# Development of an *In Vitro* Model to Study Oxidative DNA Damage in Human Coronary Smooth Muscle Cells

*Ludwika Kreja and Gerald Finking*
Institut für Arbeits-, Sozial- und Umweltmedizin; Universitätsklinikum D-Ulm

Summary
*Oxidative damage in the vascular system has been linked to the development of atherosclerosis. The aim of the present study was to establish a cell culture model for the investigation of in vitro induced oxidative DNA injury, its modulation and repair kinetics. Primary cultures of human coronary artery smooth muscle cells were used as target cells. The cells were exposed to hydrogen peroxide ($H_2O_2$). DNA damage was quantified by the alkaline single-cell gel electrophoresis assay (comet assay). This method allows quantification of DNA single and double strand breaks and of alkali-labile sites in individual cells. In the present study $H_2O_2$ concentration-responses and dependence of damage on exposure time and temperature were evaluated and the repair kinetics were studied. The results show that this cell culture model can be employed to study oxidative DNA injury in the cells of the human cardiovascular system, a promoter of cardiovascular disease. Further it offers the possibility to investigate pharmacological modulators of such oxidative effects. Such modulators are antioxidants like vitamin C and E, steroid hormones, phytoestrogens, or synthetic agents. Today, pigs, rabbits or rats are used to test systemic and local administration of the named modulating substances, the latter by use of special catheters or stents placed inside the arteries. These experiments require treatment observation over a number of weeks and killing of the animals at the end of the experiment. By using the proposed cell culture model such animal experiments could be partly avoided.*

Zusammenfassung: Etablierung eines *in vitro* Models zur Untersuchung von oxidativen DNA-Schädigungen in menschlichen koronaren glatten Muskelzellen
*Oxidative Prozesse in der Gefäßwand werden ursächlich mit der Atherogenese in Verbindung gebracht. Ziel unserer Arbeit war es, ein Zellkulturmodell zu etablieren, in dem in vitro induzierte oxidative DNA Schädigungen, ihre Beeinflussung sowie ihre Reparaturkinetik untersucht werden können. Als Zielzellen wurden Primärkulturen von humanen koronaren glatten Muskelzellen eingesetzt. Die DNA Schädigung wurde zunächst mit Hydrogenperoxid ($H_2O_2$) induziert und mit der alkalischen Einzelzell-Gelelektrophorese-Technik (comet assay) quantifiziert. Diese Methode ermöglicht es, die DNA Einzelstrang- und Doppelstrangbrüche sowie alkalisensitive Stellen in einzelnen Zellen zu erfassen. In der vorliegenden Studie wurden Konzentrations-Wirkungs-Beziehungen der DNA Schäden in Abhängigkeit von der Expositionszeit und Temperatur, sowie die anschließende Reparaturkinetik untersucht. Die Ergebnisse zeigen, dass dieses Zellkulturmodell für die Überprüfung der in vitro induzierten oxidativen DNA Schädigung in den Zellen menschlicher Koronararterien geeignet ist. Damit steht ein Modell für die Untersuchung dieses ursächlichen Teilaspektes bei menschlichen Gefäßwandveränderungen zur Verfügung, das auch für Fragestellungen der Modulation der oxidativ induzierten Prozesse eingesetzt werden kann. Als Modulatoren geeignet sind beispielsweise antioxidativ wirksame Substanzen wie die Vitamine C und E, menschliche und pflanzliche Östrogene sowie synthetische Agenzien. Diese Substanzen werden in Tierversuchen (Schweinen, Ratten und Kaninchen) derzeit systemisch oder auch lokal appliziert, letzteres durch Einbringen der Agenzien mittels bestimmter Katheter und beschichteter Stents. Diese Experimente haben eine mehrwöchige Behandlung, Beobachtung und letztendlich Tötung der Tiere zur Folge. Durch Einsatz dieses Zellkulturmodells wird es möglich sein, auf einen Teil dieser Tierversuche zu verzichten.*

*Keywords: 3R-coronary vascular smooth muscle cells, atherosclerosis, oxidative DNA damage, comet assay*

## 1 Introduction

Cardiovascular diseases (CVD) are still the leading causes of death in North America and Europe. The major pathology of CVD is atherosclerosis, an occlusive condition that develops in small vessels like coronary and cerebral arteries. The principal lesion associated with this condition is the atherosclerotic plaque which arises, partly as a result of cell proliferation and lipid accumulation, in the intimal region of the artery wall, between the single layer of luminal-facing endothelium and the smooth muscle (medial) layer. The predominant cell type in plaque is the smooth muscle cell, and proliferation of smooth muscle cells

(SMC) is essential to plaques formation and development (Ross, 1986; Fuster et al., 1992; Fuster et al., 1992; Davies and Woolf, 1993; Ross, 1993).

