Mutagenic and carcinogenic potential of menadione*

C. COJOCEL[1], L. NOVOTNY[2], A. VACHALKOVA[3]

[1]Kuwait University, Faculty of Medicine, e-mail: cojocel@hsc.edu.kw, and [2]Faculty of Pharmacy, P.O. Box 24923, Safat 13110, Kuwait; [3]Cancer Research Institute, Slovak Academy of Science, SK-833 91 Bratislava, Slovak Republic

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Menadione (2-methyl-l,4-naphthoquinone) or vitamin K_3 is a lipid-soluble substance and promotes the hepatic biosynthesis of blood clotting factors. Carcinogenic potential of menadione was determined by a DC polarography method in strictly anhydrous N,N-dimethylformamide (DMF) in the presence of α-lipoic acid. Superoxide anion formation was measured after incubation of rat lung, liver and kidney microsomes with menadione. The genotoxic potential of menadione was investigated using the unscheduled DNA synthesis (UDS) and alkaline elution assays.

The parameter of potential menadione carcinogenicity tg α was 0.0025 indicating no carcinogenic activity of menadione. Superoxide anion was generated in a concentration- and time-dependent manner when menadione was incubated with microsomes. In the mammalian cells (A 549) used for alkaline elution and UDS assays, menadione was cytotoxic at concentrations above 20 nmol/ml. The use of S9 mix (metabolic activation) fractions decreased the cytotoxicity of menadione. In the concentration range of above 20 nmol/ml menadione was genotoxic in the UDS test in absence of metabolic activation. In the presence of metabolic activation the menadione-induced DNA damage and repair was greatly reduced. Treatment of A 549 lung cells with 4-nitroquinoline-N-oxide (NQO) caused significant formation of DNA single-strand breaks both in the absence and presence of metabolic activation. Treatment of A 549 lung cells with menadione caused formation of DNA single-strand breaks in the absence of S9 mix. In the presence of metabolic activation menadione caused no significant formation of DNA strand breaks. Menadione-induced DNA repair in A 549 cells was concentration-, time-, and temperature-dependent. Measurement of unscheduled DNA (UDS) synthesis (repair) following treatment with NQO and menadione yielded strong UDS responses in the absence of S9 mix.

Taken together the results of these studies suggest the mutagenic potential of NQO and menadione. These results indicate that menadione undergoes redox cycling with formation of reactive oxygen species which cause DNA damage and repair without having a carcinogenic potential.

Key words: menadione, polarography, alkaline elution, UDS test, single-stranded DNA, mutagenicity, carcinogenicity

Vitamin K_1 is a fat-soluble substance present in the chloroplasts of plant leaves and in many vegetable oils. The animal feces contain large amounts of vitamin K_2 since the intestinal bacteria synthesize it. Vitamin K_3 (menadione) is a dietary synthetic component, and it can be converted into vitamin K_2 in the intestinal tract of the body. Menadione (2-methyl-1,4-naphthoquinone) is among the quinone derivatives presenting vitamin K activity, which is to promote the hepatic biosynthesis of blood clotting factors. The administration of large doses of menadione to animals has resulted in the production of anemia, polycythemia, splenomegaly, renal and hepatic damage and death [1–3]. The reactivity of quinones resides in their ability to undergo redox-cycling [4] and or to react directly with cellular nucleophiles such as protein and DNA. Menadione has also been shown to induce cytochrome P_{450} [5] and in combination therapy with vitamin C exhibits synergistic antitumor activity [6].

The model carcinogen NQO has historically been characterized as "UV-mimetic" with respect to its genotoxic properties. Recent evidence indicates that NQO, unlike 254 nm UV light, may exert significant cytotoxic and/or mutagenic potential via the generation of reactive oxygen species [7, 8] such as superoxide. In the present study NQO was used as positive control.

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The aim of the present study was designed to investigate the menadione genotoxic and carcinogenic potential. Further, the menadione-induced oxidative stress (superoxide production) and DNA damage and repair were investigated. While the carcinogenic potential was investigated by polarography, DNA damage was measured by alkaline elution, and DNA repair was measured by unscheduled DNA synthesis (UDS).

Material and methods

Chemicals. N, N-dimethylformamide (DMF) was of a commercial origin from Fluka (Fluka, Buchs, Switzerland). DMF was additionally purified prior to all electrochemical measurements by double vacuum distillation in a dry nitrogen atmosphere [9]. DMF water content did not exceed 0.01 wt. %. Tetrabutylammonium perchlorate (TBAP) was purchased from Fluka and was used as the supporting electrolyte at a concentration of 0.15 mol^{-1}. α-Lipoic acid which is also known as (D,L-6,8-thioctic acid) was purchased from Koch Light Laboratories, Colnbrook, United Kingdom. Vitamin K_3 (menadione, CAS Nr.: 58-27-5) and 4-nitroquinoline-N-oxide (NQO) were purchased from Sigma, Munich, Germany. 4-Nitroquinoline 1-oxide (4-NQO, CAS Nr.: 56-57-5) is classified among the hydroxylamines, amine-N-oxides, and is a well known mutagenic and carcinogenic compound, which produces adducts with DNA bases via oxidative damages. In the present study NQO was used as positive control.

Polarographic measurements. Carcinogenic potential of menadione was determined by DC polarography method in strictly anhydrous N,N-dimethylformamide (DMF) in the presence of α-lipoic acid (LA).

DMF was additionally purified prior to all electrochemical measurements by double vacuum distillation in a dry nitrogen atmosphere [9]. All polarographic measurements were performed using the three-electrode setting at the polarographic analyzer PA 2 equipped with the two-line recorder XY 4106 from Laboratorni pristroje, Prague, Czech Republic. Polarographic experiments were performed in a polarographic cell adapted for the work in anhydrous system (Fig. 1).

