Influence of natural food compounds on DNA stability

Einfluss natürlicher Nahrungsbestandteile auf die DNA Stabilität

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Submitted by

Nina Glaser

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Submitted on: _____

Members of the Promotionskommitee:

Chairperson:	Prof. Dr. Ulrike Holzgrabe
Primary Supervisor	Prof. Dr. Helga Stopper
Supervisor (Second)	Prof. Dr. Leane Lehmann
Supervisor (Third)	Prof. Dr. Karl-Heinz Wagner

Date of Public Defense: _____

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ABBREVIATIONS

8-oxo-dG	8-oxo-2'-deoxyguanosine
ABC	avidin-biotin-complex
ACE	angiotensin converting enzyme
AP-sites	apurinic/apyrimidinic sites
ARE	antioxidant response element
AT_1	angiotensin II receptor subtype 1
BER	base excision repair
BN	binucleated
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CBPI	cytokinesis block proliferation index
CHL	Chinese hamster lung
*Cis*pt	cis-platin
Co.	control
COX-2	cyclooxygenase-2
CREST-serum	serum from patients with: Calcinosis, Raynaud's syndrome, Esophageal dysmotility, Sclerodactyly or Telangiectasia
DAB	3,3'-diaminobenzidine
DCF	2,7-dichlordihydrofluorescein
DES	diethylstilbestrol
DHE	dihydroethidium
DMSO	dimethyl sulfoxide
DOCA	deoxycorticosterone acetate
FBS	fetal bovine serum
FDA	fluorescein diacetate
FITC	fluorescein isothiocyanate
FPG	formamidopyrimidine DNA glycosylase
FRAP	ferric reducing ability of plasma / ferric reducing antioxidant power
GC-MS	mass spectrometry coupled gas-chromatography
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
FRAP	ferric reducing ability of plasma
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HUMN	human micronucleus project
IARC	International Agency for Research on Cancer
JCR	Joint Research Centre
JECFA	Joint Expert Committee on Food Additives
LC-MS	mass spectrometry coupled liquid-chromatography
LD50	lethal dose, 50%
LDL	low density lipoprotein
MCB	monochlorobimane
MDA	malondialdehyde
MEM	minimum essential medium
MMR	mismatch repair

MMS	methyl methane sulfonate
MN	micronuclei
Na_2EDTA	dinatrium-ethylendiamintetraacetat-dihydrat
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NER	nucleotide excision repair
NIH	National Institutes of Health
NO synthetase	nitric oxide synthetase
NOAEL	no observed adverse effect level
NPB	nucleoplasmic bridge
NQO1	NAD(P)H quinone oxidoreductase 1
ORAC	oxygen radical absorbance capacity
Pat	patulin
PBMC	peripheral blood mononuclear cell
PMTDI	provisional maximum tolerable daily intake
PBS	phosphate buffered saline
Res	resveratrol
RAS	renin-angiotensin system
ROS	reactive oxygen species
RSA	reactive scavenging activity
SCOOP	Scientific Cooperation
SD rat	Sprague Dawley rat
SIRT1	sirtuine 1
TBARS	thiobarbituric acid reactive substances
Tris	tris(hydroxymethyl)aminomethane
VEGF	vascular endothelial growth factor
WHO	World Health Organization

1 INTRODUCTION

1.1 DNA DAMAGE

Genomic damage can be caused by a variety of physical and chemical agents such as ultraviolet and ionizating radiation, xenobiotics and endogenous reactive oxygen species (ROS) that accumulate in cells due to natural metabolic processes. DNA damage occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day. While this constitutes only a small part of the 6 billion bases, unrepaired or misrepaired lesions in critical genes (such as tumor suppression genes) can impede cellular functions and increase the likelihood of tumor formation [1].

Common types of DNA damage are: base loss, base deamination, base alkylation, base dimerization, base oxidation and single/double-strand breakage [2]. If this DNA damage is converted to mutations three types of lesions can be differentiated: (1) Gene or point mutations affect one single gene and are mainly based on substitution, insertion or deletion of a few nucleotides; (2) Chromosomal mutations describe larger lesions e.g. translocations of gene sequences between or within chromosomal regions; (3) genomic mutations refer to changes of chromosome number within a cell.

The formation of ROS inside the cell can lead to oxidized DNA bases, apurinic/apyrimidinic (AP) sites or DNA strand breaks. The most common oxidized base lesion is the highly mutagenic 8-oxo-2'-deoxyguanosine (8-oxo-dG). 8-oxo-dG is unstable and can react with compounds such as peroxynitrate to even more mutagenic lesions.

Electrophilic alkylating agents can bind to nucleophilic sites of DNA, such as N7 position of guanine, N3 position of adenine or O6 position of guanine.

DNA damage provokes three possible cellular responses: (1) DNA repair or excision of lesion, (2) DNA damage tolerance by error-free or error prone (mutagenic) mechanism and (3) apoptosis. Examples for cellular repair systems are base excision repair (BER), nucleotide excision repair (NER) or mismatch repair (MMR) [3].

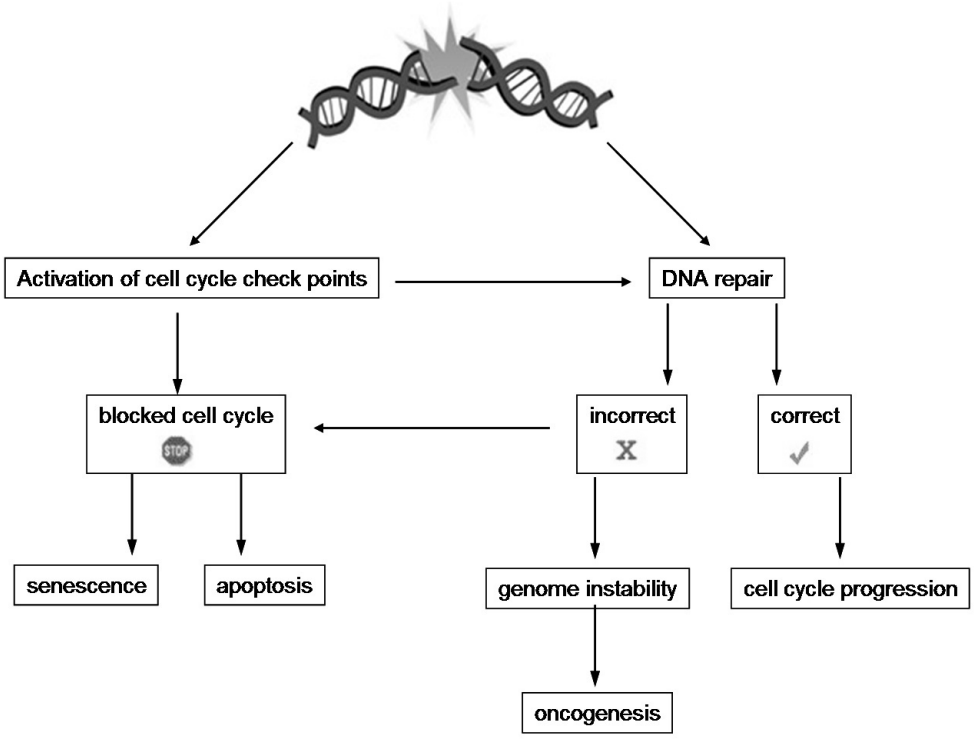


Figure 1: Cellular response to DNA damage leads in proliferating cells to a cell cycle arrest to provide the cell the possibility of DNA repair. After completion of repair the cell may proceed in its cell cycle. In resting/terminally differentiated cells, DNA repair will be initiated directly. Cell cycle can be blocked permanently if the damage cannot be repaired, leading to apoptosis or a senescent state of the cell. If unrepaired damage remains undetected, lesions may lead to mutations and genomic instability that ultimately can lead to oncogenesis. Modified after Houtgraaf et al. [4]

1.2 OXIDATIVE STRESS

The group of ROS includes among others hydroxyl radical ($^{\cdot}OH$), superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). ROS-mediated reactions have been shown to be involved in various pathogenic processes [5, 6] and therefore play an important role in the development of certain diseases [7, 8].

All cells in eukaryotic organisms contain a powerful endogenous antioxidative enzyme system. The three major classes of antioxidant enzymes are superoxide dismutases, catalases and glutathione peroxidases. Non-enzymatic antioxidative defense comprises the endogenous molecules glutathione (GSH), ascorbic acid, tocopherol and uric acid [9]. Many natural food compounds such as vitamins, polyphenols (e.g. resveratrol, anthocyanins) and flavonoids show also antioxidative properties [10]. When the defense system is compromised due to excessive oxidative stress, redox imbalance may take place [11]. ROS have also been shown to play an important role in carcinogenesis by damaging DNA and acting as tumor promoters [5, 6, 12]. Further processes and

diseases related to oxidative stress are aging [13], cardiovascular injury [14] and neurodegenerative diseases [15].

1.3 GSH

GSH is a tripeptide synthesized from the amino acids L-cysteine, L-glutamic acid and L-glycine. It is an antioxidant, preventing damage to cellular components caused by ROS such as free radicals and peroxides [16].

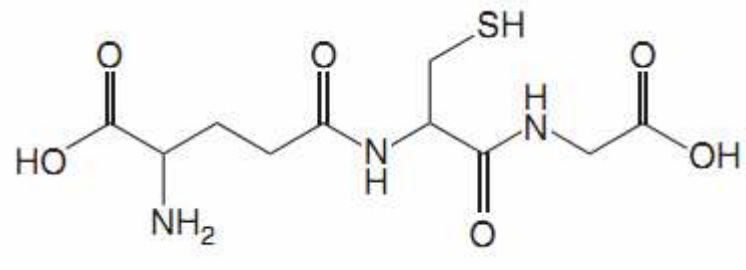


Figure 2: Structure of glutathione (GSH) [17]

The thiol group (SH) of cysteine serves as an electron donor and is responsible for the biological activity. GSH offers reducing equivalents to unstable molecules such as ROS. In this process, GSH is converted to its oxidized form glutathione disulfide (GSSG). GSSG can be again reduced by glutathione reductase, using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. In healthy cells and tissues, more than 90% of the total glutathione pool is in the reduced form and less than 10% exists in the disulfide form. The ratio of GSH to GSSG within cells is often used as a marker for oxidative stress and cellular toxicity [18].

Reaction of cysteine with L-glutamate catalyzed by ɣ-glutamylcysteine synthetase is the rate-limiting factor in GSH synthesis by the cells, since the availability of cysteine is low [19]. Treatment with buthionine sulfoximine (BSO), an inhibitor of *γ*-glutamylcysteine synthetase leads to decreased cellular GSH levels and its application provides a useful experimental model of GSH deficiency [20]. Oxidative stress can lead to an induction of *γ*-glutamylcysteine synthetase indicating an adaptive cellular response [21].

GSH occurs in high concentrations (0.5 to 10 mM) in virtually all mammalian cells [22]. It is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamin C and E in their reduced (active) forms [23].

1.4 NATURAL FOOD COMPOUNDS

Each year, several million people are diagnosed with cancer around the world and more than half of the patients eventually die from it [24]. Several lines of evidence indicate that nutrition contributes to human cancer risk [25, 26]. Nutrition has been thought to account for about 30% of cancers in Western countries. Therefore, diet contributes to equal amount to lifestyle provoked cancer as smoking [27].

Mutagens and carcinogens consumed with human diet can be classified into three groups: naturally occurring chemicals, synthetic substances and compounds produced by cooking. Examples for the first group are plant alkaloids and mycotoxins such as patulin, the second group includes food additives and pesticides and the third category comprises for example polycyclic aromatic hydrocarbons and heterocyclic amines [28].

Additionally, food mutagens can be categorized into genotoxic and non-genotoxic agents regarding their mechanistic way of action. Genotoxic substances cause DNA damage through several mechanisms, e.g. gene point mutations and chromosomal aberrations. Non-genotoxic agents are presumed to indirectly affect the cell as tumor promoters [29].

However, it should be recognized that nutrition delivers both mutagens and components that decrease the cancer risk. Dietary components could reduce the risk through protection of DNA from electrophiles or detoxification of carcinogenic substances [25, 30].

There is growing scientific evidence that antioxidants in general and particularly polyphenols such as resveratrol help lower the incidence of cancer and have beneficial effects on other negative aspects of human health such as cardiovascular and neurodegenerative diseases, DNA damage and aging. On the other hand, questions remain as to whether some antioxidants or phytochemicals potentially could do more harm than good [31].

PART I: INFLUENCE OF PATULIN AND RESVERATROL ON DNA STABILITY

1.4.1 Patulin

1.4.1.1 General aspects

The mycotoxin patulin (4-hydroxy-4H-furo (3,2C) pyran-2(6H)-one) is a secondary metabolite of fungal species, including *Penicillum*, *Aspergillus* and *Byssochlamys*.
Patulin is a colorless crystalline substance with a molecular weight of 154 Da and a melting point of 111 °C.
Patulin's chemical structure was determined by Birkinshaw et al. 1943 [32] when interest was high in its antibiotic properties [33]. Patulin was subsequently tested in a large study, which is sometimes declared as the first properly controlled multicentrical trial in the history of medicine, however the substance was not found to be effective in common cold [34].

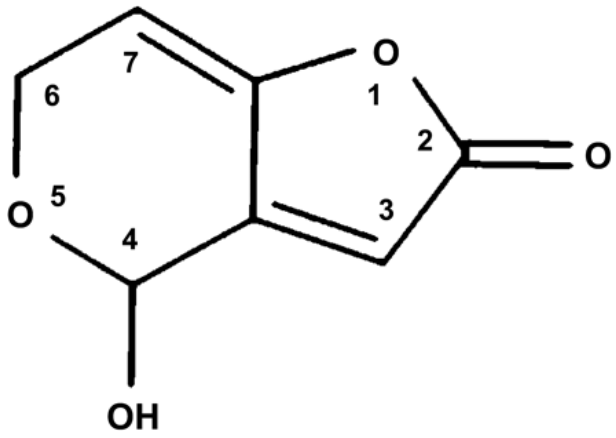


Figure 3: Chemical structure of patulin [35])

1.4.1.2 Sources and impact

It is a frequently found contaminant in spoiled fruits, especially apples and related products. Many other fruits, including grapes, pears, peaches, berries, tomatoes, other vegetables and cereals [36] have also been shown to contain patulin. Several studies have been performed on the occurrence and the toxicity of patulin. Patulin was found to

be stable to heat processing at pH < 6. It is gradually destroyed during storage in the presence of sulphites, sulfhydryl groups and ascorbic acid [37]. Removing of mouldy tissue does not necessarily remove all patulin present in fruit since some may have diffused into apparently healthy tissue [38].

A liquid chromatography method for determination of patulin in apple juice which has been validated by MacDonald et al. [39] was taken over by the Joint Research Centre of the European Union (JRC/IRMMT) [40]. Alternatively, patulin can also be measured with mass spectrometry coupled gas chromatography (GC-MS) [41]. Detection with an antibody-based system is not possible due to the small molecule size of patulin.

Safety assessments have been made by international organizations like the World Health Organization (WHO) and the Joint Expert Committee on Food Additives (JECFA). The World Health Organisation has established a safety level of 50 µg/L for apple juice [42] which was taken over by the European Union and many other countries [43]. However, several publications documented the exceedance of this safety level [44-46].

Data about the intake of patulin and other mycotoxins in the European Union are regularly collected and used for risk assessment by the Scientific Cooperation (SCOOP) Task Reports. Most products do not reach the threshold but single samples exceed the specified value up to twenty times. The no observed adverse effect level (NOAEL) was determined to be 43 µg/kg/d [47] leading to a Provisional Maximum Tolerable Daily Intake (PMTDI) of 4 µg/kg bw/d. By evaluation of questionnaires from the member states the average daily intake of patulin was calculated to be about 3 ng/kg bw/d and thereby to be below the PMTDI [45].

Due to their body size and the increased consumption of apple juice, children have a higher risk to reach this value. Therefore the European Union has set a value of 10 µg/L especially for products dedicated to children. However, home made products or food and drinks from other states do not necessarily underlie controls and may exceed the specified values.

1.4.1.3 Kinetics

Overall, very little is known about pharmacokinetic behaviour and metabolism of patulin [47]. When contaminated food is ingested, the intestine is the first organ coming in contact with mycotoxins. The toxic effects of mycotoxins on intestinal epithelia cells

have been reported in several studies [48-50]. After the resorption patulin is relatively fast metabolized and effectively excreted within 24 h after oral consumption [51, 52]. No free patulin was found after the voluntary consumption of apple juice containing patulin indicating a possible fast degradation by the big excess of GSH [53]. However, the activity of metabolites has not yet been fully elucidated e.g. patulin-cysteine adducts have been shown to be still partially bacteriostatic and capable of enzyme inhibition [54].

The major retention sites of patulin were erythrocytes and blood rich organs like spleen, lung, liver and kidney [52]. The enhanced DNA damage observed in liver, kidneys and brain could be associated with increased cellular accumulation of patulin in these tissues, mediated by specific membrane transport of this hydrophilic compound [55]. Such interactions of organic ion transporters with mycotoxins have been identified by Tachampa et al. [56]. These transporters have been found mainly in the kidneys, liver and recently in the blood–brain barrier [55].

Patulin reacts fast with sulfhydryl groups and more slowly with amino functions of proteins and glutathione [57, 58]. Up to three molecules of GSH can bind to one molecule patulin. The structures of the main reaction products were reported in different studies [59, 60].

1.4.1.4 Acute and chronical toxicity

Several cases of lethal mycotoxicosis in cattle by patulin-contaminated forage have been reported by Ciegler [54]. *In vivo* patulin caused severe damage in several organ systems like kidney, intestinal tissue [61, 62] and immune system [63]. Acute toxicity in mice, rats and hamsters ranged from 9-55 mg/kg bw. Agitation, convulsions in some cases, dyspnoea, pulmonary congestion and oedema and ulcerations, hyperaemia and dilatation of the gastrointestinal tract were reported in several studies [64]. Another symptom seen in almost all the studies was a loss of body weight [61, 62]. A high mortality in rats was attributed to severe dilatation of the gut and/or pneumonia [47]. Problems might be related to the antibiotic effect of patulin against Gram-positive bacteria thereby giving a selective advantage to pathogenic Gram-negative bacteria in the gut [64]. Besides the kidney, liver is one of the major target organs of patulin. It reduces the activity of hepatic aldolase [65] and inhibits protein synthesis and consequently cell growth in cultured hepatic cells [66].

1.4.1.5 Genotoxicity

Regarding carcinogenicity, the International Agency for Research on Cancer (IARC) assigned patulin to category C, since the evidence of carcinogenicity was considered limited in experimental animal studies [67]. After oral administration patulin did not induce any noticeable tumors in Wistar rats and Swiss mice [68, 69]. However, Oswald et al. detected adenomas in Sprague-Daley (SD) rats after gavage [69]. Dickens and Jones found local sarcomas after subcutaneous injection of patulin [70] and Saxena et al. identified patulin as a tumor initiator after topical application [71]. Additionally to embryotoxic effects [72, 73] patulin was shown to have also weak teratogenic properties [72-75].

Genotoxicity of patulin was shown in various studies. De Melo et al. applied an *in vivo* comet assay and found a dose-dependent increase of strand breaks in brain liver and kidney [55]. *In vitro* mutagenicity was shown in different mammalian cell types like Chinese hamster lung fibroblast V79 cells, mouse lymphoma L5178Y cells [76] or mouse mammary carcinoma FM3A cells [77].

Patulin did not increase revertant frequency in the Ames test using *Salmonella typhimurium* [78, 79] but was mutagenic in *Saccharomyces cerevisiae* [78].

The frequency of chromosomal aberrations in HepG2 [80] and V79 cells [81] was increased after patulin treatment. An elevated level of sister-chromatid exchanges was detected in CHO cells and human lymphocytes [82] but not in V79-E cells [83]. Patulin induced both kinetochore-positive and -negative micronuclei in V79 cells [58]. The clastogenic properties of patulin were described by Alves et al. and Liu et al. [58, 81, 82]. Cytogenetic studies *in vivo* suggest the induction of chromosomal aberrations and mitosis disturbance in mice and Chinese hamster [74, 84, 85]. The genotoxic and cytotoxic [76, 81] properties are believed to be due to the high reactivity of patulin to cellular nucleophiles. A reduction of the cellular GSH content by the GSH synthesis inhibitor BSO is known to increase the cytotoxicity [86] and genotoxicity of patulin [76, 87].

1.4.2 Resveratrol

1.4.2.1 General aspects

Resveratrol is a plant polyphenol found in the skins of red grapes and several other foods. It is a member of the stilbene family and can be found either in a glycosylated form or as the parent molecule. Resveratrol exists as *cis* and *trans* isomeric form, with significant higher concentrations of the latter. *Trans*-resveratrol is relatively stable if protected from light and high pH .*Trans* to *cis* isomerization is facilitated by UV light [88].

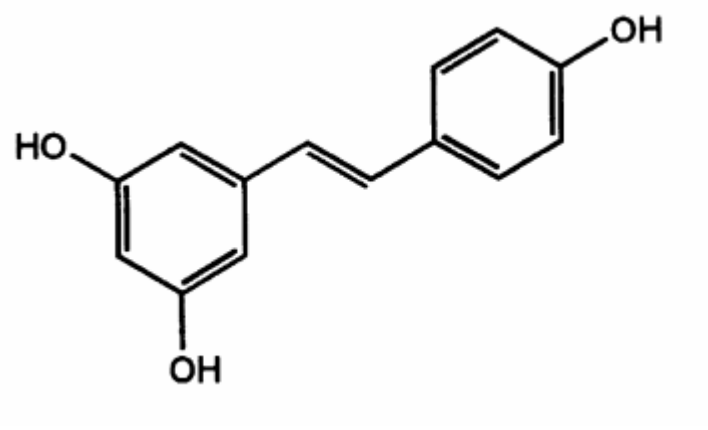


Figure 4: Structure of resveratrol [89]

1.4.2.2 Sources and Impact

High concentrations of resveratrol are found in grapes, peanuts and Japanese knotweed. The content in wine is higher than in grape juice due to the increased solubility of resveratrol in ethanol compared to water. In red wines concentrations of resveratrol range between 1-18 mg/L [90]. The majority of the stilbene is present as aglycone rather than glycoside due to sugar cleavage presumably occurring during vinification [91]. Red wines contain much higher resveratrol concentrations than white wines. This may, at least in part, be explained by the fact that skins are removed in white wine immediately after pressing while in red wine production the grape skins are left with the freshly pressed red wine for a while in order to extract aromatic compounds. Variations of resveratrol concentrations in red wine are explained by differences in wine processing, type of grapes and vintage, as well as climatic factors. Levels are generally higher in cooler climates because resveratrol is thought to play a role in the Defense of

plants against fungal infections [92]. Resveratrol synthesis in plants is also increased as a response to UV irridation [93].

1.4.2.3 Kinetics

Although there has been remarkable evidence for resveratrol as a potent chemopreventive agent *in vitro*, it seems that the low bioavailability of resveratrol in humans could interfere with a successful *in vivo* treatment. After oral administration resveratrol is absorbed in large parts but bioavailability is quite low due to extremely rapid sulphate conjugation by the intestine/liver within 30 min [94-96]. A 30-fold enrichment of resveratrol over serum concentrations has been observed in the intestinal mucosa [95]. Significant accumulation of resveratrol was also found in the bile, the stomach, the liver and the kidney [97]. Serum half-life of total resveratrol metabolites was 9.2 h, indicating that exposure to modified forms is much higher than that of unchanged resveratrol. However, it is not known whether metabolites exert health promoting effects or not [96].

Doses used to reveal the various effects reported for resveratrol (~32 nM–100 μM *in vitro* and 100 ng–1,500 mg per kg bw in animals) raise the questions about the concentrations that are achievable in humans. Assuming a consistent daily intake of 375 mL, or about two glasses of wine, a person weighing 70 kg would receive a dose of ~ 27 μg/kg bw each day. The detrimental effects of alcohol are likely to mask any health benefits achieved with higher intake [98]. However, nowadays a lot of food supplements are available delivering up to 1,000 mg resveratrol per day. Administering such high doses to improve efficacy might not be expedient since toxic effects have been observed at or above 1 g/kg bw [99]. No serious adverse effects were detected in any human study [100-102]. The highest doses reported were 5 g/ 70 kg bw for a single intake [101] or 0.9 g/d for repetitive application [100].

1.4.2.4 Health promoting effects

Resveratrol has been cited in many recent investigations for its possible protective effects against certain forms of oxidative stress related diseases. The health promoting properties of resveratrol are primarily attributed to the antioxidant effects of resveratrol. Resveratrol was found to be an effective scavenger of hydroxyl, superoxide and metal-induced radicals. It exhibits a protective effect against lipid peroxidation in cell

membranes and DNA damage caused by ROS. Resveratrol showed also a significant inhibitory effect on the NF-κB signaling pathway after cellular exposure to metal-induced radicals [103].

Resveratrol has been suggested to be one of the major compounds being responsible for the so called “French Paradoxon”. The French paradox refers to the observation that French people suffer a relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats [104]. It has been proposed that regular consumption of red wine in moderate amounts may explain this phenomenon [105]. Besides acting as antioxidant, resveratrol can further inhibit platelet aggregation [106].

On the basis of the structural similarity of resveratrol (*trans*-3,5,4'-trihydroxystilbene) to the synthetic estrogen diethylstilbestrol (4,4'-dihydroxy-α,β-diethyl-stilbene) resveratrol might work through the same cardioprotective mechanisms as estrogens [107]. However, resveratrol showed in different studies estrogenic, superestrogenic and anti-estrogenic effects dependent on cell type, receptor type and presence of 17β-estradiol [108]. *Trans*-resveratrol was found to competitively inhibit binding of [^{3}H] estradiol to type 1 estrogen receptors in estrogen-positive MCF-7 human breast cancer cells. This ability to antagonize estrogen binding provides a rationale for the possible use of *trans* resveratrol in the prevention or treatment of breast cancer [109].

Resveratrol was shown to extend life in yeast, worms and flies in a *SIR2* (SIRT1 homolog)-dependent manner [110, 111].

Prolongation of lifespan was attributed to imitation of transcriptional response to caloric restriction including improvements in insulin sensitivity, endurance and overall survival in obese mice [8, 112]. Whether these effects are related to a potential activation of SIRT1 is still controversially discussed [113]. However, treatment of mice on a normal diet did not produce any further extension of lifespan, indicating that resveratrol might mainly be counteracting the deleterious consequences of obesity, rather than slowing aging in a caloric restriction-like manner [114]. Several follow-up studies have confirmed that resveratrol does not elongate lifespan in healthy mice. Increasing the dose of resveratrol to approximately 200 mg/kg had no effect on survival and intake about 1.5 g/kg revealed toxic effects [112].

Recent data give clear evidence that resveratrol can act as a chemopreventive agent as well. Kraft et al. [115] have reviewed the anticarcinogenic effects of resveratrol. Tumor initiation, promotion and progression are affected by resveratrol via multiple pathways. Resveratrol has anti-inflammatory effects by counteracting NF-κB and AP-1

transcription. It prevents bioactivation of procarcinogens [116], constrains the initiation of tumors and inhibits the metastasis of carcinomas through prevention of angiogenesis by inhibiting vascular endothelial growth factor (VEGF) and matrix metalloproteases [117]. Induction of apoptosis and cell cycle arrest which are important mechanisms for cancer therapy, are stimulated by resveratrol through different mechanisms including activation of p53 and modulation of cell cycle proteins [118].

1.5 PART II : EFFECTS OF AN ANTHOCYANIN RICH EXCTRACT ON HYPERTENSIVE RATS

1.5.1 General aspects of anthocyanins

Anthocyanins (from Greek: *ἀνθός (anthos)* = flower + *κυανός (kyanos)* = blue) are water-soluble plant pigments that may appear red, purple or blue according to the pH. Anthocyanins are a separate group of over 635 compounds [119] belonging to the class of flavonoids. They are derivatives of 2-phenylbenzopyrlium and consist of two benzoyl rings (A and B) separated by a heterocyclic (C) ring. The skeleton is usually bound to saccharide residues such as glucose, galactose, rhamnose or arabinose as 3-glycosides or 3,5-diglycosides. Anthocyanidins are the sugarfree derivatives of anthocyanins, being very unstable at physiological pH.

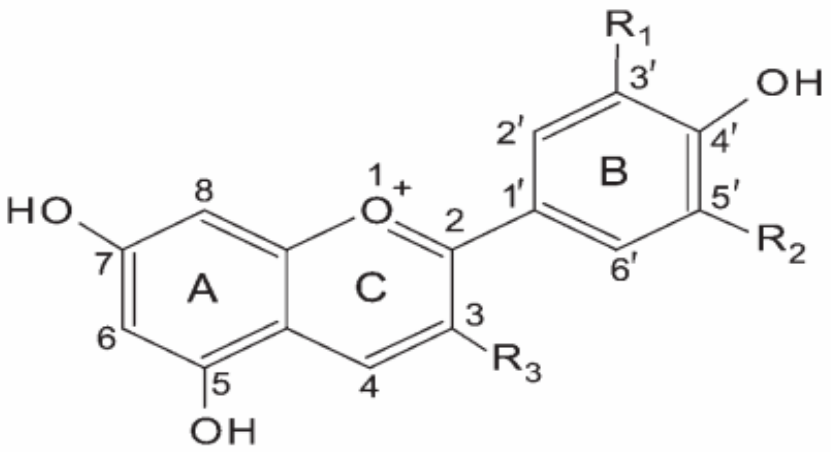
The strong antioxidant power of anthocyanins is dependent on the chemical structure particularly on (the number of) hydroxyl groups. The presence of a hydroxyl group at ring C enables also the chelation of metal ions, e.g. iron and copper [120].

They are odorless and nearly flavorless, contributing to taste as a moderately astringent sensation [121]. Anthocyanins are reactive compounds, which degrade readily to colourless or brown substances under the influence of various enzymes, oxygen, hydrolyzes, high temperatures or light [122].

