DNA damage by respiratory quartz particle in rat lung epithelial cells: mechanisms and consequences *in vitro*

Inaugural-Dissertation

zur Erlangung des Doktorgrades der
Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
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Düsseldorf/2006

Aus dem Institut für Umweltmedizinische Forschung(IUF)
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. R. Kahl
Korreferent: Prof. Dr. F. Wunderlich
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Tag der mündlichen Prüfung: 27.11.2006

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Düsseldorf, den 27.11.2006

Hui Li

For my mother country, my family and all people I love

... the dust, which is stirred and beaten up by digging, penetrates into the windpipes and lungs, and produces difficulty in breathing... If the dust has corrosive qualities, it eats away the lungs and implants consumption in the body. In the Carpatian mines, women are found to have married seven husbands, all of whom this terrible consumption has carried away.

---- *De Re Metallica*. Basel, 1556 by GEORG AGRICOLA(1494 – 1555).

CONTENTS

Abbreviations:

5'dRP	5' abasic deoxyribose phosphate
8-OHdG	8-hydroxy guanosine
AL	Aluminium lactate
ATP	adenosine triphosphate
BER	base excision repair
CARET	Beta-Carotene and Retinol Efficacy Trial
COPD	Chronic obstructive pulmonary disease
DAB	Diaminobenzidine
DEP	diesel exhaust particles
DQ12	Quartz, MIU batch
EPR	Electron Paramagnetic Resonance
ESCODD	The European Standards Committee on Oxidative DNA Damage
FPG	formamidopyrimidine DNA N-glycosylase
GC-MS	Gas Chromatography coupled mass spectrometry
HNO_2	Nitrous acid
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPLC/ECD	high performance liquid chromatography and electrochemical detection
IARC	International Agency for Research on Cancer
LOONO	Alkyl peroxynitrite
MDA	malodialdehyde
MnSOD	manganese superoxide dismutase
$NO^{\cdot}$	Nitric oxide radical
$NO_2^{\cdot}$	Nitrogen dioxide radical
NO_2	Nitrite
N_2O_3	Nitrous anhydride
$ONOO^-$	Peroxynitrite
O_3	Ozone
$O_2^{\cdot -}$	Singlet oxygen, superoxide
$OH^{\cdot}$	Hydroxyl radical
PVNO	Polyvinylpyridine-N-oxide
RLE	rat lung type II epithelial cells
RNS	Reactive nitrogen species
$RO^{\cdot}$	Alkoxyl
$RO_2^{\cdot}$	Peroxyl
ROS	Reactive oxygen species
SiO_2	silicon dioxide
SOD	superoxide dismutase
TMTU	tetramethyl thiourea
UV	Ultraviolet

ABSTRACT

Chronic inhalation of high concentrations of respirable quartz particles has been associated with various lung diseases including pulmonary fibrosis and lung cancer. Generation of reactive oxygen and nitrogen species (ROS/RNS) and oxidative stress have been implicated as a major aspect of the toxicity of quartz. Nevertheless, the mechanisms involved in carcinogenesis by quartz exposure have been only partly elucidated. Both primary and secondary genotoxicity have been discussed as pathways of quartz induced lung cancer. Primary genotoxicity is defined as genetic damage elicited by the particle itself (either direct or indirect), whereas secondary genotoxicity is defined as the genetic damage resulting from ROS/RNS (and possibly other mediators) that are generated during particle-elicited inflammation.

In the first part of the thesis, the mechanisms underlying the indirect pathways of primary DNA damage induced by respirable standard DQ12 quartz particles in rat lung epithelial type II cells (RLE) have been investigated. Transmission electron microscopy and flow-cytometry analysis showed a rapid uptake (30 minutes to 4 hours) of quartz particles by the RLE cells, but particles were not found to translocate into cell nuclei. This indicates that the DNA strand breakage and induction of 8-hydroxydeoxyguanosine (8-OHdG) which is also observed in these cells during these treatment intervals- did not result from direct physical interactions between particles and the DNA, or from short-lived particle surface-derived ROS. DNA damage caused by quartz was significantly reduced in the presence of the mitochondrial inhibitors rotenone and antimycin-A. In the absence of quartz, these inhibitors did not affect DNA damage, but they reduced cellular oxygen consumption. No signs of apoptosis were observed up to 4 h after quartz treatment. Flow-cytometric analysis indicated that the reduced DNA damage by rotenone was not due to a possible mitochondria-mediated reduction of particle uptake by the RLE cells. Further proof for the role of mitochondria was shown by the failure of quartz to cause DNA damage in mitochondria-depleted 143B (rho-0) osteosarcoma cells, at concentrations where it elicited DNA damage in the parental 143B cell line. In conclusion, our data show that respirable quartz particles can elicit oxidative DNA damage without entering the nuclei of lung epithelial type II cells, which are considered to be important target cells in quartz carcinogenesis. Furthermore, our observations indicate that such indirect DNA damage involves the mitochondrial electron transport chain function, by an as-yet-to-be elucidated mechanism.

In the second part of this thesis, the antioxidant curcumin was used to investigate its capacity to protect lung epithelial cells from the genotoxic and inflammatory effects associated with quartz exposure. Electron paramagnetic resonance measurements using the spin-trap DMPO demonstrated that curcumin reduces hydrogen peroxide-dependent hydroxyl-radical formation by quartz. In association with these observations, curcumin was found to reduce quartz-induced cytotoxicity in rat lung epithelial cells. Curcumin also inhibited the release of macrophage inflammatory protein-2 (MIP-2) from RLE cells as observed upon treatment with the pro-inflammatory cytokines interleukin-1 beta (IL-1β) and Tumour Necrosis Factor-alpha (TNFα). DQ12 caused an increase in oxidative DNA damage in the RLE cells as determined by formamidopyrimidine glycosylase (FPG)-modified comet assay as well as by immunocytochemical analysis of 8-OHdG. However, curcumin failed to protect the RLE cells from oxidative DNA damage by quartz, instead it was found to be a strong inducer of oxidative DNA damage itself at non-cytotoxic and anti-inflammatory concentrations.

In summary, the findings in this thesis suggest that mitochondria are important mediators of quartz induced DNA-damage. These data provide an alternative pathway of genotoxicity caused by quartz whereby DNA damage is induced by particles without entering into the nucleus. Though further investigations are needed on detailed mechanisms of mitochondrial function after particle uptake, it indicates that consideration should be taken in particle risk assessment i.e. that particles may still be genotoxic despite their absence in the nucleus. Whether this plays a role in *vivo* in lung cancer induced by silica, remains to be studied.

With regard to such studies, the data with curcumin as an antioxidant intervention show that *in vitro* data cannot be simply converted into *in vivo* applications. The reasons for the difference between acellular and cellular effects of curcumin remain to be elucidated, but could be associated with metabolic conversion products that are formed in cellular incubations.

ZUSAMMENFASSUNG

Die chronische Inhalation hoher Konzentrationen respirabler Quarzpartikel wird mit verschiedenen Lungenerkrankungen einschließlich pulmonaler Fibrose und Lungentumoren in Verbindung gebracht. Die Generierung von reaktiven Sauerstoff- und Stickstoffspezies (engl.: reactive oxygen species, ROS; reactive nitrogen species, RNS) und oxidativem Stress werden als Hauptaspekt bei der Toxizität von Quarzpartikeln diskutiert. Dennoch wurden die Mechanismen, die in die quarzinduzierte Karzinogenese involviert sind, bislang nur teilweise aufgeklärt. Beides, sowohl primäre als auch sekundäre Gentoxizität werden als Möglichkeiten für quarzinduzierte Lungentumoren betrachtet. Primäre Genotoxizität wird als genetische Schädigung durch die Partikel selbst – entweder direkt oder indirekt – definiert. Dagegen wird als sekundäre Genotoxizität eine genetische Schädigung bezeichnet, die durch ROS/RNS (oder mögliche andere Mediatoren) während einer partikelinduzierten Entzündungsantwort verursacht wird.

Im ersten Teil der vorliegenden Arbeit wurden die Mechanismen der indirekten Induktion von primären DNA Schäden durch die respirablen Standardquarzpartikel DQ12 in epithelialen Typ-II-Zellen der Rattenlunge (RLE) untersucht.

Transmissionselektronenmikroskopie und flowzytometrische Analysen zeigten eine schnelle Aufnahme (30 min bis 4 h) der Quarzpartikel in RLE-Zellen, jedoch wurden keine Partikel im Zellkern nachgewiesen. Dies weist darauf hin, dass die Induktion von DNA-Strandbrüchen und 8-Hydroxy-Deoxyguanosin (8-OHdG), die ebenfalls in den Zellen nach Quarzbelastung innerhalb gleicher Intervalle auftraten, nicht aus der direkten physischen Interaktion von Partikeln mit der DNA oder kurzlebiger ROS der Partikeloberfläche resultierte. Die quarzinduzierte DNA-Schädigung wurde in Gegenwart der mitochondrialen Inhibitoren Rotenon und Aktinomyzin A signifikant reduziert. In Abwesenheit von Quarz beeinflussten diese Inhibitoren die DNA-Schädigung nicht, sie reduzierten jedoch den zellulären Sauerstoffverbrauch. Bis zu 4 h nach der Quarzbelastung wurden keine Merkmale der Induktion von Apoptose beobachtet. Flowzytometrische Untersuchungen zeigten, dass die reduzierten DNA-Schäden in Gegenwart von Rotenon nicht durch eine mögliche verminderte zelluläre Partikelaufnahme verursacht wurden. Ein weiterer Beweis für die Rolle der Mitochondrien wurde mit dem Fehlen quarzinduzierter DNA-Schäden in Osteosarkomzellen mit verminderter Mitochondrienanzahl 143B (rho-0) bei Quarzkonzentrationen gezeigt, die in der parentalen 143B-Zelllinie DNA-Schäden

induzierten. Zusammenfassend zeigen die Daten, dass respirable Quarzpartikel ohne Präsenz im Zellkern oxidative DNA-Schäden in Typ-II-Lungenepithelzellen induzieren, einem Zelltyp der in der quarzinduzierten Karzinogenese als bedeutende Zielzelle angesehen wird. Weiterhin weisen die Untersuchungen darauf hin, dass in solche indirekten DNA-Schädigungen Funktionen der mitochondralen Elektronentransportkette involviert sind, wobei die hierfür verantwortlichen Mechanismen noch zu klären sind.

Im zweiten Teil der Dissertation wurde das Antioxidans Curcumin hinsichtlich seiner Kapazität, Lungenepithelzellen vor quarzinduzierten gentoxischen und entzündlichen Effekten zu schützen, untersucht. Experimente mittels Elektronen-Paramagnetischer Resonanz unter Nutzung des Spintraps DMPO zeigten, dass Curcumin die Wasserstoffperoxid-abhängige Hydroxylradikal-Formation durch Quarz reduziert. In Verbindung mit diesen Ergebnissen wurde auch demonstriert, dass Curcumin die quarzinduzierte Zelltoxizität in RLE-Zellen vermindert. Des weiteren inhibiert Curcumin die Ausschüttung des Makrophagen-inflammatorischen Proteins-2 (MIP-2) von RLE-Zellen, die nach Behandlung mit den proinflammatorischen Zytokinen Interleukin-1 beta (IL-1β) und Tumor Nekrose Faktor-alpha (TNFα) beobachtet wurde. DQ12 verursacht einen Anstieg der oxidativen DNA-Schädigung in RLE-Zellen, der sowohl mittels Formamidopyrimidin-Glykosylase (FPG)-modifiziertem Kometassay als auch durch immunozytochemische Analysen von 8-OHdG nachgewiesen wurde. Die Vorbehandlung mit Curcumin verhinderte jedoch die quarzinduzierte DNA-Schädigung nicht, im Gegenteil, Curcumin selbst verursachte bei nicht-zytotoxischen und nicht-inflammatorischen Dosen eine starke DNA-Schädigung.

Zusammenfassend weisen die Ergebnisse der Dissertation darauf hin, dass Mitochondrien bedeutende Mediatoren quarzinduzierter DNA-Schäden sind. Dies unterstützt die Hypothese einer alternativen Induktion von Gentoxizität, wobei DNA-Schäden durch Partikel induziert werden, ohne dass diese selbst in den Zellkern gelangen. Weitere Untersuchungen hinsichtlich der detaillierten Mechanismen der mitochondrialen Funktion nach Partikelaufnahme sind jedoch erforderlich. Insbesondere sollte bei der Risikoabschätzung von Partikeln berücksichtigt werden, dass diese auch - unabhängig von ihrer Präsenz im Zellkern - gentoxische Wirkungen haben können. Ob dies auch eine Rolle bei der Induktion quarzbedingter Tumoren spielt, sollte in weiteren Studien untersucht werden. Hinsichtlich solcher

Studien zeigen die Daten mit Curcumin als Antioxidanz, dass *in vitro* Ergebnisse nicht ohne weiteres auf *in vivo* Anwendungen übertragen werden können. Ursachen für die Differenzen zwischen azellulären und zellulären Effekten von Curcumin bleiben weiterhin zu untersuchen, Ursache könnte jedoch die Bildung toxischer Metabolite im zellulären System sein.

CHAPTER 1.

General Introduction

1.1 Historical review of quartz exposure and disease

The importance of daily fresh and clean air to human health is well known to people, but still millions of workers are exposed to dust filled air world-wide during occupational activities like mining or welding. The adverse effect from dust inhalation was known already many years ago, when thousands of miners worked without any protection and died from the chronic consequences of respirable dust inhalation, including emphysema and silicosis. A book written by Agricola in 1556 (*De Re Metallica*. Basel) about miners in Bohemia during the mid-1500s, contained the following text:

> ... the dust, which is stirred and beaten up by digging, penetrates into the windpipes and lungs, and produces difficulty in breathing... If the dust has corrosive qualities, it eats away the lungs and implants consumption in the body. In the Carpatian mines, women are found to have married seven husbands, all of whom this terrible consumption has carried away.

It took decades to realise that several serious adverse health effects caused by inhalation of dust after occupational exposure were also associated with increased reduction of lung function, in pathogenic conditions like Silicosis, Tuberculosis, Lung Cancer, Chronic obstructive pulmonary disease (COPD), Autoimmune disease, Chronic Bronchitis, Emphysema, and abnormalities in pulmonary function tests. A lot of research has been done on the relationship between occupational exposures to various types of dust and health effects in epidemiological and toxicological studies. Crystalline silica is one of most important toxic particles because occupational exposures to respirable crystalline silica occur in a variety of industries and occupations.

Silica refers to the chemical compound silicon dioxide (SiO_2), which exists as a crystalline or noncrystalline (amorphous) form (summarized in Table 1.1). Crystalline silica may be found in more than one form (polymorphism). The polymorphic forms of crystalline silica are alpha quartz, beta quartz, tridymite, cristobalite, keatite, coesite, stishovite, and moganite (Guthrie and Heaney, 1995; IARC, 1997; NIOSH hazard review, 2002). Each polymorph is unique in its spacing, lattice structure, and regular

relationship of the atoms. In nature, the alpha form of quartz is the most common. This form is so abundant that the term quartz is often used instead of the general term crystalline silica. The other polymorphs (i.e., keatite, coesite, stishovite, and moganite) are rarely or never observed in nature (NIOSH hazard review, 2002).

Table 1.1 Classification of Silica (adapted from IARC, 1997)

Amorphous forms	*Crystalline forms*
Natural: Opal, biogenic silica, diatomaceous earths, silica fibres (bio-genic), vitreous silica	Natural: quartz, tridymite, cristobalite, coesite, stishovite, moganite
Synthetic: fused silica, pyrogenic(fumed) silica, precipitated silica, colloidal silica, silica gel	Synthetic: keatite, silica W, porosils (zeosils and clathrasils)

Workers in a large variety of industries and occupations may be exposed to crystalline silica because of its widespread natural occurrence and the large range of materials and products containing it. Silica is a ubiquitous component of soil and rocks. It is one of the most important and basic materials for construction and foundries, and is used widely for the rapidly developing world with daily arising high buildings and high ways. Although the prevalence of silicosis has decreased steadily in modern mining, outbreaks of silica-related disease still regularly occur in unexpected conditions. At least 1.7 million U.S. workers are potentially exposed to respirable crystalline silica (NIOSH, 1991). In Turkey, sandblasting of jeans in small workshops has been recently recognized as a cause of silicosis (Akgun *et al*., 2006). Another survey among Dutch construction workers (Tjoe-Nij *et al*., 2003) revealed that around 15% of workers had a mild form of mixed-dust pneumoconiosis.

Epidemiology is the primary science used to study silica-related disease in workers. Abundant epidemiological studies indicate that workers exposed to respirable crystalline silica have an increased risk of developing silicosis (Ziskind *et al*., 1976), lung cancer (Goldsmith *et al*., 1995; Finkelstein *et al*., 1982; McDonald, 1989), pulmonary tuberculosis (Ziskind *et al*., 1976; Ng and Chan, 1991), and other airway diseases.

The possible carcinogenicity of crystalline silica became a subject of considerable and intense debate in the scientific community in the 1990s, especially after (1)

publication of new information presented at a 1984 symposium in North Carolina (Goldsmith *et al*., 1986), (2) epidemiological studies by Westerholm (1980) and Finkelstein *et al*.(1982), and (3) a literature review by Goldsmith *et al*. (1982). Many epidemiological studies of cancer mortality and morbidity in silica-exposed workers were published and reviewed by the IARC (International Agency for Research on Cancer) in 1994 and respirable crystalline silica was classified as a group I carcinogen in 1997.

However, the exact mechanisms of silica-induced carcinogenesis are still partially unknown. For instance, up to now it remains unclear whether silicosis is an intermediate step towards this carcinoma, or whether silicosis is simply a marker for the inhaled amount of silica-containing dust. Many questions in quartz-induced adverse effects still remain unanswered. As mentioned that “quartz is a carcinogen in some occupational circumstances (IARC (1997)”, and this has been the subject of a number of reviews (Donaldson & Borm, 1998; Fubini, 1998) and later studies on its genotoxicity (Seiler *et al*., 2001).

In this thesis, the mechanisms and consequences of genotoxicity induced by respirable quartz particles were investigated. The elucidation of these mechanisms may help to understand why quartz is a carcinogen in some occupational circumstances (IARC (1997)) and may also increase our understanding of the effects of other particles such as ambient particulate matter on human health. This again may help to design preventive strategies or treatments to combat the adverse effects of particulate air pollution.

1.2 Morphology of Respirable quartz particles and its target cells in human lung tissue

Notwithstanding the general reduction in mining in Western countries, research on quartz does continue.The size of the quartz particle is the first factor contributing to its toxicity. Respirable particles are defined as particles which are smaller than 10µm. As shown in Figure 1.1, a standard respirable quartz particle DQ12 is visualised using electronic microscopy. The name DQ12 comes fromthe region where the quartz mineral was quarried (Dorentrupp). It is a very fibrogenic dust.

