Optimization of the isolation procedure and culturing conditions for hepatic stellate cell obtained from mouse

Minh Thanh Dang, MSc\textsuperscript{1,2,3}, Van Trinh Le, MSc\textsuperscript{1,2#}, Quang Huy Do, MSc\textsuperscript{1,2}, Van Thuan Nguyen, PhD\textsuperscript{2,3}, Ai Xuan Le Holterman, MD, PhD\textsuperscript{4}, Loan Tung Thi Dang, PhD\textsuperscript{2,5}, Nhan Chinh Lu Phan, MSc\textsuperscript{2,6}, Phuc Van Pham, PhD\textsuperscript{1,2}, Son Nghia Hoang, PhD\textsuperscript{7}, Long Thanh Le, PhD\textsuperscript{7}, Gabriele Grassi, PhD\textsuperscript{8}, Nhung Hai Truong, PhD\textsuperscript{1,2,5*}

\textsuperscript{1}Laboratory of Stem Cell Research and Application, University of Science-VNUHCM, Vietnam

\textsuperscript{2}Viet Nam National University, Ho Chi Minh City, Vietnam

\textsuperscript{3}School of Biotechnology, International University- VNUHCM, Vietnam

\textsuperscript{4}Department of Pediatrics and Surgery, U of Illinois College of Medicine, Chicago and Peoria, IL, United States of America

\textsuperscript{5}Faculty of Biology and Biotechnology, University of Science- VNUHCM, Vietnam

\textsuperscript{6}Stem cell Institute, University of Science-VNUHCM, Vietnam

\textsuperscript{7}Animal Biotechnology Department, Institute of Tropical Biology, Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam

\textsuperscript{8}Department of Life Sciences, Cattinara University Hospital, Trieste University, Trieste, Italy

# Equal contribution to the work

*Corresponding author

Nhung Hai Truong, PhD.

Faculty of Biology and Biotechnology, University of Science- VNUHCM, Vietnam
Laboratory of Stem Cell Research and Application, University of Science-VNUHCM, Vietnam

227, Nguyen Van Cu str., Ward 04, District 05, Ho Chi Minh city, Vietnam
Tel: +84-907-974-904. Email: thnhung@hcmus.edu.vn
ABSTRACT

Liver fibrosis (LF) mortality rate is approximately 2 million per year. Irrespective of the etiology of LF, a key element in its development is the transition of Hepatic stellate cells (HSCs) from a quiescent phenotype to a myofibroblast-like cell with the production of fibrotic proteins. It is necessary to define optimal isolation and culturing conditions for good HSCs yield and proper phenotype preservation for studying the activation of HSCs in vitro. In this study, the optimal conditions of HSC isolation and culture were examined to maintain the HSC’s undifferentiated phenotype. HSCs were isolated from Balb/c mice liver using Nycodenz, 8%, 9.6%, and 11%. The efficiency of the isolation procedure was evaluated by cell counting and purity determination by flow cytometry. Quiescent HSCs were cultured in test media supplemented with different combinations of fetal bovine serum, glutamine, vitamin A, insulin, and glucose. The cells were assessed at day 3, and day 7 of culture by evaluating the morphology, proliferation using cell counting kit – 8, lipid storage using oil red o staining, expression of a-smooth muscle actin, collagen I and lecithin-retinol acyltransferase by qRT PCR and immunocytochemistry (ICC). The results showed that Nycodenz, at 9.6%, yielded the best purity and quantity of HSCs. Maintenance of HSC undifferentiated phenotype was achieved optimizing culturing conditions (serum-free DMEM supplemented with glucose (100mg/dL), glutamine (0.5mM), vitamin A (100µM) and insulin (50 ng/ml)) with a certain degree of proliferation allowing their perpetuation in culture. In conclusion, we have defined optimal conditions for HSCs isolation and culture.

Key words: Quiescent stellate cells, Hepatic Stellate Cells, liver fibrosis, Nycodenz, HSCs activation

1. INTRODUCTION

Hepatic stellate cells (HSCs) are liver-specific mesenchymal cells that reside within the sub-endothelial space of Disse around the liver sinusoid [1, 2, 3]. In healthy condition, HSCs remain in the quiescent form and function primarily as fat-storing cell representing the largest reservoir of vitamin A in the body [4]. In their quiescent state, HSCs express Glial fibrillary acidic protein (GFAP) [5-8] and desmin [9, 10]. Following toxicity-induced or viral-induced liver injury, HSCs undergo activation to a myofibroblast-like cell [11] characterized by the loss of vitamin A (vitA)-containing lipid droplets (LDs) and by increased expression of many fibrogenic proteins including collagen type 1 α1 (Col1α1),
alpha-smooth muscle actin (α-SMA), all considered to be primary markers of the stellate cell activation [4, 12-14]. Activated HSCs have become recognized as the primary cell contributor to hepatic fibrosis [15]. A precise characterization of HSC’s features together with the determination of the underlying mechanisms responsible for their trans-differentiation, is a prerequisite for the development of novel therapeutic intervention to liver fibrosis.

HSCs isolation and culture are valuable tools to study the HSCs transdifferentiation process. Density gradient centrifugation has been considered the ‘gold standard’ to separate HSCs with high isolation efficiency [16-21]. Many current studies have used various gradient substances such as Stractan [22, 23], Percoll [24-26], Optiprep [27-30], Metrizamide [31], but the most popular is Nycodenz. However, the yield and purity of the isolated HSCs varies greatly among the published works mainly due to the wide range of Nycodenz concentrations used.

