Ezhilarasan Devaraj et al. / Journal of Pharmacy Research 2014,8(8),1126-1131

Research Article
ISSN: 0974-6943

Available online through
http://jprsolutions.info

Silibinin treatment to hepatic stellate cells: an exploration of apoptosis and cellular senescence

Ezhilarasan Devaraj [1], Sivanesan Karthikeyan [2], Etienne Sokal [1], Mustapha Najimi [1*]

[1] Institut de Recherche Expérimentale et Clinique (IREC),
Laboratory of Pediatric Hepatology and Cell Therapy, Université Catholique de Louvain, Brussels, Belgium.
[2] Department of Pharmacology and Environmental Toxicology, Food and Hepatotoxicology Laboratory,
Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai, India.

Received on:06-06-2014; Revised on: 09-07-2014; Accepted on:08-08-2014

ABSTRACT

Objective: Proliferation and activation of hepatic stellate cells play pivotal role in the progression of hepatic fibrosis consequent to chronic liver injury. Thus, the inhibition and clearance of activated HSCs from the injured liver by apoptosis and cellular senescence is clearly an appropriate approach for therapeutic treatment for hepatic fibrosis. To evaluate the pro-apoptotic and cellular senescence inducing potential of silibinin, a flavonoid compound in LX-2 cells. **Methods:** LX-2 cells treated with various concentration of silibinin (10, 50 and 100 µM) treatment for 96 h, cytotoxicity test, cell counting pro-apoptotic and cellular senescence induction properties of silibinin were evaluated. **Results:** The present study demonstrates that SBN treatment shows a dose-dependent fall in active proliferation of immortalized LX-2 cells in serum supplemented medium with out affecting its cell viability. Further, SBN treatments neither induce apoptosis nor cellular senescence in the human hepatic stellate LX-2 cells. **Conclusion:** The anti-proliferative effect of SBN reported in this study might be due its cell cycle arresting properties.

KEYWORDS: Silibinin, hepatic stellate cells, apoptosis, cellular senescence, proliferation

1. INTRODUCTION

Fibrosis is a reversible scarring response that occurs in almost all patients with chronic liver injury. Ultimately, hepatic fibrosis leads to cirrhosis, associated with nodule formation and organ contraction. It is an established fact that activation and proliferation of hepatic stellate cells (HSCs) play a vital role in development and progression of hepatic fibrosis [1,2]. Experimental and clinical studies have shown that suppression of HSCs proliferation could be an index towards the reversal of hepatic fibrosis [3,4]. It is suggested that suppression of HSCs proliferation could be achieved by enhancing i) apoptosis and ii) cellular senescence [5]. Any one of the above mechanisms or together might be employed effectively towards clearance of actively proliferating HSCs from fibrotic liver, and thereby the suppression of hepatic fibrosis.

Experimental evidences have concretely proved that apoptosis of activated HSCs play a vital role towards recovery from CCl_4 [6] and bile duct ligation-induced [7] hepatic fibrosis in rats. On the other hand, prolonging survival of activated HSCs or increasing resistance to apoptosis have been shown to contribute towards progression of hepatic fibrosis. It has been proved experimentally by administration of gliotoxin, an agent that provokes selective apoptosis of HSCs in both *in vivo* and *in vitro* studies has been shown to retard hepatic fibrosis [8]. Hence, promoting the clearance of proliferated and activated HSCs by enhancing its apoptosis is considered as an effective strategy towards mitigation of hepatic fibrosis.

Further, cellular senescence is the process of preventing cell division and it is controlled by expression of certain genes and this could occur once the cells exceed a finite proliferative capacity. Senescent cells could be distinguished morphologically from quiescent cells by expression of senescence associated factor i.e., ß-galactosidase (ß-gal) enzyme activity [9]. Cellular senescence down regulates genes involved in cell proliferation, extracellular matrix (ECM) production, up regulation of inflammatory cytokines and other molecules known to modulate the microenvironment or immune response in proliferating cells [10]. In view of these reports, enhancing cellular senescence is now being considered as a means for delaying or retarding the process of fibrosis.

