Journal of Reports in Pharmaceutical Sciences 2017, 6(2), 142-149

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

Shirin Darabi [a], Hooshang Rafighdoost [a], Amaneh Mohammadi Roushandeh[b]*

[a]Anatomical Sciences Department, Medicine Faculty, Zahedan University of Medical Sciences, Zahedan, Iran
[b]Anatomical Sciences Department, Medicine Faculty, Hamadan University of Medical Sciences, Hamadan, Iran

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 2017-06-02
Revised: 2017-07-25
Accepted: 2017-08-01
ePublished: 2017-08-08

***Keywords*:**
Apoptosis
Digoxin
Mesenchymal stem cell
Proliferation
Preconditioning

A B S T R A C T

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.

*Corresponding Author: Amaneh Mohammadi Roushandeh, E-mail: a.mohammadiroshandeh@umsha.ac.ir

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% CO2. 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). Passage4 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 2×10^4cells/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 mMβ-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 2×10^4 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].

Hochest 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 Hochest 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% H_2o_2in 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).

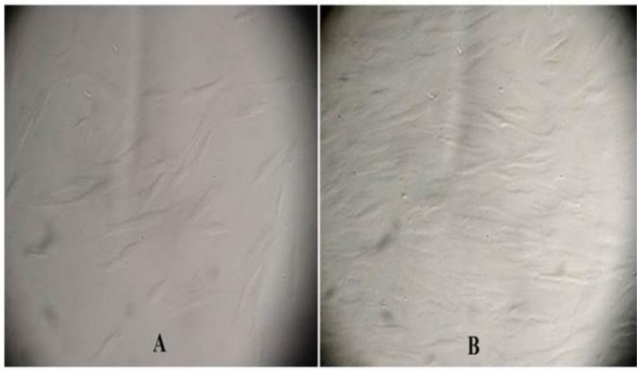

Fig. 1. Themesenchymal stem cells morphology 1 week after primary culture (A) and at passage 4 (B).

Differentiation of MSCs cells

The cells were cultured with differentiation media for osteogenesis for21days and adiopogenesis for 15 days. As it is illustrated in figure 2 the MSCs differentiated to adipose and bone tissues (Figure 2 A-B).

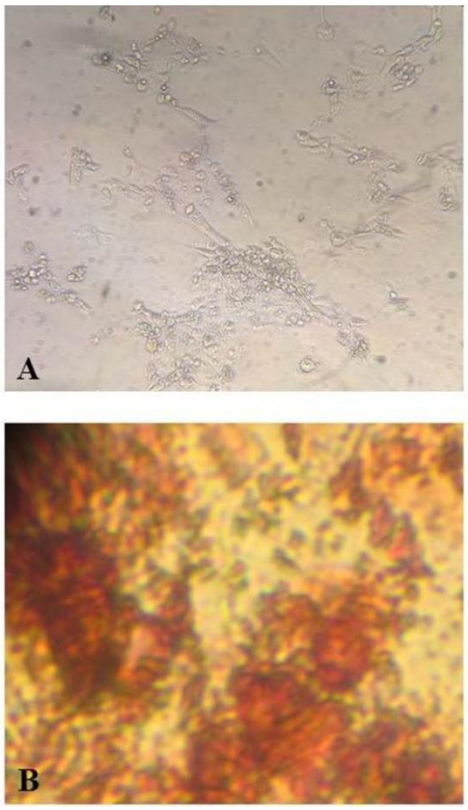

Fig. 2. The cells were cultured in differentiation media for adipogenesis for 15 days (A), osteogenesis for 21 days (B).

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 propertiesin 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).

