ISSN: 2832-7705

Research Article

International Journal of Clinical and Medical Education Research

# The Effect of MSCs with/without Silymarin Against Liver Fibrosis in HepG2 Cell Line with Focused on Caspase3, NFKβ, Apoptosis, Proliferation, Necrosis, and Collagen

**Paulus Kusnanto[1*], Bambang Purwanto[1], Brian Wasita[2] and Vitri Widyaningsih[3]**

[1]*Department of Internal Medicine, Faculty of Medicine, Sebelas Maret University, Surakarta, Central Java Province, Indonesia*

[2]*Department of Pathology, Faculty of Medicine, Sebelas Maret University; Surakarta, Central Java Province, Indonesia*

[3]*Department of Statistics, Faculty of Medicine, Sebelas Maret University; Surakarta, Central Java Province, Indonesia*

**[*]Corresponding Author**
Paulus Kusnanto, Department of Internal Medicine, Faculty of Medicine, Sebelas Maret University, Surakarta, Central Java Province, Indonesia.

**Submitted**: 2023, Mar 03; **Accepted**: 2023, May 28; **Published**: 2023, June 23

**Citation**: Kusnanto, P., Purwanto, B., Wasita, B., Widyaningsih, V. (2023). The Effect of MSCs With/Without Silymarin Against Liver Fibrosis in HepG2 Cell Line with Focused on Caspase3, NFKβ, Apoptosis, Proliferation, Necrosis, and Collagen. *Int J Clin Med Edu Res, 2*(6), 164-178.

## Abstract

***Background:*** *Liver fibrosis is characterized by irreversible damage to the liver parenchyma. The mechanism of fibrosis is based on the activation of collagen, nuclear factor kappa (NF-kβ) activation, and inhibition of ne-crosis, apoptosis, caspase-3 and proliferation. The HepG2 cell line is an evolved human liver cancer cell that exhibits a human hormone response. Hepatocyte stem cells have antifibrotic, anti-inflammatory, and im-munomodulatory properties, can suppress apoptosis and necrosis, and promote proliferation.*

***Methods:*** *This is an experimental in vitro study using pre-test and post-test. Hepatocyte stem cells were obtained from the neonatal umbilical cord. The cell lines were divided into four groups. Analysis was performed on below variable including caspase-3, NF-kβ, necrosis, apoptosis, proliferation and cytopathological capacity.*

***Results:*** *Cytopathological studies of the hepG2 cell line showed fibroblast growth. Mesenchymal stem cells reduced NF-kβ activity, but not caspase 3 activity. Mesenchymal stem cells also significantly reduced apoptosis and necrotic activity, but silymarin was not significantly affected. Mesenchymal stem cells also significantly increased proliferation ($p < 0.001$), but silymarin was not significantly affected.*

***Conclusion:*** *Mesenchymal stem cells significantly inhibited NF-kβ, necrosis, apoptosis, and proliferation ac-tivation, but caspase-3 inhibition was not significantly affected.*

**Keywords:** Pathogenesis of Liver Fibrosis, Mesenchymal Stem Cells, HepG2 Cell Line

## 1. Introduction

Liver cirrhosis is an advanced process of liver parenchymal damage which is char-acterized by changes in the liver parenchyma, becoming denser or fibrotic [1]. Liver fi-brosis itself, which was previously assumed to be irreversible, is in fact a still-in-development process with a good reversible capability. The pathogenesis of fi-brosis, in terms of inflammation, was preceded by NF-kβ activity, which then activates collagen, necrosis, Caspase3 and apoptosis, as well as inhibits proliferation [2]. Fibrosis in liver chirrosis will be increase from mild to severe levels (Metavir degrees F0 to F4), molecular and cellular changes will occur [3,4]. Liver fibrosis which was previously assumed to have irreversible, in develop, it turns out has good reversible power, especially in liver parenchyma cells that have no experienced severe fibrosis.

The HepG2 cell line is a human liver cancer cell line, which owns an inherited im-mortal cell line, responding to human growth hormone stimulation [5]. Stem cells, especially stem cell hepatocytes, are cell products that comprise of an-ti-fibrotic, anti-

inflammatory (immunomodulating) action, inhibition of apoptosis and increase proliferation. Several tests/methods regarding the benefits of stem cells for dis-orders of the heart and pancreas have been carried out, among others, by injecting or implanting them into the target organ with promising results. Hepatocyte Growth Factor is a powerful mitogen mainly associated with hepatocyte proliferation and VEGF in en-hancing angiogenesis which is important for liver regeneration. Mesenchymal stem cells also directly interfere with the immune response through direct cell-to-cell interactions and secretion of soluble factors [6].

The terms MSC and MSC are used to describe cells and cell populations of pluripotent stem / progenitor cells commonly referred to as mesenchymal stem cells, pluripotent mesenchymal cells, mesenchymal progenitor cells, and mesenchymal stem cells,. It became a preferred acronym for. MSCs can differentiate into important strains under in vitro defined conditions and in limited circumstances after in vivo transplantation [7]. Over the last decade, there is increasing evidence that MSCs are derived from perivascular cells expressing cell markers (CD10, CD13, CD44, CD73, CD90, CD105, and CD146) [8]. Therefore, MSC does not differentiate when associated with damaged tissue, but directs tissue-specific progenitor cells involved in the regeneration of damaged tissue [7]. Silymarin-Eurocil 85 is a silymarin formulation with high oral bioavailability and modest antioxidant activity. Silymarin regulates enzymes involved in the development of cell damage, fibrosis, and cirrhosis, and acts as a free radical scavenger [9]. Cirrhosis has been treated in a variety of ways, but with the least expected results. The potential benefits of mesenchymal stem cells in fibrosis, as well as the increased incidence and inadequate treatment of cirrhosis, have been seen. The aim of this study was to evaluate the role of Mesenchymal Stem Cells against liver fibrosis.

## 2. Materials and Methods

### 2.1. Research Design

This study were used in vitro with experimental pre and post test group design. Data were collected from Gastroentero Hepatology Division, Department of Internal Medicine, Dr. Moewardi Teaching Hospital, Faculty of Medicine, Sebelas Maret University. Laboratory process were conducted in Anatomy and Pathology Laboratory, Faculty of Medicine, Sebelas Maret University from March 2020 to March 2021.