***Corresponding author.**
Dr. Mustapha Najimi,
Laboratory of Pediatric Hepatology and Cell Therapy
Avenue Hippocrate 10/1301
1200, Brussels, Belgium

Mr. Ezhilarasan Devaraj,
ERASMUS MUNDUS Research Fellow,
Laboratory of Pediatric Hepatology and Cell Therapy,
Université Catholique de Louvain,
Avenue Hippocrate 10/1301,
1200, Brussels, Belgium.
Email:drezhild@yahoo.com

Dr. Sivanesan Karthikeyan

Silibinin (SBN) is a natural polyphenolic flavonolignan extracted from the seeds of the medicinal plant *Silybum marianum* and it is reported to possess strong anti-proliferative and pro-apoptotic activities and it also possess pro-apoptotic properties in actively proliferating cancer cells of prostate, skin and bladder [11,12,13]. The mechanism of SBN-induced apoptosis varies depending upon the cell type in study. For instance, SBN-induce apoptosis by down regulation of "survivin" protein expression in lung cancer cells [14]. by activating caspase-mediated cleavage of p21 protein in RT4 bladder cancer cell lines, and by suppression growth in ECV304 human umbilical vein endothelial cell lines. SBN is also reported to induce apoptosis by enhancing ROS formation in various cell lines [11,12,13]. In the light of these reports, it is likely that SBN exposure would produce anti-proliferative effects by inducing apoptosis and cellular senescence in LX-2 cells. To investigate whether or not the above mechanisms play a role in inducing anti-proliferative effect, nuclear morphology and cellular senescence were investigated in LX-2 cell lines upon SBN exposure. In the current study, we report that SBN treatments dose dependently inhibit the proliferation of LX-2 cells with out inducing cytotoxicity, apoptosis and cellular senescence.

2. MATERIALS AND METHODS

2.1. Reagents and Drugs

Silibinin ($C_{25}H_{22}O_{10}$; CAS No. 22888-70-6) was purchased from Sigma Chemical Co. (Belgium) is a mixture of two diastereoisomeric compounds (silybin A and silybin B).

2.2. Cell culture

LX-2 cells used for this study were kindly provided by Dr. S. L. Friedman, Mount Sinai School of Medicine, New York. These cells are derived from normal human HSC that are spontaneously immortalized. LX-2 cells exhibit the typical features of HSCs in primary culture, expressing desmin, glial acidic fibrillary protein, and response to platelet-derived growth factor BB and TGF-ß1. It express a- smooth muscle actin (α-SMA) under all culture conditions and therefore were regarded as at least partially activated even after immediate replating [15]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% of fetal bovine serum (Gibco, Belgium) and penicillin-streptomycin (Life Technologies, Belgium) and maintained at 37° C in a fully humidified atmosphere containing 5% CO_2 . In the present study, cells in passages 6-10 were used. The protocol and experiments were approved by the ethical committees of the St-Luc Hospital and faculty of Medicine of Université Catholique de Louvain, Brussels, Belgium.

2.3. Cell treatment

SBN was dissolved in 0.1% DMSO (v/v). LX-2 cells were plated at 10,000 cells/cm^2, and after 24 h, fed with fresh expansion culture medium supplemented or not with different final concentrations of SBN (10, 50 and 100 µM). After 96 h of treatment, cells were collected by 0.05% trypsin (Life technologies, Belgium) application. Total cell number was determined by counting each sample in triplicate using a KOVA Glasstic® Slide 10 under Leica DMIL inverted microscope.

2.4. Analysis of cytotoxicity

Confluent LX-2 cells were incubated with expansion culture medium supplemented or not with different final concentrations of SBN (10, 50 and 100 µM) for 96 h. Cell viability was evaluated by the trypan blue dye exclusion test.