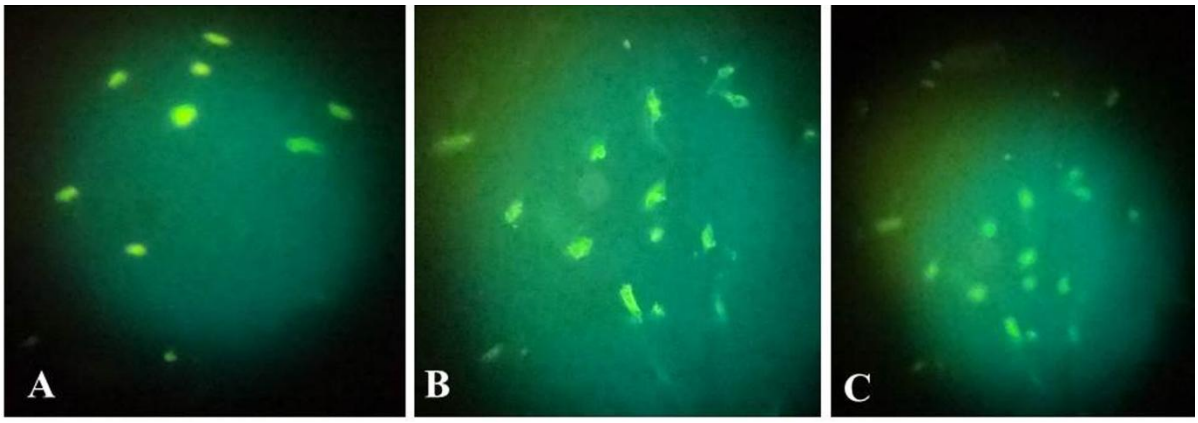

Fig. 3. Hochest staining in control (A), group with 30µM digoxin (B) and 40µM digoxin (C).