Alterations at the DNA level in cells of the artery walls may contribute to the development of atherosclerosis as well. The idea of a monoclonal origin of smooth muscle cells involved in the formation of the atherosclerotic plaque originated from Benditt and Benditt as a result of their comparing observations in atherosclerotic and non-atherosclerotic human vessel walls (Benditt and Benditt, 1973). While modern concepts of atherosclerosis development have turned from a mechanistic "response to injury hypothesis" to immunological and inflammatory interpretations (Ross, 1999), systematic investigations on genetic alterations/mutations in this regard are still rare (Penn et al., 1986; Penn, 1990; Spragg, 1991; Andreassi et al., 2000; Binková et al., 2001). Such alterations can be partially initiated by genotoxic action of environmental chemicals or oxidative damage induced by ROS (reactive oxygen species). ROS production is associated with normal cellular metabolism as well. ROS may also be generated by exogenous sources like ionising and UV radiation and a number of xenobiotics (Janssen et al., 1993; Kehrer, 1993).

Studies over the last 20 years have demonstrated that ROS may play an important role in the pathogenesis of a variety of diseases and disorders including aging, neurodegeneration, cardiovascular disease and cancer (Farber et al., 1990; Janssen et al., 1993; Kehrer, 1993; Lieber, 1998). We therefore wanted to establish a cell culture model for further investigations on ROS-induced DNA damage, repair kinetics, and possible pharmacological modulation of such oxidative effects in cardiovascular target cells. In this first step we used primary cultures of human coronary artery smooth muscle cells. Oxidative DNA damage was induced by standardised hydrogen peroxide treatment. The amount of DNA damage was quantified by determination of DNA single and double strand breaks (DNA-SSB and DSB) and of alkali-labile sites in individual cells, using the alkaline single-cell gel electrophoresis assay (comet assay) (Singh et al., 1988; Singh et al., 1991; Rojas et al., 1999; Singh, 2000; Tice et al., 2000).

## 2 Methods

### 2.1 Cell culture

Cryopreserved normal human coronary artery smooth muscle cells from a 58 year old male were purchased from BioWhittaker Europe at third passage. The experiments were performed only with cells from one donor. For the stock culture the cells were initially cultured in smooth muscle growth medium (SMGM-2 Bullet Kit™, BioWhittaker, Europe) in 25 $cm^2$ bottles (Nunc, D-Wiesbaden) following the supplier's directions, trypsinised with a ready-made trypsin solution (ReagentPack™, BioWhittaker, Europe) and cryopreserved. All experiments were performed on cells at passage 5-7 which were cultured in growth medium consisting of Dulbecco's modified Eagle Medium and nutrient mixture Ham's F-12 (DMEM/Ham's F-12, 1:1 mixture; Biochrom KG) supplemented with 10% pre-tested fetal bovine serum, 4 mM L-glutamin and 100 IU/ml penicillin and streptomycin and 0.25 µg/ml amphotericin B.

### 2.2 Treatment with hydrogen peroxide ($H_2O_2$)

Smooth muscle cells (SMC) were seeded in triplicate in 24-well plates (10.000-20.000 cells/well in 2 ml growth medium). Exponentially growing cells (about 80% confluence) were washed with 2 ml HEPES buffered saline (UltraSaline A, BioWhittaker, Europe) and incubated for 10 minutes at 20°C or 37°C with 25 µM - 100 µM $H_2O_2$ applied in 0.4 ml UltraSaline A. After treatment the cells were washed twice, trypsinised, centrifuged (300g, 7 min), resuspended in medium and immediately used for the comet assay. Cell viability was monitored by trypan blue exclusion. In all experiments the percentage of dead cells was below 10%.

For the investigation of the damage repair kinetics the cells were exposed to $H_2O_2$ for 10 min at 20°C, then washed twice, trypsinised, embedded in agarose on a microscope slide, and lysed immediately as described before (0 minutes) or allowed to incubate in 2 ml of fresh culture medium for a period of 30, 60 and 120 minutes at 37°C and 5% $CO_2$.

### 2.3 Alkaline single-cell gel electrophoresis assay (comet assay)

The comet assay was performed as described by Singh et al. with slight modifications (Fig. 1) (Singh et al., 1988). Briefly, a level thin layer of agarose gel was prepared by dipping the slides in 1% warm agarose in PBS. 15 µl cell suspension (1-2 x $10^4$ cells) were mixed with 85 µl of 0.5% low melting point agarose (LMPA) in PBS, placed on the first agarose gel layer, levelled with a cover slip, and allowed to solidify at 4°C for 5 minutes. The cover glass was removed and a third layer of 0.5% LMPA (100 µl) with a cover glass was then applied. After the agarose solidified, the cover glass was removed and the glass slide was submerged in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO; pH 10) and kept for at least 60 minutes at 4°C to lyse the cells. The slide was transferred to an electrophoresis gel box filled with alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH 13) for 20 minutes at room temperature to allow unwinding of DNA. Electrophoresis was conducted for 20 minutes at 25 V (0.8 V/cm) and 300 mA. Slides were washed in neutralisation puffer (0.4 M Trizma Baser, pH 7.5) for 15 minutes and fixed in absolute ethanol for 5 minutes. The dried slides could be stored in the dark for several weeks. Before analysis the slide was stained with 50 µl ethidium bromide (20 µg/ml in distilled water) and covered with a cover slip.

