Comparison of MSCs With/Without Silymarin Against Liver Fibrosis in Cell Line HepG2 With Study on NFKβ, Caspase3, Apoptosis, Necrosis, Proliferation,and Collagen

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Article

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Comparison of MSCs With/Without Silymarin Against Liver Fibrosis in Cell Line HepG2 With Study on NFKβ, Caspase3, Apoptosis, Necrosis, Proliferation,and Collagen

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Abstract

Background: Liver fibrosis is characterized by irreversible damage to liver parenchyma. The mechanism of fibrosis is through activation of nuclear factor-kappa (NF-kβ), collagen, activation of necrosis, caspase-3, apoptosis and inhibition of proliferation. The HepG2 cell line is a human liver cancer cell, which is developed and has a human hormonal response. Hepatocyte stem cells have anti-fibrotic, anti-inflammatory, immunomodulating properties, inhibit apoptosis and necrosis, as well as increase proliferation.

Methods: This study is an in vitro pre and posttest experimental study. Hepatocyte stem cells were taken from the umbilical cord of newborns. Cell lines are made into 4 groups. Examination was carried out on NF-kβ, Caspase-3, Apoptosis, Necrosis, Proliferation, and cytopathologically.

Results: Cytopathological test of cell line hepG2 showed fibroblast growth. Mesenchymal stem cells decreased NF-kβ activity. Mesenchymal stem cells did not decrease Caspase3 activity. Mesenchymal Stem Cells significantly decreased Apoptotic activity. Mesenchymal Stem Cells decreased Necrosis activity, while sylimarin was not significant. Mesenchymal Stem Cells increased the Proliferation significantly ($p<0.001$), while sylimarin was not significant.

Conclusion: Mesenchymal stem cells significantly inhibited NF-kβ, necrosis, apoptosis, and proliferation activation, whereas caspase-3 inhibition was not significant.

KEYWORDS: pathogenesis of liver fibrosis, mesenchymal stem cells, hepG2 cell line

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INTRODUCTION

Liver cirrhosis is an advanced process of liver parenchymal damage which is characterized by changes in the liver parenchyma to become dense or fibrotic[1]. Liver fibrosis itself, which

was previously assumed to have irreversible properties, is in fact in development it still has good reversible power. The pathogenesis of fibrosis in terms of inflammation, found NF-kβ activity which activates collagen, necrosis, Caspase3 and apoptosis as well as inhibits proliferation[2].

The HepG2 cell line is a human liver cancer cell line, which has an inherited immortal cell line, responding to stimulation with human growth hormone[3].

Stem cells, especially stem cell hepatocytes, are cell products that have anti-fibrotic, anti-inflammatory (immunomodulating) action, inhibition of apoptosis and increase proliferation. Several tests/methods regarding the benefits of stem cells for disorders 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 enhancing 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[4].

Silymarin-Eurosil 85 is a formulation of silymarin with high oral bioavailability and potent antioxidant effect in mode Silymarin acts as a free radical scavenger, along with modulating enzymes responsible for the development of cell damage, fibrosis and cirrhosis[5].

Treatment of cirrhosis has been done with many different methods, but maximum results have not been obtained. Potential benefit of Mesenchymal Stem Cell on fibrosis, increasing incidence of liver cirrhosis, and unsatisfactory treatment of liver cirrhosis were seen. The aim of this study was to evaluate the role of Mesenchymal Stem Cells against liver fibrosis.

METHODS

Data source and study design

This research is an experimental study of pre and post-test, which was conducted in vitro. Subjects

