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Antitumoral Effects of Digoxin in The Liver Cancer Cell Line is mediated through Induction of Apoptosis and Down Regulation of HIF-A Expression

Atefeh Tahervand[a], Minoo Mahmoodi[a], Mehryar Habibi Roudkenar[b], Amaneh Mohammadi Roushandeh [b *]

[a] Biology Department, Islamic Azad University, Hamadan Branch, Hamadan, Iran
[b] Medical Biotechnology Research Center, Paramedicine Faculty, Guilan University of Medical Sciences, Rasht, Iran

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Based on epidemiological evidences, cardiac glycosides such as digoxin have been recently proposed as an anticancer drug. However, the outcomes are controversial and even application of digoxin as an anticancer agent is doubtful. Hence, in the present study, the anticancer properties of digoxin and its potential mechanisms have been investigated in a liver cancer cell line i.e. HepG2. hep G2 cell line was cultivated in RPMI-1640 with %15 FBS and 2µM digoxin for 6, 12, 24 and 48 hours. Apoptosis was detected in the cells treated with digoxin using in situ cell detection kit. Furthermore, the cells were stained by Giemsa to study the occurrence of senescence following administration of digoxin. Expression of HIF- α was determined by RT-PCR after the cells exposed to digoxin as well. Our findings revealed that digoxin induces apoptosis in a time dependent manner. A significant number of apoptotic cells were observed after 48hours exposure to digoxin ($P<0.05$). It seems that digoxin elicits senescence in hepG2 cell line in time dependent manner as well. Following digoxin treatment, down regulation of HIF1-α was considerably detected in the liver cancer cell line. Again, the result was prominent after 48hrs. Our findings strongly suggest that digoxin exerts its antitumor properties through inducing of apoptosis, cellular senescence and changing of HIF1- α gene profile. Due to cytotoxic effects of digoxin on the cells, it would be essential to determine the minimum toxicity dose and the drug mechanisms before its application in clinics for the patients.

*Corresponding Author: Amaneh Mohammadi Roushandeh, E-mail: mohammadi_roushandeh@gums.ac.ir

Introduction

Cardiac glycosides (e.g., digitalis, digoxin, digitoxin, ouabain) have been used for decades in the remedy of congestive heart failure and cardiac arrhythmias. Their cardiac effects are mediated through the inhibition of Na/K ATPase leading to increased intracellular calcium concentration and increased cardiac contractility [1].

Digoxin is a purified cardiac glycoside extracted from the foxglove plant [2]. Recently, digoxin has been proposed as a potential therapeutic agent in cancer therapy *as well as* cancer prevention agent [3].

Epidemiological evidence has indicated that prevalence of cancers such as lung, colon, prostate and ovary is lower in patients who receive digoxin, while, it increases the risk of breast and uterus cancers [4]. It seems that in cancers including breast and uterus that their occurance are hormone dependant, the prevalence will increase 30% to 50% in women who have digoxin in their treatment regime. [5-8]. Therefore, the safety and efficiency of digoxin in treatment of the cancers are under doubt and needs more explorations about its nontoxic dose and the mechanisms by which the drug influence the tumor cells.

Hepatocellular carcinoma is the fifth most common cancer and has second rank among cancer-related death worldwide [9]. It has been shown that digoxin modulates cholesterol biosynthesis in liver. This process is cytotoxic for the hepatocytes and leads to pathophysiological consequences on liver [10]. As it is mentioned earlier in this section, the behavior of digoxin is different from cancer to cancer; therefore, we want to know what will happen if the hepG2 cells encounter with digoxin. Of note, the number of studies dealing with cytotoxic effects of digoxin or its mechanisms on liver and/or liver cancer cell lines is rare and insufficient.

It is noteworthy that, the mechanisms underlying digoxin exerts its cytotoxic effect are not fully understood. Proliferation, metastasis and senescence are the three main processes that influence the cancers fate and prognosis. Hence, most of anticancer agents target the cells at the stage of cell division, induce apoptosis or try to decrease the metastasis [11].

It is revealed that cardiac glycosides could induce apoptosis; f to therefore, another aim of our study is to determine whether digoxin exerts its therapeutic effects through induction of apoptosis or not?

On the other side, digoxin can influence cancer cells by altering of genes expression profile. A body of studies has indicated that cancer occurs after deregulation in expression of some genes. One of these genes that have critical effects on cancer progression is Hypoxia inducer factor-1a (HIF-1α). Recently, researchers have found that digoxin inhibits HIF-1α (). It is a transcription factor which is highly implicated in cancer development. Therefore, therapeutic strategies in which HIF- α expression comes down might be an asset [12-13]. Hence, we investigate the expression of HIF- α in HepG2 cell line following treatment with digoxin.