Depending on nutrition customs, the intake of anthocyanins in Germany was estimated in 2002 to be 2.7 mg/d with a personal variety of 0-76 mg [123].

Anthocyanin sources include colored fruits such as berries, cherries, peaches, grapes, plums any many dark colored vegetables such as red onion, eggplant and black beans [124]. Although they occur particularly in flowers and fruits they are also present in leaves, stems and storage organs [119].

If not mentioned otherwise the term anthocyanins includes hereafter also anthocyanidins.



Aglycone	R_1	R_2	R_3
Delphinidin	OH	OH	OH
Cyanidin	OH	H	OH
Petunidin	OCH_3	OH	OH
Peonidin	OCH_3	H	OH
Malvidin	OCH_3	OCH_3	OH
Pelargonidin	H	H	OH

Figure 5: Structure of the most common anthocyanidins [125]

1.5.2 Kinetic

The bioavailability of anthocyanins is consistently very low across all studies with often less than 0.1% of the ingested dose appearing in the urine. It has to be remarked that there is currently no method available which would be able to detect alternative molecular structures of anthocyanins which are very probable to be formed under *in vivo* conditions. Most studies use a high-performance liquid chromatography (HPLC) based method to detect the flavylium cation which is not likely to be present at neutral pH *in vivo* [122]. *In vitro* absorption of anthocyanins was low, but anthocyanins were detectable inside the cell [126] and glycosides showed a higher transport efficiency than

aglycons [127]. The absorption *in vivo* occurs quickly with a t_{max} of 15-60 min, suggesting an uptake of anthocyanins from stomach [128], but the major site for flavonoid absorption is the small intestine [119]. Passamonti et al. showed an efficient absorption of anthocyanins by the small intestine of rats after *in situ* perfusion, which might be related to an interaction with bilitranslocase [129]. Changing pH and microbial flora in the gastrointestinal tract may modify the molecular structure of anthocyanins. In colon neutral pH and a different microbial population lead to a fast degradation of anthocyanins to their phenolic acids and aldehydes [130]. The concentrations found in humans after ingestion of anthocyanin-rich products are located in the lower nanomolar range [123].

Most animal studies found that anthocyanins were absorbed mainly in their intact glycosylated form. Different studies describe the detection of intact anthocyanins but, neither aglycons nor conjugates in plasma [131, 132] and urine [133, 134]. However, these last years, methylated derivatives, glucuronides and glycoside glucuronides have been identified in urine and plasma by the use of HPLC combined with mass spectrometry [135, 136]. Some of the metabolites of flavonoids have comparable or even higher activity than the precursors [137].

Anthocyanins are largely excreted in urine but portions of them may reenter the jejunum by bile and be excreted through faeces together with the unabsorbed anthocyanins [138]. Excretion was usually completed after 6-8 h [122].

1.5.3 Acute and chronic toxicity

For the evaluation of acute toxicity test animals were administered anthocyanins (cyanidin, petunidin and delphinidin mixture extracted from currants, blueberries and elderberries) in doses from 0 to 25,000 mg/kg for mice and 0 to 20,000 mg/kg for rats [139]. Following i.p. or i.v. application toxic doses lead to sedation, convulsions and finally death. The LD50 value ranged between 240 (i.v.) to 20 000 mg/kg bw (oral) for rats.

Short-term studies with diets containing very high concentrations of anthocyanins showed no adverse effects in rats and guinea pigs [139] or dogs [140]. Anthocyanins were not found to be mutagenic [141-143] or teratogenic [139]. A two-generation study showed no difference in reproduction performance or pup viability between control and treated group [144].

1.5.4 Health-promoting properties

Results from various cell line studies, animal models and human clinical trials give indication for the anti-inflammatory and anti-carcinogenic activity, cardiovascular disease prevention, obesity control and diabetes alleviation. These health promoting effects of anthocyanins are at least in part related to their antioxidative properties. Epidemiological studies suggest a lower incidence of many chronic diseases for people consuming a polyphenol rich diet [119]. As already described, anthocyanins in wine might also contribute to the low incidence of cardiovascular disease in France known as French Paradoxon [145].

Due to their polyphenolic structure anthocyanins can scavenge effectively ROS such as superoxide, singlet oxygen, peroxide, hydrogen peroxide and hydroxyl radicals [146]. The scavenging properties of anthocyanidins are superior to those of the respective anthocyanins. Additionally, they possess the ability to chelate metal ions such as Fe and Cu and inhibit thereby their prooxidative effects. Antioxidant activity of anthocyanins was proved *in vitro* with the ORAC (oxygen radical absorbance capacity) assay showing the highest values for cyanosin-3-glycoside [147].

A decreased level of biomarkers related to oxidative stress was also found *in vivo* [148, 149]. Different anthocyanins inhibit proliferation of cancer cells derived from various tissues [150] and tumor formation *in vivo* [151]. However, in these studies anthocyanins were used in supra-natural doses.

Anthocyanins were shown to be antimutagenic in both the Ames Test and sister chromatid exchange test [152]. Oxidative DNA damage such as the highly mutagenic 8-oxo-dG was decreased more than 80% in the urine from animals treated with raspberry extract and azoxymethane [153].

Further suggested mechanisms are the inhibition of carcinogen activation and induction of phase II enzymes for detoxification [154, 155], cell cycle arrest [156], antiangiogenese [157], induction of apoptosis [158] and inhibition of cyclooxygenase-2 (COX-2) enzymes.

The antiinflammatory properties of cyanidin and other anthocyanins were comparable to commercial products in a COX activity assay [159].

Anthocyanins reduce also the oxidation of low density lipoprotein (LDL) [160] and show vasodilating activity [161]. Therefore, they are suggested to prevent cardiovascular diseases. Further discussed health promoting effects are the prevention of metabolic syndrome, obesity [162], diabetes [163] and the improvement of eye vision [164].

1.5.5 Dacapo extract

We used in this study an extract of Dacapo grapes from Geisenheim Research Centre (Geisenheim Research Centre, Geisenheim Germany) which possesses an extremely high amount of anthocyanins (231 mg/g) and polyphenols (640 mg/g). Dacapo is a crossing of Deckrot with Blauer Portugieser. It is characterized by blue-black berries that result in a dark red juice. After harvesting, grapes were ground, treated with pectolytic enzymes, pressed and further processed. The liquid extract was then rinsed through a chromatography column with an adsorber resin (SP70, Resindion/Mitsubishi, Mailand). The exact anthocyanin profile is published in Deutsche Lebensmittelrundschau [165]. Briefly, the most abundant anthocyanins found by HPLC/MS were the 3-glycosides and 3-(6''-O-acetyl) glycosides of delphinidin, cyanidin, petunidin, peonidin and malvidin, with malvidin-3-glucoside and malvidin-3-(6''-O-acetyl)glucoside accounting for more than 50% of the anthocyanins. Further components of the extract are polysaccharides (19.5%) and amino acids (4.6%). With the applied methods only ~ 50% of the extract could be structurally characterized. The unidentified part consists mainly of monomeric and polymeric polyphenols as detected by Folin-Ciocalteu measurement [165].

1.5.6 Renin-angiotensin system

The renin-angiotensin system (RAS) is a cascade of enzymatic reactions involved in the regulation of blood pressure:
Renin is an aspartyl protease synthesized and secreted as the inactive proenzyme pro-renin, which matures in the myoepithelioid cells of the juxtaglomerular apparatus (JGA). Angiotensinogen is cleaved by renin to generate angiotensin I. Angiotensin I is then converted to angiotensin II by the angiotensin-converting enzyme (ACE). A local angiotensin II synthesis exists in tissues such as the brain, heart, eye, adipose tissue and kidney. The vasoconstrictor angiotensin II increases blood pressure through binding to its AT_1 receptor. Additionally, it has a regulatory impact on homeostasis of the body's water content by stimulating the release of aldosteron and anti-diuretic hormone (ADH). Reduction of angiotensin II level by inhibition of ACE with drugs such as ramipril is therefore one of the major approaches in therapie of hypertension.

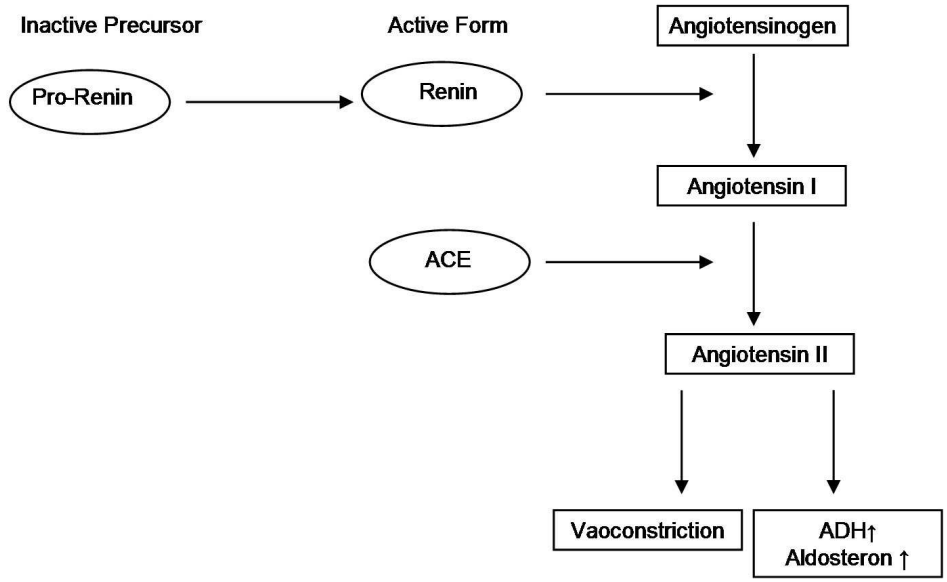


Figure 6: The renin-angiotensin system

1.5.7 Ren-2 rats

Over the past 50 years various animal models of hypertension have been developed, including spontaneously hypertensive rat, deoxycorticosterone acetate (DOCA) –salt rat and transgenic Ren-2 rat. Rats from all hypertensive models exhibit cardiac hypertrophy and show reduced endothelium-dependent relaxation of isolated arteries [166]. Accelerated hypertension is usually accompanied by organ damage. End organ damage is not only related to high blood pressure itself, but also to the underlying biochemical alterations. Perturbed kidney function is reflected in proteinuria and high serum creatine levels [167, 168].

Ren-2 rats are a rat strain transgenic for the murine Ren-2 renin gene suffering from angiotensin II-dependent hypertension [169, 170]. Homozygous male animals show blood pressure values up to 300 mmHg whereas females have around 20-30 mmHg lower values [171].

Ren-2 rats show also significant differences in albuminuria, lipid peroxidation ((malondialdehyde (MDA)) and nitrotyrosine-staining compared to Sprague Dawley (SD) rats [172]. In contrast to Ren-1 Ren-2 codes for a non-glycosylated renin protein, which is especially expressed in extrarenal tissue. The mechanism for high blood pressure might be based on an adrenal gland-induced activation of adrenal steroids. Transgenic rats are characterized by unchanged or even suppressed concentrations of active renin,

angiotensinogen, angiotensin I and angiotensin II in plasma, whereas the plasma levels of pro-renin are much higher in Ren-2 rats [169, 173]. Previous studies have proven that hypertension in Ren-2 rats is angiotensin II-dependent and that activation of angiotensin II receptor subtype 1 (AT_1) substantially contributes to the development of hypertension [174, 175]. Local increased generation of angiotensin II in organs might be also involved in end organ damage [176, 177]. Despite the known genetic alterations, the exact mechanism underlying the hypertension remains elusive [166].

1.6 OBJECTIVES

Nutrition has been identified to be one of the main factors of lifestyle-induced cancers in Western countries. Besides synthetic contaminations in food and substances arised from heating processes natural food compounds are one of the major sources of mutagens.

An important source of diet derived mutagens is contamination of food with mycotoxins. In comparison to more prominent substance of this group such as aflatoxin B1, the genotoxic effects of patulin are less clarified. One aim of this study was therefore to investigate further steps of patulin-induced genotoxicity.

However, nutrition delivers not only mutagens but also compounds that decrease the risk for cancer. Resveratrol has been vaunt as a miracle drug against many diseases, cancer and aging. Therefore, we wanted to evaluate a potential protective effect of the antioxidant resveratrol on patulin-induced genomic damage. Considering that polyphenols and other antioxidants can not only exert beneficial effects but also cause DNA damage in higher concentrations, a further issue of our study was the revision of potential genotoxic effects of resveratrol.

In the second part of this thesis we wanted to investigate the effects of an anthocyanin-rich extract on hypertension and oxidative stress.

Anthocyanins are very potent antioxidants, which have shown many health promoting effects, including the prevention of cancer and cardiovascular diseases. An anthocyanin-rich Dacapo grape extract was given to a subgroup of hypertensive Ren-2 rats. Blood pressure and markers of oxidative stress and DNA damage were measured and compared with the results of untreated Ren-2 rats and a subgroup receiving antihypertensive medication.

The approach was as follows:

Part 1: Effects of patulin and resveratrol in V79 cells

Investigation of:

- cell viability after patulin or resveratrol treatment: fluorescein diacetate/ Gel Red-staining
- genotoxicity after patulin or resveratrol treatment: micronucleus assay, kinetochore-staining; for patulin additionally: comet assay, cross-link comet assay, mitosis disturbance analysis
- oxidative stress after patulin/resveratrol treatment: 2,7-dichlordihydrofluorescein (DCF) assay, GSH measurement
- effects of resveratrol on patulin-induced damage: micronucleus assay

Part 2: Effects of an anthocyanin-rich Dacapo grape extract on hypertensive Ren-2 rats

Investigation of:

- health status: body weight, food and water intake
- blood pressure: measurement of systole, diastole and pulse
- oxidative stress: dihydroethidium (DHE)-staining, FRAP assay
- genotoxicity: comet assay, ɣ-H2AX-staining

2 EXPERIMENTAL PROCEDURES

2.1 PART I: INFLUENCE OF PATULIN AND RESVERATROL ON DNA STABILITY

2.1.1 Materials

If not otherwise mentioned, chemicals were purchased from Sigma–Aldrich, (Taufkirchen, Germany) or Carl Roth GmbH (Karlsruhe, Germany). Cell culture medium and supplements were purchased from PAA (Pasching, Austria), fetal bovine serum (FBS) was from Biochrom (Berlin, Germany). Patulin, cytochalasin B and *cis*-platin (*cis*pt) were dissolved in dimethyl sulfoxide (DMSO), resveratrol was dissolved in ethanol and H_2O_2 and BSO were dissolved in phosphate buffered saline (PBS). Compounds were added to the medium to a final solvent concentration of ≤ 1%. Control experiments were carried out with equal amount of solvent without test compound.

2.1.2 Cell culture

Experiments were carried out using V79 cells, a standard cell line for genotoxicity testing. V79 fibroblasts derived originally from the lung of a male Chinese hamster (Cricetulus griseus, 2n=22) were used for *in vitro* experiments. The adherent cell line offers of a number of desirable properties for mutagenesis assays. Due to their rapid growth rate V79 cells double every 12-16 h. They possess a stable karyotype with a modal chromosomal number of 22 ± 1 [178]. Furthermore V79 cells lack major types of xenobiotic metabolizing enzymes [179].

Cells were routinely grown in MEM (Minimum Essential Medium Eagle) with 10% fetal bovine serum, 1% L-glutamine and 1% antibiotics (penicillin, streptomycin) at 37 °C in a water-saturated atmosphere containing 5% CO_2. Cells were routinely split three times per week. For treatments the indicated number of cells were seeded the day before in 6-well-plates (tissue culture plate, flat bottom cell +; Sarstedt,

Nümbrecht, Germany) containing 3 mL of medium. Cell number was calculated using a CasyTM cell counter (Innovatis, Reutlingen, Germany).

2.1.3 Viability assay

Viability assay was used to prove that the applied concentrations of a substance has no cytotoxic effects. This is important to ensure that the investigated outcomes are real genotoxic and not unspecific cytotoxic effects. Corresponding to the incubation conditions of the micronucleus assay, cells were treated for 4 h with the indicated concentration of patulin, resveratrol or H_2O_2, followed by an 20 h postincubation with cytochalasin B (5 µg/L). Afterward cells were treated with an premixed solution of fluorescein diacetat (30 µg/mL) and Gel Red (Biochrom, Berlin, Germany; 1 µL/mL)) to distinguish between viable cells and dead cells.

Living cells actively convert the non-fluorescent fluorescein diacetate (FDA) into the green fluorescent compound fluorescein by esterases, a sign of viability; while membrane-compromised cells take up the dye Gel Red, indicating cell death. 200 cells per concentration were counted with an Eclipse 55i microscope (Nikon GmbH, Düsseldorf, Germany) at 200 x magnification.

2.1.4 Micronucleus assay

Cytokinesis blocked micronucleus assay was used to investigate the potential of the applied substances to generate micronuclei and nucleoplasmic bridges.
The micronucleus assay is a widely used and well established test, to evaluate potential genotoxic effects of substances. Micronuclei mainly originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei during mitosis because they did not attach properly with the spindle during the segregation process. Displaced chromosomes or chromosome fragment which were enclosed by a nuclear membrane form micronuclei that are morphologically similar to nuclei but smaller in size [180].
Do investigate a potential dose response of patulin V79 cells (2×10^5 cells, seeded the day before) were incubated for 4 h with the indicated concentrations of patulin,

followed by 24 h substance free post-incubation and the number of micronuclei was evaluated in 1000 cells from each of two slides.

Further micronuclei experiments were carried out in cytokinesis blocked assays.

To investigate the effects of GSH depletion 2×10^5 cells, seeded the day before in 3 mL well plates, were incubated for 20 h with 20 µM BSO. Subsequently, cells were washed and treated with 0.5 µM patulin. After 4 h patulin was removed and the cytokinesis inhibitor cytochalasin B (5 µg/mL) was added for further 20 h.

For experiments with resveratrol 2×10^5 cells, seeded the day before in 3 mL well plates, were preincubated for 30 min with the indicated concentration of resveratrol or solvent. Then 0.5 µM patulin or solvent was added for further 4 h to the cells. After 4 h the substances were removed and the cytokinesis inhibitor cytochalasin B (5 µg/mL) was added for further 24 h.

By limiting the analysis to such binucleated cells, it can be ensured that these cells have actively divided since the treatment. For the time course of micronuclei and nucleoplasmic bridge formation, cells seeded the day before, were incubated for the indicated time with 0.5 µM patulin and 5 µg/mL cytochalasin B simultaneously. Cells were brought onto glass slides by cytospin centrifugation and fixed in methanol (-20°C, ≥ 1h). Slides were stained with Gel Green (Biochrom, 1:1000 in PBS for 3 min). From each of two slides, 1000 binucleated cells were evaluated with regard to frequencies of micronuclei-containing and nucleoplasmic bridge-containing cells.

Cytokinesis block proliferation index (CBPI) was calculated in 1000 cells per slide using the formula CBPI = (MI + 2MII + 3 (MIII +MIV)) with MI-MIV representing the number of cells with one to four nuclei [181].

Micronuclei and nucleoplamic bridges were scored according to the criteria defined by the members of HUman MicroNucleus (HUMN) project [182]. Structures were defined as micronuclei if they were round or oval, had the same staining intensity as the main nuclei and were not connected to them. The main size of micronucei in binucleated cells was between 1/256 and 1/9 of one of the main nuclei. Continuous links between the nuclei in binucleated cells were scored as nucleoplasmic bridges if their width did not exceed one-fourth of the diameter of the nuclei within the cell. Cells containing more than one micronucleus or nucleoplasmic bridge were frequently seen and scored as cell with one or more micronuclei or bridge respectively [183].

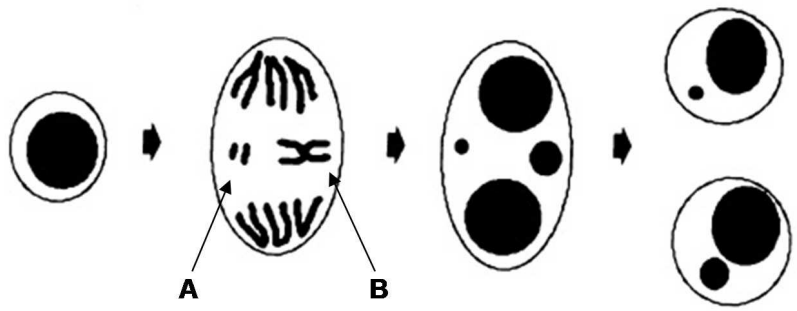


Figure 7: Mechanism of micronuclei generation
Micronuclei originate from either chromosome fragments (A) or lagging chromosomes (B). If cytokinesis is blocked by cytochalasin B the last step does not occur. Modified after Fenech et al. [184]

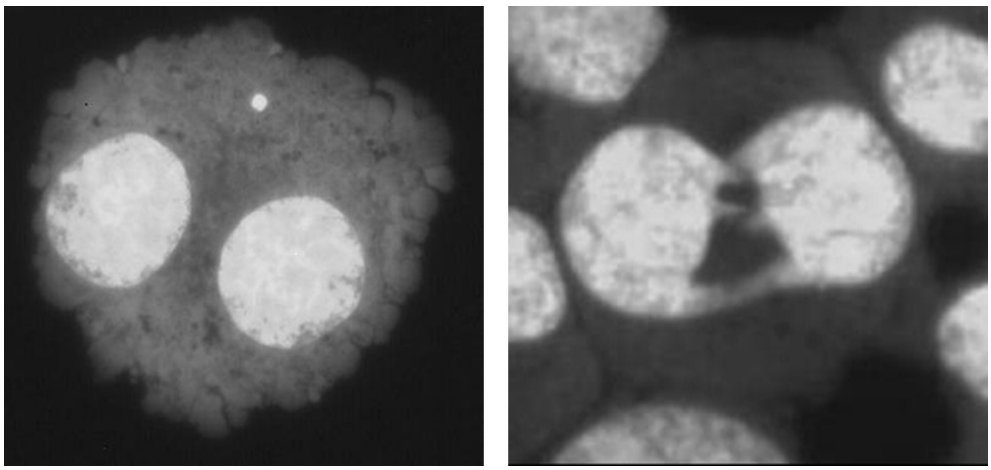


Figure 8: Representative picture of a micronucleus (left side) and nucleoplasmic bridges (right side)

2.1.5 Kinetochore-staining

The nature of micronuclei can be differentiated by kinetochore-staining. Kinetochore-negative micronuclei indicate for the potential strand breaking properties of a substance (Figure 7 (A)) whereas kinetochore-positive micronuclei consist mainly of whole chromosomes (Figure 7 (B)) not distributed to one of the daughter nuclei during mitosis. For kinetochore analysis cells were treated for 4 h with 0.5 µM patulin, resveratrol or solvent followed by 20 h post-incubation with cytochalasin B (5 µg/mL). Cells were brought onto glass slides by cytospin centrifugation and fixed in methanol (-20 °C, ≥ 1h). Kinetochores were stained with a primary antibody against centrosomes (Positive Control Serum (Centromere), Antibodies Incorporated, Davis, USA; undiluted, 37 °C, over night) and a rhodamine-conjugated secondary antibody (sc-2457, Santa Cruz Biotechnology, Santa Cruz, USA; 1:20, 37 °C, 2 h). Counter-staining of nuclei was done with chromomycin A (50 µM, 3 min). In total more than

5000 cells per concentration cells were evaluated for the presence of kinetochore-positive or –negative micronuclei, using an Eclipse 55i microscope (Nikon GmbH, Düsseldorf, Germany) at 200 x magnification.

2.1.6 Comet assay

The comet assay (also known as single cell gel electrophoresis assay) is a standard technique for the detection of DNA damage at the level of the individual eukaryotic cell. It was first described by Singh *et al.* in 1988 [185].

Single and double strand breaks as well as alkali labile sites and apurinic or apyrimidinic sites can be detected by this method. The technique involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline conditions and electrophoresis of the suspended lyzed cells. The lysis process removes membranes, cytoplasm and also histones but leaves supercoiled DNA and some DNA-associated proteins. Electrophoresis allows the migration of broken DNA strands and relaxed DNA toward the positive pole of the electrophoresis field resulting in a comet shape formation of damaged cells. After staining of DNA, comets can be observed by fluorescence microscopy and the intensity of the comet tail relative to the head reflects the number of DNA breaks. Determination of DNA damage can be performed by manual scoring or automatically by imaging software [186].

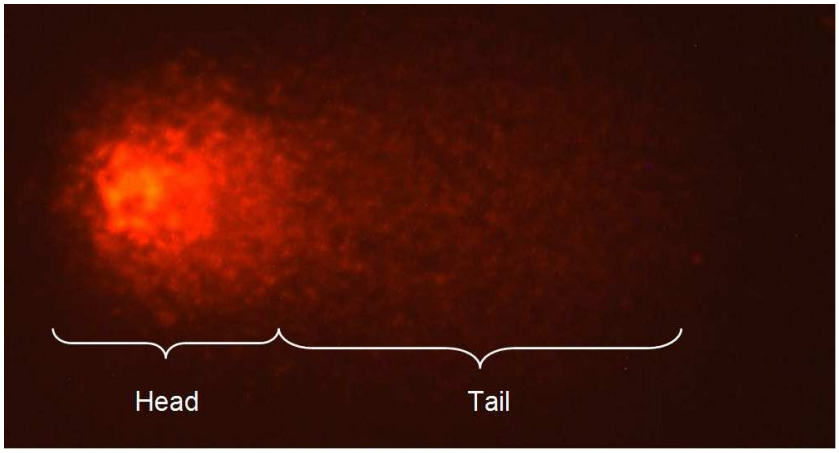


Figure 9: DNA damage in comet assay
The head of a cell is mainly composed of intact genomic DNA, whereas any fragmented or damaged DNA is concentrated within and towards the tail. Damaged DNA such as small fragments and relaxed loops move faster than larger fragments and intact DNA. Comets are quantified microscopically after DNA-staining.

For the dose response to patulin V79 cells (5×10^5), seeded the day before were treated for 4 h with the indicated concentrations of patulin. In a second assay cells were first pretreated with 20 µM BSO for 20 h to investigate the effect of GSH on patulin -induced damage in comet assay.
Subsequently, for all comet assays the cells were harvested and suspended in 180 µLof low-melting-point agarose (0.5% diluted in calcium and magnesium-free PBS). 45 µL of the suspension was embedded on frosted microscope slides, which have been coated with a layer of high-melting-point agarose (1.5%, diluted in calcium-and magnesium-free PBS). Cells were lyzed in a jar containing fresh cold lysing solution (1% Triton X-100, 10% DMSO and 89% lysis buffer containing 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 10; 1% Na-sarcosine; 2.5 M NaCl; and 100 mM dinatrium-ethylendiamintetraacetat-dihydrat (Na_2EDTA) at 4 °C in a dark chamber for 1 h. Afterwards, slides were placed into a horizontal electrophoresis tank filled with an alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA, pH 13). DNA was allowed to unwind for 20 min at 4 °C in the dark. Electrophoresis was carried out, at 4 °C in the dark, for 20 min in a 25-V and 300-mA electrical field. Afterward, the slides were neutralized for 5 min in 0.4 M Tris (pH 7.5), fixed in methanol and dried. A fluorescence microscope at 200-fold magnification and a computer-aided image analysis system (Komet 5; Kinetic Imaging, Bromborough, UK) were used for analysis. 25 cells from each of two slides stained with Gel Red (20 µg/mL in PBS) were measured, with percent tail DNA as the evaluation parameter.

For the detection of cross-links a modified protocol has be proposed by Olive et al. [187]. By creating DNA-cross-links DNA fragments resulting from treatment with radiation or strand breaking agents are artificially increased in size and their migration in an electrical field is impeded.

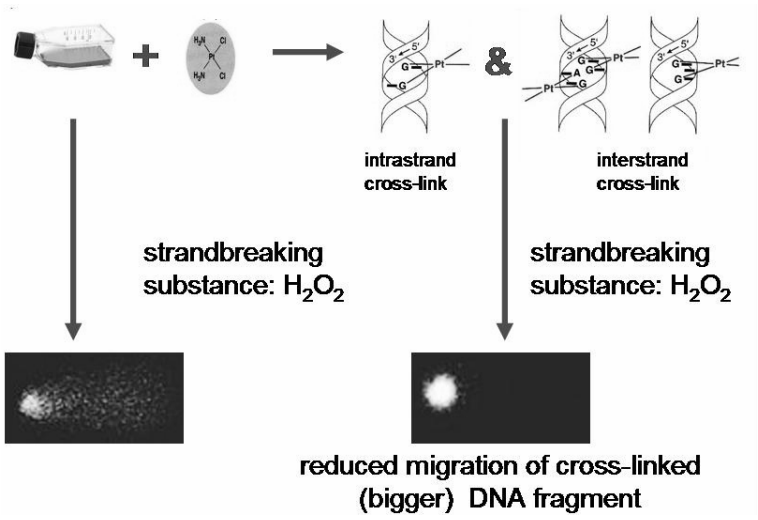


Figure 10: Detection of cross-links by a modified version of comet assay
Cross-linked DNA results in bigger fragments after treatment with strand breaking agents. Bigger fragments move slower in the electrical field and result therefore in smaller comets after DNA-staining.

V79 cells (5 × 105) seeded the day before, were treated for 4 h with 0.5 µM patulin or the known cross-linker cis-platin (10 µM). After a washing step 100 µM H_2O_2 was added for 30 min [183]. Rest of the experiment was carried out as describes above.

2.1.7 α/ɣ-tubulin-staining

α-tubulin-staining was used to visualize mitotic spindles and structure of cytoskeleton. ɣ-tubulin-staining was applied to evaluate the number of centrosomes in mitotic cells.

