Digoxin as a Cardiac Glycosoids induced Apoptosis in Mesenchymal Stem Cells: an in Vitro Study

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ABSTRACT

Low number of engrafted cells is the main challenge in stem cell therapy. The cells should overcome with reactive oxygen species, food deprivation and toxicity of the pharmacological agents that patients take during their treatment. As an example, cardiac glycosides such as digoxin can inhibit the cell proliferation and lead to apoptosis. Therefore, in this study, we are trying to know stem cell behavior following digoxin treatment. Mesenchymal Stem Cells were treated with different concentrations of digoxin for 6, 12, 24 and 48 hours. Cell viability was detected with trypan blue. Hoechst staining and tunnel assay were conducted to evaluate nuclear configuration and apoptosis in MSCs. Cell viability decreased after digoxin treatment in all groups during 6, 12, 24 and 48h significantly (P<0.05). After 6 hours, rate of nuclear fragmentation was significantly higher in30 and 40μM digoxin than control group (P< 0.001). Treatment with 20, 30 and 40μM digoxin led to nuclear damage significantly compare to control group after 12h (P< 0.001). Also, after 24 and 48 hours, nuclear damage significantly increased in 15, 20, 30 and 40μM of digoxin (P< 0.001).Digoxin induced apoptosis significantly in all groups in time and dose dependent, so that the highest rate of cell death was found after 48h.It is suggested that digoxin might lead to decline in cell survival and increase cell apoptosis in a dose and time dependent and interfere with stem cell therapy. Therefore, it is recommended to consider application of glycosides in concurrence with stem cell therapy.

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**Introduction**

Bone marrow mesenchymal stem cells (MSCs) are defined as a class of the multipotent adult stromal stem cells that differentiate into a variety of tissues including cartilage, bone, fat as well as myocytes like cardiomyocytes in vitro and in vivo\[1\]. This property makes them as a promising source for cell-based therapy. Recently, several studies have demonstrated that bone marrow mesenchymal stem cells transplantation is safe and has great potential in regenerating infracted myocardium and restoring the impaired heart function \[2–6\]. Myocardial infarction or heart failure is one of the major causes of cardiovascular mortality \[1, 7\]. Stem cell based therapy has generated significant interests and to date preclinical researches have shown its therapeutic potential. As yet, clinical trials have reported that this therapeutic modality may lead to an overall improvement of cardiac function \[1, 8, 9\]. In spite of more benefits of stem cell therapy, its application in clinics encountered to several obstacles such as cell death and low survival rate due to harsh environments resulted in free radicals, low nutrition and also pharmacological interference. Cardiac glycosides such as digoxin or digitoxin are the natural products that traditionally used to increase cardiac contractile force in patients with heart failure and cardiac arrhythmias \[10, 11\]. It is reported that glycosides including digoxin have cytotoxicity on cells and their effects on tumor cells has been confirmed \[10, 12\]. Moreover, it has shown that digoxin can directly inhibit the proliferation of tumor cells. Indeed, its efficacy is done by elevation in intracellular Ca\(^{2+}\) and apoptosis induction in cancer \[13\]. It is considered that Stem cells have ability to proliferate similar to cancer cells and differentiate to different tissues and might affect by interference with some medicines such as digoxin. Therefore, their efficiency decreases after cell therapy in patients with cardiac failure. Since the stem cell therapy is recommended for cardiac patients and its therapeutic results are promising, but its problems regard to drug interference is indissoluble yet \[14\]. Improvement of transplanted cells viability, proliferation and settlement are the strategies that pave the application of stem cell therapy in clinics. It is necessary to study the drug interaction with stem cell therapy to optimize the dose and time and also understand the mechanisms through which the drug exerts its effects. Therefore, the present study follows cytotoxic effects of digoxin as glycosides on mesenchymal stem cells behavior such as survival and apoptosis in vitro condition.