As the indicating electrode, a dropping mercury electrode (DME) was used with a drop time of 3 s and a flow rate of 2.27 $mg.s^{-1}$ at a mercury column hight h_{Hg} of 81 cm. As the reference electrode, a saturated calomel electrode (SCE) modified for anhydrous conditions was used. The auxiliary electrode was a platinum electrode. All polarographic mesurements were carried out at room temperature in a stream of dry nitrogen in order to exclude atmospheric oxygen and humidity described previously [10, 11]. The potential carcinogenic activity of menadione was determined based on the its ability to form complexes with LA.

Animal maintenance. Male Wistar rats (200–220 g) were used to prepare lung, liver and renal microsomes and S9 mix for metabolic activation. Animals were maintained and cared for as described in the "Guide for the care and use of laboratory animals" (Kuwait University). Rats received standard laboratory diet and water ad libitum and were housed in an environmentally controlled animal facility operating on a 12 h dark/light cycle and 55% humidity.

Isolation of lung, liver and renal cortical microsomes. Lung, liver and renal cortical microsomes were isolated from male Wistar rats (200 g) as previously described [12, 13] using a phosphate buffer [14]. At least 5 rats were used to harvest microsomes for each investigated experimental group.

Microsomal protein was determined by the method of SCHACTERLE and POLLACK [15] in a Beckman DU-70 spectrophotometer. Unless otherwise specified, the reaction mixture contained microsomes (20–200 μg/ml), 0.1 μmol NADPH and 163 μmol $MgCl_2$. For the microsome preparation the Beckman J2-MI centrifuge and the Beckman XL-80 ultracentrifuge were used.

Superoxide assay. Superoxide ($O^{2-\cdot}$) formation was measured as the reduction of acetylated, cythocrome c using the extinction coefficient of 21 $mM^{-1}.cm^{-1}$ [16].

Cytochrome c was acetylated by the method of AZZI et al [17]. Absorption spectra were recorded with a Beckman DU-70 spectrophotometer. Acetylation of cytochrome c was followed by a gel filtration on a Bio-Gel P6 column (Biorad Laboratories, Richmond, CA, USA). Acetylated cytochrome c was stored at –20 °C. Incubation mixtures contained menadione (5, 15, 30 or 50 nmoles/ml) and 21 μg/ml microsomal protein in 0.1 M phosphate buffer (pH 7.4), which contained NADPH, $MgCl_2$ and 50 μM acetylated cytochrome c. Incubations were done in 1 ml at 37 °C for 15 min. The amount of superoxide formed was calculated as the difference in the cytochrome c reduced in the absence and presence of 100 μg/ml superoxide dismutase. Since cytochrome c contains a small amount of the reduced form, the absorbance of cytochrome c formed in the reaction was corrected by subtracting the initial absorbance from the observed one.

Cell culture for alkaline elution and UDS experiments. A549 (human type II lung tumor derived) cells were used for the in vitro experiments carried out with menadione in alkaline elution tests. The A549 cells were a subclone (ATCC No. CCL 185) of the line described [18]. A549 cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

Cells were routinely cultured using 75-cm^2 flasks in 13 ml Dulbecco's modified Eagle medium containing 10% newborn calf serum (Gibco, Karlsruhe, Germany) and 200 mM glutamine. Cells used in early passages were determined to be mycoplasma-free and cultured in monolayer in 10% CO_2 in a humid atmosphere at 37 °C.

Cells were passed by trypsinization when approaching confluence. For determination of the cell number, cells were removed by trypsinization and counted with a Coulter counter (Coulter Electronics, Kempen-Huls, Germany).

For experiments, $4x10^5$ viable cells/35-mm dish were seeded in 2 ml Dulbecco's modified Eagle medium containing 10% newborn calf serum and 200 mM glutamine and

were incubated in 10% CO_2 at 37 °C for 24 h to allow attachment.

At the end of the 24-h attachment interval, the incubation medium was discarded and cells were cultured for an additional 48 h. During this incubation time cell cultures were washed twice (every 24 h) with an arginine-free Dulbecco's modified Eagle medium supplemented with 2% dialyzed fetal calf serum and 200 mM glutamine. At the end of the additional 48-h incubation time, the arginine-free Dulbecco's modified Eagle medium was discarded and replaced, just prior to the cell treatment, with 2 ml Dulbecco's Eagle medium supplemented with 2% dialyzed fetal calf serum, 10 mM hydroxyurea and 200 mM glutamine.

Reduction of the serum concentration, addition of hydroxyurea and arginine-free medium were used in order to inhibit the semi-conservative DNA replication (S phase). This test system for human cell cultures has been described by MARTIN et al [19].

The use of the human cell line A549 (ATCC No. CCL 185) to determine DNA damage and repair was carried out as described and recommended by the OECD guidelines [20] and MITCHELL et al [21]. The present experiments were conducted according to these guidelines.

The S9 mix. A preparation of rat liver 9000 x g supernatant (S9) was used as a metabolizing mixture for all test systems. Male Sprague-Dawly rats were pretreated with Aroclor 1254 (500 mg/kg). Preparation of the S9 fractions and of the cofactor solution (mix) was carried out as described by MARON and AMES [22]. For the S9 mix preparation a Beckman J2-MI centrifuge was used. Protein concentration was determined by the method of SCHACTERLE and POLLACK [15] using bovine serum albumin as standard.

Treatment of cultures. Stock solutions of the positive control, 4-nitroquinoline-N-oxide (NQO) and of menadione(K_3) were prepared in dimethylsulfoxide (DMSO). Stock solutions were filtered sterile through a 0.2-µm filter (Schleicher & Schull, Dassel, Germany) before use. To achieve the final concentrations, 20 µl from each stock solution was added to each of the dishes used to test a certain concentration of the test substance.

