, dass diese Substanzen über ihre Fähigkeit Strangbrüche in der DNA zu induzieren, eine Rolle in der Initiation und frühen Progression des Kolonkrebses spielen können.

Der Einsatz komplexer Umweltproben im Comet Assay war bisher am Lehrstuhl noch nicht etabliert. Da über das Wirkungsspektrum, die Wirkungshöhe und die effektiven Konzentrationen keine Informationen vorlagen, wurden in Voruntersuchungen zunächst alle 3 Wismutwässer in einem breiten Konzentrationsbereich in der Karzinomzelllinie HT29 clone 19A eingesetzt (0-20 %). Alle Proben wurden mengenmäßig in gleichen Konzentrationen getestet (% in Zellkulturmedium), um die Wirkung der unterschiedlichen Kontaminationsgrade zu erfassen. Diese Voruntersuchungen dienten der ersten genotoxischen Einstufung der Proben und der Definition geeigneter Konzentrationen. Nur die Proben B und C erwiesen sich als genotoxisch, weshalb nur diese in die weiterführenden Untersuchungen einbezogen wurden.

Die in den Voruntersuchungen als genotoxisch eingestuften Wismutproben B und C zeigten in allen 3 Zelllinien einen Dosis-Wirkungszusammenhang **(Publikationsmanuskript IV)**. In Primärzellen induzierte die Wismutprobe B in den Konzentrationen 7,5 und 10 % signifikante DNA-Schäden, die stark kontaminierte Probe C bereits ab 2,5 %. In der Adenomzelllinie wurden signifikante Werte nur in den höchsten Konzentrationen (7,5 und 10 % Wismutprobe C, 10 % Wismutprobe B) erreicht. In HT29 clone 19A-Zellen wurde eine vergleichbare Wirkhöhe nachgewiesen. Die Probe B war signifikant genotoxisch ab 5 % der Probe im Inkubationsmedium, die Probe C schon bei geringeren Konzentrationen (ab 2 %). Die in Primärzellen und LT97 mitgeführte Positivkontrolle (500 µM Fe-NTA) induzierte erwartungsgemäß signifikante Schäden. Hinsichtlich der Sensitivität der Zellen gegenüber der Wismutprobe C

konnten signifikanten Unterschiede für die Konzentration 7,5 % in Medium im Vergleich der Primärzellen mit den anderen beiden Zelltypen festgestellt werden.
Die effektiven Konzentrationen der Wismutproben, bezogen auf den Gehalt an Eisen und Uran (Hauptkontaminanten und Vergleichsubstanzen) sind um ein Vielfaches niedriger als die in den Versuchen mit den Einzelnoxen Fe-NTA und U-NTA ermittelten Konzentrationen. Die Wismutprobe B erreichte mit einer 23-fach geringeren Eisenkonzentration im Vergleich zu 500 µM der Einzelnoxe Fe-NTA und einer 25000-fach geringeren Urankonzentration im Vergleich zu 500 µM der Einzelnoxe U-NTA eine ähnlich hohe Schädigung (TI). Für die Wismutprobe C reichten eine 2,5-fach niedrigere Eisenkonzentration und 6500-fach niedrigere Urankonzentration für ein vergleichbares Schädigungsprofil aus. Die in den komplexen Proben enthaltenen anorganischen Kontaminanten verstärken bzw. addieren ihre Wirkungen gegenseitig, so dass für die vergleichsweise hohe Genotoxizität nicht nur Eisen und Uran verantwortlich sind.

4.4 Führen die Umweltproben und die Leitsubstanzen zu einer veränderten Sensitivität der Zellen gegenüber weiteren Noxen bzw. wird die Reparatur induzierter Schäden beeinflusst?

Eine Vielzahl von Untersuchungen berichten über den Einfluss von Schwermetallen auf DNA-Reparaturprozesse. Die Metalle Nickel, Cadmium, Cobalt oder Arsen haben zum Teil selbst nur ein geringes DNA-schädigendes Potential, werden jedoch wegen ihrer Fähigkeit in geringen, nicht-zytotoxischen Konzentrationen, die Reparatur entstandener Schäden zu hemmen, als karzinogen eingestuft [Hartwig A., 2002; Hartwig *et al.*, 1996; Hartwig, 1998; Hartwig & Beyersmann, 1989a; Hartwig & Schwerdtle, 2002]. So konnte für Nickel eine Hemmung der Nukleotid-Exzisions-Reparatur von UV-induzierten DNA-Schäden nachgewiesen werden [Hartwig & Beyersmann, 1989b]. Verschiedene Arbeitsgruppen beobachteten, dass Cadmium die Reparatur von UV-, Benzo[a]pyren- und Methylmethansulfonat-induzierten DNA-Schäden in humanen Blutzellen und humanen Kulturzellen (SV40-transformierte Fibroblasten, Zervixkarzinomzellen HeLa) hemmt [Dally & Hartwig, 1997; Hartmann & Speit, 1996; Nocentini, 1987]. Im Gegensatz dazu stellten Abalea et al. fest, dass Fe-NTA in geringen Konzentrationen (10 und 100 µM) die Reparatur in Leberzellkulturen nicht inhibiert, sondern aktiviert [Abalea *et al.*, 1998]. Die stimulierte Reparatur-

aktivität reichte jedoch nicht aus, um eine Anreicherung hoch mutagener, oxidativer Metaboliten zu verhindern. Eine Exposition gegenüber hohen Eisenkonzentrationen, z. B. im Falle einer Eisenüberladung, ist jedoch im Kolon wahrscheinlicher als in der Leber, wo die Exposition nur systemisch erfolgt. In den vorliegenden Studien sollte deshalb untersucht werden, wie Fe-NTA, U-NTA und eine ausgewählte Wismutprobe die Reparatur in humanen Kolonzellen moduliert. Hierfür wurden HT29 clone 19A-Zellen über 24 Stunden mit geringen, nicht zyto- und genotoxischen Konzentrationen dieser Substanzen vorinkubiert und anschließend mit verschiedenen, als kolorektale Risikofaktoren geltenden Modellsubstanzen, geschädigt. Wasserstoffperoxid (H_2O_2) als Produkt des oxidativen Stresses und 4-Hydroxynonenal als Produkt der Lipidperoxidation wirken in Kolonzellen genotoxisch [Abrahamse *et al.*, 1999; Schäferhenrich *et al.*, 2003a]. 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridin (NOH-PhIP) entsteht als Protein-Pyrolyseprodukt beim Erhitzen von Fleisch und Fisch und ist als Modellsubstanz für heterozyklische Amine anerkannt [Layton *et al.*, 1995].

U-NTA hatte keinen Effekt auf die Persistenz H_2O_2-, HNE- und NOH-PhIP-induzierter DNA-Schäden **(Publikationen III)**. D. h., die induzierten Schäden wurden unabhängig von der Vorinkubation partiell repariert. Auch für Fe-NTA konnte weder eine Hemmung der Reparatur, wie es für Nickel oder Cadmium beschrieben wurde [Dally & Hartwig, 1997], noch eine erhöhte Reparatur, wie von Abalea et al. ermittelt, nachgewiesen werden [Abalea *et al.*, 1998]. Jedoch erhöhte die Vorinkubation mit Eisen die HNE-induzierten Schäden **(Publikation I)**. Die Schadensrate war auch nach 120 Minuten Reparaturzeit noch signifikant höher als in Zellen ohne Vorinkubation. Demzufolge scheinen geringe Eisenkonzentrationen in Verbindung mit HNE additiv zu wirken. Als Produkt der Lipidperoxidation verursacht HNE v. a. Schäden in Membranen [Abrahamse *et al.*, 1999; Chen & Yu, 1994]. In humanen Kolonzellen wurden aber auch globale DNA-Schäden und genspezifische Schäden nachgewiesen [Schäferhenrich *et al.*, 2003a; Schäferhenrich *et al.*, 2003b]. Über mögliche Mechanismen für die ko-genotoxische Wirkung kann derzeit nur spekuliert werden. Am wahrscheinlichsten ist die Theorie, dass HNE in Kombination mit Metallen ähnlich reagiert wie H_2O_2. Winston et al. beschrieben schon 1983, dass Alkylhydroperoxide wie HNE durch Eisenkomplexe in Alkoxylradikale umgewandelt werden können, welche toxisch sind [Winston *et al.*, 1983].

In Leberzellen bewirkte eisenvermittelter oxidativer Stress die Bildung von HNE [Hammer *et al.*, 1997]. Metalle katalysieren die Bildung von Hydroxylradikalen und

die Peroxidation von Lipiden [Goddard & Sweeney, 1983; Halliwell & Gutteridge, 1984; Sakurai & Cederbaum, 1998]. Unter den von uns gewählten Bedingungen ist eine eisenvermittelte Lipidperoxidation durchaus denkbar. In diesem Zusammenhang könnte auch HNE gebildet werden, wie es in Nieren- und Leberhomogenaten von Fe-NTA-behandelten Ratten nachgewiesen werden konnte [Lamb *et al.*, 1999; Toyokuni *et al.*, 1997]. Es ist denkbar, dass Eisen in geringen Konzentrationen *in vitro* die Lipidperoxidation in Zellen oder dem umgebenden Medium katalysiert und somit an der Bildung reaktiver Metabolite wie HNE beteiligt ist.

4.5 Induzieren Eisen, Uran und die komplexen Umweltproben DNA-Schäden in spezifischen Genen bzw. in spezifischen Chromosomen humaner Kolonzellen?

Zum Nachweis genspezifischer Effekte wurde die auf dem Comet Assay basierende Comet FISH Technik genutzt. Neben der prozentualen Verteilung in den Kometenklassen wurde auch die prozentuale Auswanderung der *TP53*-Signale in den Kometenschwanz geschädigter Zellen ermittelt. Während der Comet Assay nur Aussagen über die Schäden in der Gesamt-DNA zulässt, ermöglicht die Kombination mit einer Fluoreszenz-*in-situ*-Hybridisierung die Visualisierung und Lokalisierung der zu untersuchenden Sequenz [Rapp *et al.*, 2001]. Diese Methode liefert neue Erkenntnisse zur allgemeinen Organisation des Zellkerns [Santos *et al.*, 1999], zur Synthese und Reparatur der DNA [McKelvey-Martin *et al.*, 1998] und zur Sensitivität bestimmter Gene oder Chromosomen gegenüber unterschiedlichen Noxen. Neben physikalischen Noxen wie γ-, UV-A- oder Röntgenstrahlung [Bock *et al.*, 1999; McKelvey-Martin *et al.*, 1998; McKenna *et al.*, 2003a; McKenna *et al.*, 2003b; Rapp *et al.*, 1999; Rapp *et al.*, 2000] wurden bisher auch verschiedene chemische Agenzien mit dieser Technik untersucht. Hier sind vor allem die Lipidperoxidationsprodukte HNE und Hexenal, H_2O_2 als Metabolit des oxidativen Stresses [Schäferhenrich *et al.*, 2003a; Schäferhenrich *et al.*, 2003b] sowie Restriktionsendonukleasen und DNAse zu nennen [Menke *et al.*, 2000].

Für eine optimale Auswertung der Comet FISH Versuche ist ein stabiler, diploider Karyotyp von großer Bedeutung, um zwei theoretisch zu erwartende Hybridisierungssignale für jede Zelle zu erhalten. Aufgrund des heterogenen Karyotyps und zahlreicher chromosomaler Aberrationen konnte die Karzinomzelllinie HT29 clone

19A in die Untersuchungen zu genspezifischen Schäden deshalb nicht einbezogen werden [Kuechler *et al.*, 2003]. Bei den primären Kolonepithelzellen kann davon ausgegangen werden, dass sie einen normalen diploiden Karyotyp haben, da sie aus gesundem humanem Kolongewebe gewonnen wurden. Neben primären Kolonzellen ist auch die Adenomzelllinie LT97 gut für diese Methode geeignet. Sie hat einen stabilen, diploiden Karyotyp und das in dieser Arbeit interessierende Gen *TP53* liegt unverändert vor [Schäferhenrich *et al.*, 2003a].

Fe-NTA, U-NTA und die Wismut-Probe B zeigten sowohl in LT97-Adenomzellen als auch in Primärzellen mit steigenden Konzentrationen eine Verschiebung der Kometenklassen in Richtung geschädigter Zellen. Dies spiegelt die Dosis-Wirkungsabhängige Genotoxizität der Testsubstanzen wider, die mittels Computer-gestützter Auswertung der Fluoreszenz im Schweif (Comet Assay) in vorangegangenen Experimenten schon bewiesen wurde. Außerdem konnte beobachtet werden, dass in geschädigten Zellen konzentrationsabhängig *TP53*-Signale signifikant in den Schweif auswandern. In beiden Zelltypen waren ab 500 µM Fe-NTA mehr als 50 % der geschädigten Zellen (Kometenklassen 2-4) von der Auswanderung des *TP53* in den Schweif betroffen **(Publikation II)**. Für U-NTA war dieser Zusammenhang in Primärzellen ähnlich stark, ca. 50 % der mit 500 und 1000 µM U-NTA geschädigten Zellen zeigten eine Auswanderung von *TP53* **(Publikation III)**. Für LT97-Adenomzellen war eine signifikante *TP53*-Auswanderung nach U-NTA-Behandlung nur in der höchsten Konzentration (1000 µM) nachweisbar. Primärzellen reagierten somit tendenziell stärker als LT97-Zellen. Für die kontaminierte Umweltprobe B zeigte sich ein ähnlich starker Zusammenhang in LT97-Zellen **(Publikationsmanuskript IV)**. Schon bei sehr geringen Konzentrationen (2,5 % in Medium) waren ca. 50 % der geschädigten Zellen von einer *TP53*-Migration betroffen, bei der höchsten Konzentration (10 % in Medium), waren es sogar 60 %. Die Comet FISH Ergebnisse deuten darauf hin, dass das *TP53*-Gen bzw. die Sonde umfassende Region sehr sensitiv auf die von uns untersuchten Testsubstanzen reagiert. Die hohe Sensitivität des Gens gegenüber Eisen stützt die Hypothese, dass Eisenüberschuss bei Eisenspeicherkrankheiten (Wilson disease und Hämochromatose) Mutationen im Tumorsuppressorgen *TP53* verursacht [Hussain *et al.*, 2000]. Die Sensitivität des *TP53*-Gens gegenüber anderen Risikofaktoren des Kolorektalkrebses waren Gegenstand der Untersuchungen von Schäferhenrich und Kollegen. Sie schlussfolgerten aus einer erhöhten Migration des *TP53* in den Schweif, dass das Gen besonders sensitiv gegenüber der genotoxi-

