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ORIGINAL ARTICLE

Molecular mechanisms of leptin and pro-apoptotic signals induced by menadione in HepG2 cells

Ebtesam Al-Suhaimi *

Biology Department, College of Sciences, University of Dammam, Dammam, Saudi Arabia

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KEYWORDS

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P^{53};
Caspase-3;
ROS

Abstract Apoptosis is a significant physiological function in the cell. P^{53} is known as tumor suppressor cellular factor, executive caspases are also the most involved pathway for apoptosis. Menadione (VK3) has apoptotic action on many harmful cells, but the molecular role of adipokines is not studied enough in this regard, so the ability of menadione to modify the adipokine (leptin hormone), caspase-3 and P^{53} signals to induce its apoptotic action on HepG2 cells was studied. The study revealed that menadione has anti-viability and apoptotic effect at sub-G1 phase of HepG2 cell cycle. Its cytotoxic effect is mediated by molecular mechanisms included: inhibiting leptin expression and level, activating caspase-3 pathway and up-regulating the expression of P^{53}. Menadione exerts its apoptotic mechanisms in a concentration and time dependent way through ROS generation. In addition to the known apoptotic pathways, the results indicate that suppressing leptin pathway is a significant mechanism for menadione apoptotic effect which made it as a potential therapeutic vitamin in preventing hepatocyte survival and proliferation.

1. Introduction

Vitamin K is an important nutritional factor contributing to the blood clotting process, its different types possess an inhibiting growth effect on many tumor cells types in vivo, studies showed that menadione (Vitamin K3) has potent inhibition effect on cell growth more than K2 and K1 (Wu et al., 1993; Lamson and Plaza, 2003; Hitomi et al., 2005). Previous studies considered menadione as apoptosis (programmed cell death) and antitumor inducing factor on hepatic cells (Tong et al., 1997; Hitomi et al., 2005). Among many agents Baran et al. (2012) reported that menadione is a potent inducer of apoptosis. It shows cytotoxic effects in many types of tumor cells (Suresh et al., 2013). P^{53} (Tumor suppressor transcription factor) has an essential physiological role in cell growth regulation by its apoptotic function in the cells to achieve balanced growth. At normal circumstances, P^{53} expression is low because of its limited needs which result in its speed inactivation but it has an ability to be more stable, active and accumulative in response to activator signals such as DNA destruction, then its transcriptive and expression level increase leading to disturbance in the cell cycle which revealed the apoptotic features (Naderi et al., 2009). P^{53} prevents tumor evolution and progression and activates death of cells subjected to anti-tumor therapy which made P^{53} as "guardian angel" as described by Royds and Iacopetta, 2006. In addition to P^{53}, caspases in

* Tel.: +966 133337007.
E-mail address: ealsuhaimi@ud.edu.sa.

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general are important mediators in apoptosis, executive caspase-3 is the most involved pathway which should be generated from its inactive protein (procaspase-3), caspase 3 is required for some apoptosis features (chromatin condensation, DNA damage and apoptotic body formation) and its role may take place before cell viability suppression starts (Porter and Jänicke (1999). Caspases also have an apoptotic effect on hepatocellular carcinoma (HepG2) which was stimulated by caspase-3 gene expression (Tong et al., 1997). On the other hand, data of Samali et al. (1999) revealed that menadione induces necrosis in HepG2 cell line and suppresses Caspase activity by generation of H_2O_2 and this oxidative stress model could suppress cell death by apoptosis, but reducing the levels of ROS by catalase reversed the inhibitory effect of menadione on Caspase activity. Bonilla-Porras et al. (2011) confirmed that treatment leukemia cells with VK3 and VC individually or together activated apoptosis by molecular signals included ROS generation, activation transcription factors included P^{53}, depolarization of the mitochondria and activation of caspase-3 pathway. It is found also that VK2 and VK3 have an arresting effect on human hepatocellular carcinoma cell cycle at G1 (Hitomi et al., 2005) but Quercetin is one of the flavonoids which decreased apoptosis and prolonged cell cycle phase G_2/M arrest stimulating by radiation (Baran et al., 2012). In contrast, it seems that leptin (the adipose tissue hormone involved in energy metabolism) has anti- apoptotic effect, it could induce glial viability in dose dependent manner, but cannot abolish H_2O_2 induced oxidation in primary mixed glial cell (Kabadere et al., 2007). Leptin also stimulated ovarian cancer cell line growth through up-regulating the genes and proteins required for cell proliferation as well as down-regulating the factors related to its pro-apoptotic effect (Ptack et al., 2013). Recent study originated connection's model between adipose tissue and hepatocytes (Nishijima-Matsunobu et al., 2013). Menadione has selective effect, Suresh et al. (2013) reported that menadione is a potential candidate in oral cancer therapy because of its high cytotoxic effect on oral squamous carcinoma cells (SAS) but not on non-tumorigenic cells (HEK293 and HaCaT), in SAS, menadione treatment increased expression of apoptosis regulator (Bax and p53) as pro-apoptotic proteins and at the same time decreased anti-apoptotic proteins such as Bcl-2 and p65. Goldstein et al. (2013) described the mechanism which made p53 an inhibitor of tumorigenesis in liver cells. Menadione is a well known cytotoxic agent which has potential role against many cancer cell lines. Therefore this study was conducted to highlight its suppressing effect on HepG2 cell viability as well as to evaluate the potential molecular signaling involved in apoptosis, to be presented as a possible apoptotic approach in preventing liver cell survival and proliferation.