2×10^5 cells, seeded the day before were incubated with 0.5 µM patulin for 4 h. After 20 h substance-free post incubation cells were harvested and brought onto glass slides as described above. Fixed slides were washed with PBS containing 0.5% Tween 20 and incubated at 37 °C for 1 h with FITC-labeled Sigma mouse anti-α-tubulin (F2168) 1:50 in 5% FBS-PBS or at 4 °C overnight with Sigma mouse anti-ɣ-tubulin (T6557) 1:50 in 5% FBS-PBS. Slides for ɣ-tubulin were subsequently washed and incubated with Alexa 488-labeled goat anti-mouse antibody (Nitrogen A11001) 1:200 in 5% FBS-PBS for 3 h at room temperature. For evaluation α- and ɣ-tubulin dyed slides were counter stained with Hoechst 33258 for 3 min. 400 mitotic figures were counted and classified as normal or multipolar mitoses [183]. Higher concentrations of patulin (5 µM and 50 µM) were applied for 6 h to V79 cells to

investigate a potential compromise of cytoskeleton. Cells were stained as described above, examined and representative pictures were taken with an Eclipse 55i microscope (Nikon GmbH, Düsseldorf, Germany) at 200-fold magnification and a Fluoro Pro MP 5000 camera (Intas Science Imaging Instruments GmbH, Göttingen, Germany).

2.1.8 GSH

GSH content of cells was measured by flow cytometry. 5×10^5 cells were seeded the day before and treated for the indicated time with 1, 10 and 100 µM resveratrol;
0.5, 5 and 50 µM patulin or solvent control, trypsinized, washed in PBS and incubated with 300 µL 400 µM monochlorobimane (MCB) solution in PBS for 30 min on ice. Afterwards cells were washed twice, resuspended in PBS and analyzed by flowcytometry using a LSR I (Becton-Dickinson, Mountain View, CA, USA). Fluorescence intensities of 20,000 cells were recorded. The shift to the right of the fluorescent histograms indicates an increase of cellular GSH content. Mean intensities of peaks were used for statistical analysis. Data is shown as percentage of peak intensity compared to solvent treated control.

2.1.9 DCF

Oxidative stress in cells was measured by flow cytometry using the dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). This non-flourescent probe enters the cytoplasm, where its acetyl groups are cleaved by esterases and the dye is oxidized to its fluorescent derivate (DCF) by intracellular oxidants.
0.5×10^5 cells were seeded the day before were incubated for 4 h or 24 h with the indicated concentrations of substances. In the last 10 min of treatment cells were additionally loaded with 10 µM H_2DCFDA (Invitrogen, Oregon, USA) at 37 °C. After incubation, cells were harvested, washed twice with cold PBS and incubated for 10 min with 1 µg/mL propidium iodide on ice.
Propidium iodide-positive cells were excluded from evaluation due to the failure of esterase activity in dead cells. 20,000 cells were analyzed by flow cytometry using a LSR I (Becton-Dickinson, Mountain View, USA). The shift to the right of the fluorescent histograms indicates an increase of ROS. Mean intensities of peaks were

used for statistical analysis. Data is shown as percentage of peak intensity compared to solvent treated control.

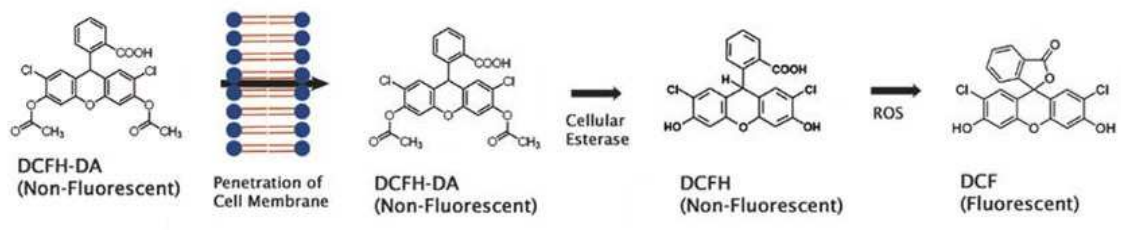


Figure 11: Mechanism of ROS-induced fluorescence of 2',7'-dichlorofluorescein
The diacetylated derivative of DCFH penetrates easily cell membranes. Intracellular esterases cleave the two ester bonds of DCFH-DA, resulting in cell membrane-impermeable product H_2DCF. This non-fluorescent molecule can be oxidized by intracellular ROS yielding the highly fluorescent product DCF, which can be detected by flow cytometry.

2.1.10 FRAP assay

FRAP assay (ferric reducing ability of plasma; also: ferric reducing antioxidant power) is an antioxidant capacity assay which uses the water-soluble derivative of vitamin E Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or ferrous sulphate as a standard. The FRAP assay is often used to measure the antioxidant capacity of plasma or solution of antioxidants.

7 µL of sample was mixed with 193 µL of water and 600 µL of FRAP reagent (ferric chloride 1.67 µM, 2,4,6-tripyridyl-s-triazine 0.83 µM, hydrochloric acid 6.67 mM and acetate buffer 250 mM). The mixture was measured after a 6 min incubation time at 593 nm with a spectrometer (Evolution 160 UV-VIS, Thermo Scientific, Dreieich, Germany). The antioxidant capacity was calculated with help of a calibration curve of ferrous sulphate (0-40 µM).

2.1.11 Cell cycle analysis

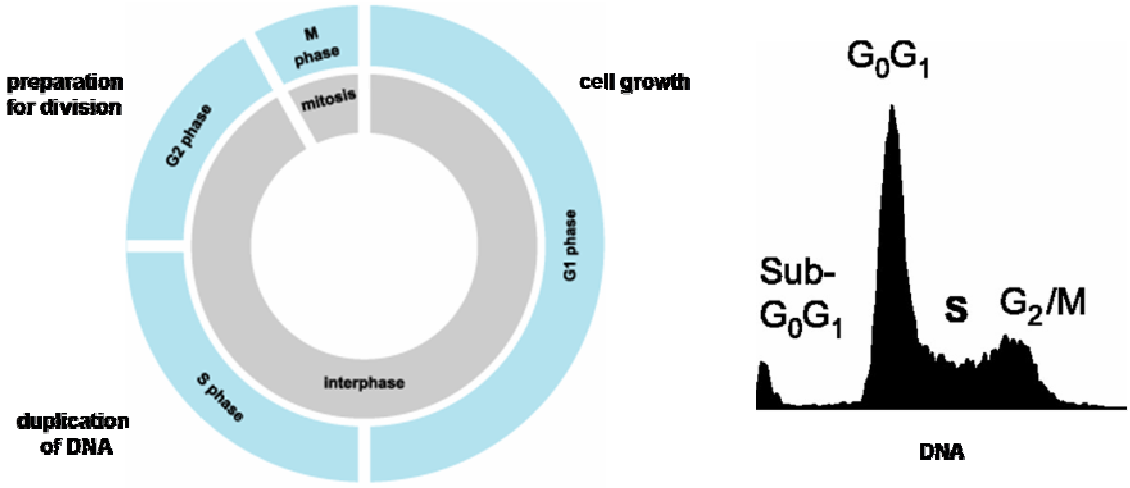


Figure 12: Cell cycle is divided into two major parts: interphase and mitosis. During interphase, the cell growth and chromosome replication takes place. The interphase is subdivided into three phases: gap phase 1 (G1), synthesis (S) and gap phase 2 (G2). Interphase is followed by mitosis (nuclear division) and cytokinesis (cell division). The sub-G1 peak contains apoptotic cells and particles whose DNA content is less of that of cells in G1. Modified after Answers™ [188]

Cell cycle analysis was carried out by fluorescence labeling of cellular DNA with Hoechst 33342. The replication state of each cell was then analyzed by measuring its fluorescence intensity with cell cytometry. Quiescent and G1 cells have one copy of DNA and will therefore have 1X fluorescence intensity. Cells in G2/M phase of the cell cycle have two copies of DNA and give therefore 2X intensity. S-phase represents cells during DNA synthesis with fluorescence values between the 1X and 2X populations.

0.5×10^5 cells, seeded the day before were treated for 6 h with 0.5 µM patulin or solvent control. BD Kit CytoPerm/CytoFix (BD Bioscience, San Diego, USA) was used for permeabilization and fixation of cells. After trypsinization cells were washed twice with PBS and resuspended in Cytofix/Cytoperm™. After 20 min of pemeabilization cells were washed in Perm/Wash ™ and resuspended in 1 mL

2.5 µM Hoechst 33342. Samples were incubated for 15 min at 37 °C. Samples were then centrifuged and suspended in a 1 % solution of bovine serum albumin (BSA). 20,000 cells were analyzed by flow cytometry using a LSR I (Becton-Dickinson, Mountain View, USA). The amount of cells in the G1/S/G2 phase was calculated by comparing the mean peak intensities.

2.2 PART II: EFFECTS OF AN ANTHOCYANIN RICH EXCTRACT ON HYPERTENSIVE RATS

2.2.1 Experimental procedure

All animal experiments were performed in accordance with the European Community guidelines for the use of experimental animals and with the German law for the protection of animals.

Homozygous female Ren-2 rats (n=23) were housed in an air-conditioned humidity-controlled environment (25 °C) with a 12 h light/dark cycle (light 7:00-19:00). Before begin of the experiment Ren-2 were treated with ACE inhibitor ramipril (Delix, Sanofi aventis, Frankfurt am Main, Germany; 1 mg/kg bw) to keep the blood pressure to the level of genetically unmodified control rats

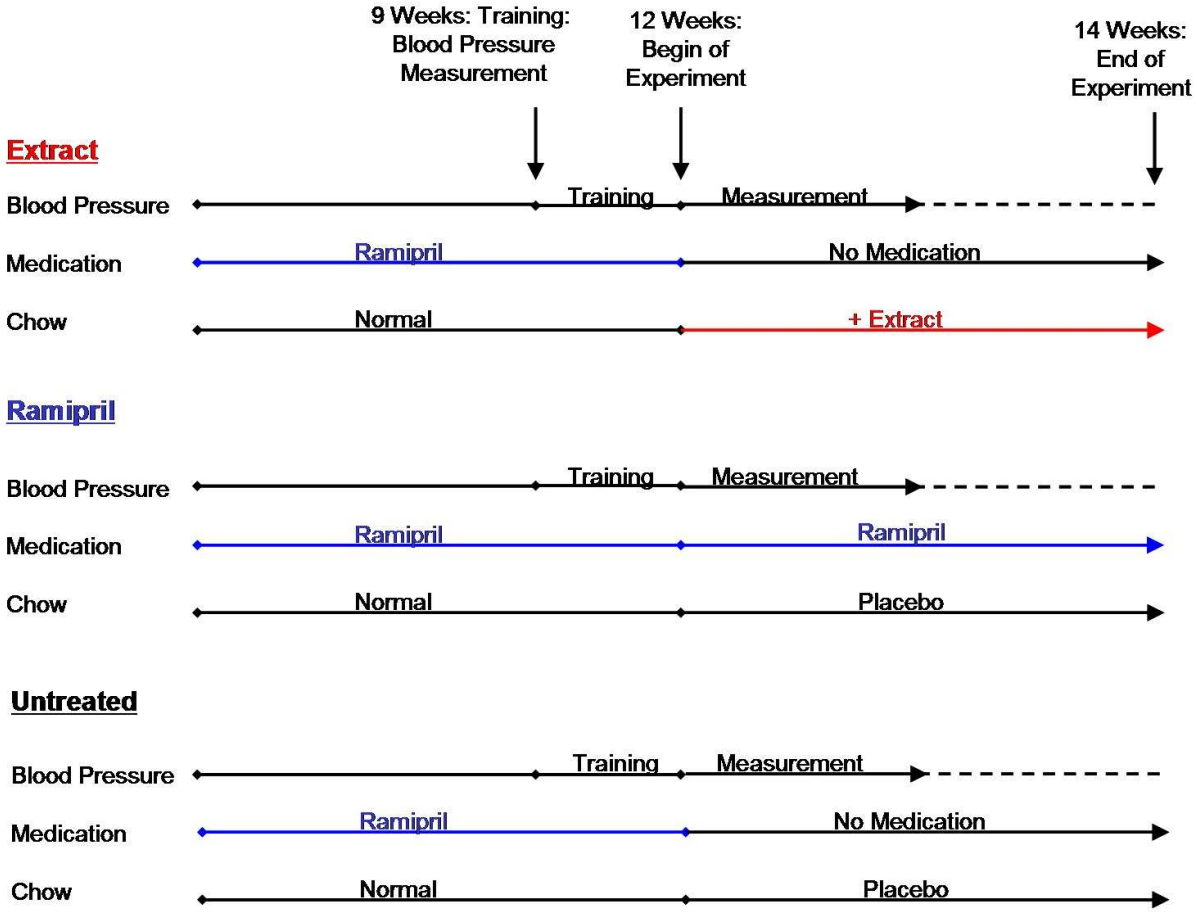


Figure 13: Treatment scheme of animal experiment

6 non-transgenic SD rats of the same age were kept under the same conditions to compare the development of body weight and digestive behaviour.

At the age of 12 weeks Ren-2 rats were randomly divided into three groups: untreated (n=9), ACE inhibitor ramipril (n=7) and Dacapo-grape extract (n=7). Three to five rats of each group were kept in one cage. They were given free access to a stock diet (ssniff, Soest, Germany, SM M-Z, 10 mm) and water. The ACE inhibitor group was further treated with 1 mg/kg bw ramipril per day dissolved in drinking water, whereas the other two groups did not obtain any medication from the beginning of the experiment.

Dacapo-grape extract was compacted to pellets in a concentration of 2.175 g/kg (≈ 0.05% anthocyanins) leading to a daily intake of approximately 10 mg/d for rats of the extract-group. The untreated group and the ramipril group were fed with placebo chow.

Body weight, food intake and water intake were controlled regularly. Before the beginning and at the end of the study rats were kept for 24 h in metabolism cages. Individual water consumption was recorded and samples of urine were stored at -80 °C for further evaluation.

Before starting the experiment rats were trained for three weeks to reduce the stress associated with the blood pressure measurements and hence reduce the variability of results. The blood pressure and the heart rate of rats were measured twice a week using the direct tail cuff method. Non-invasive blood pressures were obtained using the BP 2000 Blood Pressure Analysis System (Visitech Systems, Apex, USA). The pulse was detected on tail, distal to the tail cuff, with a photoelectric sensor. Rats were placed on a warm platform to increase blood flow to the tail in order to improve the pulse detection.

The mean of at least three successful measurements was taken as data for heart rate and blood pressure of the animal. One rat of the untreated group was excluded, due to excessive movement in all measurements.

The planned treatment time was four weeks, but the experiment was stopped ahead of schedule after the spontaneous death of four animals (two animals of the untreated group, day 11 and 12; one animal of the ramipril group, day 11; and one animal of the extract group day 11). The rats prematurely deceased were excluded from all data except of food intake, where individual data was not available. Blood pressure could not be measured successfully anymore after the first week because

the rats moved excessively and were rather agitated. Therefore, the blood pressure diagrams of this study include only the first three survey points of blood pressure measurement.

On the day of the experiment rats were anesthetized with Ketamin (0.6 mL, 10% medistar®, Ascheberg, Germany) and Xylazin (0,2 mL, Xylazin 2% cp-pharm; Burgdorf, Germany). Isofluran (Isofluran CP®, cp pharma, Burgdorf, Germany) was used to maintain the narcosis. Before surgery an isotonic saline solution (Fresenius Kabi Deutschland GmbH; Bad Homburg, Germany) was used for perfusion of rats to remove blood from organs and reduce thereby artefacts. Organs (heart, kidney, aorta, liver, lung, brain, small and large intestine) were taken out, weighted (heart, kidney), cut in pieces and frozen at -80 °C or fixed in Roti®-Histofix. Organs fixed in Roti®-Histofix were embedded in paraffin shortly after fixation.

Parts of liver and kidney tissue were used for comet assay (see 2.2.2)

Blood was withdrawn from retrobulbar vessels if possible, but the collection was just successful for 12 of 19 animals. Blood was collected in S-Monovetten (Sarstedt, Nümbrecht, Germany) with clotting inhibitor, centrifuged and serum was stored at - 20 °C until analysis.

2.2.2 Comet assay

Samples of kidney and liver tissue were choped up on ice and suspended in RPMI 1640 medium (+ 15% DMSO, + 1.8% (w/v) NaCl). The suspension was sifted through a cell strainer with a mesh pore size of 100 µm (Becton Dickinson Mountain View, USA), centrifuged for 5 min at 1000 rpm and at 4 °C and the resulting pellet was resuspended in 1 mL of the medium. Cells were kept on ice until the experiments started. Comet assay was carried out as described in 2.1.6 and 50 cells from each of two slides stained with Gel Red (20 µg/mL in PBS) were measured, with percent tail DNA as the evaluation parameter.

For logistic reasons analysis was split in two parts with equal number of animals from each group in each part. After evaluation of each part results were normalized to the ramipril treated group due to strong differences in the background damage related with variances in the experimental procedure. After the normalization data was collected in one graph.

V79 cells treated with 12.5 µg/mL methyl methan sulfonate (MMS) were used as a positive control and to prove the efficiency of the experimental conditions

2.2.3 γ-H_2AX-staining

Paraffin sections of different organs were stained with an antibody against γH2AX to visualize double strand breaks in the DNA.

Double strand breaks are highly deleterious DNA lesions as they lead to chromosomal aberrations and/or apoptosis. They can be triggered by ionizing irradiation and a variety of chemical agents, e.g. topoisomerase II poisons, heavy metal ions and ROS. The formation of double strand breaks leads to the phosphorylation of histone H2AX on Ser-139 (termed as γ-H2AX) which is probably involved in the repair of damage by holding broken DNA ends together and recruiting other repair factors [189].

At necropsy, kidneys were removed and fixed in Roti® Histofix and embedded in paraffin. The tissue blocks were cut in a microtome (LEICA RM 2165, Wetzlar, Germany) to a thickness of 2 µm and mounted on positively charged slides. Sections were deparaffinized and rehydrated in Roti® Histol and an ethanol series. Antigen retrieval was achieved by a 15 min treatment with 10 mM sodium citrate (pH 6) buffer in a microwave. Unspecific binding was avoided by blocking with donkey serum (Chemicon International, Hofheim am Taunus, Germany) for 1 h. Endogenous peroxidase activity was suppressed by 3% H_2O_2 to reduce background staining. ABC (Avidin-Biotin-Complex) method was used to enhance the signal of the applied antibody. Free avidin and biotin in the tissue was blocked in advance by incubation with 1 ppm avidin and biotin for 15 min respectively. The primary antibody (Phospho-Histone H2AX (Ser139, clone 20E3) rabbit mAb, 9718, Cellsignaling, Danvers, USA) was applied in a 1:200 concentration overnight at 4 °C. Biotinylated secondary antibody (donkey anti rabbit IgGB, sc2089, Santa Cruz Biotechnology, Santa Cruz, USA) was used afterwards in a 1:200 dilution for 45 min at room temperature. Afterwards a horseradish peroxidase (HRP) coupled ABC reagent (Vector Laboratories. Burlingame, USA) was added for 30 min.

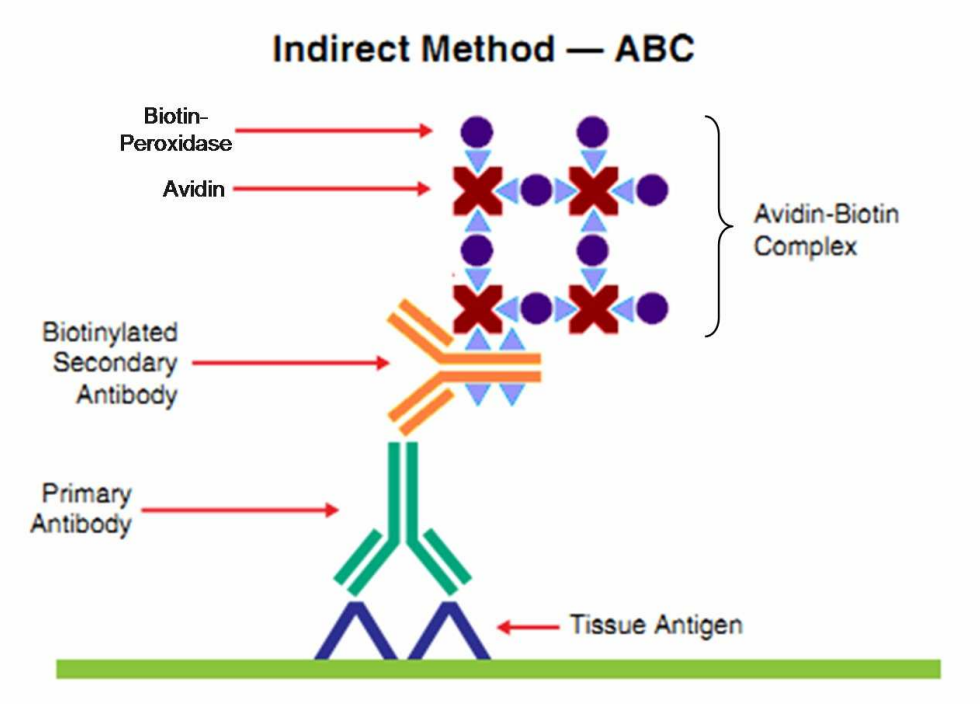


Figure 14: The amplification of signal by avidin-biotin methods rely on the strong affinity of avidin or for the vitamin biotin. Avidin (from chicken egg) possesses four binding sites for biotin. The biotin molecule is easily conjugated to antibodies and enzymes. In the avidin-biotin-complex (ABC) method the secondary antibody is conjugated to biotin and functions as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex. Modified after Key [190].

Avidin-biotin-coupled system was used to enhance the signal of the antigen. 3,3'-diaminobenzidine (DAB, Vector Laboratories. Burlingame, USA) reagent was applied for 5 min. DAB reacts with HRP in the presence of peroxide to yield an insoluble brown-colored product at locations where peroxidase-conjugated antibodies are bound to samples. Sections were counterstained with Ehrlich's haematoxilin (1 g haematoxylin , 48 mL 99.8% isopropanol, 51.9 mL H_2O d, 50 mL glycerol, 1.5 g potassium alum, 5 mL acetic acid, 0.2 g potassium iodat) for three minutes and mounted with Eukitt® after dehydration in an alcohol series and Roti® Histol. Pictures were taken with a LEICA DM750 microscope equipped with a LEICA ICC50HD camera (LEICA Camera AG, Solms, Germany).

For logistic reasons sections were split for the staining procedure in two parts with equal number of animals from each group in each part. Data was collected afterwards in one graph. Slides were analyzed using a semiquantitative scoring

Kidney was divided in papilla, medulla and cortex (Figure 15).

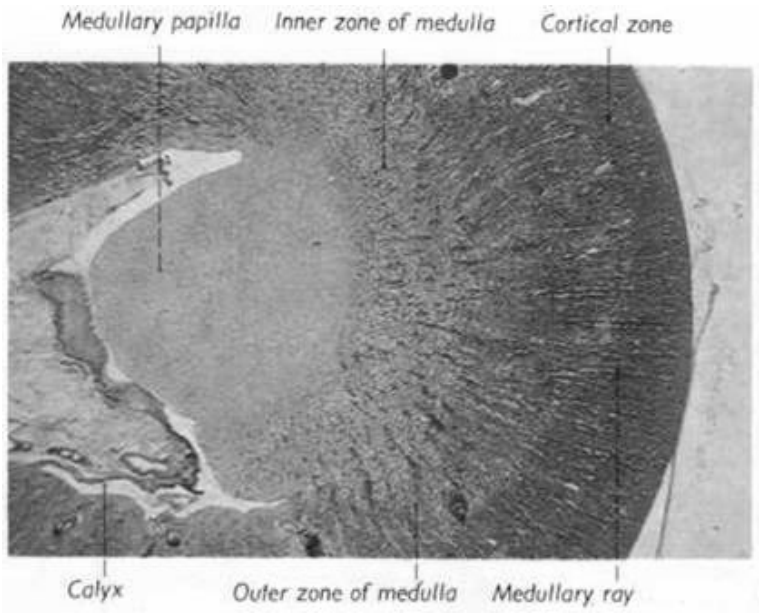


Figure 15: Cross-section of a kidney
Mallory-azan-staining; 10x; Hammersen, F. (1980) Histology, A Color Atlas of Cytology, Histology and Microscopic Anatomy 2nd Ed. Urban & Schwarzenberg [191]

The percentage of positive cells in the kidney was assessed by manual scoring of brown nuclei in minimum 5 pictures with at least 1500 cells per picture for each region and each animal. Total number of nuclei on each picture was counted by using the automatical evaluation software cellprofiler (2.0, Broad Institute, Cambridge, USA).). For the analysis of heart 5 pictures representing different parts of the heart were taken and all cells on the picture (at least 2400 per animal) were classified as negative or positive for ɣ-H2AX.

For the analysis of small intestine 1000 nuclei in crypts were counted manually for each animal and the percentage of ɣ-H2AX positive nuclei was calculated (Figure 16).

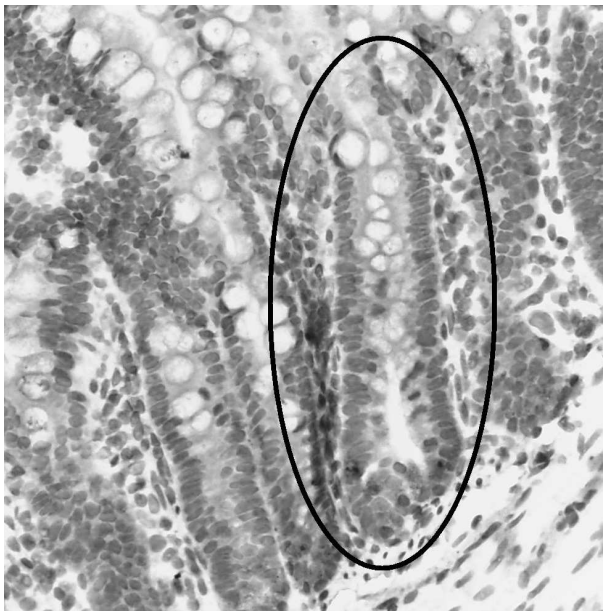


Figure 16: Crypt in a section of small intestine

2.2.4 DHE-staining

To evaluate the release of ROS and particularly superoxide anion, the cell-permeable fluorogenic probe DHE was used. Frozen tissues were embedded into Tissue-tek (Sakura, Alphen aan den Rijn, The Netherlands). The blocks were cut in a cryotome (Leica CM 3050 S, Wetzlar, Germany)) to a thickness of 3 µm. Sections were brought on a slide and stored at -80 °C.

Frozen sections were incubated for 20 min at room temperature with a 10 µM solution of DHE (Merck Bioscience GmbH, Schwalbach, Germany) in distilled water. 160 µL were added on each section and covered with a cover slip. An Eclipse 55i fluorescence microscope (Nikon GmbH, Düsseldorf, Germany) at 200-fold magnification and a Fluoro Pro MP 5000 camera (Intas Science Imaging Instruments GmbH, Göttingen, Germany) were used for analysis. At least 10 pictures were taken from each animal and the level of fluorescence intensity was calculated with the aid of the image analysis system (Cell profiler; 1.0.9717; Broad Institute, Cambridge, USA).

2.2.5 FRAP analysis

We used FRAP assay to prove the antioxidative properties of Dacapo grape extract and the antioxidative capacity of serum from experimental animals. FRAP assay was carried out as described in 2.1.10, using 7 µL of extract or 7 µL of serum.

2.3 STATISTIC

Statistical calculations were performed using Statistica 8 (StatSoft (Europe) GmbH, Hamburg, Germany). For *in vitro* experiments, if not mentioned otherwise, data from at least 3 independent experiments ± standard deviation was depicted. For the animal study each group represents 6-7 Ren-2 rats if not mentioned otherwise. Individual groups were tested using the Mann Whitney U-test and results were considered significant if the p-value was ≤ 0.05.

3 RESULTS

3.1 PART I: INFLUENCE OF PATULIN AND RESVERATROL ON DNA STABILITY

3.1.1 Viability assay after patulin treatment

Viability assay was used to exclude a potential cytotoxic effect for the concentrations of patulin used in genotoxicity assays. Figure 17 shows only slight cytotoxic effects for 0.5 µM and 5 µM patulin whereas 50 µM patulin killed almost 80% of cells.

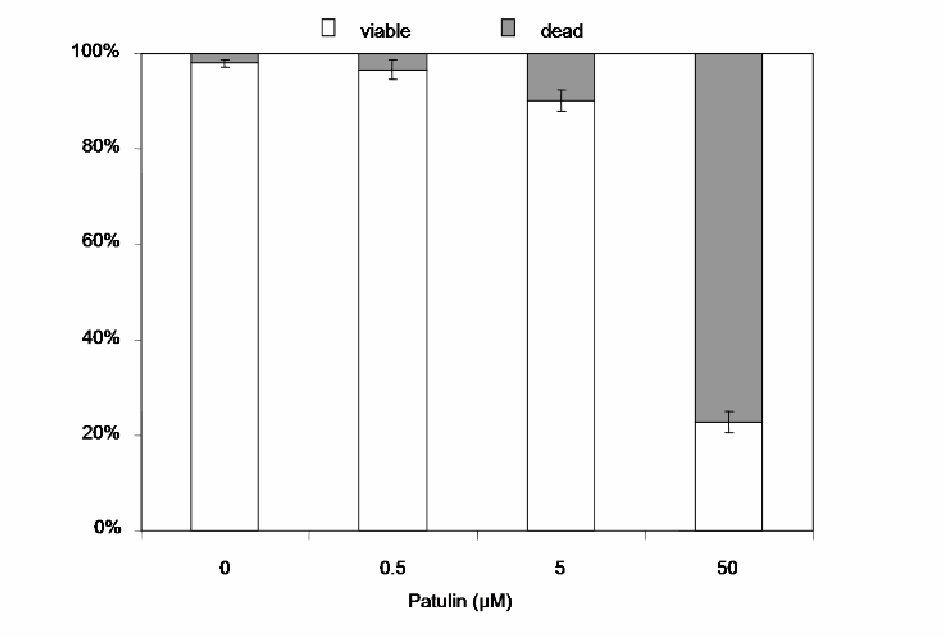


Figure 17: Viability assay with different concentrations of patulin.
V79 cells were treated for 4 h with the indicated concentrations of patulin followed by 20 h post-incubation with cytochalasin B (5 µg/mL). Cells were stained with fluorescein diacetate (viable cells) and propidium iodide (dead cells). Data represents the mean of two experiments.