schen Wirkung von HNE in LT97-Adenomzellen reagiert [Schäferhenrich *et al.*, 2003a]. Da genetische Veränderungen des Tumorsuppressorgens *TP53* im Zusammenhang mit späten Ereignissen der kolorektalen Karzinogenese stehen, sind die Hinweise auf eine hohe Sensitivität dieses Gens gegenüber kolorektalen Risikofaktoren wie Eisen von großer Bedeutung. Die erhaltenen Ergebnisse lassen darauf schließen, dass Eisen neben seiner Bedeutung für die Initiation und frühe Progression auch in späteren Ereignissen des Kolorekatalkrebses eine nicht zu vernachlässigende Rolle spielt. Die Resultate tragen somit zum besseren Verständnis der genotoxischen Wirkung von Eisen bei. Für die *in vivo*-Situation im Eisenüberschuss bedeutet dies ein karzinogenes Potential in der kolorektalen Karzinogenese. Schäferhenrich et al., die neben dem *TP53* auch weitere an der kolorektalen Karzinogenese beteiligte Gene untersuchten, stellten außerdem fest, dass das *TP53*-Gen sensitiver auf genspezifische Schäden reagiert als *APC* oder *K-RAS*-Gen [Schäferhenrich, 2004]. Dies bestätigt, dass in Tumoren das *TP53*-Gen am häufigsten Mutationen aufweist. Setzt man diese Ergebnisse jedoch in Zusammenhang mit dem Mehrstufenmodel der Kolonkarzinogenese von Fearon und Vogelstein (1990) entsteht ein Widerspruch, denn hiernach müsste das *APC*-Gen am empfindlichsten reagieren, da es im Karzinogeneseprozess an erster Stelle steht [Fearon & Vogelstein, 1990].

Für die Primärzellen wurde gegenüber den LT97-Adenomzellen eine tendenziell höhere Sensitivität gegenüber Fe-NTA und U-NTA ermittelt. Dies stimmt mit den Ergebnissen von Schäferhenrich et al. überein, die für primäre Kolonzellen im Vergleich zu den Adenomzellen eine relativ hohe *TP53*-Sensitvität gegenüber H_2O_2 als ROS-bildende Noxe feststellten [Schäferhenrich *et al.*, 2003b]. Im Comet Assay hingegen, der die Schädigung der Gesamt-DNA erfasst, erwiesen sich die Kolonkarzinomzellen (HT29 clone 19A) als besonders sensitiv gegenüber Fe-NTA. Dies stimmt gut mit der Tatsache überein, dass es im Laufe der Karzinogenese neben Mutationen in den bereits beschriebenen Tumorsuppressorgenen und Protoonkogenen auch zu Veränderungen in DNA-Reparaturgenen und somit zu einer zunehmenden genomischen Instabilität und generellen Anhäufung von Mutationen in der gesamten DNA kommt [Vogelstein *et al.*, 2000]. Die Verteilung der durch bestimmte Noxen induzierten DNA-Schäden im Genom erfolgt nicht zufällig, sondern ist abhängig von der Chromosomenstruktur, der Aktivität der Gene und der lokal spezifischen Reparaturaktivität [Cremer *et al.*, 1996; Diculescu, 1997; Johnson *et al.*, 1999; Wolf-

fe, 1994]. So konnte für strahlungsinduzierte Schäden in Comet FISH Versuchen mit Ganzchromosomenproben ein Zusammenhang mit der Dichte aktiver Gene auf den jeweiligen Chromosomen nachgewiesen werden. Verschiedene Arbeitsgruppen fanden für genärmere Chromosomen nach UV-A-Bestrahlung häufiger Chromosomenbruchstücke im Kometenschweif, als für genreichere Chromosomen [Bock *et al.*, 1999; Deloukas et al., 1998; Rapp *et al.*, 2000]. Röntgenstrahlen hingegen setzten vor allem in genreichen Chromosomenabschnitten Schäden, was für grundsätzlich unterschiedliche Schädigungsmechanismen spricht [Slijpcevic, 2004; Tucker & Senft, 1994]. In den Untersuchungen von Schäferhenrich et al. nahmen die genspezifischen Schäden mit der Dichte aktiver Gene auf den Chromosomen zu [Schäferhenrich, 2004]. So zeigte das *TP53* Gen, welches auf Chromosom 17 mit hoher Dichte aktiver Gene lokalisiert ist, höhere Schäden als die Gene *APC* und *K-RAS*, die wiederum auf Chromosomen mit geringerer Dichte aktiver Gene zu finden sind (Chromosom 5 bzw. 12). Die aufgrund der Dichte aktiver Gene auf den Chromosomen zu erwartenden Unterschiede in der genspezifischen Sensitivität der Gene *APC* und *K-RAS* (*APC*<*K-RAS*) konnten von Schäferhenrich et al. jedoch nicht bestätigt werden.
In zukünftigen Untersuchungen bleibt zu klären, ob eine verstärkte Migration des *TP53* mit direkten Mutationen dieses Gens verbunden ist und wie Eisen und wismutrelevante Verbindungen das Mutationspektrum modulieren. Erste Anhaltspunkte hierfür liefern die im Folgenden diskutierten Untersuchungen mittels 24-Farben-FISH.

Der 24-Farben-FISH ermöglicht, jedes der 24 Chromosomen (22 Autosomen, X- und Y-Chromosom) mit einer spezifischen Kombination von Fluoreszenzfarbstoffen zu markieren und somit den Ursprung des chromosomalen Materials zu identifizieren [Speicher *et al.*, 1996]. Durch eine vorherige Inkubation der Zellen mit den zu testenden Substanzen kann mit dieser Methode das klastogene Potential der Testsubstanzen untersucht werden. Die im Comet Assay messbare DNA-Schädigung stellt nur den momentanen Zustand der DNA dar. Diese Schäden können repariert werden. Spricht man also von einer mittels Comet Assay ermittelten Genotoxizität einer Substanz, kann man nicht davon ausgehen, dass sich diese Schäden auch auf chromosomaler Ebene manifestieren. Der 24-Farben-FISH ist in der Lage diese stabilen Mutationen zu erfassen. Durch das Inkubationsregime erhalten die Zellen die Möglichkeit einen kompletten Zyklus zu durchlaufen, so dass dann die geneti-

schen Veränderungen messbar sind, die nicht mehr reparabel sind und in stabile chromosomale Aberrationen konvertiert wurden.

Knoll et al. nutzten den 24-Farben-FISH erstmals *in vitro*, um substanzinduzierte zytogenetische Veränderungen in Kolonzellen nachzuweisen [Knoll *et al.*, 2006]. Sie wiesen für 2-Dodecylcyclobutanon (2dDCB), ein durch die Bestrahlung von fetthaltigen Lebensmitteln entstehendes Alkylcyclobutanon, zytogenetische Eigenschaften in humanen Kolonadenomzellen (LT97) nach. In der vorliegenden Arbeit ist es nun erstmals gelungen, die durch eine Umweltnoxe verursachten genotoxischen Effekte in humanen Kolonzellen mittels 24-Farben-FISH auch auf chromosomaler Ebene nachzuweisen. So konnte gezeigt werden, dass U-NTA vor allem Deletionen und Translokationen verursacht **(Publikation III)**. Das für U-NTA beobachtete Mutationsspektrum unterscheidet sich von denen durch 2dDCB induzierten Mutationen, welche sich vor allem als Isochromosomen und Deletionen manifestieren (Knoll et al, 2006). Die Entstehung der durch U-NTA verursachten Translokationen erfolgte konzentrationsabhängig. Mit steigenden Konzentrationen konnte ein erhöhter Anteil aberranter Metaphasen nachgewiesen werden. Die durch U-NTA induzierten und mittels 24-Farben-FISH nachgewiesenen chromosomalen Aberrationen verdeutlichen, dass sich ein großer Teil der im Comet Assay ermittelten DNA-Schäden auch auf chromosomaler Ebene manifestiert haben. U-NTA wirkt somit genotoxisch und klastogen.

Ein interessantes Bild ergab sich bei näherer Betrachtung der Chromosomen 5, 12 und 17, auf denen die tumorrelevanten Gene *APC*, *K-RAS* und *TP53* lokalisiert sind. Der Anteil dieser Chromosomen an der Gesamtanzahl geschädigter Chromosomen, verursacht durch die höchste Konzentration U-NTA (2000 µM), betrug etwa 30 %. Diese Ergebnisse stützen die Hypothese, dass chromosomale Veränderungen auch bereits in frühe Prozesse der kolorektalen Karzinogenese involviert sein können. Lin et al. untersuchten mit Uranylnitrat eine vergleichbare Verbindung [Lin *et al.*, 1993]. Sie konnten nach Exposition (10-300 µM) von Chinesische Hamster-Ovarien-Zellen (CHO-Zellen) erhöhte Frequenzen an Mikrokernen, Schwester-Chromatid-Austauschen und chromosomalen Aberrationen nachweisen. In *in vivo* Versuchen hingegen zeigten Uranverbindungen meist keine derartigen Effekte. In Lymphozyten von Golf-Krieg Veteranen, die mit abgereichertem Uran (depleted uranium) exponiert waren, konnten keine chromosomalen Aberrationen detektiert werden [McDiarmid *et al.*, 2000]. Minenarbeiter, die über einen längeren Zeitraum höheren Dosen Uranbergbau-assoziierter Verbindungen ausgesetzt waren, zeigten keine erhöhte Inzi-

denz chromosomaler Veränderungen in weißen Blutzellen [Lloyd *et al.*, 2002]. Im Gegensatz dazu konnte für eine Gruppe von Arbeitern der Wismut-Minen eine erhöhte Mikrokernrate in Lungenmakrophagen ermittelt werden [Popp *et al.*, 2000]. Mögliche Ursache für diese zum Teil sehr unterschiedlich ausfallenden Studienergebnisse könnte sein, dass den Untersuchungen unterschiedliche Expositionsarten und -höhen zugrunde lagen.

4.6 Sind Leukozyten als Biomarker zur Erfassung von hohen Eisen-Expositionen geeignet?

Neben der genotoxischen Wirkung von Fe-NTA *in vitro* in humanen Kolonzellen konnten wir auch für isolierte humane Leukozyten Fe-NTA-induzierte Strangbrüche nachweisen **(Publikationsmanuskript V)**. Die durch 500 µM Fe-NTA verursachten Schäden waren in humanen Leukozyten deutlich höher als in humanen Kolonzellen (Leukozyten: 34,1 ± 7,4 % TI; Primäre Kolonepithelzellen: 20,1 ± 1,7 % TI; LT97-Kolonadenomzellen: 20,4 ± 8,5 % TI). Leukozyten von Ratten, die *ex vivo* durch eine Eisenüberschuss-Diät exponiert wurden, zeigten ebenfalls signifikante Schäden. Humane Leukozyten sind somit als Marker hoher Eisenexpositionen geeignet. Die *ex vivo* Resultate lassen darauf schließen, dass nach einer oralen genotoxischen Belastung mit Eisen auch systemische Effekte nachweisbar sind. Diese kombinierte Studie zeigte außerdem, dass Eisenüberschuss oxidierte DNA-Basen *ex vivo* in Ratten-Leukozyten, nicht aber *in vitro* in humanen Leukozyten induziert **(Publikationsmanuskript V)**. Mögliche Ursachen für die unterschiedlichen Effekte in Leukozyten von Menschen und Ratten (bzw. *in vitro* und *ex vivo*) können die unterschiedlichen Eisenquellen (Fe-NTA bzw. $FeSO_4$) und die verschiedene Ausstattung mit antioxidativen Enzymsystemen sein. In humanen Kolonkarzinomzellen (HT29 clone 19A) konnten Glei et al. für Fe-NTA *in vitro* oxidierte Purinbasen (Fpg-sensitive Stellen) nachweisen. Dies spricht dafür, dass Fe-NTA in der Lage ist, die Oxidation von DNA-Basen in humanen Zellen auszulösen, wenn auch auf vergleichsweise geringerem Niveau [Glei *et al.*, 2002].

4.7 Kann ein polyphenolreicher Apfelextrakt chemopräventiv gegenüber Eisen und anderen Genotoxinen wirken?

Die durch Fe-NTA verursachte Schädigung der DNA kann auf oxidativen Prozessen beruhen. Antioxidative Nahrungsinhaltsstoffe sind in der Lage diesen Prozessen entgegenzuwirken und präventiv in Karzinogeneseprozesse einzugreifen [Dias *et al.*, 2005; Roig *et al.*, 2002].

Für die Untersuchungen zur Protektion genotoxischer Effekte von Eisen und anderen Noxen wurde ein Apfelextrakt mit hohem Polyphenolgehalt ausgewählt [Will *et al.*, 2004]. In Genexpressionsanalysen mit diesem Apfelextrakt erwiesen sich in HT29-Zellen und LT97-Zellen unterschiedliche Konzentrationen als effektiv. In beiden Zelllinien wurden durch den Apfelextrakt (HT29-Zellen: 30 µM; LT97-Zellen: 7,5 µM) die Gene GSTT2 und GSTP1 hoch reguliert [Veeriah *et al.*, 2006]. Diese beiden Gene kodieren für Enzyme, die zur Familie der Glutathion-S-Transferasen (GST) gehören. Als Phase-II-Enzyme sind sie an der Entgiftung toxischer Metabolite beteiligt. GSTT2 entgiftet unter anderem Cumenhydroperoxid (Cum-OOH) [Hayes *et al.*, 2005] und GSTP1 Benzo[a]pyrendiolepoxid (BPDE) [Hu *et al.*, 1999]. Neben H_2O_2, das auch endogen im Körper gebildet wird und an dessen Entgiftung verschiedene Enzyme beteiligt sind, wurden deshalb zusätzlich die beiden Substrate Cum-OOH und BPDE im Comet Assay eingesetzt, um die funktionellen Konsequenzen der GST-Induktion durch den Apfelextrakt, d.h., den potentiellen Schutz vor den durch diese Substanzen hervorgerufenen DNA-Schäden, nachzuweisen. Hierfür wurden die Zellen für 24 Stunden mit dem Apfelextrakt vorinkubiert (HT29-Zellen: 30 µM; LT97-Zellen: 7,5 µM; 37 °C) und anschließend mit Fe-NTA, BPDE (30 min, 37 °C), H_2O_2, bzw. Cum-OOH (5 min, 4 °C) geschädigt.