2. Materials and methods

2.1. Cell culturing and treatment

Hepatocellular carcinoma (HepG2) cells were obtained from American Type Cell Culturing (ATCC HB-8065) and incubated in moistened environment at 37 °C and 5% CO_2, the cells were cultured at a density of 5×10^5 in DMEM medium mixed with L-glutamine, supplemented with fetal bovine serum (FBS), penicillin Streptomycin (Gibco, Invitrogen Corporation, USA). After 70% cell gathering, the cells were sub cultured using a standard trypsin-EDTA method. After cell culturing and confluence (Lu et al., 2008; Lee et al., 2012; Yeo et al., 2012), HepG2 cells were treated with different concentrations of menadione for different periods as described below.

2.2. Effect of menadione on cell viability

Cells viability was determined by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) (Mosmann, 1983), the HepG2 cells were cultured into 96 well microtiter plates at a density of 2×10^5 cells per well. After 24 h the media were incubated with different concentrations of menadione (Sigma Aldrich) for 24 h, then treated with MTT reagents and other involved solutions. After removing unbound stain, the blue formazan crystal compound was dissolved, its absorbance was measured on an ELISA reader at 570 nm. Control growth is considered 100%, viability % was calculated (sample/control × 100) (Hsu and Yen, 2006). The cell culture was repeated to assess dose dependent effect of menadione using concentration of 5, 10, 20 and 40 μM for 24 h. 10 μM menadione was used to investigate time dependent effect (for periods 6, 12, 24 and 36 h) and for further assays.

2.3. Menadione apoptotic assay by FACS

After treatment with (10 μM) menadione at indicated time, cell cycle progress was analyzed by Fluorescence activated cell sorter (FACS), the media were centrifugated to collect the cells, then fixed, the cell suspension was kept at 4 °C for overnight to stain the fragmented molecules of DNA with Propidium iodide (PI) reagents, the stained DNA was analyzed by flow cytometer (Becton Dickinson) (Lee et al., 2012).

2.4. Western blotting for molecular signal detection

Post treatment with menadione at the indicated time as described above, the cells were washed with ice-cold 1× PBS and collected. After gathering, the cells were centrifuged, and then total protein was extracted using lysis buffer which was added to re-suspend and subjected to ultrasonication and then cells were centrifuged to separate cell debris. The supernatant was kept at −20 °C until further use (Reyes-Leon et al., 2007; Naviglio et al., 2009). Immuno blotting was carried out using antibodies against p53 (sc-99), caspase-3 (sc-7148), leptin (ab3583) and β-actin (sc-47778) (Santa Cruz Biotechnology and Abcam).

2.5. Measurement of ROS generation

Intracellular ROS generation was evaluated by the oxidant-sensitive fluorescent probe, DCFHDA and inverted microscope. HepG2 cells were incubated at a density of 2×10^5 cells per dish and pretreated for 2 h with or without N-acetylcysteine (NAC), and then treated with menadione (10 μM) for 24 and subjected to 5 μM H2DCFDA for 20 min and washed with 1× PBS. An inverted microscope (Zeiss Axiovert 200) was used for imaging DCF fluorescence (excitation 480 nm and emission 520 nm) Lee et al., 2012; Yeo et al., 2012). H2DCFDA was purchased from Invitrogen Molecular Prob.