Observations were made using a Zeiss fluorescence microscope (160x magnification) with excitation filter 515-560 nm and barrier filter 590 nm attached to a CCD camera. The extent of DNA migration was analysed using an image analysis system (Kinetic Imagining Ltd., U.K., Version 2.4). The comet assay operates on the principle that DNA strand breaks create smaller DNA fragments that migrate more rapidly than intact DNA in

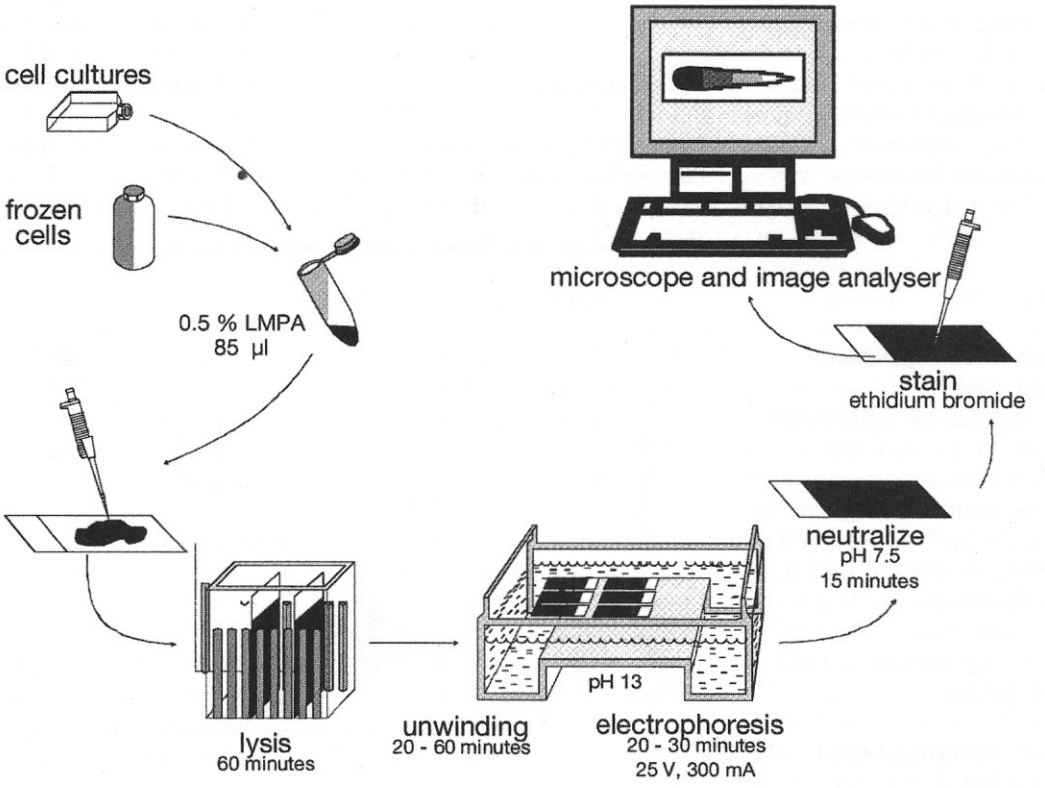


**Fig. 1: Scheme of the principle of the comet assay.**

an electric field. The rapidly migrating DNA forms a comet-shaped tail, which is morphologically distinct from the round head containing intact DNA (Fig. 2).

The comet assay essentially reflects the displacement of fluorescence from the comet head to the tail in damaged cells. The tail moment (TM) has been found to be the most informative feature, providing the best description of the overall damage to the nucleic DNA. It has been defined as the product of the percentage of DNA in the comet tail multiplied by the tail length (Olive et al., 1990).

### 2.4 Statistical evaluation

The statistical analysis was performed as recommended in the literature (Speit and Hartmann, 1999; Tice et al., 2000). For each cell the TM was calculated and the median of the 50 randomly selected cells/experimental point was determined. The mean TM (± standard deviation, SD) from 2-7 single independent experiments was calculated. Each replicate experiment was performed on a different day with separately cultured cells.

Threshold for DNA damage: Under normal conditions (control) 11± 4% of all cells show TM values >4.0 (mean±SD; n=13 experiments). Such cells are considered significantly damaged and a TM value of 4 can be applied

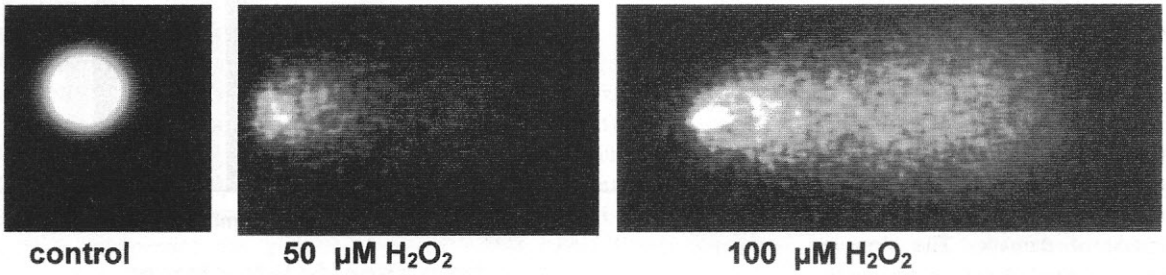


**Fig. 2: Photomicrograph of human coronary artery smooth muscle cells after single cell gel electrophoresis as described in Material and Methods.**