2.5. Cytochemical staining for ß-galactosidase

β-galactosidase staining of LX-2 cells were performed to assess the induction of cellular senescence by SBN treatment, using histochemical staining kit (Sigma-Aldrich, Belgium, Product Number: CS0030). Briefly, After 96 h of treatment, existing medium was aspirated, cells were washed twice with D-PBS and then 1.5 ml of fixation buffer was added and incubated for 6 to 7 min at room temperature. Then, the cells were rinsed 3 times with D-PBS and 1 ml of β-galactosidase staining solution was added, and was incubated at 37º C for overnight. Stained cultures were viewed under bright field phase contrast microscope (Leica) and representative fields were photographed (IM50 image analyzer).

2.6. DAPI nucleic acid staining

Condensed chromatin and nuclear fragmentation, characteristic of apoptosis was performed by using DAPI staining (Thermo Scientific, Belgium). The fluorescent dye 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI) binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity. On adding DAPI to culture cells it is rapidly taken up into cellular DNA yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence. Briefly, the cells were seeded in 24 well plates at density of 5,000 cells/cm^2 and treated with increasing concentrations of SBN for 96 h. For the nuclear analysis, at the end of the treatment period, the monolayer of cells were washed with PBS and stained with DAPI at 0.5µg/ml for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei, and condensed chromatin) were observed under a digital fluorescence microscope EVOS (AMG, USA).

2.7. Statistical analysis

The values are presented as mean ± S.D. One way Analysis of Variance (ANOVA) and the Post-hoc multiple comparisons was performed by Newman-Keul's Multiple Comparison Test using Graph Pad Prism software (version 5).

3. RESULTS

3.1. Effect of SBN treatments on the cytotoxicity of LX-2 cells

The data shows that DMSO and SBN treatments (10, 50 and 100 µM) did not produce any cytotoxic effects and the cell viability was more than 90% as compare to control during their exposure for 96 h (Fig. 1). The viable LX-2 cells were observed as cultured activated and acquired myofibroblast like phenotypes, which were the characteristic features of the activated HSCs in fibrotic liver diseases (Fig. 2).

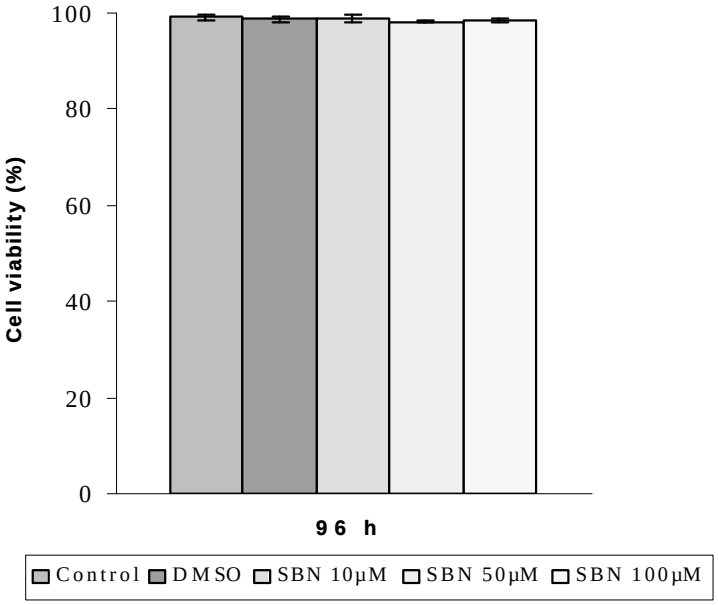

Fig. 1. Analysis of cell viabiity. Values are presented as mean ± S.D. of 4 nos. of observations for 96 h of different concentrations of SBN exposure respectively. Multiple comparisons between treatment groups were performed by Newman-Keul's test