Beyond defining the optimal isolation procedure, it is also important to find culturing conditions to maintain HSCs in the quiescent phenotype. This is not a trivial technical aspect as HSCs acquire the activated phenotype typically occurring in liver fibrosis within a few days after isolation and culture [11, 15, 32, 33]. This rapid switch to the activated phenotype represents an obstacle to study HSCs physiology in vitro. Therefore, many studies have investigated the possibility of maintaining the HSC quiescent phenotype in vitro by modulating the culture conditions. In this regard, vitamin A and insulin have been reported to maintain HSC in the quiescent state [34]; glucose and L-glutamine affect both growth and activation of HSCs [35-40]. Therefore, the appropriate combination of these elements in culture could potentially keep HSCs quiescent while promoting proliferation.

The present study was undertaken to evaluate the optimal Nycodenz concentration for isolating HSCs with high purity and yield. We also investigated the culture medium composition to maintain the quiescent phenotype while retaining the ability to proliferate.

2. METHODS

2.1. Animal

4-5 months old BALB/c mice (Pasteur Institute, HCM city, Vietnam) were kept in the Microventilation cage system (THREE-SHINE Inc., Korea), 12-hour dark/light cycle, and were fed with regular chow ad libitum. All in vivo experiments were performed at Laboratory of Animal Care and Use (Stem Cell Institute, VNU-HCM- University of Science, Vietnam) following the guidelines of the EU directive (2010/63/EU) and the permission from the
Animal Ethics Committee of the Stem Cell Institute, VNU-HCM- University of Science, Vietnam (Ref N°: 200501/SCI-AEC).

2.2. Chemicals and Reagents

The basic medium consisted of Dulbecco's Modified Eagle Medium–DMEM with low glucose concentration (100mg/dL), glutamine-free; other supplements: Fetal bovine serum–FBS and glucose solution, GlutaMAX™, purchased from Gibco, US. The primary antibodies used in Flow cytometry, ICC (immunocytochemistry) staining and Western blot were anti-desmin (ab8592), anti-SMA (ab15734), anti-GFAP (ab68428), anti-collagen I (ab21286) anti-GAPDH (ab181602). The secondary antibody was Alexa fluor 488-conjugated (ab150077) and Goat Anti-Rabbit IgG H&L (HRP) (ab6721). All antibodies were purchased from Abcam, US. The antibodies were diluted in antibody diluent, consisting of Tris Buffered Saline, 0.1% tween 20 (TBST) solution, 1% bovine serum albumin – BSA. The blocking buffer was prepared from TBST, 4% goat serum (Gibco, Massachusetts, US), 1% BSA. The permeabilization solution contained PBS and 0.1% Triton x-100. Oil red o, VitA, BSA and insulin were acquired from Sigma-Aldrich, US.

2.3. Hepatic stellate cells isolation

HSCs were isolated from BALB/c mice following the protocol published in 2015 [18] with some modifications. Briefly, mouse was given intramuscular injections of 20mg/kg of Ilium xylazil-20 (Troy laboratories, Australia) and 14mg/kg of Zoletil (Virbac, France) to induce deep anesthesia. Then, livers were digested by in situ perfusion with EGTA solution (Sigma-Aldrich, US) for 2 minutes, pronase E (Merck, Germany) and collagenase D 0.038% for 5-7 minutes each (Roche Diagnostics, Germany). The mouse was sacrificed due to the change in circulation and the opening of diaphragm for liver perfusion. Next, the liver was excised into small pieces and further digested with pronase E and collagenase D supplemented with Dnase I 1% (Roche Diagnostics, Germany) in vitro for 15 minutes. The digested solutions were then filtered through a 70µm cell strainer and subjected to low-speed centrifugation (50g for 3 minutes) to discard pelleted hepatocytes. The single-cell suspension was divided equally into three 15ml-tubes, mixed with a solution of Nycodenz (Axis-Shield, UK) to reach the final concentrations of 8%, 9.6%, and 11% separately and centrifuged at 1400g for 20 minutes. HSCs were collected from the white layer. The number of isolated cells and cell viability were determined by Trypan Blue stain (Sigma-Aldrich, US).

2.4. Purity analysis
The isolated cells were fixed with 4% paraformaldehyde (PFA) for at least 15 minutes, washed with PBS twice, permeabilized for 15 minutes, incubated with blocking buffer for 30 minutes at room temperature (RT) with shaking. Afterward, the cells were incubated with primary antibody anti-desmin (1:200) for 1 hour at RT, followed by washing twice with PBS, 5 minutes each. Then, the secondary antibody (1:500) was added and incubated at RT for 1 hour; the unbinding antibody was eliminated by washing twice with PBS, 5 minutes each. The percentage of desmin-positive cells was then analyzed using the FACS Calibur flow cytometer (BD-Biosciences, US).

2.6. Culturing of hepatic stellate cells

HSCs were cultured on plastic dishes coated with fibronectin. Quiescent HSCs were cultured in standard medium for one day. Then, the standard medium was replaced with the different test medium reported in Table 1. The cells were assessed at day 3, and day 7 after adding the test medium.