### 2.2. Materials and Methods

Materials in this study are: i) mesenchymal stem cells, which were produced from the umbilical cord of newborns (48 hours) ), ± 12 cm long, then were produced/isolated in the Prodia Stem Cell Laboratory (ProStem); ii) cell cultures that were taken from liver parenchyma biopsy in patients with liver cirrhosis (physical examination shows sign of cirrhosis, laboratory results shows thrombocytopenia, hypoalbumin, hyperbilirubinemia, increased INR, abdominal ultrasound showed signs of portal hypertension); iii) the HepG2 cell line, a cultured cell made from parenchymal liver cells that have been induced into fibrosis by ATCC, USA (figure 1); iv) ciba needle no.18, lidocaine injection, 5cc syringe, abdominal ultrasound guided by linear probe, glass tube with EDTA; v) Biosafety Cabinet, centrifuge, CO2 incubator and inversion microscope. Cell analysis facility were carried out with a flowcytometer, fluorescence microscope and Ellisa (figure2).

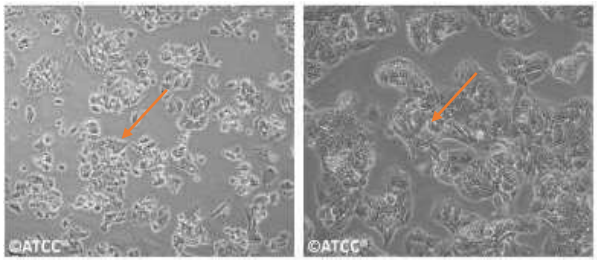



**Figure 1. Fibroblast Growth was seen in Cell Line HepG2 Isolation**

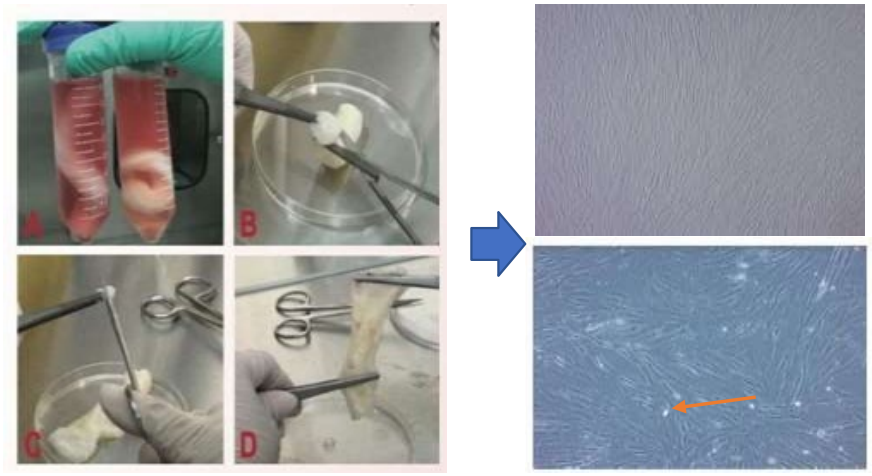



**Figure 2. Fibroblast Growth was found in Umbilical cord isolation – Mesenchymal Stem Cell**

Sample were calculated using the number of plates based on the Triplication method, where: isolation / culture of HepG2 cells were divided into four groups, consisting of NC group (negative control), PC group (positive control / sylimarin) group C1 (Mesenchymal Stem Cells), group C2 (sylimarin and Mesenchymal Stem Cells) and each group was divided into 3 plates, each plate consisted of 7 wells.

### 2.3. Research Procedure

Prepared patients with liver cirrhosis who fulfill the research inclusion reuirements, then liver biopsy was performed under ultrasound guidance abdomen, find a safe area (few blood vessels), did a disinfectant, local anesthetic with lidocaine injection then did a biopsy with a Ciba no. 18 needle. The results of the biopsy process were placed into a glass tuber filled with EDTA with previously centrifuging and adding by MMT reagent (figure19).

The results of the culture were divided into four groups, every group made three cups with seven wells in each cup.

- Negative control group (NC), three cups every seven wells, there are two samples: Ca (liver tissue bi-opsy), and Cb (HepG2 cell line)
- Positive control: PC (cell culture+sylimarin), four cups each well, called as C1
- Group C2, consist of cultured cells+mesenchymal stem cells, consisting of three plates, every plates consisting of seven wells

Negative group / Negative control will be examined to observe the fibroblast growth; after three to five days in incubator, the growth of culture will be observed by microscope, if it successfully growth, the group will be labelling as Cb (figure5), C1 (figure 8), C2 (figure 9), C3 (figure 10) and will be tested for NF-kβ, Caspase.3, Apoptosis, Necrosis, proliferation, and collagen.

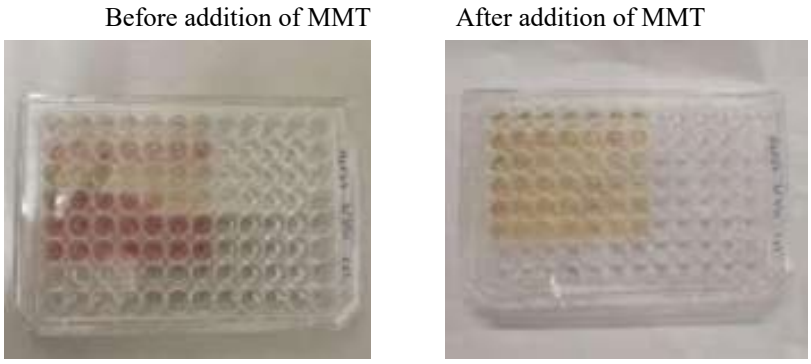



**Figure 19:** Proliferation test with MMT

**Figure 5:** Cell Line HepG2 Isolation (negative control)

**Figure 8:** Fibroblast growth was seen in cell line HepG2 with Silymarin (C1 as positive control)

**Figure 9:** Denser fibroblast growth was seen in Cell line HepG2 with MSC (C2) isolation

**Figure 10:** fibroblast growth was seen in Cell line HepG2 with MSC + Silymarin (C3) isolation

A. Isolation of hepar cell
- A number of 3 mg/ml collagenase were weighed and added by 5 mL of PBS

Collagenase solution
- Collagenase solution was filtered using a syringe filter
- Collagenase solution was stored and samples were prepared

B. Sample processing
The sample was centrifuged at a speed of 500g for 5 minutes; The supernatant will be discarded and top up with PBS for clean cells from the rest of the blood; Samples were chopped and transferred to 5 ml of Collagenase solution; Samples were incubated in an incubator at 37°C for 1 hour; Sample were centrifuged and the supernatant is discarded; Top up with 1 ml of growth medium; Cells were transferred to a petri dish and incubated in an incubator at 37°C; Every 5 days the medium will be replaced and observed under a microscope.

