Cytotoxic and apoptotic effects of menadione on rat hepatocellular carcinoma cells

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Abstract Hepatocellular carcinoma (HCC) is one of the most common cancers, which may lead to death. Menadione shows cytotoxic activity thought affecting redox cycling in cancer cells. The aim of the present study was to investigate the effects of menadione on rat hepatocellular carcinoma (H4IIE) cell morphology, cytotoxicity, apoptosis and DNA damage or repair in vitro. Cell morphology evaluated by microscopy and cell viability was determined using the 3-[4,5-dimethylthiazol-2yl]-diphenyltetrazolium bromide test. Apoptotic cell death was assessed in H4IIE cells treated with menadione by 4',6-diamidino-2-phenylindole staining. Quantitative real time polymerase chain reaction used to determine the expression level of *poly (ADP-ribose) polymerase 1 (PARP1)* gene. According to the results of this study menadione has got a cytotoxic activity (IC_{50} 25 μ M) and change the cell fate in H4IIE cells. Menadione treatments lead to *PARP1* activation in a dose dependent manner and induce DNA damage and

apoptosis, and this may suggest its use as a therapeutic agent in HCC treatment.

Keywords Menadione · HCC · Therapeutic · Cell morphology · Cytotoxicity

Introduction

Hepatocellular carcinoma (HCC) which is derived from the hepatocytes, is one of the most common cancers in the world (El-Serag and Rudolph 2007). It is the third most common cause of cancer mortality (Wong and Ng 2008). It occurs more often in men than women and it has high incidence among the people who are older than 50 years of age (Parikh and Hyman 2007). Over 80 % of HCC occur in developing countries such as Sub-Saharan Africa, East and South East Asia (Wong and Ng 2008) where as North and South America, Northern Europe, and Oceania have low-rates of this cancer (El-Serag and Rudolph 2007). HCC has found to be related with several risk factors, including chronic viral hepatitis (HBV), hepatitis C viral (HCV) infection (Kapoor and Kumar 2012), alcohol consumption, tobacco (Wong and Ng 2008) and aflatoxin ingestion (Choi et al. 2010). Chronic HBV and HCV infections are very important precursors for HCC development on a global scale (Wong and Ng 2008).

Since, most of the patients developing HCC have no symptoms; diagnosis can be difficult (Yang et al.

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2002). HCC is an aggressive tumour and its prognosis is very poor, the median survival is only a few months (Yang et al. 2002). Treatment of HCC is depending on tumour size and staging; high-grade tumours have worse prognosis (Motola-Kuba et al. 2006). Surgery is the main curative treatment for HCC, liver transplantation is another treatment option on the other hand non-surgical methods such as trans catheter, arterial chemoembolization (Liang et al. 2011) also exist. Radiotherapy or chemotherapy treatments are ineffective and the results are unsatisfactory (Leung et al. 2000; Xu et al. 2011). Since HCC has a highly chemo-resistant nature (Yang et al. 2002) more effective and less toxic therapeutic agents are needed.

Menadione, known as vitamin K3, is a synthetic derivative of vitamin K and it has been used as a cytotoxic agent for rodent and human cancer cell lines (Lamson and Plaza 2003; Acharya et al. 2009). It is shown to enhance the cytotoxic effect of some anticancer agents (Matzno et al. 2008) including action against multidrug resistant human cancer cell lines (Nutter et al. 1991). Previous studies demonstrated that menadione kills malignant cells by inducing oxidative damage (Takahashi et al. 2009; Wu et al. 2011). When menadione enters the cell, reactive oxygen species (ROS) are reduced through redox cycling of quinone. The cytotoxic effect of menadione is thought to be mediated through its one or two electron reduction to semiquinone or hydroquinone radicals, which subsequently enter redox cycle with molecular oxygen to produce ROS and oxidative stress (Ngo et al. 1999). Previous studies reported that high levels of ROS leads to cell damage and toxicity by lipid peroxidation leading to breaks in DNA, arrest in cell cycle and induces apoptosis in vitro (Akiyoshi et al. 2009; Loor et al. 2010).