At least 4 cell cultures (dishes) were used in each experiment per tested concentration. In the present study concentrations of 1.31, 2.63, and 5.26 nmol.ml^{-1} NQO were tested. Menadione concentrations used were 10, 20 and 45 nmoles.ml^{-1}.

In the experiments with metabolic activation, 100 µl of rat liver S9 mix (3 mg/ml protein) were added to each dish in the alkaline elution assays. Cells were incubated with the test substance in 10% CO_2 and at 37 °C for 3 h. At the end of the 3-h treatment, incubation medium was discarded and cells were harvested by gentle policing into 1 ml cold, phosphate-buffered saline (PBS, pH 7.4) using a rubber policeman.

Alkaline elution. The alkaline elution assay measures the rate of DNA single-strand brakes elution through a filter membrane under alkaline conditions.

The amount of DNA single-strand brakes under alkaline conditions is determined on the basis of the increase in DNA elution rate. The procedures followed in this study were essentially those previously described [23–25]. Alkaline elution was shown to be effective in detecting DNA damage [25].

UDS test. The assay for unscheduled DNA synthesis was used to detect DNA damage by measuring DNA-excision repair. This test system for human cell cultures has been described by MARTIN et al [19]. The use of the human cell line A549 (ATCC No. CCL 185) to determine DNA damage and repair in the unscheduled DNA synthesis test was carried out as described and recommended by the OECD guidelines [19, 20]. The present experiments were conducted according to these guidelines. Incorporation of [H^3]-thymidine was measured at different menadione concentrations (5, 10, 20, 30 or 45 nmoles/ml), temperature and incubation times.

The UDS test measures DNA-repair synthesis with excision and removal of DNA sequences that have been previously damaged by chemical or physical agents [26]. The endpoint of unscheduled DNA synthesis was measured in the present study by liquid scintillation counting of the incorporated radio labeled nucleoside [H^3]thymidine into the DNA of mammalian cells. In order to inhibit semi-conservative DNA replication (S phase), arginine-deficient medium, reduction of the amount of serum and addition of hydroxyurea to the medium were used. This test system for human cell cultures was used as previously described [19, 23, 27, 28]. The endpoint of unscheduled DNA synthesis was measured in the present project by liquid scintillation counting of the incorporated radiolabeled nucleoside [H^3thymidine] into the DNA of mammalian cells. The extent of [H^3]-thymidine incorporation was expressed as dpm/µg DNA]. The [H^3]-thymidine was measured in a Beckman LS 6000 TA scintillation counter. Two independent experiments in the absence or in the presence of a metabolic activation system (liver S9 mix) were performed. A compound was considered positive if it induced a reproducible and statistically significant increase of [H^3]-thymidine incorporation in comparison with the solvent control.

Calculations. Mean value, standard deviation (SD). Student's t-test and the 1-way analysis of variance were used for statistical analysis of the data. The 0.05 level of probability was used as the criterion for significance.

Results

Polarography. The reduction of menadione in strongly acidic environment is a 3-steps process ($E_{1/2I.}$–0.68 V, $E_{1/2II.}$–0.85 V $E_{1/2III.}$–1.00 V vs. SCE). The shift of pH towards more neutral values increases the definition of waves. However, a mechanism of reduction undergoes qualitative and quantitative changes and the reduction process is completed in 2 steps only. Additionally, $E_{1/2}$ values are shifted in the negative direction. In a strongly alkaline environment at pH around 12, menadione-sodium-bisulfide reduction becomes 1-step process (8/3).

In an aprotic environment of dry DMF, menadione is reduced in two well-defined one-electron steps with $E_{1/2}$ values of –0.550 V and –1.290 V vs. SCE. The presence of lipoic acid (LA) did not induce any qualitative or qualitative changes in the reduction mechanism. Values of difuse current of the 1st and 2nd wave of menadione did not change with increased LA concentrations. No new polarographic wave appeared even at the equimolar concentrations of menadione and LA). This fact indicates that no complex between menadione and LA was formed. These findings indicate that menadione does not interact with LA during DC polarography experiment in aprotic conditions. Consequently, polarographic determination indicates that menadione does not possess carcinogenic potential.

Thus, menadione is reduced in strictely anhydrous DMF on a mercury dropping electrode in two difuse well defined steps, the first step being reversible and the second irreversible. The $E_{1/2}$ values of these polarographic waves were –0.550 and –1.290 V vs. SCE. In the presence of α-lipoic acid, an increase of the difuse current of the second polarographic wave of menadione was observed. The parameter of potential carcinogenicity tg was 0.0025 indicating no carcinogenic activity.

Superoxide formation. In the present study a concentration-dependent increase of $0_2^{\cdot-}$ production was measured when liver, kidney or lung microsomes were incubated with 5, 15, 30 or 50 nmoles/ml menadione (Fig. 2).

Alkaline elution and UDS. Prior to conducting the alkaline elution and UDS tests, the cytotoxic concentration range for menadione was determined to be above 20 nmol/ml (data not shown). As shown in Figures 3 and 4 the positive control NQO induced significant formation of DNA single-strand breaks, with and without metabolic activation. DNA damage was stronger in the absence of metabolic activation.

However, in the presence of metabolic activation, NQO caused single-strand brakes of DNA in A549 cells which were detected at all investigated concentrations (Fig. 4). Significant formation of DNA strand breaks was measured without metabolic activation at menadione concentrations above

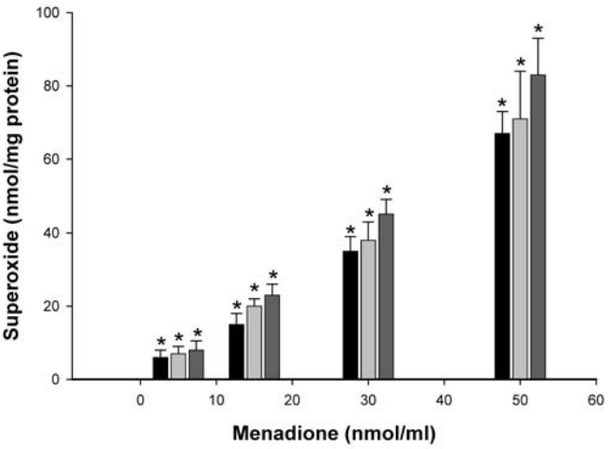

Figure 2. Concentration-dependent increase in superoxide ($O2^{\cdot-}$) production caused incubation of lung (■), kidney (□) or liver (▨) microsomes with menadione (5, 15, 30 and 50 nmoles/ml). Menadione stock solution was nitrogen-saturated.
Each column represents x ±SD of 5 experiments. *Significantly different from controls.