2.7. Lipid droplet staining and quantification

Briefly, the cultured cells were fixed with 4% PFA. The stock oil red o (ORO) 0.5% was prepared in absolute isopropanol. Before using, the stock ORO was diluted 3:2 in distilled water and left for 10 minutes at RT to obtain the final working solution. Cells were stained with the working solution for 20 minutes at RT, followed by a three-time rinse with PBS. The nuclei were counterstained with Hematoxylin for 1 minute before light microscope observation. Lipid droplet (LDs) area was measured by ImageJ software (NIH, US) that quantify the red-stained area in the image as decribed in ImageJ User Guide. A minimum of 300 cells per well (10%) were used for analyzing LDs.

2.8. Gene expression examination by qRT-PCR

Total RNA was extracted using column-based (with on-column Dnase treatment) total RNA Purification Kit (Norgen Biotek, Canada). The concentration of isolated RNA was measured using a spectrophotometer (Eppendorf, Germany). Then, 1 μg of RNA was reverse transcribed into cDNA using SensiFAST™cDNA Synthesis Kit (Bioline, UK). Subsequently, cDNA was employed as template for quantitative-PCR using SensiFAST™SYBR®Hi-ROX Kit (Bioline, UK) on the LightCycler-480 Instrument-II (Roche, Switzerland). The primer sequences used in this study are shown in Table 2. The expression level of the target genes was normalized to the housekeeping gene (Gapdh) and analyzed using the Livak method (2^-ΔΔCt).

2.9. ICC staining
The adherent cells were rinsed with PBS twice and fixed with 4% PFA. Then, the cells were permeabilized, incubated with blocking buffer for 30 minutes. Afterward, the cells were incubated with primary antibody against desmin (1:200), GFAP (1:100) or α-SMA (1:300) overnight at 4°C followed by washing two times, 5 minutes each. Then the primary antibodies were detected by incubating with secondary antibody Alexa fluor 488-conjugated (1:500) for 1 hour at RT. The nuclei were stained with DAPI (1:5, Santa Cruz, US), for 15 minutes. The fluorescent images were acquired by the Cytell-TM Cell Imaging System (GE-Healthcare, UK).

2.10. Proliferation assay

The cell count was quantified using Cell Counting Kit 8–CCK8 assay. At the assessment time point, the medium was replaced with 100µl of fresh medium followed by the addition of 10µl of CCK8 Kit reagent. The cells were then incubated in the incubator for 4 hours before the absorbance at 450nm was measured using DTX-880 microplate reader (Beckman Coulter, US). The absorbance of a series of cell concentrations was measured to serve as the standard curve.

2.11. Cell cycle analysis

Cultured HSCs were fixed with 4% PFA. The cells were stained with DNA-binding dye DAPI (Sigma-Aldrich, USA). All stages of the cell cycle were identified based on a complete single-color assay workflow of the Cytell-TM Cell Imaging System (GE-Healthcare, UK).

2.12. Western blot

The cells were lysed with ice-cold RIPA buffer (ab156034) for 10 minutes. The lysate then was centrifuged at 13000rpm for 10 minutes at 4°C, the supernatant was collected. The total protein concentration was determined using BCA protein assay kit (ab102536). 10 µg of protein samples was mixed with LDS Sample Buffer (ab119196) and heated for 10 minutes at 70°C. The protein samples were loaded to SDS-PAGE gel (ab139596) with equal amounts. Gel running was performed with Running buffer (ab119197) at 50 V for 2 hour. Protein samples were transferred to PVDF membrane (ab133411) at 90 V for 1.5 hour. The membrane was treated with Blocking buffer (ab126587) for 1 hour at room temperature with shaking. The membrane was incubated with primary antibody anti-SMA (1:2000), anti-collagen I (1:5000) overnight at 4°C. Anti-GAPDH antibody (1:10000) was used as the
control. The membrane was washed three times with TBST, 5 minutes each before incubated with secondary antibody Goat Anti-Rabbit IgG (HRP) (1:10000) at room temperature for 1 hour with shaking. ECL Kit (ab65623) was used to detect the blots and the imaging was conducted with X Ray film. All the reagents were purchased from Abcam, US.

2.13. Statistical analysis

All experiments were performed in triplicate. GraphPad Prism software was used for statistical analysis. All data were shown as mean±SEM (standard error of the mean). Statistical differences were assessed by One-way analysis (ANOVA) or Student t-test (p-value<0.05 was considered statically different), depending on the number of groups.
3. RESULTS

3.1 Determination of the optimal Nycodenz concentration for hepatic stellate cells isolation

The amount of cells isolated using Nycodenz 11% was not different from that of Nycodenz 9.6% being higher than with Nycodenz 8% (Figure 2.a). The purity data showed a significant lower (p-value<0.05) proportion of desmin-positive cells using the Nycodenz concentration of 11% compared to 8 and 9.6% (Figure 2.b and d).

The mRNA levels of clec4f (marker of Kupffer cells) and pecam1 (marker of endothelial cells) (p-value<0.05, Figure 2.c) in cells isolated from Nycodenz 11% were found to be remarkably higher than that from Nycodenz 9.6% and 8%. Based on the above results (amount of isolated cells and purity), cells isolated by Nycodenz 9.6% were considered for further testing.

3.2 Cell count and viability of HSCs from the isolation procedure

With Nycodenz 9.6%, we could recover 1.96±0.07 million HSCs per mouse. The viability of the isolated cells was higher than 95% assessed by a hemocytometer (Figure 3.I.c).