Cellular senescence was formally described more than 40 years ago as a process which limits the proliferation /growth of normal human cells in culture [14]. Absence of senescence is essential for tumor formation [15]. Finally, we investigate whether digoxin can induce senescence on HepG2 cells or not? If any, it would be helpful to develop safer and more effective treatment strategies.

Materials and methods

Cell culture and digoxin treatment

Liver cancer cell line, HepG2, was recieved from National Cell Bank of Iran (NCBI), Pasteur Institute. The cells were cultivated in RPMI-1640 supplemented with 15% FBS at 37°C with 5% CO2. The culture medium was replaced every 3 days andthe cells were sub-cultured when reached to 70-80% confluency. The cells were cultured with 2μM digoxin for 6, 12, 24 and 48 hrs [16].

Determination of senescence with Giemsa staining

The cells were cultured in a 6-well plate and treated with 2µM digoxin for 6, 12, 24 and 48 hrs. Then, the cells were washed with PBS and fixed with methanol. Thereafter, they were stained with Giemsa for 15 minutes and observed under microscope.

Apoptosis detection

The apoptotic cells were detected with *in situ* cell death detection kit (Roche, USA). HepG2 cells were seeded on a cover slide into a 6-well plate and treated with 2µM digoxin for 6, 12, 24 and 48 hrs. Then, the cells were fixed with paraformaldehyde for 1hr and soaked in blocking solution (3% H2o2 in methanol) for 10 min. After 3 times washing with PBS, the cells were permeabilized with 0.1% Triton x-100 in sodium citrate on ice for 5 min. The cells incubated with 50µl tunnel reaction for 60 min at 37ºC in moisture chamber. Then, the cells were incubated with POD solution for 30 min at 37ºC and finally developed by DAB substrate. The cells were observed with light microscope and photographed.

RT- PCR

Total RNA was extracted by RNA extraction kit (Invitrogen, Carlsbad,CA) according to the manufacturer's protocol. cDNA was synthetized by SuperScript III reverse transcriptase kit (Invitrogen) using 1µg of total RNA according to the manufacturer's protocol. PCR was performed using Pfu polymerase (Cinnagene, Tehran,Iran) in a GeneAmp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA). After an initial denaturation at 94 °C for 5 min, cDNA was subjected to 30 cycles of PCR. Primer set for HIF-α was ACCATGAGGAAATGAGAGAAATGC (forward) and TACTGTTGGTATCATATACGTGAATGTGG (reverse). Primer set for B-actin included TTCTACAATGAGCTGCGTGTGG (forward) and GTGTTGAAGGTCTCAAACATGAT (reverse).

Statistical analysis

All results were expressed as the mean ± standard deviation. Statistical analyses were performed by one-way ANOVA of variance using SPSS version 21. The differences between means was considered statistically significant when the probability level (*P*-value) was less than 0.05 ($P<0.05$).

Results

Cell senescence after digoxin treatment

Giemsa staining was performed to study senescence in hepG2 cell line. After treatment of cells by digoxin, large size (Giant cells) was detected (Figure 1). The number of giant cells were significantly observed at 12, 24 and 48 hrs following treatment by digoxin compared to control ($P<0.001$). It is noteworthy that, 6 hrs exposure of cells with digoxin did not induce senescence ($P>0.5$). (Figure2).