The cell nucleus of a non-treated control is round, indicating absence of DNA strand breaks. Cells after treatment with 50 or 100 µM of hydrogen peroxide show a comet, indicating presence of strand breaks.

as the minimum value for defining significant DNA damage. Differences between a non-exposed control group and $H_2O_2$ treated cells were tested for significance ($p<0.05$) using Student's t-test. Pair-wise comparison of each dose group against the concurrent control to identify significant effects was recommended by Tice et al. (Tice et al., 2000).

## 3 Results

### 3.1 Concentration-dependent DNA damage

Significant DNA damage could be reproducibly induced in coronary artery smooth muscle cells in a dose-dependent manner by treatment with $H_2O_2$ at concentrations of 25, 50, 75, and 100 µM for 10 minutes (Fig. 3). 100 µM $H_2O_2$ treatment resulted in damage to all cells. Under different temperature conditions of 20°C and 37°C the amount of damaged cells was comparable.

### 3.2 Exposition time-dependent DNA damage

A separate investigation with 50 µM $H_2O_2$ treatment confirmed the observation that DNA damage could be induced comparably at 20°C and 37°C (Fig. 4). However, the time of exposition had a significant influence on the grade of DNA damage and showed different kinetics at the different temperatures. At 20°C the grade of DNA damage increased from 0 to 5 to 10 minutes and decreased during 30 minutes of exposure. At 37°C the grade of DNA damage increased only over the first 5 minutes and decreased after 10 minutes and 30 minutes.

### 3.3 Repair kinetics

Focussing on DNA repair kinetics (Figure 5) again the amount of damaged cells correlated with the concentration of $H_2O_2$ (treatment over 10 minutes at 20°C). The rate of DNA repair, investigated over 30 to 120 minutes, depended on the initial grade of damage. The slowest DNA repair occured after treatment with 200 µM $H_2O_2$. However, after 120 minutes post-incubation in 37°C with fresh culture medium all cell samples initially damaged with 50, 100 or 200 µM $H_2O_2$, were repaired to a grade that was not significantly different from the control.

## 4 Discussion

In this cell culture study significant DNA damage could be induced dose-dependently with 25-200 µM $H_2O_2$ treatment over 5, 10 and 30 minutes. The influence of different temperatures (20°C or 37°C) was only slight. DNA repair depended on the initial grade of $H_2O_2$ induced damage and was completed after 120 minutes. Target cells in these experiments were human coronary artery smooth muscle cells which play a key role in human atherosclerosis (Fuster et al., 1992a;

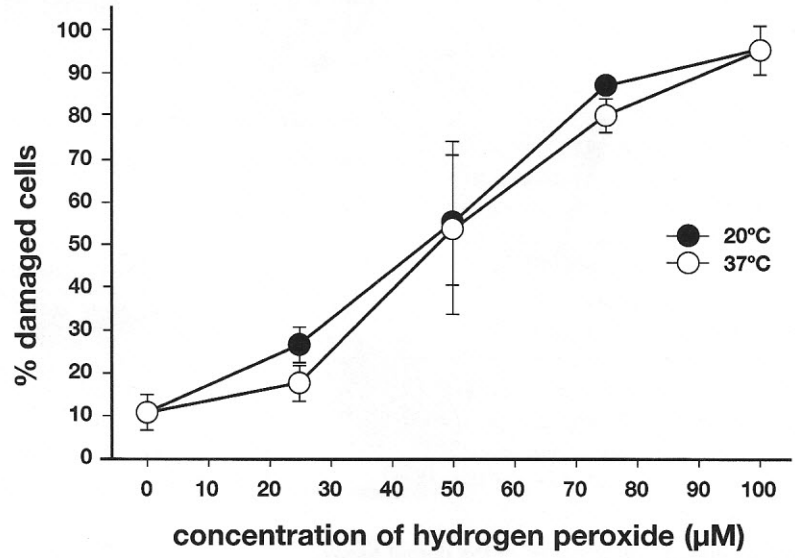


**Fig. 3: Percentage of damaged human coronary artery smooth muscle cells after treatment with varying doses of hydrogen peroxide as described in text. Values are means (± SD) of 2-7 independent experiments.**