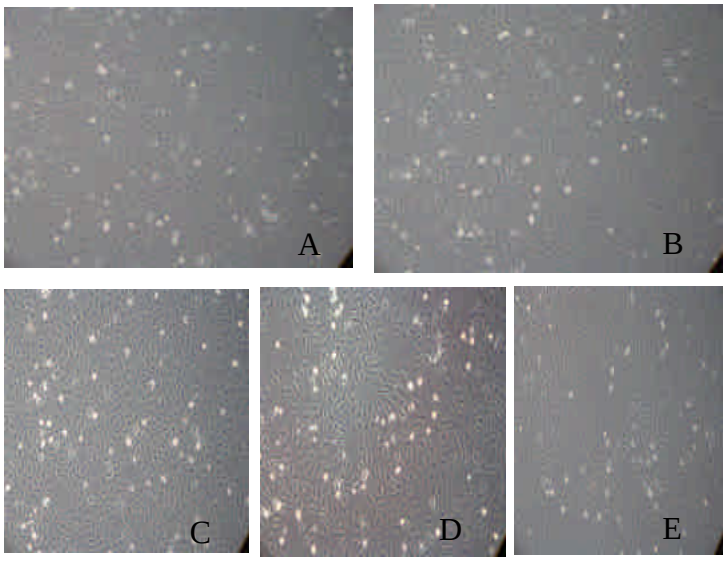

Fig. 2. Morphology of LX-2 cells treated with silibinin for 96 h. A – Control; B – DMSO; C, D, E – SBN 10, 50 and 100 ㎜ treatments respectively (100x).

3.2. Effect of SBN treatments on the proliferation of LX-2 cells

Treatment of LX-2 cells at different concentrations of SBN for 96 h in serum supplemented medium produced a dose-dependant fall in the proliferative activity of LX-2 cells as compared to control and DMSO treatments ($p<0.01$; $p<0.001$). The anti-proliferative activity of SBN on LX-2 cells was at the maximum in the highest concentration (100 µM) (Fig. 3).

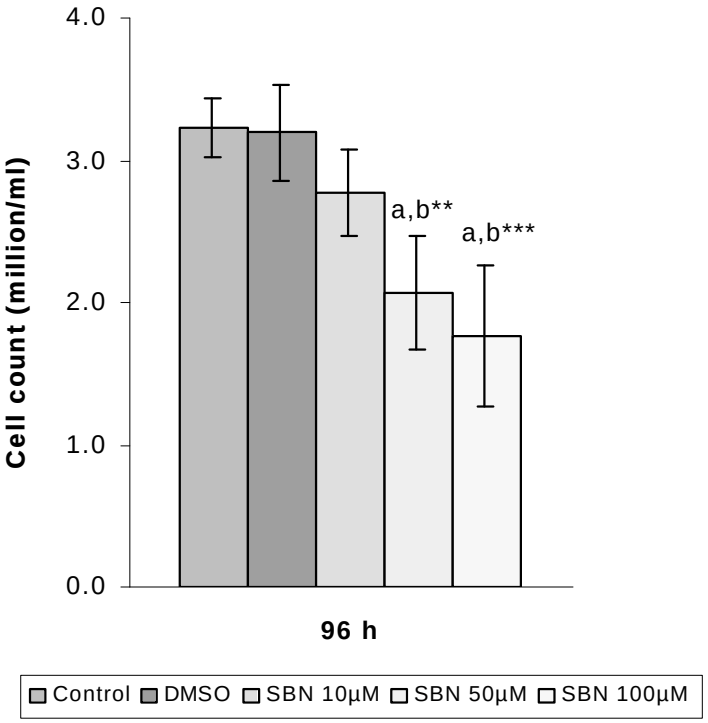

Fig. 3. Proliferation of LX-2 cells. Values are presented as mean ± S.D. of 4 nos. of observations for 96 h of different concentrations of SBN exposure respectively. Multiple comparisons between treatment groups were performed by Newman-Keul's test. a – control compared to DMSO and SBN treatment groups. b – DMSO group compared to all the SBN treated groups. ** $p<0.01$; *** $p<0.001$.