Epidemiological and experimental studies showed that alveolar type II cells are considered to be a key target in quartz carcinogenesis (IARC, 1997; Knaapen *et al*., 2004; Greim *et al*., 2001). The alveolar epithelium is composed of two different cell

types, namely type I cells (which line more than 90% of the alveolar surface and account for more than 7% of the parenchymal lung cells) and type II cells (which cover about 10% of the alveolar surface and account for 15% of all parenchymal cells). It is known that type II cells are important in carcinogenesis because it readily differentiates into type I cells when type I cells die (Johnson *et al*., 1987). Therefore, when investigating quartz-induced genotoxicity, using a cell line generated from immortalized rat alveolar type II cells is meaningful.

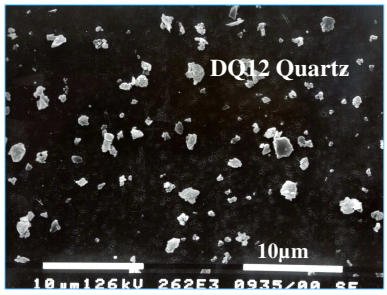

Figure 1.1 Transmission electron microscopic picture of DQ12 respirable quartz particle.

1.3 Role of surface chemistry in quartz-induced toxicity

As known that once inhaled, several particle-cell interactions take place, whereby the silica surface elicits a specific biological response. The molecular mechanism governing each step may involve different surface functionalities, so that the physicochemical features determining the overall pathogenicity of quartz partivles are probably more than one (Fubini *et al*., 1998)

The current research shows that the composition of quartz particles and especially its surface is the root of its toxicity (Vallyathan *et al*., 1998; Fubini, 1998). As know by chemical analysis, quartz particles in the environment do not consist of a single component, but are in fact a heterogeneous mixture, with varying composition. The variation depends on location and contamination with other mineral materials, for example, quartz may occur in mixtures with alumino-silicates, kaoline, and organic substrates. This so-called matrix may exert a crucial influence on the quartz surface. A simplified impression of the complex composition of the quartz particle is shown in Fig 1.2.

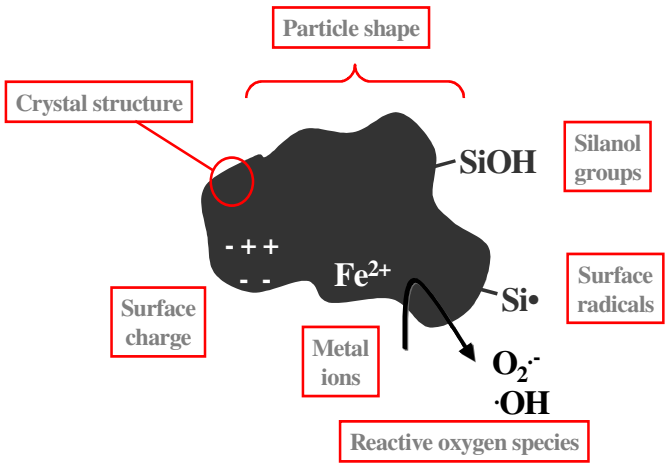

Figure 1.2 Simplified impression of the complex composition of quartz particle.

The quartz surface may contain several entities that are considered to play a role in its interaction with biological targets. These include a number of entities (metal ions, surface radicals, silanol groups) that play a role in the generation of reactive oxygen species, such as $OH^{\cdot}$, from its surface.

As shown in Figure 1.2, factors that can contribute to toxicity are particle shape, crystal structure, surface charge, silanol groups and surface radicals generated from the particle surface as well as metal ions present on its surface. Several research groups have addressed the question of variability using different approaches. A series of studies has shown the variability of the natural quartz hazard with regard to inflammation and genotoxicity (Fubini *et al.*, 2004; Bruch *et al.*, 2004; Clouter *et al.*, 2001). In another approach it was shown that coating the quartz surface with small amounts of aluminium lactate(AL), PVNO (polyvinylpyridine-N-oxide) or soluble matrix components reduced the ability of quartz to cause inflammation, DNA-damage, hemolysis and cell toxicity (Schins *et al.*, 2002; Albrecht *et al.*, 2004; Duffin *et al.*, 2002).

The cellular effects of quartz and the modulation of these effects by modification of the quartz surface was supported by chemical analysis of radical production from the quartz surface before and after treatment with AL and/or PVNO (Schins *et al.*, 2002). The Electron Paramagnetic Resonance Spectra (EPR) showed that quartz surfaces coated with PVNO were almost unable to generate OH-radicals in the presence of hydrogen peroxide (H_2O_2). These findings offer further support to the quartz surface paradigm mentioned earlier. The next paragraphs will go into detail concerning the properties, origin and function of these ROS in quartz-induced genotoxicity.

1.4 Pathways of reactive oxygen species (ROS) generation and oxidative stress by quartz in cellular systems

1.4.1 Reactive oxygen species (ROS) and oxidative stress

Reactive oxygen species (ROS) is a collective term used to describe oxygen ($RO_2^{\cdot}$) and alkoxyl radicals ($RO^{\cdot}$). Additionally, the term is used to describe certain non-radicals that are either oxidizing species or that can easily be converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). It should noted that the word 'reactive' is a relative term. The half –lives of these species range from minutes, like for hydrogen peroxide, to seconds, for the peroxide radical to about a nanosecond, in case of the hydroxyl radical. The hydroxyl radical is by far the most reactive ROS and reacts with whatever biological molecule is in its vicinity.

At steady-state, formation of pro-oxidants in cells and organs is normally achieved by a balance between the rate of their consumption by antioxidants (enzymatic or non-enzymatic) and their rate of formation. The processes of enzymatic ROS consumption in eukaryotic organisms mainly involve catalase, peroxidases, methionine sulphoxide reductase, superoxide dismutase (SOD); the non-enzymatic mechanisms involve small molecules, such as ascorbic acid, vitamine E, glutathione and uric acid. If the steady state between formation and consumption of oxidants is disturbed, oxidative stress and excessive production of radicals, including superoxide ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and peroxy radicals may occur. ROS are able to attack all biological macromolecules, i.e they will not only interact with DNA, but will also attack lipids and proteins. Proteins and polyunsaturated fatty acid residues of phospholipids, abundantly present in biomembranes, are extremely sensitive to oxidation. Alternatively, interaction of ROS with proteins may cause the induction of covalent cross-links between DNA and proteins, a process also reported to be mutagenic (Kulcharyk and Heinecke, 2001).

The concept of oxidative stress was first defined in 1991 (Sies, 1991) as a disruption of the prooxidant-antioxidant balance in favor of the former. Nowadays we know that oxidative stress is involved in many diseases including inflammation, autoimmune diseases, cancer, diabetes, neurodegenerative disease, heart attack and stroke

(Hensley and Floyd, 2002; Sorescu and Griendling, 2002). In Table 2 overview of different types of reactive oxygen and nitrogen species (ROS and RNS) is given.

Table 2. Overview of reactive oxygen and nitrogen species (ROS and RNS)

ROS	*RNS*
Superoxide ($O_2^{\cdot-}$)	Nitric oxide radical ($NO^{\cdot}$)
Hydrogen peroxide (H_2O_2)	Nitrogen dioxide radical ($NO_2^{\cdot}$)
Hydroxyl radical ($^{\cdot}OH$)	Nitrite(NO_2)
Singlet oxygen ($^1O_2$)	Peroxynitrite ($ONOO^-$)
Hypochlorous acid (HOCl)	Alkyl peroxynitrite (LOONO)
Peroxyl ($RO_2^{\cdot}$)	Nitrous acid (HNO_2)
Alkoxyl ($RO^{\cdot}$)	Nitrous anhydride (N_2O_3)
Ozone (O_3)	
Peroxynitrite ($ONOO^-$)	

1.4.2 Pathways of ROS generation by quartz particles

There are several ways in which the quartz surface can generate reactive species in the lung. Silanol groups (Si-OH) and ionised silanol groups (Si-O–) on the surface are considered to play a major role in the interaction with membranes (Nolan *et al.,* 1981). The regular Si/O tetrahedra are interrupted when the quartz is comminuted, producing both homolytic and heterolytic cleavage of the Si-O bonds that make up the basic crystalline structure of the quartz (Fubini *et al*., 1995). Homolytic cleavage results in $Si^{\cdot}$ and $Si\text{-}O^{\cdot}$ radicals whilst heterolytic cleavage produces charged Si^+ and $Si\text{-}O^-$ groups**.** In solution, for instance in lung lining fluid or in tissue fluid, these products of homolytic cleavage can give rise to $OH^{\cdot}$ and H_2O_2 (Castranova *et al*., 1996) whilst the charged products of heterolytic cleavage are involved in interactions with membranes (Fubini *et al*., 1995). Common contaminating metals such as iron and aluminium (Guthrie and Heaney,1995) may ameliorate the toxicity of quartz but Fenton chemistry-derived hydroxyl radicals may also be generated (Castranova *et al.,* 1996), adding to the oxidative stress. The ability of quartz surfaces to produce surface radicals could be ranked as follows – cristobalite > quartz > stishovite > coesite i.e. in the same order as pathogenicity (Fubini *et al*., 1995).

Therefore upon interaction with cells, quartz can induce ROS through a variety of properties and interactions. These include:

1. Complexity of the silica particle itself.
2. Its ability to disrupt membrane function or enzymes due to uptaken by cells.
3. Its bio-persistence and ability to react with other organelles in the cell.

This concept is illustrated in Figure 1.3 and the mechanisms indicated in Figure 1.3 are briefly elaborated in the next paragraphs.

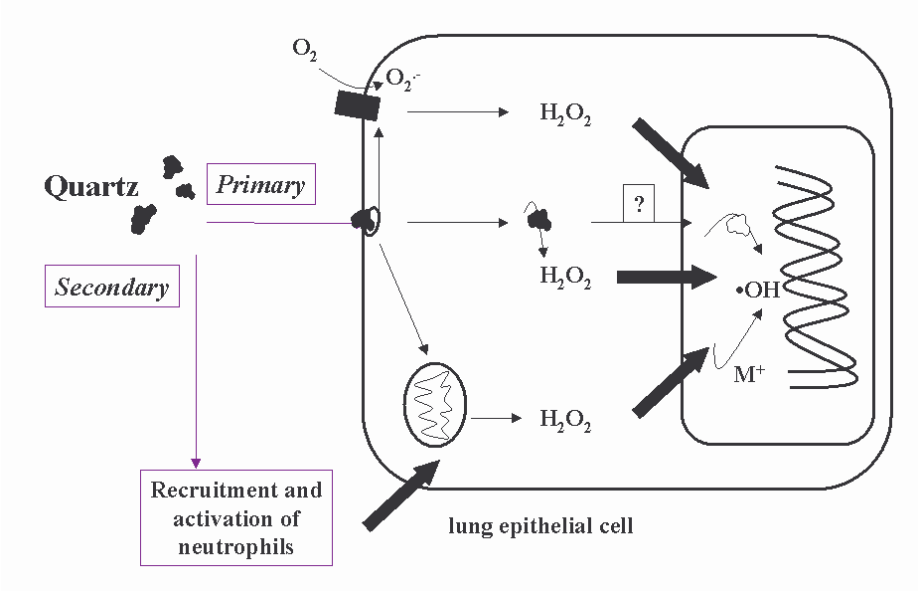

Figure 1. 3 Pathways of ROS generation by quartz particles in cellular system.

First, ROS can be generated from the quartz surface in the aqueous environment of the cell; alternatively, ROS can be generated from membranes during and after uptake, and finally intracellular ROS may be generated from the mitochondrial respiratory chain in.

1.4.2.1 Quartz particle surface chemistry and ROS in aqueous environments

Surface radicals are always present on quartz particles, Si-O· and Si· but can be excessively formed when crystal silica is freshly fractured. In solution these surface radicals can react with water allowing the following chemical reactions to take place and highly reactive hydroxyl radicals to be produced:

$Si\text{-}O^{\cdot} + H_2O \rightarrow SiOH\ (Silanol) + OH^{\cdot}$

$Si\text{-}O^{\cdot} + OH^{\cdot} \rightarrow SiOOH$

$SiOOH + H_2O \rightarrow SiOH + H_2O_2$

$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^{\cdot} + OH^{-}$

The ability to generate OH-radicals from different quartzes can be hugely different despite the fact that similar amounts of crystalline silica are present (Bruch *et al*., 2004; Fubini *et al*., 2004). As well the process difference during quartz particle preparation can lead to variation on treatment effect, this is an important point in particle work, but the discussion is not included in this thesis.

1.4.2.2. ROS generated from mitochondria after quartz particle uptake

Two major mechanisms have to be considered when relating particle uptake to ROS generation. First, the activation of the NAPDH-oxidase in the membrane of phagocytic and epithelial cells (Driscoll *et al*., 2001) is a major source of ROS during uptake of quartz particles. A second important pathway may be the uncoupling of the respiratory chain in mitochondria. Mitochondria are important to cell function, they are multifunctional organelles which regulate generation of oxidants for cell signaling,

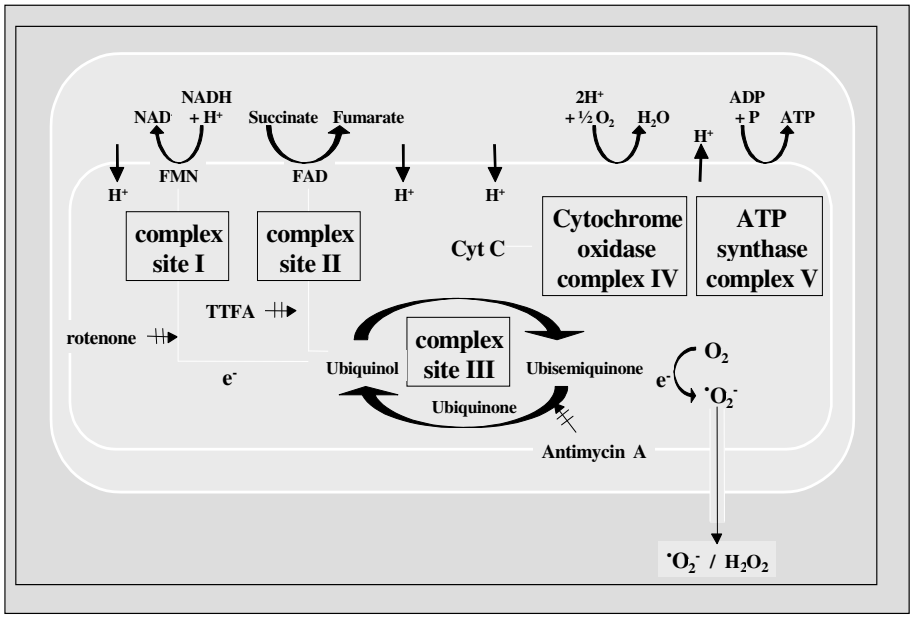

Figure 1.4. Mitochondria respiratory chain and ROS generation.

and provide essential ATP. The majority of oxygen consumed by mitochondria is converted to water by complex IV (cytochrome c oxidase), it can also pick up electrons directly from the ubiquinone site in complex III and flavin mononucleotide group of complex I to generate $O_2^{\bullet-}$(Figure 1.4). The $O_2^{\bullet-}$ generated from mitochondria can be converted to hydrogen peroxide (H_2O_2) by mitochondrial manganese superoxide dismutase (MnSOD). Under basal conditions, up to 2% of mitochondrial

oxygen consumption is used to generate H_2O_2. Being freely diffusible, mitochondrion-generated H_2O_2 can act as a signaling molecule, and can be converted to H_2O by the glutathione redox system. In the presence of transition metals, it may also react with $O_2^{\cdot -}$, thus producing highly reactive hydroxyl radicals ($OH^{\cdot}$) which may cause protein and DNA damage.

1.5 Oxidative modification of DNA bases by ROS and mutagenesis

Numerous studies were performed to investigate DNA damage induced by ROS derived from both endogenous and exogenous sources (Marnett, 2000). In the mid 80‘s Ames and co-workers discovered that oxidized DNA bases were abundantly present in tissues of both humans and rodents (Cathcart, 1984). At the same time it became clear that ROS-induced DNA damage was likely to be involved in carcinogenesis. ROS can oxidatively attack DNA bases, possibly leading to structural alterations in the DNA, such as base repair mutations, deletions, or insertions, which are all commonly observed in mutated oncogenes and tumor suppressor genes (Wiseman and Halliwell, 1996).

The chemistry of DNA damage elicited by several types of ROS has been well characterized, especially using *in vitro* systems. Generally, the reactions contributing to ROS-induced DNA damage are oxidation, nitration, depurination, methylation and deamination (Wiseman and Halliwell, 1996). It is important to note that the reactivity from various reactive species towards the DNA is extremely variable. For instance: whereas superoxide and hydrogen peroxide are thought not to react with DNA at all (Halliwell and Aruoma, 1991; Wiseman and Halliwell, 1996), $O_2^{\cdot -}$ selectively reacts with the guanine base (Van den Akker *et al*., 1994). However, the most potent ROS by far to react with DNA is the hydroxyl radical, which generates a multiplicity of DNA lesions (Pryor, 1988; Spencer *et al*., 1995). Hydroxyl radicals can be formed in several ways, but in biological systems the most significant pathway is probably the Fenton reaction, which involves hydrogen peroxide and transition metals. In 1984 Kasai discovered the DNA lesion 8-hydroxydeoxyguanosine (8-OHdG), which is produced by a hydroxylation of the C-8 position of the guanine derivate of the DNA (Kasai *et al*., 1984) as shown in Figure 1.5. The discovery that 8-OHdG was easily detectable by the use of HPLC with electrochemical detection (Floyd, 1986), made it the most abundantly studied oxidative DNA adduct in carcinogenesis research.

While it is generally believed that the OH radical is the oxidizing agent at the guanine molecule is less clear where the OH radical is formed. If a direct attack of the quartz particle on DNA is assumed the particles should translocate into the nucleus. On the other hand, hydrogen peroxide could be formed by an action of the particle outside the nucleus. Since hydrogen peroxide is stable it can freely diffuse across the nuclear membrane and react with DNA-bound transition metal to initiate a Fenton reaction in the immediate proximity of the target guanine. The hydrogen peroxide could also be formed in an inflammatory reaction because a parallel increase of phagocyte immigration and 8-OHdG formation has been shown in rat lung.

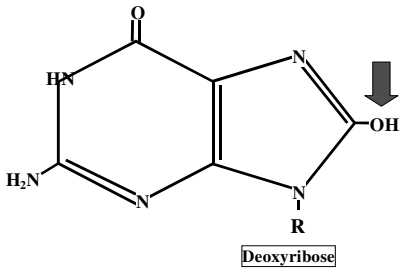

Figure 1.5 Hydroxylation of the guanine base at the C-8 position by hydroxyl radicals.