2.6. Caspase-3 activity assay

After incubation with 10 μM menadione, cell culture was washed with cold 1× PBS and treated with lysis buffer, the contents of the wells were pooled and centrifuged at 4 °C. The reaction buffer was incubated with the supernatant which also incubated with colorimetric agent Ac-DEVD-pNA (caspase-3 substrate) (Promega) at 37 °C for 1 h. Absorbance (A) of Caspase activity was measured at 405 nm, it was calculated according to "A/mg of protein in menadione treated sample/(A/mg of protein in control sample)" (Kil et al., 2007; Thayyullathil et al., 2013).

2.7. Leptin assay

A specific Kit from Cayman Chemical was used for assaying human leptin as described by Wu et al. (2007). The concentration of leptin in the supernatant of menadione treated HepG2 cell culture media was measured as per the manufacturer's instruction based on a double-antibody sandwich principle. Incubation with specific antibodies for 60 min was done twice, at first, 50 μl from culture media was added to 96-wells to incubate with pre-coated solid polyclonal antibody at room temperature, after shaking, and washing with washing buffer then the second incubation was run with peroxidase-conjugated anti-leptin antibody. The unbound reagents were removed by washing while the absorbance of the bound double-antibody complexes was measured spectrophotometrically at 450 nm.

2.8. Statistical analysis

The assays were carried out three times for the statistical evaluation. Data were introduced as mean ± SD, analysis of variance and LSD were done by SPSS ver. 19. $P < 0.05$ was considered for significance differences.

3. Results

3.1. Cytotoxicity of menadione on HepG2 cells

Menadione inhibits HepG2 cell viability. The cytotoxicity of menadione on HepG2 cells was evidenced by its ability to reduce the cell viability with concentration increasing as shown in Fig. 1A. LSD test showed significantly that most HepG2 cells were dead after incubation with 20, 40 μM for 24 h, so

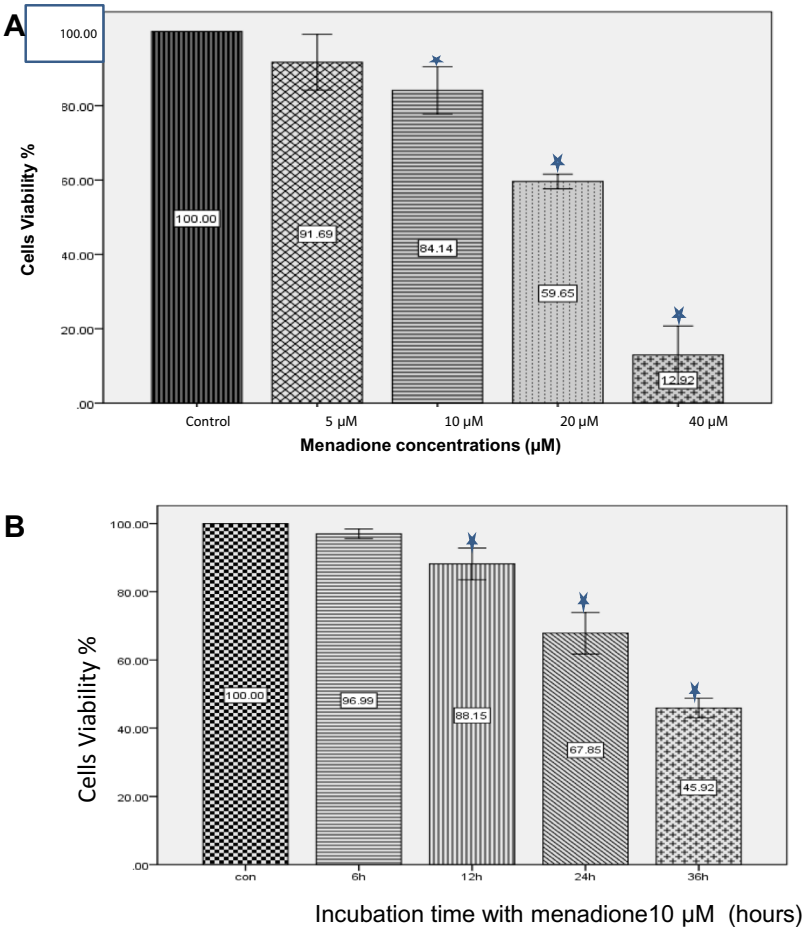

Figure 1 (A) HepG2 cells were incubated with different concentrations (5–40 μM) of menadione for 24 h. Cell viability was assessed by MTT assay. Data present mean ± SD, where $p \geqslant 0.05$. (B) HepG2 cells were incubated with menadione (10 μM) for indicated time. Cell viability was assessed by MTT assay. Data present mean ± SD, where $p \geqslant 0.05$.