3.1.2 Influence of patulin in micronucleus assay

Treatment of V79 cells with patulin for 4 h led to a significant, dose-dependent formation of micronuclei up to 96 micronuclei per 1000 cells with 0.5 µM patulin.

However, with higher doses of patulin the number of micronuclei in mononucleated cells was reduced (Figure 18).

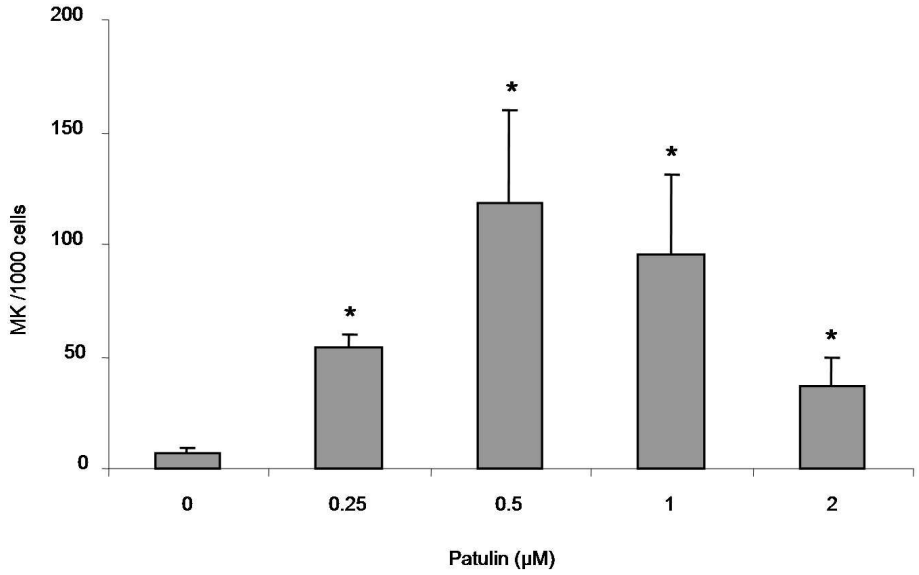


Figure 18: Micronucleus (MN) assay with different concentrations of patulin.
V79 cells were treated for 4 h with the indicated concentrations of patulin followed by 24 h substance-free post-incubation. 1000 from each of two slides per concentration were checked for the presence of micronuclei. Data show means of three experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk.

3.1.3 Influence of GSH on patulin-induced micronuclei and nucleoplasmic bridges

The micronucleus assay and an analysis of nucleoplasmic bridges were carried out to evaluate the genotoxicity of patulin in V79 cells (Figure 19). We used for the following experiments cytokinesis blocked micronucleus assay to avoid artefacts by a potential treatment-induced inhibition of proliferation. The formation of micronuclei increased dose-dependently in BSO-pretreated (GSH-depleted) cells, but increased only slightly without BSO pre-treatment. Nucleoplasmic bridges increased significantly in BSO-pretreated as well as in not pretreated cells. However, the induction of nucleoplasmic bridges occurred at lower concentrations in the BSO-pretreated cells.

Cell proliferation was slightly reduced with increasing concentrations of patulin and much stronger in BSO-pretreated cells (Figure 19a). At the highest concentration of

1 µM patulin, proliferation of the BSO-pretreated cells was almost completely blocked, making an evaluation of binucleated cells for micronuclei/nucleoplasmic bridges impossible [183].

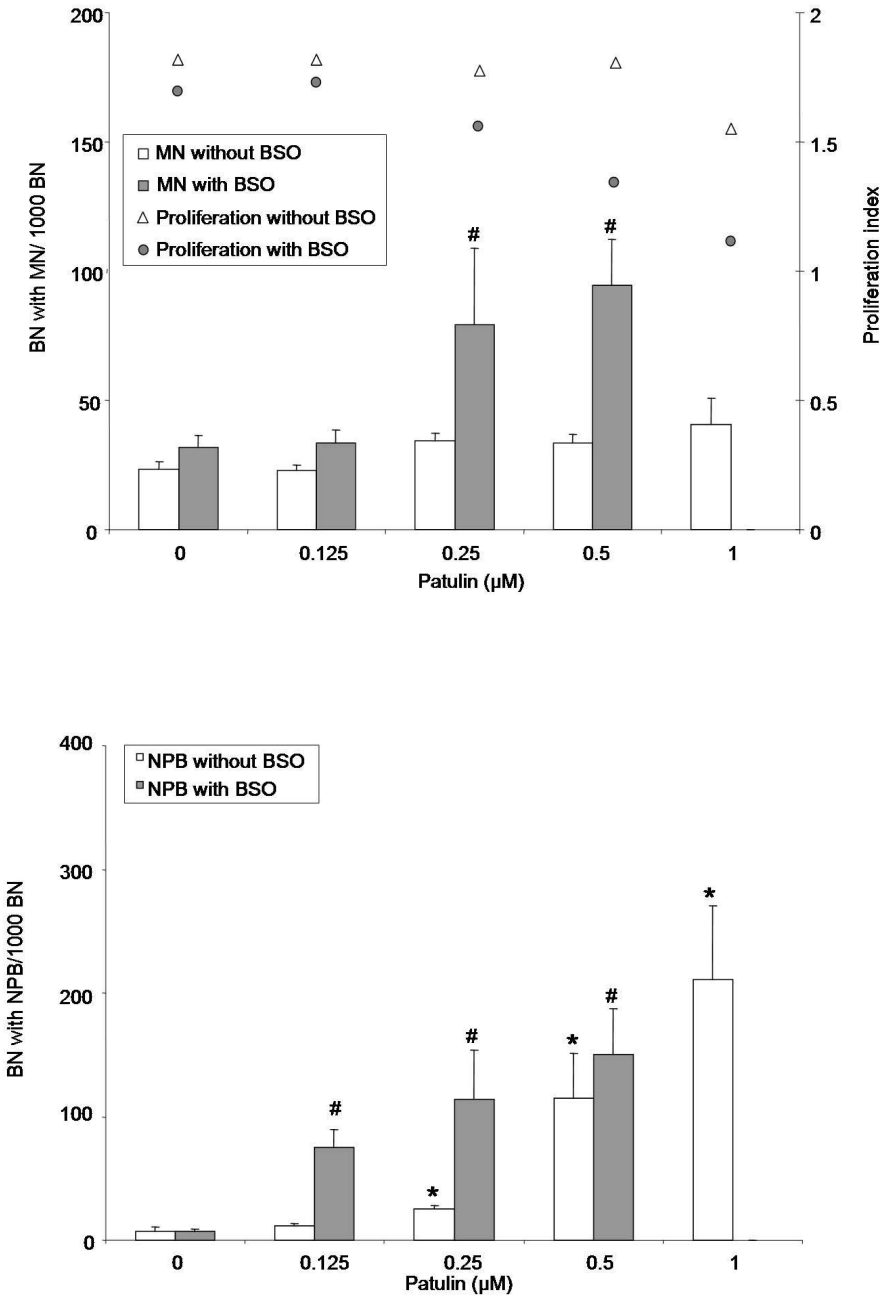


Figure 19: Induction of micronuclei (MN), proliferation index (Figure 19a) and nucleoplamic bridges (NPB) (Figure 19b) in 1000 binucleated (BN) V79 cells.
Cells were pretreated with PBS buffer (white bars) or 20 µM buthionine sulfoximine (BSO, grey bars) for 20 h and then incubated with different concentrations of patulin (4 h treatment and 20 h post-incubation with cytochalasin B (5µg/mL)). Data show means of three independent experiments + standard error of mean.
Cytokinesis block proliferation index (CBPI) was calculated in 1000 cells per slide using the formula CBPI = [MI + 2MII + 3 (MIII +MIV)] with MI-MIV representing the number of cells with one to four nuclei. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk. Significance ($p \leq 0.05$; Mann Whitney U-test) in BSO pretreated cells is indicated by a hash key [183].

3.1.4 Kinetochore analysis of patulin-induced micronuclei

Kinetochore-staining was applied to differentiate the origin of micronuclei induced by patulin. Figure 20 shows, that patulin induced both kinetochore-positive and -negative micronuclei with a higher percentage of kinetochore-negative micronuclei. Patulin-induced nucleoplasmic bridges were almost all kinetochore-negative (data not shown).

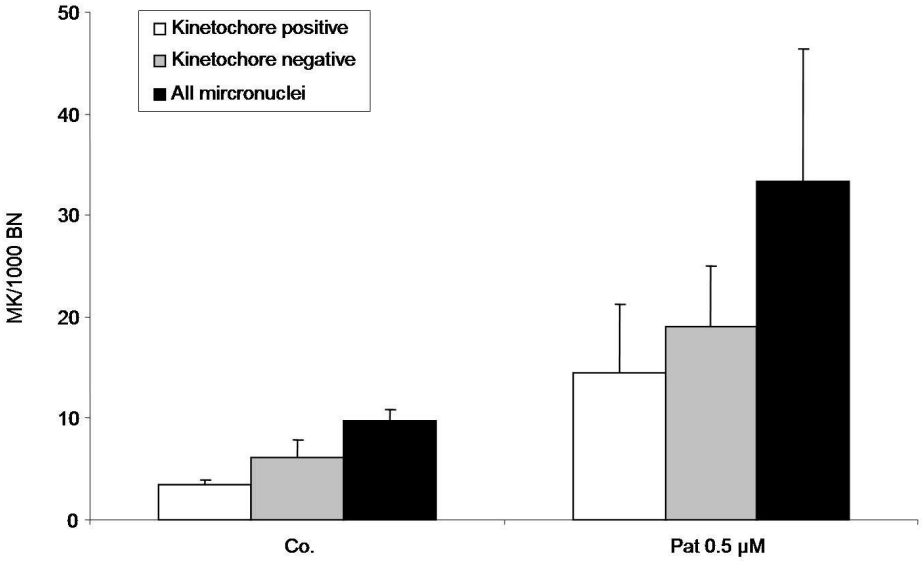


Figure 20: Kinetochore-staining after patulin treatment. V79 cells were incubated for 4 h with patulin or solvent, followed by 20 h substance free post-incubation. Kinetochores were stained with CREST serum and TRITC labeled secondary antibody. Hoechst 33258 was used for counterstaining. Data represents the number of kinetochore-positive/ -negative micronuclei per 1000 binucleated cells evaluated in two independent experiments.

3.1.5 Time course of NPB formation after patulin treatment

Time course of NPB formation after simultaneous incubation with patulin and cytochalasin B was used to investigate the generation of nucleoplasmic bridges. The time course revealed that nucleoplasmic bridges were already present after 3 h with patulin treatment. The number further increased with longer treatment time, reaching almost 20% NPB-positive cells after 6h. In contrast the level of micronuclei did not change within the first 6 hours after treatment (Figure 21).

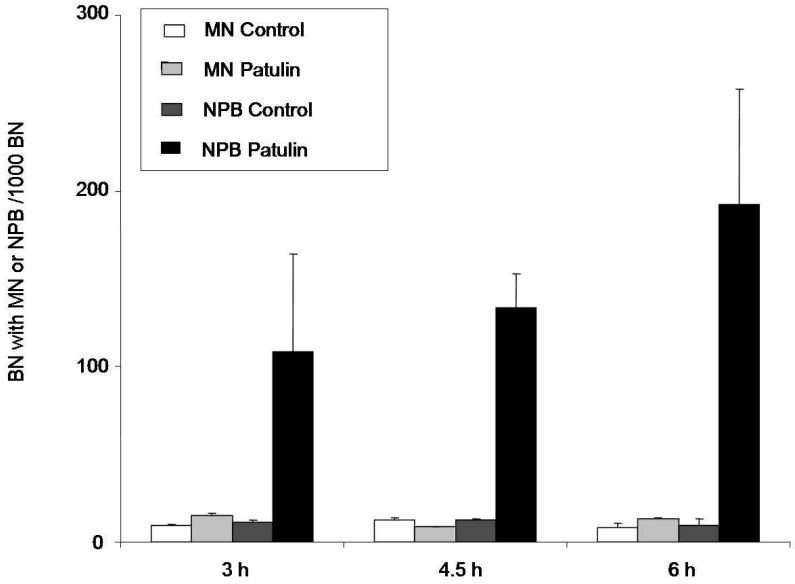


Figure 21: Induction of micronuclei (MN) and nucleoplamic bridges (NPB)
Cells were treated with 0.5 µM patulin or solvent control (DMSO) for the indicated time. Cytochalasin B (5 µg/mL) was added simultaneously with patulin to all samples. Micronuclei and nucleoplasmic bridges were counted in 1000 cells from each of two slides per concentration. Data show the means of two independent experiments + standard error of mean [183].

3.1.6 Influence of patulin in a modified version of comet assay

A modified version of alkaline comet assay was performed to investigate the potential cross-linking properties of patulin. Figure 22 shows the DNA damage induced by H_2O_2 with or without pre-treatment with the known cross-linking agent *cis*-platin or patulin. If there are cross-links, the DNA can migrate less after H_2O_2 treatment. DNA migration was decreased in patulin pretreated cells and significantly reduced in *cis*-platin-pretreated cells compared to H_2O_2 alone [183].

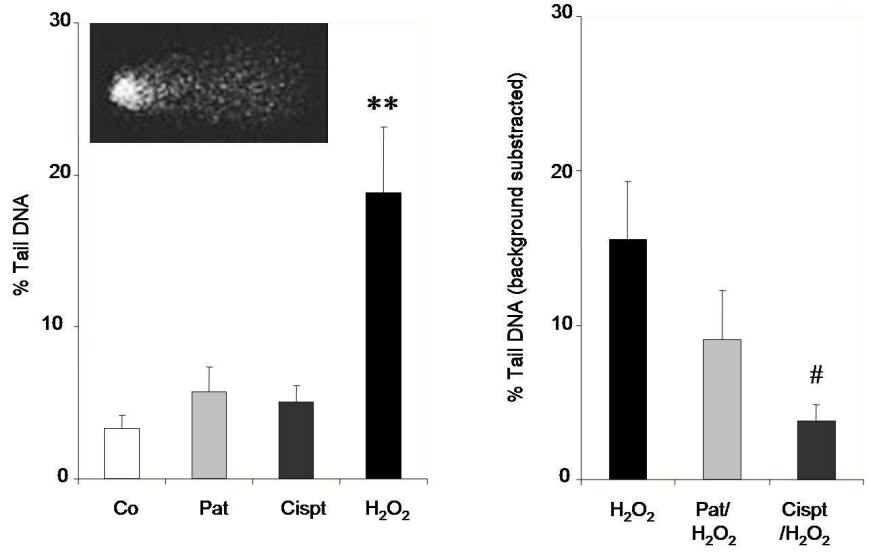


Figure 22: The influence of patulin or the known cross-linking agent *cis*-platin (*Cis*pt) on the DNA migration of H_2O_2 treated V79 cells.
Cells were treated with solvent control (Co), 0.5 µM patulin (Pat) or 10 µM *cis*-platin for 4 h. Subsequently 100 µM H_2O_2 or solvent control was added in fresh medium for 30 min. Left side: damage of control, patulin and cis-platin (post-incubation with solvent control) and control with H_2O_2-post-incubation. Right side: control, patulin and cis-platin with H_2O_2-post-incubation. Basic damage of each pre-treatment was substracted respectively from the H_2O_2 treated samples. Data represent means of five independent experiments + standard error of mean. Left side: Highly significant difference ($p \leq 0.01$; Mann Whitney U-test) compared to the control is shown by a double asterisk. Right side: Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the H_2O_2 treated control is shown by a hash key. Insert: representative picture of a cell in comet assay [183].

3.1.7 Effects of patulin on tubulin

To investigate if the high reactivity of patulin on cellular macromolecules also affects the formation of tubulin fibers from its subunits, a well known mechanism of genotoxic micronucleus forming spindle poisons such as colcemide, α and ɣ- tubulin-staining for detection of mitotic spindles (α) and centrioles (ɣ) was employed.
Microscopic inspection did not reveal a compromised formation of spindle fibers after patulin treatment at the lower concentration of 0.5 µM, whereas the higher cytotoxic concentrations showed a clear conglutination of the cytoskeleton (Figure 23).
However, an eyecatching phenomenon of after incubation of cells with 0.5 µM patulin was the very high number of mitoses containing multipolar spindles (Figure 24, left side), which was about 25 times increased compared to control cells (Figure 25, left

side). This was associated with an equal increase (Figure 25, right side) of cells with supernumerary centrosomes as shown by ɣ-tubulin-staining (Figure 24, right side).

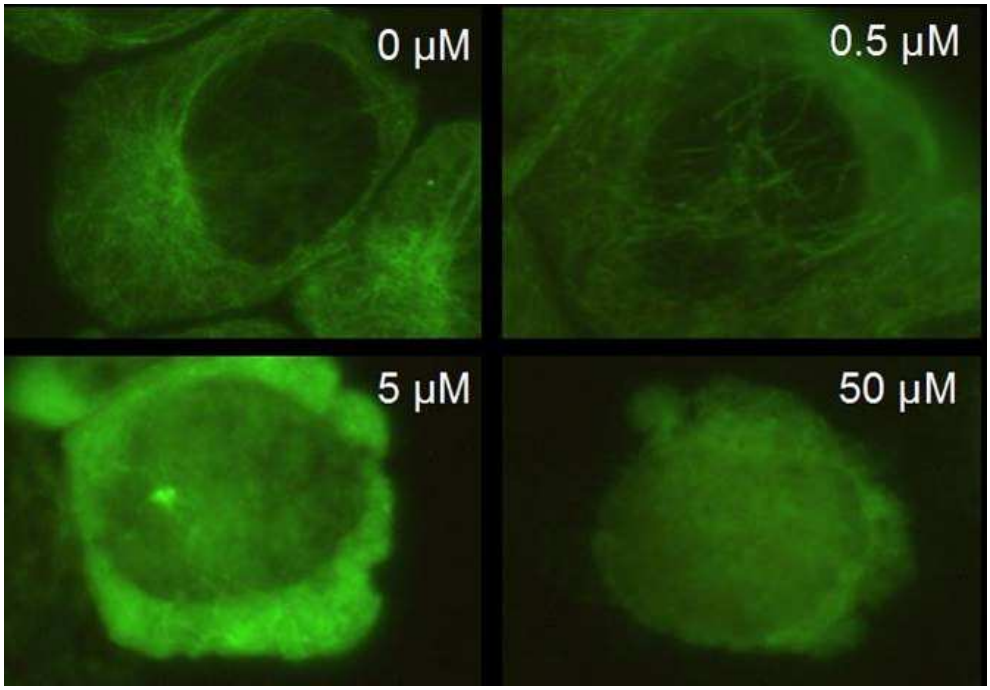


Figure 23: Effects of patulin treatment on tubulin polymerization.Cells were treated for 6 h with different concentrations of patulin. Pictures show representative cells, whose cytoskeleton was stained with a FITC labeled antibody against α-tubulin

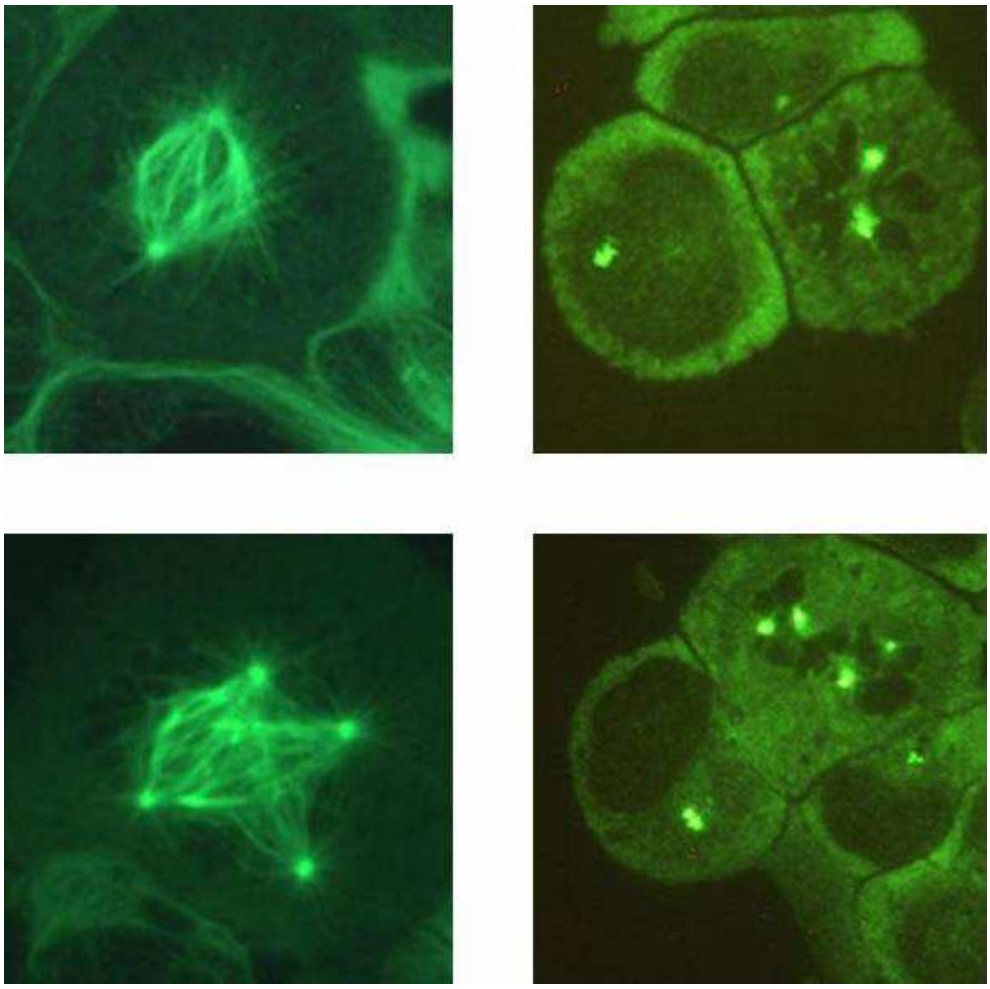


Figure 24: Binucleated and multinucleated cells (left side) and normal and multipolar mitoses (right side) after 4 h incubation followed by 20 h postincubation with cytochalasin B (5 µg/mL). Cells in the upper part were treated with solvent. Pictures of the lower part show cells treated with 0.5 µM patulin.

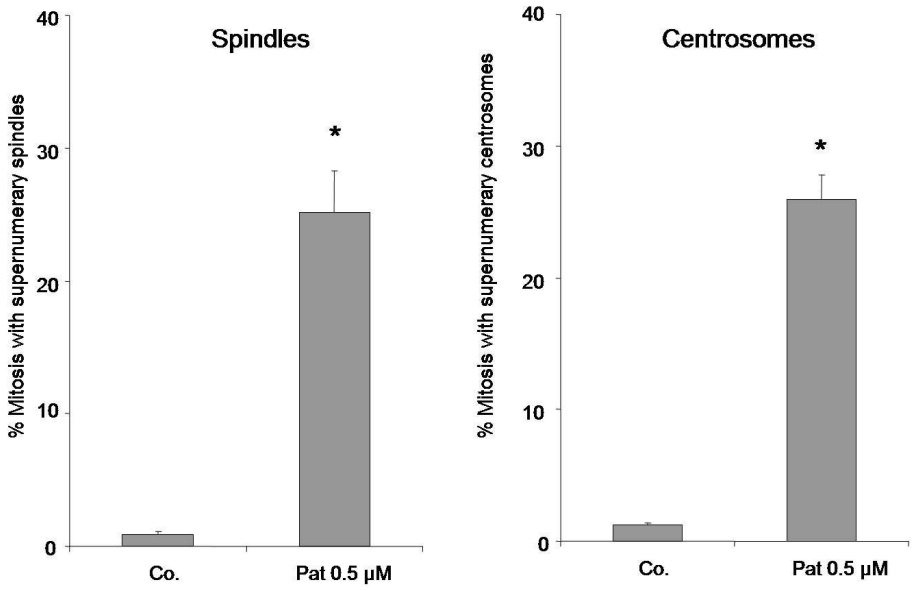


Figure 25: Induction of mitoses with > 2 spindles (left side) and > 2 centrosomes (right side) in V79 cells exposed to patulin (Pat).
Cells were treated with 0.5 µM patulin or solvent control (Co.) for 4 h, followed by 20 h substance free post-incubation. Data indicate the means of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control (Co) is shown by an asterisk [183].

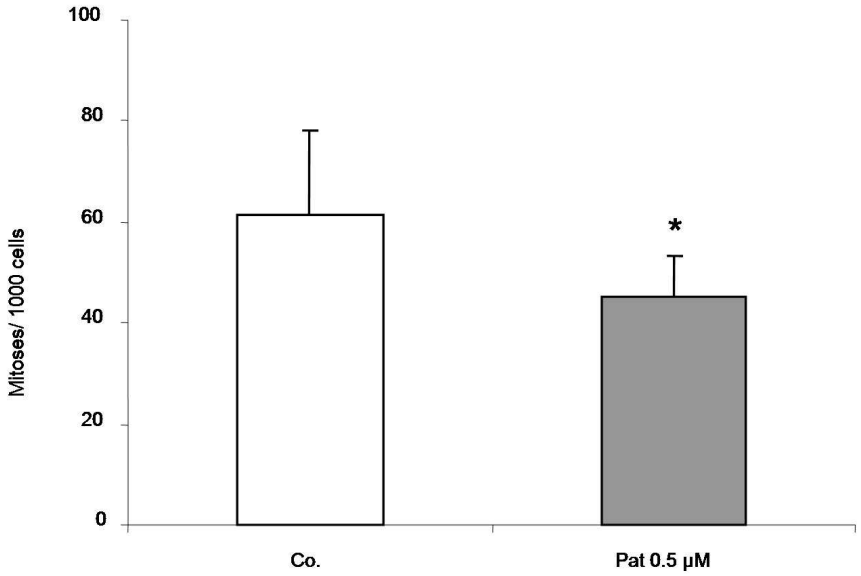


Figure 26: Number of mitoses per 1000 cells after a 4 h treatment with patulin or solvent control, followed by 20 h substance free post-incubation. Cells were stained with an FITC labeled antibody against α-tubulin. Data indicate the means of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control (Co.) is shown by an asterisk.

The mitotic index was reduced by around one quarter in patulin treated cells (Figure 26). This was also reflected in a clear increase of cells arrested in G2 phase. 45.5% of patulin treated cells were in G2 phase compared to 25.6% of the solvent treated cells, indicating a patulin-induced G2 arrest (Figure 27). Patulin incubated cells showed additionally an increased number of odd multinucleated cells and disturbed mitoses (Figure 28).

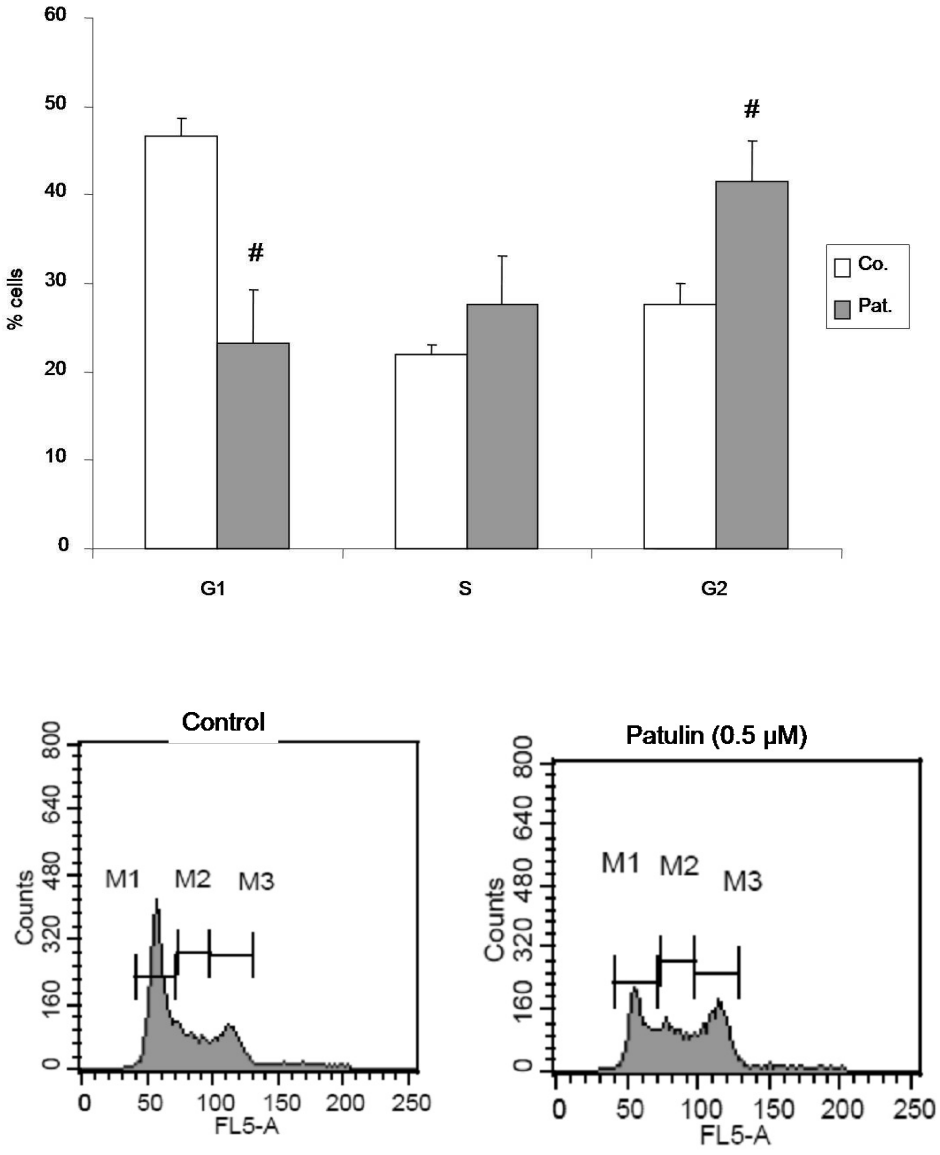


Figure 27: Cell cycle analysis
Cells were treated for 6 h with patulin. Phase of cell cycle was determined by measurement of cellular DNA concentration after staining with Hoechst 33342. Data represents the mean of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control (Co.) is shown by an asterisk.