1.6 Detection of oxidative DNA damage: Methods and comparison

The base oxidation product 8-OHdG has become the most commonly measured biomarker of oxidative DNA damage in cells. A variety of analytical procedures have been developed to measure oxidative DNA damage, these include HPLC/ECD, GC-MS, FPG-comet assay and immunocytochemistry. The methodology of these techniques will be briefly described.

High performance liquid chromatography with electrochemical detection (HPLC/ECD): DNA is isolated and then precipitated with ethanol. After drying, DNA is dissolved in buffer, and subsequently hydrolyzed enzymatically with P1 nuclease and alkaline phosphatase. Finally the hydrolysate is injected into a HPLC device using a C18 column followed by electrochemical detection of 8-OHdG, paralleled by UV detection of 8-OHdG(Floyd *et al*., 1986).

Gas Chromatography coupled mass spectrometry (GC-MS): DNA samples are hydrolyzed to bases with 60% formic acid at 130℃ for 30 min. Bases are then derivatized with bis(trimethylsilyl)-trifluoroacetamide at room temperature for up to 2h in argon or nitrogen. Nucleosides are then separated on a C18 column and injected

into a triple quadrupole mass spectrometer for identification and quantitative analysis using the so-called multiple reaction-monitoring mode.

Both the above methods are quantitative and allow the number of oxidized bases to be expressed per number of nucleotides. Typical values are between $1/10^5$ and $2/10^6$ nucleotides and are subject to considerable variation between labs, mostly due to differences in sample preparation. In addition to these chemical quantitative methods, assays are available that allow evaluation of a tissue on a cellular level. These include:

Immunocytochemistry: In this method, slides of tissue sections or cells cultured on chamber slides are fixed with acetone. Sildess are stained using a primary antibody of 8-OHdG, then detected by incubation with a secondary antibody. Staining of 8-OHdG was developed with substrate of Diaminobenzidine (DAB) and visualised under microscope.

FPG comet assay: An alternative approach to measuring base oxidation employs the enzyme FPG to convert 8-OHdG to strand breaks, which are then measured by alkaline unwinding, alkaline elution, or the comet assay.

In this method a bacterial DNA repair endonuclease, FPG, is applied to the cells. FPG creates single strand breaks in the DNA at the sites of altered purines, including 8-OHdG. These breaks are then measured using the comet assay, where cell nuclei show a comet-like appearance after electrophoresis. These enzymatic methods are intrinsically highly sensitive, as they are based on the ability of small numbers of DNA breaks to disrupt the physicochemical behaviour of larger DNA molecules. As a corollary, they detect DNA strand breaks accurately only over a limited range. In the alkaline unwinding technique, the extent of denaturation over a fixed period is increased in the presence of DNA breaks, and the fractions of single and double-stranded DNA molecules are measured by chromatography. The comet assay measures DNA breaks by their ability to relax DNA supercoiling and allow DNA to migrate under electrophoresis, taking a comet-like appearance in which the percentage of DNA present in the tail reflects break frequency. The DNA breakage assays are calibrated indirectly, by comparison with cells treated with ionizing radiation to induce breaks at a known dose rate. These enzymatic methods have produced the lowest estimates of background damage levels in human white blood cells. The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up to resolve problems in the measurement of DNA oxidation that have

resulted in varying estimates of the extent of this damage in humans (ESCODD, 2003). HPLC/ECD is capable of measuring induced 8-OHdG in cellular DNA with great accuracy, but estimation of the background level of damage is still hampered by the occurrence of highly variable amounts of oxidation during sample preparation. Compared with HPLC-ECD, the levels of base oxidation for enzymatic analysis are much lower in the FPG comet assay but it offers a limited detection range. Immunocytochemistry is also employed using a specific antibody against 8-OHdG, it is specific but non-quantitative.

1.7 Repair of oxidative DNA damage: Base Excision Repair

The cell developed several pathways for repairing different types of DNA damage which have occurred. Oxidative DNA damage (e.g. 8-OHdG) is specifically repaired by the base excision repair (BER) pathway BER (Hansen and Kelly, 2000).

This pathway can be divided into four steps. First, the lesion is enzymatically regognozed by a specific glycosylase (e.g. OGG-1 for 8-OHdG) (Boiteux and Radicella, 1999). The glycosylase cleaves the N-glycosidic bond between the damaged base and the sugar phosphate backbone, creating an AP-site. Next, the AP site is processed by AP-endonuclease-1, which cleaves the phosphodiester backbone, resulting in a 3' OH and a 5' abasic deoxyribose phosphate (5'dRP). In the following step DNA polymerase ß adds one nucleatide to the 3' of the nick and then removes the 5' dRP via its intrinsic lyase activity. In the final step, the nick in both subpathways is sealed by DNA ligase I (Wallace, 1988) (Figure 1.6).

In addition, several other repair mechanisms have been identified, including nucleotide excision repair (NER), mismatch repair and direct repair by DNA alkyltransferases.

1.8 Effect on antioxidant*s on DNA damage:*

The idea that antioxidants in natural food are potentially effective for cancer prevention was generated from observational epidemiology. It is known that free radicals released during respiration can damage DNA and that oxidation damage to DNA can result in mutation. Antioxidants present in fruits, vegetables and flavonoids are expected to decrease oxidative DNA damage. However, confirmation of this hypothesis is needed for a practical daily guideline to human health, while it remains an elusive goal of experimental scientists. Nowadays it is realized that most antioxidants also possess pro-oxidant properties and care should be taken in the

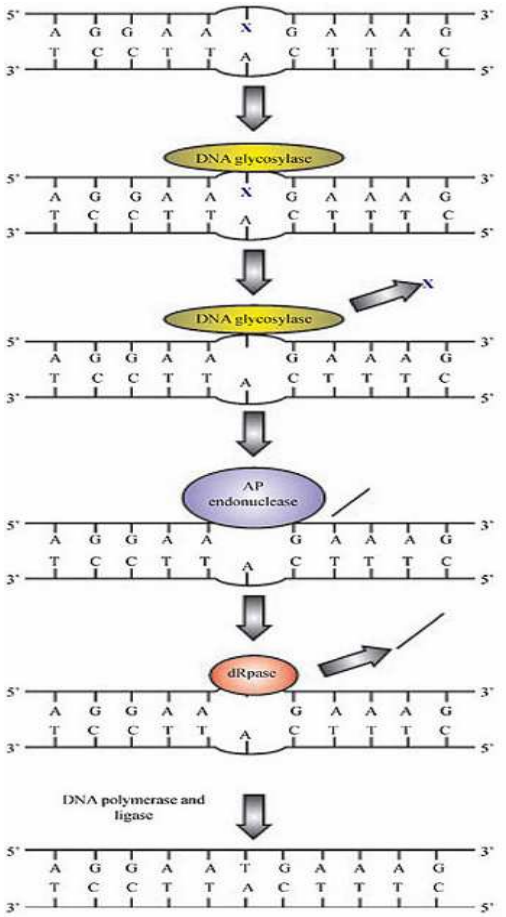

Figure 1.6 Pathway scheme of DNA base excision repair

(A). DNA damage is recognized by specific glycosylase, and glycosylase cleaves the N-glycosidic bond between damaged base and sugar phosphate backbone to form AP siite. (B). The AP site is processed by the AP-endonuclease-1, which cleaves the phosphodiester backbone and result in a 3' OH and a 5' abasic deoxyribose phosphate (5' dRP). (C). Nucleotide insertion: DNA polß add nucleotide to the 3' of the nick and remove the 5' dRP by its lyase activity. (D). The nick is sealed by the DNA ligase I.

dose and dose-route of different antioxidants (Bast *et al*., 1991). A good example of the double-edged effect of antioxidants was shown by the the Beta-Carotene and Retinol Efficacy Trial (CARET), where the effect of daily beta-carotene (30 mg) and retinyl palmitate (25,000 IU) on the incidence of lung cancer as well as other cancers and death was investigated. All 18,314 participants were at high risk for developing lung cancer due to a history of smoking or asbestos exposure. CARET was stopped ahead of schedule in January 1996 because among participants who were randomly assigned to receive the active intervention, a 28% increase in incidence of lung cancer as well as a 17% increase in incidence of death and a higher rate of

cardiovascular disease mortality compared with participants in the placebo group (Goodman *et al*., 2004) was observed.

Curcumin, a polyphenolic antioxidant derived from *Curcuma Longa* (structrue showed in Figure 1.7) is widely used as a spice in Indian cooking and has been proposed as an antioxidant which would protect against the effects of endogenous production of ROS. Various research groups considered curcumin as a non toxic phytochemical with anti-inflammatory, antioxidative and antitumorigenic effects antioxidants (Sharma et al., 2005; Rahman, et al., 2006). Our previous work on quartz-induced DNA damage has indicated that the adverse effect may be due to the capability of quartz to induce both ROS and inflammation. Curcumin is expected to protect lung epithelial cells from quartz-induced genotoxicity since it is related to its ability to generate oxidative stress and inflammation.

Figure 1.7 Chemical structure of curcumin

1.9 Aims of this thesis

Although respirable quartz has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC), the exact mechanisms by which quartz exposure might cause lung cancer are not yet fully understood. Chronic inhalation studies in rats indicate that alveolar type II cells are key target cells for quartz carcinogenesis. Previous observations by our group have suggested that formation of reactive oxygen species (ROS) by the quartz particle surface and/or by neutrophils activated during quartz-elicited inflammation may play a role in the induction of oxidative DNA damage in these cells.

As illustrated in Figure 1.8, a model was proposed involving primary versus secondary genotoxic pathways (Schins and Hei, in press). Primary genotoxicity is defined as genetic damage elicited by the particle itself (either direct or indirect), whereas secondary genotoxicity is defined as genetic damage resulting from ROS (and possibly other mediators) that are generated during particle-elicited inflammation.

The main aims of this thesis are:

(I) To study the role of mitochondria in oxidative DNA damage induction in the nucleus *in vitro* by quartz particles;

(II) To evaluate the effects of exogenous antioxidant curcumin on DNA strand breakage by quartz particles.

(I). The role of mitochondria in oxidative DNA damage formation in the nucleus *in vitro* by quartz particles

An *in vitro* model is used in which DNA damage is elicited in rat lung type II epithelial (RLE) cells by treatment with respirable quartz (DQ12). Various methods for oxidative DNA damage detection are deployed to determine 8-OHdG. Transmission electron microscopy and flow-cytometry showed a rapid particle uptake of quartz by the RLE cells, but particles were not found to translocate into the cell nuclei. Therefore, the hypothesis that quartz particles may elicit DNA damage by an alternative pathway is proposed. The role of mitochondria is elucidated in Chapter 2.

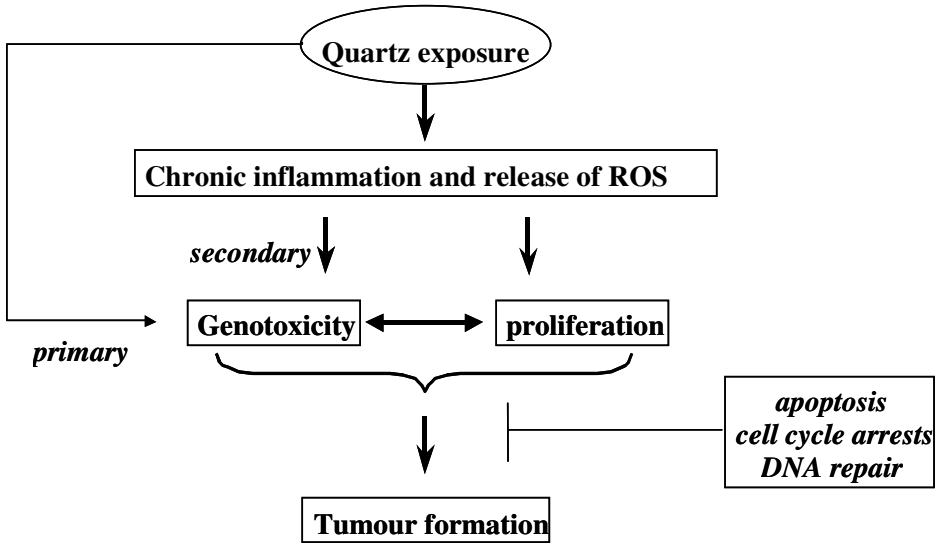

Fig.1.8 Illustration of the current paradigm of quartz induced fibrosis and cancer , and the crucial role of oxidative stress and inflammation in both of these endpoint.

(II). The effects of exogenous antioxidant curcumin on DNA strand breakage caused by quartz particles

In the second part of this thesis, experiments were carried out to determine whether curcumin can protect lung epithelial cells from DNA damage by quartz. Curcumin is a

major component of turmeric and is commonly used as a spice and food coloring material. It has been demonstrated to exhibit strong anti-inflammatory, antitumorigenic, and antioxidant properties. The effect of curcumin on quartz-induced DNA damage is investigated in Chapter 3.

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CHAPTER 2:

INHIBITION OF THE MITOCHONDRIAL RESPIRATORY CHAIN FUNCTION ABROGATES QUARTZ INDUCED DNA DAMAGE IN LUNG EPITHELIAL CELLS

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This chapter is accepted for publication in Mutation Research

ABSTRACT

Respirable quartz dust has been classified as a human carcinogen by the International Agency for Research on Cancer. The aim of our study was to investigate the mechanisms of DNA damage by DQ12 quartz in rat lung epithelial type II cells (RLE). Transmission electron microscopy and flow-cytometry analysis showed a rapid particle uptake (30 minutes to 4 hours) of quartz by the RLE cells, but particles were not found to translocate into cell nuclei. This indicates that DNA strand breakage and induction of 8-hydroxydeoxyguanosine -as also observed in these cells during these treatment intervals- did not result from direct physical interactions between particles and DNA, or from short-lived particle surface-derived reactive oxygen species. DNA damage by quartz was significantly reduced in the presence of the mitochondrial inhibitors rotenone and antimycin-A. In the absence of quartz, these inhibitors did not affect DNA damage, but they reduced cellular oxygen consumption. No signs of apoptosis were observed by quartz. Flow-cytometry analysis indicated that the reduced DNA damage by rotenone was not due to a possible mitochondria-mediated reduction of particle uptake by the RLE cells. Further proof of concept for the role of mitochondria was shown by the failure of quartz to elicit DNA damage in mitochondria-depleted 143B (rho-0) osteosarcoma cells, at concentrations where it elicited DNA damage in the parental 143B cell line. In conclusion, our data show that respirable quartz particles can elicit oxidative DNA damage without entering the nuclei of type II cells, which are considered to be important target cells in quartz carcinogenesis. Furthermore, our observations

indicate that such indirect DNA damage involves the mitochondrial electron transport chain function, by an as-yet-to-be elucidated mechanism.

2. 1. Introduction

World-wide, millions of people are occupationally exposed to crystalline silica (e.g. quartz) and this has been linked to pulmonary fibrosis and lung cancer. Respirable quartz has been classified as a human carcinogen by the International Agency for Research on Cancer [1]. Experimental studies in rats indicate that the carcinogenicity of quartz as observed at high and long-term exposure is largely driven by the DNA damaging and proliferative properties of reactive oxygen- and nitrogen species (ROS/RNS) generated from neutrophils and macrophages during quartz-elicited persistent inflammation [2,3]. In support of this concept, enhanced DNA damage and mutagenicity has indeed been demonstrated in the lungs of rats upon treatment with quartz at doses which also induce severe persistent pulmonary inflammation [2,4,5]. Importantly, these effects have been shown specifically in lung epithelial cells of these animals, among which alveolar type II cells are considered to be a key target in quartz carcinogenesis [2,3]. However, several independent investigations have also demonstrated DNA damaging capacities of quartz *in vitro*, i.e. in the absence of inflammation, although the responsible mechanism(s) is/are still poorly understood [6-10]. Hence, the relevance of these *in vitro* findings for the observed *in vivo* effects of quartz remains to be clarified.

It is well known for many years that quartz particles can generate reactive oxygen species (ROS) in aqueous environments, including the highly reactive hydroxyl-radical [10-15]. In relation to these observations, we previously showed that the radical-generating properties of quartz could play a major role in its ability to elicit DNA damage in alveolar type II cells. First, we showed that the increase in DNA single strand breaks as elicited by quartz *in vitro*, can be partly inhibited with hydroxyl-radical scavengers such as DMSO and mannitol [10]. Secondly, we showed that quartz particles can elicit formation of the oxidative, and pre-mutagenic DNA lesion 8-hydroxydeoxyguanosine (8-OHdG) [10]. Finally, we showed that modification of the surface of quartz with polyvinylpyridine-N-oxide (PVNO) or aluminium ions results in a reduction of the hydroxyl-radical generating properties of quartz, as well as of their DNA damaging effects in type II cells [16]. Using light microscopy analysis

of thin sections of these cells after quartz treatment, we showed a rapid uptake of the particles in relation to surface modification [16]. Taken together, our observations led us to suggest that the DNA damaging properties of quartz may be at least in part due to ROS generated from their surface after uptake of these particles. In line with this hypothesis, Daniel *et al*. previously demonstrated that quartz particles can oxidise naked DNA, and that this could be prevented with antioxidants [17,18]. The same investigators also reported the occasional appearance of quartz particles within the nuclei of cells as well as in mitotic spindles following long-term *in vitro* treatment and hence proposed a mechanism whereby direct interactions between particles and the genomic DNA may be implicated in its carcinogenic action [18,19]. However, the preliminary light microscopy analyses in our previous investigations in A549 human lung epithelial cells did not reveal any indication that quartz particles penetrate into the nucleus [16]. Hence, considering their extreme short half-life and accordingly limited diffusion distance, hydroxyl-radicals as generated from the quartz-surface therefore cannot explain for the oxidative damage to the nuclear DNA as observed in these cells.

To further evaluate how quartz may cause DNA damage without translocating into the nucleus we proposed several possible alternative pathways [3]. Among these, we considered a predominant role for mitochondria for a number of reasons. Firstly, mitochondria are considered as the main source for endogenous ROS [20,21]. Secondly, quartz particles have been shown to lead to enhanced H_2O_2 production by RLE rat lung epithelial cells, and this effect could be blocked by the mitochondrial inhibitor rotenone [22]. Based on these observations we hypothesised that stable mitochondria-derived ROS, (mainly H_2O_2) will diffuse into the nucleus where they can locally generate hydroxyl-radicals upon reaction with DNA-bound transition metals [3]. Recent investigations indicated that mitochondria-derived ROS are not responsible for basal levels of oxidative damage in mammalian nuclear DNA [23]. However, it is not known whether mitochondria mediate or contribute to nuclear DNA damage upon treatment of cells with exogenous oxidants such as, for instance, quartz particles. Therefore, the aim of the present study was to investigate the mechanism whereby quartz elicits DNA damage in rat lung type II epithelial cells, specifically focusing at the role of mitochondria. DNA damage by quartz was determined in RLE cells in dependence of mitochondrial inhibitors, as well as comparatively in an osteosarcoma cell line lacking functional mitochondria versus its

parental counterpart. To evaluate the role of quartz particle uptake and sub-cellular localisation in the RLE cells, we used transmission electron microscopy and flow-cytometry analysis.