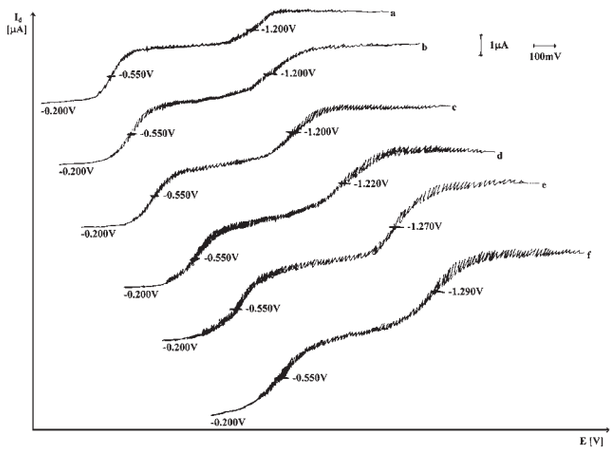

Figure 1. Polarographic reduction of menadione in anhydrous DMF in the presence of α-lipoic acid. Supporting electrolyte: 150 nmol.l^{-1} TBAP in DMF. Menadione concentration (cMEN): 5.10^{-4} mol.l^{-1}; Concentration of α-lipoic acid (cLA): a=0.00 mol.l^{-1}; b=1.19.10^{-4} mol.l^{-1}; c=1.96.10^{-4} mol.l^{-1}; d=3.47.10^{-4} mol.l^{-1}; e=4.94.10^{-4} mol.l^{-1}; f=5.66.10^{-4} mol.l^{-1}. Scanning range ($E_{|V|}$) from –0.200 V to –1830 V at a scanning rate of 10 mV.s^{-1}. The half-wave potential ($E_{1/2}$) values for menadione were –0.550 V and –1.290 V. Id = the diffuse current [A].

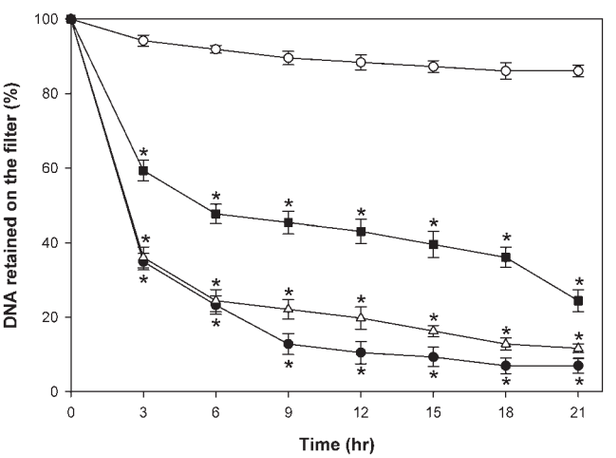

Figure 3. Alkaline elution of DNA single-strand brakes after a 3-h incubation of human lung tumor cells with different concentration of 4-nitrochinoline-N-oxide (NQO) without metabolic activation. (○) Control (DMSO); NQO, (■) 1.31 nmol.ml^{-1}; (△) 2.63 nmol.ml^{-1}; (●) 5.26 nmol.ml^{-1}.
Each point represents x ±SD of 8 experiments. 100% DNA retained on the filter corresponds to the total amount of the sample. *Significantly different from controls.

20 nmol.ml^{-1} (Fig. 5), which was greatly decreased in the presence of metabolic activation (data not shown).

Menadione induced in the absence of metabolic activation a concentration-dependent increase of DNA repair when incubated without metabolic activation (Fig. 6). DNA repair was decreased in the presence of metabolic activation. It appears that the UDS test is somewhat more sensitive than alkaline elution test (Figs. 5 and 6). Further experiments with the UDS test indicated a time-, and temperature-dependent increase in the menadione-induced DNA repair (data not shown).

Discussion

Phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone) is designated as vitamin K_1. The menaquinones, collectively known as vitamin K_2, are a large series of compounds containing an unsaturated side chain with differing numbers of isoprenyl units at the 3 position in the methyl-1,4-naphthoquinone nucleus. There exist even several synthetic water-soluble compounds containing the 2-methyl-1,4-naphthoquinone structure also exist. These include menadione (vitamin K_3), menadiol (vitamin K_4) and vitamin K_5 (2-methyl-4-amino-1-naphthol). Certain naphthoquinones, in particular the synthetic vitamin K_3, have been found to have antitumor activity *in vitro* and *in vivo*. Vitamin K_2 has been found to induce the *in vitro* differentiation of myeloid leukemic cell lines [29].

Benzo(a)pyrene is an ubiquitous environmental pollutant with potential carcinogenicity. Vitamin K_3 inhibits the conversion of the carcinogen benzo(a)pyrene to its more polar metabolites in an in vitro rat liver microsomal system [30]. Vitamin K also inhibits benzo(a)pyrene metabolism in rat liver and reduces its mutagenecity in the Ames test [31, 32].