3.3. DAPI staining for the evaluation of apoptosis and nuclear morphology

DAPI staining was performed in LX-2 cells exposed to different concentrations of SBN (10, 50 and 100 µM) for 96 h to evaluate its effect on apoptosis. DAPI staining permits its binding to cellular DNA and changes in nuclear morphology of apoptotic cells is indicated by shrunken or dot-shaped nuclear fragments when visualized under fluorescent microscope. In this study, this evaluation shows identical nuclear morphology in control, DMSO and all SBN treatments in LX-2 cells, clearly indicating that SBN exposure does not cause apoptosis in these cells and the nuclear morphology was intact (Fig. 4).

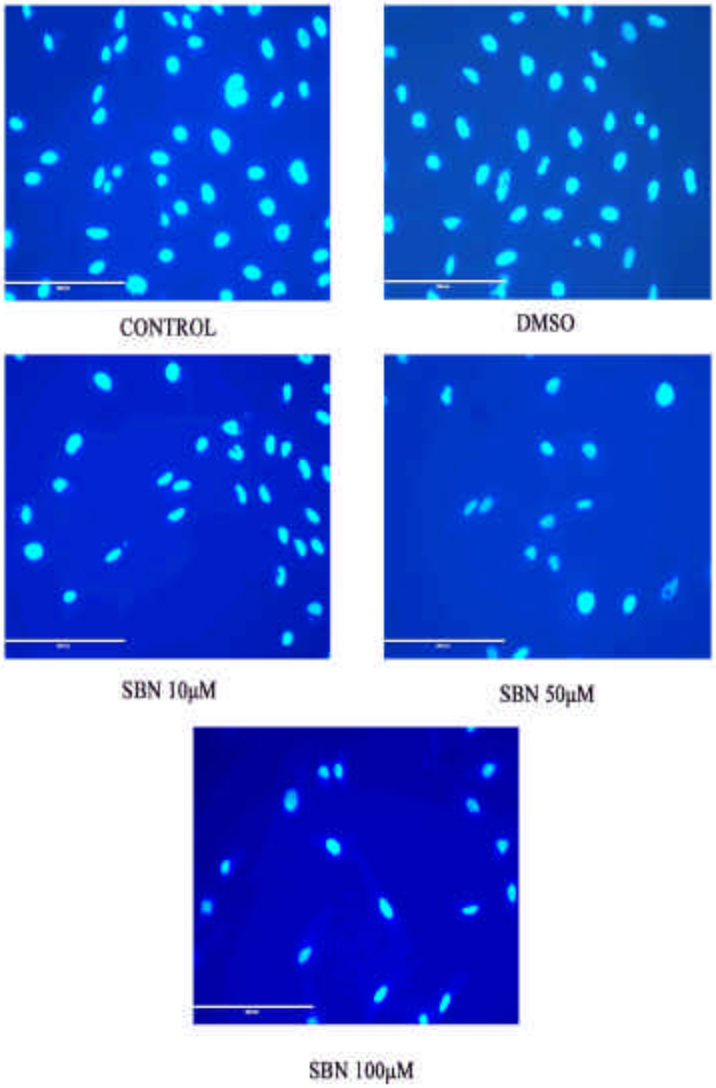

Fig. 4. DAPI staining. Nuclear morphology of LX-2 cells treated with silibinin for 96 h (100x).

3.4. ß-galactosidase (ß-gal) assay for evaluation of cellular senescence

ß-gal staining is an index to evaluate the cellular senescence and this technique was employed to investigate the senescence inducing capacity of SBN on rapidly proliferating LX-2 cells. Adult Derived Human Liver Stem Cells (ADHLSCs) of 17th passage shows positivity towards ß-gal staining indicating cellular senescence in these cells. None of the LX-2 cells of control, DMSO and SBN treatments (10, 50 and 100 µM) exhibit any positivity towards ß-gal staining during 96 h period of study. These results clearly indicate that SBN treatment does not induce cellular senescence in rapidly proliferating LX-2 cells (Fig. 5).