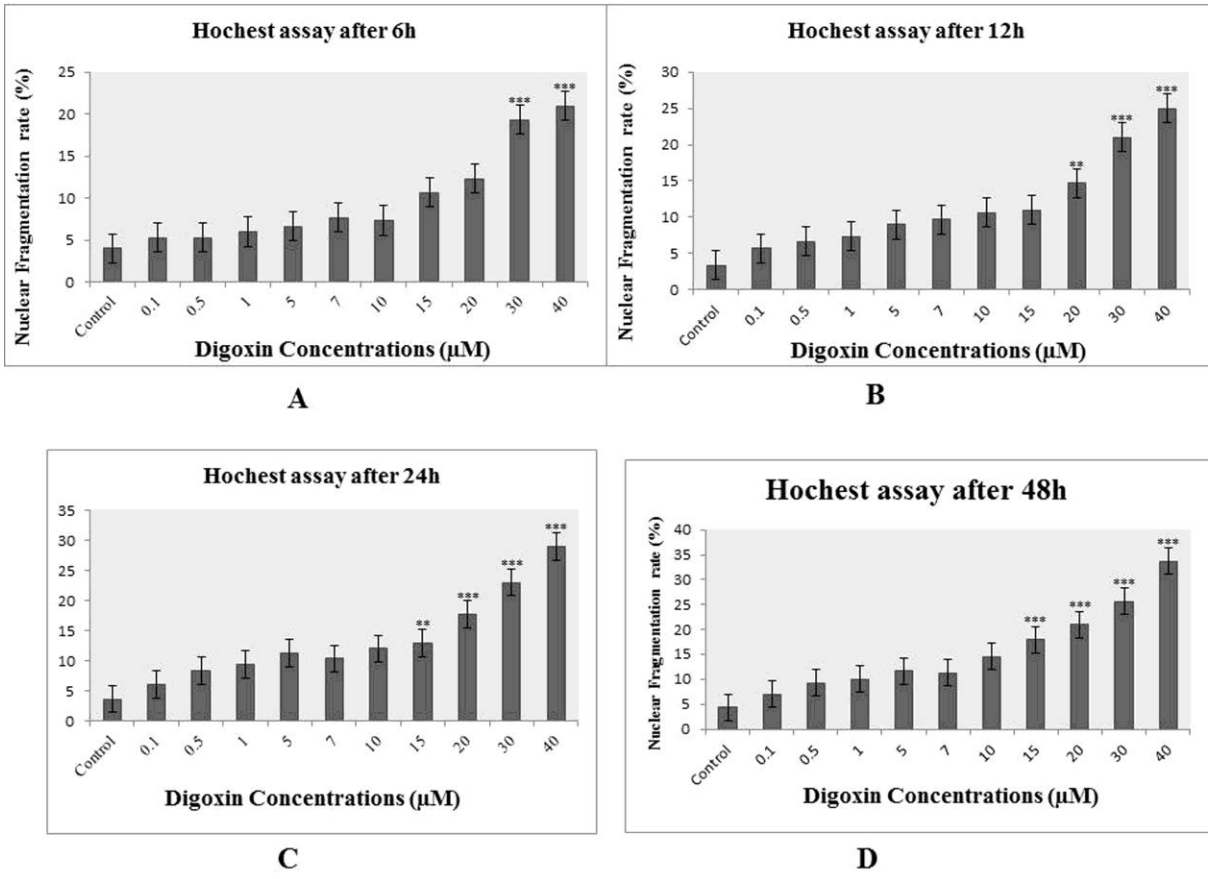

Fig. 4. Following treated the cells with digoxin, MSCs stained with hochest to detect nuclear morphology. Control (A), digoxin treated groups (B and C). The arrows indicate denser nucleus.

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.

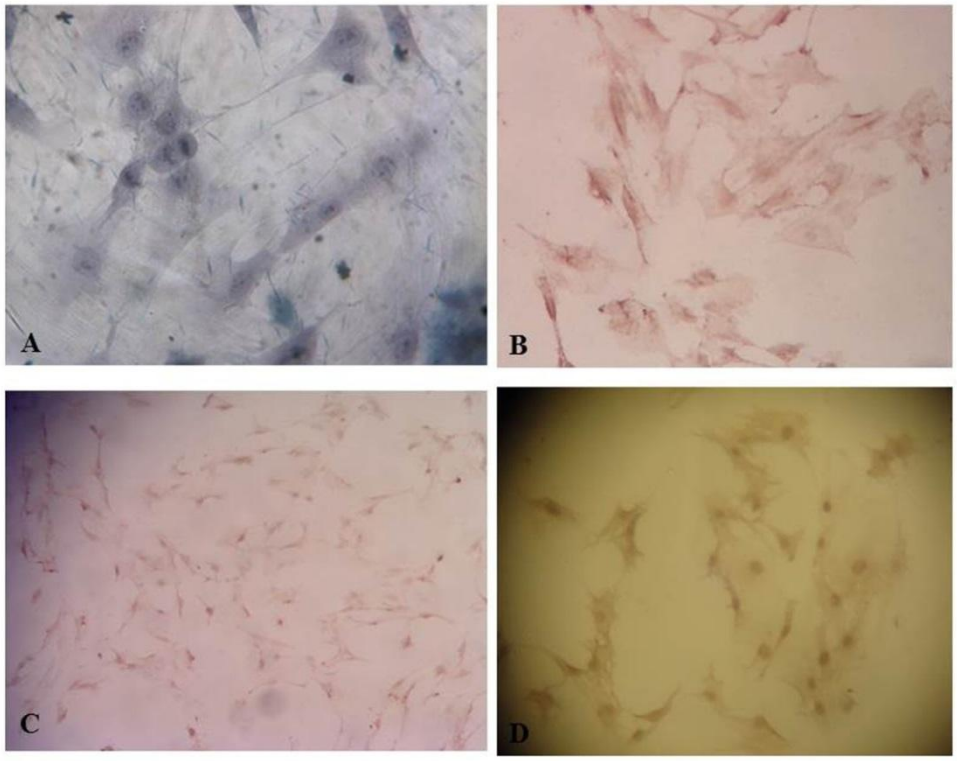

Fig. 5. the diagram shows apoptosis rate of MSCs after 6, 12, 24 and 48 h in different concentration of digoxin.