C. Isolation of hepar G2 cell line
HepG2 cells were cultured in 100 mm petri dishes using DMEM + 5% og HPL growth medium for 5 days until they reached >80% confluence; when confluent, cells were harvested by adding 3 ml of TrypLe solution and incubated at 37oC; 5% CO2 for 7 minutes; the Petri dish is gently tapped so that the cells were completely released; TrypLe was neutralized with growth medium (1:1); the entire solution is accommodated in a petri dish into a conical tube; samples were centrifuged for 5 min at 500g speed to obtain cell suspension; the supernatant was discarded and the cells were top up with 1 ml of PBS for cell count.

D. Isolation of Umbilical Cord-Mesenchymal Stem Cell and Immunophenotyping
Mesenchymal Stem Cells 1x10^6 cells/mL were prepared; take a 100µmL pipette as a sample and place it in a new cup; each tube is marked with 20µl positive cocktail (and 20µl negative cocktail); incubated for 30 minutes at room temperature. Protect from direct light; added 1 mL of PBS and centrifuged at 200g for 10 minutes; the supernatant were discarded; suspend the pellet again by adding 0.5 mL of PBS; use flow cytometry (figure 3).

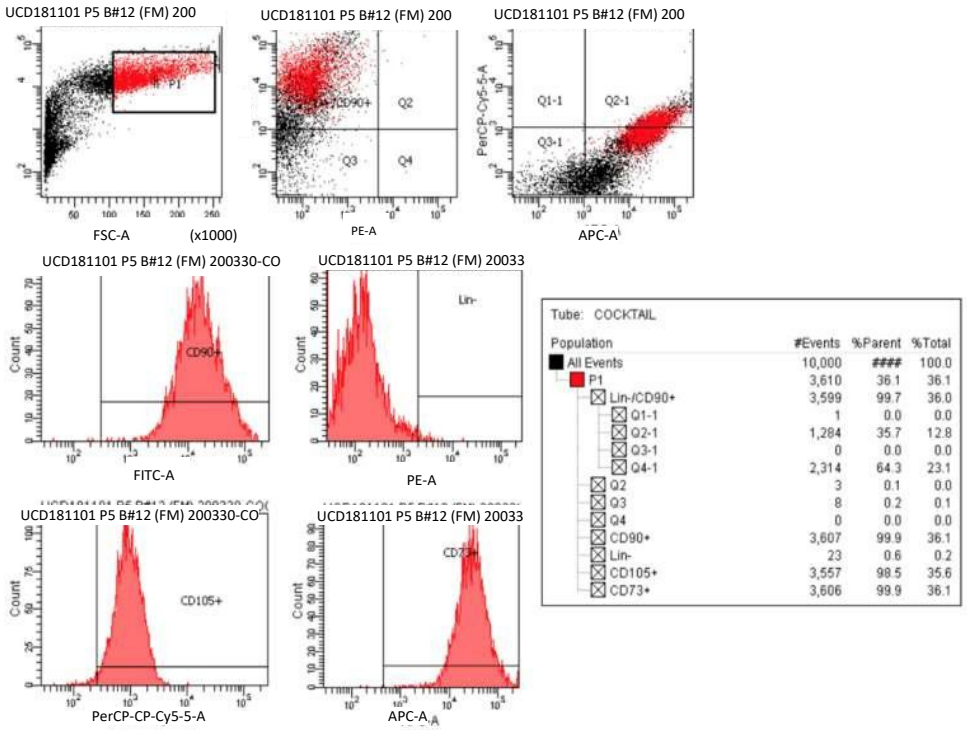



**Figure 3:** Umbilical Cord – MSC characterization

E. Implementation Tests for Proliferation, Necrosis, Apoptosis, NF-kβ, Caspase3, and Collagen

A total of 100 mL of HepG2 cell suspension was implanted in Umbilical Cord-Mesenchymal Stem Cells in 96-well plates with a density of 5 x 10^3 cells/well; cells were incubated at 37oC; 5% CO2 for 24 hours; 100 mL of 1mM Oleic Acid in each well to trigger cell fibrosis and the cells were incubated for 24 hours at 37oC; 5% CO2; 100 mL of test and control solution was put into the well (figure 6). The test solutions that were used are: i) Silymarin 250µg/ml; ii) Umbilical Cord-Mesenchymal Stem Cells 5 x 10^3 cells/well (1:1 ratio in DMEM growth medium) iii) Umbilical Cord-Mesenchymal Stem Cells in a carrier solution of Silymarin; cells were incubated at 37oC; 5% CO2 with incubation period of 24 hours and 48 hours; the solution test was discarded and controlled and then 10 mL of reagent was added by MTT with a concentration of 5 mg/mL into each well; cells were re-incubated in an incubator at 37oC; 5% CO2 for 4 hours; 100 mL of MTT solubilization solution reagent to each well; the absorbance was measured at a wavelength of 450 nm using an ELISA reader(figure 11).

**Figure 6:** Cell line HepG2 isolation with addition of Oleic Acid

**Figure 11:** Cell line HepG2 with NF-kβ reagent test using ELISA Reader

## 2.4. Data Analysis

Data were presented in the form of mean, then were analyzed using SPSS 22 for windows, with the p value were considered statistically significant if $p<0.05$. Sample were divided into four groups to determine the difference in mean between before and after treatment in one group using the ANOVA test, paired samples if the distribution was normal (if not normal, the Kruskal Wallis test was used). For different tests on the fibrosis pathogenesis variable, the chi-square test was used.

## 3. Results

The results were shown in Supplementary File. Table 1 shows the phenotype involvement, which was predominated by CD73+ and CD90+. Table 2 shows the conditioned medium identification yielded results which was indicated the higher results of IL-6 followed by SDF-1. The difference between group of C1, C2, C3, and Cb were shows significant with p-value <0.001 (Table 3). However, the Post Hoc NF-k□ variable shows the significant difference between Cb and C1,C2, C3 group (Table 4). While in Caspase-3 variable , the significant difference only shown in 48 hours treatment (Table 5) (figure 14). Post hoc caspase-3 variable were shown significant difference between C1 vs C2 in 48 hours group (Table 6). Apoptosis variables shows significant difference in 48-hours group (Table 7) (figure 16). Post-hoc apoptosis variables were shown significant difference in 24-hours treatment (Table 8). Additionally, ANOVA test and post hoc of necrosis variable was also shown significant difference (Table 9 and Table 10). Proliferation variable was also shown significant difference between 24 and 48 hours (Table 11 and Table 12) (figure 20).

| Marker | Expression |
|---|---|
| CD73+ | 99.9% |
| CD90+ | 99.9% |
| CD105+ | 98.5% |
| Lin- | 0.6% |