Treatment with NQO has been widely employed in mammalian systems as a paradigm for DNA damage-induced carcinogenesis. To exert its neoplastic effect, NQO must first undergo metabolic activation to the proximate carcinogen

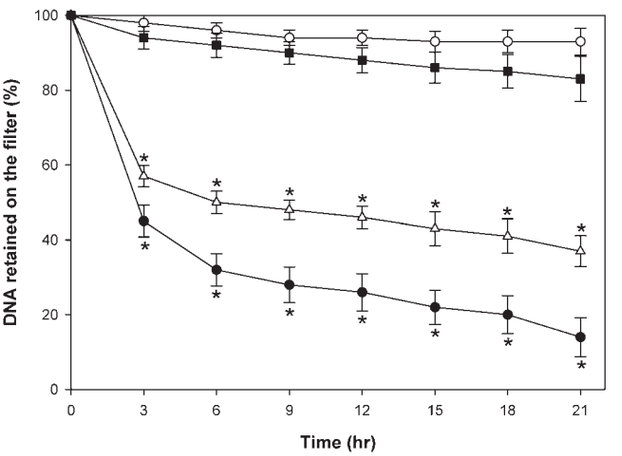

Figure 5. Alkaline elution of DNA single-strand brakes after a 3-h incubation of human lung tumor cells with different concentration of menadione without metabolic activation. (○) Control (DMSO); menadione, (■) 10 nmol.ml^{-1}; (△) 20 nmol.ml^{-1}; (●) 45 nmol.ml^{-1}.
Each point represents x ±SD of 8 experiments. 100% DNA retained on the filter corresponds to the total amount of the sample. *Significantly different from controls.

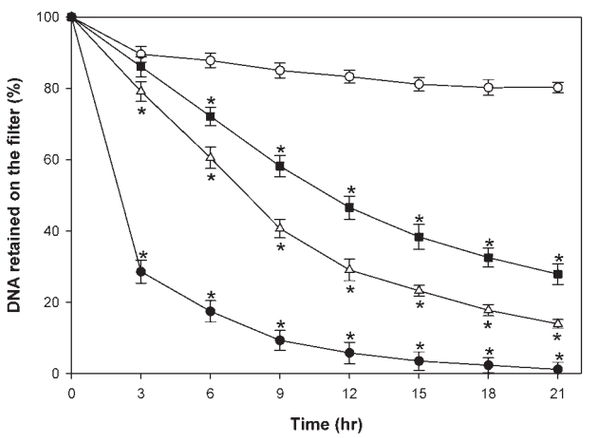

Figure 4. Alkaline elution of DNA single-strand brakes after a 3-h incubation of human lung tumor cells with different concentration of 4-nitroquinoline-N-oxide (NQO) with metabolic activation. (○) Control (DMSO); NQO, (■) 1.31 nmol.ml^{-1}; (△) 2.63 nmol.ml^{-1}; (●) 5.26 nmol.ml^{-1}.
Each point represents x ±SD of 8 experiments. 100% DNA retained on the filter corresponds to the total amount of the sample. *Significantly different from controls.

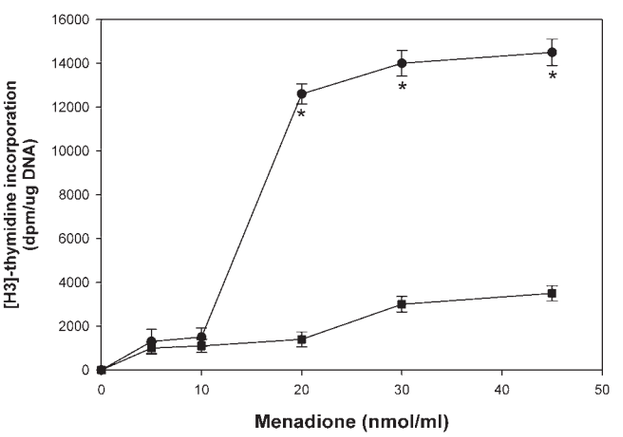

Figure 6. Unscheduled DNA synthesis (UDS) expressed as [H^3]-thymidine incorporation (dpm/µgDNA) after a 3-h incubation of human lung tumor cells with 5, 10, 15, 30 or 50 nmol.ml^{-1} of menadione with metabolic activation (■) or (●) without metabolic activation.
Each point represents x ±SD of 8 experiments. *Significantly different from controls and data resulted from incubations with metabolic activation (+ S9 mix).

4-hydroxyaminoquinoline 1-oxide, which, following acylation, reacts with DNA to form stable quinoline-purine monoaducts [33, 34]. Several studies have clearly demonstrated that NQO can generate a substantial degree of intracellular oxidative stress.

NQO undergoes redox cycling to produce genotoxic reactive oxygen species such as superoxide anion, hydroxyl radicals and singlet oxygen. This may herald significant consequences for NQO-exposed cells at the level of cell killing and/or mutagenesis [35, 8].

Combination treatment with vitamin K_3 and vitamin C without simultaneous use of chemotherapy and radiation has shown anticancer effects both *in vitro* and *in vivo* studies. When combined at the same concentrations, a synergistic inhibition was achieved. Human endometrial adenocarcinoma cells subjected to similar concentrations of vitamin C and vitamin K_3 resulted in similar inhibition of growth [36].

In vivo and *in vitro* studies showed a synergistic effect when menadione was combined with conventional chemotherapeutic agents. Combining menadione and 5-fluorouracil significantly enhanced the action against hepatoma cells [37]. Menadione was found synergistic with 5-fluorouracil, bleomycin, cisplatin, and dacarbazine in human oral epidermoid carcinoma (KB) cell culture.

Menadione in KB cell cultures also demonstrated an additive effect when combined with 10 other chemotherapeutic agents such as mercaptopurine, cytarabine, hydroxyurea, VP-16, vincristine, doxorubicin, mitoxanthine, mitomycin C, actinomycin D, and thiotepa [38].