3.3 Hepatic stellate cells characterization

3.3.1 Morphology of hepatic stellate cells

Bright-field microscope images of freshly isolated HSCs showed the irregular shape and the irregular surface from multiple cytoplasmic lipid droplets (Figure 3.I.a). Under phase-contrast microscope, the retinoid–containing lipid droplets were shining as yellowish-orange color (Figure 3.I.b). The flow cytometry analysis showed more than 82% of cells were positive for GFAP, marker of the quiescent phenotype (Figure 3.I.h). Twenty four hours after isolation, staining with ORO dye showed lipid droplets (red droplets, marker of the quiescent phenotype) in various size in adherent cells (Figure 3.I.e). Moreover, HSCs started to produce filament fibers conferring a star-shaped morphology (Figure 3. I. d). Finally, immunofluorescence (Figure 3.I.f,g) confirmed that the isolated HSCs were positive for desmin and GFAP, both markers of the quiescent phenotype. All the above observation confirms the presence of the typical quiescent phenotype for the isolated HSCs.

3.3.2 Activation of hepatic stellate cells in culture

Previous studies have reported that HSCs change their shape in vitro when acquiring the activated myofibroblast-like phenotype [15, 17, 20, 40]. Our data shows that following in vitro culturing over days 1, 3 and 7, the ‘body’ of HSCs expanded widely while the filament
fiber receded, leading to progressively enlarged cells, progressing into myofibroblast cells (Figure 3.II.i.j.k) as shown by OROstaining (Figure 3.II.l.m.n) with significant (Figure 3.II.r) loss of lipid droplets, consistent with the activated myofibroblast-like phenotype.

Our data indicate that \(\alpha\)-SMA and collagen I (markers of HSC activation) were significantly up-regulated by day 3 and 7 of culture, whereas the mRNA level of LRAT was remarkably reduced (Figure 3.II.s). Parallel increase in the expression of \(\alpha\)-SMA and collagen I over time was confirmed by immunofluorescence (Figure 3.II.o.p.q) and western blot (Figure 3.II.t).

The activated myofibroblast-like phenotype of HSCs is also characterized by increased proliferation rate [32, 40]. The cell counting showed that the number of cells rapidly increased over time (Figure 3.III.v). This was paralleled by the increase in the expression of Ki67 (marker of cell proliferation) which peaked on day 3 (Figure 3.III.w, p-value<0.05). Cell cycle analysis also indicated a higher proportion of cells in S and G2/M phases at day 3 compared with day 1 and 7 (Figure 3.III.x, p-value<0.05).

### 3.4 Definition of the culture conditions suitable for the maintenance of the quiescent phenotype of HSCs

#### 3.4.1 Effect of culture medium on the proliferation of HSCs

At day 3 the proliferation assay CCK8 showed that cells cultured in the standard medium had the highest proliferation rate (Figure 4.a). Among the remaining media, all FBS-free (Table 1), HSC in media 2 and 3 (DMEM without glutamine), had the lowest cell proliferation rate (Figure 4.a). Therefore, we excluded these media from further testing. Cell proliferation in medium 1, which was FBS-free, showed a decrease in proliferation compared with the standard medium (Figure 4.a). Medium 4, which contained DMEM with low glucose concentration (100mg/dL), vitA and insulin without FBS, enhanced the proliferation of HSCs compared to medium 1 (Figure 4.a). However, proliferation rate was inferior compared to the standard medium. Medium 5, (DMEM with vitA and insulin but high glucose concentration (450 mg/dL), had high proliferation rate, similarly as the standard medium (Figure 4.a). At day 7, cells in medium 4 and 5 displayed similar induction in proliferation, but inferior to that of the standard (Figure 4.d). Moreover, medium 4 and 5 keep their superiority in promoting cell growth compared to medium 1 (Figure 4.d).

Cell cycle analysis performed at day 3, showed that the proportion of HSCs in S and G2/M phase in medium 5 was higher than in medium 1 and 4 (Figure 4.b, p-value<0.05) but lower than standard medium. At day 7, the difference in the proportion among the different
medium was no longer apparent (Figure 4.c). These data therefore suggest that presence of FBS and/or high glucose is conducive to HSC proliferative state. In the absence of glutamine, HSC cannot proliferate.

### 3.4.2. Effect of culture medium on HSCs phenotype and activation

The bright-field images of HSCs on day 3 showed that cells in standard medium rapidly expanded their cytoplasm thus becoming well-spread compared with HSCs cultured in media 1,4,5 (Figure 5). On day 7, for media 4/5, more elongated and dendritic-like filaments become evident thus indicating the acquisition of the quiescent phenotype.

We evaluated other markers of activation, such as the loss of cytoplasmic lipid droplets. On day 3, lipid droplet contents were highest in medium 4 compared to standard or media 1 and 5 (Figure 6.a-d and r). At day 7, lipid droplets were still detectable in media 4/5 (Figure 6. e-h) and were higher in amount compared to the standard medium (Figure 6.s), suggesting more HSC in media 4 and 5 being in the quiescent state.

Consistent with the above findings, the gene expression of the pro-fibrotic genes α-SMA and collagen I was upregulated at day 3 in HSCs cultured in medium 1 and standard medium compared to media 4 and 5 (Figure 6.t). This was associated with parallel changes in the mRNA amount of LRAT, a marker for HSCs in vivo quiescence, with higher levels in HSCs maintained in media 4 and 5 compared to standard medium (Figure 6.t) or medium 1 (Figure 6.t) at day 3. At day 7, no major differences were seen among the different media for both α-SMA and collagen I genes (Figure 6.x). Immunofluorescent staining with α-SMA confirmed mRNA data showing that cells cultured in medium 1 and standard medium have acquired the myofibroblastic phenotype with the presence of α-SMA in day 3 (Figure 6.i-m) and 7 (Figure 6.n-q) compared to cells cultured in media 4 and 5.