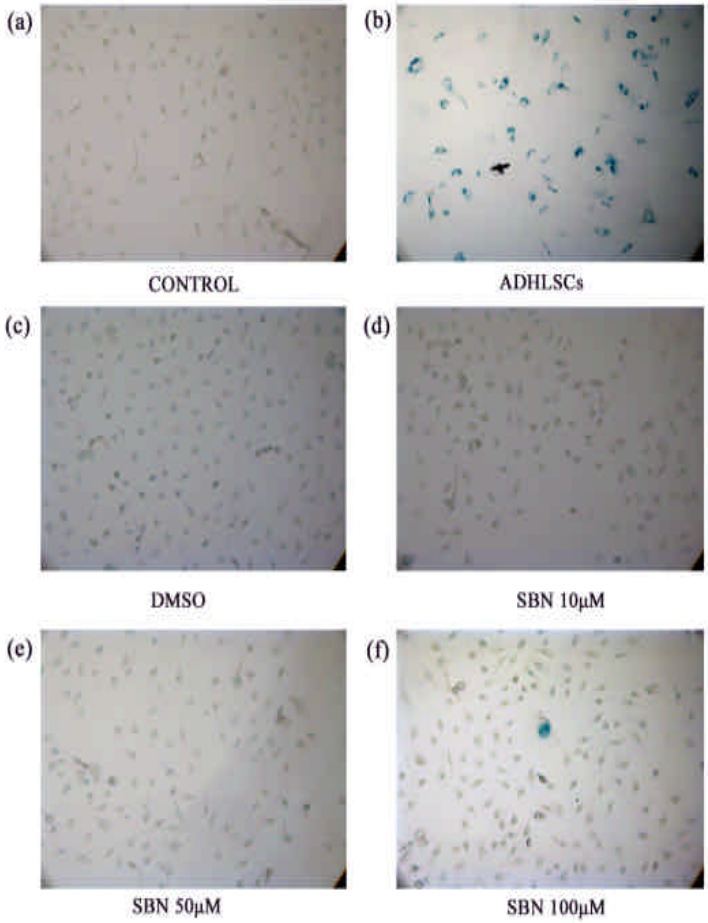

Fig. 5. Analysis of cellular senescence. b- **galactosidase staining of LX-2 cells treated with silibinin for 96 h (100x). (b) 17 passages old Adult Derived Human Liver Stem Cells (ADHLSCs) used as positive control.**

4. DISCUSSION

The flavonoid SBN is known to intervene in many cellular processes involved in a variety of pathologies, thus appearing a promising therapeutic tool [16]. Ironically, SBN exposure has been reported to induce cytotoxicity in various cell lines [17,18]. In contrast to these reports, we have observed that SBN exposure is not cytotoxic in LX-2 cells in the present study. It is likely that this discrepancy in cytotoxic response to SBN exposure could be due to different mechanisms of action of SBN on various cell types as being proposed byZhang *et al* [19]. SBN treatment shows a dose-dependent fall in active proliferation of immortalized LX-2 cells in serum supplemented medium. These observations clearly indicate that SBN treatments retard active proliferation of LX-2 cells with out affecting its cell viability.

It is suggested that the rapidly proliferating HSCs can undergo spontaneous cell death or receptor mediated death and thus they are susceptible to apoptosis [20]. Further, fibrosis is reported to stimulate apoptosis. It is proposed that certain pro-apoptotic genes are ex-

pressed in parenchymal cells, which leads to the up regulation of death receptors (FAS/FASL) leading to apoptosis during ECM accumulation in fibrosis [21,22]. Conversely, it is also reported that the ECM mediated expression of collagen l and TIMP I sends anti-apoptotic signals, rending the HSCs to be resistant to apoptotic stimuli [23]. In view of these reports, inhibiting parenchymal cell apoptosis and enhancing HSCs apoptosis could be an effective therapeutic approach for stepping down hepatic fibrosis. In the present investigation, the pro-apoptotic potentials of SBN treatments on LX-2 cells were evaluated in order to ascertain whether SBN can be a better candidate to induce apoptosis of rapidly proliferating LX-2 cells.