Studies in rats determined that menadione showed synergy with methotrexate inducing inhibition of tumor growth [39].

Substances with vitamin K activity belong chemically among derivatives of 1,4-naphthoquinone. From among the synthetic vitamin K derivatives, menadione (vitamin K_3) possesses high biological activity. Electrochemical determination of the vitamine K_3 was reported earlier by ONRUST and WÖSTMANN [40].

This method is based on petrolether extraction of menadione from samles followed by the vitamine K_3 polarographic determination ($E_{1/2}$ –0.30 V, pH 7.4). Because naphthoquinones are photosensitive, it is necessary to block light and oxygen out of polarographic cell. Polarographic behavior of vitamin K_3 (menadione sodium bisulfide) in water was investigated at various pH, since reduction depends on pH [41].

Polarographic measurements of the carcinogenic potential as the parameter tg α for N-nitroso-N-methylurea (NMU) and streptozotocin (STZ) displayed values of 0.459 and 0.400, respectively [23]. In contrast, polarographic measurements carried out in the present study indicate a tg α value of 0.0025 for menadione. These results indicate that menadione does not possess a carcinogenic potential. However, several studies have shown that vitamins K_1, K_2, K_3 and K_5 exert antitumor effects *in vivo* and *in vitro* on various types of rodent- and human-derived neoplastic cell lines [42].

After a 3 h exposure of A549 cells, in the absence of metabolic activation, both NQO and menadione caused significant DNA single-strand brakes production and incorporation of H^3-thymidine at all investigated concentrations. In the presence of metabolic activation, menadione-induced formation of DNA single-strand and DNA repair were decreased. The results of the present study underline the important role of using a metabolic activation system when the carcinogenic potential of various xenobiotics is investigated in *in vitro* studies.

The mechanism of the possible anticarcinogenic activity of vitamin K is not well understood. The effectiveness of menadione against cancer is partly due to oxidative stress via redox-cycling of the quinone to produce reactive oxygen species such as the hydroxyl radical, superoxide radical, and hydrogen peroxide [43]. The increased redox cycling of menadione and the production of reactive oxygen species surpasses the oxidative capacity of the cell, resulting in cell death. Quinones can undergo either one-electron reduction, producing semiquinone radicals, or two-electron reduction, resulting in hydroquinones. Menadione was found to be more cytotoxic at higher doses than other forms of vitamin K by directly arylating nucleophiles such as glutathione and initiating one- or two-electron redox cycling.

Menadione-induced oxidative stress trigerrs the direct arylation of thiols within the cell resulting in the depletion of glutathione and/or sulfhydryl-containing proteins [44]. Arylation refers to the introduction of aromatic groups, such as menadione, to a molecule such as glutathione.

Reactive oxygen species such superoxide anion, hydroxyl radical and hydrogen peroxide caused DNA damage and repair as also shown in the present study. Menadione increased oxidative stress in malignant cells [45]. Oxidative stress was detected by increased DNA strand breaks due to hydroxyl radicals produced by the presence of menadione in cell culture [46, 47].

Menadione is an oxidative stress inducer and its anticarcinogenic activity may, in part, be explained by induction of apoptotic cell death [48]. Results of other studies suggested that the induction of apoptosis by menadione is mediated by the Fas/Fas ligand system [49]. The expression of c-myc and c-fos proto-oncogenes are involved in the mechanism of vitamin K induced apoptosis, differentiation, and cell cycle arrest. The proto-oncogene myc (c-myc) codes for a nuclear protein transcription factor c-myc that activates other genes [4]. In the case of the oncogene c-myc, the complex is involved in transformation, immortalization, cell differentiation, and induction of apoptosis [51].

Vitamin K_3 induces cell cycle arrest and cell death [52] by inhibiting protein kinases in association with a cyclin-dependent mechanism. Cyclins are regulatory proteins of the cell cycle that activate cellular maturation-promoting factors. Cyclins complex and modulate theprotein kinase catalytic subunit of proteins such as cyclin dependent kinase 1 (CDK1). Further studies reported that menadione induces

cell cycle arrest and cell death by inhibiting Cdc25 phosphatase [53]. Vitamin K_3 has been found to act on proteins such as myc and fos, which in turn leads to growth arrest and death [54].

In conclusion, the results of the present study indicate that menadione induced superoxide production and DNA damage and repair. The DNA damage and repair was concentration-, and time-dependent in the absence of metabolic activation. The results of polarographic experiments and alkaline elution obtained in the presence of metabolic activation correlate well (tg α was 0.0025 and decreased formation of DNA strand breaks as well as decreased DNA repair) and indicate no carcinogenic potential for menadione. The results of the present study also indicate that reactive oxygen species are likely to react with DNA and cause DNA damage and repair without leading to carcinogenic effects.

References

[1] ALARCON QM, VASQUEZ F, ACOSTA A, BURGUERA GL, ORTEGA SY. Hematologic changes in rats treated with high doses of Vitamin K3. Ach Latinoam Nutr 1991; 41: 363–374.

[2] MUNDAY R, SMITH BL, MUNDAY CM. Toxicity of 2,3-dialkyl-1,4-naphthoquinones in rats: comparison with cytotoxicity in vitro. Free Radic Biol Med 1995; 19: 759–765.

[3] VASILIOU V, QAMAR L, PAPPA A, SOPHOS NA, PETERSON DR. Involvement of electrophile responsive element and p53 in the activation of hepatic stellate cells as response to electrophile menadione. Arch Biochem Biophys 2003; 413: 164–171.

[4] SMITH MT. Quinones as mutagenes, carcinogens, and anticancer agents: introduction and overview. J Toxicol Environ Health 1985; 16: 665–672.

[5] SIDOROVA YA, GRISHANOVA AY, LYAKHOVICH VV. Rat hepatic CYP1A1 and CYP1A2 induction by menadione. Toxicol Lett 2005; 155: 253–258.