BPDE schädigte die DNA beider Zelllinien signifikant, jedoch in HT29-Zellen erst ab 30 µM **(Publikationsmanuskript VI)**. Damit erwiesen sich die LT97-Adenomzellen hinsichtlich der Schädigung durch BPDE signifikant sensitiver. Die Vorinkubation mit dem Apfelextrakt bewirkte in der Kolonadenomzelllinie bei höheren BPDE-Konzentrationen einen Schutz der Zellen gegenüber der genotoxischen Wirkung dieser Substanz. In HT29-Zellen hingegen erhöhte der Apfelextrakt die genotoxische Wirkung signifikant. Die Vitalität der mit dem Apfelextrakt vorinkubierten Zellen war nach dem Challenge Assay signifikant reduziert, was jedoch nicht auf die Behand-

lung mit BPDE, sondern auf den Apfelextrakt selbst zurückzuführen war. Möglicherweise ist hierfür die in Verbindung mit dem Zellkulturmedium der HT29-Zellen nachweisbare H_2O_2-Produktion des Apfelextraktes verantwortlich. In LT97-Adenomzellen konnte zumindest bei höherer Schädigung ein protektiver Effekt nachgewiesen werden. In Verbindung mit dem MCDB-Zellkulturmedium der LT97-Zellen war keine Generation von H_2O_2 messbar. *In vitro* war Quercetin in humanen Lymphozyten in der Lage vor diesen Addukten zu schützen [Wilms *et al.*, 2005].

Das synthetische Hydroperoxid Cum-OOH erwies sich in beiden Zelllinien als genotoxisch, wobei die Kolonadenomzelllinie LT97 signifikant sensitiver als die Karzinomzelllinie (bis zu doppelt so hohe Schäden bei der maximalen Konzentration von 360 µM) reagierte **(Publikationsmanuskript VI)**. Die Vorinkubation mit dem Apfelextrakt reduzierte die genotoxische Wirkung von Cum-OOH signifikant in beiden Zelllinien. Auch gegenüber H_2O_2 zeigten LT97-Zellen eine höhere Sensitivität. Ab einer Konzentration von 37,5 µM zeigten sich erste signifikante Schäden **(Publikationsmanuskript VI)**. Der Apfelextrakt ASO2 wirkte signifikant protektiv in beiden Zellen, wobei der Effekt auch hier in LT97-Zellen eindeutig stärker war. In weiteren Versuchen bleibt zu klären, ob die gemessene Verminderung der DNA-Schädigung tatsächlich eine funktionelle Konsequenz der GSTT2-Induktion ist oder im Zusammenhang mit den durch den Apfelextrakt hervorgerufenen zytotoxischen Effekten steht. Hierbei könnte über Zytotoxizität eine Selektion resistenterer Zellen erfolgen, was sich dann im Comet Assay als verminderte DNA-Schädigung widerspiegeln würde.

Der Schutz vor oxidativen Schäden durch Polyphenole wurde bereits in verschiedenen Studien nachgewiesen. So konnten Roig und Kollegen zeigen, dass Procyanidine, die vor allem im Rotwein, aber auch in Äpfeln nachweisbar sind, Fao-Zellen vor H_2O_2-induzierten oxidativen DNA-Schäden schützen [Roig *et al.*, 2002]. Hierbei war ein Mix aus Procyanidinen effektiver als die Einzelkomponenten. Über eine Induktion der zellulären antioxidativen Aktivität und die Hemmung der Xanthin-Xanthin-Oxidase verminderten Polyphenolextrakte aus Äpfeln Schäden in humanen Magenepithelzellen *in vitro* und in der Magenmukosa von Ratten *in vivo* [Graziani *et al.*, 2005]. Chlorogensäure und Catechine, Hauptvertreter der Polyphenole in Apfelextrakten, waren als Einzelsubstanzen dabei genauso effektiv wie komplexe Apfelextrakte. Quercetin und Myricitin sind ebenfalls in Äpfeln vorkommende Polyphenole mit hohem antioxidativem Potential. Sie schützten humane Lymphozyten und Kolonozyten (Caco-2) gegenüber oxidativen Angriffen durch H_2O_2 [Duthie *et al.*, 1997a; Duthie & Dobson,

1999]. Horvathowa und Kollegen bezogen noch weitere Flavonoide (Rutin, Apigenin, Luteolin) in ihre Untersuchungen ein [Horvathova *et al.*, 2003]. Sie fanden heraus, dass Quercetin den stärksten protektiven Effekt in L1210-Leukemia-Zellen ausübt, gefolgt von Luteolin. Apigenin und Rutin zeigten keine protektive Wirkung. Apigenin war in hohen Konzentrationen sogar selbst genotoxisch. Potentielle pro-oxidative Eigenschaften einiger Polyphenole wurden auch von Szeto und Benzie demonstriert [Szeto & Benzie, 2002]. Die Grüntee-Catechine Epigallocatechin und Epigallocatechingallat induzierten über die Bildung von H_2O_2 DNA-Schäden in humanen Lymphozyten *(ex vivo)*.

Der Apfelextrakt wirkte pro-oxidativ in Verbindung mit der in dieser Arbeit im Mittelpunkt stehenden genotoxischen Testsubstanz Fe-NTA (eigene unveröffentlichte Daten). Die genotoxische Wirkung in HT 29-Zellen und dem Subklon HT29 clone 19A wurde durch den Apfelextrakt verstärkt. In vorangegangenen Untersuchungen konnte nachgewiesen werden, dass Fe-NTA in humanen Leukozyten und humanen Kolonzellen unterschiedlicher Transformationsgrade genotoxisch wirkt **(Publikationen I und II)**. Diese genotoxischen Effekte können indirekt über die Bildung von ROS über die Fenton-Reaktion induziert werden [Meneghini, 1997]. Polyphenole sind in der Lage diese radikalischen Verbindungen abzufangen und Metalle zu chelatieren. Es wurde beschrieben, dass Polyphenole in Anwesenheit von Metallen unter bestimmten Bedingungen *in vitro* die Bildung von oxidativen Schäden fördern [Sahu & Gray, 1993; Singh *et al.*, 1994]. Die in unseren Untersuchungen ermittelten pro-oxidativen Effekte des Apfelextraktes im Zusammenhang mit Fe-NTA-induzierten DNA-Schäden könnten durch eine Autoxidation der im Extrakt enthaltenen Polyphenole und der damit verbundenen Bildung von ROS zu erklären sein [Cao *et al.*, 1997; Mochizuki *et al.*, 2002]. Bei der Autoxidation werden die Polyphenole durch O_2 und ein Transitionsmetal zu den entsprechenden Qinonen oxidiert. Dabei entstehen reaktive Sauerstoffspezies wie O_2^- und H_2O_2 [Long *et al.*, 2000; Roques *et al.*, 2002]. Akagawa et al. beschrieben ebenfalls die Bildung von H_2O_2 durch Polyphenole und polyphenolreiche Getränke unter nahezu physiologischen Bedingungen [Akagawa *et al.*, 2003].

Die von anderen Forschungsgruppen demonstrierte H_2O_2-Bildung in unterschiedlichen Zellkulturmedien und Pufferlösungen durch Zugabe von Polyphenolen stimmen gut mit den von uns im FOX Assay ermittelten Ergebnissen überein [Long *et al.*, 2000; Roques *et al.*, 2002]. Im Zellkulturmedium DMEM (HT29-Zellen) generierte der

Apfelextrakt signifikant dosisabhängige Mengen von H_2O_2, nicht jedoch im MCDB-Medium (LT97). Eine Erklärung für die differierenden Ergebnisse mit beiden Zellkulturmedien könnte in den unterschiedlichen FCS-Gehalten der beiden Medien (DMEM 10 % FCS, MCDB 2 % FCS) und damit in unterschiedlichen Mengen der darin enthaltenen Transitionsmetalle (z.B. Eisen und Kupfer), begründet sein. In der Studie von Akagawa und Kollegen war der Metalchelator EDTA in der Lage, die Bildung von Metal-Polyphenol-Komplexen und die damit assoziierte Bildung von H_2O_2 um 25-30 % zu reduzieren [Akagawa *et al.*, 2003]. Die nur unvollständige Inhibierung der H_2O_2-Bildung spricht dafür, dass die Chelatierungsfähigkeit der Polyphenole höher ist, bzw. die Autoxidation der Polyphenole an der Generation von H_2O_2 beteiligt ist. Transitionsmetalle in den Medien begünstigen die Autoxidation der Polyphenole des Apfelextraktes. In Anwesenheit von Katalase war unter sonst gleichen Bedingungen kein H_2O_2 mehr nachweisbar, was bestätigt, dass H_2O_2 im Inkubationsansatz gebildet und dann vom Enzym abgebaut wird. Die anteilsmäßig am stärksten vertretene Chlorogensäure induzierte ebenfalls signifikante H_2O_2-Mengen im DMEM-Medium. Im MCDB-Medium wurde das nur durch sehr hohe Mengen an Chlorogensäure erreicht (500 µM). Die Einzelsubstanz Phloridzin scheint an der Bildung von H_2O_2 nicht beteiligt zu sein.

5. SCHLUSSFOLGERUNGEN UND AUSBLICK

5.1 Schlussfolgerungen

Unter welchen Bedingungen (Zeit-, Konzentrations-, Effektbeziehungen) beeinflussen Eisen, Uran und die komplexen Umweltproben das Wachstum von Kolonkarzinomzellen?

- Eisen beeinflusst das Wachstum von HT29 clone 19A-Zellen konzentrationsabhängig. Während geringe Eisen-Konzentrationen (25 µM) das Wachstum fördern hemmen hohe Fe-Konzentrationen (2000 µM) das Zellwachstum.
- Ein karzinogenes Potential besteht auch über niedrige, physiologische Eisen-Konzentrationen durch die Stimulierung des Zellwachstums.
- Hohe Uran-Konzentrationen (2000 µM) hemmen das Wachstum von HT29 clone 19A-Zellen nach 24 und 48 h, was auf zytotoxische Effekte hindeutet. Nach 72 h wirkt Uran schon bei geringeren Konentrationen (75 µM) zytotoxisch (EC_{50} 215 µM).
- Die komplexen Umweltproben beeinflussen das Wachstum von HT29 clone 19A-Zellen in Abhängigkeit vom Kontaminationsgrad. Hohe Gehalte an Schwermetallen und Radionukliden (Probe C) hemmten das Wachstum signifikant.

Welchen Einfluss haben Eisen, Uran und die komplexen Umweltproben auf die Bildung reaktiver Sauerstoffspezies (ROS) und den Glutathion-Status.

- Die stark mit Schwermetallen und Radionukliden belastete Gewässerprobe (Probe C) induziert die Bildung von ROS. Die ROS-Bildung ist aufgrund der hohen Eisen-Kontamination und der in Versuchen mit der Einzelnoxe Fe-NTA betätigten ROS-Generation hauptsächlich auf das darin enthaltene Eisen zurückzuführen. Uran scheint hierbei keine bzw. nur eine untergeordnete Rolle zu spielen.
- Für die genotoxische Wirkung von U-NTA spielt die Bildung von ROS keine Rolle. Es scheint daher eher der von Franklin [Franklin, 2000] beschriebene Mechanismus, bei dem Uran mit der DNA interagiert und diese hydrolysiert, von Bedeutung zu sein.
- Eisen und Uran induzieren in den humanen Kolonkarzinomzellen HT29 clone 19A eine Erhöhung intrazellulärer GSH-Gehalte.

Wirken Eisen, Uran und die komplexen Umweltproben genotoxisch in Kolonzellen unterschiedlicher Transformationsgrade?

- Eisen, Uran und stark belastete Wässer (Proben B und C) wirken genotoxisch in humanen Kolonzellen unterschiedlicher Transformationsgrade.
- Primäre Kolonepithelzellen und Kolonadenomzellen reagieren empfindlicher auf das genotoxische Potential von Eisen und Uran als Kolonkarzinomzellen. Dies spricht dafür, dass Eisen und Uran eine Rolle in der Initiation und frühen Progression der Kolonkarzinogenese spielen können.
- Die komplexen Wasserproben enthalten eine Vielzahl von Noxen, die ihre Wirkung gegenseitig verstärken bzw. addieren können.

Führen die Umweltproben und die Leitsubstanzen zu einer veränderten Sensitivität der Zellen gegenüber weiteren Noxen bzw. wird die Reparatur induzierter Schäden beeinflusst?

- Eisen und Uran haben keinen Einfluß auf die Reparatur induzierter DNA-Schäden.
- Während geringe Konzentrationen von Uran (10 µM) die Höhe induzierter Schäden nicht verändern, verstärkt Eisen (10 µM) die durch 4-Hydroxynoneneal (HNE) induzierten DNA-Schäden. Eisen wirkt also synergistisch in Verbindung mit HNE.
- Ein karzinogenes Potential kann deshalb auch aus niedrigen, physiologischen Eisen-Konzentrationen über pro-genotoxische Effekte resultieren.

Induzieren Eisen, Uran und die komplexen Umweltproben DNA-Schäden in spezifischen Genen bzw. in spezifischen Chromosomen humaner Kolonzellen?

- Das *TP53*-Gen in Primären Kolonzellen und Kolonadenomzellen reagiert sensitiv auf Eisenüberschuss. Dies stützt die These von Hussain et al. die besagt, dass Eisenüberschuss bei Eisenspeicherkrankheiten wie Hämochromatose oder Wilson Disease Mutationen im Tumorsuppressorgen *TP53* verursacht [Hussain et al., 2000].
- Eisen spielt neben Initiation und frühen Progression auch für späte Mechanismen der Kolonkarzinogenese (z. B. die mit *TP53*-Mutationen verbundene Entartung von Zellen) eine nicht zu vernachlässigende Rolle.