**Table 1. Phenotype Involvement; CD73+, CD90+, CD105, and Lin -**

| Parameter | Unit | Result |
|---|---|---|
| BDNF | pg/ml | 126.6 |
| SDF-1 | pg/ml | 861.95 |
| FGF | pg/ml | 48.13 |
| VEGF | pg/ml | 12.02 |
| PDGF | pg/ml | 1.33 |
| FGF | pg/ml | 48.13 |
| EGF | pg/ml | 6.25 |
| NGF | pg/ml | 31.52 |
| IL-10 | pg/ml | 15.4 |
| IL-1□ | pg/ml | 2.64 |
| IFN-□ | pg/ml | 4.06 |
| MCP-1 | pg/ml | 0.08 |
| IL-6 | pg/ml | 1166.4 |
| IL-12p70 | pg/ml | 30.06 |
| IL-17A | pg/ml | 4.65 |
| IL-18 | pg/ml | 13.44 |
| IL-23 | pg/ml | 22.06 |

**Table 2. Conditioned Medium identification yielded results: BDNF, SDF-1, HGF, FGF, PDGF, EGF, NGF, IL-10, IL-β, IFN-γ, MCP-1, IL-6, IL-12p70, IL-17A, IL-18, IL-23**

| Group | N | NF-kβ | |
|---|---|---|---|
| | | 24 Hours (Mean±SD) | 48 Hours ((Mean±SD) |
| C1 | 7 | 0 .108±0.013 | 0.096±0.006 |
| C2 | 7 | 0.103±0.008 | 0.112±0.011 |
| C3 | 7 | 0.107±0.007 | 0.098±0.007 |
| Cb | 7 | 0.188±0.012 | 0.218±0.015 |
| p-value | | <0.001 | <0.001 |
| Notes :<br>C1 = Silymarin 250ug/mL<br>C2 = MSC 5x103 cell/well<br>C3 = MSC + Silymarin<br>Cb = Control without intervention | | | |

**Table 3. One Way ANOVA test among NF-kβ variables from each group**

| Group | NF-Kβ | |
|---|---|---|
| | 24-hours (p-value) | 48 hours (p-value) |
| Cb vs C1 | <0.001* | <0.001* |
| Cb vs C2 | <0.001* | <0.001* |
| Cb vs C3 | <0.001* | <0.001* |
| C1 vs C2 | 0.455 | 0.009* |
| C1 vs C3 | 0.960 | 0.687 |
| C2 vs C3 | 0.486 | 0.024* |

Notes :
C1 = Silymarin 250ug/mL
C2 = MSC 5x10$^3$ cell/well
C3 = MSC + Silymarin
Cb = Control without intervention

**Table 4. Post Hoc NF-kβ variable**

| Group | N | Caspase-3 | |
|---|---|---|---|
| | | 24 hours (Mean+SD) | 48 hours (Mean+SD) |
| C1 | 7 | 0.071 ±0.004 | 0 .060 ±0.006 |
| C2 | 7 | 0.074 ±0.005 | 0 .068 ±0.004 |
| C3 | 7 | 0.075 ±0.007 | 0 .059 ±0.003 |
| Cb | 7 | 0.071 ±0.007 | 0 .063 ±0.005 |
| p-value | | 0,458 | 0,008* |

Notes :
C = Silymarin 250ug/Ml
C2 = MSC 5x103 cell/well
C3 = MSC + Silymarin
Cb = Control without intervention

**Table 5. One way ANOVA results from Caspase-3 variable in each group**

| Group | Caspase3 | |
|---|---|---|
| | 24 hours[a] (p-value) | 48 hours[b] (p-value) |
| Cb vs C1 | 0.964 | 0.053 |
| Cb vs C2 | 0.372 | 0.072 |
| Cb vs C3 | 0.185 | 0.095 |
| C1 vs C2 | 0.396 | 0.021* |
| C1 vs C3 | 0.200 | 0.847 |
| C2 vs C3 | 0.654 | 0.003 |

Notes:
C1= Silymarin 250 ug/mL
C2= MSC 5x103 cell/well
C3= MSC+ Silymarin
Cb= Control without intervention

**Table 6. Post hoc Caspase-3 variable in each group**

| Group | N | Caspase3 | |
|---|---|---|---|
| | | 24 hours[b] (Mean+SD) | 48 hours[a] (Mean+SD) |
| C1 | 7 | 3699.86 ±1741.16 | 4476.86 ±750.17 |
| C2 | 7 | 138942.86 ±60091.73 | 34302.86 ±11605.87 |
| C3 | 7 | 19142.86±4789.53 | 10510.86 ±2291.25 |
| Cb | 7 | 2763000.00 ±961994.11 | 1631028.57 ±517758.77 |
| Notes:<br>C1= Silymarin 250 ug/mL<br>C2= MSC 5x103 cell/well<br>C3= MSC+ Silymarin<br>Cb= Control without intervention | | | |

**Table 7. One Way ANOVA results of Apoptosis variables in each group**

| Group | Caspase3 | |
|---|---|---|
| | 24 hoursb (p-value) | 48 hoursa (p-value) |
| Cb vs C1 | 0.002* | <0.001* |
| Cb vs C2 | 0.002* | <0.001* |
| Cb vs C3 | 0.002* | <0.001* |
| C1 vs C2 | 0.002* | 0.831 |
| C1 vs C3 | 0.002* | 0.966 |
| C2 vs C3 | 0.002* | 0.865 |
| C1 = Silymarin 250ug/mL<br>C2 = MSC 5x103 cell/well<br>C3 = MSC + Silymarin<br>Cb = Control without intervention | | |

**Table 8. Post Hoc Apoptosis Variables in each group**

| Group | N | Caspase3 | |
|---|---|---|---|
| | | 24 hours[b] *(Mean+SD)* | 48 hours[a] (Mean+SD) |
| C1 | 7 | 11.184 ± 2.182 | 13.409 ± 1.016 |
| C2 | 7 | 1.596 ± 0.134 | 2.066 ± 0.369 |
| C3 | 7 | 15.078 ± 0.857 | 15.066 ± 0.931 |
| Cb | 7 | 9.035 ± 0.990 | 5.670 ±1.513 |
| p-value | | <0,001* | <0,001* |
| C1 = Silymarin 250ug/mL<br>C2 = MSC 5x103 cell/well<br>C3 = MSC + Silymarin<br>Cb = Control without intervention | | | |

**Table 9. One way ANOVA of Necrosis Variables in each group**

| Group | Caspase3 | |
|---|---|---|
| | 24 hoursb (p-value) | 48 hoursa (p-value) |
| Cb vs C1 | 0.085 | <0.001 |
| Cb vs C2 | 0.002 | <0.001 |
| Cb vs C3 | 0.002 | <0.001 |
| C1 vs C2 | 0.002 | <0.001 |
| C1 vs C3 | 0.004 | 0.007 |
| C2 vs C3 | 0.002 | <0.001 |