**Materials and Methods**

**Mesenchymal stem cells isolation, culture and treatment**

Mesenchymal stem cells (MSCs) were obtained from 6 weeks old wistar albino male rat by flushing its femurs and tibias with Dulbecco’s Modified Eagle’s medium (DMEM) (Gibco, Invitrogen, Germany). Then, MSCs were suspended in DMEM medium supplemented with 10% FBS (Gibco, Invitrogen, Germany) and incubated at 37°C in a humidified chamber with 5% CO\(_2\). The culture medium was completely replaced every 3 days and non-adherent cells were discarded. MSC were recognized by their ability to proliferate in culture with an attached well-spread morphology. Once cells were more than 80% confluent, adherent cells were detached and re-plated 1:3 by flask (passage1). Passage 4 of MSCs was incubated with digoxin at different concentrations (0.1, 0.5, 1, 5, 7, 10, 15, 20, 30 and 40μM) for 6, 12, 24 and 48 hours.

**Differentiation potential of stem cells**

The multi-potency of MSC was confirmed by induction of osteogenic and adipogenic differentiation using specific differentiation media as describe below.

**Osteogenic differentiation**

For osteogenic differentiation, BMMSCs were seeded at density 2×10\(^4\) cells/cm\(^2\) in 24 well plate and then induced to differentiate in an osteogenic induction medium composed of DMEM with 10%
FBS (Gibco, Invitrogen, CA, USA), 100 units/ml penicillin and 100 g/ml streptomycin, 10 nM dexamethasone, 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate. Cells were incubated for 21 days in 5% CO2 at 37º C. After 21 days Osteoblast differentiation was evaluated by 2% Alizarin Red (Sigma) staining. Briefly, Cells were washed three times with PBS (Gibco, Invitrogen, CA, USA) and then fixed for 15-30 min with 4% formaldehyde. After rinsing 3 times with distilled water, they were stained with alizarin red for 3 minutes. Then, the cells were washed several times with distilled water and observed under microscope[15].

**Adipogenic differentiation**

The cells were seeded at a density of $2 \times 10^4$ cells/cm² in 24well plate and after 80% confluency, adipogenic induction medium, consisting of DMEM, supplemented with 10% FBS, 100 units/ml penicillin and 100 g/ml streptomycin, 100nM dexamethasone, 50µg/ml indomethacin was added and cultured for 10 days and fat droplets were examined under microscope[15].

**Hoechst staining**

MSCs were cultured and treated with different concentrations (0, 0.1, 0.5, 1, 5, 7, 10, 15, 20, 30 and 40µM) of digoxin for 6, 12, 24 and 48 hours. Then, they were stained by Hoechst to evaluate the MSCs nuclear fragmentation. Briefly, after washing the MSCs by PBS, fixation with methanol and adding the triton X100, staining with Hoechst was performed. Then, MSCs were observed using fluorescent microscope (DP12; Zeiss, Gottingen, Germany).

**In situ cell death detection (Tunnel assay)**

The apoptotic cells were detected by in situ cell death detection kit. MSCs cells were seeded on a coverslide in 6-well plate and treated with digoxin for 6, 12, 24 and 48 h. Then, the slides were fixed with paraformaldehyde for 1hour 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-100in sodium citrate on ice for 5 min. The cells incubated with 50µl tunnel reaction for 60 min at37ºCin moisture chamber. Then, the cells were incubated with POD solution for 30 min at 37ºC and finally developed by DAB substrate.

**Statistical analysis**

Results were presented as mean ± SD in triplicate experiment. Differences were determined using ANOVA with the Tukey–Kramer multiple comparisons test at significant difference of 0.05.

**Results**

**Morphology of MSCs**

After one week the attached cells were found in the flasks. The cells morphology represented as polygonal and fibroblasts like (Figure 1).

![Fig. 1. Themeshenchymal stem cells morphology 1 week after primary culture (A) and at passage 4 (B).](image)

**Differentiation of MSCs cells**

The cells were cultured with differentiation media for osteogenesis for 21 days and adipogenesis for 15 days. As it is illustrated in figure 2 the MSCs differentiated to adipose and bone tissues (Figure 2 A-B).
Fig. 2. The cells were cultured in differentiation media for adipogenesis for 15 days (A), osteogenesis for 21 days (B).