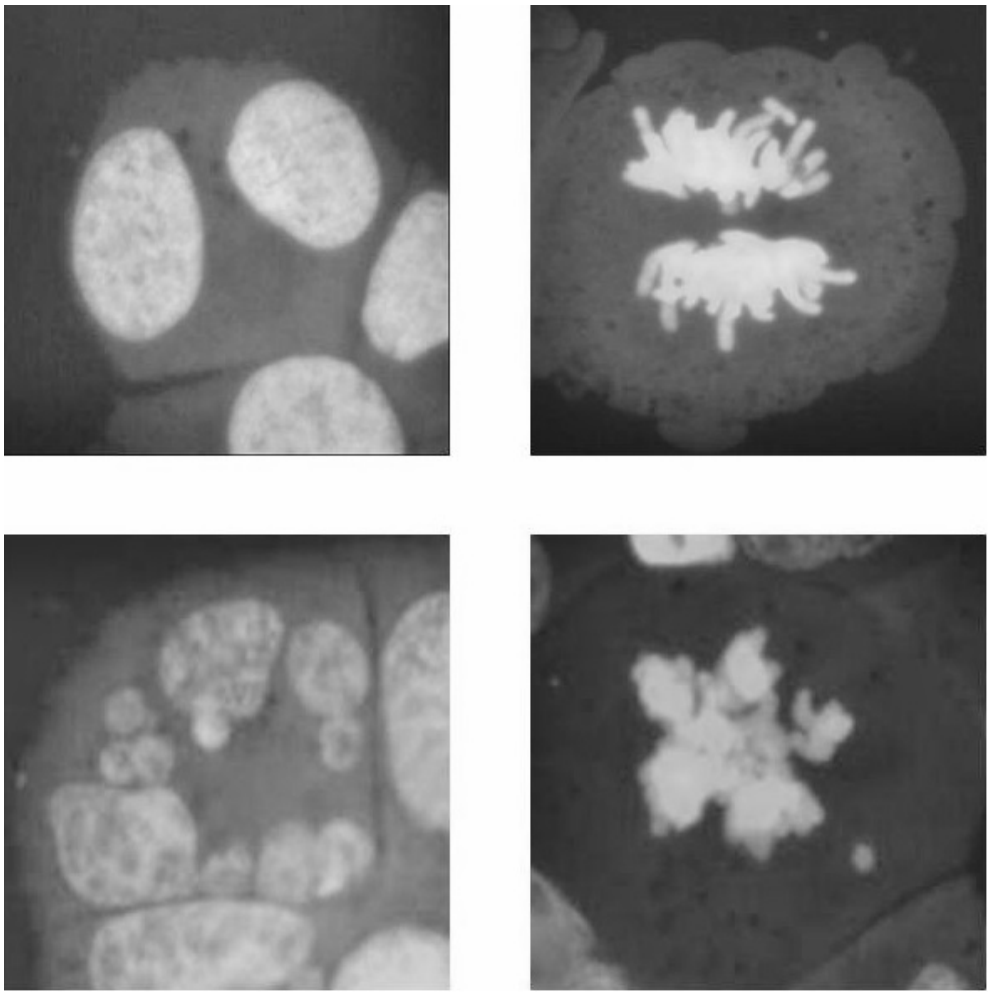


Figure 28: Upper part: binucleated cell (left side) and control mitosis (right side) after 4 h solvent treatment and 20 h post-incuabtion with cytochalasin B (5 µg/mL). Lower part: multinucleated cell (left side) and disturbed mitosis (right side) after 4 h treatment with 0.5 µM patulin and 20 h post-incubation with cytochalasin B (5 µg/mL).

3.1.8 Comet assay with patulin

We used comet assay to investigate the potential strand breaking properties of patulin at different concentrations. Patulin induced a significant, dose-dependent increase of DNA damage in comet assay (Figure 29). The higher concentration of 50 µM led to the formation of so called ghost cells in which DNA damage can not be evaluated properly. This concentration was therefore excluded from analysis (data not shown).

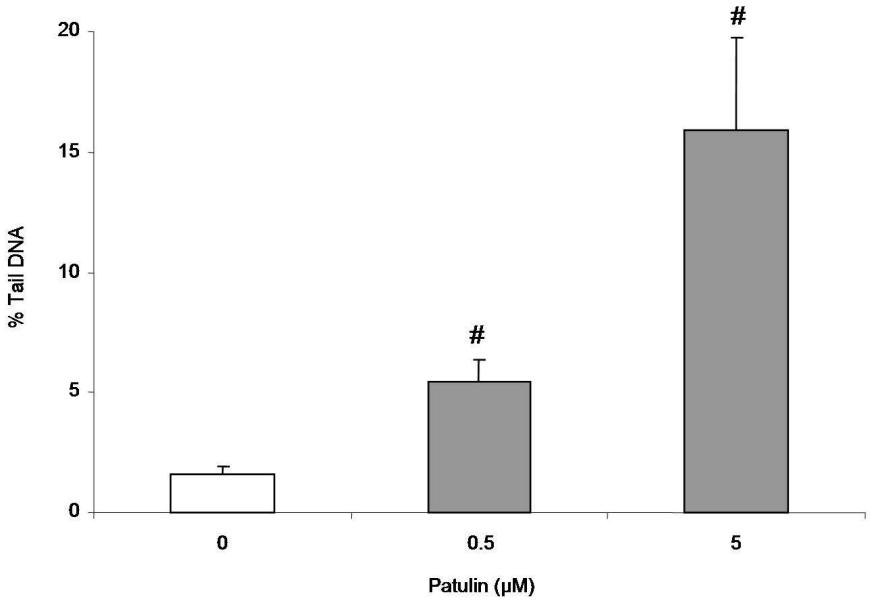


Figure 29: Measurement of DNA damage in comet assay
V79 cells were treated for 4 h with indicated concentrations of patulin. Data represent means of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk

3.1.9 Influence of GSH in comet assay

To further investigate the influence of GSH on DNA stability we used comet assay in BSO-pretreated cells. BSO treatment and patulin treatment at low concentrations (0.5 µM) induced an increasement of strand breaks of around 100%, whereas a higher damage was seen in cells treated with 5 µM patulin. Genotoxicity was increased in a synergistic manner when patulin 0.5 µM treated cells were preincubated with BSO. No difference was seen for patulin 5 µM treated cells with or without BSO pre-treatment (Figure 30).

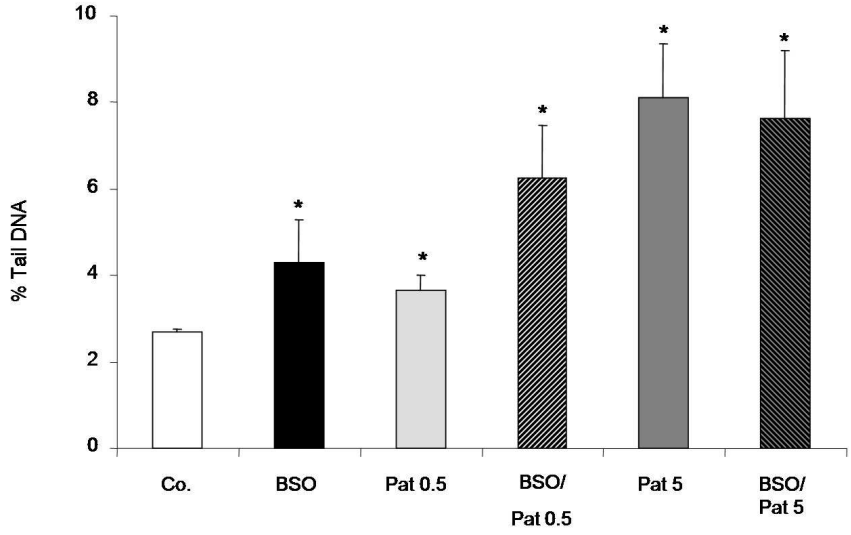


Figure 30: Measurement of DNA damage in Comet assay
V79 cells were pretreated with PBS buffer or 20 µM buthionine sulfoximine (BSO) for 20 h and then incubated with different concentrations of patulin (4 h treatment). Data represent means of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk.

3.1.10 Oxidative stress measurement after patulin treatment

We used DCF measurement to check if the patulin-induced damage in comet assay is also reflected in an increased oxidative stress after patulin treatment. In contrast to the results from comet assay patulin at a concentration of 0.5 µM patulin did not cause any effect, whereas the incubation with 5 µM doubled the level of oxidative stress in cells (Figure 31).

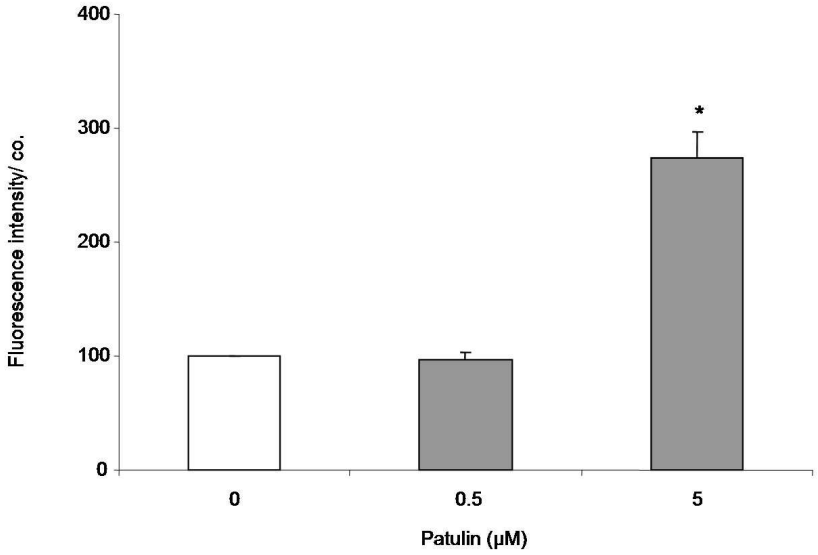


Figure 31: Measurement of oxidative stress
V79 cells were treated for 4 h with indicated concentrations of patulin. Data represent means of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk

3.1.11 Cellular GSH level after patulin treatment

We used cell cytometry to investigate the influence of patulin on cellular glutathione content after different incubation times. Figure 32 shows a dose-dependent decrease of glutathione level after 30 min incubation with patulin. However, after 24 h patulin led to a significant induction of cellular glutathione synthesis (Figure 33). Due to the high cytotoxicity of patulin and the decreased proliferation at a concentration of 50 µM, the glutathione level in these cells could not be evaluated after 24 h.

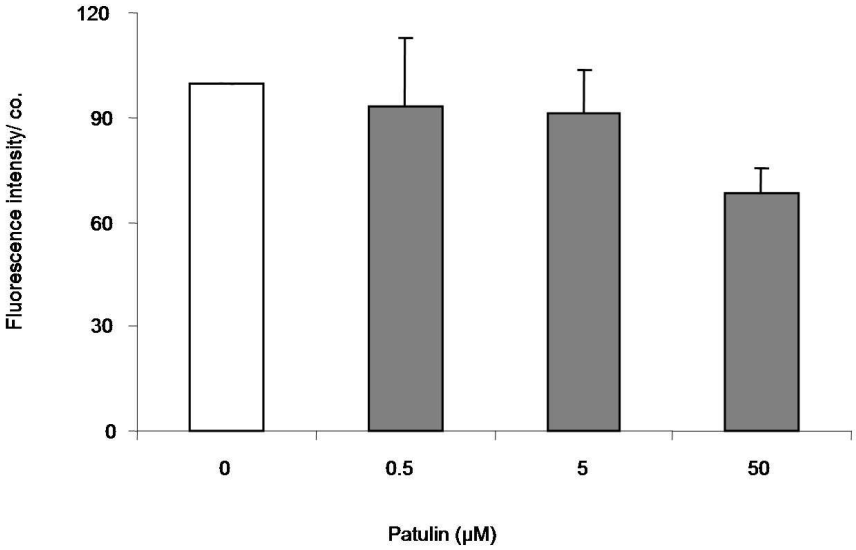


Figure 32: Cellular GSH level after 30 min incubation with the indicated concentrations of patulin. Analysis was done by flow cytometry using the dye monochlorobimane. Data represent means of three independent experiments + standard error of mean.

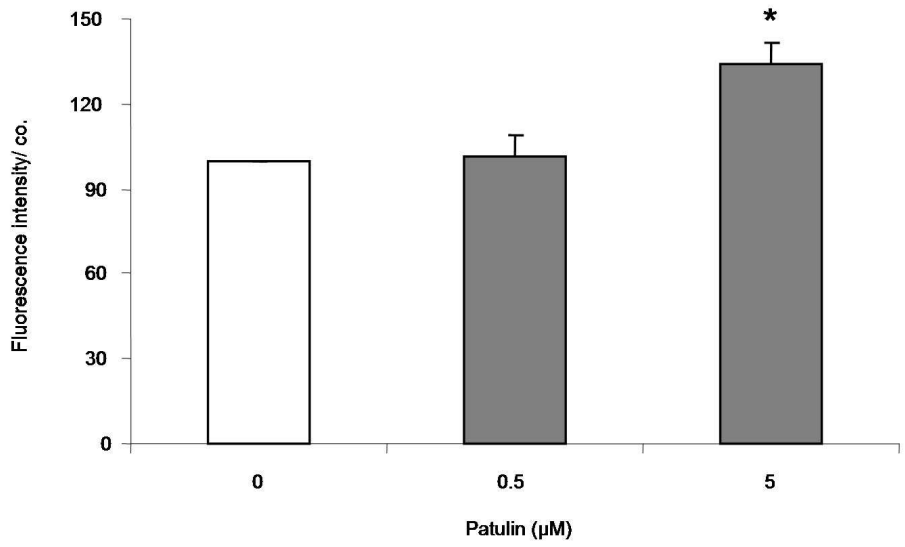


Figure 33: Cellular GSH level after 24 h incubation with the indicated concentrations of patulin. Analysis was done by flow cytometry using the dye monochlorobimane. Data represent means of four independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk.

3.1.12 Effects of resveratrol on patulin-induced micronucleus formation

We incubated V79 cells with 0.5 µM patulin and different concentrations of resveratrol to investigate a potential protective effect of the antioxidant resveratrol on patulin-induced genotoxicity. With 1 µM resveratrol no protective effect was observed whereas resveratrol in a concentration of 10 µM showed a small reduction of patulin-induced micronuclei formation. However, resveratrol in higher concentration led to a strong micronuclei formation itself and had no more protective effects on micronuclei induction by patulin. Proliferation of cells was reduced by patulin (0.5 µM) and resveratrol in higher concentrations (100 µM) as well (Figure 34).

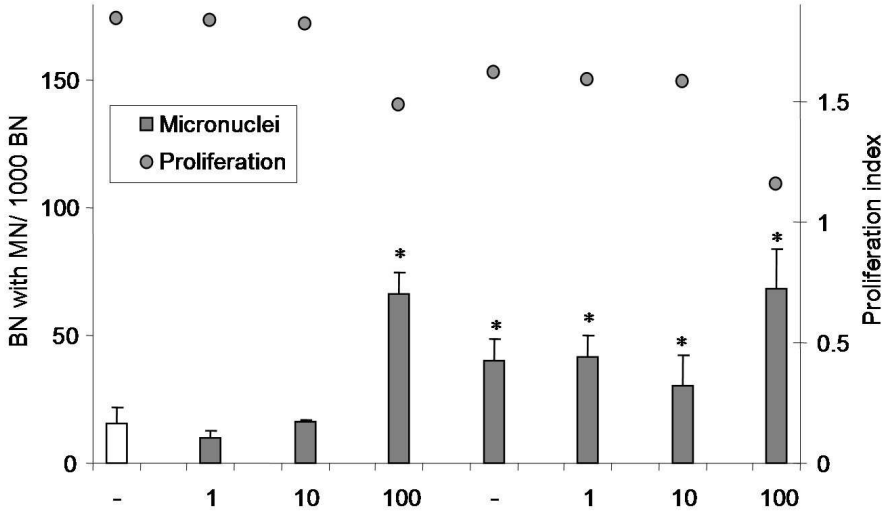


Figure 34: Effects of resveratrol on patulin-induced micronucleus (MN) formation.
V79 cells were incubated with the indicated concentrations of resveratrol (4.5 h), patulin (4 h) or combinations of both. After the treatment cells were incubated for further 24 h with cytochalasin (5 µg/mL). Micronulei frequency was evaluated in 1000 binucleated (BN) cells from each of two slides. Cytokinesis block proliferation index (CBPI) was calculated in 1000 cells per slide using the formula CBPI = [MI + 2MII + 3 (MIII +MIV)] with MI-MIV representing the number of cells with one to four nuclei. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk.

3.1.13 Kinetochore analysis of resveratrol-induced micronuclei

Treatment with higher concentrations led to an increased number of micronuclei after 20 h post-incubation. Kinetochore-analysis with a CREST serum and rhodamine labeled antibody proved that the resveratrol-induced micronuclei were apparently all

negative. Compared to the control level no kinetochore-positive micronuclei were induced (Figure 35).

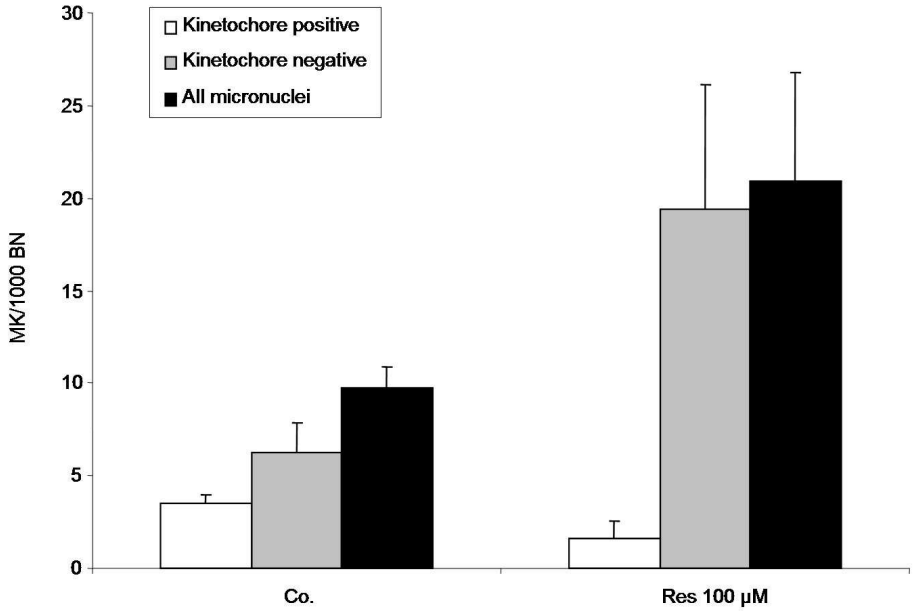


Figure 35: Kinetochore-staining after resveratrol treatment
V79 cells were incubated for 4 h with patulin or solvent, followed by 20 h substance free post-incubation. Data represents the number of kinetochore-positive/ -negative micronuclei per 1000 binucleated cells.

3.1.14 Viability after resveratrol treatment

Viability assay with resveratrol under the conditions of micronucleus assay (4 h incubation, 20 h post-incubations with cytochalasin B (5 µg/mL)) indicated a slight cytotoxic effect of resveratrol at higher concentrations (Figure 36).

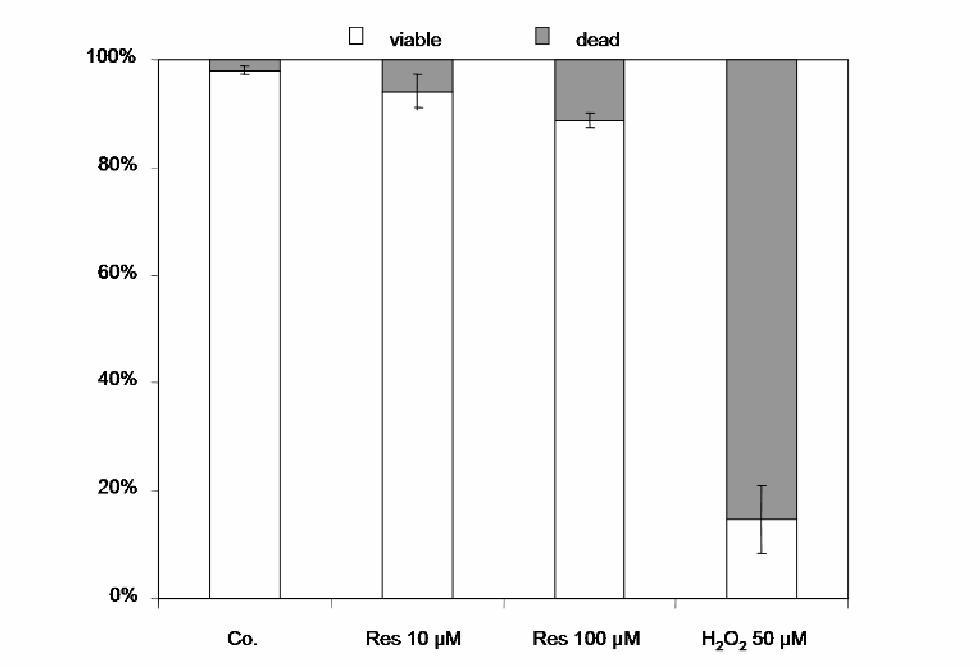


Figure 36: Viability assay with different concentrations of resveratrol
V79 cells were treated for 4 h with the indicated concentrations of resveratrol or H_2O_2 as a positive control for cytotoxicity, followed by 20 h post-incubation with cytochalasin B (5 µg/mL). Cells were stained with fluorescein diacetate (viable cells) and Gel Red (dead cells). Data represents the mean of two experiments + standard error of mean.

3.1.15 FRAP assay with resveratrol

We used FRAP assay to prove the the antioxidant properties of resveratrol, which are attributed to this polyphenol in literature. Figure 37 shows a dose-dependent increase of antioxidative capacity. Higher concentrations of resveratrol led to a saturation of absorbance (data not shown).

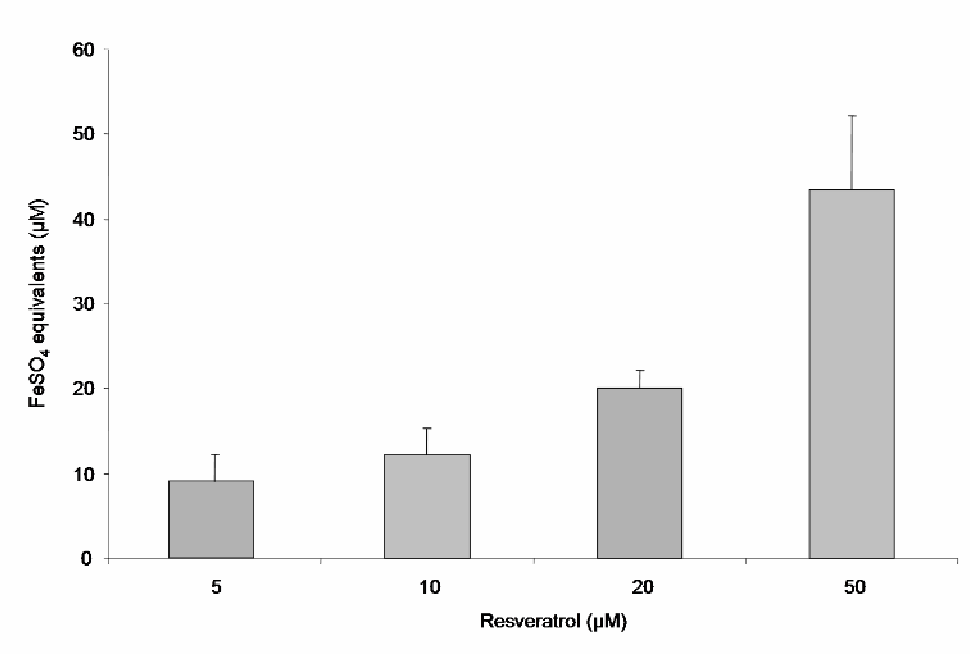


Figure 37: Measurement of antioxidative capacity of resveratrol with FRAP-Assay *(*ferric reducing ability of plasma). Indicated concentration were incubated for 6 min with FRAP-reagent and analyzed at 593 nm with a spectrophotometer. Data represents the mean of three experiments + standard error of mean

3.1.16 Oxidative stress after resveratrol treatment

DCF assay was used to investigate if the antioxidative properties shown in a cell free environment are also present on cellular level. Figure 38 shows that 24 h after treatment resveratrol did not exert any antioxidative properties but led to a slight increased oxidative stress in V79 cells as shown by an increased fluorescence of difluorescein diacetate.

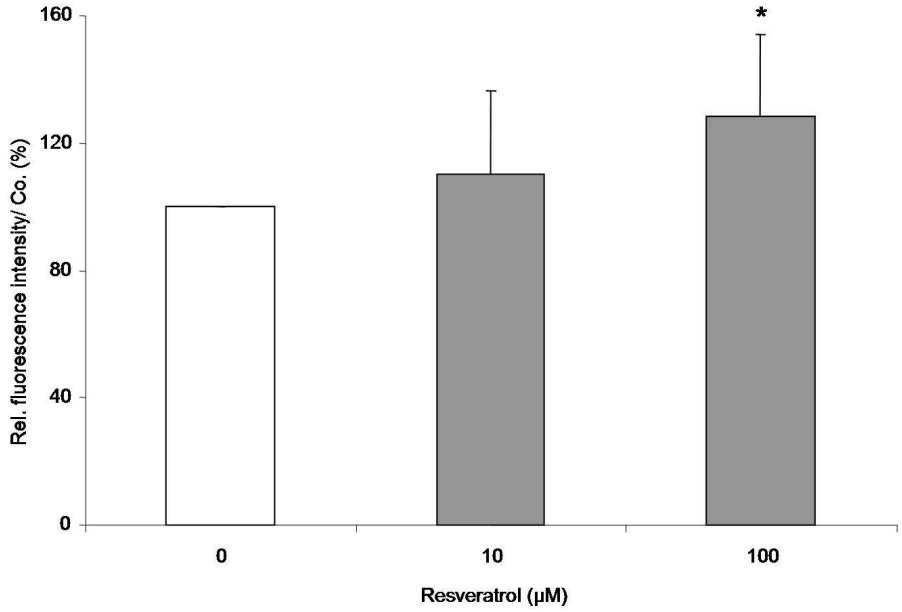


Figure 38: Measurement of oxidative stress
V79 cells were treated for 24 h with indicated concentrations of resveratrol. Analysis was done by flow cytometry using the dye 2',7'-dichlorodihydrofluorescein diacetate. Data represent means of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk.

3.1.17 Cellular GSH level after resveratrol treatment

To investigate a potential relationship of resveratrol-induced oxidative stress and cellular GSH level, we repeated the experiment under the same incubation conditions, followed by GSH measurement. Figure 39 shows that resveratrol-induced stress was accompanied by an increase of cellular GSH concentration. Thereby incubation of V79 cells with 100 µM caused almost a doubling of cellular GSH level.

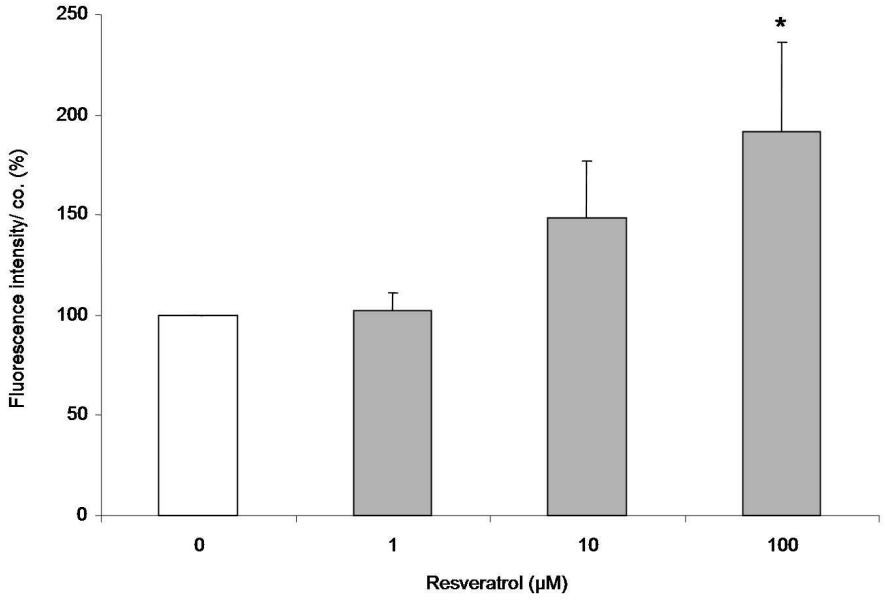


Figure 39: Cellular GSH level after 24 h incubation with resveratrol. Analysis was done by flow cytometry using the dye monochlorobimane. Data represent means of three independent experiments + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the control is shown by an asterisk.

3.2 PART II: EFFECTS OF AN ANTHOCYANINS RICH EXCTRACT ON HYPERTENSIVE RATS

The aim of this study was to investigate the potential protective effects of an anthocyanin-rich Dacapo grape extract in hypertensive Ren-2 rats as a model for oxidative stress *in vivo*.

3.2.1 General physical conditions

After two weeks small differences in the body weight were observable, with ramipril treated animals showing little higher values than the other two groups without medication (Figure 40). SD-rats of the same age showed a body weight of 318 ± 16 g (mean ± SEM). Ren-2 rats without medication also showed a continuous decrease in food intake over the study time, whereas the food consumption of ramipril treated rats was more stable (Figure 41). In contrast the untreated group and the anthocyanin group showed an elevated intake of water (Figure42).