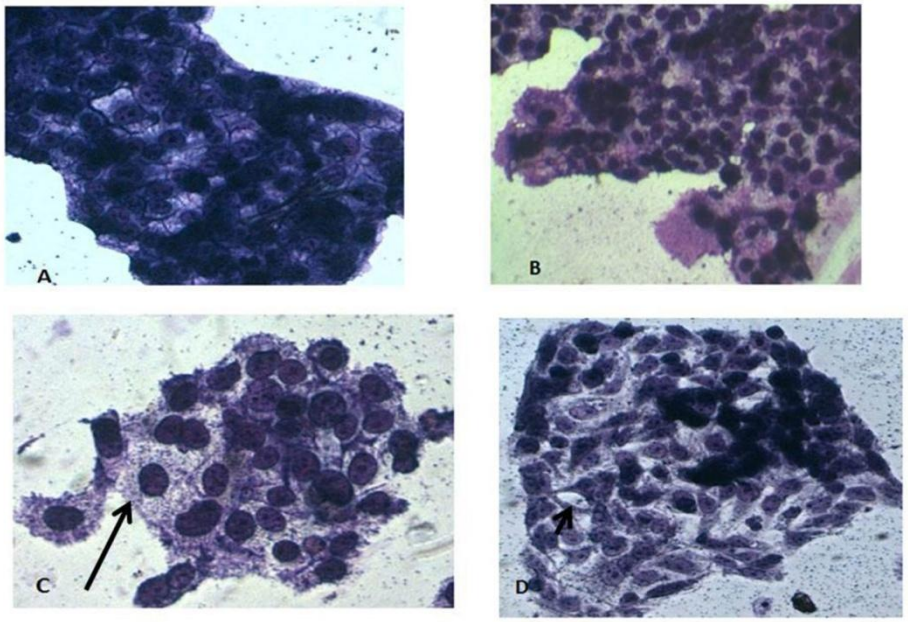

Fig. 1. The cells were cultured with digoxin and stained with Giemsa to detect senescence. (A) The cells shape is regular and homogenous and represents normal size. (B-D) the cells size increased in groups received digoxin (black arrow) after different 12 (B), 24 (C) and 48(D) hours.

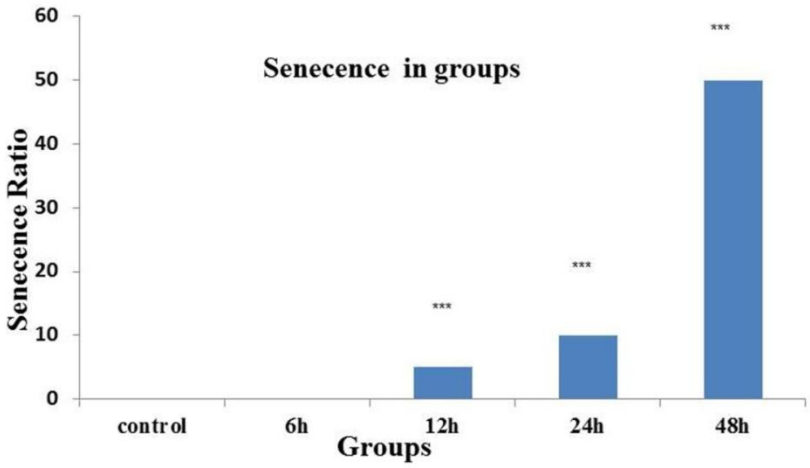

Fig. 2. The diagram shows the number of senescence like cells. The number of senescent cells increased with time of exposure. (Mean ± SD, *p ≤0.1, ** p ≤0.01, *** p ≤0.001)

Digoxin exerts its antitumor properties through apoptosis

Cultivation of HepG2 cells with 2μM digoxin resulted in increasing of apoptotic cells after 6, 12, 24 and 48hours compared to the control group. The most apoptotic cells were observed after 48h ($p<0.001$). As shown in figure 3, brown cells increased along with increase in the time of digoxin treatment; so that, after 48h the higher number of brown cells were observed under light microscope (Figure 4)