2.2. Materials and methods

2.2.1. Materials

DQ12 (batch 6, IUF, Germany) quartz was used for all experiments. HAM F-12 medium, Trypsin, Dulbecco's Ca^{2+}/Mg^{2+} free PBS, Agarose, Low melting point (LMP) agarose, Triton X-100, Ethidium bromide, rotenone, antimycin-A and tenoyltrifluoroacetone (TTFA), Fetal calf serum (FCS), Uridine, Albumin, RNAse, Hydrogen peroxide, 3,3'-Diaminobenzidine tetrahydrochloride (DAB) were all purchased from Sigma (Germany). Dulbecco's modified Eagle's high glucose medium (DMEM), Sodium-Pyruvate, Penicillin/Streptomycin and Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS) were obtained from Gibco (Germany). Other chemicals were from Merck (Germany).

2.2.2 *Culture and treatment of RLE cells, 143B cells, and 143B-p^0 cells*

RLE rat lung epithelial type II cells were kindly provided by Dr. K. Driscoll (Procter & Gamble, Cincinnati, USA), and were cultured as described previously [10]. Experiments were always performed between cell passages 45 to 60. Functional mitochondria depleted 143B-p^0 osteosarcoma cells, as well as the parental 143B cell line (a generous gift from Dr. R.J. Wiesner, University of Cologne, Germany), were cultured in DMEM containing 5% FCS, 100 µg/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml uridine, and 1 mM sodium pyruvate. Cells were always treated at near confluence. For treatment, the DQ12 particles were suspended in complete culture medium. The suspension was vortexed for 2 minutes, sonicated (Sonorex TK52 water-bath; 60 Watt, 35 kHz) for 2 times 5 minutes and then directly added to the cells at the indicated concentrations. The mitochondrial inhibitors rotenone, antimycin-A and TTFA were used at final concentrations of 1 µM, 10 µM, and 10 µM respectively. All inhibitors were initially dissolved in DMSO, diluted in culture medium and then immediately added to the cells in a volume of 500 µl. The final DMSO concentration was always ≤ 0.1 %. Thirty minutes later, a further volume of 500 µl, either with or without DQ12 particles was added to the cells at the indicated

concentrations and incubated for 4 hours. For all assays, cell monolayers were rinsed repeatedly with PBS or serum free medium immediately after the end of the treatments. This was done to remove excess of extracellular particles that might interfere with the various assays, as well to remove detached (death) cells and cell debris.

2.2.3. Alkaline comet assay

Oxidative DNA damage induced by quartz particles was determined by the alkaline comet assay [24]. Briefly, after treatment of RLE cells or 143B and 143B-p^0 cells in 24-well culture plates, the cells were rinsed twice with PBS, detached by trypsination and immediately suspended in culture medium. Cells were centrifuged for 5 min at 400 g and resuspended in medium at a concentration of $1.5x10^6$ cells/ml. A mixture of 10 µl cell suspension with 120 µl 0.5% LMP agarose was added onto 1.5% agarose pre-coated slides. Following 5 min of solidification in 4°C refrigerator, slides were lysed for at least 1h or overnight at 4°C in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10% DMSO and 1% Triton X-100). Slides were then transferred into the electrophoresis tank followed by denaturation for 40 minutes. Subsequent electrophoresis was conducted at 300 mA and 25 V for 10 min. Slides were neutralised 3 x 5 min using neutralisation buffer (0.4 M Tris, pH 7.5). Before analysis slides were stained with ethidium bromide (10µg/ml, 40 µl per slide). Comet appearances were analysed using an Olympus BX60 fluorescence microscope at 200 x magnification. A comet image analysis software program (Comet Assay II, Perceptive Instruments, Haverhill, UK) was used for quantification of DNA damage (i.e. Olive tail moment).

2.2.4. Determination of 8-OHdG formation in RLE cells

Formation of 8-hydrodeoxyguanosine (8-OHdG) was determined by immuno-cytochemistry as described previously [10]. Briefly, RLE cells were seeded in 4-Chamber Slides (Falcon) at a concentration of 120,000 cells/chamber. After 48 hours, cells were exposed to quartz suspension for 4 hours. At the end of the incubations, cell monolayers were rinsed two times in PBS. Immuno-cytochemistry was performed using a monoclonal 8-OHdG antibody (N45.1, Japan Institute for the

Control of Aging, Japan), and the Vectastain-ABC kit (Vector Laboratories, Burlingame, CA).

2.2.5. Transmission electron microscopy

The morphology of the RLE cells and the intracellular distribution of the quartz particles were investigated using transmission electron microscopy (TEM). Therefore, RLE cells were grown in 35 mm culture dishes until 85 % confluence. Cells were washed twice with serum free medium and treated with DQ12 for different time intervals at the indicated concentrations. At the end of each incubation the monolayers were rapidly washed three times with serum free medium and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4°C. After postfixation in 2% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h at 4°C, the samples were en bloc stained with 1.5% uranylacetate dihydrate and phosphotungstic acid, dehydrated in ethanol series and embedded in epoxy resin (Epon Serva, Heidelberg, Germany) [25]. The morphology of the cells and the intracellular distribution of the quartz particles were investigated using ultra-thin sections (50 nm) placed on 150 mesh grids, stained with uranylacetate and lead citrate [26] and examined by transmission electron microscopy (STEM CM12, Philips, Eindhoven, Netherlands). EDX analysis with elemental mapping was used to confirm intracellular localisation of the DQ12 particles.

2.2.6. Oxygen consumption

A Clarke-electrode based method was used for oxygen consumption determination as described by Berneburg *et al*. [27]. Briefly, after treatment with DQ12 or mitochondrial inhibitors, cells were rinsed with PBS and detached by trypsination. The cell number was adjusted to a concentration of 1.5 x 10^6 cells/ml, and added into the incubation chamber. The oxygen amount was measured at 37°C under continuous agitation. Culture medium in the absence of cells was used as control. The consumption curve was recorded by a recorder and calculated as oxygen concentration versus time. Oxygen consumption was expressed as a percentage of control cells.

2.2.7. Uptake of quartz by RLE cells measured by flow-cytometry

To determine the effect of the mitochondrial inhibitors on the uptake of quartz particles, instead of electron microscopy analysis, a flow cytometry based-measurement was used based on the method as developed by Stringer *et al*. [28]. Although this the flow-cytometry method is only semi-quantitative, its advantage is that it allows for a rapid screening of a large cell population to determine uptake, in contrast to the laborious analysis of multiple TEM fields. Briefly, after treatment the cells were rinsed two times, then collected by trypsination, centrifuged at 900 g for 5 min at 4°C and washed twice with 1 ml of ice cold HBSS. After resuspension of the cells in 400 µl HBSS, they were kept on ice until flow-cytometry measurement using FACS*Calibur* (fluorescence-activated cell sorter, Becton Dickinson, Heidelberg, Germany). The side-scatter (SSC) and forward-scatter (FSC) angle of 10,000 cell-counts were determined, whereby the cell granularity as determined by SSC was used as a measure of particle uptake. The software Cell Quest 3.3 software (Becton Dickinson) was used for data analysis. Data were calculated from the median of SSC from gated SSC-histograms and are expressed as a percentage of control. Experiments were done in triplicate and repeated twice.

2.2.8. Statistical analysis

Data are expressed as mean ± SD from three independent experiments unless stated otherwise. Mean values were compared statistically using Student's t-test or Analysis of Variance (ANOVA). Dose- and time- dependent effects were determined using ANOVA with Dunett-T post hoc analysis. Statistical analysis was performed with SPPS for Windows (version 12.0.01).

2.3. Results

2.3.1.Induction of DNA strand breakage and 8-hydroxydeoxyguanosine in RLE cells by quartz.

The effects of quartz particles on DNA strand breakage in RLE cells are shown in Figure 1. As can be seen in the figure, clear dose- and time-dependent increases in strand breakage were observed with DQ12. Based on these investigations, for subsequent investigations a 4 hour treatment time was chosen at a dose of 100 µg/cm^2. Immunocytochemical analysis also indicated enhanced 8-hydroxydeoxyguanosine (8-OHdG) formation in the RLE cells upon treatment with DQ12 at this concentration (see Figure 1C).

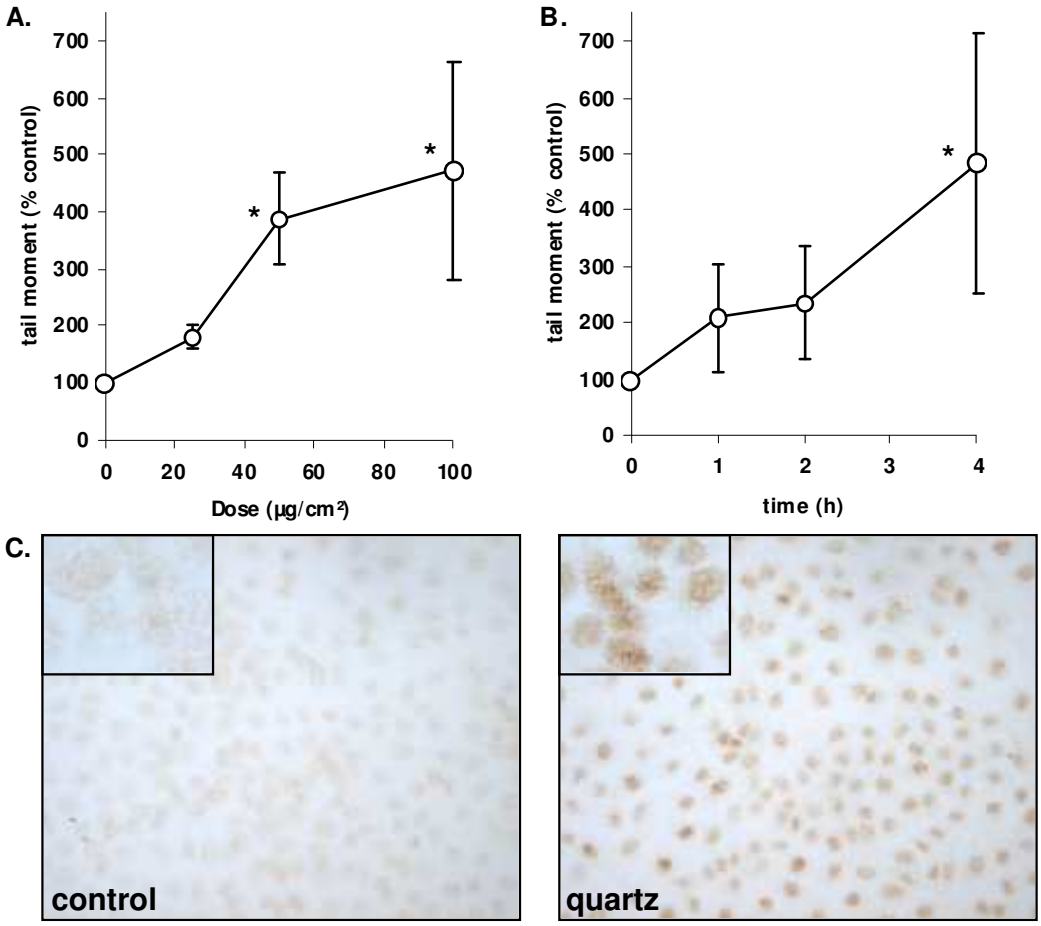

Fig. 1. DNA strand breakage and 8-OHdG induction by DQ12 quartz in RLE cells.

(A) Dose-dependent effect of DQ12 quartz particles on DNA strand breakage induction in RLE cells after 4 h treatment. **(B)** Time dependent effect of DQ12 quartz particles on DNA strand breakage induction in RLE cells. Cells were treated for the indicated time intervals with 100µg/cm² DQ12. **(C)** Immuno-cytochemical appearance of 8-OHdG in RLE cells after 4 h treatment with 100µg/cm² DQ12: (a) = control; (b) = DQ12. Magnification: 400x , insert: 1000x. *p<0.05 versus control.

2.3.2. Intracellular localisation of DQ12 particles after treatment in RLE cells

To find out whether quartz particles would translocate into the nucleus of the RLE cells upon treatment, we assessed sub-cellular presence of DQ12 within the RLE cells by TEM analysis. Representative pictures are shown in Figure 2.

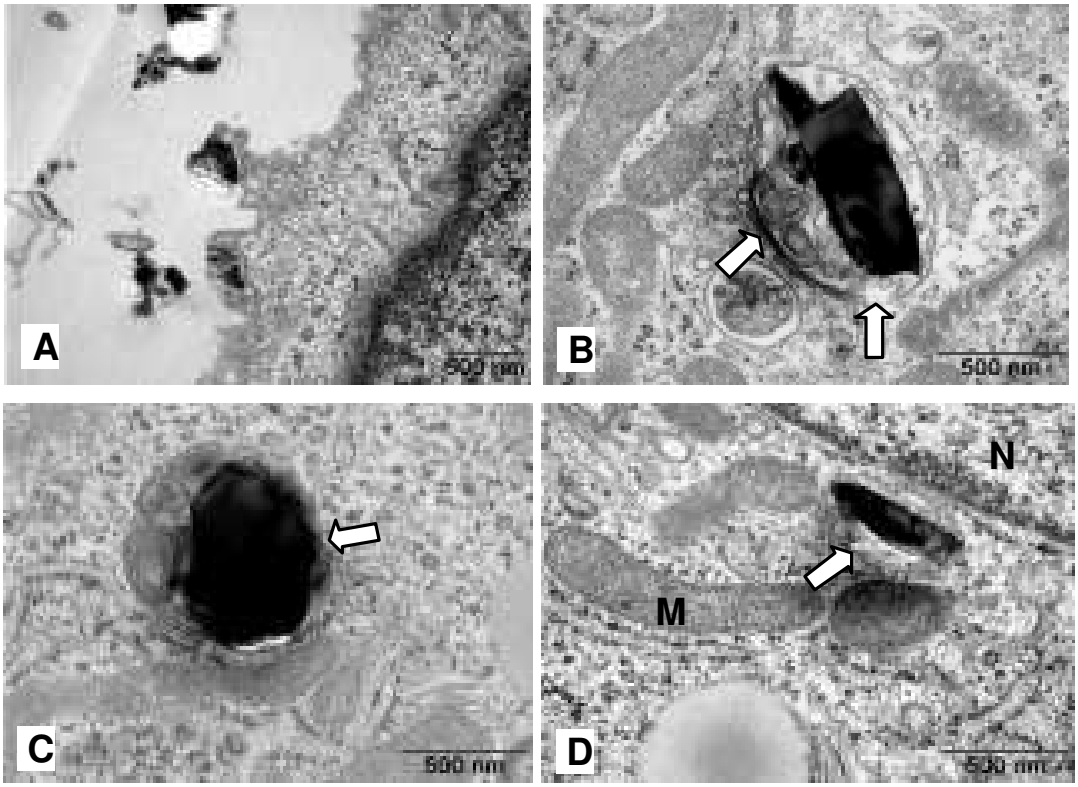

Fig. 2. Representative pictures from transmission electron microscopic analysis of RLE cells upon treatment with DQ12 particles.

Electron micrographs of transcytosis of DQ12 particles in RLE cells. The quartz particles were engulfed by microvilli for endocytosis (A). In the cytoplasma particles were enclosed by membranes (D), in phagosomes/phagolysosomes (B) and in lamellar bodies (C). Particles enclosed with membranes or in large vacuoles were observed near the nucleus (**=N**) and mitochondria (**=M**) (D). Arrows indicate membranes enclosing quartz particles.

The TEM analysis revealed a significant uptake within 2 hours of DQ12 particles into the RLE cells. The particles were found to be engulfed by surface protrusions of the cells. Particles were frequently observed associated with the plasma membrane and internalised into the cell cytosol. EDX analysis with elemental mapping demonstrated that the particles which were added to the cell cultures were identical to those particles observed inside the different cell compartments, thus confirming the internalisation of the quartz particles (data not shown). Quartz particles were mostly found incorporated in membrane-surrounded vacuoles usually containing two or more particles, and were seldom observed freely in the cytoplasm without enclosing membrane. Quartz particles were also found to be associated with lamellar bodies, in larger vesicles and in phago-lysosomes mixed with residual membranous material and other cell debris. Although particles were occasionally found located close to the

nuclei, we did never observe any DQ12-particle within the nucleus. Particles were also found in close proximity to mitochondria, suggesting interaction with these organelles, although they were never observed within these organelles.

2.3.3. Effects of mitochondrial inhibitors on oxygen consumption and DQ12-induced DNA damage in RLE cells

To determine the role of mitochondria in DNA damage induced by quartz particles, three different mitochondrial respiratory chain inhibitors were used, i.e. rotenone, TTFA, and antimycin A, which respectively inhibit complex I, II and III. As a fist step we analysed the specificity and capacity of these inhibitors to alter oxygen consumption by the RLE cells. As shown in Figure 3A, all three inhibitors were able to decrease oxygen consumption by the cells at the concentration used. Rotenone and antimycin showed comparatively stronger effects than TTFA in the RLE cells. The effects of these inhibitors on DQ12-induced DNA damage is shown in Figure 3B. As can be seen in the figure, the DNA damaging effect of DQ12 could be clearly prevented upon pre-treatment with rotenone as well as antimycin. Although the complex II inhibitor TTFA also tended to reduce DQ12-induced DNA damage, this effect did not reach statistical significance. In the absence of particles, neither of the inhibitors was found to induce DNA damage at the concentrations used. The effect of quartz (in either absence or presence of the inhibitors) on oxygen consumption was also measured, but could not be properly determined due to marked binding of the particles to the electrode membrane.