10 μM was selected for menadione time dependent effect, it showed an inhibitory action on cell viability and increases with time at $P < 0.05$ as shown in Fig. 1B. The dose was also used for further assays.

3.2. Menadione induced time dependent apoptosis

HepG2 cells were treated with 10 μM menadione for 3, 6, 12 and 24 h and staining with PI, then the cell's DNA content was analyzed by flow cytometry. FACS assay showed that menadione increased cell fraction at sub-G1 phase in a time dependent way (Fig. 2A).

3.3. Meadione modifies molecular leptin and other signals expression

Menadione treatment decreases leptin expression (Fig. 4B), while there was an up-regulation in the expression of P^{53} with increasing time (Fig. 2B). The results also revealed activation in caspase-3 in a time-dependent manner at protein level as the enzyme cleavages from its inactive form pro-caspase 3, which exhibited a decrease in its expression (Fig. 3B).

3.4. Menadione activiates caspase-3

Post 10 μM menadione treatment, caspase-3 activity of the treated sample was elevated in a time dependent way compared with control sample as shown in Fig. 3A.

3.5. Menadione decreases leptin levels

Leptin levels in menadione treated culture media were measured by ELISA and revealed that menadione (10 μM) decreased leptin levels in a time dependent way (Fig. 4A).

3.6. ROS generation by menadione

Cell fluorescence was imaged by an inverted microscope and revealed that 10 μM menadione alone induces oxidative stress in HepG2 cells compared with control cells while treatment of cells with (menadione – pre treated with NAC) followed by incubation with H2DCFDA reversed menadione ROS generation effect as shown in Fig. 5.

4. Discussion

It has been shown that menadione has cytotoxic effect on many harmful cells types, but some certain signals for its mechanism of action are not fully studied such as the adipokine leptin molecular signal in association with other proapoptotic factors. The study confirmed menadione viability reducing effect on HepG2 cells which agreed with the finding of Chen and Cederbaum (1997) who found the cytotoxic effect of menadione on HepG2 cells. Also Wu et al. (1993) showed that VK3 antiproliferative activity was more potent than VK1 and VK2 in many cell lines including HepG2 cells by two assays (MTT and SRB (sulforhodamine B). Such action was reported for VK2 and VK3 on human hepatocellular carcinoma (Hitomi et al., 2005).

MTT assay showed that the viability of HepG2 decreased with increasing concentration of menadione, 10 μM concentration was used for time dependent effect and further analysis. Our result indicated that menadione has an antiviability effect on the cells in a concentration–time dependent way, the result agreed with Sata et al. (1997). Concentrations more than 10 μM were not used in order to prevent any potential side effects because Sata et al. (1997) found that although menadione at low concentration 1–20 μM and time dependent way inhibited cell proliferation and caused apoptosis evidenced by DNA indicative indicative but higher concentration of menadione (100 μM) revealed cell death more than 90% during 4 h and caused necrosis.

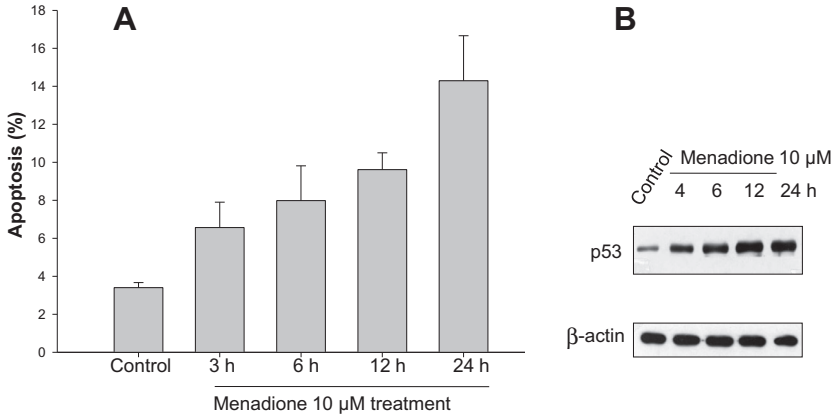

Figure 2 (A) HepG2 cells were treated with 10 μM of menadione for indicated time and stained with P1 and their DNA content was analyzed by FACS. Menadione increases cell fraction at sub-G1 phase in a time dependent way, which is indicative of apoptosis. The sub-G1 apoptotic cells (%) were placed in the graph against the indicated time of 10 μM menadione effect. Data express mean ± SD at $p \geqslant 0.05$. (B) At the indicated time, menadione (10 μM) treatment induces the expression of P53 up-regulation in a time dependent way. β-actin level is used as a loading control.