In this study we aimed to investigate the therapeutic potential of menadione on rat hepatocellular carcinoma (H4IIE) cell line in vitro and showed an enhanced DNA damage and up regulated *poly (ADP-ribose) polymerase 1 (PARP1)* activity in the context of menadione's cytotoxic effect.

Materials and methods

Chemicals, cell culture and treatments

Rat hepatocellular carcinoma (H4IIE) cells were obtained from American Type Culture Collection

(ATCCC, Manassas, VA, USA). All chemicals were purchased from Sigma (St. Louis, MO, USA). H4IIE cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) containing 10 % foetal bovine serum (FBS), 1 % penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C in 5 % CO₂ atmosphere. When confluence was achieved, cells were seeded in 96 well micro titre plates (1×10^4 cells/well) and incubated for 24 h. Cell viability was accessed by trypan blue dye exclusion and found to be higher than 98 %.

Water-soluble form of menadione (sodium bisulphite) was dissolved in DMEM. 5-Fluorouracil (5-FU) was used as positive control and dissolved in dimethyl sulfoxide (DMSO), then diluted in DMEM at a ratio of 1:10 and the maximum concentration of DMSO was adjusted to 0.01 %. Freshly prepared test compound concentrations for menadione were as follows: 1, 10, 25, 50, 75, and 100 µM and for 5-FU 10, 50, 100, 150, 200 and 250 µM were added to the growth medium for 24 h. Each tested concentration was inoculated in eight wells and each experiment was performed in triplicates.

Effects of menadione doses on cell morphology

To determine morphological effects of menadione on H4IIE cells, 1×10^6 cells in 5 mL medium were seeded into 25 cm² flasks. The cells were treated with 1–100 µM menadione doses for 24 h and morphological effects of menadione were detected under an inverted light microscope (Olympus CK2, Tokyo, Japan).

MTT assay

Menadione toxicity was determined by using the 3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide (MTT) colorimetric assay (Mosmann 1983). MTT dye (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. MTT solution has been converted into blue formazan by the mitochondrial dehydrogenase activity of the viable cells. The resulting blue formazan crystals were dissolved in 100 µL DMSO and measured at 550 nm wavelength (ELx 800, Bio-Tek Instruments, Winooski, VT, USA). Percentage of the viable cells was calculated with the following formula: Absorbance of treated cells in each well $\times$ 100/the mean absorbance of control cells (Cao et al. 2010).

Control wells included native cells and medium. Data were given as the mean percent fraction of

control $\pm$ standard error of mean (SEM). Statistical significance was ascertained by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. The Statistical Package for the Social Sciences (SPSS 12.0 for Windows) was used for statistical analyses. A p value less than 0.05 was considered to be significant.

Detection of apoptosis

Alterations in the morphology and chromatin structure of H4IIE cells which underwent apoptosis, were examined by fluorescence dye 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (Li et al. 2009). A previously reported protocol was used for DAPI staining (Park et al. 2007). Cells were seeded in a 24 well plate (3×10^4 cells/well) and cultured for 24 h and were treated with or without 25 and 50 μ M menadione. At the end of 24 h, cells were collected and fixed in 0.5 % paraformaldehyde for 2–3 h at room temperature. After fixation, slides were incubated in 70 % cold ethanol for 15 min and washed with phosphate buffer saline (PBS) for 10 min. Slides were stained with 1 μ g/mL DAPI in the dark for 10 min and were rinsed with PBS 3 times for 10 min. At least 300 cells per condition were subjected to examination using a digital fluorescence microscope (Leica CTR 6000, Wetzlar, Germany).