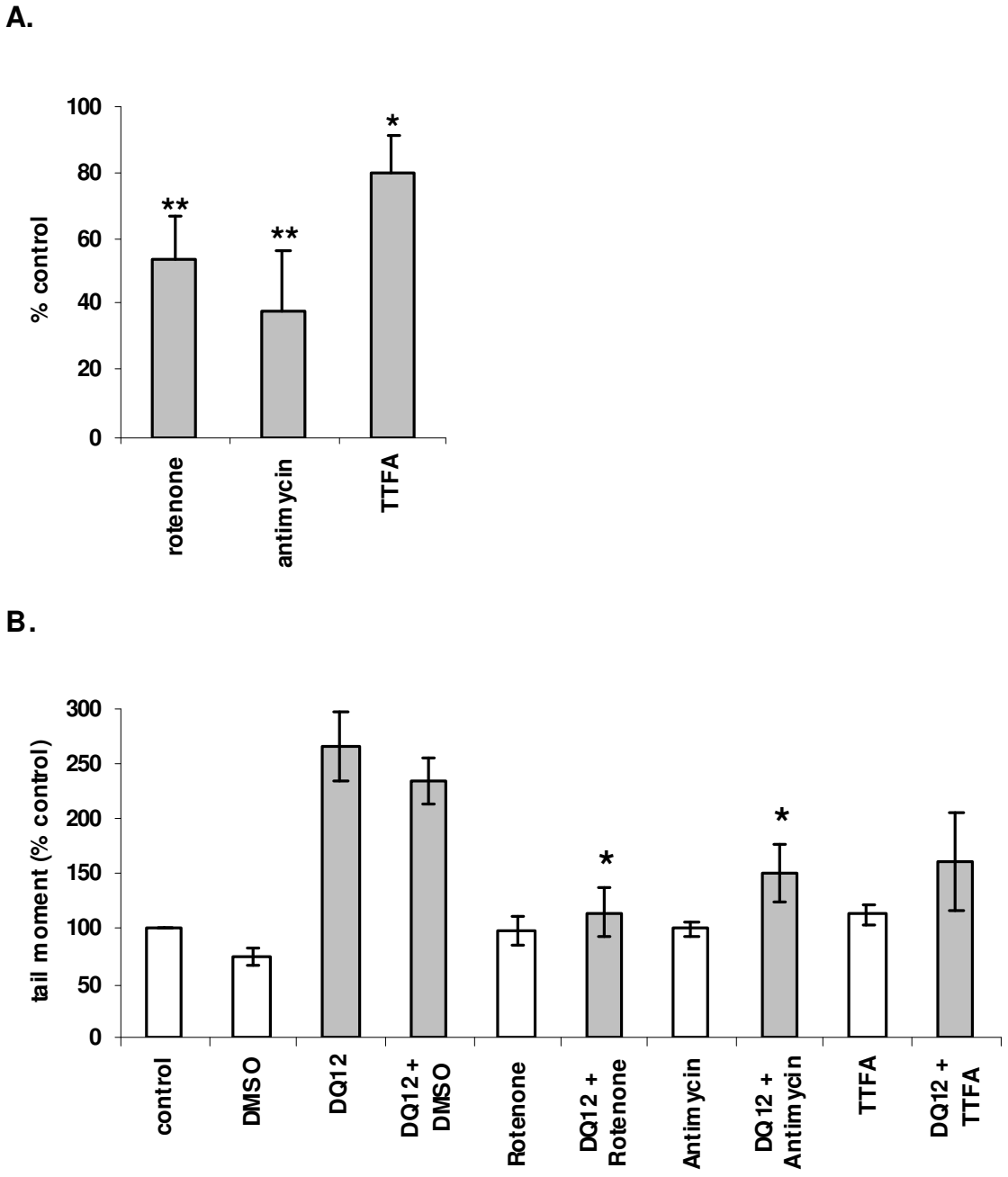

Fig. 3. Effect of mitochondrial respiratory chain function inhibitors on oxygen consumption and DQ12-induced DNA damage in RLE cells.

(A) Oxygen consumption by RLE cells upon treatment with the mitochondrial respiratory chain inhibitors rotenone (1µM), antimycin (10µM) or TTFA (10µM) as determined by a Clark electrode. **p<0.01 and *p<0.05 vs. control cells (B) DNA damage by 100 µg/cm^2 DQ12 in RLE cells in the presence or absence of mitochondrial respiratory chain inhibitors. *p<0.05 Vs DQ12+DMSO.

2.3.4. DNA damage by quartz in cells lacking functional mitochondria

To further investigate the role of mitochondrial function in quartz-induced DNA damage in RLE cells, the effects of DQ12 were comparatively evaluated in rho-0 143B cells, which lack functional mitochondria, and the parental 143B cell line. The contrasting functional status of these cell lines was verified using oxygen consumption measurements, as depicted in Figure 4A. The effect of DQ12 quartz particles on DNA damage in both cell lines, as tested by the comet assay, is shown in Figure 4B.

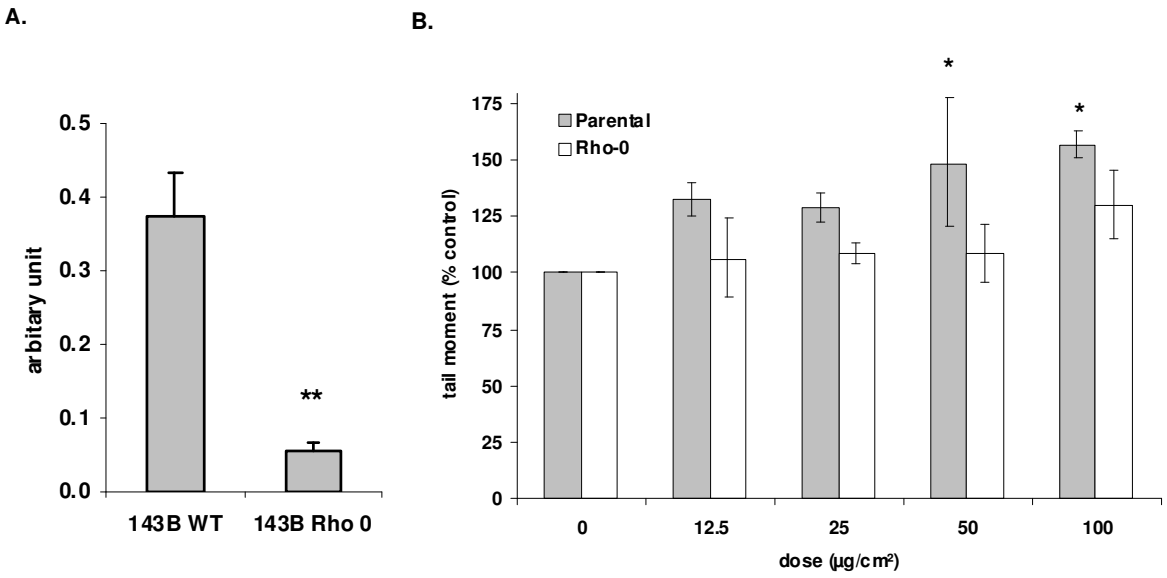

Fig. 4. DNA damage by quartz particles in 143B osteosarcoma cells without or with lacking functional mitochondria (rho-0 cells).

(A) Oxygen consumption in 143B and rho-0 143B cells as determined by a Clark electrode. **p<0.01

(B) DNA damage in 143B and rho-0 143B cells as determined by the comet assay following 4 h treatment with DQ12. *p<0.05.

As shown in this figure, DQ12 particles were found to cause a significant dose-dependent increase in DNA strand breakage in the parental cell line at concentrations where these particles failed to induce significant DNA damage in the rho-0 cell line. Notably, the effects of quartz were less pronounced in the wildtype 143B cell line than the RLE cell line.

2.3.5. Particle uptake in absence and presence of rotenone

Our previous investigations indicate that particle-uptake might be important for quartz-induced DNA damage [16]. Therefore, we also investigated the role of mitochondrial respiratory chain inhibition on particle uptake, specifically using rotenone which in our hands showed the strongest inhibitory effect on quartz-induced DNA damage in the RLE cells (see Figure 3B). The results of the uptake analysis experiments are shown in Figure 5.

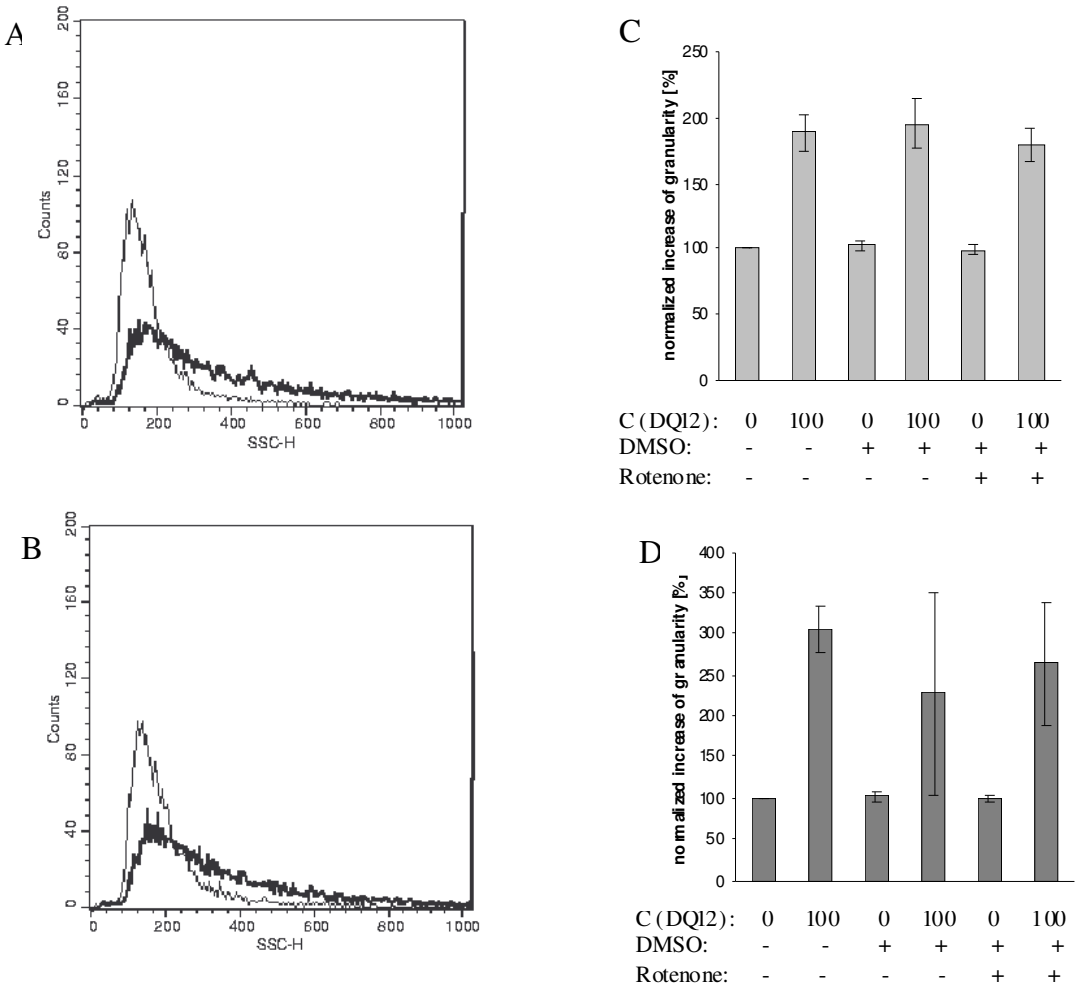

Fig. 5. Flow-cytometry measurement of quartz particle uptake in RLE cells in the presence or absence of rotenone.

Panels A and B display representative univariant gated histograms of RLE cell number (counts) and SSC (Side Scatter Angle) without (A) and with (B) 30 minutes pre-treatment of 1µM rotenone. In both figures the thin lines represents untreated cells, and the thick lines represent cells treated with for 30 min with 100 µg/cm^2 DQ12. Panels C and D represent the effects of rotenone on quartz uptake in RLE cells shown as median SSC values, upon 30 minutes (C) and 2 hours (D) treatment with 100 µg/cm^2

DQ12 quartz. Data represent the normalised increase of granularity as percentage of control, in the presence or absence of rotenone or its solvent DMPO. Granularity was significantly different ($p<0.001$) between control and DQ12 treated samples, irrespectively of the presence or absence of DMSO or rotenone+DMSO. No significant differences were observed in cell granularity after treatment with DMSO+quartz or rotenone/DMSO+quartz, in comparison to quartz alone ($p>0.1$).

Uptake of DQ12 quartz particles was visualised as a shift in distribution of cell granularity (see panels A and B). Significant uptake of quartz particles was observed already after 30 minutes in concordance with the TEM analysis. No significant effects on particle uptake of rotenone or its solvent control DMSO were observed after 30 minutes as well as 2 hours treatment (see graphs C and D). Hence, the inhibitory effect of rotenone on quartz-induced DNA damage as observed in the RLE cells could not be fully explained by a reduced particle uptake.

2.4. Discussion

The present study was undertaken to unravel the mechanisms whereby respirable quartz particles can elicit DNA damage in lung epithelial cells. About ten years ago, quartz has been classified as a human carcinogen by IARC [1]. Herein a mechanism whereby the observed tumours in rodents are driven by genotoxic and proliferative ROS generated during excessive persistent lung inflammation has been judged as the most plausible [1-3,29]. Such mechanism has indeed been supported by several *in vivo* studies where oxidative DNA damage and mutagenesis was found to be associated with pulmonary inflammation [2,4,5]. However, genotoxic and mutagenic effects of quartz have also been shown *in vitro*, i.e. independent of inflammation [6-10]. It is important to notice that over all fairly high concentrations have been used in those *in vitro* investigations as well as in our current study (i.e. up to 100µg/cm^2), hampering direct extrapolation of these conditions to exposure levels in experimental animals or humans [30]. Nevertheless, elucidation of the molecular mechanisms involved in these observed *in vitro* effects is important, as this will contribute to an improved understanding of the carcinogenic action of quartz as well as other particulate toxicants, and may hence benefit health risk assessment strategies.

Regarding the various toxicity and pathogenicity aspects of quartz particles, considerable research has contributed to the identification of its surface reactivity as

a central hallmark [14]. In aqueous environment, quartz has been shown to generate various ROS including the highly DNA reactive hydroxyl-radical [10-15,19], and early studies addressing the potential carcinogenic mechanisms of quartz showed that this mineral dust could damage naked DNA in an oxidant dependent manner [18,19]. A mechanism of quartz-induced carcinogenesis was hence proposed which involved binding of the crystalline silica surface to the nuclear DNA and subsequent attack of target DNA nucleotides by particle generated ROS [18,19]. Following up on these investigations, we previously demonstrated that also damage to the genomic DNA of intact mammalian lung epithelial cells by quartz occurs in a surface reactivity-associated and oxidant dependent manner [10,16]. However, in the present study we demonstrated that DNA strand breakage and induction of the oxidative and pre-mutagenic DNA lesion 8-OHdG can occur in the absence of direct physical interactions with genomic DNA, and/or via DNA attack by highly reactive, limited diffusable ROS that are generated from the particle surface. Specifically, we showed by both TEM and flow-cytometry analysis that quartz particles rapidly enter the rat lung epithelial cells. However, despite thorough TEM analysis, we did never observe nuclear translocation of any single particle for incubation times up to 24 hours. Yet, as early as 1 h, quartz particles already tended to enhance strand breakage in these cells. Quartz particles have occasionally been reported within the nucleus of cells, as well as in mitotic spindles after long-term exposures [18,19]. Although these observations indicate that DNA damage by quartz particles may occur via direct interactions, such mechanism cannot fully explain for our current observations. First, our cells were treated for relatively short time intervals at confluence, which virtually excludes mitotic processes in our culture. Moreover, the rapid induction of DNA damage (i.e. within 4 hours) as observed in our hands occurred in a large proportion of cells as evidenced by comet assay and immuno-cytochemistry data. Although we cannot rule out that quartz particles occasionally may have been translocated into the nucleus, and by chance not captured throughout our over all TEM analysis, it appears likely that the observed oxidative DNA damage predominantly resulted from quartz acting "at a distance".

In the knowledge that DNA-reactive ROS as generated from quartz surfaces have extremely limited diffusion distance, we focused on the role of mitochondria in the quartz genotoxicity [3]. Mitochondria represent a major endogenous source for intracellular ROS [20,21] predominantly H_2O_2 which is formed upon spontaneous or

enzymatic dismutation of superoxide anion leaking from the respiratory chain. Although mitochondria-derived ROS were previously demonstrated not to be responsible for the basal oxidation of nuclear DNA [23], we considered that this might be different in cells after treatment with respirable particles such as quartz. Specifically, we hypothesised that interactions between mitochondria and quartz particles causes formation of H_2O_2, which then diffuses into the nucleus, reacts with DNA-bound transition metals and thereby yields highly DNA-reactive hydroxyl radicals [3]. In line with this hypothesis, the mitochondrial complex I inhibitor rotenone was previously shown to abrogate enhanced H_2O_2 production in quartz-treated RLE cells [22]. Indeed, our current investigations provided support for such possible mechanism, since DNA damage as elicited by quartz in RLE cells could be prevented by rotenone. Moreover, inhibition of quartz-induced DNA damage also occurred with the complex III inhibitor antimycin-A as well as with the complex II inhibitor TTFA, although the latter did not reach significance. Interestingly, the differential capacity of these compounds to inhibit quartz-induced DNA damage tended to correlate with their ability to suppress oxygen consumption by the cells. The role of mitochondrial function in the genotoxicity of quartz, was independently verified using the 143B rho-0 osteosarcoma cell line and its parental cell line. Indeed, we observed a clearly reduced DNA damage by quartz in the rho-0 cells, which lack functional mitochondria, when compared to the normal cells. The clear functional impairment of the mitochondria of the 143B rho-0 cells was demonstrated in the form of their severely impaired oxygen consumption.

Although studies with isolated mitochondria have indicated that complexes I and III are major sources of superoxide, contrasting effects of electron chain inhibitors including rotenone, antimycin and TTFA have been reported in intact cells [23,31,32]. Such contrasts have been related to differences in cell types as well as the in the applied concentrations of the inhibitors. Importantly in this regard, in contrast to the investigations by Driscoll *et al.*, we failed to measure detectable H_2O_2 levels in the RLE cell cultures either in the presence or absence of quartz and/or rotenone using the same biochemical assay as used in their study [22]. In subsequent attempts, we were able to verify the clear inhibitory role of rotenone on endogenous ROS production in the RLE cells by flow-cytometry analysis of dihydrorhodamine 123 (DHR) oxidation. Also here however, we could not determine the effect of quartz due to the lacking sensitivity of this dye in the RLE cells in combination with the abundant

presence of quartz particles and associated scattering effects (data not shown). As such, it remains to be elucidated whether mitochondria-derived ROS are responsible for the observed effects of quartz in our RLE cells.

Since our previous work suggested a possible role for particle-uptake in quartz-induced DNA damage, we also determined the relation between mitochondria function and quartz uptake. We found that the rapid uptake of quartz particles by the RLE cells was not modified by rotenone. As such, this indicates that the inhibition of DNA damage by rotenone was not due to a reduced particle uptake and hence intracellular particle load. Beyond this, our observations also provided some first evidence that a significant inhibition of the mitochondrial respiratory chain function and associated reduction in oxygen consumption does not necessarily affect endocytosis of quartz particles by lung epithelial cells *in vitro*, at least for the time interval considered.