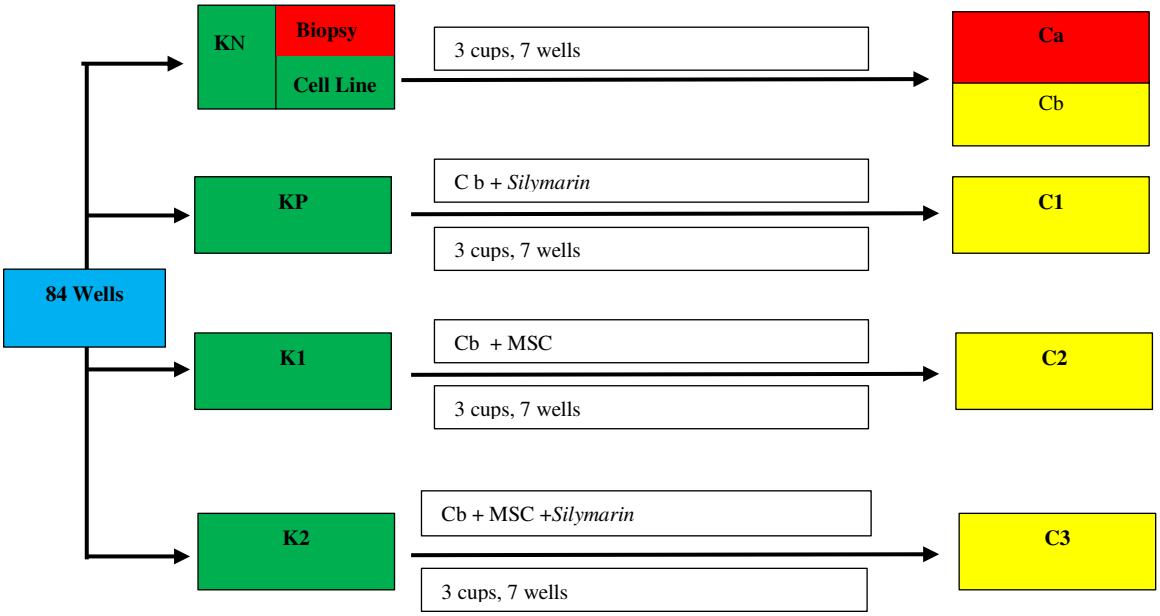


Fig. 1 Research Design. **KP:** Negative oontrol, no treatment but checked according to research objectives (NF-kβ, Casepae-3, Apoptosis, Necrosis, Proliferation and Collagen). **Ca:** Negative control group without treatment. Liver tissue biopsy results, no fibroblasts growing. **Cb**: The negative control group without treatment, from lineHepG2 cells, fibroblasts grew well. **KP:** Positive control, negative control (Cb) given Silymarin (C1). **K1:** Second treatment group, negative control (Cb) added Mesenchymal Stem Cells (C2). **K2 :** Third treatment group, negative control (Cb) added with Mesenchymal Stem Cells and Silymarin (C3). **C1:** Positive control group. **C2**: Second treatment group. **C3**: Third treatment

are outpatients and inpatients in the Gastroenterology division of RSUD dr. Moewardi in March 2020-March 2021, aged >18 years with liver cirrhosis which was shown clinically, laboratory, abdominal ultrasound, fibroscan, and willing to do a liver biopsy. Hepatocyte stem cells were taken from the Prodia stem cell laboratory, which came from the umbilical cord of newborns. Cell culture cell line hep.G2 liver cirrhosis patients was produced by ATCC, USA. Research Design was shown in Fig 1.

Samples were taken at random, met the inclusion and exclusion criteria. All participants agreed to and signed the written informed consent form prior to enrolment into the study. Written informed consesnt was given by all participants. All methods were carried out in accordance with relevant guidelines and regulations Before observing the growth of fibroblasts, liver cell biopsy was isolated. From the isolation of liver biopsy culture cells, it turned out that there was no growth/formation of fibroblasts, the work had been done 3 times (no fibroblasts occurred) so the researchers used the Hep.G2 cell line. The detailed pictures of fibroblast growth was shown in additional file 1.

Isolation of the HepG2 / Umbilical Cord-Mesenchymal Stem Cell line culture was isolated with DMEM + 5% HPL medium for 5 days The sample was centrifuged for 5 minutes and the supernatant was discarded. Cell top up was done with 1 ml of PBS for cell count.

Immunophenotyping and assessment of Umbilical Cord-Mesenchymal Stem Cell characteristics were performed using the Human Mesenchymal SC Analysis Kit.

Statistical Analysis

The sample was 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 Kruscal Wallis test was used).

RESULT

Hep.G2 cell line

The results showed that there was fibroblast growth in the Hep.G2 cell line (Fig.2). Additional file 1 showed Isolation of Umbilical Cord-Mesenchymal Stem Cells Isolation of UC-MSCs was obtained with phenotypes CD 73+, CD90+, CD105+ and Lin- (Fig.3,4) and parameters/ condotioned medium BDNF, SDF1, FGF, VEGF, PDGP, EGF, NGF, IL10, IL1beta, INF alpha, MCP1, IL6, IL12p70, IL17A, IL18, IL23 (Fig. 5), all of which are standard content of Mesenchymal Stem Cells for cell therapy as anti-fibrosis, anti-inflammatory, immunomodulatory, anti-necrotizing and anti-apoptotic.