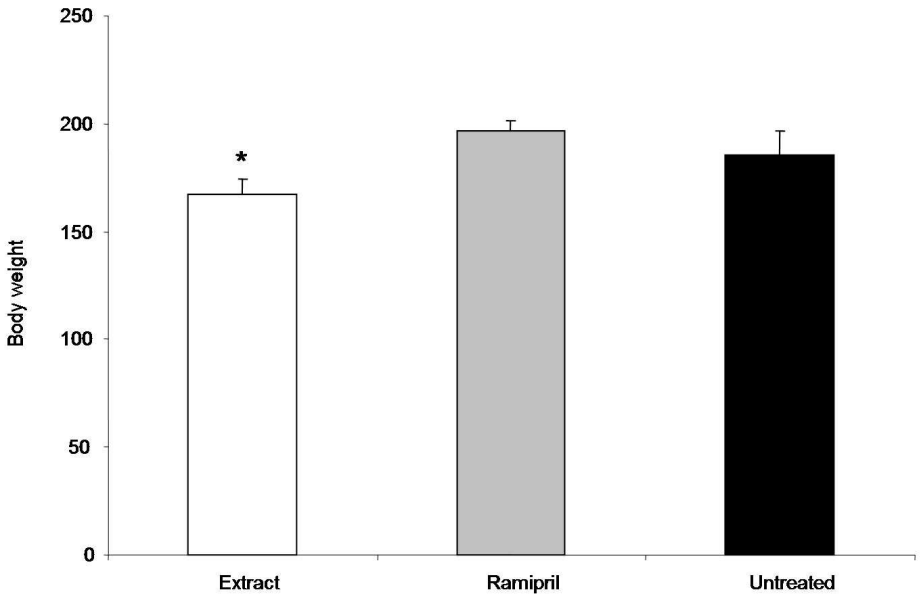


Figure 40: Body weight of Ren-2 rats after 2 week treatment with Dacapo grape extract, ramipril or without treatment. Data represents the mean bodyweight of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to the untreated group is shown by an asterisk.

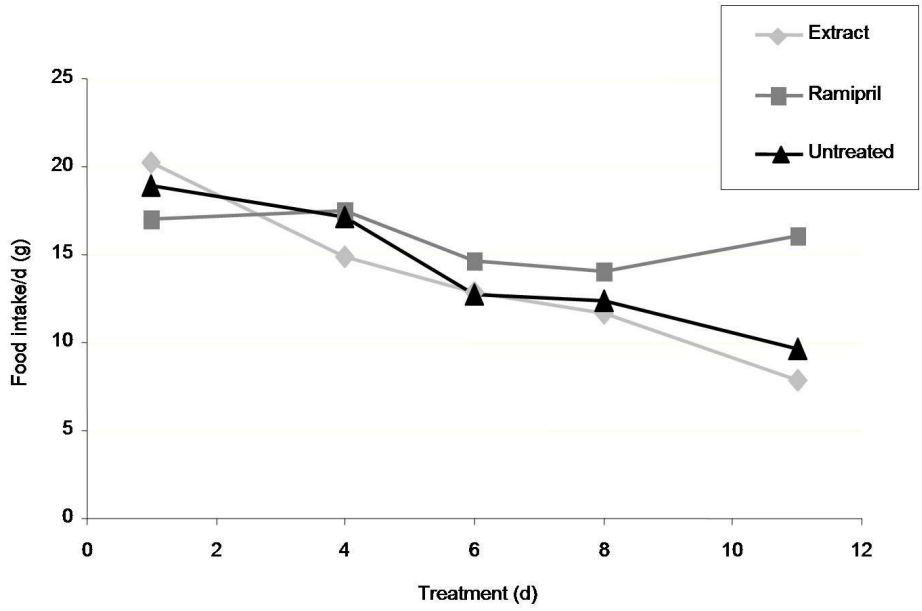


Figure 41: Daily food intake (g) of Ren-2 rats during 2 week treatment with Dacapo grape extract, ramipril or without treatment. Data represents the mean food intake of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats.

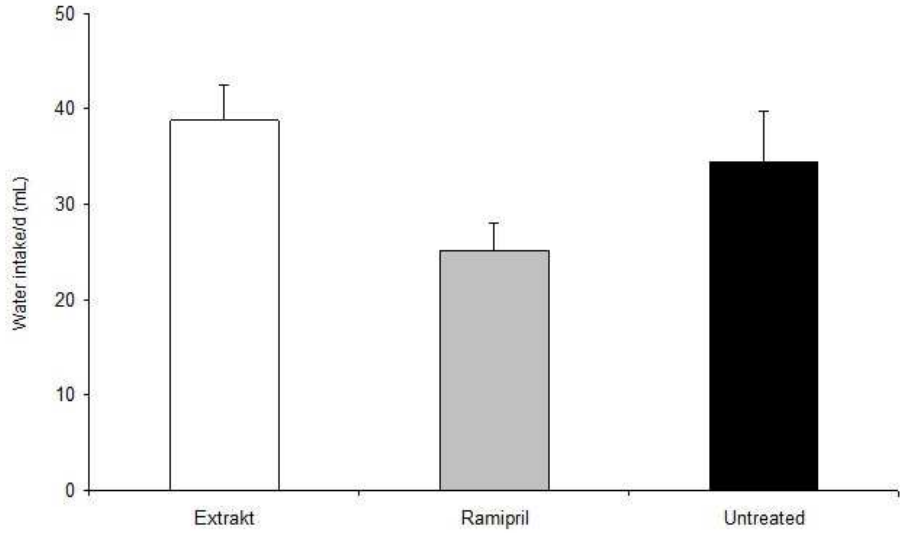


Figure 42: Water consumption of Ren-2 rats over 24 h after 2 week treatment with Dacapo grape extract, ramipril or without treatment. Individual water consumption was measured during 24 h metabolism cage housing. Data represents the mean water consumption of 6 extract treated rats, 6 ramipril treated rats and 6 untreated rats

Heart size of Ren-2 rats without medication was increased in comparison to the ramipril treated animals (Figure 43). The weight of kidneys was nearly equal for all three treatment groups (data not shown).

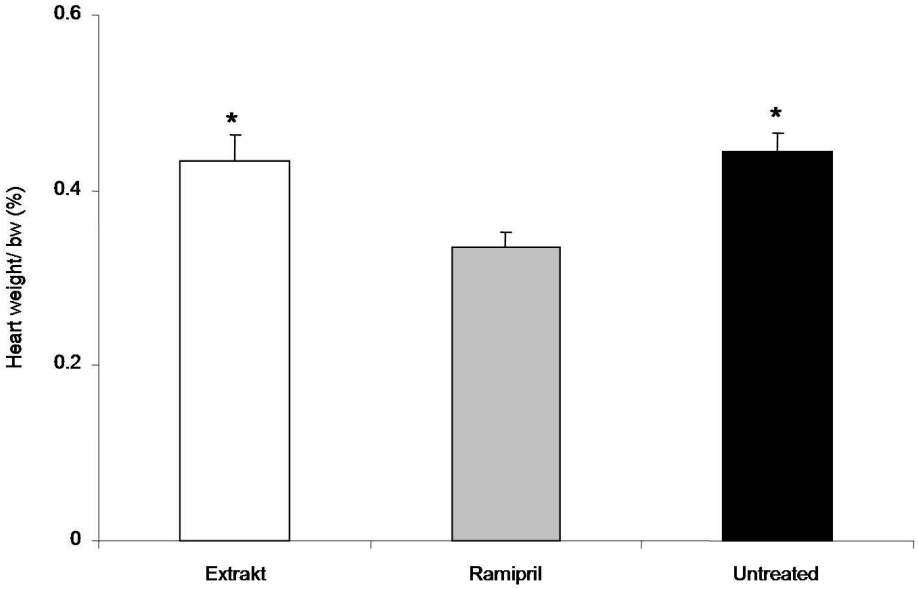


Figure 43: Heart weight in % of body weight of Ren-2 rats after 2 week treatment with Dacapo grape extract, ramipril or without treatment. Data represents the mean heart weight of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to ramipril treated animals is shown by an asterisk.

3.2.2 Blood pressure

Changes in blood pressure were tracked over the first week of the experiment (Figure 44). The stop of antihypertensive treatment in the untreated group induced a significant increase of systolic (Figure 45) and diastolic (Figure 46) blood pressure compared to the ramipril group, whose blood pressure kept nearly constant. The extract treated group showed also an elevation of blood pressure, which was however less prominent than in the untreated group.

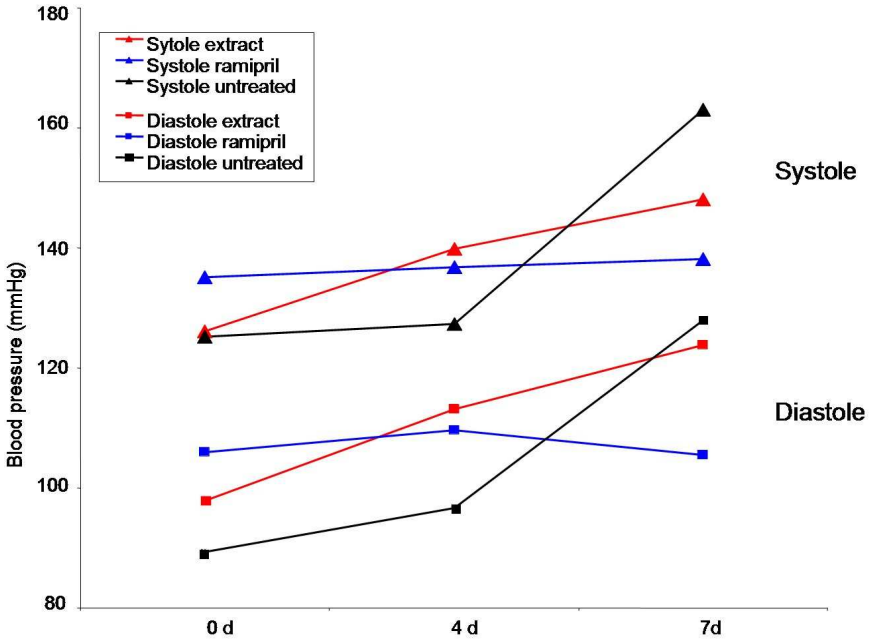


Figure 44: Change in systolic and diastolic blood pressure of Ren-2 rats over one week treatment with Dacapo grape extract, ramipril or without treatment. Data represents the mean systolic or diastolic blood pressure values of 6 extract treated rats, 6 ramipril treated rats and 6 untreated rats + standard error of mean.

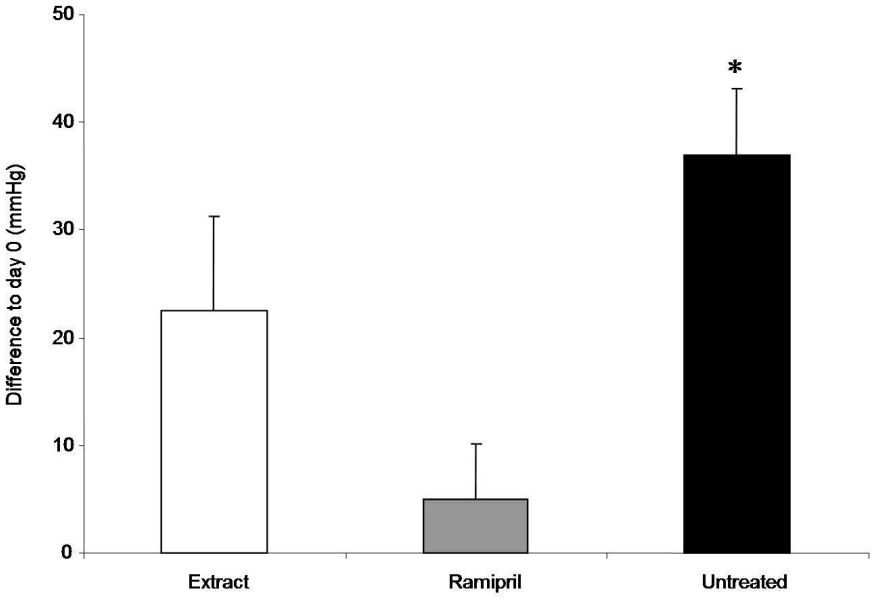


Figure 45: Change in systolic blood pressure of Ren-2 rats after one week treatment with Dacapo grape extract, ramipril or without treatment. Data represents the mean change of systolic blood pressure of 6 extract treated rats, 6 ramipril treated rats and 6 untreated rats + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to ramipril treated animals is shown by an asterisk.

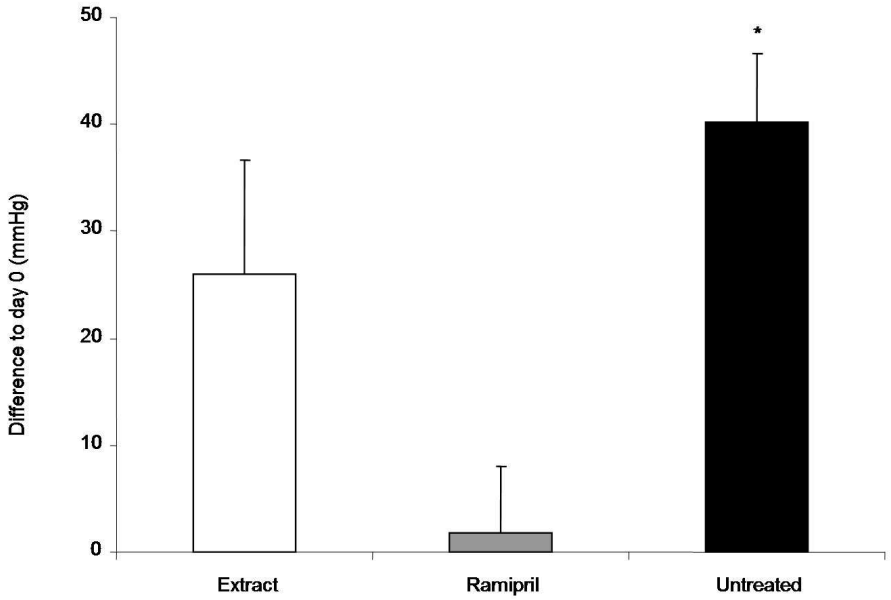


Figure 46: Change in diastolic blood pressure of Ren-2 rats after one week treatment with Dacapo grape extract, ramipril or without treatment. Data represents the mean change of systolic blood pressure of 6 extract treated rats, 6 ramipril treated rats and 6 untreated rats + standard error of mean. Significance ($p \leq 0.05$; Mann Whitney U-test) compared to ramipril treated animals is shown by an asterisk.

3.2.3 Comet assay

We used isolated cells from kidney and liver to investigate DNA damage in comet assay. The results from comet assay show a small variance of damage for kidney cells. However, there was a significant lower DNA damage in extract treated rats compared to the untreated group (Figure 47).

Similar results were obtained in the comet assay with liver cells, indicating a small reduction of DNA damage in the extract group compared to the other two groups.

MMS treated V79 cells were used as a positive control and proved the efficiency of the experimental conditions (data not shown).

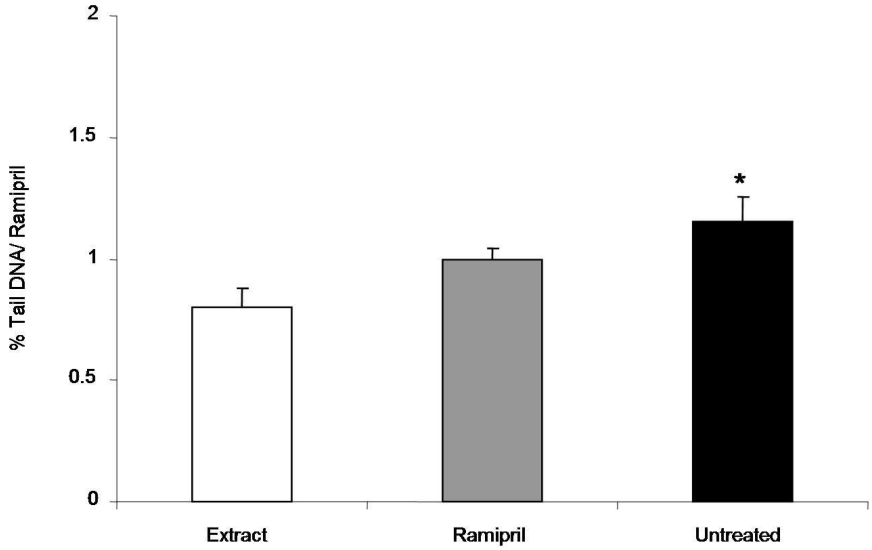


Figure 47: Comet assay with kidney cells. 50 cells from each of two slides per individuum were evaluated for the percentage of DNA in tail. Data is normalized to the mean of ramipril treated group and represents the mean damage of 6 extract treated rats, 6 ramipril treated rats and 6 untreated rats + standard error of mean Significance ($p \leq 0.05$; Mann Whitney U-test) compared to extract treated animals is shown by an asterisk.

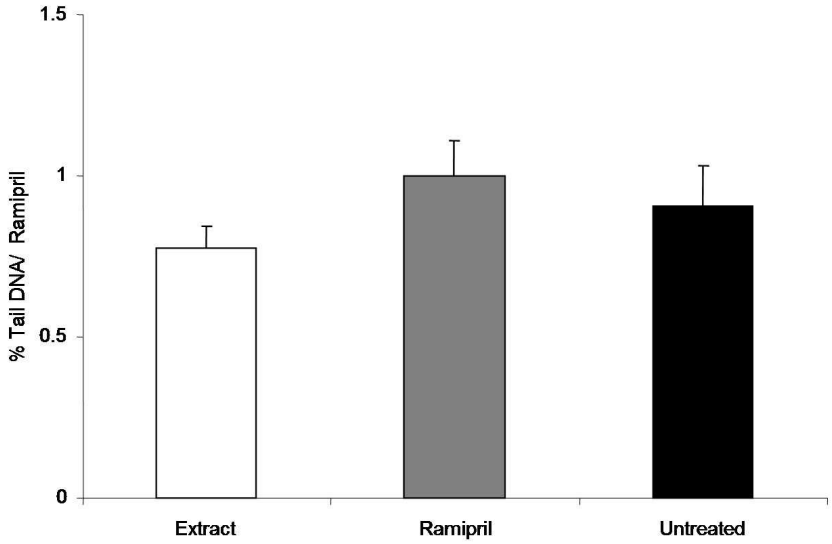


Figure 48: Comet assay with liver cells. 50 cells from each of two slides per individuum were evaluated for the percentage of DNA in tail. Data is normalized to the mean of ramipril treated group and represents the mean damage of 6 extract treated rats, 6 ramipril treated rats and 6 untreated rats + standard error of mean.

3.2.4 ɣ-H2AX-staining

ɣ-H2AX-staining was applied to investigate DNA damage in kidney, heart and small intestine of the experimental animals. To equalize the results from immunohistochemistry kidney was divided into different sections. In average extract treated animals show the lowest damage of all three groups. Standard error of mean was big between the individual animals from each group resulting from the high variability of damage between single regions of one section. Results from papilla show for the anthocyanin fed animals a half as large damage as for the other groups. Similar results were seen for medulla. In cortex the number of ɣ-H2AX positive cells in extract fed rats and ramipril treated rats was on an equal level, whereas untreated rats showed a twofold higher damage (Figure 49).

The result of ɣ-H2AX-staining in the heart indicate clearly a higher amount of double strand breaks in untreated rats compared to the extract group. The values for rats with ramipril medication range in the middle between the other two groups (Figure 50).

ɣ-H2AX-staining of small intestine showed an around threefold higher DNA damage in extract treated group compared to the ramipril medicated animals, whereas the number of ɣ-H2AX positive cells was only slightly increased in the untreated group (Figure 51).

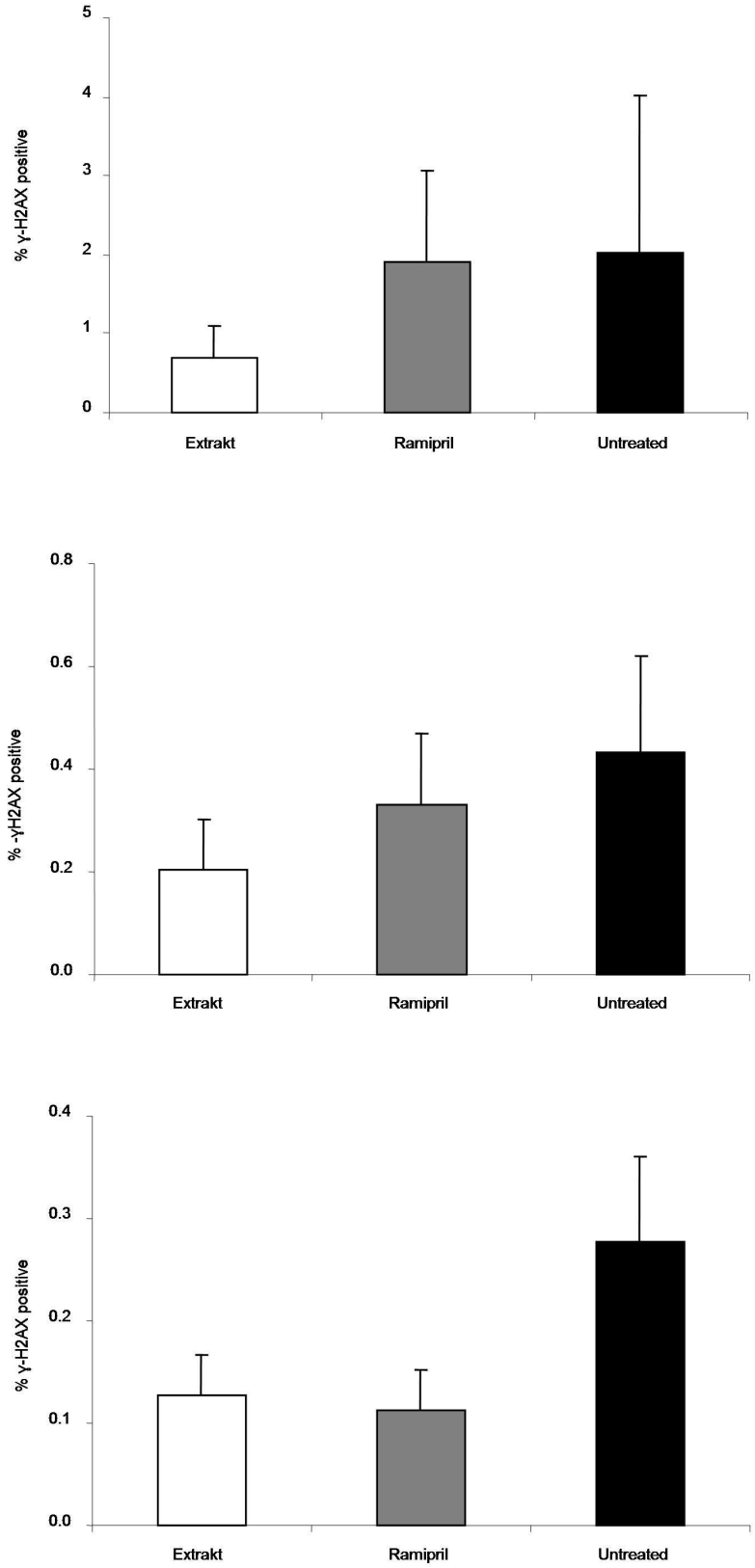


Figure 49: Percentage of ɣ-H2AX positive cells in papilla (upper figure), medulla (middle figure) and cortex (lower figure). Paraffin sections were stained with an antibody against ɣ-H2AX to check for double strand breaks. Data represents the mean of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean.

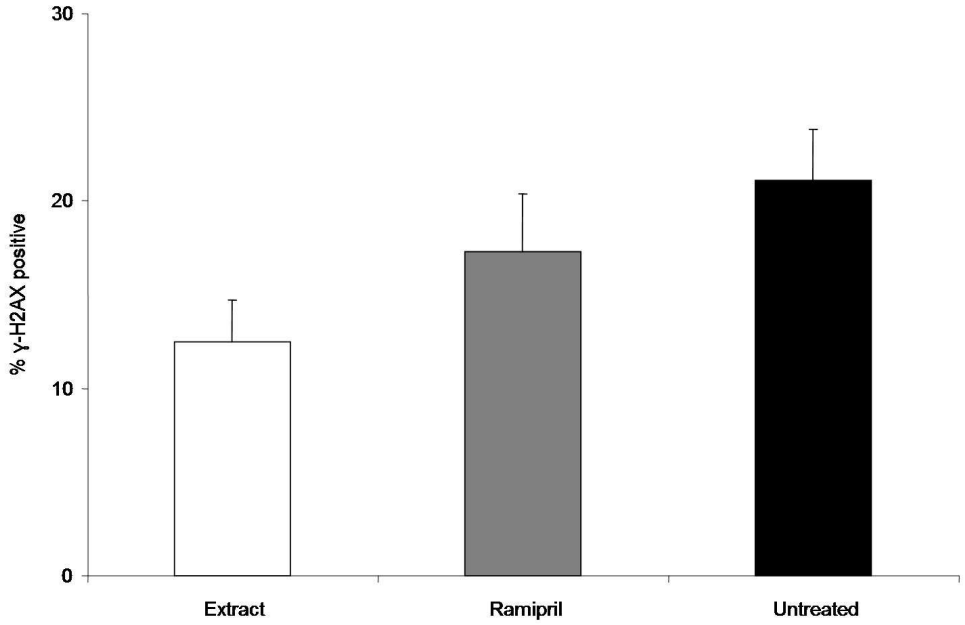


Figure 50: Percentage of ɣ-H2AX positive cells in heart. Paraffin sections were stained with an antibody against ɣ-H2AX to check for double strand breaks. Data represents the mean of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean.

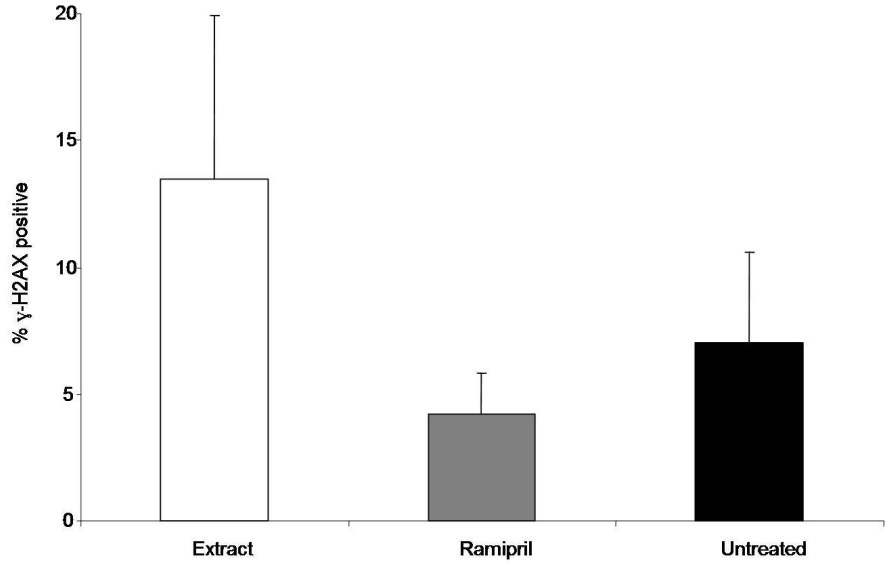


Figure 51: Percentage of ɣ-H2AX positive cells in small intestine. Paraffin sections were stained with an antibody against ɣ-H2AX to check for double strand breaks. Data represents the mean of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean.

3.2.5 DHE-staining

DHE-staining of kidney, heart and small intestine was used to investigate potential differences in the level of ROS in experimental animals. DHE-staining of tissues did not reveal significant differences between the different treatment groups. There was a slightly reduced level of oxidative stress in the group treated with the antioxidant grape extract.

In the renal cortex levels for ramipril medicated animals and extract treated animal were nearly equal and the fluorescence for untreated rats was slightly increased (Figure 52). In heart (Figure 53) and small intestine (Figure 54) levels for Ren-2 rats with ramipril medication and untreated rats were equal and just the group fed with extract showed a slightly reduced DHE fluorescence.

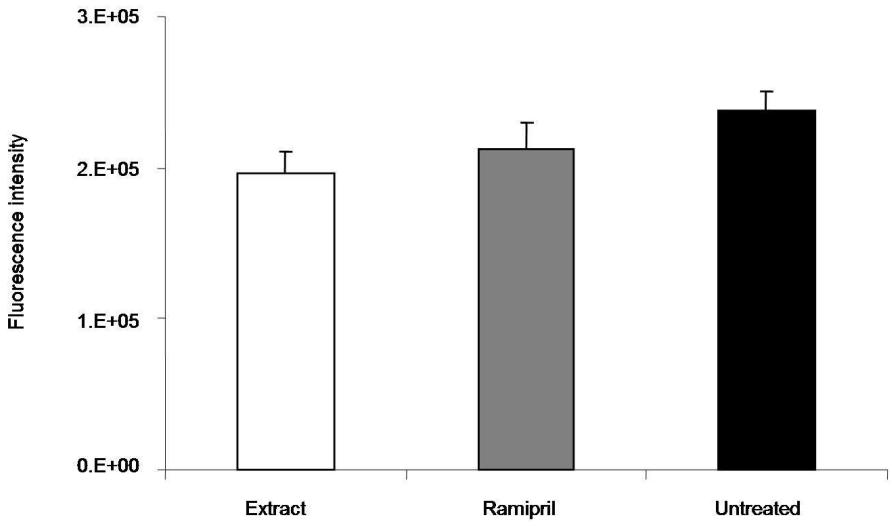


Figure 52: DHE-staining of kidney tissue. Frozen sections were stained with DHE to evaluate the cellular level of ROS. Data represents the mean of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean.