SBN is reported to possess apoptotic inducing properties in rapidly proliferating cancerous cells and hence it was suggested as better candidate for suppressing cancer [12,13,19]. DAPI fluorescent stain binds to the fragmented nuclear debris (apoptotic bodies), which could be visualized clearly by dot shaped nuclear fragments [19,24]. SBN treated LX-2 cells did not show any alteration in their nuclear morphology, clearly indicating that these cells has not undergone apoptosis. Briefly, in the present study, DAPI staining fluorescent microscopic studies have clearly shown that SBN exposure at different concentrations does not have any apoptotic effect on rapidly proliferating LX-2 cells. Gonzalez *et al* [25] reported that the higher incidence to resistance of human primary HSCs by expressing pro-apoptotic protein Bcl-2 (B-cell lymphoma 2) and our results are in agreement with this report.

Cellular senescence is the process of irreversible arrest of cell growth and hence it is a major barrier for cell proliferation [26,27]. ß-gal staining is prominent technique developed to evaluate the senescent stage of cells. The senescent cells take up this dye and yield blue color when visualized under microscope and non-senescence and actively proliferating cells will not take up this dye. It is the most sensitive and appropriate cytochemical technique for evaluating cellular senescence. Exposure of LX-2 cells to various concentrations of SBN did not show any positivity towards ß-gal staining and this cytochemical evaluation clearly indicates the fact that SBN does not promote cellular senescence. This novel observation is not reported elsewhere in the literature. In conclusion, the present study demonstrates that DAPI staining technique has shown that SBN treatment does not have any apoptotic effect on LX-2 cells. ß-gal staining has shown that LX-2 cells does not under go cellular senescence during SBN treatments.

5. CONCLUSION

From these experimental evidences, we conclude that SBN treatments neither induce apoptosis nor cellular senescence in the human hepatic stellate LX-2 cells. The anti-proliferative effect of SBN reported in this study might be due its cell cycle arresting properties and further studies are warranted on these lines.

ACKNOWLEDGMENTS

Mr. Ezhilarasan Devaraj gratefully acknowledges the support of a 20-month scholarship obtained thanks to the MAHEVA ERASMUS MUNDUS Action 2 European project (Agreement No. 2010-2372 / 001-001-EMA2, coordinator Montpellier2).

REFERENCES

1. Zhou WC, Zhang QB, Qiao L. Pathogenesis of liver cirrhosis. *World J Gastroenterol.* 2014; 20(23): 7312-7324.
2. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology.* 2008; 134(6): 1655-1669.
3. Wang F, Liu S, DU T, Chen H, Li Z, Yan J. NF-κB inhibition alleviates carbon tetrachloride-induced liver fibrosis via suppression of activated hepatic stellate cells. *Exp Ther Med.* 2014; 8(1): 95-99.
4. Sakata R, Ueno T, Nakamura T, Sakamoto M, Torimura T, Sata M. Green tea polyphenol epigallocatechin-3-gallate inhibits platelet-derived growth factor-induced proliferation of human hepatic stellate cell line LI90. *J Hepatol.* 2004; 40(1): 52-59.
5. Kuo LM, Kuo CY, Lin CY, Hung MF, Shen JJ, Hwang TL. Intracellular glutathione depletion by oridonin leads to apoptosis in hepatic stellate cells. *Molecules.* 2014; 19(3): 3327-3344.
6. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJ. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J Clin Invest.* 1998; 102(3): 538-549.
7. Iredale JP. Hepatic stellate cell behavior during resolution of liver injury. *Semin Liver Dis.* 2001; 21(3): 427-436.
8. Wright MC, Issa R, Smart DE, Trim N, Murray GI, Primrose JN, Arthur MJ, Iredale JP, Mann DA. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. *Gastroenterology*. 2001; 121(3): 685-698.
9. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*. 2007; 8(9): 729-740.
10. Schnabl B, Purbeck CA, Choi YH, Hagedorn CH, Brenner D. Replicative senescence of activated human hepatic stellate cells is accompanied by a pronounced inflammatory but less fibrogenic phenotype. *Hepatology*. 2003; 37(3): 653-664.