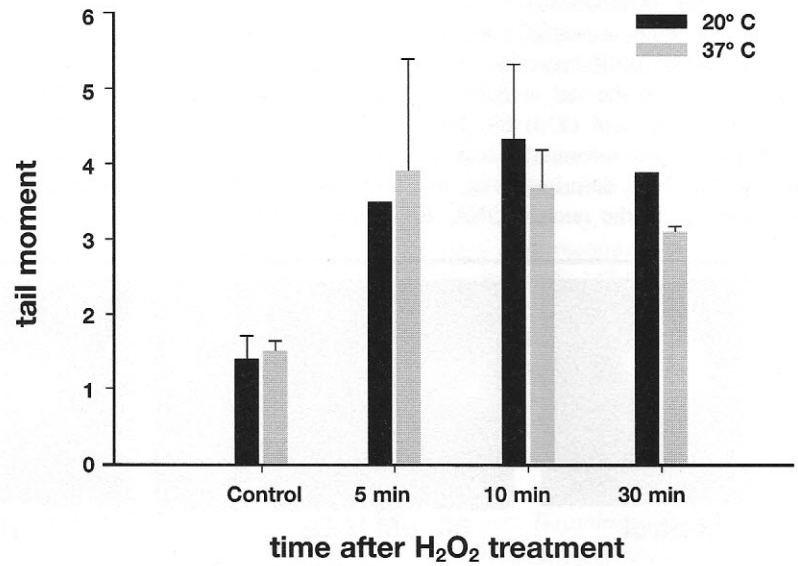


**Fig. 4: Response of human coronary artery smooth muscle cells to treatment with 50 µM of hydrogen peroxide at 20°C and 37°C for 5–30 minutes. Values are means (± SD) of 2-7 independent experiments.**

Fuster et al., 1992b; Ross, 1993). The current idea is that vascular smooth muscle cells from the medial tissue – due to a stimulatory effect – migrate to the intimal vascular tissue where they proliferate and produce connective tissue. Altered DNA capable of causing the cell transformation and proliferation has been found in atherosclerotic lesions of human coronary arteries (Penn et al., 1986). Recently, significantly higher DNA adduct levels have been found in the thoracic aortas of sudden death subjects with frequent atherosclerotic changes in the whole body compared with the samples from subjects with rare atherosclerotic changes (Binková et al., 2001). Another clinical trial found that coronary artery disease in humans is a condition characterised by and correlating with an increase of DNA damage in peripheral blood lymphocytes (Botto et al., 2001). Therefore it is of interest, whether free radicals and reactive oxygen species (ROS), which are frequently found metabolites in the human organism, contribute to such serious alterations (Farber et al., 1990; Janssen et al., 1993; Kehrer, 1993; Gutterige, 1994). Oxidative lesions in DNA caused by ROS or $H_2O_2$ (which was used for reasons of reproducibility in our experiments) are DNA single strand breaks (SSB) and double strand breaks (DSB), cross linking of DNA, alkali labile sites and base damage (Janssen et al., 1993). Double strand breaks (DSB) are more important but less frequent lesions in DNA. Their repair is complicated and even a few unrepaired breaks lead to cell death (Cantoni et al., 1996).

DNA single strand breaks (SSB) and alkali labile sites are easiest to detect and make up by far the largest numbers of lesions in DNA in general. Among numerous techniques for their estimation the most sensitive is the alkaline microgel electrophoresis technique known as the single-cell gel electrophoresis assay or comet assay, first introduced by Singh et al. (Singh et al., 1988).

Eucaryotic DNA molecules are several centimeters in length and they are tightly condensed to be accommodated within the confines of the 5-10 µm wide nucleus. If breaks occur in the DNA strands, the supercoiled chromosomal DNA relaxes, and this damage can be monitored by microgel electrophoresis (Östling and Johanson, 1984). The introduction of alkaline conditions prior to and during the electrophoresis to unwind the condensed nuclear DNA increases the sensitivity for detecting DNA damage and forms the basis of widely used comet assay protocols (Singh et al., 1988).

Comet assay is increasingly used in diverse applications such as genotoxicity testing, DNA damage and repair studies, environmental biomonitoring, human population monitoring and clinical applications (Rojas et al., 1999; Speit and Hartmann, 1999). Sensitivity and specificity of the test are considered to be very high and recommendations for the conduct of the comet assay in genetic toxicology have been published recently (Singh, 2000; Tice et al., 2000).

The aim of our present *in vitro* study was to establish a cell culture system for investigations of oxidative DNA damage, repair kinetics, and their possible pharmacological modulation in coronary artery smooth muscle cells. In these first experiments we found that significant DNA damage could be induced with 50 µM $H_2O_2$ after 5 minutes of treatment. After 10 minutes (at 37°C) or 30 minutes (at 20°C and 37°C) of treatment decreasing amounts of DNA damage were seen which can be explained by the start of repair processes (Figure 4). During the treatment the $H_2O_2$ concentration also decreased due to inactivation and disaggregation. DNA damage induced by 10 minutes exposition with 50-200 µM $H_2O_2$ at 20°C was completely repaired (when compared with controls) after 120 minutes of cultivation with medium at 37°C (Fig. 5).