Quantitative real time PCR (qRT-PCR)

Total RNA was isolated by QiagenRNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) RNA quality and quantity were checked with Nanodrop 1000 (Thermo Fisher Scientific, Schwerte, Germany) and cDNA was synthesized by random hexamers and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, from 1 μ g of total RNA according to manufacturer protocol (MBI Fermentase Life Sciences, Vilnius, Lithuania). Quantitative Real Time PCR (qRT-PCR) was carried out on Light Cycler 480 Instrument (Roche Applied Sciences, Mannheim, Germany). *PARP1* expression levels were normalized to *Cyclophilin A* (*CYPA*) gene. The specific primer-probe sets were designed and used from Roche Universal Probe Library (probe no #22 for *PARP1* and probe no:#48 for *CYPA*). The primers of *PARP1* were as follows: forward, 5' TCTTTGATGTGGAAAGTATG AAGAA 3'; and reverse, 5' GGCATCTTCTGAAGG

TCGAT 3'. The primers of *CYPA* were as follows: forward, 5' CCTAAAGCATACGGGTCCTG 3'; and reverse, 5' CACTTTGCCAAACACCACAT 3'. The real time amplification was performed as described by the manufacturer's protocol (Roche Applied Sciences). Relative mRNA levels were calculated according to the delta Ct method, based on the mathematical model (Livak and Schmittgen 2001). Differences between the relative expression levels of controls and treatment doses were tested with two-sided Mann–Whitney U test by GraphPad Prism V (USA). A p value of 0.05 or less was considered as statistically significant.

Results and discussion

Effects of menadione on cell morphology

In the present study, menadione doses of 1 and 10 μ M did not effect on cell morphology or count compared to the controls. Cell count started to decrease at 25 μ M. Menadione concentrations of 50, 75 and 100 μ M changed cell morphology and declined the cell count. Moreover, cells were observed more spherical and smaller at these concentrations (Fig. 1). Increased concentrations of menadione causes rounding and small cell morphology as well as declined cell count. Several studies also support our findings. For example, Abe and Saito demonstrated that menadione led to change in the cell morphology and count of rat cortical astrocytes in a dose-and time-dependent manner (Abe and Saito 1996). Another study by Warren et al. (2000) reported morphological changes in the endothelial cells (ECs) after treatment with 35 μ M menadione within 18 h.

Determination of cytotoxicity by MTT assay

Menadione concentrations of 1 and 10 μ M were compared to the controls and no significant differences were observed in the H4IIE growth ($p > 0.05$). However, at 25, 50, 75 and 100 μ M menadione concentrations inhibited cell survival for 24 h ($p < 0.001$, Fig. 2). At the concentrations of 25, 50, 75 and 100 μ M, decreased H4IIE cell viability was observed at 24 h (50.6, 75.0, 72.9 and 71.6 %, respectively). IC_{50} value of menadione for H4IIE was estimated at 25 μ M. The cytotoxic activity of

Fig. 1 Cellular morphological changes in the H4IIE cells after treatment with menadione doses for 24 h (C control, Scale bar 40 μ m)

Fig. 2 The effects of menadione on H4IIE cell survival for 24 h (C control, *** $p < 0.001$ by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests). An asterisks (*) represents a significant difference $p < 0.05$ from the control cells

Fig. 3 The effects of 5-FU on H4IIE cell survival for 24 h (C control, *** $p < 0.001$ one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests). An asterisks (*) represents a significant difference $p < 0.05$ from the control cells

menadione has been shown in a number of studies using both rodent and human cancer lines (Ogawa et al. 2007; Osada et al. 2008; Acharya et al. 2009). Menadione has anti-proliferative effect on human hepatoma cell lines and IC_{50} value was determined as 10 μ M for human hepatoma (Hep3B) cells for 72 h (Nishikawa et al. 1995), 8 μ M for 18 h (Sasaki et al. 2008) and 13.7 μ M for human hepatoblastoma (HepG2) cells (Matzno et al. 2008) for 24 h. There are no previous data for menadione toxicity on rat hepatoma cell lines. 5-FU was used as positive control and a cytotoxic effect started at 10 μ M. Although 250 μ M is the highest concentration of 5-FU, inhibited cell viability was only 32 % ($p < 0.001$, Fig. 3). While 100 μ M menadione reduced the cell viability up to 72 %, the highest dose of 5-FU (250 μ M) reduced the cell viability up to 32 % in 24 h. Dimethyl sulfoxide (DMSO) had no effect on cell viability at all

concentrations tested. These findings, showed that menadione is more effective than 5-FU. It is possible to conclude that growth inhibitory effects of menadione change over time, cell types and culture conditions.