### 4. DISCUSSION

Primary HSCs have become a necessary tool in drug development for the treatment of hepatic fibrosis. However, the reproducibility of current isolation procedures is inconsistent, and the maintenance of original characteristics of primary HSCs in culture is also suboptimal. While Nycodenz is the most commonly used agent in density gradient centrifugation, its concentrations vary significantly between studies, with variable efficiency of cell yield and purity. Our results suggest that Nycodenz at the 11% concentration has the best cell yield but
at the expense of more cell contaminants. The first finding in our quest for the optimal isolation approach is that Nycodenz at 9.6% is optimal for HSC capture with the least contamination.

Our second finding is that HSCs isolated by 9.6% Nycodenz better retain the undifferentiated phenotype. Post-isolation HSCs are circular, ranging from 12 µm to 20 µm in diameter, are rich of lipid droplets, thus perfectly resembling the HSCs isolated from previous groups [18, 33, 41]. They also express desmin, a widely used marker for HSCs [17, 42, 43].

As expected, HSCs in culture switch from the quiescent to the activated state with changes in their morphology from star-like shape to myofibroblast-like shape and progressive loss of the lipid droplets. Their activation is characterized by the induction of the fibrogenic genes α-SMA and collagen I, and the down regulation of the quiescent-related gene LRAT. Thus, our method of HSCs isolation favors the maintenance of HSC in a quiescent state. This, in turn, allows to study their transition from the quiescent to the activated state in a simplified model of liver fibrosis whose molecular mechanisms have not been fully understood.

The third finding of our investigation is related to the possibility to maintain the HSCs in a quiescent state without cell loss for a prolonged time in culture by modulating the culture medium composition. First, FBS has to be completely excluded from the culture medium as TGFβ contained in FBS may promote cell activation. Second, the optimal combination of media supplements such as glucose, glutamine, insulin, and vitamin A previously shown to have effects on HSCs behaviors [34, 35, 37, 40] is critical. Indeed, treatment with vitamin A has been reported to increase the accumulation of LDs (a feature of quiescent HSCs) and to up-regulate the GFAP (a marker for quiescent HSC) in HSC [34]. In addition, a certain degree of proliferation is needed to allow HSCs propagation in culture. High glucose concentration (450mg/dL) promotes proliferation together with the expression of collagen in rat and human HSC [35]. To enhance the proliferation of HSC, also the supplement of L-Glutamine has been explored. Glutamine (GLN) is one of the essential amino acids which plays a crucial and unique metabolic function [36]. Many studies have shown the effect of glutamine on the proliferation of different cell lines in vitro [37-40] including HSCs. The proliferation of LX2 (an immortalized cell line of activated-HSCs) and of activated rat HSC was reduced markedly in the condition of glutamine depletion [40].
Although many previous studies have shown the effect of those individual elements in HSCs culture medium, the optimal combination of these elements in the culture medium has not been explored. Therefore, we hypothesize that an appropriate combination of these elements in culture could potentially keep HSCs quiescent while promoting proliferation better than a typical medium. Our data show that the medium combination (medium 4) composed of DMEM supplemented with glucose at low concentration (100mg/dL), FBS-free, glutamine (0.5mM), vitA (100µM) and insulin (50ng/ml) is the best to maintain HSCs in a quiescent phenotype without losing the ability for some proliferative activity, thus allowing the cells to be perpetuated in culture. In this respect, glutamine is necessary as its deprivation (such as in media 2 and 3) inhibits HSCs proliferation [40, 44]. Vitamin A and insulin have been shown to be important for the quiescent phenotype [34]. In their absence (such as in medium 1) (but with glutamine) HSC acquired the activated phenotype, with the loss of the lipid droplets, the increase of the fibrogenic genes \(\alpha\)-SMA and collagen I and the reduction of LRAT expression. With regard to the glucose concentration, cells cultured in the lower glucose concentration of 100mg/dL (medium 4) better maintain their inactivated state as shown by lower lipid content and a decrease in expression of the fibrogenic markers \(\alpha\)-SMA and collagen I compared to high glucose concentration of 450mg/dL (medium 5).

In conclusion, Nycodenz at 9.6% is the most effective concentration to employ for HSCs isolation procedure. The optimal culture condition to maintain HSCs in the quiescent phenotype can be achieved with DMEM supplemented with glucose at low concentration (100 mg/dL), glutamine (0.5mM), vitA (100µM) and insulin (50ng/ml) in the absence of FBS. Under these conditions, HSC biology can be manipulated in vitro to further understand the mechanism of HSCs activation in hepatic fibrosis and to develop novel therapeutic strategies for this pathological condition.

ACKNOWLEDGEMENT

This research was funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 108.05-2017.30.

DISCLOSURE STATEMENT

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Minh Thanh Dang and Trinh Van Le conducted the experiment and acquisition of data. Minh Thanh Dang took the lead in writing the manuscript. Huy Quang Do and Loan Tung Thi Dang contributed to conduct gene/protein evaluation. Van Thuan Nguyen supported for immunocytochemistry. Son Nghia Hoang and Long Thanh Le supported for capturing and
analyzing on Cytell-TM Cell Imaging System. Nhan Chinh Lu Phan and Phuc Van Pham contributed to carry out flow cytometry. Ai-Xuan Le Holterman and Gabriele Grassi helped supervise the project and manuscript editing. Being corresponding author, Nhung Hai Truong contributed to original idea, planned the study and gave final approval of the manuscript to be submitted and any revised version.