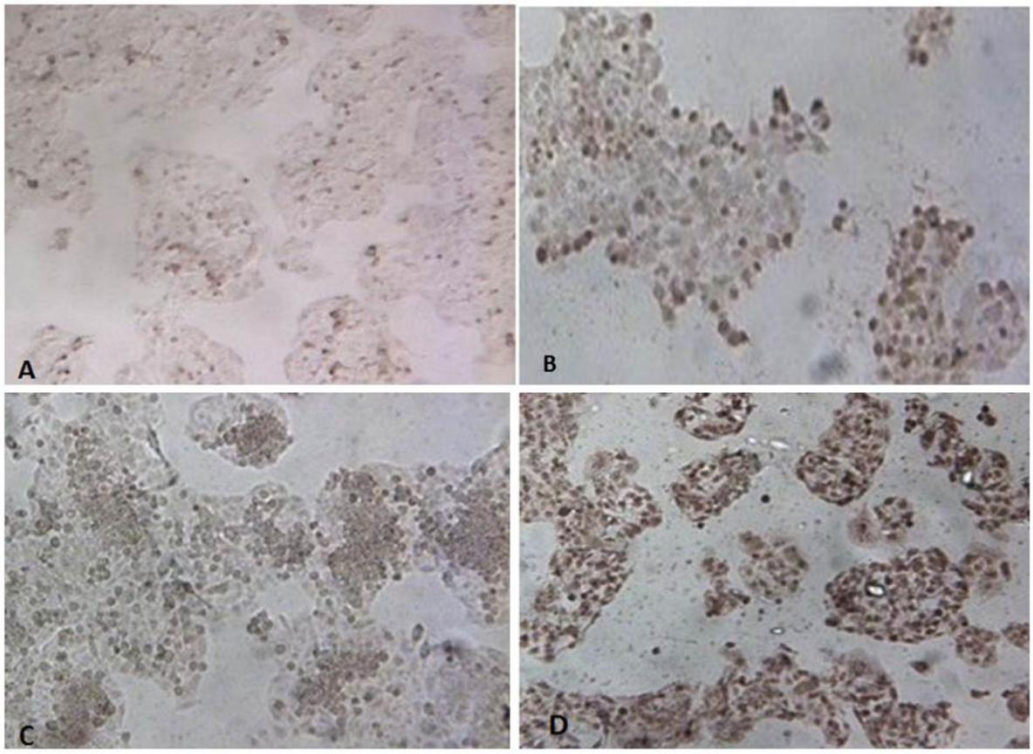

Fig. 3. The microphotograph of cells treated with TUNEL reagent to detect apoptosis after cultivating with 0.2μM digoxin at different time intervals. The brown nuclei determine the apoptotic cells. The higher number of apoptotic cells can be observed after 48h of the treatment (D). (A) Control, (B, C and D) 12, 24 and 48 hours after digoxin treatment respectively.

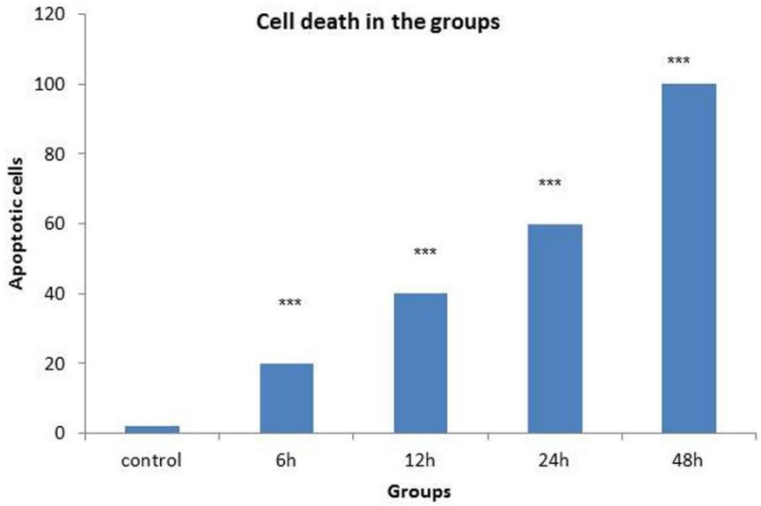

Fig. 4. Apoptotic cell number in control and experimental groups after 6, 12, 24 and 48 hrs. Induction of apoptosis was increased when time of treatment increased.

Digoxin was down regulated expression of HIF-α

Our results revealed that digoxin acts its anticancer properties via down regulation of HIF-α expression. Following 6, 12, 24 and 48h treatment of cells with digoxin, down regulation of HIF- α was detected by RT-PCR. However, the down regulation was prominent after 24h and 48h compared to other groups (Figure 5).

Fig. 5. RT-PCR was employed to detect HIF-α expression in control and experimental groups at different time intervals. The expression has been shown in digoxin group after 48h (lane 1), 24h (lane 2), 12h (lane 3), 6h (lane 4) and control group (lane 5).

Discussion

It has been shown that digoxin decreased the viability and proliferative competency of the cancer cells [17].

Interestingly, it has been reported that in heart failure patients who received digoxin in their therapeutic regimefor several years, incidence of cancer was lower in comparison with patients who received other therapeutic agents suggesting anticancer potential of digoxin.

Digoxin is one of the most prescribed drugs in treatment of patients with cardiac heart failure and certain types of cardiac arrhythmias [18].

In an attempt to investigate the anticancer potential of digoxin on HepG2 cell line and clarify the some possible mechanisms, this study was conducted.

Our findings revealed that digoxin acts as an anticancer agent on HepG2 cell line.

Our results also indicated that digoxin induces apoptosis and senescence in HepG2 cell line.

Based on several studies, digoxin decreases the incidence and metastasis of some cancers.

However, according to others, digoxin resulted in higher occurrence of cancers [4-8, 19]. Recent studies have also shown that the use of digoxin significantly increases the risk of breast and uterus cancers that both of them are estrogen-sensitive [6-8]. In contrast, no incensement was observed in risk of ovary and cervix cancers in which they are relatively estrogen-insensitive [6-8].