The introduced cell culture model offers a variety of opportunities to work systematically on questions of potentially ROS-induced and mutagenic aspects of human coronary atherosclerosis and their modulation by pharmacological treatment. Steroid hormones like 17ß-estradiol (Finking et al., 2001) or phytoestrogens (Finking et al., 1999) which have antioxidant properties can be investigated as modulators. It has frequently been demonstrated that such agents are antiatherogenic and antiproliferative in mouse (Sullivan et al., 1995), rat (Oparil et al., 1997), rabbit (Hanke et al., 1996; Finking and Hanke, 1997; Finking et al., 2001), pig (Shi et al., 1996), and primate (Holm et al., 1998) animal models as well as in organ culture experiments

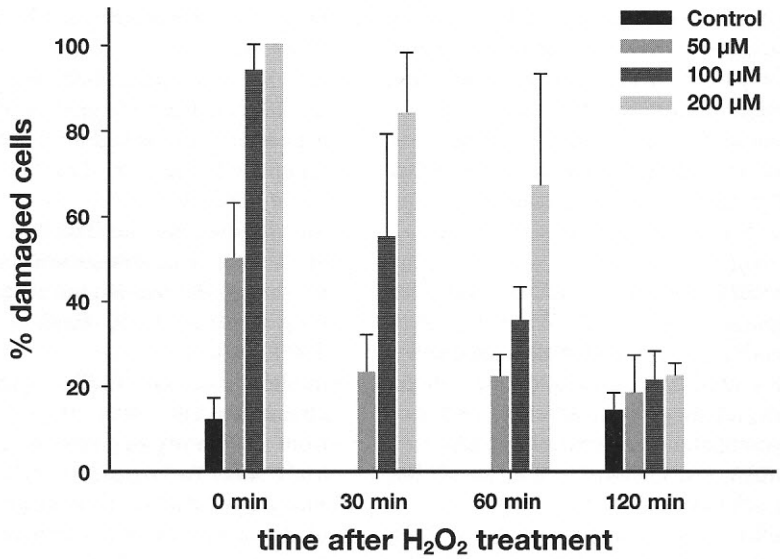


**Fig. 5: Repair kinetics of DNA damage induced in human coronary artery smooth muscle after treatment with 50-200 µM of hydrogen peroxide for 10 minutes at 20°C.** Following treatment, cells were either trypsinised and lysed immediately (0 minutes) or allowed to incubate in culture medium for a period of 30-120 minutes Values are means (± SD) of 2-6 independent experiments. After 120 minutes incubation at 37°C the percent of damaged cells in treated groups was not significantly different to the control.

(Vargas et al., 1993; Finking et al., 1999; Finking et al., 2000). Other potent antioxidative agents to be investigated are vitamin C and E or artificial antioxidants like probucol (Bhavnani et al., 2001). However, experiments with rabbits, because of their complexity, yield rather controversial results regarding antioxidative vitamin actions and their potential in vivo effects (Upston et al., 2002; Yoshida et al., 2002). This should be a reason to carry out more investigations on distinct pathophysiological aspects using a suitable cell culture model first.

Current animal experiments try to increase concentrations of modulating agents at the target site by local intravascular administration using special (balloon) catheters or stents (Gershlick, 2002). Special targets of treatment are vessels with hypercholesterolemia-induced atherosclerotic alterations (Tepe et al., 2002) and those vascular sites after mechanical (i.e. by vasodilation) injury (Kalinowski et al., 1999; Yoon et al., 2002; Chandrasekar et al., 2001) or after surgical intervention (Motomura et al., 1997). These experiments, too, require treatment and observations over weeks and regular killing of the animals at the end of the experiment.

Oxidised LDL-cholesterol is the most important agent causing hypercholesterolemia-induced atherosclerotic and proliferative alterations. Little is known about DNA damage as a promoting process. Until today animal experiments focus on these alterations. As an alternative or additional experimental design our present cell culture model may partly avoid these animal experiments. Experiments on vascular injury may be of interest as well because ROS are frequently found under inflammatory conditions. ROS contribute to DNA damage and proliferation, too. In order to standardise our cell culture model hydrogen peroxide ($H_2O_2$) was used as the oxidative agent. $H_2O_2$ represents one important and frequently found reactive agent in human and animal organisms. However, in further experiments oxidised LDL-cholesterol can also be used in order to investigate another *ex vivo* derived oxidant. Using this cell culture model it may be possible to get more important insights into oxidative, mutagenic actions in smooth muscle cells involved in atherogenesis. Pharmacological strategies to modulate these oxidative and mutagenic effects can also be investigated. Thus, animal experiments in this field using systemic or local drug administration can be partly reduced or avoided.

## References


Andreassi, M. G., Botto N., Colombo M. G. et al. (2000). Genetic instability and atherosclerosis: can somatic mutations account for the development of cardiovascular diseases? *Environ. Mol. Mutagen. 35(4)*, 265-9.

Benditt, E. P. and Benditt, J. M. (1973). Evidence for a monoclonal origin of human atherosclerotic plaques. *Proc. Natl. Acad. Sci. USA 70(6)*, 1753-6.