- Uran induziert chromosomale Aberrationen. Die durch Uran induzierten DNA-Schäden manifestieren sich auch auf chromosomaler Ebene. U-NTA wirkt somit genotoxisch und klastogen.
- Die durch Uran verursachten chromosomalen Veränderungen betreffen vor allem Chromosomen, auf den die an der Kolonkarzinogenese beteiligten Gene *APC*, *K-RAS* und *TP53* lokalisiert sind.
- Gelangen Verbindungen wie Eisen und Uran über eine Umweltkontamination von Böden und/oder Gewässern in ausreichender Menge in die Nahrungskette, besteht die Gefahr einer erheblichen oralen Exposition und somit einer genotoxischen Belastung.

Sind Leukozyten als Biomarker zur Erfassung von hohen Eisen-Expositionen geeignet?

- Humane Leukozyten sind geeignete Marker für die Erfassung von hohen Eisen-Expositionen. Die Messung von DNA-Schäden in humanen Leukozyten könnte als sensitiver Biomarker in kontrollierten humanen Studien genutzt werden, um zu prüfen, ob eine verabreichte Eisen-Supplementation mit einem genotoxischen Risiko verbunden ist.
- Eine orale Exposition mit Eisen kann sowohl lokale (Darmzellen), als auch systemische Effekte (Leukozyten) induzieren.

Kann ein polyphenolreicher Apfelextrakt chemopräventiv gegenüber Eisen und anderen Genotoxine wirken?

- Der Apfelextrakt wirkt pro-genotoxisch in Verbindung mit BPDE und Eisen in HT29-Kolonkarzinomzellen. Die pro-genotoxischen Effekte sind auf die Generation von H_2O_2 im Zellkulturmedium DMEM zurückzuführen.
- Der Apfelextrakt schützt vor BPDE-induzierten Schäden in LT97-Adenomzellen und vor genotoxischen Schäden, die durch H_2O_2 und Cum-OOH in HT29-Kolonkarzinomzellen und LT97-Kolonadenomzellen verursacht werden.
- Der Verzehr polyphenolreicher Obst- und Gemüsesorten, wie z. B. Äpfel kann zum Schutz gegenüber bestimmten Kolonrisikofaktoren beitragen.

5.2 Ausblick

Die im Rahmen dieser Arbeit durchgeführten Untersuchungen tragen dazu bei, die Mechanismen hinter den genotoxischen Wirkungen wismut-relevanter Verbindungen, speziell Eisen und Uran, aufzuklären.

In humanen Interventionsstudien sollten DNA-Schäden in Leukozyten herangezogen werden, um eine Grenze zwischen adäquater Eisen-Supplementation und Eisenüberladung zu definieren bzw. Risikogruppen (z. B. Personen mit Eisenspeicherkrankheiten) zu definieren.

Es bleibt außerdem zu klären, ob eine durch Eisen verursachte verstärkte Migration des *TP53* mit direkten, stabilen Mutationen dieses Gens verbunden ist und wie Eisen und andere wismut-relevante Verbindungen das Mutationsspektrum modulieren. Hierbei könnte die in dieser Arbeit vorgestellte innovative Methode des 24-Farben-FISH Anwendung finden. Diese Untersuchungen könnten eine Bestätigung dafür liefern, dass sich durch Eisen oder andere Noxen induzierte genspezifische Schäden auch als Mutationen manifestieren. Die Einbeziehung weiterer Gene, die in die Kolonkrebsentstehung involviert sind (z. B. *APC*, *K-RAS*, *MCC*), wäre in diesem Zusammenhang auch sehr interessant.

Das Potential der Polyphenole unter bestimmten Bedingungen H_2O_2 zu generieren und pro-oxidativ zu wirken, sollte in Zukunft stärker in chemopräventive und toxikologische Untersuchungen einbezogen werden, nur so können komplexe Zusammenhänge aufgedeckt werden.

In weiterführenden Untersuchungen wäre es sinnvoll, den FOX Assay auch im zellulären System durchzuführen, um die Aufnahme, die Stoffkinetik, den Metabolismus und die Ausscheidung einer Noxe aus der Zelle zu berücksichtigen, da diese für die Art und den Umfang der Wirkung von großer Bedeutung sind.

6. ZUSAMMENFASSUNG

6.1 Zusammenfassung

Hintergrund: Durch die ehemaligen Uranbergbaubetriebe der SDAG „Wismut“ hat sich in der dortigen Umgebung eine besondere Umweltsituation ergeben, die es notwendig macht, die Auswirkungen der Stoffflüsse auf die Biosphäre zu prüfen. Die in den geologischen Kompartimenten enthaltenen Radionuklide und Schwermetallsalze können über verschiedene Transportprozesse tierische und menschliche Zielzellen erreichen und dort über die Bildung von ROS und anderen Mechanismen zu besonderen Interaktionen führen.

Zielstellung: Ziel der vorliegenden Arbeit war es, ausgewählte Wasserproben aus dem ehemaligen Ronneburger Uranbergbaugebiet bzw. die Leitsubstanzen Eisen und Uran hinsichtlich ihrer lokalen bzw. systemischen Wirkungspotentiale in humanen Kolonzellen und Leukozyten zu charakterisieren und grundlegende Erkenntnisse über die beteiligten Wirkungsmechanismen zu erhalten. Des Weiteren sollte geprüft werden, ob Apfelpolyphenole chemoprotektiv gegenüber Eisen und anderen Genotoxinen wirken.

Material und Methoden: In humanen Kolonzellen unterschiedlicher Transformationsgrade (Primäre Kolonepithelzellen, Kolonadenomzellen und Kolonkarzinomzellen) und zum Teil in humanen Leukozyten wurden Konzentrations- und Effektbeziehungen (DAPI-Assay), das ROS-bildende Potential (ROS Assay, FOX Assay), der GSH-Status (fluoreszenzphotometrischer Assay), das genotoxische Potential (Comet Assay) der Einzelnoxen Fe-NTA und U-NTA sowie komplexer Gewässerproben und deren Einfluß auf die Reparatur induzierter Schäden (Comet Assay mit Reparatur) untersucht. Weitere Studien befassten sich mit der Frage, ob Eisen, Uran und die komplexen Umweltproben DNA-Schäden in spezifischen Genen bzw. Chromosomen induziert (Comet FISH, 24-Farben-FISH). Mittels Challenge Assay wurde das chemopräventive Potential eines polyphenolreichen Apfelextraktes in humanen Kolonzellen ermittelt.

Ergebnisse und Schlussfolgerungen Eisen: Für geringe, physiologische Eisendosen konnte eine Verstärkung des Zellwachtums in Kolonkarzinomzellen beobachtet werden, sehr hohe Dosen hingegen führten zu einem Wachstumsstopp und erhöhten intrazellularen GSH-Konzentrationen und zeitabhängig zu einer signifikanten Bildung

von ROS. Das genotoxische Potential für Fe-NTA konnte für alle Zelltypen (Primäre Kolonepithelzellen, LT97-Kolonadenomzellen, HT29 clone 19A-Kolonkarzinomzellen, humane Leukozyten) nachgewiesen werden. In primären Kolonepithelzellen, LT97-Zellen und humanen Leucozyten war Fe-NTA darüber hinaus in der Lage spezifisch Schäden in der Region des Tumorsuppressorgens *TP53* zu induzieren, was für eine besondere Sensitivität dieses Gens gegenüber Eisen spricht. Fe-NTA zeigte keinen Einfluss auf die Reparatur induzierter DNA-Schäden in HT29 clone 19A-Zellen. Interessant war jedoch der synergistische Effekt nicht genotoxischer Eisendosen im Hinblick auf 4-Hydroxynonenal induzierte DNA-Schäden.

Ergebnisse und Schlussfolgerungen Uran: U-NTA inhibierte das Wachstum von HT29 clone 19A-Zellen bereits bei im Vergleich zu Eisen geringen Konzentrationen. In hohen Konzentrationen wirkte U-NTA genotoxisch, wobei die Karzinomzellen signifikant weniger sensitiv waren als primäre Kolonepithelzellen und Kolonadenomzellen. In primären Kolonzellen und LT97-Zellen reagierte das *TP53*-Gen besonders sensitiv. In den LT97-Zellen induzierten 0,5-2 mM U-NTA chromosomale Veränderungen in den Chromosomen 5, 12 und 17, die die tumorrelevanten Gene *APC*, *K-RAS* und *TP53* tragen. Dies ist einer der ersten Arbeiten, in der es mittels 24-Farben-FISH gelang das klastogene Potential von Risikosubstanzen, in unserem Fall Uran, *in vitro* in humanen Kolonzellen nachzuweisen.

Ergebnisse und Schlussfolgerungen komplexe Gewässerproben: Die kontaminierten Wasserproben haben das Potential zur Bildung von ROS und schädigen die DNA humaner Kolonzellen, wobei das Tumorsuppressorgen *TP53* eine hohe Sensitivität aufweist. Das Wachstum der Kolonkarzinomzellen HT29 clone 19A wurde durch die am stärksten verunreinigte Probe gehemmt. Erreichen Verbindungen wie Uran, Eisen, Nickel oder Cadmium die humane Nahrungskette können sie das genotoxische Belastungspotential für die Bevölkerung erhöhen.

Ergebnisse und Schlussfolgerungen Apfelextrakt: Der Apfelextrakt verminderte die genotoxischen Wirkungen der Peroxide H_2O_2 und Cum-OOH. Den durch BPDE induzierten DNA-Schäden konnte in den Adenomzellen entgegen gewirkt werden, in den Kolonkarzinomzellen wirkte der Apfelextrakt jedoch pro-oxidativ. Ebenfalls pro-oxidativ wirkte der polyphenolreiche Extrakt bezüglich Fe-NTA-induzierter Schäden. Der Verzehr polyphenolreicher Obst- und Gemüsesorten, wie z. B. Äpfel ist effektiver Schutz gegenüber ausgewählten Kolonrisikofaktoren.

6.2 Summary

Backround: Previous extensive uranium mining in the „Wismut“ region of Ronneburg (Thuringia, Germany) has led to an enhanced environmental distribution of heavy metals and radionuclids. The migration of radionuclids and uranyl compounds from uranium mining is associated with the transfer of these compounds into the food chain and water cycle. It is necessary to investigate the effects of the flow of these compounds to the biosphere. The radionuclides and heavy metals in the geological compartiments are able to migrate into animal and human target cells. Through the formation of ROS and other mechanisms they lead to special interactions in these cells.

Objektive: Thus, the objective of the present investigations was to characterize the local and systemic actions of complex contaminated water samples from the former uranium mining area around Ronneburg (Thuringia, Germany) and the single noxes iron and uranium in human colon cells and leucocytes to obtain fundamental findings about the involved mechanisms. Furthermore we wanted to know, if apple polyphenols are chemopreventive against iron and other genotoxins.

Material and Methods: In human colon cells with different stages of transformation (primary human colon epithelial cells, colon adenoma cells, colon carcinoma cells) and partial in human leucocytes we investigested concentration-effect-relationships (DAPI-Assay), the potential to generate ROS (ROS Assay, FOX Assay), the GSH homeostasis (fluorescence photometrical assay), the genotoxic potenital (Comet Assay) of the single noxes Fe-NTA and U-NTA and of complex water samples and their influence on the repair of induced damage (Comet Assay with repair). Additional studies dealed with the question if iron, uranium and the complex environmental samples induce DNA damage in specific genes or chromosomes (Comet FISH, 24-colour-FISH). With the Challenge Assay we measured the chemopreventive potential of a polyphenol rich apple extract in human colon cells.

Results and conclusions iron: Lower, physiological concentrations could promote tumor cell growth. In contrast, high concentrations of Fe-NTA inhibited cell growth, enhanced GSH and resulted in a significant time-dependent formation of ROS. Fe-NTA was genotoxic in all cell types (primary human colon epithelial cells, LT97 colon adenoma cells, HT29 clone 19A colon carcinoma cells). In primary colon cells, LT97 cells and human leucocytes it was especially sensitive in the region around the tumor

suppressor gene *TP53*. The repair of DNA damage induced due to other genotoxic compounds in HT29 clone 19A cells was not influenced. An interesting effect of low, non genotoxic concentrations of iron was the synergistic action with HNE.

Results and conclusions uranium: In comparison to Fe-NTA smaller concentrations of U-NTA are necessary to inhibit the growth of HT29 clone 19A cells. High concentrations were genotoxic, whereas the carcinoma cell line was less sensitive than the adenoma cell line and primary colon cells. In primary colon cells and LT97 cells the *TP53* gene was especially sensitive. In LT97 cells 0.5-2 mM U-NTA increased chromosomal aberrations in chromosomes 5, 12 and 17, which harbour the tumour-related genes *APC, K-RAS* and *TP53*. To our knowledge, this is one of the first reports on a cellular *in vitro* system utilizing human colon cells to investigate the clastogenic potential of risk compounds, in our case induced by uranium.

Results and conclusions complex water samples: The contaminated water samples and thus the related compounds have the potential to produce ROS and damage global and *TP53*-specific DNA. Non cytotoxic concentrations of the strongly contaminated water sample inhibited the growth of HT29 clone 19A cells. Thus, if compounds like uranium, iron, nickel or cadmium reach the nutritional cycle via contaminated water or food they can increase the risk of alimentary genotoxic exposure in the population.

Results and conclusions apple extract: The apple extract depleted effective the genotoxic action of the two peroxides H_2O_2 and Cum-OOH. The extract could reduce BPDE-induced DNA damage in LT97 cells, whereas in HT29 carcinoma cells the extract was pro-oxidative. ASO2 was also pro-oxidative in the case of Fe-NTA-induced DNA damage. The consumption of polyphenol rich fruits and vegetables for examples apples can protect from selective colon cancer risk factors.

LITERATURVERZEICHNIS

Abalea V, Cilliard J, Dubos MP, Anger JP, Cilliard P & Morel I (1998) Iron-induced oxidative DNA damage and its repair in primary rat hepatocyte culture. *Carcinogenesis* **19**, 1053-1059.