Smolarczyket al in 2018 had a combination therpy with MXAA (5,6-Dimethylxanthenone-4-acetic Acid; also known as: ASA404, Vadimezan) and digoxin in murine melanoma. They revealed that both of the drugs inhibited the cancer growth when asministered alone but, drug combination inhibitory effect was higher. They showed that the digoxin acts as a HIF-a1 inhibitor and the other drug decline the angiogenesis. Also, they exert their antitumoral effect through regulation of immune cells [20]. . In a recent published paper in 2018, the Muhannad Abu-Remaileh et al described that digoxin can inhibit HIF-a1 and significantly delayed Hepatocellular carcinoma (HCC) formation. They found that digoxin controls the WW domain-containing oxidoreducatase (*WWOX*) gene expression. This gene is critical for several cell functions including affect genome integrity, apoptosis, cell growth and extracellular matrix signaling, and glucose metabolism[21].

Although the inhibitory effects of digoxin on cancer growth have been reported with number of epidemiological evidence, its application in clinic as a therapeutic agent is not approved. There are some deficiencies regarding to the drug dose and time optimizations and absence of integrated results are the reasons that limit its application . Overdose of digoxin and other glycosides leads to cytotoxicity and even death in some cases. Therefore, it is necessary to determine the therapeutic dose of digoxin and the mechanisms by which digoxin exerts its antitumoral effects.

Several important mechanisms of cardiac glycosides were described in the cancer treatments including the inhibition of Na+/K+ ATPase, activation of ERK1/2, inhibition of topoisomerases I and II and downregulation of some genes such as HIF1-α, regulation of immune cells, angiogenesis and apoptosis [20-25].

Hypoxia is a common condition in solid cancers and is especially frequent in Hepatocellular carcinoma (HCC) mainly due to its rapid growth. HIF1-α - is a key transcription factor which enables cancer cells to survive under hypoxia condition. It is consists of two subunits; a stable β subunit and the oxygen-sensitive α subunit [26]. Interestingly, it targets several genes which contribute in apoptosis and cell proliferation [26].

Digoxin and other cardiac glycosides inhibit HIF-1α synthesis and block tumor growth. It is suggested that cardiac glycosides are potential inhibitors of HIF-1α synthesis [20-21].

Within our assumption, our results revealed that digoxin down regulates the expression of HIF-α in HepG2 cell line especially after 48 hrs. In most human solid tumors, adaptation to decreased O_2 availability is achieved primarily through the activity of two hypoxia-inducible factors i.e. HIF-1 and HIF-2 which regulate the expression of over hundred target genes [25].

In consistence with our findings Bielawski et al reported that digoxin down regulates the tumor xenograft growth of P493-Myc, PC3, and Hep3B in mice in a dose-dependent manner along with down regulating of HIF-1α expression [27].

Moreover, Zhang et al showed that Digoxin induces cell cycle arrest and apoptosis via HIF-1α inhibition in PC3 prostate cancer cells [27].
Although the inhibition of HIF-1 synthesis by cardiac glycosides is well known effect, its exact mechanisms remains are studies.
The ability of cardiac glycosides to induce apoptosis in human prostate cancer cell line, PC-3, correlates with their role in inhibiting the expression of four prostate cancer target genes including the transcription factors Hoxb-13, hPSE/PDEF, hepatocyte nuclear factor-3α, and the inhibitor of apoptosis [28].
Zao et al in 2017 found that digoxin decreased the proliferation of breast cancer. Digoxin increased the apoptosis and reduced Bcl-2 expression and elevated Bax level in MM231 cells, characterized by increased Bax/Bcl-2 ratio [29]. []. Meanwhile, many studies indicated that HIF-1 plays a critical role in the proliferation of A549 cells under hypoxic condition [27]. Taken together, our results strongly suggest that digoxin is potential antitumor agent against HepG2 cell line.
We think that digoxin via down-regulation of HIF-α, regulates induction of apoptosis and more probably senescence in liver cancer cell line. However, further studies are required to address this issues as well as studies dealing with *in vivo* and clinical trials.

Conclusion

Overall, our findings indicate that digoxin effectively induces apoptosis and senescence in a liver cancer cell line. In other words, it might be considered as a novel antitumor agent against HepG2 cell line. Furthermore, we found that digoxin down-regulates expression of HIF-1α and in turn could inhibit apoptosis induction. It means that we took initial steps toward clarifying the mechanisms underlying digoxin exerts its cytotoxicity effects on the cancer cell line.
Finally, our results showed that digoxin induces senescence in HepG2 cell line which highlights the importance of digoxin as a safe and more effective therapeutic agent in future. However further and comprehensive studies are required in these regards.

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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