In line with our observations on the importance of mitochondrial function for the genotoxicity of quartz, we occasionally observed quartz particles located in close proximity to the mitochondria of the RLE cells (e.g. Figure 2D). However, we did not observe quartz particles inside mitochondria, and no signs of structural damage to these organelles were observed. The molecular mechanism underlying mitochondrial activation after quartz exposure remains thus to be elucidated. Nel and co-workers previously demonstrated that ambient ultrafine as well as fine particulate matter localise within mitochondria, in association with a clear induction of structural damage to these organelles [33]. However, further studies indicated that various functional disturbances of mitochondria (e.g. superoxide generation, membrane potential reduction) were merely driven by the organic constituents on these particles [34].

Functional disturbance of mitochondria was however also demonstrated for asbestos [35,36]. In concordance with quartz, asbestos is also well-known for its ability to generate ROS from their surface [37,38]. In line with our observations with quartz, intracellular ROS production as well as DNA fragmentation by asbestos was markedly stronger in A549 human lung epithelial cells than in A549 cells depleted of functional mitochondria [36]. Notably, these asbestos studies specifically addressed mechanisms of apoptosis (determined as DNA fragmentation at 24h), and accordingly revealed major novel findings on the activation of its intrinsic mitochondria and caspase-9 triggered pathway [36]. In contrast, our current study aimed at elucidation of the mechanisms involved in early oxidative DNA damage by

quartz (i.e. ≤ 4 hours), irrespective of the various later responses to such damage. These responses, which apart from mitochondria-triggered apoptosis, can include cell cycle arrest and the induction of DNA repair and/or mutations, are topics of our ongoing investigations. In this regard it is also interesting to note that DNA damage, and specifically 8-OHdG formation, as shown here with quartz, as well as elsewhere with asbestos and ambient particulate matter has been implicated in mutagenesis [3,39-41].

It should be emphasised that the endpoints in our study, i.e. DNA strand breakage, oxidative DNA damage induction and uptake of quartz, were determined after stringent washing of the monolayers. Hence, damaged cells including those who possibly underwent rapid apoptosis, but most importantly also necrotic cells in which DNA artificially can be oxidized through non-physiological mechanisms, are removed from our analyses. This aspect may also explain why relatively high concentrations of quartz were required to elicit significant DNA damage in contrast to other studies [7]. This washing procedure also provided further explanation for the fact that we failed to observe clear apoptotic cells with quartz, as might have been identified by the comet assay (i.e. "teardrop"-comet appearances), electron microscopy analysis (e.g. membrane blebbing, mitochondrial damage/swelling), or DAPI staining (not shown). Therefore, it should be emphasised that the effects as observed in our current study in terms of DNA damage induction, particle uptake and the mediating role of mitochondria herein, reflect effects in viable, non-apoptotic/non-necrotic cells.

In conclusion, in this study we could show that quartz elicits oxidative DNA damage in rat lung epithelial cells without being localised within nuclei, i.e. close or adjacent to the DNA. Our findings also suggest that the genotoxic properties of quartz in these cells depend on the mitochondrial respiratory chain function. In general, our data thus identify mitochondria as being potentially crucial host cell modifiers of quartz-induced intracellular oxidative stress and associated genotoxicity. In future studies we will concentrate on further elucidation of this mitochondria-mediated induction of DNA damage and its cellular consequences (e.g. DNA repair, apoptosis, mutagenesis) as this will contribute to our understanding of the mechanisms and *in vivo* relevance of the genotoxicity of this carcinogenic compound.

ACKNOWLEDGEMENTS

This study is supported by the German Research Council (Deutsche Forschungsgemeinschaft/DFG - International Graduate College 738). We acknowledge Prof. Rudolf Wiesner (Institute of Vegetative Physiology, University of Cologne, Germany) for providing us with the rho-0 and parental 143B cell lines. A.M. Knaapen is a postdoctoral research fellow of the Netherlands Organisation for Scientific Research (NWO, grant 916.46.092).

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CHAPTER 3:

Curcumin protects against cytotoxic and inflammatory effects of quartz particles but causes oxidative DNA damage in rat lung epithelial cells

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ABSTRACT

Chronic inhalation of high concentrations of respirable quartz particles has been implicated in various lung diseases including pulmonary fibrosis and lung cancer. Generation of reactive oxygen species (ROS) and oxidative stress has been implicated as a major aspect of the toxicity of quartz. Curcumin, a yellow pigment from *Curcuma Longa*, is commonly used as a spice and food colouring material. Because of its strong anti-inflammatory, antitumour, and antioxidant properties is has been considered as nutraceutical. The aim of our present study was to investigate whether curcumin can protect lung epithelial cells from the toxic and inflammatory associated with quartz exposure. Electron paramagnetic resonance measurements using the spin-trap DMPO demonstrated that curcumin reduces hydrogen peroxide-dependent hydroxyl-radical formation by quartz. In association with these observations, curcumin was found to reduce quartz-induced cytotoxicity in rat lung epithelial cells (RLE). Curcumin also inhibited the release of macrophage inflammatory protein-2 (MIP-2) from RLE cells as observed upon treatment with the pro-inflammatory cytokines interleukin-1 beta (IL-1β) and Tumor Necrosis-alpha (TNFα).. DQ12 caused a dose- and time dependent increase in oxidative DNA damage in the RLE cells as determined by formamidopyrimidine glycosylase (fpg)-modified comet assay as well as by immunocytochemical analysis of 8-hydroxydeoxyguanosine. However, curcumin failed to protect the RLE cells from oxidative DNA damage by quartz, but was found to be a strong inducer of oxidative DNA damage itself at non-cytotoxic and anti-inflammatory concentrations. Taken together, these observations indicate that one should be cautious about its clinical use in lung diseases associated with quartz exposure.

3.1 INTRODUCTION

World-wide, millions of people are occupationally exposed to crystalline silica (e.g. quartz). Chronic inhalation of high concentrations of respirable quartz particles has been implicated in various lung diseases including pulmonary fibrosis and lung cancer (IARC, 1997; Mossman and Churg, 1998; Albrecht *et al*., 2004a). Inhalation studies in experimental animals have demonstrated that the fibrogenic and tumourigenic effects of quartz are associated with persistent inflammatory and proliferative effects. The ability of quartz particles to generate free radicals in aqueous environments has been implicated in the molecular mechanisms which are considered to orchestrate these adverse health responses. Early investigations using electron spin resonance (ESR) analysis of quartz samples have indicated a role of surface-mediated free radical generation in the cytotoxic effects of quartz, via processes of cell membrane damage and lipid peroxidation processes (Vallyathan *et al*., 1998; Fubini, 1998; Dalal *et al*., 1990; Fubini and Hubbard, 2003). The generation of reactive oxygen species (ROS) by the quartz particles surface and/or particle-exposed cells is nowadays also considered to trigger the activation of transcription factors such as Nuclear Factor kappa B (NFκB) and activator protein-1 (AP-1) which bind promotor regions of a number of intermediate genes that govern inflammation, proliferation and/or apoptosis (Mossman and Churg, 1998; Fubini and Hubbard, 2003; Albrecht *et al*., 2004a). With regard to the inflammatory effects of quartz particles, the NFκB pathway has been considered as a central pathway. Quartz particles have been demonstrated to cause activation of NFκB in pulmonary macrophages and associated release of inflammatory mediators *in vitro* and *in vivo* (Schins and Donaldson, 2000; Duffin *et al*., 2001b; Albrecht *et al*., 2004b). *In vitro* studies have also demonstrated activation by quartz of NFκB and NFκB-regulated inflammatory genes such as the potent neutrophil chemokine interleukin-8 (IL-8) (Schins *et al*., 2000) or its rat equivalent macrophage inflammatory protein -2 (MIP-2) (Driscoll *et al*., 1997a) in lung epithelial cells. However, the activation of NFκB as observed in lung epithelium *in vivo* by quartz has been considered to be merely driven by inflammatory mediators from activated macrophage such as TNFα, IL-1β and/or other yet-to-be elucidated factors (Driscoll *et al*., 1997a; Albrecht *et al*., 2004). Finally, ROS have also been implicated in the genotoxic effects of quartz particles as observed *in vitro* (Schins *et al*., 2002) and *in vivo* (Seiler *et al*., 2001; Knaapen *et al*.,

2002). Although *in vivo* investigations indicate that oxidative DNA damage and mutagenesis is driven by the excessive formation of ROS during quartz-elicited inflammation (Driscoll *et al*., 1997; Seiler *et al*., 2001; Knaapen *et al*., 2002), *in vitro* investigations have demonstrated the genotoxic potential of quartz in the absence of inflammatory cells in relation to its ROS-generating properties (Zhong *et al*., 1997; Liu *et al*., 1998; Schins *et al*., 2002). The various mechanisms implicated in quartz toxicity as described above, are schematically shown in Figure 1.

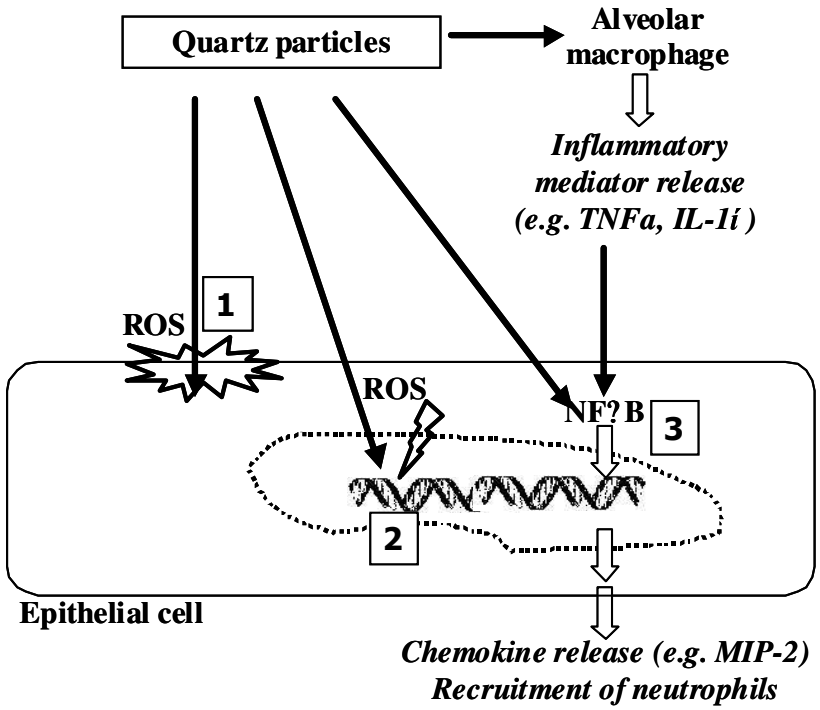

Figure 1. Simplified scheme of role particle mediated ROS formation and induction of oxidative stress in the inflammogenic, cytotoxic and genotoxic effects of quartz particles in lung epithelial cells.
Quartz particles have been shown to generate reactive oxygen species (ROS) and this has been implicated in cytotoxicity (1) and the induction of oxidative DNA damage (2) in lung epithelial cells. Quartz-elicited ROS generation has also been implicated in the induction of the transcription factor NFκB (3) and associated induction of NFκB controlled genes which are considered to orchestrate inflammation (e.g. the neutropihl chemokine MIP-2). Quartz particles are also known to activate alveolar macropahges for the release of inflammatory cytokines swhich are known as strong NFκB activators.

Curcumin (diferuloylmethane), a yellow pigment of the rhizome of the plant *Curcuma Longa*, is a major component of turmeric. It is used as a spice, food colouring

material and food preservative. The polyphenolic compound curcumin has been demonstrated to exhibit strong anti-inflammatory, antitumour, and antioxidant properties (Aggarwal *et al*., 2003). Accordingly, curcumin is currently used as nutriceutical for the treatment of various diseases including cancer, cardiovascular diseases, Crohn's disease, arthritis and neurodegenerative diseases as reviewed elsewhere (Aggarwall *et al*., 2003; Shishodia *et al*., 2005; Ramassamy, 2006).

For a number of reasons, curcumin might also represent a potential neutraceutical in quartz-associated lung disease. Firstly, curcumin has been demonstrated to have marked antioxidant properties. Curcumin has been for instance shown to scavenge superoxide radicals, hydrogen peroxide and nitric oxide from activated macrophages (Joe and Lokesh, 1994;). Others have shown protection from singlet oxygen-induced DNA strand breakage in plasmid DNA by curcumin (Subramanian *et al*., 1994). Hence, curcumin may offer protection against quartz-induced cell toxicity and oxidative DNA damage (see Figure 1). Secondly, curcumin is known as a potent inhibitor of signalling cascades, including NFκB, AP-1 and the c-Jun N-terminal Kinase pathway (Singh and Aggarwal, 1995; Jobin *et al*., 1999; Han *et al*., 2002; Chen and Tan, 1998). These pathways have also been implicated in quartz-induced lung disease (reviewed in Mossman and Churg, 1998; Albrecht *et al*., 2004b). Among these, NFκB has been considered as the transcription factor which critically drives the pulmonary inflammatory responses upon quartz exposure (Duffin *et al*., 2001; Albrecht *et al*., 2004) in association with an enhanced pulmonary expression of NFκB regulated genes encoding cytokines and chemokines. Indeed, curcumin has been found *in vitro* to reduce quartz-induced mRNA expression and protein release of interleukin-8 from A549 human lung epithelial cells (Schins *et al*., 2000). Furthermore, in view of the potential role of macrophage mediators in NFkB pathway activation *in vivo* following quartz-exposure (Driscoll *et al*., 1997a), curcumin has been demonstrated to block cytokine stimulated expression of IL-8 (Chaudhary and Avioli, 1996; Hidaka *et al*., 2002) (see Figure 1). Finally, although its availability to the respiratory tract is considered to be low upon oral administration (Sharma *et al*., 2005), curcumin has been shown previously to offer protection against pulmonary toxicity and fibrosis in a rats after bleomycin application (Venkatesan *et al*., 1997; Punithavathi *et al*.,2000). Recently, curcumin was shown to induce glutathione biosynthesis and inhibition of NFkB and AP-1 activation and IL-8 release in A549 human lung epithelial cells after treatment with H_2O_2 or IL-1β (Biswas *et al*., 2005),

and hence has been proposed along with other dietary polyphenols in the treatment of chronic obstructive pulmonary disease (Rahman and Adcock, 2006).

Taken together, the natural occuring spice curcumin may offer protection from quartz associated health effects in multiple ways, i.e. via its potential to inhibit cytotoxicity, to protect from oxidative DNA damage, as well as to inhibit NFkB pathway activation by quartz or macrophage-derived inflammatory mediators (see Figure 1). Therefore, the aim of our present study was to investigate the potential neutraceutical effects of curcumin in lung epithelial cells.

3.2 Materials and methods

3.2.1 Materials

DQ12 (batch 6, IUF, Germany) quartz was used for all experiments. HAM F-12 medium, Trypsin, Dulbecco's Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS), Agarose, Low melting point (LMP) agarose, Triton X-100, Ethidium bromide, Fetal calf serum (FCS), Albumin, RNAse, Hydrogen peroxide, 3,3'-Diaminobenzidine tetrahydrochloride (DAB) were all purchased from Sigma (Germany). Dulbecco's modified Eagle's high glucose medium (DMEM), Sodium-Pyruvate, Penicillin/Streptomycin and Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS) were obtained from Gibco (Germany). Curcumin was obtained from Sigma and had a purity of 94%. Recombinant rat IL-1β and TNFα were purchased from R&D Systems (Wiesbaden, Germany). Fpg-enzyme was kindly provided by Dr. Andrew Collins, Institute for Nutrition Research, University of Oslo, Norway. All other chemicals were from Merck (Germany).

3.2.2 Electron paramagnetic resonance

The ability of the quartz particles to generate hydroxyl radicals in the presence of hydrogen peroxide, was evaluated by Electron paramagnetic resonance (EPR) with the use of the spin-trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), as described previously (Schins *et al*., 2002). Briefly, 50 µl of distilled deionized water, 50 µl of 0.5M H_2O_2 in PBS and 100µl of the spin trap 0.05M of 5,5-dimethyl-1-

pyrroline-N-oxide (DMPO) in distilled deionized water were added to 4mg of DQ12. This suspension was incubated in a shaking water bath for 15 min at 37°C and filtered through a Sartorius 0.22 µm pore filter. The filtrate was then transferred to a capillary and DMPO–OH adduct formation was measured with a Miniscope ESR spectrometer (Magnettech, Berlin, Germany) as described previously (Schins *et al*., 2002). The effect of curcumin on the quartz-mediated hydroxyl-radical generation was determined by addition of curcumin to the above reagent mixture at the final concentratrations of 10 or 100 µM.

3.2.3 Culture and treatment of RLE cells

The rat lung epithelial type II cell line RLE-6TN (obtained from Dr. K. Driscoll, Procter & Gamble, Cincinnati, USA), was grown in HAM F12 supplemented with 5% heat inactivated FCS, L-glutamine, 1% 1M HEPES buffer and 30 IU/ml penicillin–streptomycin. For experiments, cells were trypsinised at near confluency, seeded into 24 well tissue culture plates and grown until near confluency. Experiments were always performed between cell passages 45 to 60. For treatment, the DQ12 particles were suspended in complete culture medium. The suspension was vortexed for 2 minutes, sonicated (Sonorex TK52 water-bath; 60 Watt, 35 kHz) for 2 times 5 minutes and then directly added to the cells at the indicated concentrations. Experiments with curcumin were performed as follows: Curcumin was dissolved in ethanol at the stock concentration of 10mM and then further diluted into culture medium at a final concentration of 5 or 10µM and immediately added to the cells in a volume of 500µl. Thirty minutes later, a further volume of 500µl medium, either with or without DQ12 particles, was added to the cells at the indicated concentrations and incubation time. For all assays, cell monolayers were rinsed repeatedly with PBS or serum free medium immediately after the end of the treatments. This was done to remove excess of extracellular particles that might interfere with the various assays, as well to remove detached (death) cells and cell debris.

3.2.4 Cytotoxicity

Cytotoxicity was determined by lactacte dehydrogenase (LDH)-assay assay as a marker of cell membrane integrity. LDH activity in the cell free supernatants was measured using a commercial diagnostic kit (Merck, Darmstadt, Germany). Therefore, cells RLE cells were treated at the indicated concentrations and time

intervals with quartz in the presence or absence of curcumin as described above, and at the end of the incubations cell free supernatants were collected by aspiration and centrifugation. The supernatants were immediately stored at 4℃ (for a maximum of 24 hours) and analysed for LDH.

3.2.5 Effect of curcumin on MIP-2 release from RLE cells after treatment with quartz or pro-inflammatory cytokines.

To determine the effect of curcumin on activation of the NFkappaB pathway in RLE cells, we evaluated the release of the NFkB-regulated neutrophil chemokine MIP-2 after treatment of the cells with quartz or a mixture of recombinant IL-1β (0.5ng/ml) and TNFα (1ng/ml). The release of Macrophage-Inflammatory Protein-2 (MIP-2) was measured in cell free supernatant using a commercial ELISA kit (R&D) according the manufacturer's instructions.