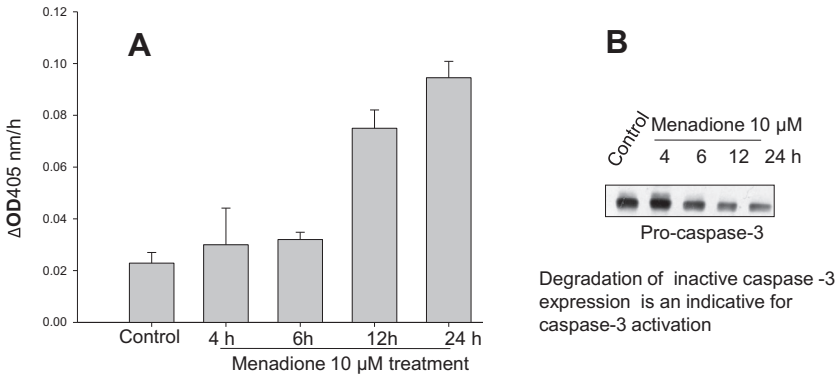

Figure 3 Shows the ability of menadione to activate caspase 3 signal pathway. (A) Menadione induces caspase-3 activity in a time dependent way after incubation HepG2 cells with the enzyme substrate. Data express mean ± SD at $p \geqslant 0.05$. (B) At the indicated time, menadione treatment decreases procaspase-3 expression, which is an indicative for casapse-3 activation in a time-dependent manner.

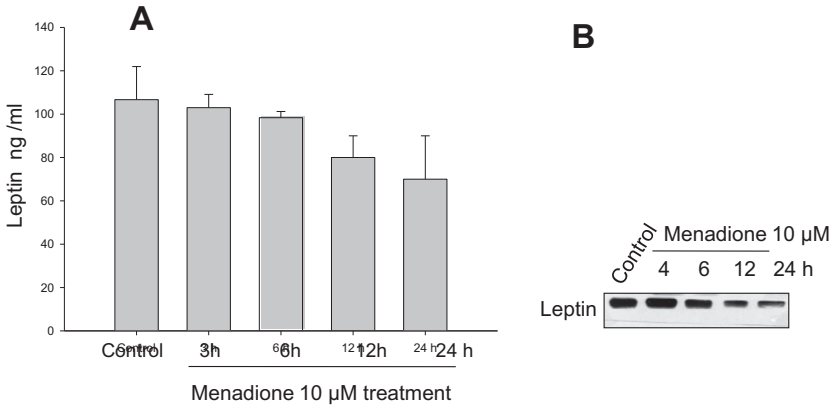

Figure 4 Menadione inhibits leptin pathway. (A) Leptin levels in menadione treated HepG2 cell culture media were measured by ELISA and revealed that menadione (10 μM) decreases leptin levels in a time dependent way. (B) Also Western blot analysis demonstrated a decrease in leptin expression with time.

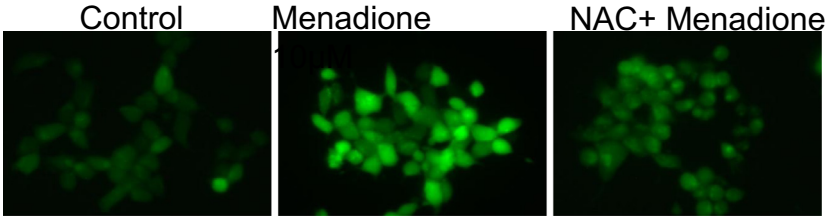

Figure 5 Shows that menadione (10 μM) alone induced ROS generation in HepG2 cells compared with control cells. But treating the cells with NAC followed by 10 μM menadione inhibited ROS generation.

FACS assay showed that menadione increased cell fraction at sub-G1 phase in a time dependent way which made it as an apoptotic agent on HepG2, the result coincides with its potent antitumor effects on human hepatocellular carcinoma by inducing cell cycle arrest at G1 phase (Hitomi et al., 2005), also incubation of human bladder tumor cells with combination of Vitamins C and K3 induced antitumor activity and arrested the cell growth at the G_0/G_1-S-phase (McGuire et al., 2013).