[6] VON GRUENIGEN VE, JAMISON JM, GILLOTEAUX J, LORIMER HE, SUMMERS M et al. The in vitro antitumor activity of vitamins C andK3 against ovarian carcinoma. Anticancer Res 2003; 23: 3279 -3287.

[7] NUNOSHIBA T, DEMPLE B. Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide. Cancer Res 1993; 53: 3250–3252.

[8] YAMAMOTO K, INOUE S, KAWANISHI S. Site-specific DNA damage and 8-hydroxydeoxyguanosine formation by hydroxylamine and 4-hydroxyaminoquinoline 1-oxide in the presence of Cu (II): role of active oxygen species. Carcinogenesis 1993; 14: 1397–1401.

[9] RIDDICK JA, BUNGER WB. In: A. Weissberger, editor. Organic solvents, Vol. II. N,N-dimethylformamid. New York, Wiley-Interscience 1970: 836–840.

[10] NOVOTNY L, VACHALKOVA A. Some electrochemical characteristics of synthetic analogs of nucleic acid components. I. Derivates of cytidine and arabinosylcytosine. An attempt to find correlation among some of their properties. Neoplasma 1990; 37: 377–386.

[11] VACHALKOVA A, NOVOTNY L. Polarographic testing of carcinogenicity of some chemotherapeutics. Neoplasma 1997; 44: 389–394.

[12] NETTER KJ. A method for the direct measurement of O-demethylation in liver microsomes and its use in studying microsome inhibition by SKF-525-A. Naunyn-Schmiedebergs Arch Exp Path Pahrmacol 1960; 238: 292–300.

[13] ORRENIUS S, ERNSTER, L. Interaction between liver microsomes and compounds capable of undergoing enzymic hydroxylation. Life Sci 1967; 6: 1473–1482.

[14] DENT JG, GRAICHEN ME, SCHNELL S, LASKER J. Constitutive and induced hepatic microsomal cytochrome P-450 monooxygenase activities in male Fischer-344 and CD rats A comparative study. Toxicol Appl Pharmacol 1980; 52: 45–53.

[15] SCHACTERLE GR, POLLACK RL. A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal Biochem 1973; 51: 654–655.

[16] MASSEY V. The microestimation of succinate and the extinction coefficient of cytochrome c. Biochim Biophys Acta 1959; 34: 255–256.

[17] AZZI A, MONTECUCCO C, RICHTER C. The use of acetylated ferrycytochrome c for the detection of superoxide radicals produced in biological membranes. Biochem Biophys Res Commun 1975; 65: 597–603.

[18] GIARD DJ, AARONSON SA, TODARO GJ, AMSTEIN P, KERSEY JH et al In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973; 51: 1417–1423.

[19] MARTIN CN, MCDERMID AC, GARNER RC. Testing of known carcinogens and noncarcinogenes for their ability to induce unscheduled DNA synthesis in HeLA cells. Cancer Res 1978; 38: 2621–2727.

[20] OECD Guideline for Testing of Chemical Genetic Toxicology. DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells in Vitro 1986; 482: 1–6.

[21] MITCHELL AD, CASCIANO DA, MELLZ ML, ROBINSON DE, SAN RHC et al. Unscheduled DNA synthesis test: a report of the U.S. Environmental Protection Agency Gene Tox Program. Mutation Res 1983; 123: 363–410.

[22] MARON DM, AMES BN. Revised methods for the Salmonella mutagenicity test. Mutation Res 1983: 113: 173–215.

[23] COJOCEL C, NOVOTNY L, VACHALKOVA A, KNAUF B. Comparison of the carcinogenic potential of streptozotocin by polarography and alkaline elution. Neoplasma 2003; 50: 110–116.

[24] HINEGARDNER RT. An improved fluorometric assay for DNA. Anal Biochem 1971; 39: 197–201.

[25] GABELOVA A, SLAMENOVA D, RUZEKOVA L, FARKASOVA T, HORVATHOVA E. Measurement of DNA strand breakage and DNA repair induced with hydrogen peroxide using single cell gel electrophoresis, alkaline DNA unwinding and alkaline elution of DNA. Neoplasma 1997; 44(6): 380–388.

[26] TYSON CK, MIRSALIS JC. Measurement of unscheduled DNA synthesis in rat kidney cells following in vivo treatment with genotoxic agents. Environ Mutagen 1985; 7: 889–899.

[27] JUNG R, COJOCEL C, MULLER W, BOTGER D, LUCK E. Evaluation of the toxic potential of sorbic acid and potassium sorbate. Fd Chem Toxic 1992; 30: 1–7.

[28] MULLER W, COJOCEL C, KRAMER W, MAYER, D. Absence of genotoxic activity of penbutolol in bacterial and mammalian cell screening systems. Mutation Res 1990; 242: 135–142.

[29] FUNATO K,MIYAZAWA K, YAGUCHI M, GOTOH A, OHYASHIKI K. Combination of 22-oxa-1,25-dihydroxyvitamin D(3), a vitamin D(3) derivative, with vitamin K(2) (VK2) synergistically enhances cell differentiation but suppresses VK2-inducing apoptosis in HL-60cells. Leukemia 2002; 16(8): 1519–1527.

[30] GELBOIN HV. Benzo[a]pyrene metabolism, activation and carcinogenesis. Role and egulation of mixed-function oxidases and related enzymes. Physiol Rev 1980; 60: 1107–1166.

[31] AMES BN, MCCANN J, YAMASAKI E. Methods for detecting carcinogenesis and mutagenesis with the Salmonella/mammalian microsome mutagenecity tests. Mutation Res 1975; 31: 347–364

[32] WILLS ED. Effects of vitamin K and naphthoquinones on lipid peroxide formation and oxidative demethylation by liver microsomes. Biochem Pharmacol 1972; 21: 1879–1883.