**DATA AVAILABILITY STATEMENT**

All data generated or analysed during this study are included in this published article (and its supplementary information files).
5. References

1. March S, Graupera M, Sarrias MR, Lozano F, Pizcueta P, Bosch J, et al. Identification and functional characterization of the hepatic stellate cell CD38 cell surface molecule. 2007;170(1):176-87.

2. Friedman SLJNEJoM. The cellular basis of hepatic fibrosis--mechanisms and treatment strategies. 1993;328(25):1828-35.

3. Geerts A, editor. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Seminars in liver disease; 2001: Copyright© 2001 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New ….

4. Blaner WS, O’Byrne SM, Wongsiriroj N, Kluwe J, D’Ambrosio DM, Jiang H, et al. Hepatic stellate cell lipid droplets: a specialized lipid droplet for retinoid storage. 2009;1791(6):467-73.

5. Gard AL, White FP, Dutton GRJJon. Extra-neural glial fibrillary acidic protein (GFAP) immunoreactivity in perisinusoidal stellate cells of rat liver. 1985;8:359-75.

6. Neubauer K, Knittel T, Aurisch S, Fellmer P, Ramadori GJJoh. Glial fibrillary acidic protein—a cell type specific marker for Ito cells in vivo and in vitro. 1996;24(6):719-30.

7. Buniatian G, Gebhardt R, Schrenk D, Hamprecht BJEnocb. Colocalization of three types of intermediate filament proteins in perisinusoidal stellate cells: glial fibrillary acidic protein as a new cellular marker. 1996;70(1):23-32.

8. Niki T, De Bleser PJ, Xu G, Van der Berg K, Wisse E, Geerts AJH. Comparison of glial fibrillary acidic protein and desmin staining in normal and CCl4-induced fibrotic rat livers. 1996;23(6):1538-45.

9. Yokoi Y, Namihisa T, Kuroda H, Komatsu I, Miyazaki A, Watanabe S, et al. Immunocytochemical detection of desmin in fat-storing cells (Ito cells). 1984;4(4):709-14.

10. Geerts A, Lazou JM, de Bleser P, Wisse EJH. Tissue distribution, quantitation and proliferation kinetics of fat-storing cells in carbon tetrachloride–injured rat liver. 1991;13(6):1193-202.

11. Atzori L, Poli G, Perra AJTijob, biology c. Hepatic stellate cell: a star cell in the liver. 2009;41(8-9):1639-42.
12. Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, et al. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. 2013;4:2823.

13. Choi SS, Sicklick JK, Ma Q, Yang L, Huang J, Qi Y, et al. Sustained activation of Rac1 in hepatic stellate cells promotes liver injury and fibrosis in mice. 2006;44(5):1267-77.

14. Bae M, Kim M-B, Lee J-Y. Carotenoids and Retinoids.

15. Reeves HL, Friedman SLJFB. Activation of hepatic stellate cells—a key issue in liver fibrosis. 2002;7(4):808-26.

16. Zhai X, Wang W, Dou D, Ma Y, Gang D, Jiang Z, et al. A novel technique to prepare a single cell suspension of isolated quiescent human hepatic stellate cells. 2019;9(1):1-9.

17. Maschmeyer P, Flach M, Winau FJJ. Seven steps to stellate cells. 2011(51):e2710.

18. Mederacke I, Dapito DH, Affò S, Uchinami H, Schwabe RFJNp. High-yield and high-purity isolation of hepatic stellate cells from normal and fibrotic mouse livers. 2015;10(2):305.

19. Chang W, Yang M, Song L, Shen K, Wang H, Gao X, et al. Isolation and culture of hepatic stellate cells from mouse liver. 2014;46(4):291-8.

20. Bartneck M, Warzecha KT, Tag CG, Sauer-Lehnen S, Heymann F, Trautwein C, et al. Isolation and time lapse microscopy of highly pure hepatic stellate cells. 2015;2015.

21. Weiskirchen R, Gressner AM. Isolation and culture of hepatic stellate cells. Fibrosis research: Springer; 2005. p. 99-113.

22. Pinzani M, Gesualdo L, Sabbah GM, Abboud H. Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. The Journal of clinical investigation. 1989;84(6):1786-93.

23. Friedman SL, Roll FJ. Isolation and culture of hepatic lipocytes, Kupffer cells, and sinusoidal endothelial cells by density gradient centrifugation with Stractan. Analytical biochemistry. 1987;161(1):207-18.

24. Tsai T-H, Shih S-C, Ho T-C, Ma H-I, Liu M-Y, Chen S-L, et al. Pigment epithelium-derived factor 34-mer peptide prevents liver fibrosis and hepatic stellate cell activation through down-regulation of the PDGF receptor. 2014;9(4):e95443.
25. Bhatt S, Shen G-Q, Li Y, Qian S, Ragni MV, Lu LJ. Hepatic stellate cell–conditioned myeloid cells provide a novel therapy for prevention of factor VIII antibody formation in mice. 2015;43(4):277-85.