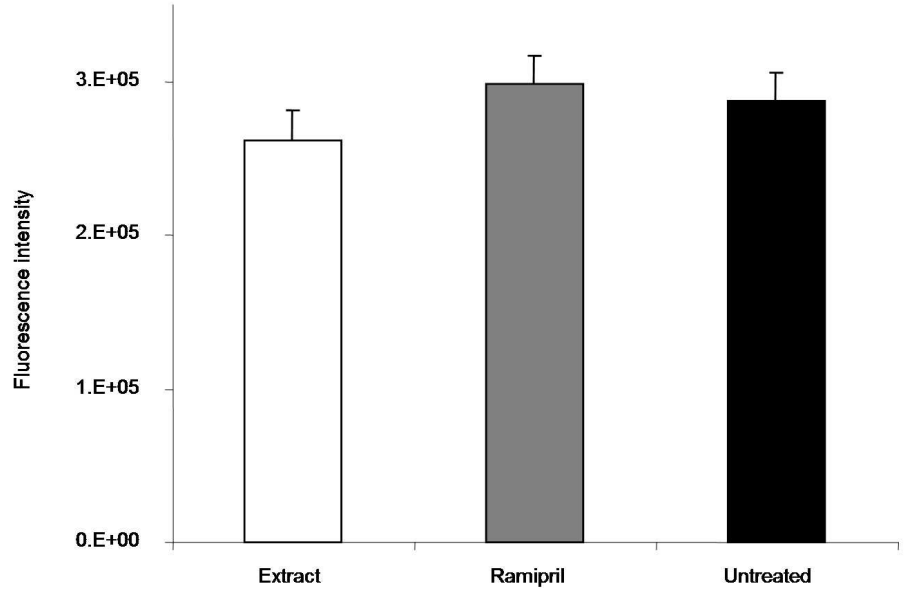


Figure 53: DHE-staining of heart tissue. Frozen sections were stained with DHE to evaluate the cellular level of ROS. Data represents the mean of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean.

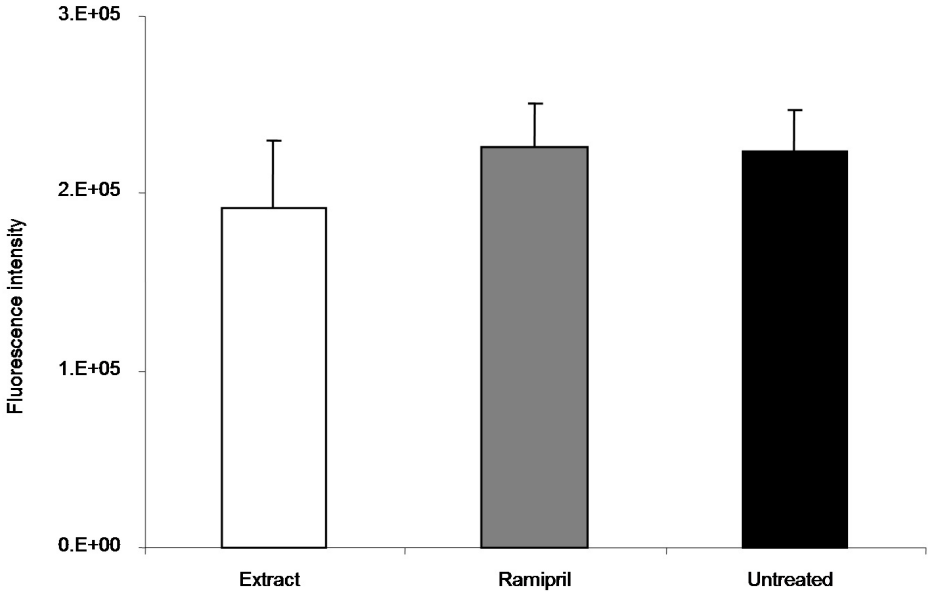


Figure 54: DHE-staining of tissue from small intestine. Frozen sections were stained with DHE to evaluate the cellular level ROS. Data represents the mean of 6 extract treated rats, 6 ramipril treated rats and 7 untreated rats + standard error of mean.

3.2.6 Frap extract

We used FRAP assay to investigate the antioxidative properties of anthocyanin-rich Dacapo grape extract *in vitro.* Figure 55 shows a dose-dependent increase of antioxidative capacity with increasing concentration of extract.

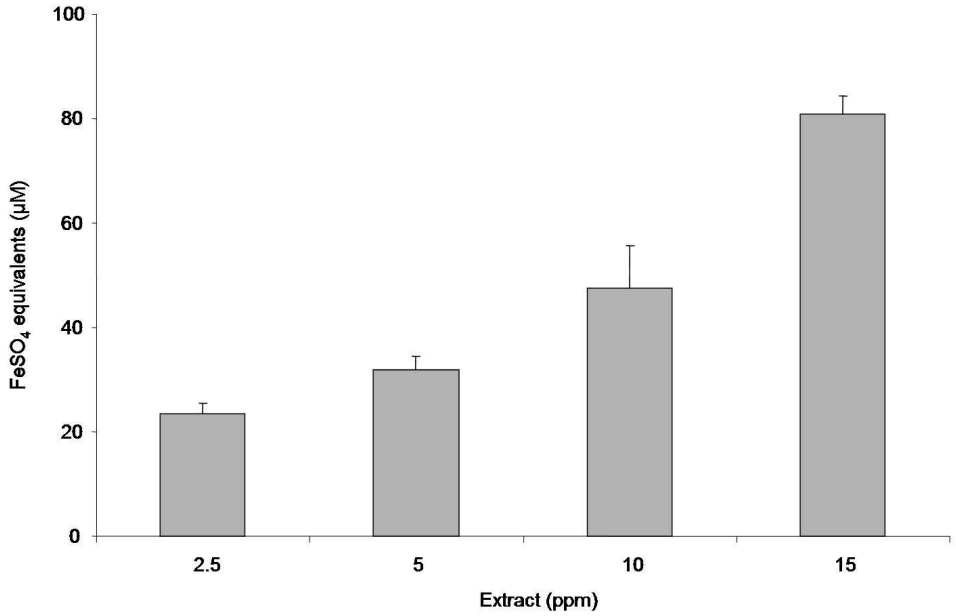


Figure 55: Measurement of antioxidative capacity of Dacapo grape extract with FRAP-assay. Indicated concentration were incubated for 6 min with FRAP-reagent and analyzed at 593 nm with a spectrophotometer. Data represents the mean of three experiments + standard error of mean

3.2.7 Plasma antioxidant capacity assessed by the FRAP assay

We applied FRAP assay also to investigate if the antioxidative properties seen *in vitro* were also reflected in an increased antioxidative capacity in serum. However, no increased ferric reducing ability was detected in extract fed animals of our study (Figure 56).

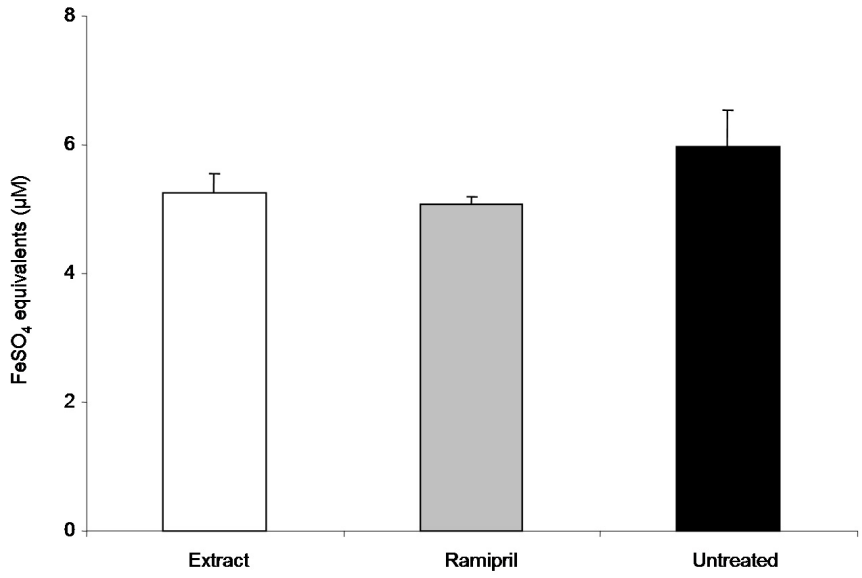


Figure 56: Measurement of antioxidative capacity of serum with FRAP assay. Serum from each animal was incubated for 6 min with FRAP reagent and analyzed at 593 nm with a spectrophotometer. Data represents the mean FRAP value from animals of each group (extract: n=4, ramipril: n=2, untreated: n=6).

4 DISCUSSION

4.1 PART I: INFLUENCE OF PATULIN AND RESVERATROL ON DNA STABILITY

The mycotoxins patulin is a well-known mutagenic substance, which is frequently found in spoilt fruits and related products. However, the genotoxic effects of patulin are not yet completely clarified. The aim of the first part of this study was therefore to investigate further steps of patulin-induced genotoxicity.

The micronucleus assay revealed a dose-dependent induction of micronuclei. However, at high doses the number of micronuclei decreased, indicating a reduced cell proliferation. This was also reflected in viability assay, showing a dose-dependent increase of dead cells after patulin treatment. Therefore, the following micronucleus experiments were carried out with cytochalasin B to ensure that all evaluated cells have passed mitosis since the treatment.

After staining centromeres with an TRITC labeled antibody, kinetochore-positive and –negative micronuclei were present, which is in agreement with the results of Pfeiffer et al. [58]. Microscopic evaluation revealed a striking number of nucleoplasmic bridges, which were formed directly after patulin treatment. Nucleoplasmic bridges are generally explained by the disturbed distribution of dicentric chromosomes during mitosis. Dicentric chromosomes which are pulled to opposite poles during mitosis lead to the formation of anaphase bridges, which in the absence of rupture form nucleoplasmic bridges. However, for the formation of dicentric chromosomes, a breakage and reunion event is needed [192]. This cannot be passed off within 3 h, the shortest time after which nucleoplasmic bridges were observed in our study. Furthermore, the mechanism of breakage-fusion-bridge-cycles is thought to include the formation of micronuclei accompanying the generation of nucleoplasmic bridges [192]. Such simultaneous appearance of micronuclei and nucleoplasmic bridges was rarely seen during microscopic evaluation of patulin-treated cells in our experiments.

Therefore, another mechanism must be responsible for the formation of nucleoplasmic bridges by patulin. We hypothesized that cross-linking of sister-chromatids by patulin provides an explanation [183]. If sister chromatids cannot

separate, but are pulled to the two opposite spindle poles, the chromatin must either rupture or a bridge is formed. The cross-linking ability of patulin was proved in a modified version of comet assay [183] and is in agreement with previous publications. Fliege and Metzler reported that patulin causes protein-protein cross-links. Patulin was not only able to react with the thiol group of cysteine but also with side chains of lysine, histidine and α-aminogroups. After the first Michael-like addition the resulting primary monoadduct was shown to be even more reactive with further nucleophiles [193]. Later, the same working group has shown as well that the treatment of V79 cells with patulin lead to irreversible DNA-DNA cross-links [194]. Moreover, analysis of the yeast transcriptome upon challenge with patulin has revealed the induction of genes involved in repair of alkylation damage among others [195]. An increased cellular DNA repair was also reported by Lee and Roschenthaler [57]. In case of unsuccessful DNA repair a later rupture of bridges may lead to the formation of chromatin fragments, which are then enclosed into micronuclei [196]. This might particularly be the case if cytokinesis is not impeded by cytochalasin B and could explain why substance-free post-incubation of cells leads to higher numbers of micronuclei compared to experiments with cytochalasin B treatment after patulin incubation.

This hypothesis of action by patulin through cross-linking would also be in agreement with our observations made after GSH depletion with the synthesis inhibitor BSO, which significantly increased the formation of micronuclei and nucleoplasmic bridges in cells incubated with patulin [183]. GSH has been supposed to protect cells by inactivation of substances through direct binding, increased metabolism and detoxification of free radicals. The protective effect of cellular GSH against cross-linking agents has been described in several studies. It was suggested that GSH either inhibits the reaction of cross-linking agents with DNA or prevents the monoadducts from rearranging to bifunctional adducts [197]. Cells exposed to BSO before drug treatment showed a significant increase of DNA interstrand cross-links [198]. The high susceptibility of V79 cells to patulin might also be based on their relative low level of GSH (11.3 nmol/mg cellular protein) [58]. Cells with an high GSH level in comparison to V79 such as HepG2 [199] showed a lower level of micronucleus and nucleoplasmic bridge formation upon patulin treatment. Under the given incubation conditions patulin led to a 1.3 times higher number of micronuclei

and 2.7 times higher number of NPB in HepG2, but to an elevation of 2.3 times more micronuclei and 9.8 times more NPB in V79 (unpublished data).

One of the molecules which might be affected by the high reactivity of patulin is tubulin, as an important constituent of the mitotic apparatus. Patulin impeded cell-free microtubule polymerization at higher concentrations (50-200 µM) [58]. It is known that accessible sulfhydryl groups are essential for polymerization of microtubuli subunits [200]. In cultured hepatoma cells patulin treatment (30 µM) led to disorganization of the cytoplasmatic microfilaments similar to effects caused by colchicin [201]. Anaphase bridges [58] and entangled chromatids [76] indicate a potential mitotic disturbance by patulin.

Therefore, we applied staining of α-tubulin to investigate the effects of patulin on mitotic spindles. Cells were exposed to patulin under similar conditions as in micronucleus assay. The formation of fibers from the tubulin subunits did not seem to be affected at the concentration used in genotoxicity assays, but cytoskeleton was compromised at higher patulin concentrations. However, the number of multipolar mitoses was strikingly elevated after patulin treatment. Staining of ɣ-tubulin was carried out to show that supernumerary spindles were connected with centrosome amplification [183].

Centrosomes consist during G1 phase of two centrioles which are supplemented during S phase by two procentrioles. In late G2 phase, the two centrosomes each containing a parental and a daughter centriole separate to the two poles of the mitotic spindle [202].

Centrosome amplification can be a passive consequence of an elongated cell cycle [204]. The generation of supernumerary centrosomes has been described for an extended S-phase [205, 206] as well as for an elongated G2-phase [204]. We detected a patulin-induced G2 arrest after 6 h, which is an accordance with the results of Pfeiffer et al. [58] and Schumacher et al. [76], who describe a mitotic and/or G2 arrest for patulin-treated V79 cells. Thus, patulin might lead to centrosome amplification via DNA damage-related cell cycle arrest. Patulin was also reported to repress RAD51 expression in BY4743 yeast cells [207]. Deficiency of RAD51 is known to lead to the formation of supernumerary centrosomes, possibly by the reduced ability of RAD51 deficient cells to repair DNA damage and thereby suffering from an arrest in G2 phase [204].

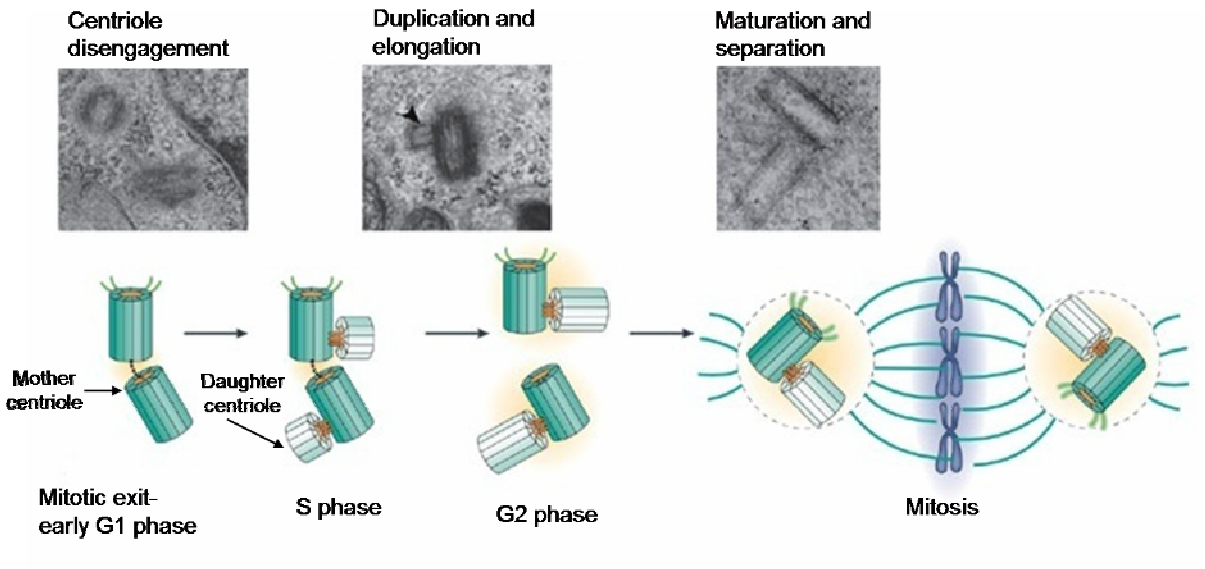


Figure 57: The centrosome cycle
Schematic illustration of the main phases of the centrosome cycle (centriole disengagement, centriole duplication and elongation, centriole maturation and centrosome separation). A pair of mother centrioles is supplemented during S phase by two daughter centrioles. After elongation of daughter centrioles centrosomes split in G2 phase and migrate to the opposite spindle poles. Modified after Bettencourt-Dias and Glover [203].

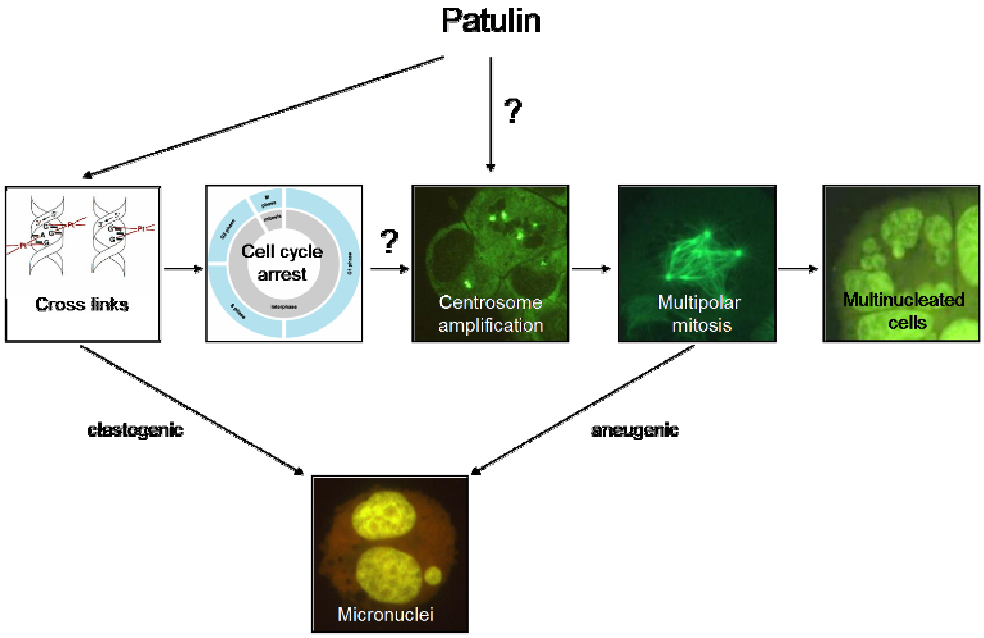


Figure 58: Potential pathway of patulin-induced DNA damage
Patulin-induced centrosome amplification might be caused by a direct interaction of patulin with centrosome proteins or by a patulin-induced DNA damage resulting in cell cycle arrest and therefore desynchronisation of DNA synthesis and centrosome doubling

As another possible pathway for centromsome amplification, cells lacking the tumor suppressor p53 overduplicate centrosomes, if they are arrested in S phase with

substances such as hydroxyurea or aphidicolin [205, 208]. The absence of functional p53 in V79 cells [209] might therefore contribute to the patulin-induced formation of supernumerary centrosomes.

Induction of p53 by patulin was reported in various species and cell lines with functional p53 [71, 80, 210] and might indicate a p53 mediated cell cycle arrest or apoptosis as a response to patulin-induced DNA damage. We used comet assay to evaluate a potential dose response relationship for patulin. The induction of strand breaks at low concentrations supports the finding of clastogenic effects seen in kinetochore-staining. This is in accordance with the results of previous studies which also described the strand breaking properties of patulin [77, 87, 211, 212]

The pre-treatment of cells with BSO increased DNA damage of lower doses patulin whereas there was no difference with 5 µM patulin for BSO and solvent pretreated cells. This indicates that the cellular GSH can detoxify parts of the dose at lower patulin concentrations, whereas the protecting impact of natural GSH in cells was negligible at higher levels of patulin. The protective effects of cellular GSH against patulin-induced damage were also described in some other studies [76, 86, 87, 210]. Zhou et al. attribute the protective effects of GSH on oxidative stress-induced by patulin. Indeed a few studies report the ability of patulin to induce oxidative stress. Measurement of ROS with DCF assay showed in a few studies an induction of oxidative stress after patulin treatment [35, 80, 210, 213, 214].

The incubation of cells with patulin leads to an amplified TBARS (thiobarbituric acid reactive substances) formation indicating an increase in lipid peroxidation [210, 213-215]. The raise of ROS also led to oxidative DNA damage, as shown in FPG (formamidopyrimidine DNA glycosylase) comet and 8-oxo-dG measurement [82, 214].

However, almost all the above mentioned studies were accomplished with concentrations in a range from 5 – 100 µM patulin. A publication of Schumacher et al. does not show any clastogenicity or induction of FPG-labil sites at non-cytotoxic concentrations [194].

We applied DCF assay to investigate the potential prooxidative effects of patulin in low doses under our experimental conditions. No effects were seen with 0.5 µM, the concentration generally used in genotoxicity assays of our study.

The impact of cellular GSH on patulin-induced genotoxicity has been reported in several studies. Depletion of GSH led to elevated number of micronuclei [87],

increased damage in comet assay [210], cell cycle delay, enhanced mutant number and a lower viability [76]. Therefore we investigated the effects of patulin on GSH homeostasis of V79 cells. We saw a slight reduction of cellular GSH level after incubation of cells with high doses of patulin (50 µM) for 30 min and a later increase of GSH concentrations. This is in accordance with the results of Schumacher et al., who also detected 24 h after patulin treatment a dose-dependent increase in GSH level [76]. Other studies – most of them with a short incubation time - report a depletion of GSH in association with patulin treatment [55, 210, 214-217].

The authors mostly suppose a potential consumption of cellular GSH by patulin-induced free radicals, whereas Schumacher et al. suppose a loss of GSH due to a decrease in structural integrity after patulin treatment, referring to the discrepancy between the reported effects and ratio of patulin and cellular GSH level [76].

However, it seems to be reasonable that depletion of GSH is a short time effect, whereas after 24 h the level increases due to an enhanced biosynthesis of GSH as an adaptive response.

We hypothize that cellular levels of reduced GSH are not mainly decreased by an oxidation to GSSG but by a direct reaction of GSH with patulin or a loss of GSH due to patulin-induced membrane permeability. This would also explain, why de Melo et al. found a depletion of cellular GSH but no increased level of GSSG after patulin treatment, how one would expect after a ROS-induced oxidation of GSH [55].

Therefore, we assume, that patulin has no prooxidative effects at the low genotoxic concentrations applied in our study. The impact of glutathione against patulin-induced damage might be explained by a direct reaction of glutathione with patulin leading to the formation of less genotoxic products.

As mechanism for cytogenetic damage induced by patulin in V79 cells, we suggest that cross-linked sister chromatids do not segregate well during mitosis and are pulled to the opposite poles forming an anaphase bridge, which converts to a nucleoplasmic bridge during cytokinesis. DNA-damage-induced cell cycle disturbances may then lead to centrosome amplifications, which cause multipolar spindles. The kinetochore-negative micronuclei might be generated by rupture of bridges or during repair and replication processes of cross-linked DNA, whereas the kinetochore-positive micronuclei are likely the result of the mitotic disturbances. Whether this mechanism occurs *in vivo* after consumption of patulin contaminated

food products, for example in individuals with reduced glutathione levels caused by inflammation, hypoxia, or enzyme polymorphisms remains to be determined.

A few studies describe the protective effects of antioxidants on patulin-induced genotoxicity and oxidative stress [80, 81, 214]. These studies attribute the protective properties of the employed substances mostly to the scavenging of free radicals induced by the potential prooxidant patulin. Therefore we used resveratrol to investigate if this antioxidant substance can also reduce the genotoxic effects of patulin in lower, non-prooxidative concentrations.

Small reductions of micronucleus frequencies were seen by co-incubation with 10 µM resveratrol. There are several possible mechanisms explaining the protecting effects of resveratrol at this concentration. Assuming that patulin is not prooxidative at a concentration of 0.5 µM the direct antioxidative properties of resveratrol as a radical scavenger [89] might be non-relevant however resveratrol might increase detoxification of patulin by inducing enzymes of glutathione system such as glutamate cysteine ligase [218], glutathione peroxidase [219] or glutathione S-transferase [220]. Resveratrol was also described to decrease markedly the oxidation of thiol groups after incubation with cis-platin [221]. Similar mechanism might influence the high reactivity of patulin towards thiol groups. Resveratrol-induced phase II enzyme activity [222] could accelerate the metabolism of xenobiotics such as patulin.

Resveratrol affects many aspects of DNA metabolism such as replication, recombination, repair, relaxation and telomere maintenance. Resveratrol, though itself not intercalating with DNA, has been shown to revert DNA intercalation by stabilization of helical structure and protect DNA therefore against mutagenic substances such as patulin [89]. By a putative activation of sirtuin 1 resveratrol might accelerate proteins involved in DNA repair such as p53, KU70, NF-κB and FOXO proteins. [89]. Resveratrol might counteract the patulin-induced RAD51 suppression [207] by its RAD51 upregulating properties [223]. Resveratrol-induced cell cycle arrest [224-226] and apoptosis [89] prevents additionally the proliferation of cells with genomic damage.

However, higher concentrations of resveratrol-induced themselves the formation of micronuclei and proliferation inhibition in our experiments. The role of resveratrol on genomic damage is controversially discussed. Several publications describe a protective effect of resveratrol [222, 227-229]. In contrast there are also many publications reporting a resveratrol-induced DNA damage. Resveratrol mediated strand scissions were strongly dependent on the presence of copper [230-236]. A ternary complex between resveratrol, copper (II) and DNA has been proposed to be responsible for DNA cleavage [237].

Matsuoka et al. described a resveratrol-induced increase in sister chromatid exchange and micronuclei [226]. Due to its structural similarity to the synthetic estrogen diethylstilbestrol resveratrol was suspected to share its aneugenic properties. However, Matsuoka et al. report only a very weak increase of numerical chromosome aberrations in Chinese Hamster lung (CHL) cell line [226]. Mitotic chromosome displacement was described in L5178Y mouse lymphoma cells but not in V79 cells [224]. This is in accordance with our results from kinetochore analysis and the data from Schmitt et al. [224] showing only an elevated level of kinetochore-negative micronuclei but no kinetochore-positive micronuclei in V79 cells.

Incubation of V79 cells with 100 µM resveratrol led to a marked reduction of viable cells. This is in agreement with the results of Matsuoka et al. who also reported a dose-dependent decrease of viability in CHL cell line after incubation with low concentrations of resveratrol [226].

Despite its antioxidative properties proved in FRAP assay, measurement of DCF fluorescence showed the induction of oxidative stress in a cellular system. As every antioxidant has in fact redox properties prooxidative effects have been described for several classes of plant-derived polyphenols [237]. These antioxidants can turn into prooxidants via interaction with transition metal ions. The prooxidative properties of resveratrol have been investigated by de la Lastra et al. and Heiss et al. [237, 238]. Under certain conditions even physiological concentrations of resveratrol (100 pM – 100 nM) can result in oxidative stress [239]. Incubation of V79 cells with resveratrol for 24 h led to a dose-dependent increase of cellular GSH. This is in agreement with the results of other studies [218, 222, 240, 241] and might be explained by an activation of Nrf2 [240]. Nrf2 can be induced by oxidative stress [242]. This raises the general question whether disturbance of glutathione homeostasis results from to oxidative stress or leads to oxidative stress [243].

We proved in our study a slight protective effect of resveratrol on patulin-induced DNA damage. However, higher concentrations of resveratrol showed genotoxic and prooxidative effects themselves. Although it is difficult to compare directly, the effective concentrations used in our study (10/ 100 µM ≈ 2.3/ 23 mg/L) correspond to the higher concentrations of resveratrol in red wine (1-18 mg/L) [90] and may potentially exert deleterious effects in gastro intestinal tract, which is exposed to high concentrations of resveratrol after oral ingestion. Due to the low bioavailability only concentrations in the lower nano- and micromolar range of unmodified resveratrol are reached in plasma. However, concentrations of metabolites can be > 10 times higher and potentially take over the effects attributed to resveratrol.

In this context the intake of resveratrol in high doses e.g. as food supplement should be assessed very carefully, since the potential prooxidative and genotoxic properties, of resveratrol have not been fully elucidated yet.

4.2 PART II: EFFECTS OF AN ANTHOCYANINS RICH EXCTRACT ON HYPERTENSIVE RATS

Epidemiological data as well as *in vivo* and *in vitro* studies indicate that diets rich in fruits and vegetables may exert protective effects against the development of cancer and cardiovascular diseases [244, 245]. These protective effects have often been attributed to antioxidative compounds of vegetables. Anthocyanins are well-known antioxidants, but there is a controversial discussion about their benefit on health aspects. Therefore we tried to investigate in this part of the study the effects of an anthocyanin-rich diet on hypertension, oxidative stress and DNA damage in Ren-2 rats.

The planned period for the treatment and blood pressure measurement was four weeks, but the experiment was stopped ahead of schedule after aggravation of health status and premature decease of four animals. A higher mortality in Ren-2 rats was also described by Langheinrich et al. [173] and Pinto et al. [177]. Homozygous Ren-2 rats developed excessive hypertension and died from cardiovascular complications such as heart and kidney failure or stroke, if they were not medicated with an ACE inhibitor [177]. Animals show functional and biochemical markers of a cardiac insufficiency and cardiovascular hypertrophy in their study. Inhibition of the renin-angiotensin system by ACE inhibitors and AT_1 receptor antagonists effectively lowered blood pressure, attenuated the development of cardiac hypertrophy and improved endothelium dependent relaxation [166].