Our study confirmed that menadione induces cell apoptosis by activation of caspase 3 signal. Caspases are found as inactive pro-enzymes, once the initial signal of caspase activation has been triggered, inactive forms (certain procaspases includes procaspases-3) are subjected to proteolytic steps to produce the active form (Earnshaw et al., 1999; Alnemri et al., 1996; HGNC, 1504), the proteolytic process which could explain the disappearance of procaspase 3 expression and the increase in caspse-3 activity with increasing time observed in this study after incubating HepG2 cells with 10 μM menadione. Cleavage of the active form of the enzyme is a conversion that allows initiation of apoptosis in colon tumor cells (Putt et al., 2006). So procaspase-3 cleavage was used as an apoptotic indicator (Kato et al., 2013), our results harmonize

with those of Tong et al. (1997) who found that VK3 inhibits the proliferation of HepG2 and induces apoptosis through caspase-3 signal, and with findings of Hu et al. (2006) who found that procaspase-3 was significantly decreased after treatment with oroxylin A causing apoptosis in HepG2 cells, the authors described it as a promising antitumor drug. Also pro-caspase-3 expression was reduced and activation of caspase-3 increased by Berberine in HepG2 cells (Hyun et al., 2010). It was noticed in this study that the decrease in procaspase-3 expression and increasing caspase-3 activity in a time dependent way after menadione treatment are coincided with decreasing viability of hepG2 cells, these results may attributed to the enzyme action before or at the phase when loss of cell viability occurs (Porter and Jänicke, 1999). Also Putt et al. (2006) found that procaspase-3 concentration was significantly higher in certain tumor cells than that in non-tumor control cells, and they concluded that the activation of the executioner caspases is considered a beneficial approach in treating many tumors characterized by high levels of procaspase-3.

Immunoblotting analysis demonstrated that menadione has up-regulating effect on P^{53} expression which corresponds with cell apoptosis in a time dependent manner, these results agreed with the significant role of P^{53} in the regulation of cell cycle and growth described by previous study (Erlacher et al., 2005). It is worth to mention that menadione apoptotic effect depends on its concentration, Sata et al. (1997) reported that low levels (1–20 μM) of menadione induced wild type P^{53}, but (100 μM) had a little effect on it and induced necrosis. P^{53} may be considered as an inhibitor of tumorigenesis through its ability in regulating glucose release in both human liver cells and primary mouse HepG2 cells (Goldstein et al., 2013). In contrast, other vitamin like VD compounds activate apoptosis through inhibition of caspase and p^{53} pathway (Erlacher et al., 2005).

Leptin is an anti-obesity hormone. Obesity is considered a significant factor for hepatocellular carcinoma complicated with some liver diseases, leptin prevented apoptosis by ethanol (Balasubramaniyan et al., 2007), our results indicated that menadione apoptotic action on HepG2 cells is mediated by inhibiting leptin expression and decreasing its levels at the indicated time, the result coincides with recent researches in obese individuals, their data indicated the benefit of inhibiting leptin signals in preventing breast cancer (Gillespie et al., 2012), also, leptin seems to be an antiapoptotic hormone and the correlation between ovarian cancer risk and high leptin levels should be considered in obese women (Ptack et al., 2013). Our result indicated that menadione promotes HepG2 cells apoptosis depending on leptin- signal suppressing.

NAC is a well known antioxidant and inhibitor of intracellular ROS (Sun, 2010). Therefore we used NAC in the current experiment as negative control to assure menadione specific effect in ROS generation, so we confirm the inverting effect of menadione by addition of NAC before treating the cells with menadione. The results showed that 10 μM menadione apoptotic action is mediated by inducing oxidative stress in HepG2 cells, while treating the cells with NAC inhibited menadione ROS generation effect in this study, the result agreed with study of Chen and Cederbaum (1997), also is supported by data of Koka et al. (2010) who found that pretreatment prostate cancer cell with NAC suppressed thymoquinone-ROS generation inducing and growth inhibition effects. Although the current study confirmed that menadione suppresses the cell viability by activation caspase-3 and ROS pathways, but in contrast Samali et al. (1999) found that menadione inactivates caspase by the generation of H_2O_2 which may prevent cell death. The results concluded that menadione has a dose and time dependent anti- viability effect by suppressing leptin pathway and activation caspase 3 and P^{53} signals depending on ROS generation. It is worth to mention that leptin should be focused in the apoptosis studies because of its opposite direction to apoptosis. So regulating leptin, caspase-3 and P^{53} molecular signals by medical nutrition may be of significant benefit in managing anti-proliferative therapy.

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