**Table 10.Post Hoc of Necrosis variables in each group**

| Group | N | Poliferation | |
|---|---|---|---|
| | | 24 hours (Mean±SD) | 48 hours (Mean±SD) |
| C1 | 7 | 0.187±0.039 | 0.232±0.075 |
| C2 | 7 | 0.777±0.123 | 1.031±0.180 |
| C3 | 7 | 0.258±0.04 | 0.290±0.048 |
| Cb - | 7 | 0.657±0.196 | 1.084±0.031 |
| p-value | | <0.001* | <0.001* |
| Notes:<br>C1 = Silymarin 250ug/mL<br>C2 = MSC 5x103 cell/well<br>C3 = MSC + Silymarin<br>Cb = Control without intervention | | | |

**Table 11. One Way Anova of proliferation test in each group**

| Group | Caspase3 | |
|---|---|---|
| | 24 hoursb (p-value) | 48 hoursa (p-value) |
| Cb vs C1 | 0.002* | 0.002* |
| Cb vs C2 | 0.338 | 0.337 |
| Cb vs C3 | 0.002* | 0.002* |
| C1 vs C2 | 0.002* | 0.002* |
| C1 vs C3 | 0.013* | 0.085 |
| C2 vs C3 | 0.002* | 0.002* |
| C1 = Silymarin 250ug/mL<br>C2 = MSC 5x103 cell/well<br>C3 = MSC + Silymarin<br>Cb = Control without intervention | | |

**Table 10.Post Hoc of Necrosis variables in each group**

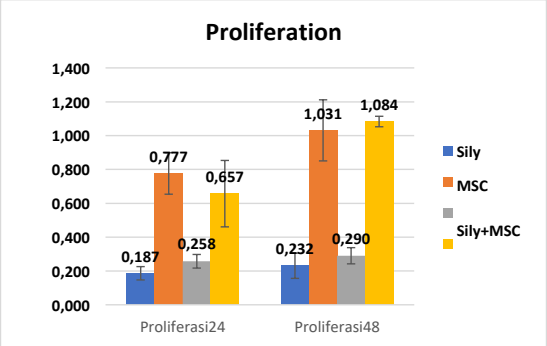



**Figure 20:** Bar Chart of Proliferation test in each group

## 5. Discussion

In the In-Vitro test, the material were obtained from the tissue should be isolated in the form of culture cells previously to form culture cells (fibroblasts). The researcher used Hep.G2 cell line that were obtained from ATCC, USA, as the research variable. Likewise, the Umbilical Cord-Mesenchymal Stem Cells that were isolated/cell cultured to obtain fibroblasts/collagen and then the immunophenotyping test was also performed.

The purposes of assesing the stage and the fibrosis rate are: i) estimating the response of a therapy; ii) provide therapy as needes, if only a small fibrosis rate is observed over a relatively long observation interval, then antiviral treatment can be delayed until therapy is thought to be more effective and tolerable; iii) estimate the time occurrence of liver cirrhosis[10]. Several things may cause fibroblasts not to occur; such as liver parenchyma tissue that is damaged either when the biopsy is performed or the time interval for cell culture is performed; the media that were not suitable for growth; the emergence of growth complications (infection); unsuitable growth reagents for the growth of cell culture [10].

Isolation of Umbillical Cord-Mesenchymal Stem Cell culture showed fibroblast growth (figure 7), then proceed with Immunophenotyping and parameters by testing the characteristics of the condotioned medium using the Mesenchymal Stem Cell Kit. Mesenchymal stem cells obtained results. There are CD 73+, CD90+, CD105+ and Lin- phenotypes and parameters/ condotioned medium BDNF, SDF1, HGF, FGF, VEGF, EGF, NGF, IL10, IL1-β, INF-α, MCP1, IL6, IL12p70, IL17A, IL18, IL23. The International Society of Cell Therapy (ISCT) stipulates that Mesenchymal Stem Cells must meet the following criteria. First, cells need to be adhered to plastic under normal/standard culture conditions. Second, they need to expressing positive CD105, CD73, and CD90 and do not express (negative) CD11b, CD14, CD34, CD45, CD79a and HLADR [11].

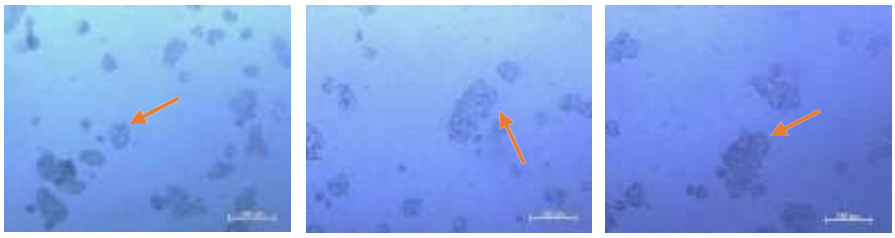


**Figure 7:** UC – MSC Isolation (Fibroblast growth was found)

Mesenchymal stem cell phenotypes are not necessarily the same in all types of can-cer. Thus, it is necessary to identify specific biomarkers including cell surface markers to determine the prognosis and survival of patients. Collagen is one of the proteins that formed the human body. Its presence is approximately 30% of all protein contained in the human body. Collagen is the organic structure that builds bones, teeth, joints, muscles, and skin. Collagen fibers have a strong resistance to pressure. The word "collagen" itself comes from the Greek language which means (sticky or produces adhesive/sticky) [12]. Overall, the conclusions are in accordance with the theory of the fibrosis mechanism, but there are indeed variations in the strength of the treatment variables. This is happened because of variable reasons, including the dose used is too small, the materials and reagents used are damaged, the time used to evaluate is too short, and any differentiation effect of Mesen-chymal Stem Cell.

According to the WHO, liver cirrhosis was noted as the 11th leading cause of death in the world. In 2019. Many types of liver disease can progress to cirrhosis, liver fibrosis is the major pathological symptoms of various causes including toxic injury, viral infectious diseases, as well as metabolic and hereditary diseases. Liver fibrosis was characterized by excessive synthesis, reduced extracellular matrix (ECM) synthesis and degradation. Hepatocytes death, activation of hepatic stellate cells (HSCs), and inflammation are important signs of liver fibrosis. The process of fibrosis is also closely associated with the metabolic and immune disorders. These are usually triggered by the disruption of oxygen homeostasis including mitochondrial dysfunction, oxidative stress, and activation of hypoxic pathways. Mitochondria are organelles that are important in the production and metabolism of energy. Hypoxia-inducible factor (HIF) is an important factor that is activated when hypoxia occurs and considered to be an essential factor for cirrhosis. The effects of oxygen imbalance on metabolism and immunity of cirrhosis is a potential new target for anti-fibrotic therapyv [13].