Body weight of Ren-2 rats in our study was in general low and values were reduced in comparison to non-transgenic age-matched littermates. This is contradictory to the data observed in other studies [166, 246]. Discrepancies might reflect the different genetical background of Ren-2 strains or the effects of longtime inbreeding.

Small differences in body weight were observed after two weeks of treatment. The slightly reduced body weight of untreated and extract treated group corresponds to the decreased food intake by these animals and might be related with general aggravation of health status after the start of the experiment.

Untreated or extract treated Ren-2 rats consumed greater amount of water compared to the group with ACE inhibitor medication. This might be explained by the elevated levels of angiotensin II, which is well known for its dipsogenic properties [247].

Szczepanska-Sadowska et al. described for Ren-2 rats increased concentrations of angiotensin II in brain regions, involved in regulation of body fluid balance [248]. However, this study found also an increased food ingestion by Ren-2 rats compared to age-matched control rats, which was not present in our study (data not shown).

Weighting of organs at the end of the experiment revealed an increased heart size of untreated and extract treated group compared to the ramipril medicated group. Similar heart to body weight ratio in untreated Ren-2 rats was also described by Tschudi et al. [246]. Pinto et al. observed the development of cardiac hypertrophy in untreated transgenic Ren-2 rats [177]. Some authors attribute cardiac hypertrophy and end organ damage to the raised RAS system [177]. Others place responsibility on the increased blood pressure [249] or describe a contribution of elevated blood pressure and augmented RAS system [176].

The increased heart size of not-medicated animals might therefore reflect an adaptive response to the increased burden by higher blood pressure or is related on potentially augmented concentrations of angiotensin II in various tissues compared to the ACE inhibitor treated group.

Johnson et al. [250] have investigated sexual dimorphism in the cardiovascular parameters between male and female SD and Ren-2 rats. They describe for nearly all the endpoints a worse prognosis for Ren-2 rats compared to the SD control animals and between the two genders worse values for the male Ren-2 rats. The study describes the relative protection of females compared with males in development of hypertension, autonomic dysfunction (e.g. baroreflex sensitivity and heart rate variability) and associated end organ damage. Female Ren-2 rats demonstrated a 20-30 mmHg lower systolic blood pressure compared to their male counterparts.

An early onset of hypertension in homozygotous Ren-2 rats was reported by Lee et al. [176] and Mullins et al. [251], who describes a beginning of hypertension at the age of four weeks reaching a maximum by nine weeks. In our study blood pressure increased significantly in Ren-2 rats after one week deprivation of antihypertensive medication. Systolic blood pressure of untreated rats reached one week after the beginning of experiment values of 163 ± 7 mmHg (mean ± SEM). These values are much lower than the blood pressure of 239 ± 8 mmHg (mean ± SEM) in 12 week old Ren-2 rats reported by Tschudi et al. [246]. However, in their study, rats were grown

up without medication and it seems reasonable, that blood pressure would have further increased in our study if the unmedicated period had been longer.

Unfortunately, blood pressure could not be measured successfully anymore after the first week because rats moved excessively and were rather agitated.

Raised activity of the renin-angiotensin system in Ren-2 rats was reported to increase anxiety, which could be reversed by a treatment with the ACE inhibitor ramipril [252]. Elevated anxiety particularly in unmedicated Ren-2 rats might have influenced the problems of blood pressure measurement but also the blood pressure values themselves.

Activation of the renin-angiotensin system in tissue enhances the vascular production of ROS in part through activation of membrane bound NADH and NADPH oxidase [253]. Angiotensin II leads to an increased formation of ROS in vascular tissue [254] and kidney [255, 256]. Superoxide anion and H_2O_2 can act as second messengers in angiotensin II mediated signaling but in an excess level they lead to inflammation and cellular dysfunction [257].

The protective effects of the anthocyanin-rich extract in our study might be related with its antioxidative properties. Hypertension is associated with increased oxidative stress. However, there is still a debate whether oxidative stress is a cause or a result of hypertension [258]. Several studies describe the important role of oxidative stress for the pathogenesis of essential hypertension [259, 260]

Oxidative stress may contribute to the generation of hypertension via a number of possible mechanisms. These include among others quenching of the vasodilatator nitric oxide, generation of vasoconstricting lipid peroxidation products, stimulation of inflammation and increased intracellular free calcium concentration [258]. Tempol, a superoxide dismutase mimeticum attenuates the development of hypertension via scavenging of ROS [261]. However, there are also many contrarious studies, failing to prove an amelioration of hypertension by application of antioxidants [262-264]. Ren-2 rats are known to express significantly higher amounts of Nox1 and Nox4 in aorta and kidney compared to tissues from normotensive wild-type animals which leads to a enhanced generation of superoxide [265]. Results from previous studies suggest that high superoxide levels in Ren-2 rats might contribute to the pathophysiology of hypertension but treatment of Ren-2 rats with the known antioxidants apocyanin and tempol did not alter systolic blood pressure or angiotensin II level in studies conduced by Kopkan et al. [266] and Wei et al. [267].

They conclude therefore that hypertension in Ren-2 rats is dependent on angiotensin II but independent from the elevated oxidative stress level.

On the contrary antihypertensive drug therapy has additionally to the blood pressure lowering properties also beneficial effects on oxidative stress and endothelial function [268, 269]. Therefore Grossman concludes in his review that oxygen stress is not the cause, but rather a consequence, of hypertension [258]. Ren-2 rats treated with Dacapo grape extract showed obviously a lower blood pressure than animals of the untreated group. Anthocyanins rich plant extracts have been shown to reduce blood pressure in various studies with hypertensive individuums. [270, 271]. In contrast, no effects were seen after treatment of healthy volunteers [272].

Despite the known antioxidative properties anthocyanins may exceed their protective effects also by a few other mechanisms. The results of Dell' Agli et al. indicates that an inhibition of phosphodiesterases might contribute to the vasorelaxing effects of anthocyanins [273].

Additionally, anthocyanins are known to activate endothelial nitric-oxide synthetase leading to a NO-mediated vasorelaxation [274-276], which might contribute to their hypotensive properties.

Beside the potential factors mentioned above anthocyanins exceed also a direct inhibitory effect on ACE. There are several reports describing the inhibitory effects of anthocyanins [277, 278] and other flavonoids [279, 280] on the activity of ACE.

Therefore, it is possible that the protective effects of anthocyanin-rich Dacapo extract are not only due to the antioxidative properties of anthocyanins but also related with a potential reduction of angiotensin II by inhibition of ACE, inhibition of phosphodiesterases or activation of endothelial NO synthetase.

Al-Awwadi et al. [271] tested different polyphenol rich plant extracts in hypertensive high-fructose-fed rats. Only the anthocyanin enriched extract was able to reduce the blood pressure of high-fructose-fed rats to the level of the control group with normal diet, indicating the impact of anthocyanins for health-promoting effects of such plant extracts.

The protective effects of Dacapo grape extract on DNA damage were shown by Comet assay and γ-H2AX-staining in our study. The results of comet assay in kidney and liver indicate only small differences between the treatment groups. The anthocyanin-rich diet proved slight protecting properties, whereas there was only a negligible effect in ramipril treated animals. In contrast, γ-H2AX-staining showed a

strong protective effect on DNA damage in heart and kidney of the anthocyanin treated group and smaller effects for the ramipril medicated group. This is accordance with several other studies, which investigated the effect of anthocyanins on DNA damage *in vivo*. Weisel et al. investigated in healthy probands the effect of an anthocyanin and polyphenol rich juice, which was also developed by Research Institute Geisenheim. Intake of the fruit juice markedly reduced DNA damage of peripheral blood mononuclear cells (PBMCs) in comet assay and increased GSH level already at the first blood sampling time point after one weak. This might result from direct antioxidant effects, such as scavenging of ROS, chelating of transition metals, increased synthesis of cellular antioxidants or enhanced DNA repair activity [281]. Similar results were also reported by the same working group after treatment of hemodialysis patients with a red fruit juice derived from Research Institute Geisenheim [282]. Luceri et al. [283] used in their study different Arabidopsis thaliana mutants with a contrasted flavonoid profile. They compared in rats four different diets containing flavonols, flavonols and proathocyanidins, flavonols and anthocyanins or none of these flavonoids. Rats fed with the diet containing anthocyanins showed a strong decrease in DNA damage compared to rats with an anthocyanin free diet.

Thus the protective effect of this extract and similar preparations might be mainly based on the presence of anthocyanins in the diet.

The protective effect of anthocyanin-rich extract could not only be related to a direct radical scavenging activity of anthocyanins or polyphenols, but it could also be due to a modulation of gene expression of the antioxidant response element (ARE) and/or of enzymes involved in DNA repair. Cyanidin was shown to exert its activities by increasing ATM, topoisomerase II, HSP70 and p53 expression and influencing thereby genome integrity [156].

The results of Shih et al. in rat liver Clone 9 cells showed that treatment with anthocyanins (particularly delphinidin and cyanidin) leads to elevation of antioxidant capacity, including augmented activation of glutathione-related enzymes (glutathione reductase, glutathione peroxidase and glutathione *S*-transferase) and increased GSH/GSSG ratio. In addition, the expression of NAD(P)H quinone oxidoreductase 1 (NQO1) was also promoted by activation of antioxidant response element (ARE). However, this important aspect seems also to be dependent on the time of exposure to the dietary components. Boateng et al. [284] reported a significant increase in liver glutathione S-transferase (GST) activity of rats after 13 weeks of a freeze-dried

blueberry supplementation, while no effect was evident on the level of GST, quinone reductase and UDP-glucuronosyltransferase after a period of three weeks, as reported by Dulebohn et al. [285].

Del Bo' et al. treated rats with an anthocyanin rich (24.0 ± 5.2 mg/day) wild blueberry extract and assessed resistance to oxidative DNA damage by H_2O_2 afterwards *ex vivo* in comet assay. Level of DNA damage was significantly lower in rats fed with the wild blueberry diet compared with those on the control diet after eight weeks but not after four weeks [286]. Similar effects were also seen by Dulebohn et al., who observed after a three weak treatment with a diet highly concentrated in anthocyanins (1%) only slight effects in comet assay with liver cells [285]. In this context one could suspect that the treatment time of two weeks in our experiment was maybe not enough for pointing out differences in comet assay between the treatment groups. Several studies describe a lower sensitivity for the comet assay compared to γ-H2AX-staining [287, 288]. Additionally the relative high background DNA damage of cells in the *in vivo* comet assay might mask smaller differences between the groups.

Contradictory results were obtained by γ-H2AX-staining of small intestine, where animals treated with Dacapo grape extract showed much more DNA damage than animals of the other two groups. There are also other conflicting studies available describing the prooxidative and strand breaking properties of anthocyanins *in vitro* [289-291] or the increased damage in comet assay after ingestion of an anthocyanin-rich diet *in vivo* [292]. Hanif et al. proved the ability of anthocyanins and other flavonoids to cause oxidative strand breakage in DNA either alone or in the presence of chromatin bound copper. Structure–activity studies indicated that the presence of orthodihydroxy phenol groups on the B-ring of anthocyanidins (Delphinidin and Cyanidin) appears to be essential for apoptosis and oxidative degradation of DNA in the presence of copper ions [291]. Anthocyanins have been described by Habermeyer et al. and Esselen et al. to be catalytic inhibitors of topoisomerases I and II. Topoisomerases change DNA topology by introducing transient single (I) or double (II) strand breaks in the phosphodiester backbone of the DNA, enabling the release of torsion stress associated with replication, transcription, translation and recombination. Catalytic inhibitors bind to topoisomerases prior to DNA binding, thus inhibiting the formation of the cleavable complex. Habermeyer et al. investigated the potential catalytic inhibition of topoisomerase II in a decatenation assay. Catalytic

activity was found to be completely blocked with 10 µM cyanidin or delphinidin. Malvidin, pelagonidin and petunidin showed no effect on the catalytic activity of topoisomerase IIα and IIβ up to 100 µM. Data suggest that inhibitory properties are limited to analogues possessing vicinal hydroxy groups at the B-ring (cyanidin, delphinidin). At low micromolar concentrations anthocyanidins showed no effect on DNA integrity, whereas at higher doses (50 µM) all anthocyanidins tested induced at least a slight but significant increase of DNA strand breaks, with delphinidin being the most potent derivate. They speculate that DNA-breaking properties might be due to increased torsion stress resulting from inhibited activity of topoisomerases. Therefore, the apparent protective effects of anthocyanins in low concentrations regarding topoisomerase I might cross over to breaking of double strands by inhibition of topoisomerase II in higher concentrations (50 µM) [293, 294]. Such concentrations might be exceeded in our study by the intake of fed enriched in anthocyanins.

Structural analysis showed that delphinidin and other flavonoids bind weakly to adenine, guanine and thymine bases, as well as to the backbone phosphate group. Low flavonoid concentration induces helical stabilization, whereas high anthocyanin content causes helix opening [295].

Felgines et al. found 3 h after gavage more than 50% of the administered ^{14}C labeled cyanidin 3-O-glucoside in the small intestine. Concentrations in heart and kidney were much lower, showing nearly no accumulation in these organs [296].

This might explain why the extract showed protective effects in heart and kidney whereas in small intestine where higher local concentrations of anthocyanins were reached the extract induced DNA damage in crypt. The high number of double strand breaks found in small intestine tissue might be related with ther effects of anthocyanins on topoisomerases and their ability to cause oxidative stress.

However, it should be remarked that only 50% of the extract was structurally characterized, the other half consist of undefined polyphenols, which could also exert negative effects. One possible example for such polyphenols in grape extract might be resveratrol, whose strand breaking properties were shown in the first part of our study.

The antioxidant potential of glycosides was generally lower than that of the corresponding anthocyanidins [297]. Structural factors modulate the stability and polarity of anthocyanins and also their ability to scavenge free radicals and chelate

reactive metals. In the aqueous environment of RSA (reactive scavenging activity) assay anthocyanins with orthodihydroxy phenol groups on the B-ring (delphinidin and cyanidin) possessed the highest activity [297, 298]. However, in a oil-water-mixture (methyl linoleate emulsion) or in an assay with human LDL (low density lipoprotein) malvidin - the most prominent anthocyanin in Dacapo grape extract - showed also a very high antioxidative capacity, what might be related to the fact that it has better access to the lipophilic phase due to its methoxy groups in the B ring [297].

We compared the extract in a 10 ppm concentration (corresponding to an anthocyanin concentration of ~ 5 µM) with single anthocyanidins (10 µM) and found even with this low concentration a 6 times higher value in FRAP assay compared to delphinidin, which showed the highest antioxidative capacity of the single anthocyanidins [299]. Other uncharacterized polyphenols and flavonoids might contribute therefore to antioxidative properties of Dacapo extract.

We used FRAP assay also, to check if the antioxidative properties of the extract found *in vitro* were also detectable in serum of the respective animals.

Anthocyanins are fast metabolized and disappear from the blood stream around 4 h after the intake. As rats are nocturnal animals the last ingestion of feed happened likely several hours before the time of sacrifice, explaining the absence of anthocyanins in the plasma and therefore, the difficulty to detect an increased antioxidant capacity in plasma, as evaluated by the FRAP assay. However, it has to be remarked that the withdrawal of blood was only successful for a few animals of each group and therefore not necessarily representive.

No protective effect was also detected in DHE assay with sections of different organs. It might be possible that there were no significant differences between the groups (yet). Another option would be that this assay was inappropriate for the issue and other methods, e.g. 8-oxo-dG measurement with mass spectrometry coupled liquid-chromatography (LC-MS) would be more sensitive in the detection of oxidative damage.

Our study proved the health promoting effects of a diet enhanced in anthocyanins on hypertension and DNA damage in Ren-2 rats. Recently, preparations enriched in anthocyanins as natural antioxidants have gained increasing popularity on the fast expanding market of food supplements. Products are available enabling a several fold augmentation of daily intake above the ordinary amount, raising the question whether such an enhanced intake might potentially be related with adverse health

effects. Considering the low bioavailability the oral intake of anthocyanins with the diet should not result in plasma concentrations in the range where DNA strand breaks were observed *in vitro*. On the other hand, enhanced local concentrations in the gastrointestinal tract or in tissues with increased uptake might have to be considered. Our data indicate that at least locally in the gastrointestinal tract concentrations might be reached, at which the reported strand breaking effects of anthocyanins or other compounds of the extract might be of relevance. On the other side, under the same conditions a reduction of the hypertension related DNA damage in the heart and even more in the kidney was observed. Therefore, in our opinion before any dietary recommendation can be made, in-depth analysis of exposure conditions is required.

5 SUMMARY

Cancer is one of the leading causes of death all over the world. Malnutrition and toxic contaminations of food with substances such as mycotoxins have been thought to account for a high percentage of cancers. However, human diet can deliver both mutagens and components that decrease the cancer risk. Genomic damage could be reduced by food components through different mechanisms such as scavenging of reactive oxygen species.

In the first part of this study we tried to investigate the effects of patulin and resveratrol on DNA stability in V79 cells. Patulin is a mycotoxin, which is frequently found in spoiled apples and other fruits. The WHO has established a safety level of 50 µg/L, which is indeed not observed by all manufacturers. The acute toxicity of patulin in high concentrations is well known, however its potential carcinogenicity is still a matter of debate. Therefore we wanted to investigate further steps in the mechanism of patulin-induced genotoxicity. Patulin caused the formation of micronuclei and nucleoplasmic bridges in a dose-dependent manner. Further analysis revealed that patulin induced both kinetochore-negative and positive micronuclei. Time course of incubation indicate a new mechanism for patulin-induced nucleoplasmic bridge formation. We hypothized a mechanism via cross-linking of DNA, which was confirmed by a modified version of comet assay. Incubations of cells with patulin led to an increased number of multinucleated cells and multipolar mitoses. Cell cytometry revealed a G2 arrest by patulin, which might explain the amplification of centrosomes and patulin-induced aneuploidy. Patulin cause a dose-dependent DNA damage in comet assay which was influenced by the cellular GSH content. However, an induction of oxidative stress was just seen with higher concentrations of patulin. Levels of cellular glutathione were increased after 24 h incubation indicating an adaptive response to patulin-induced stress.

There is growing interest in polyphenols such as resveratrol which have shown many positive effects on human health. The beneficial properties are partially attributed to their ability to scavenge reactive oxygen species.

Co-incubation of V79 cells with patulin and 10 µM of the antioxidant resveratrol led to a slight reduction of micronucleus frequency compared to cells which were just

treated with patulin. However, in higher concentrations resveratrol themselves caused the formation of micronuclei in V79 cells. Kinetochore analysis indicated only clastogenic properties for resveratrol but no disturbance of mitosis. The antioxidant properties of resveratrol were shown in ferric reducing antioxidant power (FRAP) assay. However, in cellular system resveratrol in higher concentrations revealed also prooxidative properties, as shown in 2,7-dichlordihydrofluorescein (DCF) assay. The increased level of glutathione after resveratrol treatment might reflect an adaptive response to resveratrol-induced oxidative stress.

For the second part of this thesis we investigated the effects of an anthocyanin-rich grape extract on hypertensive Ren-2 rats.

Ren-2 rats are an accepted genetically modified rat model for the investigation of hypertension and increased oxidative stress. We divided 23 female Ren-2 rats into three groups. One group was fed with an anthocyanin-rich Dacapo grape extract, one group was treated with the angiotensin converting enzyme (ACE) inhibitor ramipril and the third group was kept without medication during the experiment. After one week untreated group showed a clear increase in systolic and diastolic blood pressure compared to the ramipril treated rats. This was in part attenuated in the animals fed with anthocyanin-rich Dacapo grape extract. Effects on blood pressure were also reflected in an increased thirst of untreated and extract fed animals. Comet assay with cells of kidney and liver revealed a slight protective impact of Dacapo extract on DNA damage compared to the other groups. Similar results were obtained after evaluation of ɣ-H2AX-staining of kidney and heart sections. However, in the small intestine oppositional effects were seen, indicating an increased number of double strand breaks probably due to the high local concentration of polyphenols after oral ingestion. Antioxidative properties of the extract were shown in FRAP assay. However, this effect was not reflected in an increased antioxidative capacity in serum or a protective impact in the dihydroethidium (DHE) assay.

The extract showed protective effects on DNA damage in comet assay and ɣ-H2AX-staining, but was not able to reduce hypertension back to the control level of ramipril treated animals. High local concentrations could also result in an increased damage of the affected tissue. Therefore, the administration of such concentrated compounds should be handled with care.

6 ZUSAMMENFASSUNG

Krebs ist eine der häufigsten weltweiten Todesursachen. Fehlernährung und Kontaminationen der Nahrungsmittel mit Toxinen wie Schimmelpilzgift tragen zu einem hohen Prozentsatz zu Krebserkrankungen bei. Allerdings enthält die Nahrung neben Mutagenen auch Bestandteile, die dazu beitragen das Krebsrisiko zu senken.
Schäden am Genom können durch Nahrungsbestandteile über verschiedene Mechanismen, wie zum Beispiel das Abfangen von freien Radikalen reduziert werden.
Im ersten Teil dieser Studie haben wir versucht die Effekte von Patulin und Resveratrol auf die DNA Stabilität von V79 Zellen zu untersuchen. Patulin ist ein Schimmelpilztoxin, welches häufig in verfaulten Äpfeln und anderen Früchten gefunden wird. Die WHO hat einen Grenzwert von 50 µg/L festgelegt, der jedoch nicht von allen Herstellern eingehalten wird. Die akute Giftwirkung von Patulin in hohen Dosen ist gut bekannt, wohingegen seine potentielle Kanzerogenität immer noch umstritten ist. Daher wollten wir weitere Schritte der Patulin induzierten Genotoxizität aufdecken. Patulin führte zu einer dosisabhängigen Bildung von Mikrokernen und Nucleoplasmic Bridges. Weitere Untersuchungen zeigten, dass Patulin sowohl kinetochor-positive wie auch kinetochor-negative Mikrokerne verursacht.
Bei der Analyse des Zeitverlaufs einer Patulininkubation deutete sich ein neuer Mechanismus für die Patulin induzierte Bildung von Nucleoplasmic Bridges an.
Wir haben die Hypothese einer Quervernetzung von DNA-Strängen aufgestellt, die durch eine modifizierte Version des Comet Assays bestätigt wurde. Die Inkubation mit Patulin führte zudem zu einer erhöhten Anzahl von vielkernigen Zellen und multipolaren Mitosen. Mittels Durchflusszytometrie konnten wir einen durch Patulin verursachten G2 Arrest nachweisen, der die Amplifikation von Centrosomen und die Patulin induzierte Aneuploidie erklären könnte. Patulin verursachte einen dosisabhängigen Schaden im Comet Assay, der durch den zellulären Glutathiongehalt beeinflusst ist. Eine Auslösung von oxidativem Stress wurde dagegen erst bei höheren Konzentrationen an Patulin beobachtet. Der zelluläre

Gluathiongehalt war nach 24 h Inkubationszeit erhöht, was auf eine adaptive Antwort auf den durch Patulin verursachten zellulären Stress hindeutet.
Polyphenole wie Resveratrol gewinnen zunehmend an Bedeutung, da zahlreiche positive Effekte auf die menschliche Gesundheit bewiesen wurden. Diese vorteilhaften Eigenschaften werden zum Teil ihrer Eigenschaft als Radikalfänger zugeschrieben. Die Co-Inkubation von V79 Zellen mit Patulin und Resveratrol führte zu einer leichten Reduktion der Mikrokernfrequenz im Vergleich zu Zellen, die nur mit Patulin inkubiert wurden. Allerdings löste Resveratrol in höheren Konzentrationen selbst die Bildung von Mikrokernen aus. Die Kinetochor-Analyse zeigte für Resveratrol clastogene Eigenschaften aber keine störende Effekte auf den Ablauf der Mitose. Die antioxidativen Eigenschaften von Resveratrol wurden im FRAP (ferric reducing antioxidant power) -Assay nachgewiesen. Im Gegensatz dazu wurden im zellulären System mittels DCF (2,7-Dichlordihydro-fluorescein) -Assay in höheren Konzentrationen auch prooxidative Eigenschaften festgestellt.
Der erhöhte zelluläre Glutathionspiegel nach Resveratrol-Behandlung könnte dabei auf eine adaptive Anwort auf den durch Resveratrol ausgelösten oxidativen Stress hindeuten

Im zweiten Teil dieser Doktorabeit haben wir die Effekte eines anthocyanreichen Traubenextrakts auf hypertensive Ren-2 Ratten untersucht.
Ren-2 Ratten sind ein anerkanntes genetisch modifiziertes Rattenmodell zur Untersuchung von Bluthochdruck und erhöhtem oxidativem Stress. Wir haben 23 weibliche Ren-2 Ratten in 3 Gruppen geteilt. Eine Gruppe wurde mit einem anthocyan-reichen Dacapo Traubenextrakt gefüttert, eine Gruppe wurde mit dem ACE (angiotensin converting enzyme) Inhibitor Ramipril behandelt und eine dritte Gruppe wurde während dem Experiment nicht medikamentös behandelt. Nach einer Woche zeigte die nicht therapierte Gruppe einen deutlichen Anstieg des systolischen und diastolischen Blutdrucks. Dieser Anstieg war bei der mit anthocyanreichem Dacapo Traubenextrakt gefütterten Gruppe abgeschwächt. Die Effekte auf den Blutdruck spiegelten sich auch in einer erhöhten Trinkmenge der unbehandelten und mit Extrakt behandelten Tiere wider. Ein Comet Assay mit Nieren- und Leberzellen zeigte einen schwachen schützenden Einfluß des Dacapoextrakts auf den DNA Schaden im Vergleich zu den anderen Behandlungsgruppen. Ähnliche Ergebnisse wurden auch bei der Auswertung der ɣ-H2AX Färbung in Nieren- und Herzschnitten

erzielt. Im Dünndarm wurden dagegen gegensätzliche Effekte beobachtet, die auf eine erhöhte Doppelstrangfrequenz durch die hohe lokale Konzentration an Polyphenolen nach oraler Aufnahme hindeuten. Die antioxidative Eigenschaften des Extrakts wurden im FRAP_Assay nachgewiesen. Diese Effekte spiegelten sich jedoch nicht in einer erhöhten antioxidativen Kapazität des Serums oder einem schützenden Effekt im DHE-Assay wider.

Der Extrakt zeigte schützende Eigenschaften im Comet Assay und in der ɣ-H2AX-Färbung, war aber nicht in der Lage den Bluthochdruck auf das Kontrollniveau der Ramipril-behandelten Tiere herabzusenken.

Hohe lokale Konzentrationen können auch zu einem erhöhten Schaden des betroffenen Gewebes führen. Daher sollte die Anwendung solcher hochkonzentrierter Präparate mit Vorsicht bedacht werden.

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9 CURRICULUM VITAE

Nina Glaser
* August 2nd 1983 in Saarbruecken, Germany

Germany

Education

01.2009 – present	Ph.D. in Toxicology, Institute of Pharmacology and Toxicology, Department of Toxicology, University of Wuerzburg, Germany
04.2012	Degree: Pharmacist specialised in Toxicology and Ecology
12.2008	Pharmaceutical examination, liscence as pharmacist
10.2003-11.2008	Study of pharmacy, Saarland University; Germany
05.2008-10.2008	Second part of practical year: Thomas Mann pharmacy, Schiffweiler-Saar, Germany
11.2007-04.2008	First part of practical year: Diploma thesis, Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, Saarland University, Germany
07.1994-07.2003	Secondary school, Illtal-Gymnasium, Illingen, Germany Degree: General qualification for university entrance (Abitur)
08.1990-06.1994	Primary school, Marpingen, Germany

Publications

Glaser N., Stopper H.
Patulin: Mechanism of Genotoxicity
Food and Chemical Toxicology, 50 (5), 1796-1801

Abstracts

Glaser N., Stopper H.:
Patulin induced oxidative stress and genotoxicity in V79 cells; 6th International Symposium of the Graduate School of Live Sciences; October 2011; Wuerzburg, Germany

Glaser N., Stopper H.:
Patulin induced oxidative stress and genotoxicity in V79 cells; Annual Meeting of the European Enviromental Mutagen Society; July 2011; Barcelona, Spain

Glaser N., Stopper H.
Patulin induced oxidative stress and genotoxicity in V79 cells; 77. Jahrestagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie; April 2011; Frankfurt, Germany

Glaser N., Stopper H.
Patulin induced oxidative stress and genotoxicity in V79 cells; 7th GUM ^{32}P-postlabeling Workshop & UKEMS / Dutch EMS- sponsored Workshop on Biomarker of Exposure and Oxidative DNA damage; March 2011; Muenster, Germany

Glaser N., Stopper H.;
Effects of Resveratrol and Patulin on DNA damage in V79 cells; 25. Tagung der Gesellschaft für Umwelt- und Mutationsforschung; October 2010; Potsdam, Germany

Glaser N., Stopper H.;
Effects of Resveratrol on Patulin induced DNA-Damage in V79 cells; Society for Free Radical Research Europe; September 2010; Oslo; Norway;
Oral presentation

Bozena Czarkowska-Paczek, Nina Glaser, Nicole Schupp, Malgorzata Zendzian-Piotrowska, Helga Stopper;
Acute bout of exercise induces oxidative stress in the liver in rats; 4th International Symposium of the Graduate School of Live Sciences; March 2009; Wuerzburg, Germany

Bozena Czarkowska-Paczek, Nina Glaser, Nicole Schupp, Malgorzata Zendzian-Piotrowska, Helga Stopper;
Acute bout of exercise induces oxidative stress in the liver in rats; 24. Tagung der Gesellschaft für Umwelt- und Mutationsforschung; February 2009; Vienna, Austria

10 AFFIDAVIT

I hereby declare that my thesis entitled “Influence of natural food compound on DNA stability“is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, June 26th, 2012

Nina Glaser