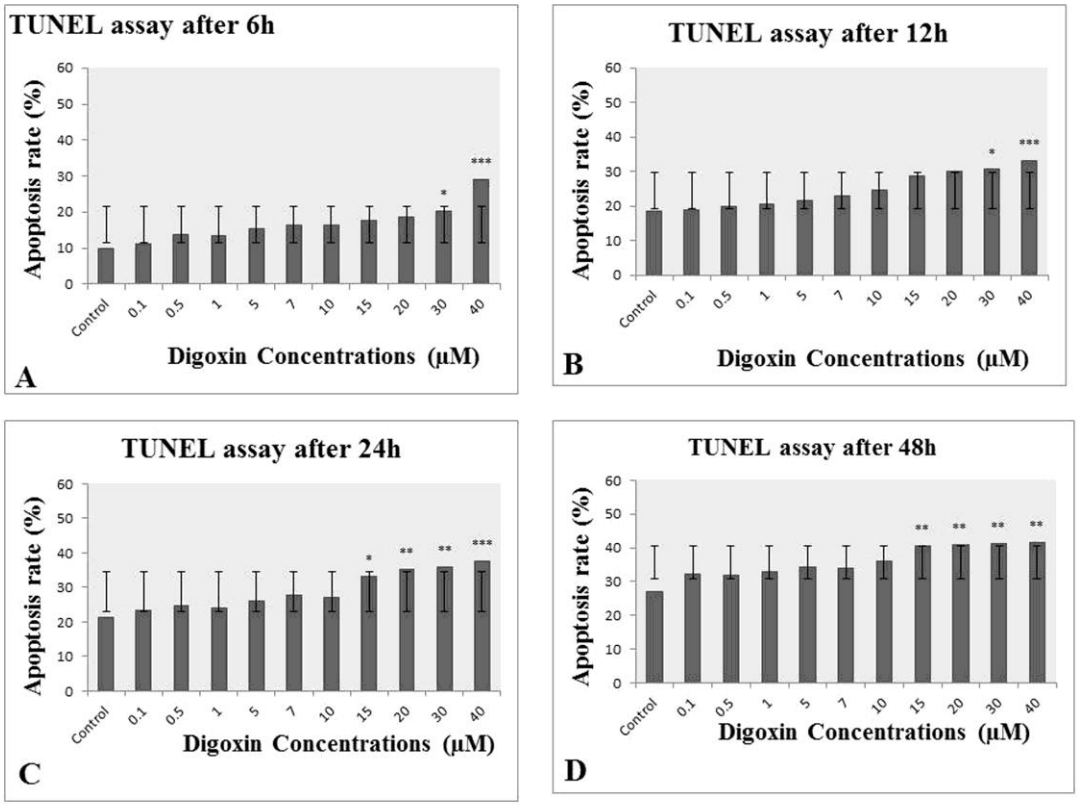

Fig. 6. The diagram indicates rate of apoptosis (%) in different concentrations of digoxin at 6, 12, 24 and 48hr after culture of the MSCs.

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.

References

[1] Mirotsou M, Jayawardena TM, Schmeckpeper J, Gnecchi M, Dzau VJ. Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. *J Mol Cell Cardiol*. 2011;50:280-9.

[2] Berry MF, Engler AJ, Woo YJ, Pirolli TJ, Bish LT, Jayasankar V, *et al.* Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. *Am J Physiol-Heart Circulate Physiol*. 2006;290:H2196-H203.

[3] Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, *et al.* Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nature Med*. 2006;12:459-65.

[4] Wang T, Tang W, Sun S, Ristagno G, Xu T, Weil MH. Improved outcomes of cardiopulmonary resuscitation in rats with myocardial infarction treated with allogenic bone marrow mesenchymal stem cells. Crit Care Med. 2009;37:833-9.

[5] Wang T, Tang W, Sun S, Wan Z, Xu T, Huang Z, *et al.* Mesenchymal stem cells improve outcomes of cardiopulmonary resuscitation in myocardial infarcted rats. J Mol Cell Cardiol. 2009;46:378-84.

[6] Wen Z, Zheng S, Zhou C, Wang J, Wang T. Repair mechanisms of bone marrow mesenchymal stem cells in myocardial infarction. *J Cell Mol Med*. 2011;15:1032-43.