**Digoxin induced apoptosis in MSCs**

The results from Hoechst staining showed that after 6 hours, rate of nuclear fragmentation in 30 and 40μM digoxin were significantly higher than control group (p < 0.05). Moreover, treatment with different concentrations of digoxin for 12 hours led to increase in nuclear damage in 20, 30 and 40μM digoxin compare with control group, significantly (p < 0.05). Also, after 24 and 48 hours, nuclear damaging rate in 15, 20, 30 and 40μM concentrations of digoxin showed significant increase toward control group (p < 0.05). (Figure 3 and 4)

In situ cell death detection confirmed the results obtained with Hoechst staining, as the number of brown cells increased at higher doses (30 and 40μM) after 6 and 12hr. Also, the apoptotic cells increased significantly at 15, 20, 30 and 40μM after 24 and 48hr (P<0.001)(Fig 5 and 6). It is detected that apoptosis induction increased in dose and time dependent.

**Discussion**

Recently, cell transplantation therapy with bone marrow mesenchymal stem cells to treat heart failure has been interested. The therapeutic efficacy of mesenchymal stem cells is dependent on their survival and differentiation ability in the target tissue\[^{16}\]. On the other hands, many of patients with cardiac disease consume cardiac glycosides such as digitalin and digoxin. A variety of reports suggested that cardiac glycosides may have anticancer properties in breast\[^{17-21}\], prostate\[^{22}\] and lung cancers\[^{23}\]. Probably, reduction in transplanted MSCs viability in heart failure is due to apoptotic effects of cardiac glycosides. Hence, present paper evaluated the effects of different concentrations of digoxin on mesenchymal stem cells.

Our results showed that digoxin decreases the cell viability and also increases the cell apoptosis in a dose-time dependent manner. Several studies have confirmed the apoptotic and cytotoxicity effects of digoxin\[^{24, 25}\]. In a recent study, digoxin decreased the viability and proliferation in HepG2 cell line\[^{26}\]. Based on Pubmed and another medical Databases, there is no enough informations regard to effects of glycosoids on stem cells behavior(www.pubmedand www.scholar.com).
The cardiac glycosides like digoxin, acts by inhibition the activity of the Na+/K+-ATPase. Inhibiting Na+/K+-ATPase leads to higher levels of intracellular Ca2+. However, decrease in intracellular K+ and increase in intracellular Na+ and Ca2+ following inhibition of the Na+/K+-ATPase are early key steps in apoptosis (27-29). Moreover, inhibition of IL-8 production and the
TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway are the mechanisms describes the effects of digoxin\[18\]. However, finding optimizes concentrations of digoxin with lower apoptotic effects is an efficient strategy in stem cell therapy in cardiac patients. Lopez-Lazaro et al suggested that Digitoxin inhibits the growth of cancer cell lines at concentrations commonly found in cardiac patients\[24\]. We examined the effect of different concentrations of digoxin on mesenchymal stem cells. According to MTT and apoptosis assays, obtained results showed that 0.1, 0.5, 1, 5 and 7 μM of digoxin in time periods of 6, 12, 24 and 48 hours had not cytotoxic effects on mesenchymal stem cells. However, application of low doses of cardiac glycosoids should not disturb its cardiac therapeutic effects. Therefore, more studies are required to understand this issue in In vivo experiments.

**Fig. 5.** the diagram shows apoptosis rate of MSCs after 6, 12, 24 and 48 h in different concentration of digoxin.
Fig. 6. The diagram indicates rate of apoptosis (%) in different concentrations of digoxin at 6, 12, 24 and 48 hr after culture of the MSCs.

Conclusion

It seems that pharmacological interaction with stem cell therapy is a great challenging in cardiac patients. More in vitro and in vivo studies are necessary to know mesenchymal stem cells behavior when encounter with heart drugs such as digoxin.

Conflict of Interests

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

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