Detection of apoptosis

Cells were treated with 25 or 50 μ M menadione and stained with DAPI to detect the nuclear morphology after 24 h. Although untreated cells displayed a normal nuclear size, menadione treated and stained cells showed typical morphological features of apoptotic cells, with condensed and fragmented nuclei. Apoptotic changes have started at 25 μ M dose and increased parallel with the dose increase to 50 μ M. The results indicated that menadione induced apoptosis in a dose dependent manner (Fig. 4). Similarly,

Fig. 4 Nuclei were stained with DAPI (blue). **a** Control cells (no treatment). 25 and 50 μM menadione treated cells showed condensed and fragmented chromatin (**b**, **c**). Arrows demonstrate

apoptotic nuclei with condensed chromatin. Original magnification $\times 1,000$. (Color figure online)

Warren et al. (2000) monitored apoptotic ECs cells after menadione treatment (35 and 75 μM) by DAPI staining and Zhang et al. (2003) showed membrane blebbing on leukemic cells and primary retina cells which were treated with 50 μM menadione for 6 h.

Detection of *PARP1* mRNA levels by qRT-PCR

PARP1 mRNA level was examined and compared in the menadione treated (25 and 50 μM) and non-treated cells. *PARP1* mRNA levels were found to be significantly higher (3.5 fold increase for 25 μM and 4.5 fold increase for 50 μM) ($p < 0.001$, by Mann–Whitney test) in the cells treated with menadione (Fig. 5). *PARP1* gene is coding a nuclear DNA repair enzyme whose activity increases in response to DNA damage or single and double stranded DNA breaks and apoptosis (Hong et al. 2004; Barton et al. 2009; Gonçalves et al. 2011). *PARP1* protein associates with chromatin structure and accumulated in the nucleoli. *PARP1* binds to oxidative damage-induced strand break thorough two zinc finger motifs and becomes activated (Altmayer and Hottiger 2009). In vivo and in vitro studies showed that *PARP1* activation made chromatin denser and compact that may down regulate tumour related gene expression (Ji and Tulin 2010). It has been known that menadione induced oxidative damage on DNA causes to excessive *PARP1* activation, triggers cytochrome c, AIF release from mitochondria and initiates the early stages of apoptotic activation (Loor et al. 2010). To test this hypothesis, we performed a qRT-PCR analysis and determined up

Fig. 5 Relative levels of *PARP1* mRNA expression in H4IIE cells treated with 25 and 50 μM menadione at 24 h. (C control, ** $p < 0.01$, by Mann–Whitney test). An asterisks (*) represents a significant difference $p < 0.05$ from the control cells

regulated *PARP1* gene expression by 25 and 50 μM doses of menadione. According to the results of this study, menadione treatment led to *PARP1* activation in a dose dependent manner and induced DNA damage and apoptosis. We can conclude that this activation causes the repression of this transcriptional machinery in rat hepatoma cells. Comparable data were reported previously that about 50 μM menadione increased the *PARP1* cleavage in primary rat hepatocyte for 9 h (Conde de la Rosa et al. 2006).

Conclusion

In this study a new anticancer agent, menadione, was examined for the first time in rat hepatoma cells and

obtained some preliminary results about the cytotoxic and apoptotic effects of this agent. This study also provided some evidence about the menadione induced ROS mediated activation of *PARP1*, which will be addressed in further functional research.

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