26. Li Y, Kim B-G, Qian S, Letterio JJ, Fung JJ, Lu L, et al. Hepatic Stellate Cells Inhibit T Cells through Active TGF-β1 from a Cell Surface–Bound Latent TGF-β1/GARP Complex. 2015;195(6):2648-56.

27. Wang Z, Liu F, Tu W, Chang Y, Yao J, Wu W, et al. Embryonic liver fodrin involved in hepatic stellate cell activation and formation of regenerative nodule in liver cirrhosis. 2012;16(1):118-28.

28. Zhang X, Tan Z, Wang Y, Tang J, Jiang R, Hou J, et al. PTPRO-associated hepatic stellate cell activation plays a critical role in liver fibrosis. 2015;35(3):885-98.

29. Page A, Paoli P, Salvador EM, White S, French J, Mann JJ. Hepatic stellate cell transdifferentiation involves genome-wide remodeling of the DNA methylation landscape. 2016;64(3):661-73.

30. He L, Gubbins J, Peng Z, Medina V, Fei F, Asahina K, et al. Activation of hepatic stellate cell in Pten null liver injury model. 2016;9(1):8.

31. Knook D, Seffelaar A, De Leeuw AE. Fat-storing cells of the rat liver: their isolation and purification. 1982;139(2):468-71.

32. Tacke F, Weiskirchen R. Hepatology. Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques. 2012;6(1):67-80.

33. Weiskirchen S, Tag CG, Sauer-Lehnen S, Tacke F, Weiskirchen R. Isolation and culture of primary murine hepatic stellate cells. Fibrosis: Springer; 2017. p. 165-91.

34. Yoneda A, Sakai-Sawada K, Niitsu Y, Tamura Y. Vitamin A and insulin are required for the maintenance of hepatic stellate cell quiescence. 2016;341(1):8-17.

35. Sugimoto R, Enjoji M, Kohjima M, Tsuruta S, Fukushima M, Iwao M, et al. High glucose stimulates hepatic stellate cells to proliferate and to produce collagen through free radical production and activation of mitogen-activated protein kinase. 2005;25(5):1018-26.

36. Wang Y, Bai C, Ruan Y, Liu M, Chu Q, Qiu L, et al. Coordinative metabolism of glutamine carbon and nitrogen in proliferating cancer cells under hypoxia. 2019;10(1):201.
37. Yu Y, Newman H, Shen L, Sharma D, Hu G, Mirando AJ, et al. Glutamine metabolism regulates proliferation and lineage allocation in skeletal stem cells. 2019;29(4):966-78. e4.

38. Choi Y-K, Park K-GJB, therapeutics. Targeting glutamine metabolism for cancer treatment. 2018;26(1):19.

39. Jiang J, Srivastava S, Zhang JJC. Starve Cancer Cells of Glutamine: Break the Spell or Make a Hungry Monster? 2019;11(6):804.

40. Li J, Ghazwani M, Liu K, Huang Y, Chang N, Fan J, et al. Regulation of hepatic stellate cell proliferation and activation by glutamine metabolism. 2017;12(8):e0182679.

41. Riccalton-Banks L, Bhandari R, Fry J, Shakesheff KMJM, biochemistry c. A simple method for the simultaneous isolation of stellate cells and hepatocytes from rat liver tissue. 2003;248(1-2):97-102.

42. Minchenko DO, Tsymbal D, Yavorovsky O, Solokha N, Minchenko OJEr. Expression of genes encoding IGFBPs, SNARK, CD36, and PECAM1 in the liver of mice treated with chromium disilicide and titanium nitride nanoparticles. 2017;51(2):84-95.

43. Nitou M, Ishikawa K, Shiojiri NJTJoA. Immunohistochemical analysis of development of desmin-positive hepatic stellate cells in mouse liver. 2000;197(4):635-46.

44. Smith RJJJoP, Nutrition E. Glutamine metabolism and its physiologic importance. 1990;14:40S-4S.

ABBREVIATION

| Abbreviation | Description |
|--------------|-------------|
| LRAT         | Lecithin-retinol Acyltransferase |
| α-SMA        | Alpha - Smooth Muscle Actin |
| vitA         | Vitamin A |
| FBS          | Fetal Bovine Serum |
| DMEM         | Dulbecco’s Modified Eagle Medium |
| LDs          | Lipid Droplets |
| Pecam1       | Platelet Endothelial Cell Adhesion Molecule-1 |
| Clec4f       | C-Type Lectin Domain Family 4 Member F |
| HSCs         | Hepatic Stellate Cells |
| COL1         | Type I Collagen |
| Gene   | Forward (5'-3')          | Reverse (5'-3')          | ID               |
|--------|--------------------------|--------------------------|------------------|
| Gapdh  | AAGTTGTCATGGGATGACG      | TCACCATCTTTCCAGGAGC      | NM_008084.3      |
| α-SMA  | GCATCCACGAAACCACCATA     | CACGAGTAACAAATCAAGC      | NM_007392.3      |
| Collagen I | CAATGCCACGGCTGTCGG      | AGCACTCGCCCTCCCCGCTCT    | NM_007742.4      |
| Lrat   | CTGACCAATGACAGAGGACGACCTC | CTAATCCAAGACAGCCGAGCAAGAC | NM_023624.4      |
| Pecam1 | AGTCAGAGTCTTCTTCTGCC     | AGTTCAAGATGGAGCCAGC      | NM_00103237.8.2  |
| Clec4f | CTTCGGGGAAGCAACAA        | CAAGCAACTGCAACAGAGAG     | NM_016751.3      |