Determination of the degree of fibrosis has an important role in the treatment of chronic liver disease, because in general, chronic liver disease develops into severe fibrosis and can end up with cirrhosis. Besides being important for prognosis, determining the degree of liver fibrosis can reveal the natural history of the disease and risk factors associated with disease progression to be used as a guide for variations in antifibrotic therapy to be given [14]. Results of nuclear factor kappa Beta (NF-kβ) were obtained first, Statistically, Um-bilical Cord-Mesenchymal Stem Cells and/ or Silymarin gave significant suppression to the control NF-kβ, but when compared with each research variable, there were no significant difference observed. The most effective treatment group in reducing NF-kβ at 24-hour was group C2 (Mesenchymal Stem Cells 5x10^3 cells/well) with the lowest levels of NF-kβ, with an average of 0.103 +0.008. Yet, it did not show any difference with the group C1 (p=0.455) and group C3 (p=0.486). The most effective treatment group in re-ducing NF-kβ at 48-hour was group C1 (Silymarin 250ug/mL) with the lowest levels of NF-kβ, averaging 0.096 +0.006. It also showed a significant difference

in group C2 (p=0.009) and did not show a significant difference in the C3 group (p=0.687) (In ac-cordance with the theory of Umbilical Cord-Mesenchymal Stem Cells and/or silymarin able to significantly suppressed NF-kβ (figure 13).

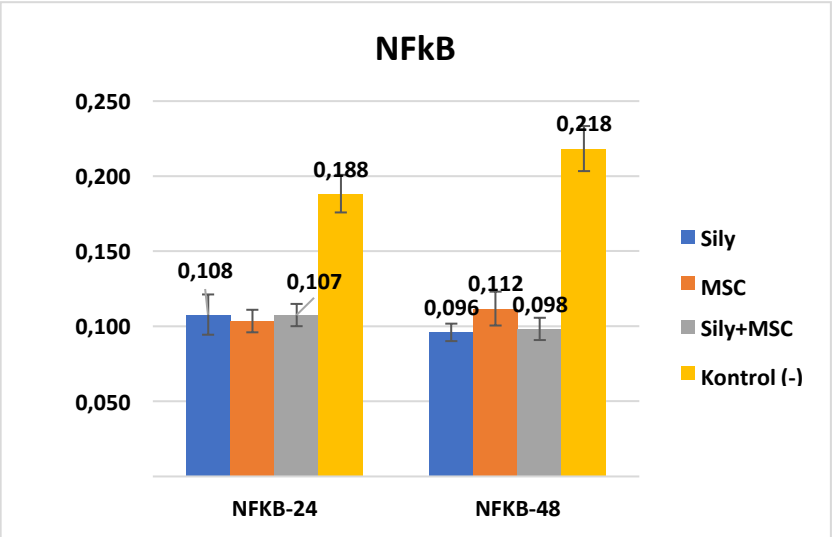



**Figure 13:** Bar chart of NF-kβ levels in each group

Second, Caspase3 Umbilical Cord-Mesenchymal Stem Cell Tests and/or silymarin against caspase3 in the pathogenesis of HepG2 cell line fibrosis in vitro, between study and control variables, were not significantly different. This may occur because of the mechanism of caspase inhibition in the pathogenesis of fibrosis. It can occur due to sev-eral reasons, including intrinsic and extrinsic factors, the NF-kβ pathway, Calcium Influx, and DNA cell mitosis. The role of casepase-3 in activating apoptosis through the TRAIL pathway is inhibited by c-FLIP so that casepase-8 and casepase-10 may activate casepase-3. Its role is carried out by mitochondria, which is activated by tBid, Bcl-2 Bcl-X, casepase-8, and casepase-10. Mitochondria also activates apaf-1 and then sequentially activate casepase-9 to activate casepase-3, influencing caspase3 apoptosis. This process is also known as apoptotic executor[15] (figure 15).

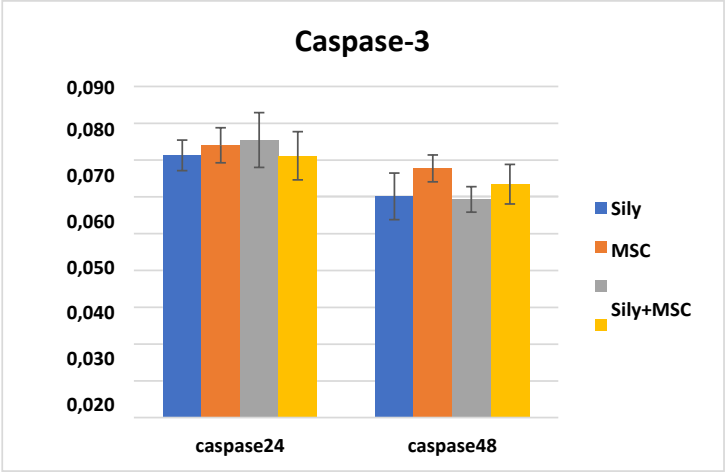



**Figure 15.** Bar Chart of Caspase-3 Levels in each group

Third, the apoptosis process is happening. The effect of Umbilical Cord-Mesenchymal Stem Cells and/or silymarin was shown to significantly suppress apoptosis at either 24 or 48-hour compared to controls. For each treatment variable at 24-hour, there were strong suppression of silymarin variables compared to Umbilical Cord-Mesenchymal Stem Cells and the combination (Umbilical Cord-Mesenchymal Stem Cells + silymarin) that significantly different. However, the suppression between variables did not differ at 48-hour (according to the theory that underlines mesenchymal stem cells and/or silymarin significant role in suppressing apoptosis) (figure 17). Apoptosis is directly in-fluenced by the value of caspase 3. In this study, caspase3 showed insignificant results, so that even apoptosis the results obtained were not stronger when associated with caspase3. Silymarin possess dual effects in terms of proliferation and apoptotic effects [9].