Abrahamse SL, Pool-Zobel BL & Rechkemmer G (1999) Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration in isolated rat colon cells. *Carcinogenesis* **20**, 629-634.

Akagawa M, Shigemitsu T & Suyama K (2003) Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. *Biosci Biotechnol Biochem* **67**, 2632-2640.

Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ & Young J (2001) Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* **85**, 692-696.

Arand M & Oesch F (2004) Fremdstoffmetabolismus. In *Lehrbuch der Toxikologie*, pp. 89-116 [H Marquardt and S Schäfer, Hrsg.]. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH.

ATSDR (Agency for Toxic Substances and Disease Registry) (1999) *Toxicological profile for uranium.* Atlanta, GA: US Public Health Service, Department of Health & Human Services.

Au WW, McConnell HM, Wilkinson GS, Ramanujam VMS & Alcock N (1998) Population monitoring: experience with residents exposed to uranium mining / milling waste. *Mut Res* **405**, 237-245.

Augeron C & Laboisse CL (1984) Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. *Cancer Res* **44**, 3961-3969.

Babbs CF (1992) Oxygen radicals in ulcerative colitis. *Free Radic Biol Med* **13**, 169-181.

Bergmann H, Machelett B, Leinhos V & Eckert H (1996) Effect of natural amino alcohols on stress tolerance and food quality in stressed crops. Royal Soc. of Chem. *Royal Soc Chem (UK)* **179**, 71-74.

Bergmann H, Machelett B, Lippmann B & Friedrich Y (2001) Influence of heavy metals on the accumulation of trimethylglycine, putrescine and spermine in food plants. *Amino Acids* **20**, 325-329.

Bergmann H, Machelett B, Lippmann B, Tiroke S & Rahn K (1999) Influence of heavy metals on contents of non-protein amino acids and polyamines in food plants. *Amino Acids* **17**, 100.

Beyer-Sehlmeyer G, Glei M, Hartmann F, Hughes R, Persin C, Böhm V, Rowland IR, Schubert R, Jahreis G & Pool-Zobel BL (2003) Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources. *Br J Nutr* **90**, 1057-1070.

Bingham SA, Day NE, Luen R*, et al.* (2003) Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *The Lancet* **361**, 1496-1501.

Block G, Patterson B & Subar A (1992) Fruit vegetables, and cancer prevention: A review of the epidemiological evidence. *Nutr Cancer* **18**, 1-29.

Bock C, Rapp A, Dittmar H, Monajembashi S & Greulich KO (1999) Localisation of specific sequences and DNA single strand breaks in individual UV-A irradiated human lymphocytes by COMET FISH. *J Biomed Opt* **3568**, 207-217.

Bos JL (1989) ras oncogenes in human cancer: a review. *Cancer Res* **49**, 4682-4689.

Boutron-Ruault M-C, Senesse P, Méance S, Belghiti C & Faivre J (2001) Energy intake, body mass index, physical activity and the colorectal adenoma-carcinoma sequence. *Nutr Cancer* **39**, 50-57.

Bouwer EJ, McKlveen JW & McDowell WJ (1978) Uranium assay of phosphate fertilizers an other phosphatic materials. *Health Phys* **34**, 345-352.

Boyle P & Ferlay J (2005) Cancer incidence and mortality in Europe, 2004. *Ann Oncol* **16**, 481-488.

Bray TM & Taylor CG (1995) Tissue glutathione, nutrition and oxidative stress. *Can J Physiol Pharmacol* **71**, 746-751.

Breuer-Katschinski B, Nemes K, Marr A, Rump B, Leiendecker B, Breuer N & Goebel H (2001) Colorectal adenomas and diet: a case-control study. Colorectal Adenoma Study Group. *Dig Dis Sci* **46**, 86-95.

Briviba K, Pan L & Rechkemmer G (2002) Red Wine Polyphenols Inhibit the Growth of Colon Carcinoma Cells and Modulate the Activation Pattern of Mitogen-Activated Protein Kinases. *J Nutr* **132**, 2814-2818.

Burkart W (1991) *Uranium, thorium and decay products.* Metals and their compounds in the environment, occurence, analysis and biological relevance. Waldheim: VCH Verlagsgesellschaft.

Cai L, Tsiapalis G & Cherian G (1998) Protective role of zinc-metallothionein on DNA damage in vitro by ferric nitrilotriacetate (Fe-NTA) and derric salts. *Chem Biol Interact* **115**, 141-151.

Cao G, Sofic E & Prior RL (1997) Antioxidant and prooxidant behaviour of flavonoids: structure-activity relationships. *Free Radic Biol Med* **22**, 749-760.

Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, Franklin RA & McCubrey JA (2003) Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* **17**, 1263-1293.

Chen JJ & Yu BP (1994) Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radic Biol Med* **17**, 411-418.

Chin TA & Templeton DM (1993) Protective elevation of glutathione and metallothionein in cadmium-exposed mesangial cells. *Toxicology* **77**, 145-156.

Chinnici F, Gaiani A, Natali N, Riponi C & Galassi S (2004) Improved HPLC determination of phenolic compounds in cv. golden delicious apples using a monolithic column. *J Agric Food Chem* **52**, 3-7.

Christmann M, Tomicic MT, Roos WP & Kaina B (2003) Mechanisms of human DNA repair: an update. *Toxicology* **193**, 3-34.

Clifford MN (2004) Diet-derived phenols in plasma and tissue and their implications for health. *Planta Med* **70**, 1103-1114.

Cooke MS, Evans MD, Dizdaroglu M & Lunec J (2003) Oxidative DNA damage: mechanisms, mutation, and desease. *FASEB J* **17**, 1195-1214.

Crawford D, Zbinden I, Amstad P & Cerutti PA (1988) Oxidant stress induces the proto-oncogenes c-*fos* and c-*myc* in mouse epidermal cells. *Oncogene* **3**, 27-32.

Cremer C, Munkel C, Granzow M*, et al.* (1996) Nuclear architecture and the induction of chromosomal aberrations. *Mut Res* **366**, 97-116.

Dally H & Hartwig A (1997) Induction and repair inhibition of oxidative DNA damage by nickel (II) and cadmium (II) in mammalian cells. *Carcinogenesis* **18**, 1021-1026.

Dang HS & Pullat VR (1993) Normal concentration and excretion ratio of uranium in serum of normal indiviuals in India. *Health Phys* **65**, 303-305.

Davies RJ, Miller R & Coleman N (2005) Colorectal cancer screening: prospects for molecular stool analysis. *Nat Rev Cancer* **5**, 199-209.

Deloukas et al. (1998) A physical map of 30,000 human genes. *Science* **282**, 744-746.

Dias AS, Porawski M, Alonso M, Marroni N, Collado PS & Gonzalez-Gallego J (2005) Quercetin decreases oxidative stress, NF-kappaB activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. *J Nutr* **135**, 2299-2304.

Dickinson DA & Forman HJ (2002) Cellular glutathione and thiols metabolism. *Biochem Pharmacol* **64**, 1019-1026.

Diculescu GL (1997) The sources of variation in the human genome and genome instability in human cancers. *Rom J Physiol* **34**, 3-17.

Dryden GW, Song M & McClain C (2006) Polyphenols and gastrointestinal diseases. *Curr Opin Gastroenterol* **22**, 165-170.

Duthie SJ, Collins AR, Duthie GG & Dobson VL (1997a) Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. *Mutat Res* **393**, 223-231.

Duthie SJ & Dobson VL (1999) Dietary flavonoids protect human colonocyte DNA from oxidative attack *in vitro*. *Eur J Nutr* **38**, 28-34.

Duthie SJ, Johnson W & Dobson VL (1997b) The effect of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimidines) and growth in human cells. *Mutat Res* **390**, 141-151.

Ebert MN, Beyer-Sehlmeyer G, Liegibel UM, Kautenburger T, Becker TW & Pool-Zobel BL (2001) Butyrate-induces glutathione S-transferase in human colon cells and protects from genetic damage by 4-hydroxynonenal. *Nutr Canc* **41**, 156-164.

Ebert MN, Klinder A, Schäferhenrich A, Peters WHM, Sendt W, Scheele J & Pool-Zobel BL (2003) Expression of glutathione S-transferases (GST) in human colon cells and inducibility of GSTM2 by butyrate. *Carcinogenesis* **24**, 1637-1644.

Esterbauer H, Eckl P & Ortner A (1990) Possible mutagens derived from lipids and lipid precursors. *Mutat Res* **238**, 223-233.

Fearon ER (1994) Molecular genetic studies of the adenoma-carcinoma sequence. *Adv Intern Med* **39**, 123-147.

Fearon ER & Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* **61**, 759-767.

Fisenne IM, Perry PM, Decker KM & Keller HW (1987) The daily intake of $^{234, 235, 238}$U, $^{228, 230, 232}$Th and $^{226, 228}$Ra by New York City residents. *Health Phys* **53**, 357-364.

Fodde R, Smits R & Clevers H (2001) APC signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer* **1**, 55-67.

Fogh J & Trempe X (1975) Human Tumor Cells in Vitro. In *Human tumor cells in vitro*, pp. 115-159 [J Fogh, editor]. New York: Plenum Press.

Franklin SJ (2001) Lanthanide-mediated DNA hydrolysis. *Curr Opin Chem Biol* **5**, 201-208.

Giang N, Shiraishi K, Sinh NM, Kimura S, Tuan NN & Arae H (2001) Estimation of dietary ^{232}Th, ^{238}U, cesium, and strontium intakes in Vietnamese peoples from different geographical regions. *Health Phys* **80**, 605-611.

Glei M, Klenow S, Sauer J, Wegewitz U, Richter K & Pool-Zobel BL (2006) Hemoglobin and hemin induce DNA damage in human colon tumor cells HT29 clone 19A and in primary human colonocytes. *Mutat Res* **594**, 162-171.

Glei M, Klinder A, Latunde-Dada GO, Becker TW, Hermann U, Voigt K & Pool-Zobel BL (2002) Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A . *Mutat Res* **519**, 151-161.

Goddard JG & Sweeney GD (1983) Fe-NTA: a potent stimulant of *in vivo* lipid peroxidation in mice. *Biochem Pharmacol* **32**, 3879-3882.

Graziani G, D´ Argenio G, Tuccillo C, Loguercio C, Ritieni A, Morisco F, Del Vecchio Blanco C, Fogliano V & Romano M (2005) Apple polyphenol extracts prevent damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo. Gut* **54**, 193-200.

Griffith OW (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med* **27**, 922-935.

Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, Joslyn G, Stevens J & Spirio L (1991) Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66**, 589-600.

Hahn SA, Schutte M, Hoque AT*, et al.* (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**, 350-353.

Halliwell B & Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals, and disease. *Biochem J* **219**, 1-14.

Hamilton MM, Ejnik JW & Carmichael AJ (1997) Uranium reactions with hydrogen peroxide studies by EPR-spin trapping with DMPO. *J Chem Soc Perkin Trans* **2**, 2491-2494.

Hammer A, Ferro M, Tillian HM, Tatzber F, Zollner H, Schauenstein E & Schaur RJ (1997) Effect of oxidative stress by iron on 4-hydroxynonenal formatopn and proliferative activity in hepatomas of different degrees of differentiation. *Free Radic Biol Med* **23**, 26-33.

Hartmann AM & Speit G (1996) Effect of arsenic and cadmium on the persistence of mutagen-induced DNA lesions in human cells. *Environ Mol Mutagen* **27**, 98-104.

Hartwig A, Asmuss M, Ehleleben I, Herzer U, Kostelac D, Pelzer A, Schwerdtle T & Burkle A (2002) Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanism. *Environ Health Perspect* **110**, 797-799.

Hartwig A (1994) Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: a review. *Environ Health Perspect* **102**, 45-50.

Hartwig A (1998) Carcinogenity of metal compounds: possible role of DNA repair inhibition. *Toxicol Lett* **28**, 235-239.

Hartwig A & Beyersmann D (1989a) Comutagenicity and inhibition of DNA repair by metal ions in mammalian cells. *Biol Trace Elem Res* **21**, 359-365.

Hartwig A & Beyersmann D (1989b) Enhancement of UV-induced mutagenesis and sister-chromatid exchanges by nickel ions in V79 cells: evidence for inhibition of DNA repair. *Mutat Res* **217**, 65-73.

Hartwig A, Klyszcz-Nasko H, Schlepegrell R & Beyersmann D (1993) Cellular damage by ferric nitrilotriactate and ferric citrate in V79 cells: interrelationship between lipid peroxidation, DNA strand breaks and sister chromatid exchanges. *Carcinogenesis* **14**, 107-112.

Hartwig A & Schlepegrell R (1995) Induction of oxidative DNA damage by ferric iron in mammalian cells. *Carcinogenesis* **16**, 3009-3013.

Hartwig A, Schlepegrell R, Dally H & Hartmann M (1996) Interaction of carcinogenic metal compounds with deoxyribonucleic acid repair process. *Ann Clin Lab Sci* **26**, 31-38.

Hartwig A & Schwerdtle T (2002) Interactions by carcinogenic metal compounds with DNA repair processes: toxicological implications. *Toxicol Lett* **127**, 47-54.

Hata Y, Kawabe T, Hiraishi H, Ota S, Terano A & Ivey KJ (1997) Hydrogen peroxide-mediated cytotoxicity to cultured colonic epithelial cells. *Life Sci* **60**, 2221-2230.

HVBG (Hauptverband der gewerblichen Berufsgenossenschaften) (1997) *Fachkolloquium: Berufsgenossenschaftliche Aktivitäten und Forschungsvorhaben zum Thema Wismut - Erkenntnisstand und Perspektiven*. Sankt Augustin: DCM-Druck Center Meckenheim.

Hayes JD, Flanagan JU & Jowsey IR (2005) Glutathione transferases. *Annu Rev Pharmocol Toxicol* **45**, 51-88.

Hei TK, Liu SX & Waldren C (1998) Mutagenicity of arsenic in mammalian cells: role of ractive oxygen species. *Proc Natl Acad Sci USA* **95**, 8103-8107.