† FBS: fetal bovine serum
‡ VitA: vitamin A
CTC | AAC
---|---
† Gapdh; glyceraldehyde 3-phosphate dehydrogenase
‡ α-SMA; alpha-smooth muscle actin
§ Lrat; lecithin-retinol Acyltransferase
¶ Pecam1; platelet Endothelial Cell Adhesion Molecule-1
¥ Clec4f; c-Type Lectin Domain Family 4 Member F

**Figure legends**

**Figure 1. The flowchart of the isolation procedure of HSCs from mouse livers (a) and experimental design (b)**

**Figure 2. Evaluation of different Nycodenz concentrations on HSCs purification.** The cell yield using Nycodenz concentrations at 8%, 9.6%, and 11% was evaluated by cell counting (a). Enrichment in HSCs cells was determined by cytofluorimetry (d) for desmin-positive cells as quantified in (b). The amount of contaminating cells (Kupffer and endothelial cells) was determined measuring the mRNA expression levels of clec4f and pecam1 genes, as markers of Kupffer and endothelial cells (c) after normalizing to the housekeeping gene Gapdh. Data are shown as means ± SEM, n=3, *p<0.05.

**Figure 3. HSCs characterization.** Freshly isolated cells (I) and day 1 and 7 isolated cells (II) were evaluated by phase-contrast microscopy (a, b, d, i, j, k), by oil red o dye staining for lipid droplets (e, l, m, n, r), by hemocytometer and trypan blue staining for viability (c), by flow cytometry for GFAP marker (h), by desmin immunofluorescence (f), GFAP (g) and α-SMA (o, p, q) and mRNA levels of α-SMA, COL I and LRAT (s), western blot for α-SMA and collagen I (t). (III) HSCs proliferation evaluated by cell counting (u), by proliferation rate with cck8 assay (v), by ki67 mRNA levels normalized to the housekeeping gene Gapdh (w), by cell cycle analysis (x). Data are shown as means ± SEM, n=3, *p<0.05, scale bar: 50 µm were not noted

**Figure 4. Proliferation of HSCs in different culture media.** HSCs cultured at day 3 (a, b) and day 7 (c, d) in standard medium and media 1-5 were evaluated for proliferation rate by the CCK8 assay and percentages of cells in the different phases of the cell cycles by cell cycle analysis (n=3), *p<0.05.

**Figure 5. Changes in HSCs morphology by differences in culture media.** Representative images of the morphology by light microscope of day 3 (a – d and e – h) for X100 and X200
magnification, respectively) and day 7 HSCs cultured (i–m, n–q and r–u for X100, X200 and X400 magnification, respectively) in standard medium and media 1-5.

Figure 6. Effects of different culture media on the activation of primary HSCs. Quiescent HSCs cultured under different test media (standard, 1-5) were examined for activation by lipid droplet content using oil red o dye staining at day 3 (a), (b), (c), (d) and day 7 (e), (f), (g), (h); by immunofluorescent staining for α-SMA day 3, (n), (o), (p), (q) and day 7 (i), (k), (l), (m). Quantification of lipid droplets accumulation at day 3 (r), and day 7 (s) was shown. mRNA levels of the activation-related genes (α-SMA, COL I) and quiescent-related gene (LRAT) at day 3 (t), and day 7(x). Data are shown as means ± SEM, n=3. *p<0.05. The arrowheads show the oil red o-stained area. Scale bar: 50 µm were not noted.
**a**

**1/ In situ perfusion**
- BALB/c mice 4-5 months old
  - EGTA solution: 2 min
  - Pronase E: 0.038% / 5-7 min
  - Collagenase D: 0.038% / 5-7 min

**Perfused liver**

**2/ In vitro digestion**
- Pronase E / Collagenase D / Dnase I 1%: 15 min

**Total single cells**

- Wash with GBSS/B: 580g / 10 min x 2
- Low speed centrifugation: 50g / 3 min

**discard**

**Hepatocyte pellet**

**collect**

**non-parenchymal cells**

**3/ Nycodenz centrifugation**
- Nycodenz 9.6% solution
- Centrifugation: 1400g / 17 min

**HSC**

**b**

1. Determination of the optimal Nycodenz concentration (6 mice)
   - BALB/c mice 4-5 months old
   - Liver perfusion
   - Nycodenz centrifugation
   - 8%
   - 9.6%
   - 11%

2. HSC characterization (12 mice)
   - Isolated HSC
   - Day of culture
     - Day 0
     - Day 3
     - Day 7

3. Definition of the culture conditions (9 mice)
   - Isolated HSC
   - Standard medium
   - Medium 1
   - Medium 5
   - Day 3
   - Day 7
   - Day of culture
a) Cell count

b) % desmin-positive

c) mRNA levels

---

d) Control - Unlabel

HSC - Desmin

Nycodenz-8%

HSCs 32.0

desmin positive 0.37

desmin positive 86.2

Nycodenz-9.6%

HSCs 31.4

desmin positive 0.37

desmin positive 92.0

Nycodenz-11%

HSCs 28.8

desmin positive 0.37

desmin positive 81.6
CCK8 assay

Day 3

Proliferation rate

Day 7

Proliferation rate

Cell cycle

Percentage of cells (%)

G0/G1, S, G2/M

Day 0

Standard

Med 1

Med 2

Med 3

Med 4

Med 5
Gapdh

Collagen I

α-SMA