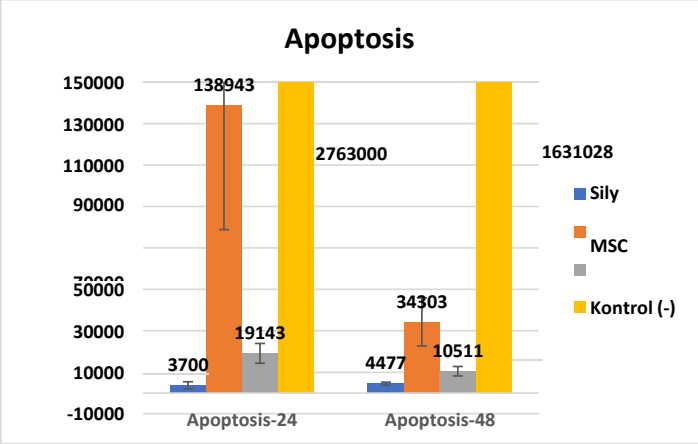



**Figure 17:** Bar Chart of Apoptosis Levels in each group

The last result is necrosis. Umbilical Cord-Mesenchymal Stem Cells gave stronger suppressive effect than the control at 24-hour groups. Meanwhile, silymarin and Umbil-ical Cord-Mesenchymal Stem Cell + silymarin did not have any suppressive effect on necrosis compared to controls (according to the research objective, the effect of Umbilical Cord-Mesenchymal Stem Cell is to inhibit necrosis)(figure 18). Silymarin, which contains flavonoids and silibinin, has the following effects: (i) as an antioxidant, scavenger, and regulator of intracellular glutathione content; (ii) as a cell membrane stabilizer and permeability regulator, preventing hepatotoxic agents from entering hepatocytes; (iii) as a promoter of ribosomal RNA synthesis, stimulates liver regeneration; and (iv) as an inhibitor of the transformation of stellate hepatocytes into myofibroblasts, the process responsible for the deposition of collagen fibres leading to cirrhosis.

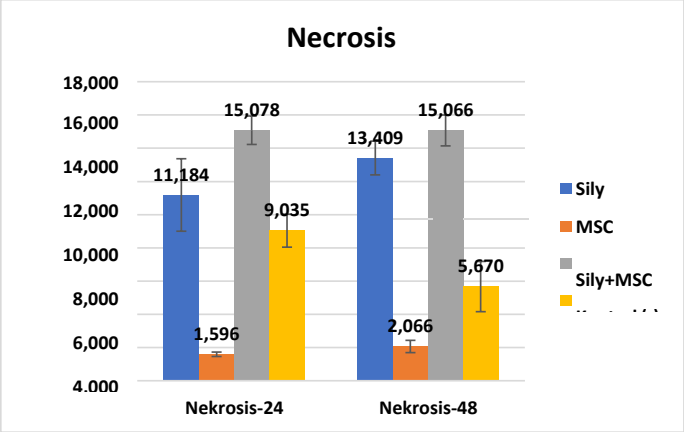



**Figure 18:** Bar Chart of Necrosis Levels in each group

Several journals suggest using silymarin as early as possible. It is because the longer the silymarin were used, the less effective will it be. It is also because the toxic effects had been reported [2]. Acute toxicity of silymarin has been studied in rats, rabbits, and dogs after intravenous injection. The lethal dose value of 50% (LD50) was 400 mg/kg in rats, 385 mg/kg in rats, and 140 mg/kg in rabbits and dogs due to toxic effects, resulting in necrotic results that were not significant (silymarin did not inhibit hepatocyte necrosis) [16]. Umbilical cord- mesenchymal stem cells strongly increased proliferation compared to control. Meanwhile, silymarin and the combination (Umbilical Cord-Mesenchymal Stem Cell + silymarin) did not significantly increase the weak proliferation compared to controls. Proliferation begins with the interaction of ligand molecules growth factor with the receptor of tyrosine kinases, which then activates the embryonic signal transduction pathway and/or MAPK, and ends with the activation of various proliferative genes [17]. Silymarin did not stimulate proliferative growth compared to control because of its weak proliferative effect and toxic effect [9].

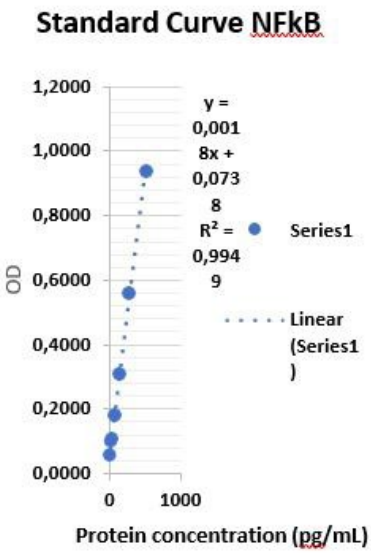
Cirrhosis is defined as the histological development of a regenerative nodule surrounded by a fibrous band in response to chronic liver injury leading to portal hypertension and end-stage liver disease. Recent advances in understanding the natural history and pathophysiology of cirrhosis and managing its complications have resulted in improved care, quality of life, and life expectancy for patients with cirrhosis. Currently, liver transplantation is the only treatment option for selected patient groups, but pharmacological therapies that may stop the progression to decompensated or reverse cirrhosis are being developed. This brief overview focuses

on the diagnosis, complications, treatment of cirrhosis, and new clinical and scientific developments [18]. The exact mechanism of MSC transplantation remains a mystery, but MSCs have been shown to prevent the progression of liver damage and improve liver function. MSCs can self-replicate by migrating to the site of injury, dividing, and differentiating into multiple cell types, including hepatocytes. In addition, MSC has immunomodulatory properties and releases paracrine soluble factors. In fact, the safety and efficacy of MSC therapy in animal liver disease has been demonstrated. However, preclinical and clinical studies are primarily required to confirm its safety and efficacy prior to large-scale clinical use [19].

Experimental models represent useful tools for studying the different stages of liver cancer and help to develop new pharmacologic treatments. Each model in vivo and in vitro has several characteristics and advantages to offer for the study of this disease [20]. A diverse family of NF-kB transcription factors is widely characterized in organisms from flies to humans. However, homologues of NF-kB and many upstream signaling components are unicellular, including cnidarians (isoginchak, coral, hydra, jellyfish), polyfera (sponge), and Capsaspora turkey and some choanoflagellates. Recently characterized by basal strains containing protists [21]. There are numerous limitations found in this study. For example, mesenchymal stem cells were used as independent variables in the study, which were made from the umbilical cord. This still allows them to grow into various cell differentiations. Then, isolation of cultured cells from liver biopsy tissue did not grow sufficiently (figure4). There are several possibilities of fibroblast growth failure, including the tissues and the reagents used. It also needs a suitable medium as well as additional certain vitamins that help growth. The dose used in this study was based on a previous study. A total of 100 µL of HepG2 cell suspension was grown in 96-well plates with a density of 5 x 10^3 cells/well. Examination of protein concentration was using ELISA and flow cytometry methods with short assessment time (figure 12). Fibrous remodeling is a highly conserved protecting response to tissue damage, essential for maintaining structural and functional organizational integrity. Cirrhosis can also be seen as a wound healing response to liver injury balance between liver repair and scarring [22].