Bhavnani, B. R., Cecutti, A., Gerulath, A. et al. (2001). Comparison of the antioxidant effects of equine estrogens, red wine components, vitamin E, and probucol on low-density lipoprotein oxidation in postmenopausal women. *Menopause 8(6)*, 408-19.

Binková, B., Strejc, P., Boubelik, O. et al. (2001). DNA adducts and human atherosclerotic lesions. *Int. J. Environ. Health 204*, 49-54.

Botto, N., Rizza, A., Colombo, M. G. et al. (2001). Evidence for DNA damage in patients with coronary artery disease. *Mutat. Res. 493(1-2)*, 23-30.

Cantoni, O., Sestili, P., Guidarelli, A. et al. (1996). Cytotoxic impact of DNA single vs. double strand breaks in oxidatively injured cells. *Arch. Toxicol. Suppl. 18*, 223-235.

Chandrasekar, B., Nattel, S. and Tanguay, J. F. (2001). Coronary artery endothelial protection after local delivery of 17beta-estradiol during balloon angioplasty in a porcine model: a potential new pharmacologic approach to improve endothelial function. *J. Am. Coll. Cardiol. 38(5)*, 1570-6.

Davies, M. J. and Woolf, N. (1993). Atherosclerosis: what is it and why does it occur? *Br. Heart J. 69(1 Suppl)*, 3-11.

Farber, J. L., Kyle, M. E. and Coleman, J. B. (1990). Mechanisms of cell injury by activated oxygen species. *Lab. Invest. 62(6)*, 670-9.

Finking, G., Gohar, M. H., Lenz, C. and Hanke, H. (2001). Cardiovascular oestrogen effects in women. *J. Obstet. Gynaecol. 21(4)*, 340-349.

Finking, G. and Hanke, H. (1997). Nikolaj Nikolajewitsch Anitschkow (1885-1964) established the cholesterol-fed rabbit as a model for atherosclerosis research. *Atherosclerosis 135(1)*, 1-7.

Finking, G., Hess, B. and Hanke, H. (1999). The value of phytoestrogens as a possible therapeutic option in postmenopausal women with coronary heart disease. *Journal of Obstetrics and Gynaecology 19*, 455-459.

Finking, G., Krauss, N., Römer S. et al. (2001). 17beta-estradiol, gender independently, reduces atheroma development but not neointimal proliferation after balloon injury in the rabbit aorta. *Atherosclerosis 154(1)*, 39-49.

Finking, G., Wohlfrom, M., Lenz, C. et al. (1999). The phytoestrogens Genistein and Daidzein, and 17 beta-estradiol inhibit development of neointima in aortas from male and female rabbits in vitro after injury. *Coron. Artery. Dis. 10(8)*, 607-15.

Finking, G., Wolkenhauer, M., Lenz, C. and Hanke, H. (2000). Post-injury ex vivo model to investigate effects and toxicity of pharmacological treatment in rings of rabbit aortic vessels. *ALTEX 17(2)*, 67-74.

Fuster, V., Badimon, L., Badimon, J. J. et al. (1992a). The pathogenesis of coronary artery disease and the acute coronary syndromes. *N. Engl. J. Med. 326(4)*, 242-50.

Fuster, V., Badimon, L., Badimon, J. J. et al. (1992b). The pathogenesis of coronary artery disease and the acute coronary syndromes. *N. Engl. J. Med. 326(5)*, 310-8.

Gershlick, A. H. (2002). Treating atherosclerosis: local drug delivery from laboratory studies to clinical trials. *Atherosclerosis 160(2)*, 259-71.

Gutterige, J. (1994). Biological origin of free radicals and mechanism of antioxidant protection. *Chem. Biol. Interac. 91*, 133-140.

Hanke, H., Hanke, S., Finking, G. et al. (1996). Different effects of estrogen and progesterone on experimental atherosclerosis in female versus male rabbits. Quantification of cellular

proliferation by bromodeoxyuridine. *Circulation 94(2)*, 175-81.

Holm, P., Andersen, H. L., Arre, G. and Stender, S. (1998). Gender gap in aortic cholesterol accumulation in cholesterol-clamped rabbits: role of the endothelium and mononuclear-endothelial cell interaction. *Circulation 98(24)*, 2731-7.

Janssen, Y. M. W., Van-Houten, B., Borm, P. J. A. and Mossmann, B. T. (1993). Cell and tissue responses to oxidative damage. *Lab. Invest. 69(3)*, 261-74.

Kalinowski, M., Tepe, G., Schieber, A. et al. (1999). Local administration of ramiprilat is less effective than oral ramipril in preventing restenosis after balloon angioplasty in an animal model. *J. Vasc. Interv. Radiol. 10(10)*, 1397-404.

Kehrer, J. P. (1993). Free radicals as mediators of tissue injury and disease. *Crit. Rev. Toxicol. 23*, 21-48.

Lieber, M. (1998). Pathological and physiological double-strand breaks: roles in cancer, aging, and the immun system. *Am. J. Pathol. 153*, 1323-1332.