Hermann-Kunz E & Thamm M (1999) Dietary recommendations and prevailing food and nutrition intakes in Germany. *Br J Nutr* **81**, S61-S69.

Herrinton LJ, Friedman GD, Baer D & Selby JV (1995) Tranferrin saturation and risk of cancer. *Am J Epidemiol* **142**, 692-698.

Hirayama K & Yasutake A (1998) Free radicals and trace elements. *J Trace Elem Exp Med* **11**, 209-217.

Hong WK & Sporn MB (1997) Recent advances in chemoprevention of cancer. *Science* **278**, 1073-1078.

Horvathova K, Novotny L & Vachalkova A (2003) The free radical scavenging activity of four flavonoids determined by the comet assay. *Neoplasma* **50**, 291-295.

Hu Q & Shoupeng Z (1990) Induction of chromosomal aberrations in male mouse germ cells by uranyl fluoride containing enriched uranium. *Mutat Res* **244**, 209-214.

Hu X, Herzog C, Zimniak P & Singh SV (1999) Differential protection against benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide-induced DNA damage in HepG2 Cells stably transfected with allelic variants of π class human glutathone S-transferases. *Cancer Res* **59**, 2358-2362.

Hughes R, Cross AJ, Pollock JRA & Bingham S (2001) Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation. *Carcinogenesis* **22**, 199.

Hussain SP, Raja K, Amstad PA*, et al.* (2000) Increased p53 mutation load in non-tumorous human liver of Wilson disease and hemochromatosis: Oxyradical overload diseases. *Med Sci* **97**, 12770-12775.

IARC (International Agency for Research on Cancer) (1994) Monographs volumes 1-60.

Imlay J, Chin SM & Linn S (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science*, 640-642.

Iqbal M, Giri U & Athar M (1995) Ferric nitrilotriacetate (Fe-NTA) is a potent hepatic tumor promotor and acts through the generation of oxidative stress. *Biochem Biophys Res Commun* **212**, 557-563.

Izak-Biran T, Schlesinger T, Weingarten R, Even O, Shamai Y & Israeli M (1989) Concentrations of uranium and polonium in animal feed supplements, in poultry meat and in eggs. *Health Phys* **56**, 315-320.

Jacob P, Pröhl G, Schneider K & Voß J-U (1997) *Machbarkeitsstudie zur Verknüpfung der Bewertung radiologischer und chemisch-toxischer Wirkungen von Altlasten.* Berlin: Umweltbundesamt.

Jaeger J, Sorensen K & Wolff SP (1994) Peroxide accumulation in detergents. *J Biochem Biophys Methods* **29**, 77-81.

Jaworowski Z & Grzybowska D (1996) Natural radionuclides in industrial and rural soils. *Sci Total Environ* **7**, 45-52.

Jen J, Powell SM, Papadopoulos N, Smith KJ, Hamilton SR, Vogelstein B & Kinzler KW (1994) Molecular determinants of dysplasia in colorectal lesions. *Cancer Res* **54**, 5523-5526.

Johnson KL, Brenner DJ, Nath J, Tucker JD & Geard CR (1999) Radiation-induced breakpoint misrejoining in human chromosomes: random or non-random? *Int J Radiat Biol* **75**, 131-141.

Joossens JV & Kesteloot H (2001) Epidemiology of cancer mortality. *Verh K Acad Geneeskd Belg* **63**, 123-135.

Jostes RE (1996) Genetic, cytogenetic, and carcinogenic effects of radon: a review. *Mutat Res* **340**, 125-139.

Kathren RL (2001) Tissue studies of persons with intakes of the actinide elements: the U.S.transuranium and uranium registries. *Occup Med* **16**, 317-329.

Kawabata T, Awai M & Kohno M (1986) Generation of active oxygen species by iron nitrilotriacetate (Fe-NTA). *Acta Med Okayama* **40**, 163-173.

Kim YI & Mason JB (1996) Nutrition chemoprevention of gastrointestinal cancers: a critical review. *Nutr Rev* **54**, 259-279.

Kinzler KW & Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170.

Klinder A, Gietl E, Hughes R*, et al.* (2004) Gut fermentation products of inulin-derived prebiotics inhibit markers of tumour progression in human colon tumour cells. *Int J Cancer Prev* **1**, 19-32.

Knekt P, Jarvinen R, Seppanen R & et al. (1997) Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *Am J Epidemiol* **146**, 223-230.

Knekt P, Reunanen A, Takkunen H, Aromaa A, Heliovaara M & Hakulinen T (1994) Body iron stores and risk of cancer. *Int J Cancer* **56**, 379-382.

Knoll N, Weise A, Claussen U, Sendt W, Marian B, Glei M & Pool-Zobel BL (2006) 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary human colon cells and in cells from preneoplastic lesions. *Mutat Res* **594**, 10-19.

Ko LJ & Prives C (1996) p53: puzzle and paradigm. *Genes Dev* **10**, 1054-1072.

Kuechler A, Weise A, Michel S, Schäferhenrich A, Pool-Zobel BL, Claussen U & Liehr T (2003) Precise breakpoint characterization of the colon adenocarcinoma cell line HT-29 clone 19A by means of 24-color FISH and multicolor banding (MCB). *Genes Chromosomes Cancer* **36**, 207-210.

Kune GA & Vitetta L (1992) Alcohol consumption and the etiology of colorectal cancer: A review of the scientific evidence from 1957 to 1991. *Nutr Cancer* **18**, 97-111.

Kuntz S, Wenzel U & Daniel H (1999) Comparative analysis of the effcts of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. *Eur J Nutr* **38**, 133-142.

Küppers C & Schmidt G (1994) Strahlenschutzaspekte bei Altlasten des Uranbergbaus in Thüringen und Sachsen. Öko-Institut e. V.. *Werkstattreihe* **86**.

La Vecchia C, Franceschi S & Levi F (2003) Epidemiological research on cancer with a focus on Europe. *Eur J Cancer Prev* **12**, 5-14.

Lamb N, Quinlan GJ, Mumby S, Evans TW & Gutteridge MC (1999) Haem oxygenase shows pro-oxidant activity in microsomal and cellular systems: implications for the release of low-molecular-mass iron. *Biochem J* **344**, 153-158.

Lane DP (1992) Cancer. *TP53*, guardian of the genome. *Nature* **358**, 15-16.

Layton DW, Bogen KT, Knize MG, Hatch FT, Johnson VM & Felton JS (1995) Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research. *Carcinogenesis* **16**, 39-52.

Leinhos V & Bergmann H (1995) Changes in yield, lignin content and protein pattern of barley (Hordeum vulgare cv. Alexis) induced by drought stress. *J Appl Bot* **69**, 206-210.

Lengauer C, Kinzler KW & Vogelstein B (1997) Genetic instability in colorectal cancers. *Nature* **386**, 623-627.

Leslie A, Carey FA, Pratt NR & Steele RJ (2002) The colorectal adenoma-carcinoma sequence. *Br J Surg* **89**, 845-860.

Levi F, Pasche C, Lucchini F & La Vecchia C (2000) Selected micronutrients and colorectal cancer, a case-control study from the canton of Vaud, Switzerland. *Eur J Cancer* **36**, 2115-2119.

Leyval C, Turnau K & Haselwandter K (1997) Effects of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza* **7**, 139-153.

Li W & Chou I-N (1992) Effects of arsenite on the cytoskeleton and cellular glutathione levels in cultured cells. *Toxicol Appl Pharmacol* **114**, 132-139.

Lin JK (2002) Cancer chemoprevention by tea polyphenols through modulating signal transduction pathways. *Arch Pharm Res* **25**, 561-571.

Lin RH, Wu LJ, Lee CH & Lin-Shiau SY (1993) Cytogenetic toxicity of uranyl nitrate in Chinese hamster ovary cells. *Mutat Res* **319**, 197-203.

Lloyd DC, Lucas JN, Edwards AA, Deng W, Valente E, Hone PA & Moquet JE (2002) A study to verify a reported excess of chromosomal aberrations in blood lymphocytes of Namibian uranium miners. *Radiat Res* **155**, 809-817.

Long LH, Clement MV & Halliwell B (2000) Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Biophys Res Commun* **273**, 50-53.

Lund EK, Wharf SG, Fairweather-Tait SJ & Johnson IT (1998) Increases in the concentrations of available iron in response to dietary iron supplementation are associated with changes in crypt cell proliferation in rat large intestine. *J Nutr* **128**, 175-179.

Lund EK, Wharf SG, Fairweather-Tait SJ & Johnson IT (1999) Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. *Am J Clin Nutr* **69**, 250-255.

Machelett B, Brandt D, Schubert R & Bergmann H (1996) Heavy metal accumulation in food plants of contaminated soils studied in Phaseolus vulgaris L. *Zeitschrift für Ernährungswiss* **35**, 87.

Manach C, Scalbert A, Morand C, Rémésy C & Jiménez L (2004) Polyphenols: Food sources and bioavailability. *Am J Clin Nutr* **79**, 727-747.

Martinez-Aguirre A, Garciá-Leon M & Ivanovich M (1994) U and Th distribution in solution and suspended matter from rivers affected by phosphate rock processing in southwestern Spain. *Nucl Instr Meth* **A339**, 287-293.

May P & May E (1999) Twenty years of *TP53* research: structural and functional aspects of the *TP53* protein. *Oncogene* **18**, 7621-7636.

McCormick F (1995) Ras-related proteins in signal transduction and growth control. *Mol Reprod Dev* **42**, 500-506.

McDiarmid MA, Keogh JP, Hooper FJ*, et al.* (2000) Health Effects of dpleted uranium on exposed gulf war veterans. *Environ Res Section A* **82**, 168-180.

McKay BC, Chen F, Perumalswami CR, Zhang F & Ljungman M (2000) The tumor suppressor p53 can both stimulate and inhibit ultraviolet light-induced apoptosis. *Mol Biol Cell* **11**, 2543-2551.

McKelvey-Martin VJ, Ho ET, McKeown SR, Johnston SR, McCarthy PJ, Rajab NF & Downes CS (1998) Emerging applications of the single cell gel electrophoresis (Comet) assay. I. Management of invasive transitional cell human bladder carcinoma. II Fluorescent in situ hybridization Comets for the identification of damaged and repaired DNA sequences in individual cells. *Mutagenesis* **13**, 1-8.

McKenna DJ, Gallus M, McKeown SR, Downes CS & McKelvey-Martin VJ (2003a) Modification of the alkaline Comet assay to allow simultaneous evaluation of mitomycin C-induced DNA cross-link damage and repair of specific DNA sequences in RT4 cells. *DNA Repair (Amst)* **2**, 879-890.

McKenna DJ, Rajab NF, McKeown SR, McKerr G & McKelvey-Martin VJ (2003b) Use of the comet-FISH assay to demonstrate repair of the *TP53* gene region in two human bladder carcinoma cell lines. *Radiat Res* **159**, 49-56.

Meinrath A, Schneider P & Meinrath G (2003) Uranium ores and depleted uranium in the environment, with a reference to uranium in the biosphere from Erzgebirge/Sachsen, Germany. *J Environ Radioact* **64**, 175-193.

Mendelsohn JEd (2001) *The Molecular Basis of Cancer. 2nd Ed. Saunders Company Philadelphia.*

Meneghini R (1997) Iron homeostasis, oxidative stress, and DNA damage. *Free Radic Biol Med* **23**, 783-792.

Menke M, Meister A & Schubert I (2000) N-Methyl-N-nitrosourea-induced DNA damage detected by the comet assay in Vicia faba nuclei during all interphase stages is not restricted to chromatid aberration hot spots. *Mutagenesis* **15**, 503-506.

Michels KB, Fuchs CS, Giovannucci E, Colditz GA, Hunter DJ, Stampfer MJ & Willet WC (2005) Fiber intake and incidence of colorectal cancer among 76.947 women and 47.279 men. *Cancer Epidemiol Biomarkers Prev* **14**, 842.

Miller AC, Blakely WF, Livengood D*, et al.* (1998) Transformation of human osteoblast cells to the tumorigenic phenotype by depleted uranium-uranyl chloride. *Environ Health Perspect* **106**, 465-471.

Miller AC, Stewart M, Brooks K, Shi L & Page N (2002a) Depleted uranium-catalyzed oxidative DNA damage: absence of significant alpha particle decay. *J Inorg Biochem* **91**, 246-252.

Miller AC, Xu J, Stewart M, Brooks K, Hodge S, Shi L, Page N & McClain D (2002b) Observation of radiation-specific damage in human cells exposed to depleted uranium: dicentric frequency and neoplastic transfoermation as endpoints. *Radiat Prot Dosim* **99**, 275-278.

Miyaki M, Konishi M, Kikuchi-Yanoshita R, Enomoto M, Igari T, Tanaka K, Muraoka M, Takahashi H, Amada Y & Fukayama M (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. *Cancer Res* **54**, 3011-3020.

Mobarhan S (1999) Calcium and the colon: recent findings. *Nutr Rev* **57**, 124-129.

Mochizuki M, Yamazaki S, Kano K & Ideka T (2002) Kinetic analysis and mechanistic aspects of autoxidation of catechins. *Biochim Biophys Acta* **1569**, 35-44.

Nelson RL (2001) Iron and colorectal cancer risk: human studies. *Nutr Rev* **59**, 140-148.

Nocentini S (1987) Inhibition of DNA replication and repair by cadmium in mammalian cells. Protective interaction of zinc. *Nucleic Acids Res* **15**, 4211-4225.

Norat T, Bingham S, Ferrari P*, et al.* (2005) Meat, fish, and colorectal cancer risk: The European Prospective Investigation into Cancer and Nutrition. *J Natl Cancer Inst* **97**, 906-916.

Norat T & Riboli E (2001) Meat consumption and colorectal cancer: a review of epidemiologic evidence. *Nutr Rev* **59**, 37-47.

Norat T & Riboli E (2002) Fruit and vegetable consumption and risk of cancer of the digestive tract: meta-analysis of published case-control and cohort studies. *IARC Sci Publ* **156**, 123-125.

Okada S (2003) Prevention of free-radical mediated tissue damage and carcinogenesis induced by low-molecular-weight iron. *Biometals* **16**, 99-101.