3.2.6 Formamidopyrimidine glycosylase (fpg)-modified comet assay

The Fpg-modified comet assay was used to measure oxidative DNA damage in the RLE cells, according to the method as described by Speit *et al*. (2003). Briefly, after treatment the RLE cells were rinsed twice with PBS, detached by trypsination and immediately suspended in culture medium. Cells were centrifuged for 5 min at 400 g and resuspended in medium at a concentration of $1.5x10^6$ cells/ml. A mixture of 10 µl cell suspension with 120 µl 0.5% LMP agarose was added onto 1.5% agarose pre-coated slides. Following 5 min of solidification in 4℃ refrigerator, slides were lysed for at least 1h or overnight at 4℃ in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH =10, containing 10% DMSO and 1% Triton X-100). The slides were washed three times for 5 minutes in Fpg-enzme buffer (40mM HEPES, 100mM KCl, 0.5mM EDTA, 0.2mg/ml BSA, pH=8) and then covered with 100 µl of either buffer or Fpg in buffer, sealed with a coverslip and incubated for 30 min at 37°C. Slides with and without Fpg post-treatment were then transferred into the electrophoresis tank andsubjected to 40 minutes denaturation and subsequent electrophoresis for 10 minutes at 300 mA and 25 V. Slides were neutralised 3 x 5 min using neutralisation buffer (0.4 M Tris, pH 7.5). Before analysis slides were stained with ethidium bromide (10µg/ml, 40 µl per slide). Comet appearances were analysed using an Olympus BX60 fluorescence microscope at 200 x magnification. A comet image analysis

software program (Comet Assay II, Perceptive Instruments, Haverhill, UK) was used for quantification of DNA damage by analysis of tail moments.

3.2.7 Determination of 8-OHdG formation in RLE cells

As an independent approach to determin oxidative DNA damage, immuno-cytochemistry was performed using a monoclonal 8-hydrodeoxyguanosine (8-OHdG) antibody. For this method RLE cells were seeded in 4-Chamber Slides (Falcon) at a concentration of 120,000 cells/chamber. After 48 hours, cells were exposed to quartz and/or curcumin at the indicated concentrations and exposure intervals. At the end of the incubations, cell monolayers were rinsed two times in PBS. Immuno-cytochemistry was performed using the monoclonal 8-OHdG antibody N45.1 (Japan Institute for the Control of Aging), and the Vectastain-ABC kit (Vector Laboratories, Burlingame, CA).

3.3 RESULTS

In relation to the well-known ability of quartz particles to generate free radicals, EPR was used to investigate the potential protective effects of curcumin. Specifically, the effect of curcumin on the hydrogen peroxide-dependent generation of hydroxyl-radicals was determined with the use of the spin-trap DMPO. As shown in Figure 2, the typical •OH specific 1:2:2:1 quartet pattern as observed for quartz could be reduced by curcumin in a dose-dependent manner. A marked reduction was observed in the presence of 10µM curcumin, whereas the ESR signal could be completely abrogated in the presence of 100µM curcumin (see Fig. 2). These acellular results were indicative of the potential of curcumin to inhibit oxidative effects of quartz in lung epithelial cells.

To determine whether in relation to these above findings, curcumin could protect RLE cells from the toxicity of quartz via its antioxidative properties, the LDH-assay was used as a marker of membrane damage. As shown in Figure 3, quartz caused both a dose- and time dependent increase in membrane damage. This toxic effect of quartz particles could be significantly reduced in cells that were also treated with curcumin. Notably, at 24h treatment, curcumin itself was found to show slight cytotoxicity (See Fig. 3).

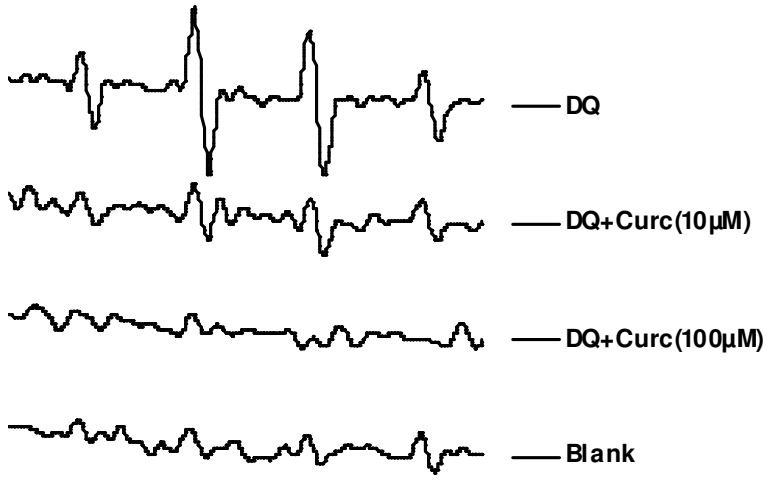

Figure 2. Effect of curcumin on hydroxyl-radical generating properties of respirable quartz particles.

The graphs represent ESR signals as observed upon 15 min incubation of quartz, DMPO and hydrogen peroxide in the presence or absence of curcumin.

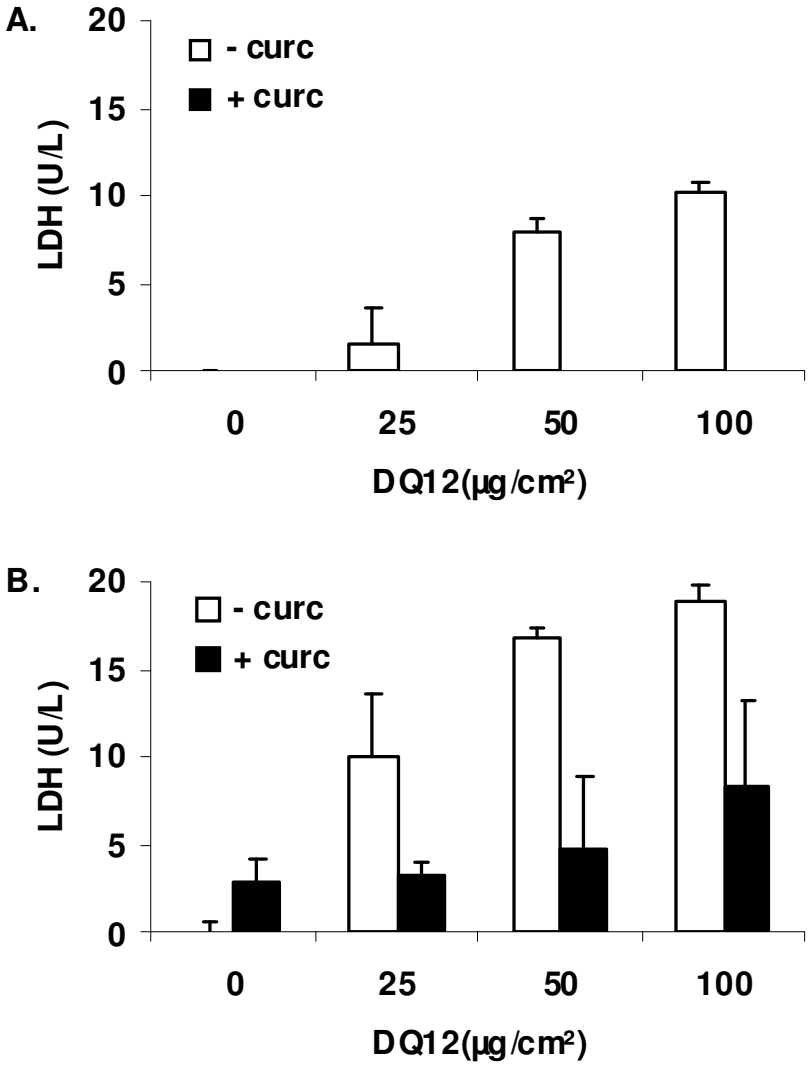

Figure 3. Effect of curcumin on cytotoxicity of quartz in RLE cells.

RLE cells were treated for 4 h (A) or 24 h (B) with quartz in the absence (-curc) or presence of curcumin (+curc).

Since curcumin has been identified by various investigators as a potent inhibitor of the NFkB pathway and associated inflammatory responses, we investigated the effect of this natural pigment on the release of the NFkappaB regulated chemokine MIP-2 from RLE cells after treatment with quartz with IL-1β plus TNFα. The results of these experiments are shown in Figure 4. Treatment of the RLE lung epithelial cells with quartz did not results in a significant increase in MIP-2 release. However, treatment of the cells with IL-1β/TNFα lead to an enhanced release of MIP-2. In cells that were co-treated with curcumin, the MIP-2 release as induced by IL-1β/TNFα was significantly reduced (see Figure 4).

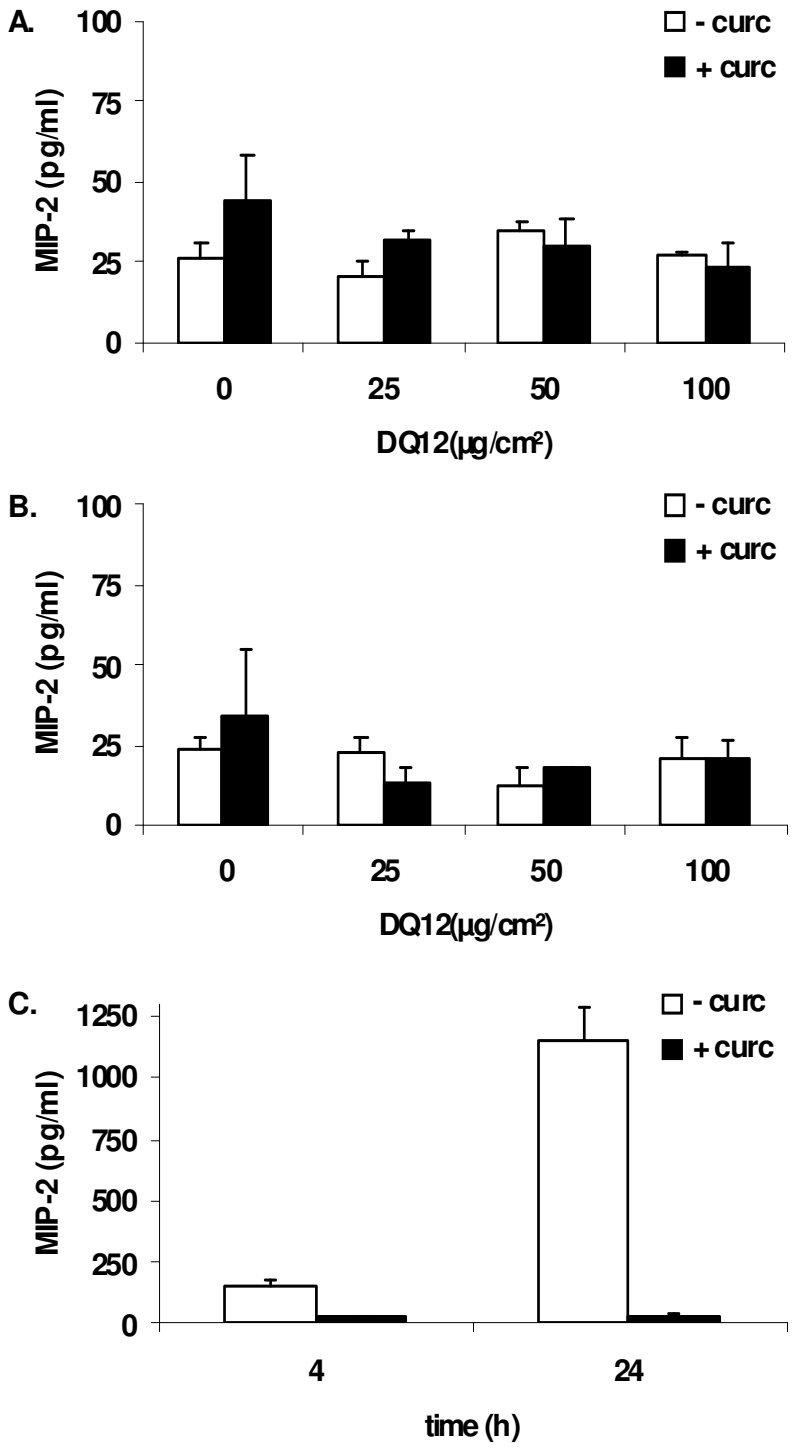

Figure 4. Effect of curcumin on MIP-2 release from RLE cells after treatment with quartz or TNFα/IL-1β mixture.

RLE cells were treated for 4 or 24 hours with quartz or by IL-1β/TNFα in the presence or absence of curcumin. (A) 4h treatment with quartz (B) 24h treatment with quartz (C) treatment with IL-1β/TNFα for 4h or 24h, in the absence (-curc) or presence (+curc) of curcumin.

In view of the oxidative properties of quartz, the fpg-modified comet assay was used to determine the induction of oxidative DNA damage in the RLE cells upon exposure to quartz and/or curcumin. Results are shown in Figure 5. Quartz particles were found to induce a dose-dependent increase in oxidative DNA damage in the RLE cells. Notably however, curcumin failed to protect the RLE cells from quartz-induced oxidative DNA damage (see Fig 5). In fact, curcumin was found to elicited a marked induction of oxidative DNA damage in the absence of quartz. Moreover, quartz-induced DNA damage was further enhanced in the presence of curcumin. In order to confirm these unexpected observations, as an independent approach we also evaluated the effects of quartz and/or curcumin on the formation of 8-OHdG using immuno-cytochemistry. The results of these analysis are shown in Figure 6. The results of this analysis were in concordance with the findings using the fpg-comet assay. However, in contrast to the fpg-modified assay no clear dose-dependency could be established with the immunohistochemical method. This most likely results from to lower sensitivity and semi-quantitative nature of this method.

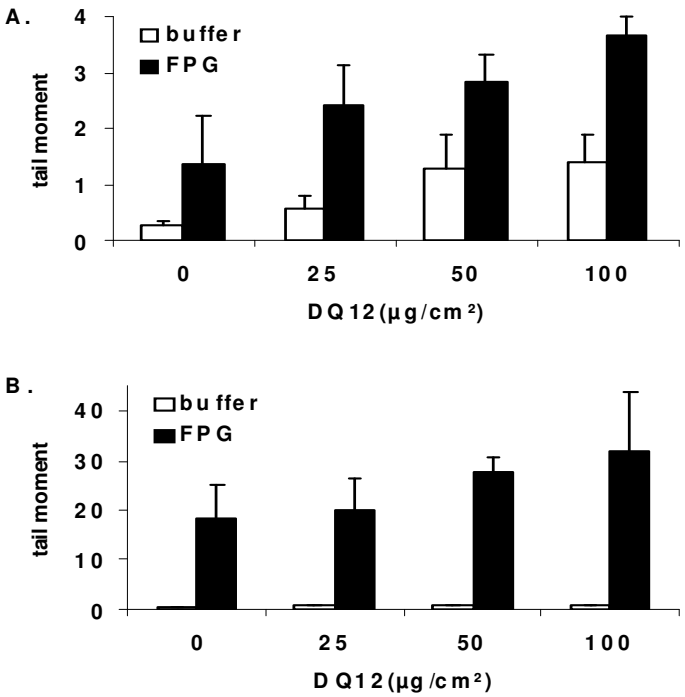

Figure 5. Effect of curcumin on quartz-induced oxidative DNA damage using fpg-comet assay.
Oxidative DNA damage was determined in RLE cells by fpg-modified comet assay following 4 h treatment of quartz. (A) Dose-response of quartz; (B) Dose-response of quartz in the presence of curcumin.

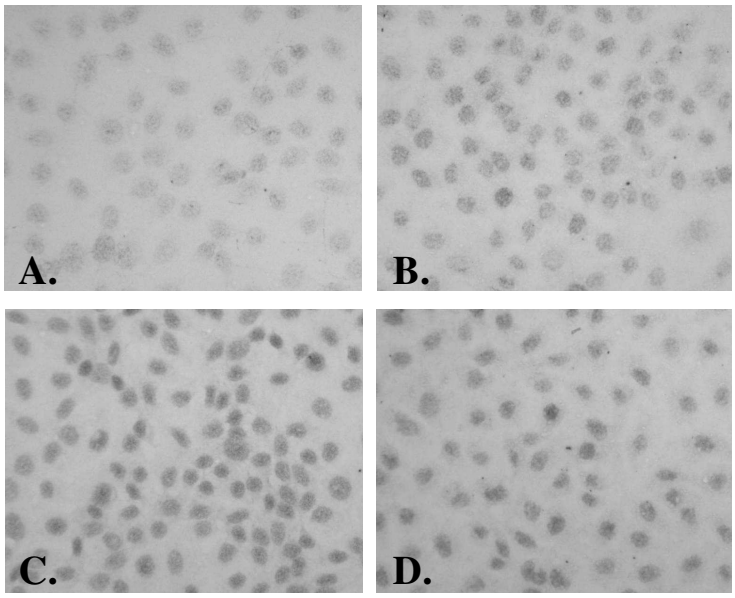

Figure 6. Effect of curcumin on quartz-induced 8-OHdG formation using immuno-histochemistry.

Immuno-cytochemical analysis was performed to determine the formation of 8-OHdG. (A) Control, (A) control, (B) 100µg/cm2 DQ12, (C) 10µM Curcumin + 100µg/cm2 DQ12, (D) 10µM Curcumin.

To further evaluate the oxidative effects of curcumin, a series of dose- and time dependent experiments were performed (not shown). These investigations revealed that curcumin causes a rapid induction of oxidative DNA damage. As early as 30 minutes after initial treatment, curcumin was found to cause a significant induction of oxidative DNA damage in a concentration-dependent manner(see Figure 7).

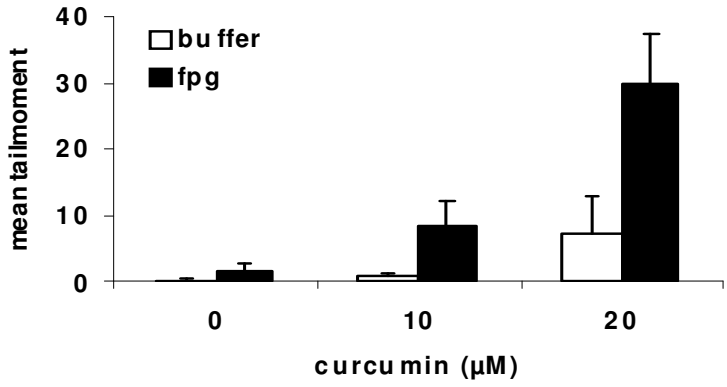

Figure 7. Effects of curcumin on oxidative DNA damage induction in RLE cells.

Oxidative DNA damage by curcumin (30 min treatment) was determined in RLE cells by fpg-modified comet assay.