Motomura, N., Lou, H., Hong, M. et al. (1997). Local administration of estrogen inhibits transplant arteriosclerosis in rat aorta accelerated by topical exposure to IGF-I. *Transplant. Proc. 29(1-2)*, 1118-20.

Olive, P. L., Banáth, J. P. and Durand, R. E. (1990). Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet assay". *Radiat. Res. 122*, 86-94.

Oparil, S., Levine, R. L., Chen, S. J. et al. (1997). Sexually dimorphic response of the balloon-injured rat carotid artery to hormone treatment. *Circulation 95(5)*, 1301-7.

Östling, O. and Johanson, K. (1984). Microelectrophoretic study of radiation-induced DNA damage in individual mammalian cells. *Bioch. Biophys. Res. Commun. 123*, 291-298.

Penn, A. (1990). International Commission for Protection Against Environmental Mutagens and Carcinogens. ICPEMC Working Paper 7/1/1. Mutational events in the etiology of arteriosclerotic plaques. *Mutat. Res. 239(3)*, 149-62.

Penn, A., Garte, S. J., Warren, L. et al. (1986). Transforming gene in human atherosclerotic plaque DNA. *Proc. Natl. Acad. Sci. (USA) 83*, 7951-7955.

Rojas, E., Lopez, C. M. and Valverde, M. (1999). Single cell gel electrophoresis assay: methodology and applications. *J. Chromatogr. B. Biomed. Sci. Appl. 722(1-2)*, 225-54.

Ross, R. (1986). The pathogenesis of atherosclerosis – an update. *N. Engl. J. Med. 314(8)*, 488-500.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature 362(6423)*, 801-9.

Ross, R. (1999). Atherosclerosis – an inflammatory disease. *N. Engl. J. Med. 340(2)*, 115-26.

Shi, Y., O'Brien jr., J. E., Fard, A. et al. (1996). Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. *Circulation 94(7)*, 1655-64.

Singh, N. P. (2000). Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat. Res. 455*, 111-127.

Singh, N. P., McCoy, M. T., Tice, R. R and Schneider, E. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell. Res. 175(1)*, 184-91.

Singh, N. P., Tice, R. R., Stephens, R. E. and Schneider, E. L. (1991). A microgel electrophoresis technique for the direct quantitation of DNA damage and repair in individual fibroblasts cultured on microscopic slides. *Mutat. Res. 252*, 289-296.

Speit, G. and A. Hartmann (1999). The comet assay (single-cell gel test). A sensitive genotoxicity test for the detection of DNA damage and repair. *Methods Mol. Biol. 113*, 203-212.

Spragg, R. G. (1991). DNA strand break formation following exposure of bovine pulmonary artery and aortic endothelial cells to reactive oxygen products. *Am. J. Respir. Cell Mol. Biol. 4(1)*, 4-10.

Sullivan jr., T. R., Karas, R. H., Aronovitz, M. et al. (1995). Estrogen inhibits the response-to-injury in a mouse carotid artery model. *J. Clin. Invest. 96(5)*, 2482-8.

Tepe, G., Brehme, U., Seeger, H. et al. (2002). Endothelin A receptor antagonist LU 135252 inhibits hypercholesterolemia-induced, but not deendothelialization-induced, atherosclerosis in rabbit arteries. *Invest. Radiol. 37(6)*, 349-355.

Tice, R. R., Agurell, E., Anderson, D. et al. (2000). Single cell gell/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environ. and Molecular Mutagenesis 35*, 206-221.

Upston, J. M., Terentis, A. C., Morris, K. et al. (2002). Oxidized lipid accumulates in the presence of alpha-tocopherol in atherosclerosis. *Biochem. J. 363(3)*, 753-760.

Vargas, R., Wroblewska, B., Rego, A. et al. (1993). Oestradiol inhibits smooth muscle cell proliferation of pig coronary artery. *Br. J. Pharmacol. 109(3)*, 612-7.

Yoon, J. H., Wu, C.-J., Homme, J. et al. (2002). Local delivery of nitric oxide from an eluting stent to inhibit neointimal thickening in a porcine coronary injury model. *Yonsei Med. J. 43(2)*, 242-251.

Yoshida, N., Murase, H., Kunieda, T. et al. (2002). Inhibitory effect of a novel water-soluble vitamin E derivate on atherosclerosis in rabbits. *Atherosclerosis 162(1)*, 111-117.

**Acknowledgements**

This study was supported by a grant from the "Institute for the evaluation of alternative models for animal experiments" (Zentralstelle für die Erfassung und Beurteilung von Ersatz- und Ergänzungsmethoden zum Tierversuch – ZEBET), Berlin.

The authors thank Mrs. Gisela Baur for excellent technical assistance and Mr. Peter Plecity for editorial help.

**Correspondence to**

Dr. med. Gerald Finking
Institut für Arbeits-, Sozial- und Umweltmedizin
Frauensteige 10
D-89075 Ulm
Tel.: +49-731-500 33126
Fax: +49-731-500 33125
E-mail:
gerald.finking@medizin.uni-ulm.de