Oren M & Rotter V (1999) Introduction: *TP53*--the first twenty years. *Cell Mol Life Sci* **55**, 9-11.

Pan Z & Godwin K (2005) *Oncogenes. Encyclopedia of Molecular Cell Biology and Molecular Medicine.* Weinheim: Wiley-VCH Verlag.

Peters U, Sinha R, Chaterjee N*, et al.* (2003) Dietary fibre and colorectal adenoma in a colorectal cancer early detection programme. *The Lancet* **361**, 1401-1405.

Pool-Zobel BL (2005) Inulin-type fructans and reduction in colon cancer risk: Review of experimental and human data. *Br J Nutr* **93**, S73-S90.

Pool-Zobel BL, Abrahamse SL, Collins AR, Kark W, Gugler R, Oberreuther D, Siegel EG, Treptow van Lishaut S & Rechkemmer G (1999) Analysis of DNA strand breaks, oxidized bases, and glutathione S-transferase P1 ih human colon cells from biopsies. *Cancer Epidemiol Biomarkers Prev* **8**, 609-614.

Pool-Zobel BL, Van Loo J, Rowland IR & Roberfroid MB (2002) Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer. *Br J Nutr* **87**, S273-S281.

Pool-Zobel BL, Veeriah S & Böhmer F-D (2005a) Modulation of xenobiotic metabolising enzymes by anticarcinogens - focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. *Mutat Res* **591**, 74-92.

Pool-Zobel BL, Veeriah S, Kautenburger T, Kiefer J, Richter KK, Sauer J, Soom M & Wölfl S (2005b) Butyrate may enhance toxicological defence in primary, adenoma and tumour human colon cells by favourably modulating expression of glutathione S-transferases genes. *Carcinogenesis* **26**, 1064-1076.

Popp W, Plappert U, Müller WU*, et al.* (2000) Biomarkers of genetic damage and inflammation in blood and brochoalveolar lavage fluid among former German uranium miners: A pilot study. *Radiat Environ Biophys* **39**, 275-282.

Potter JD (1999) Colorectal cancer: Molecules and populations. *J Natl Cancer Inst* **91**, 916-932.

Prabhavathi PA, Fatima SK, Rao S & Reddy PP (2000) Analysis of chromosomal aberration frequencies in the peripheral blood lymphocytes of smokers exposed to uranyl compounds. *Mutat Res* **466**, 37-41.

Quig D (1998) Cysteine metabolism and metal toxicity. *Altern Med Rev* **3**, 262-270.

Rapp A, Bock C, Dittmar H & Greulich KO (1999) Comet-FISH used to detect UV-A sensitive regions in the whole human genome and on chromosome 8. *Neoplasma* **46**, S99-S101.

Rapp A, Bock C, Dittmar H & Greulich KO (2000) UV-A breakage sensitivity of human chromosomes measured by COMET-FISH depends on gene density and not on the chromosome size. *J Photochem Photobiol* **53**, 109-117.

Rapp A, Havenaar R, Pool-Zobel BL & Greulich KO (2001) Comet Assay and Comet FISH for the detection of individual radiation and toxine sensitivities of genome regions. In *Strahlenbiologie und Strahlenschutz - Individuelle Strahlenempfindlichkeit und ihre Bedeutung für den Strahlenschutz*, 114-131 [G Geinemann and WV Müller, Hrsg.]. Köln: TUV Rheinland.

Rashid A, Zahurak M, Goodman SN & Hamilton SR (1999) Genetic epidemiology of mutated *KRAS* proto-oncogene, altered suppressor genes, and microsatellite instability in colorectal adenomas. *Gut* **44**, 826-833.

Rice-Evans CA, Miller NJ & Paganga G (1996) Structure-antioxidant activity relationship of flavonoids and phenolic acids. *Free Radic Biol Chem* **20**, 933-956.

Rice-Evans CA, Miller NJ & Paganga G (1997) Antooxidant properties of phenolic compounds. *Trend Plant Sci* **2**, 152-159.

Richardson DR & Ponka P (1997) The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochim Biophys Acta* **1331**, 1-40.

Richter M, Jurek D, Wrba F, Kaserer K, Wurzer G, Karner-Hanusch J & Marian B (2002) Cells obtained from colorectal microadenomas mirror early premalignant growth patterns *in vitro. Eur J Cancer* **38**, 1937-1945.

Robert Koch Institut (2003) *Dachdokumentation Krebs, Interaktives Datenangebot zur Epidemiologie von Krebserkrankungen in Deutschland.* Berlin.

Robert Koch Institut (2006) *Dachdokumentation Krebs, Interaktives Datenangebot zur Epidemiologie von Krebserkrankungen in Deutschland.* 5. überarbeitete Auflage. Saarbrücken.

Roig R, Cascon E, Arola L, Blade C & Salvado MJ (2002) Procyanidins porotect Fao cells against hydrogen peroxide-induced oxidative stress. *Biochim Biophys Acta* **1572**, 25-30.

Roques SC, Landrault N, Teissédre P-L, Laurent C, Besancon P, Rouanet J-M & Caporiccio B (2002) Hydrogen peroxide generation in Caco-2 cell culture medium by addition of phenolic compounds: effect of ascorbic acid. *Free Radic Res* **36**, 593-599.

Rumble MA & Bjugstad AJ (1986) Uranium and radium concentrations in plants growing on uranium mill tailings in South Dakota, USA. *Recl Revegetat Res* **4**, 271-278.

Sahu SC & Gray GC (1993) Interactions of flavonoids, trace metals, and oxygen: nuclear DNA damage and lipid peroxidation induced by myricetin. *Cancer Lett* **70**, 73-79.

Sakurai K & Cederbaum A (1998) Oxidative stress and cytotoxicity induced by ferric-nitrilotriacetate in HepG2 cells that express Cytochrome P450 2E1. *Mol Pharmacol* **54**, 1024-1035.

Sandhu MS, White IR & McPherson K (2001) Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. *Cancer Epidemiol Biomarkers Prev* **10**, 439-446.

Santos SJ, Singh NP & Natarajan AT (1999) Fluorescence *in situ* hybridization with comets. *Exp Cell Res* **232**, 407-411.

Scalbert A & Williamson G (2000) Dietary intake and bioavailability of polyphenols. *J Nutr* **130**, 2073S-2085S.

Schäferhenrich A (2004) Untersuchungen zu Mechanismen der ernährungsbedingten Kolonkarzinogenese: Genspezifische Schäden in humanen Kolonzellen durch endogene Risikofaktoren. Dissertation an der Friedrich-Schiller Universität Jena.

Schäferhenrich A, Beyer-Sehlmeyer G, Festag G*, et al.* (2003a) Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. *Mutat Res* **9496**, 1-14.

Schäferhenrich A, Sendt W, Scheele J, Kuechler A, Liehr T, Claussen U, Rapp A, Greulich K-O & Pool-Zobel BL (2003b) Putative colon cancer risk factors damage global DNA and *TP53* in primary human colon cells isolated from surgical samples. *Food Chem Toxicol* **41**, 655-664.

Scott N, Bell SM, Sager P, Blair GE, Dixon MF & Quirke P (1993) *TP53* expression and *KRAS* mutation in colorectal adenomas. *Gut* **34**, 621-624.

Seeber O (1998) Das Angebot, die Aufnahme und die Bilanz des toxischen Schwermetalls Uran bei erwachsenen Mischköstlern in Deutschland. Dissertation an der Friedrich-Schiller-University Jena.

Seymen O, Seven A, Candan G, Yigit G, Hatemi S & Hatemi H (1997) The effect of iron supplementation on GSH levels, GSH-Px, and SOD activities of erythrocytes in L-thyroxine administration. *Acta Med Okayama* **51**, 129-133.

Sharpe CR, Siemiatycki JA & Rachet BP (2002) The effects of smoking on the risk of colorectal cancer. *Dis Colon Rectum* **45**, 1041-1050.

Sheehan D, Meade G, Foley VM & Dowd CA (2001) Structure, function and evolution of glutathion transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J* **360**, 1-16.

Shiraishi K & Yamamoto M (1995) Dietary ^{232}Th and ^{238}U intakes for Japanese as obtained in a market basket study and contributions of importnat foods to inetrnal doses. *Radioanalyt Nuclear Chem* **196**, 89-96.

Silberberg M, Mornad C, Mathevon T, Besson C, Manach C, Scalbert A & Remesy C (2006) The bioavailability of polyphenols is highly governed by the capacity of the intestine and of the liver to secrete conjugated metabolites. *Eur J Nutr* **45**, 88-96.

Singh V, Ahmad S & Rao GS (1994) Prooxidant and antioxidant properties of iron-hydroquinone and iron-1,2,4-benzenetriol complex. Implications for benzene toxicity. *Toxicology* **89**, 25-33.

Sinha R, Kulldorff M, Chow WH, Denobile J & Rothman N (2001) Dietary intake of heterocyclic amines, meat--derived mutagenic activity, and risk of colorectal adenomas. *Cancer Epidemiol Biomarkers Prev* **10**, 559-562.

Slattery ML (2000) Diet, lifestyle, and colon cancer. *Semin Gastrointest Dis* **11**, 142-146.

Slattery ML, Anderson K, Curtin K, Khe-Ni M, Schaffer D & Samowitz W (2001) Dietary intake and microsatellite instability in colon tumors. *Int J Cancer* **93**, 601-607.

Slattery ML & Potter JD (2002) Physical activity and colon cancer: confounding or interaction? *Med Sci Sports Exerc* **34**, 913-919.

Slattery ML, Potter JD, Duncan DM & Berry TD (1997) Dietary fats and colon cancer: assessment of risk associated with specific fatty acids. *Int J Cancer* **73**, 670-677.

Slijpcevic P (2004) Is there a link between telomere maintenance and radiosensitivity? *Radiat Res* **161**, 82-86.

Smerhovsky Z, Landa K, Rossner P, Juzova D, Brabec M, Zudova Z, Hola N, Zarska H & Nevsimalova E (2002) Increased risk of cancer in radon-exposed miners with elevated frequency of chromosomal aberrations. *Mutat Res* **514**, 165-176.

Speicher MR, Ballard SG & Ward DS (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat Genet* **12**, 368-375.

Sporn MB & Newton DL (1979) Chemoprevention of cancer with retinoids. *Fed Proc* **38**, 2528-2534.

Standeven AM & Wetterhahn KE (1991) Possible role of glutathione in chromium (VI) metabolism and toxicity in rats. *Pharmacol Toxicol* **69**, 469-476.

Szeto YT & Benzie IF (2002) Effects of dietary antioxidants on human DNA *ex vivo*. *Free Radic Res* **36**, 113-118.

Takagi Y, Koumura H, Futamura M, Aoki S, Yamaguchi K, Kida H, Tanemura H, Shimokava K & Saji S (1998) Somatic alterations of the SMAD-2 gene in human colorectal cancers. *Br J Cancer* **78**, 1152-1155.

Tanaka T, Kohno H & Mori H (2001) Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. *Asian Pac J Cancer Prev* **2**, 165-177.

Taqui Khan MM & Martell AE (1969) Kinetics of metal ion and metal chelate catalyzed oxidation of ascorbic acid. IV. Uranyl ion catalyzed oxidation. *J Am Chem Soc* **91**, 4668-4672.

Thiel C, Heinemann L & Thai DM (1996) Die Ernährungssituation der erwachsenen Bevölkerung nach der Wende in den neuen Bundesländern. *Ernährungsforschung* **41**, 239-248.

Toyokuni S, Luo XP, Tanaka T, Uchida K, Hiai H & Lehotay DC (1997) Induction of a wide range of C(2-12) aldehydes and C(7-12) acylions in the kidney of Wistar rats after treatment with a renal carcinogen, ferric nitrilotriacetate. *Free Radic Biol Med* **22**, 1019-1027.

Toyokuni S, Okamoto K, Yudoi J & Hiai H (1995) Persistent oxidative stress in cancer. *FEBS Letters* **358**, 1-3.

Tracy BL, Prantl FA & Quinn JM (1983) Transfer of ^{226}Ra, ^{210}Pb and uranium from soil to garden products: assessments of risk. *Health Phys* **44**, 469-477.

Tucker JD & Senft JR (1994) Analysis of naturally occurring and radiation-induced breakpoint locations in human chromosomes 1, 2 and 4. *Radiat Res* **140**, 31-36.

UNSCEAR (United Nations Scientific Committee on the effects of Atomic Radiation) (2000) *Sources and effects of ionizing radiation.* New York:United Nations: UNSCEAR.

Veeriah S, Kautenburger T, Saauer J, Dietrich H, Will F & Pool-Zobel BL (2006) Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. *Mol Carcinog* **45**, 164-174.

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AMM & Bos JL (1988) Genetic alteration during colorectal-tumor development. *N Engl J Med* **319**, 525-532.

Vogelstein B, Lane D & Levine AJ (2000) Surfing the p53 network. *Nature* **408**, 307-310.

Voorrips LE, Goldbohm RA, van Poppel G, Sturmans F, Hermus RJ & van den Brandt PA (2000) Vegetable and fruit consumption and risk of colon and rectal cancer in a prospective cohort study: The Netherlands Cohort Study on Diet and Cancer. *Am J Epidemiol* **152**, 1081-1092.

WCFR (1997) *Food, nutrition and the prevention of cancer: a global perspective.* Washington DC: American Institute for Cancer Research.

Weinberg ED (1996) The role of iron in cancer. *Eur J Cancer Prev* **5**, 19-36.

Weir E (2004) Uranium in drinking water, naturally. *Can Med Assoc J* **170**, 951-952.

WHO (1998) *Guidelines for drinking water quality.Health criteria and other supporting information, second ed.* Geneva, Switzerland: WHO.

Wiechen A (1970) Die Bestimmung des Urangehaltes biologischer Materialien durch die Kernspaltspurenmethode am Beispiel der Milch. *Milchwirtschaft* **25**, 95-98.

Will F, Bauckhage K & Dietrich H (2004) Apple pomace liquefaction with pectinases and cellulases - Analytical data of the corresponding juices. *Eur Food Res Technol* **211**, 291-297.

Willet WC (2001) Diet and cancer: one view at the start of the Millenium. *Cancer Epidemiol Biomarkers Prev* **10**, 3-8.