3.4 DISCUSSION

In this study, we have investigated the effects of curcumin on quartz-induced toxicity in lung epithelial cells. Specifically, we investigated whether curcumin could protect against the cytotoxic, genotoxic and inflammatory effects of quartz particles. Since free radical generation by quartz particles has been implicated in each of these effects, we also determined the potential direct scavenging effect of curcumin herein using ESR with spin-trapping. Curcumin was found to reduce the H_2O_2-mediated generation of hydroxyl radicals from DQ12 in a dose-dependent manner. At a concentration of 100µM curcumin the ESR-signal could be completely abrogated. Our findings are in line with the reported antioxidant properties of curcumin (Joe and Lokesh, 1994; Subramanian *et al*., 1994). Recently, Biswas *et al*. (2005) showed by ESR that curcumin interacts with superoxide anions as well as hydroxyl-radicals (Biswas *et al*., 2005). Our current ESR-findings indicated that curcumin could also protect cells from the cytotoxic effects of quartz, specifically to membranolytic effects, since these have been contributed to the ROS generating properties of these particles (Dalal *et al*., 1990; Fubini and Hubbard, 2003). Indeed, our experiments showed that curcumin could abrogate the release of LDH from the RLE cells as observed in dose- and time dependent manner with quartz.

Apart from a role in cell membrane damage, ROS, as generated from quartz particles and/or their target cells, are also implicated in the activation of the NFkB pathway and associated induction of inflammatory mediator release. Therefore we investigated whether curcumin could block the release of MIP-2 from the RLE cells upon direct treatment with quartz, or with by IL-1β/TNFα. MIP-2 was specifically analysed for two main reasons. First, its known to be regulated by NFkB in lung epithelial cells (Driscoll *et al*., 1997a; Schins and Donaldson, 2000). Secondly, MIP-2 a major chemokine with regard to the neutrophilic inflammation, typically observed with quartz (Driscoll *et al*., 1997a; Duffin *et al*., 2001). In the present study, quartz was not found to elicit significant increase in MIP-2 release from the RLE cells, and therefore the potential inhibitory effect of curcumin could not be established. In contrast to our current observations, DQ12 quartz has been shown to enhance both the mRNA expression and protein release of the human neutrophil chemokine interleukin-8 (Schins *et al*., 2000). Earlier, Driscoll *et al*. (1997a) showed enhanced MIP-2 mRNA expression by Min-U-Sil quartz in RLE cells, the same as used in our current study.

Further investigations are needed to determine whether these current contrasting observations are due to differences in quartz samples, or due to cell and/or gene specific contrasts in post-transcriptional pathways, i.e. of MIP-2 in the RLE cells versus IL-8 in the A549 cells. In line with the inhibitory effect of curcumin on quartz-induced IL-8 release from A549 cells (Schins *et al*., 2000), curcumin has recently been also found to inhibit NFkB activation and IL-8 release in these cells after treatment with H_2O_2 or IL-1β (Biswas *et al*., 2005). In our current study, treatment of the RLE cells with a mixture of IL-1β/TNFα was found to induce MIP-2 release, and this release could be reduced by curcumin. These cytokines were evaluated in this study because of several reasons. First, both IL-1β and TNFα are well-known activators of the NFkB pathway (Schins and Donaldson, 2000). Furthermore, these cytokines have been implicated in the pathogenic effects of quartz (Driscoll *et al*., 1997; Fubini and Hubbard, 2003). Finally, activation of the NFkB pathway in the lung *in vivo* by quartz as well as other inflammatory mediators is considered to be crucially driven by inflammatory mediators released from activated macrophages (Lentsch *et al*., 1999; Driscoll *et al*., 1997; Koay *et al*., 2002; Albrecht *et al*., 2004). Taken together, our current findings suggest that curcumin, among various other dietary polyphenols, may have anti-inflammatory properties in the respiratory tract (Rahman and Adcock, 2006). In relation to its potential nutriceutical effect in the lung, curcumin was found to cause apoptosis in scleroderma lung fibroblasts but not in normal lung fibroblasts, suggesting its potential use in the treatment of fibrotic lung disease (Tourkina *et al*., 2004). More recently, curcumin was also found to inhibit cigarette smoke-induced NFkB activation and expression of the NFkB related genes cyclooxygenase-2 and matrix metalloproteinase-9 in human lung carcinoma cells (Shihodia *et al*., 2003).

Our current data in lung epithelial cells, however, show that curcumin concentrations which resulted in prevention of cytotoxicity and inflammatory mediator release, also elicited oxidative DNA damage. These observations were made in an attempt to determine the potential inhibitory effect of curcumin on quartz-induced oxidative DNA damage. Previously, we already demonstrated that quartz can elicit oxidative DNA damage in A549 human and RLE rat lung epithelial cells using the alkaline comet assay and immunohistochemical analysis of the oxidative and pre-mutagenic lesion 8-OHdG (Schins *et al*., 2002a; 2002b). In support of these findings, presently we also demonstrate enhanced fpg-sensitive DNA lesions in RLE cells. Using the same

sensitive assay to detect oxidative DNA damage, we found that curcumin itself represent a strong pro-oxidative agent in these lung epithelial cells. In fact, the effects of curcumin by far exceeded the effects of quartz. The ability of curcumin to elicit oxidative DNA damage was independently shown by immunohistochemistry for 8-OHdG. Our current findings with are in line with several other recent investigations. Kelly *et al*. (2001) observed that curcumin causes DNA damage in Jurkat T-lymphocytes. In this study, the DNA damaging properties of curcumin were linked to a mechanism involving oxygen radical generation (Kelly *et al*., 2001). Recently, Cao *et al*. (2006) showed 8-OHdG formation and DNA strand breakage in human hepathoma G2 cells after curcumin treatment. In the latter study, apart from nuclear DNA damage, also mitochondrial DNA damage was observed, which was suggested to relate to the pro-apoptotic effects of curcumin. Notably, in our present study the oxidative DNA damage by curcumin was already evident at 30 minutes. It remains to be investigated whether the observed oxidative DNA damage by curcumin in the RLE cells culminates into apoptosis or whether mutagenesis may occur, depending on the type and extent of DNA damage responses. Although apoptosis by curcumin has been described in several cells, this effects occurs at high doses (Jiang *et al*., 1996), and tends to be relatively lower in normal cells versus transformed/malignant cells (e.g. Tourkina *et al*., 2004). Furthermore, curcumin has been shown to enhance chromosomal damage in Chinese Hamster Ovary (CHO) cells (Antunes *et al*., 1999; Araujo *et al*., 1999).

In conclusion, the data from our present study indicate in support with other investigations that curcumin has potential beneficial effects in relation to prevention and/or treatment of inflammatory lung diseases. However, we also showed that relatively low concentrations of curcumin cause a marked induction of oxidative DNA damage in lung epithelial cells, suggesting that one should be cautious about its considerations for clinical trials

Acknowledgements

The authors thank Dr. Andrew Collins (Institute for Nutrition Research, Oslo, Norway) for kindly providing the fpg-enzyme. This study was supported by the German Research council (DFG) International Graduate College 738.

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CHAPTER 4:

SUMMARY AND GENERAL DISCUSSION

Numerous particles including particulate matter (PM) and crystalline silica have been associated with increased prevalence of lung cancer in humans (Dockery *et al*., 1993; Cohen *et al*., 1995; Pope *et al*., 2002; Nikula *et al*., 1995; IARC, 1997) and rats (Borm *et al*., 2004). Nevertheless, the mechanisms involved in carcinogenesis after particle exposure have only partly been elucidated (Knaapen *et al*., 2004). Carcinogenesis is a complicated multistep process and specifically in the initiation stage, genotoxic events are thought to play a crucial role. *In vitro* studies indicated that the genotoxic properties of quartz particles may depend on characteristics such as size, shape, chemical, composition, surface reactivity, crystallinity, hydrophobicity of the particle surface, and solubility of elements in the particle (Schins *et al*., 2002). In this thesis, mechanisms of DNA damage induced by DQ12, a standard respirable quartz particle, was studied in rat lung type II epithelial cell line.

In Chapter 2, we concentrated on the role of mitochondria in DNA damage elicited by quartz. It was shown that quartz induces oxidative DNA damage in rat lung epithelial cells without being localised inside the nucleus, whereas DNA damage caused by quartz was significantly reduced in presence of mitochondrial inhibitors. Further proof for the role of mitochondria was shown by the inability of quartz to elicit DNA damage in mitochondria-depleted 143B (rho-0) osteosarcoma cells, treated with a concentration which is capable of eliciting DNA damage in the parental 143B cell line. These findings suggest that the genotoxic properties of quartz depend on the mitochondrial respiratory chain function.

In Chapter 3, the antioxidant effect on quartz induced DNA damage was investigated using curcumin. As oxidative stress and chronic inflammation are important features in the pathogenesis of chronic pulmonary disease, antioxidant and/or anti-inflammatory agents such as curcumin and quercetin have been suggested for therapeutic administration to target oxidative stress or to boost the endogenous levels of antioxidants (Sharma *et al*., 2005; Rahman, *et al*., 2006). Curcumin has been reported to control NF-kappaB activation, regulation of glutathione biosynthesis genes, chromatin remodeling and hence inflammatory gene expression. We were able to show that the antioxidative properties of curcumin can reduce ROS

generation induced by quartz particles. It also inhibits MIP-2 secretion by RLE cells stimulated with TNFα/IL1-β which suggests a significant inhibition of neutrophils chemotaxis. However, an additive effect of curcumin on oxidative DNA damage was found when measured by FPG comet assay. The enhancement of quartz induced DNA damage by curcumin suggests that a one to one extrapolation of *in vitro* anti-oxidative protective effect is not without risk.

An extensive discussion has been held on the carcinogenicity of crystalline silica, especially after its classification as a human carcinogen by the IARC (1997). The arguments have focused on the variable entity (Donaldon & Borm, 1998) of the quartz surface which was experimentally underscored by studies with model (e.g. Schins *et al*., 2002) and commercially available quartzes (Cakmak *et al*., 2004; Seiler *et al*., 2004). Although in the IARC monograph considerable attention was given to the mechanisms of quartz induced DNA-damage, few studies have been published on this issue since. Our studies demonstrate a rapid uptake of quartz particles and the induction of DNA damage (i.e. within 4 hours) in lung epithelial type II cell lines. This arises the question whether DNA damage is induced by a direct interaction between the particle and the nuclear DNA. Our TEM analysis showed that quartz particles are not present in the nuclear compartment, which indicates that the observed oxidative DNA damage predominantly has to result from quartz acting "at a distance". Whereas considering their extreme short half-life and accordingly limited diffusion distance, hydroxyl-radicals generated from the quartz-surface cannot explain for the oxidative DNA damage observed in these cells.

The hypothesis of the mitochondrial role in quartz induced DNA damage was proposed for reasons that, firstly, mitochondria are considered as the main source for endogenous ROS (Turrens *et al*., 2003; Chen *et al*., 2003). Secondly, quartz particles have been shown to enhance H_2O_2 production by rat lung epithelial cells, an effect that could be blocked by the mitochondrial inhibitor rotenone (Driscoll *et al*., 2001). It is hypothesized that stable mitochondria-derived ROS (mainly H_2O_2) can diffuse into the nucleus where they locally generate hydroxyl-radicals upon reaction with DNA-bound transition metals (Henle and Linn, 1997; Rodriquez *et al*., 1997; Knaapen *et al*., 2004). Our current experiments indicate that the mitochondrial function is possibly involved in quartz induced genotoxicity. This finding offers an explanation as to how particles can generate DNA damage without entering the nucleus considering the half-life and diffusion distance of hydroxyl-radicals generated from the quartz.

However, the exact mechanisms behind the influence on mitochondria of quartz particles remain unclear. Further studies are needed to investigate mitochondrial function and its modification by quartz particle treatment. It should be emphasised that the endpoints in our study, i.e. uptake of quartz, DNA strand breakage, and abrogation of DNA damage by mitochondrial inhibitors were determined *in vitro* after treatment with a dose of 100µg/cm². These concentrations are much higher than the concentrations that occur in humans lungs during exposure to the current quartz exposure limit (50 to 75 ug/m3 silica in humans) .

The extrapolation to humans, generally exposed to much lower particle concentrations is a subject for ongoing debate. In fact exposure to high doses can occur in reality based on the fact that quartz is poorly soluble and always aggregates in lung tissue. Though not included in this thesis, additional oxidative DNA damage was found in RLE cells pre-treated with low dose of quartz particles followed by Ro 19-8022 sensitisation which can induce specific oxidative DNA damage. It leads to the question whether there is a synergistic effect from quartz particle uptake and inflammation, as known the complexity chemistry of quartz that different mechanisms may be involved in quartz induced carcinogenesis.

A second, major finding in this work is curcumin enhanced quartz induced DNA-damage despite its scavenging OH-radicals in acellular systems. Antioxidant therapy is applied in many conditions where oxidative stress and chronic inflammation are features of the pathogenesis of diseases and ageing. We found that curcumin, which is a common antioxidant in food and food flavours, can abrogate ROS generation by quartz particle in an acellular system which was measured by EPR, and inhibits MIP-2 secretion induced by TNFα/ IL-1β on rat lung epithelial cells which indicates a reduction of inflammatory response. But it does induce a dose dependent oxidative DNA damage in rat lung epithelial cells in a dose dependent manner and consequently increases quartz induced oxidative DNA damage.

Though this finding seems quite unexpected, it merely indicates a different effect of curcumin measured under acellular and cellular conditions. This can be explained by the fact that ESR is a simple method to measure ROS generation in acellular systems, however cell structures are much more complicated than the ESR system, since ROS generated by chemicals have to get past the cell membrane, mitochondria and the antioxidant defence system to induce any biological effect.

As we showed that curcumin is capable of eliciting oxidative DNA damage in alveolar epithelial cells and considering that oxidative DNA damage(8-OHdG) is a premutagenic event, our observations on curcumin suggest that antioxidants may have a potential adverse effect in certain conditions i.e. at high concentrations or in certain organs. Though further studies are needed to determine the metabolic pathway of curcumin in the human body and the final concentration it can reach in lung tissue, it still implicates that one should be cautious when applying antioxidants as they may have a dual effect.

Moreover, the cellular response to oxidative DNA damage in terms of apoptosis induction or DNA repair activation induced by curcumin as well by other antioxidants need to be further addressed both *in vitro* and *in vivo*.

A major question remains to be answered, is whether the mechanisms of quartz induced DNA damage discussed in this thesis are relevant for human situation. As such, quartz particles were found to elicit DNA damage without translocating into cell nuclei. Our data show that respirable quartz particles can elicit oxidative DNA damage without entering the nuclei of type II cells, which are considered to be important target cells for quartz carcinogenesis. The finding that particles are capable to induce genotoxicity by an epigenetic mechanism leads to a new insight in particle risk assessment. This may also be important for genotoxicity determination of nanoparticles which are generated by recently developed technology. A number of publications have shown that silica-nanoparticles are translocated into the nucleus (Chen and von Mikecz, 2005; Geiser *et al*., 2005) and may interfere with normal DNA machinery and enzymes linked to its repair and production. However, the results from chapter 2, show that particles can also damage DNA without going into the nucleus. This means that the concept of particles only being dangerous when capable of entering the nucleus needs to be reconsidered.

Is this the only implication? If particles elicit genotoxicity by an epigenetic mechanism this also has implications for risk assessment. Current exposure limits set to prevent lung cancer by silica exposure are based on dose-response curves for quartz exposure and silicosis, thereby assuming that silicosis occurs much earlier than lung cancer. However, it will open up new pathways for risk assessment if quartz induced cancer acts independent from the fibrotic response via an epigenetic mechanism. A third meaning of mitochondria mediated DNA-damage is for test methods and protocols used to determine particle induced genotoxicity. So far, most soluble

chemicals have been tested in bacterial and *in vivo* mammalian systems, depending on the outcome in the bacterial system. Although no gold standard is available for particle testing, our findings certainly suggest that pre-testing needs to be done in a metabolically competent cell type. Finally, as a general outcome it sets the stage for further thinking and experiments to explain cancer in relation to nutritional intake, energy production and more physiological functions related to mitochondrial input and output.

Moreover, our studies on curcumin showed an opposite effect between acellular and cellular effect of quartz as it can reduce ROS generation by quartz particle in ESR measurement, while it induces oxidative DNA damage in alveolar epithelial cells. These findings suggest that one should be cautious to interpret data derived from acellular systems considering the complexity of biological matter.

Notes should also put on other antioxidants which are going to be applied as preventive strategy to human. Previous intervention studies with ß-carotene in chronic smokers had to be ceased because of additive effects of ß-carotene on lung cancer the heavy smokers group (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994). Nowadays, there is a common awareness among scientists that also antioxidants have an optimal dose, and that increasing the dose just to be sure, may be a hazardous strategy.

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Acknowledgements:

I would like to first sincerely thank my entire family member for giving me their support and encouragement to do my PhD study. Especially to my husband, Xueliang Zhou and my son, Yuewu Zhou. It will be impossible to finish my PhD study without their accompanying with love in Germany.

Then I'd like to thank the German Research Council (Deutsche Forschungs Gemeinschaft-DFG) for providing the stipend, giving me the chance to be a PhD student in Düsseldorf. As well thanks to the institute of environmental medicine (Institut für umweltmedizinische Forschung-IUF) where my lab is located, providing me with a working environment and allowing me to extend my project with one year to follow-up on my experiments.

Many sincerely thanks to my supervisors: Prof. Dr. Paul Borm, Prof. Dr. Regine Kahl, Prof. Dr. Frank Wunderlich and Dr. Roel Schins for their guidance during my study.

Thanks to Prof. Dr. Borm for providing the structure suggestion during my thesis writing and his help in the beginning I came to Germany. It was a big loss for our group when he chose to return to the Netherlands.

Prof. Dr. Kahl is always there for me when I meet trouble or difficulties, not only work-related but also general issues involving my VISA for instance. I am very impressed by the clear and well-prepared lectures she gave in the courses of toxicology. I am very glad to be able to witness the great job she did when organising the International Graduate College.

Specifically, many thanks to my supervisor Roel Schins for his patient and enthusiastic week-discussion. That is where I learned most of my new knowledge and get to know how a real scientist is working.

All of my dear colleagues: Dr. Catrin Albrecht, Dr. Rodger Duffin, Dr. Karim Bagate, Dr. Tingming Shi, Dr. Seema Singh, Dr. Petra Haberzettl, Damien van Berlo, Anton Wessels, and James Meiring, they are not only helped me greatly in my work, but also provided me a nice atmosphere for work and study. The nice friendship and sweet memories will be cherished for the rest of my life.

Astrid Winzer, who is both a nice colleague and good friend of mine, I want to thank a lot for her kind care to me and my family. Technical assistants in our lab, Mrs. Ledermann and Mrs. Weishaupt, I would like give my thanks for all the work they did

for me and our group. Finally, thanks to all of my friends in Düsseldorf for being with me, to overcome the cultural shock I experienced.

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