[7] Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, *et al.* Heart disease and stroke statistics—2010 update A report from the American Heart Association. *Circulation*. 2010;121:e46-e215.
[8] Laflamme MA, Murry CE. Regenerating the heart. *Nature Biotech*. 2005;23:845-56.
[9] Melo LG, Pachori AS, Kong D, Gnecchi M, Wang K, Pratt RE, *et al.* Molecular and cell-based therapies for protection, rescue, and repair of ischemic myocardium reasons for cautious optimism. *Circulation*. 2004;109:2386-93.
[10] Winnicka K, Bielawski K, Bielawska A. Cardiac glycosides in cancer research and cancer therapy. *Acta Pol Pharm*. 2006;63:109-15.
[11] Abarquez J, Ramon F. Microvascular disease relevance in the hypertension syndrome. Clin Hemorheol Microcirc. 2003;29(3):295-300.
[12] Johansson S, Lindholm P, Gullbo J, Larsson R, Bohlin L, Claeson P. Cytotoxicity of digitoxin and related cardiac glycosides in human tumor cells. *Anti-Cancer Drugs*. 2001;12(5):475-83.
[13] Yeh JY, Huang WJ, Kan SF, Wang PS. Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells. *The Prostate*. 2003;54:112-24.
[14] Schächinger V, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, Hölschermann H, *et al.* Intracoronary bone marrow–derived progenitor cells in acute myocardial infarction. N Engl J Med. 2006;355:1210-21.
[15] Amiri F, Halabian R, Dehgan Harati M, Bahadori M, Mehdipour A, Mohammadi Roushandeh A, *et al.* Positive selection of Wharton's jelly-derived CD105+ cells by MACS technique and their subsequent cultivation under suspension culture condition: A simple, versatile culturing method to enhance the multipotentiality of mesenchymal stem cells. *Hematology*. 2015;20:208-16.
[16] Pasha Z, Wang Y, Sheikh R, Zhang D, Zhao T, Ashraf M. Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium. *Cardiovascular Res*. 2008;77:134-42.
[17] Banuls LMY, Katz A, Miklos W, Cimmino A, Tal DM, Ainbinder E, *et al.* Hellebrin and its aglycone form hellebrigenin display similar in vitro growth inhibitory effects in cancer cells and binding profiles to the alpha subunits of the Na+/K+-ATPase. *Mol Cancer*. 2013;12:33.
[18] Slingerland M, Cerella C, Guchelaar H, Diederich M, Gelderblom H. Cardiac glycosides in cancer therapy: from preclinical investigations towards clinical trials. *IND*. 2013;31:1087-94.
[19] Stenkvist B, Bengtsson E, Dahlqvist B, Eriksson O, Jarkrans T, Nordin B. Cardiac glycosides and breast cancer, revisited. N Engl J Med. 1982;306:484.
[20] Stenkvist B, Bengtsson E, Eklund G, Eriksson O, Holmquist J, Nordin B, *et al.* Evidence of a modifying influence of heart glucosides on the development of breast cancer. *AQCH*. 1979;2:49-54.
[21] Li BH, Yuan L, Shi RR, Wang JG. [Reversal of adriamycin resistance by digoxin in human breast cancer cell line MCF-7/adriamycin and its mechanism]. *Sheng Li Xue Bao*. [English Abstract Research Support, Non-U S Gov't]. 2015;67(6):611-7.
[22] Platz EA, Yegnasubramanian S, Liu JO, Chong CR, Shim JS, Kenfield SA, *et al.* A novel two-stage, transdisciplinary study identifies digoxin as a possible drug for prostate cancer treatment. *CANCER DISCOV*. 2011;1:68-77.
[23] Lin S-Y, Chang H-H, Lai Y-H, Lin C-H, Chen M-H, Chang G-C, *et al.* Digoxin suppresses tumor malignancy through inhibiting multiple src-related signaling pathways in non-small cell lung cancer. *PloS one*. 2015;10:e0123305.
[24] López-Lázaro M, Pastor N, Azrak SS, Ayuso MJ, Austin CA, Cortés F. Digitoxin inhibits the growth of cancer cell lines at concentrations commonly found in cardiac patients. *Journal of natural products*. 2005;68:1642-5.
[25] Svensson A, Azarbayjani F, Bäckman U, Matsumoto T, Christofferson R. Digoxin inhibits neuroblastoma tumor growth in mice. *Anticancer Res*. 2005;25:207-12.
[26] Tahervand A, Mahmoodi M, Mohammadi A. Digoxin effectively decreased proliferation of liver cancer cell line. *cancer Ther*. 2016;11:12.
[27] Yu SP. Regulation and critical role of potassium homeostasis in apoptosis. *Prog neurobiol*. 2003;70:363-86.
[28] Bortner CD, Cidlowski JA. The role of apoptotic volume decrease and ionic homeostasis in the activation and repression of apoptosis. *Pflügers Archiv*. 2004;448:313-8.
[29] Panayiotidis M, Bortner C, Cidlowski J. On the mechanism of ionic regulation of apoptosis: would the Na+/K+-ATPase please stand up? *Acta Physiologica*. 2006;187:205-15.