Williamson G & Manach C (2005) Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* **81**, 243S-255S.

Wilms LC, Hollmann PCH, Boots AW & Kleinjans JCS (2005) Protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage and formation of BPDE-DNA adducts in human lymphocytes. *Mutat Res* **582**, 155-162.

Winston GW, Harvey W, Berl L & Cederbaum AL (1983) The generation of hydroxy and alkoxyl radicals from the interaction of ferrous bipyridyl with peroxides. *Biochem J* **216**, 421.

Witte JS, Longnecker MP, Bird CL, Lee ER, Frankl HD & Haile RW (1996) Relation of vegetable, fruit, and grain consumption to colorectal adenomatous polyps. *Am J Epidemiol* **144**, 1015-1025.

Wolffe AP (1994) Inheritance of chromatin states. *Dev Genet* **15**, 463-470.

Wurzelmann JI, Silver A, Schreinemachers DM, Sandler RS & Everson RB (1996) Iron intake and risk of colon cancer. *Cancer Epidemiol Biomerkers Prev* **5**, 507.

Yanagihara K, Ito A, Toge T & Numoto M (1993) Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res* **53**, 5815-5821.

Yasutake A & Hirayama K (1994) Acute effects of methylmercury on hepatic and renal glutathione metabolism in mice. *Arch Toxicol* **68**, 512-516.

Yazzie M, Gamble SL, Civitello ER & Stearns DM (2003) Uranyl acetate causes DNA single strand breaks in vitro in the presence of ascorbate (vitamin C). *Chem Res Toxicol* **16**, 524-530.

Yoon J-H & Baek SJ (2005) Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei Medic J* **46**, 585-596.

Younes M (2004) Freie Radikale und reaktive Sauerstoffspezies. In *Lehrbuch der Toxikologie.*, pp. 117-131 [H Marquart and S Schäfer, Hrsg.]. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH.

Zhang D, Okada S, Yu Y, Zheng P, Yamaguchi R & Kasai H (1997) Vitamin E inhibits apoptosis, DNA modification, and cancer incidence induced by iron-mediated peroxidation in Wistar rat kidney. *Cancer Res* **57**, 2410-2414.

DANKSAGUNG

Ich möchte mich bei allen bedanken, die direkt und indirekt zum Gelingen dieser Arbeit beigetragen haben und mir während meiner Promotion unterstützend zur Seite standen.

Mein besonderer Dank gilt Frau Prof. Dr. Beatrice L. Pool-Zobel, die mir die Möglichkeit gegeben hat, dieses interessante und vielseitige Promotionsthema am Lehrstuhl für Ernährungstoxikologie zu bearbeiten. Prof. Pool-Zobel hat mir durch ihre Bereitschaft zur wissenschaftlichen Diskussion stets neue Impulse für meine Arbeit gegeben und dadurch wesentlich zum Gelingen dieser Dissertation beigetragen.

Die Finanzierung des Projektes oblag der Deutschen Forschungsgemeinschaft (DFG, PO 284/6-1 and PO 284/6-2). Die Weiterführung meiner Arbeit nach der Geburt meiner Tochter wurde mir durch ein Wiedereinstiegsstipendium der Graduiertenförderung Thüringen ermöglicht. Vielen Dank für die finanzielle Unterstützung. Mein Dank gilt auch dem Bundesministerium für Bildung und Forschung für die Finanzierung des Projektes zur Chemoprävention von Apfelpolyphenolen (BMBF, FKZ.01EA0103).

Besonders möchte ich auch Herrn PD Dr. Michael Glei für seine Diskussionsbereitschaft und die Unterstützung beim Schreiben der Publikationen bedanken. Auch für andere Probleme hatte er immer ein offenes Ohr und half dadurch sehr, die Motivation und Freude an der Arbeit trotz einiger Hürden aufrechtzuerhalten.

Mein Dank gilt OA PD Dr. Richter aus der Klinik für Allgemeine und Viszerale Chirurgie der FSU Jena für die Bereitstellung der Kolongewebeproben. Frau Prof. Dr. Marian, Institut für Krebsforschung der Universität Wien, danke ich für die Überlassung der LT97-Zellen.

Ebenfalls möchte ich mich bei Dr. Anja Weise und Prof. Dr. Uwe Claussen vom Institut für Humangenetik und Anthropologie der FSU Jena bedanken, die mich durch die Herstellung der Gen-Sonden für die Comet-FISH-Versuche und die Durchführung der 24-Farben-FISH-Versuche hilfreich unterstützt haben.

Mein Dank gilt auch Frau Dr. Anja Schäferhenrich, die mir bei der Erlernung des Comet FISH mit Rat und Tat zur Seite stand.

Herrn Dr. Dirk Merten vom Institut für Geowissenschaften, Lehrstuhl für Angewandte Geologie danke ich herzlich für die Unterstützung bei der Probennahme und die chemische Charakterisierung der Wismutproben.

Bei Frau Eva Möller, Frau Edda Lösch, Frau Esther Woschee, Frau Dr. Kerstin Osswald, Frau Stefanie Klenow, Frau Nina Habermann und Frau Nadine Knoll bedanke ich mich für die tatkräftige Unterstützung im Labor und dafür, dass die Projektweiterführung auch während meiner Elternzeit gewährleistet war.

Außerdem danke ich allen ehemaligen und derzeitigen Mitarbeitern des Lehrstuhls für Ernährungstoxikologie für die stets gute Zusammenarbeit und freundliche Arbeitsatmosphäre.

An meine Freundinnen Annett Strauß und Annett Knoth geht mein Dank für die Korrektur und hilfreiche Diskussion.

Ganz herzlich möchte ich mich auch bei meiner Familie bedanken, meinen Eltern Joachim und Helga Knöbel, für ihre moralische Unterstützung und ihr Verständnis dafür, dass ich diesen Weg gewählt habe. Meinem Verlobten Mario Neudeck und unserem Sonnenschein Lucy Victoria danke ich für die immerwährende Unterstützung während der Anfertigung dieser Arbeit und ihr Verständnis dafür, dass unsere gemeinsame Zeit vor allem im Endspurt etwas zu kurz kam.

LEBENSLAUF

Name:	Yvonne Knöbel
Geboren:	02.12.1977 in Gera
Familienstand:	ledig

Schulbildung

09/84 - 08/93	Staatliche Regelschule am Zaschberg, Greiz
09/93 - 07/96	Pestalozzigymnasium Greiz Abschluss: Abitur (Note 1,7)

Studium

09/96 - 03/01	Studium der Ernährungswissenschaften, Friedrich-Schiller-Universität Jena, Abschluss: Diplom Trophologe (Note 1,7) während des Studiums aktives Mitglied in „STEP“ – Studentische Ernährungsprojekte

Postgraduale Ausbildung

04/01-09/03	Dissertation am Institut für Ernährungswissenschaften der Friedrich-Schiller-Universität in Jena, Promotion zum Thema " Untersuchungen zum toxikologischen Potential von mit dem Uranabbau assoziierter Verbindungen und kontaminierter Gewässerproben aus dem Ronneburger Wismut-Gebiet in humanen Kolonzellen "
10/03-09/04	Elternzeit
10/04-09/06	Weiterführung der wissenschaftlichen Tätigkeit, Dissertation
seit 09/06	Qualitätsmanagement-Beauftragte der EWU Thüringer Fleischverarbeitungs GmbH

Stipendien

03/03	44. Frühjahrstagung der DGPT und 20. Tagung der GUM, Mainz, 17.-20. März 2003 - Reisestipendium
03/05	46. Frühjahrstagung der DGPT, Mainz, 15.-17. März 2005 – Reisestipendium
09/04-08/05	Wiedereinstiegsstipendium der Graduiertenförderung des Thüringer Landes

PUBLIKATIONSLISTE

1. Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells

   Y. Knöbel, M. Glei, K. Osswald, B.L. Pool-Zobel

   Toxicology In Vitro, 2006, Vol. 20(6), 793-800

2. Ferric iron is genotoxic in non transformed and preneoplastic human colon cells

   Y. Knöbel, A. Weise, M. Glei, W. Sendt, U. Claussen, B.L. Pool-Zobel

   zur Publikation angenommen bei Food and Chemical Toxicology am 30. Oktober 2006, online publiziert doi:10.1016/j.fct.2006.10028

3. Uranyl nitrilotriacetate (U-NTA), a stabilized salt of uranium, is genotoxic in nontransformed human colon cells and in the human colon adenoma cell line LT97

   Y. Knöbel, M. Glei, A. Weise, K. Osswald, A. Schäferhenrich, K. Richter, W. Sendt, U. Claussen, B.L. Pool-Zobel

   Toxicological Sciences, 2006, Vol. 93(2), 286-297

4. Blood mononucleocytes are sensitive to the DNA damaging effects of iron overload – *in vitro* and ex *vivo* results with Fe-NTA

   E. Park, M. Glei, Y. Knöbel, B. L. Pool-Zobel

   zur Publikation angenommen bei Mutation Research am 26. Januar 2007

5. Heavy metal- and radionuclid-contaminated water samples have genotoxic potential to human colon cells

   Y. Knöbel, M. Glei, K. Osswald, K. Richter, D. Merten, B.L. Pool-Zobel

   Manuskript in Vorbereitung für Toxicological Sciences

6. Apple polyphenols modulate the genotoxic effects of different cancer risk factors in human colon cells

   S. Klenow*, S. Veeriah*, Y. Knöbel*, B.L. Pool-Zobel (* 3 Erstautoren)

   Manuskript in Vorbereitung für Mutation Research

VORTRÄGE

Annual Minisymposium zum DFG-Verbundprojekt "Bio-Geo-Interaktionen", Jena, 07.-8. März 2002
Vortrag: „Gen-Umwelt-Interaktionen wismutrelevanter Wasserproben und Metallsalze an Humanzellen".

Internationaler Comet Assay Workshop, Warschau, 22.-24. September 2005
Vortrag: Knöbel, Y.; Weise, A.; Glei, M.; Sendt, W.; Claussen, U.; Pool-Zobel, B. L.: „Application of the Comet Assay, Comet FISH and 24-colour-FISH to detect genotoxic effects of uranyl nitrilotriacetate and related environmental samples in human primary colon cells and colon adenoma cells".

Projekttreffen Nutrition Net, Ernährung und Darmgesundheit, Kaiserslautern, 15. Oktober 2005
"Assessment of the modulation of detoxifying enzyme systems as biomarkers of chemoprevention in human colon cells".

Institutskolloquium, Jena, 10. Januar 2006:
Vortrag: "Gene-environmental interactions of wismut-relevant compounds in human colon cells - toxicology of relevant uranium mining compounds".

36. Annual Meeting der European Environmental Mutagen Society (EEMS): From Genes to Molecular Epidemiology, ECNIS symposium: "Food mutagens impact on health", Prag, 02.-06. Juli 2006,
Vortrag: Pool-Zobel, B. L.; Hofmann, T.; Klenow, S.; Knöbel, Y.; Raschke, M.; Sauer, J.; Steiner, C.; Veeriah S.: "Evaluation of genotoxicity and antigenotoxicity by food-derived compounds in human colon, prostate, breast and blood cells using novel genomics and transcriptomics-based methods".

POSTER

40. Wissenschaftlicher Kongress der DGE, Potsdam, 13.-14. März 2003
Poster: Knöbel, Y.; Glei, M.; Pool-Zobel, B. L.: „Schwermetallbelastetes Wasser induziert DNA-Schäden – *in vitro*-Untersuchungen an humanen Kolonzellen"

44. Frühjahrstagung der DGPT und 20. Tagung der GUM, Mainz, 17.-20. März 2003 - Reisestipendium
Poster: Knöbel, Y.; Glei, M.; Marian, B.; Sendt, W.; Scheele, J.; Pool-Zobel, B. L.: „Schwermetall- und Radionuklid-belastete Gewässerproben induzieren DNA-Schäden *in vitro*-Untersuchungen an humanen Kolonzellen"

4. International Conference on Environmental Mutagens in Human Populations, Florianopolis, Brasilien, 04.-08. Mai 2003

3. Sanierungskolloqium, Jena, 03.-06. Oktober 2004
Poster: Knöbel, Y.; Pool-Zobel, B. L.; Glei, M.; Claussen, U.; Weise, A.: "U-NTA and a heavy metal- and radionuclide-contaminated water sample induce DNA damage – *in vitro* investigations on different human colon cells".

46. Frühjahrstagung der DGPT, Mainz, 15.-17. März 2005 - Reisestipendium
Poster: Knöbel, Y.; Weise, A.; Glei, M.; Sendt, W.; Claussen, U.; Pool-Zobel, B. L.: „ Umweltproben aus einem ehemaligen Uranbergbaugebiet in Deutschland sowie Uran sind genotoxisch in humanen Kolonzellen“.

22. GUM-Tagung, Darmstadt, 21.-24. Februar 2006
Poster: Knöbel, Y.; Glei, M.; Veeriah, S.; Pool-Zobel, B. L.: „Investigations on DNA damage in the human colon carcinoma cell line HT 29 – modification of toxic effects by an apple extract”.

PUBLIZIERTE KURZFASSUNGEN

Knöbel, Y.; Glei, M.; Pool-Zobel, B. L. (2003): „Schwermetallbelastetes Wasser induziert DNA-Schäden – *in vitro*-Untersuchungen an humanen Kolonzellen“. Proc. Germ. Nutr. Soc., Vol. 5 (2003)

Knöbel, Y.; Glei, M.; Marian, B.; Sendt, W.; Scheele, J.; Pool-Zobel, B. L. (2003): „Heavy metal and radionuclide-contaminated water samples induce DNA damage – in vitro investigations at different human colon cells”. Archieves of Pharmacology, Vol. 367, Suppl. 1 (2003), R 167

Knöbel, Y.; Weise, A.; Glei, M.; Sendt, W.; Claussen, U.; Pool-Zobel, B. L. (2005): „Environmental samples collected near a former uranium mining area in Germany as well as uranium are genotoxic in human colon cells“. Archieves of Pharmacology, Vol. 371, Suppl. 1 (2005), R 133

SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt habe.

Jena, den 22.02.2007 Yvonne Knöbel