[33] GALIEGUE-ZOUITINA S, BAILLEUL B, GINOT YM, PERLY B, VIGNY P, LOUCHEUX-LEFEBRE MH. N2-guanyl and N6-adenylarylation of chicken erythrocyte DNA by the ultimate carcinogen 4-nitroquinoline 1-oxide. Cancer Res 1986; 46(4): 1858–1863.

[34] SUGIMURA T, OKABE K, NAGAO M. The metabolism of 4-nitroquinoline-1-oxide, a carcinogen. 3. An enzyme catalyzing the conversion of 4-nitroquinolie-1-oxide to 4-hydroxyaminoquinoline-1- oxide in rat liver and hepatomas. Cancer Res 1966; 26(8): 1717 -1721.

[35] KOHDA K, TADA M, KASAI H, NISHIMURA S, KAWAZOE Y. Formating of 8-hydroxyguanine residues in cellular DNA exposed to the carcinogen 4-nitroquinoline 1-oxide. Biochem Res Commun 1986; 139(2): 626 -632.

[36] NOTO V, TAPER HS, JIANG YH, JANSSENS J, BONTE J, DE LOECKER W. Effects of sodium ascorbate (vitamin C) and 2-methyl 1,4-naphthoquinone (vitamin K3) treatment on human tumor cell growth in vitro. I. Synergism of combined vitamin C and K3 action. Cancer 1989; 63: 901–906.

[37] WAXMAN S, BRUCKNER H. The enhancement of 5-fluorouracil anti-metabolic activity by leucovorin, menadione and alpha-tocopherol. Eur J Cancer Clin Oncol 1982; 18: 685–692.

[38] NUTTER LM, CHENG AL, HUNG HL, HSICH RK, NGO EO, LIU TW. Menadione: spectrum of anticancer activity and effects on nucleotide metabolism in human neoplastic cell lines. Biochem Pharmacol 1991; 41: 1283–1292.

[39] GOLD J. In vivo synergy of vitamin K3 and methotrexate in tumor-bearing animals. Cancer Treat Rep 1986; 70: 1433–1435.

[40] ONRUST H, WOSTMANN B. Rec Trav Chim 1950; 69: 1207. In: J. Prozst, V. Cielszki, K. Gyorbiro: Polarographie mit besonderer berucksichtigung der klassischen Methoden. Akademiai Kiado, Budapest 1967: 445–446.

[41] MNOUCEK K. Contribution to polarographic and spectral studies of water-soluble vitamine K3 (menadione-natriumbisulfite). Ceskoslovenska farmacie 1970; XIX (4): 133–138 (in Czech).

[42] HITOMI M, NONOMURA T, YOKOYAMA F, YOSHIJI H, OGAWA M et al. In vitro and in vivo antitumor effects of vitamin K5 on hepatocellular carcinoma. Int J Oncol 2005; 26: 1337–1344.

[43] GANT TW, RAO DN, MASON RP, COHEN GM. Redox cycling and sulphydryl acrylation; their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. Chem Biol Interact 1988; 65: 157–173.

[44] NISHIKAWA Y, CARR BI, WANG M, WANG M, KAR S et al. Growth inhibition of hepatoma cells induced by vitamin K and its analogs. J Biol Chem 1995; 270: 28304–28310.

[45] BROWN PC, DULIK DM, JONES TW. The toxicity of menadione (2-methyl-1,4-naphthoquinone) and two thioether conjugates studied with isolated renal epithelial cells. Arch Biochem Biophys 1991: 285: 187–196.

[46] NUTTER LM, NGO EO, FISHER GR, GUTIERREZ PL. DNA strand scission and free radical production in menadione-treated cells. Correlation with cytotoxicity and role of NADPH quinone acceptor oxidoreductase. J Biol Chem 1992; 267: 2474–2479.

[47] ROSS D, THOR H, THREADGILL MD, SANDY MS, SMITH M et al. The role of oxidative processes in the cytotoxicity of substituted 1,4-naphthoquinones in isolated hepatocytes. Arch Biochem Biophys 1986; 248: 460–466.

[48] NIEMCZYK E, MAJCZAK A, HALLMANN A, KEDZIOR J, WOZNIAK M, WAKABAYASHI T. A possible involvement of plasma membrane NAD(P)H oxidase in the switch mechanism of the cell death mode from apoptosis to necrosis in menadione-induced cell injury. Acta Biochim Pol 2004; 51(4): 1015–1022.

[49] CARICCHIO R, KOVALENKO D, KAUFMANN WK, COHEN PL. Apoptosis provoked by the oxidative stress inducer menadione (Vitamin K(3)) is mediated by the Fas/Fas ligand system. Clin Immunol 1999; 93: 65–74.

[50] BOUCHARD C, STALLER P, EILERS M. Control of cell proliferation by Myc. Trends Cell Biol 1998; 8: 202–206.

[51] COLE MD, MCMAHON SB. The Myc oncoprotein: a critical evaluation of transactivation and target gene regulation. Oncogene 1999; 18: 2916–2924.

[52] JAMISON JM, GILLOTEAUX J, NASSIRI MR, VENUGOPAL M, NEAL DR, SUMMERS JL. Cell cycle arrest and autoschizis in a human bladder carcinoma cell line following Vitamin C and Vitamin K3 treatment. Biochem Pharmacol 2004; 67: 337–351.

[53] WU FY, SUN TP. Vitamin K3 induces cell cycle arrest and cell death by inhibiting Cdc25 phosphatase. Eur J Cancer 1999; 35: 1388–1393.

[54] WU FY, CHANG NT, CHEN WJ, JUAN CC. Vitamin K3-induced cell cycle arrest and apoptotic cell death are accompanied by altered expression of c-fos and c-myc in nasopharyngeal carcinoma cells. Oncogene 1993; 8: 2237–2244.