Ca Ca Ca

**Figure 4:** Liver Cell Isolation from Biopsy (no fibroblast growth)




| Standard Curve | OD 1 | OD 2 | Average |
|---|---|---|---|
| 0 | 0,059 | 0,060 | 0,0595 |
| 15,625 | 0,103 | 0,101 | 0,1023 |
| 31,25 | 0,108 | 0,110 | 0,1091 |
| 62,5 | 0,184 | 0,185 | 0,1845 |
| 125 | 0,324 | 0,297 | 0,3108 |
| 250 | 0,557 | 0,560 | 0,5586 |
| 500 | 0,939 | 0,934 | 0,9364 |

**Figure 12:** Protein Concentration Distribution NF-kβ

## 5. Conclusions

Administration of Mesenchymal Stem Cells 5 x 10^3 cells/well and/or sylimarin will improve the Pathogenesis of HepG2 Cell Line Fibrosis in vitro by growing collagen cells, decreasing NF-kβ, apoptosis, necrosis activity, and by increasing proliferation process. This result highlights potential benefit of mesenchymal stem cells against liver fibrosis.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

**Author Contributions:** P.K for manuscript preparation and data collection. B.P for supervisor and manu-script preparation. B.W for data collection and laboratory method. V.W for research methodology and man-uscript preparation.

**Funding:** Please add: "This research received no external funding".

**Institutional Review Board Statement:** "The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board. This research study have been approved by Ethi-cal Committee of Dr. Moewardi General Hospital with the registered number 178/I/HREC/2020.

**Informed Consent Statement:** Yes

**Data Availability Statement:** Not Applicable

**Acknowledgments:** We would like thanks to Department of Internal Medicine, Faculty of Medicine, Uni-versities Sebelas Maret for all of support.

**Conflicts of Interest:** "The authors declare no conflict of interest."

## Appendix A

Table Page

## Appendix B

Figure Page

## References

1. Nurjanah. Buku Ajar Ilmu Penyakit Dalam: Sirosis Hati. In: Edisi ke-6. Jakarta: Departemen Ilmu Penyakit Dalam Fakultas Kedokteran Universitas Indonesia; 2014. p. 1978–83.
2. Ebrahimi, H., Naderian, M., & Sohrabpour, A. A. (2016). New concepts on pathogenesis and diagnosis of liver fibrosis; a review article. Middle East journal of digestive diseases, 8(3), 166.
3. Tarantino, G., Conca, P., Pasanisi, F., Ariello, M., Mastrolia, M., Arena, A., ... & Vecchione, R. (2009). Could inflammatory markers help diagnose nonalcoholic steatohepatitis?. European journal of gastroenterology & hepatology, 21(5), 504-511.
4. Arthur, M. J. (2000). Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. American Journal of Physiology-Gastrointestinal and Liver Physiology, 279(2), G245-G249.
5. Donato, M. T., Tolosa, L., & Gómez-Lechón, M. J. (2015). Culture and functional characterization of human hepatoma HepG2 cells. Protocols in in vitro hepatocyte research, 77-93.
6. Halim, D., Harry, M., Ferry, S., Arief, B., Tono, D., & Boenjamin, S. Stem Cell "Dasar Teori dan Aplikasi Klinis". 2010. Jakarta: Erlangga.
7. Pittenger, M. F., Discher, D. E., Péault, B. M., Phinney, D. G., Hare, J. M., & Caplan, A. I. (2019). Mesenchymal stem cell perspective: cell biology to clinical progress. NPJ Regenerative medicine, 4(1), 22.
8. Crisan, M., Yap, S., Casteilla, L., Chen, C. W., Corselli, M., Park, T. S., ... & Péault, B. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell stem cell, 3(3), 301-313.
9. Gillessen, A., & Schmidt, H. H. J. (2020). Silymarin as supportive treatment in liver diseases: A narrative review. Advances in therapy, 37(4), 1279-1301.
10. Hartl, J., Denzer, U., Ehlken, H., Zenouzi, R., Peiseler, M., Sebode, M., ... & Schramm, C. (2016). Transient elastography in autoimmune hepatitis: timing determines the impact of inflammation and fibrosis. Journal of hepatology, 65(4), 769-775.
11. Myers, R. P., Crotty, P., Pomier-Layrargues, G., Ma, M., Urbanski, S. J., & Elkashab, M. (2010). Prevalence, risk factors and causes of discordance in fibrosis staging by transient elastography and liver biopsy. Liver International, 30(10), 1471-1480.
12. Bolke, L., Schlippe, G., Gerß, J., & Voss, W. (2019). A collagen supplement improves skin hydration, elasticity, roughness, and density: Results of a randomized, placebo-controlled, blind study. Nutrients, 11(10), 2494.
13. Li, X., Zhang, Q., Wang, Z., Zhuang, Q., & Zhao, M. (2022). Immune and Metabolic Alterations in Liver Fibrosis: A Disruption of Oxygen Homeostasis?. Frontiers in Molecular Biosciences, 8, 1348.
14. Wahyuni, R. D. (2016). Analisis derajat fibrosis hati dengan fibroscan, indeks FIB4, King's Score dan APRI Score pada penyakit hepatitis kronis. Healthy Tadulako Journal (Jurnal Kesehatan Tadulako), 2(2), 42-52.
15. Huebert, R. C., & Rakela, J. (2014, March). Cellular therapy for liver disease. In Mayo Clinic Proceedings (Vol. 89, No. 3, pp. 414-424). Elsevier.
16. Fraschini, F., Demartini, G., & Esposti, D. (2002). Pharmacology of silymarin. Clinical drug investigation, 22, 51-65.
17. Putra, A. (2019). Basic Molecular Stem Cell.
18. Schuppan, D., & Afdhal, N. H. (2008). Liver cirrhosis. The Lancet, 371(9615), 838-851.
19. Wu, M. C., & Meng, Q. H. (2021). Current understanding of mesenchymal stem cells in liver diseases. World Journal of Stem Cells, 13(9), 1349.
20. Galicia-Moreno, M., Silva-Gomez, J. A., Lucano-Landeros,

S., Santos, A., Monroy-Ramirez, H. C., & Armendariz-Borunda, J. (2021). Liver cancer: therapeutic challenges and the importance of experimental models. Canadian Journal of Gastroenterology and Hepatology, 2021.
21. B LDN-, Williams LM, Gilmore D. (2020). Looking Down on NF-?B Leah. 40(15):1–13.
22. Seitz, T., & Hellerbrand, C. (2021). Role of fibroblast growth factor signalling in hepatic fibrosis. Liver International, 41(6), 1201-1215.