11. Katiyar SK, Korman NJ, Mukhtar H, Agarwal R. Protective effects of silymarin against photocarcinogenesis in a mouse skin model. *J Natl Cancer Inst.* 1997; 89(8): 556-566.
12. Singh RP, Tyagi AK, Zhao J and Agarwal R. Silymarin inhibits its growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis. *Carcinogenesis.* 2002; 23(3): 499-510.
13. Deep G, Agarwal R. Antimetastatic efficacy of silibinin: molecular mechanisms and therapeutic potential against cancer. *Cancer Metastasis Rev*. 2010; 29(3): 447-463.
14. Kim JY, Kim EH, Kim SU, Kwon TK, Choi KS. Capsaicin sensitizes malignant glioma cells to TRAIL-mediated apoptosis via DR5 upregulation and survivin downregulation. *Carcinogenesis.* 2010; 31(3): 367-375.
15. Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, Mukherjee P, Friedman SL, Eng FJ. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut.* 2005 ; 54(1): 142-151.
16. Grillo C, Chichiarelli S, Gaucci E, Altieri F, Turano C, Cervoni L. The binding of silibinin to ERp57. *Chem Biol Interact.* 2014; 25(213): 37-43.
17. Deep G, Singh RP, Agarwal C, Kroll DJ, Agarwal R. Silymarin and silibinin cause G1 and G2-M cell cycle arrest via distinct circuitries in human prostate cancer PC3 cells: a comparison of flavanone silibinin with flavanolignan mixture silymarin. *Oncogene*. 2006; 25(7): 1053-1069.
18. Li L, Gao Y, Zhang L, Zeng J, He D, Sun Y. Silibinin inhibits cell growth and induces apoptosis by caspase activation, down-regulating survivin and blocking EGFR-ERK activation in renal cell carcinoma. *Cancer Lett.* 2008; 272(1): 61-69.
19. Zhang Y, Li Q, Ge Y, Chen Y, Chen J, Dong Y, Shi W. Silibinin triggers apoptosis and cell-cycle arrest of SGC7901 cells. *Phytother Res.* 2013; 27(3): 397-403.
20. Kisseleva T, Brenner DA. Hepatic stellate cells and the reversal of fibrosis. *J Gastroenterol Hepatol.* 2006; 21: S84-87.
21. Razzaque MS, Ahsan N, Taguchi T. Role of apoptosis in fibrogenesis. *Nephron.* 2002; 90(4): 365-372.
22. Razzaque MS, Koji T, Kumatori A, Taguchi T. Cisplatin-induced apoptosis in human proximal tubular epithelial cells is associated with the activation of the Fas/Fas ligand system. *Histochem Cell Biol.* 1999 ; 111(5): 359-365.
23. Holt AP, Haughton EL, Lalor PF, Filer A, Buckley CD, Adams DH. Liver myofibroblasts regulate infiltration and positioning of lymphocytes in human liver. *Gastroenterology.* 2009; 136(2): 705-714.
24. Souza IC, Martins LA, Coelho BP, Grivicich I, Guaragna RM, Gottfried C, Borojevic R, Guma FC. Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells. *Mol Cell Biochem*. 2008; 15(1-2): 1-7.
25. Gonzalez SA, Fiel MI, Sauk J, Canchis PW, Liu RC, Chiriboga L, Yee HT, Jacobson IM, Talal AH. Inverse association between hepatic stellate cell apoptosis and fibrosis in chronic hepatitis C virus infection. *J Viral Hepat.* 2009; 16(2): 141-148.
26. Smith JR, Pereira-Smith OM. Replicative senescence: implications for *in vivo* aging and tumor suppression. *Science*. 1996; 273(5271): 63-67.
27. Campisi J. Cancer, aging and cellular senescence. *In Vivo. 2000;* 14(1): 183-188.

Source of support: Nil, Conflict of interest: None Declared