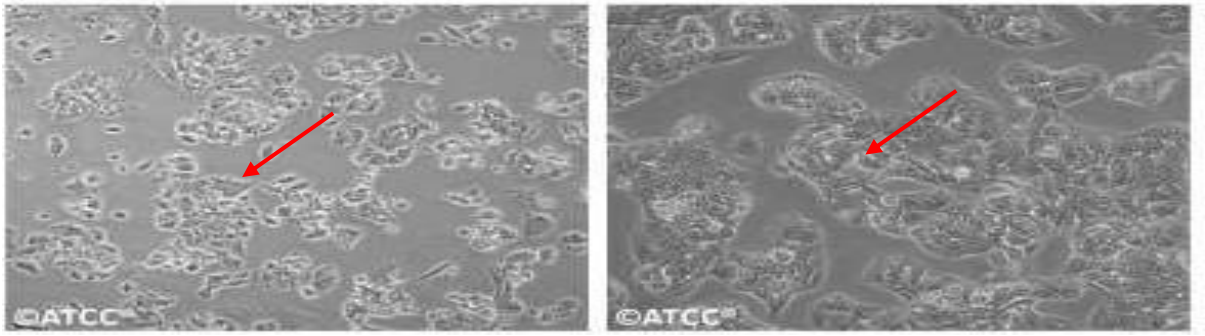


Fig. 2 Fibroblast growth was seen in cell line HepG2 isolation

Marker	Ekspresi
CD73+	99.9%
CD90+	99.9%
CD105+	98.5%
Lin-	0.6%

Fig. 3. UC MSC phenotypes

Immunophenotyping
UC-MSC Conditioned Medium Identification
Mesenchymal Stem Cells + Silymarin

Molecular Study Examination Results

Effect of Mesenchymal Stem Cells and/or Silymarin on NF-kβ on HepG2 Cell Line

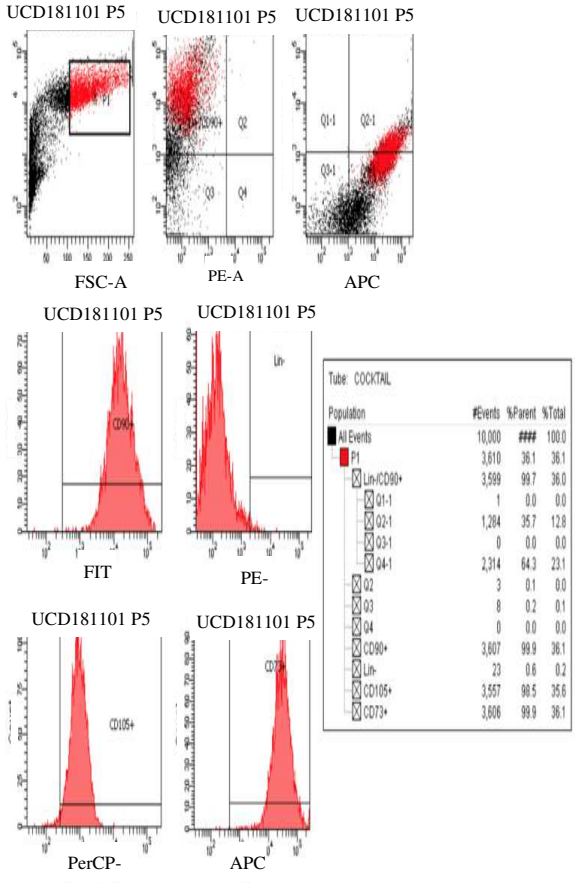


Fig. 4 UC MSC phenotypes

ANOVA test

At 24th hours after treatment, the lowest NF-kβ level was in the C2 treatment group, which was 0.103 +0.008 on average, and the highest NF-KB level was in the Cb group is 0.188 +0.012 (Table 2).

At 48th hours after treatment, the lowest NF-k level was in the C1 treatment group (Silymarin 250ug/mL) which was an average of 0.096 +0.006 nmol/ml, and the highest NF-KB level was in the Cb group (Control without Treatment). Which is 0.218 +0.015. The results of statistical tests at the 24th hour and 48th hour got p value = <0.001 (p<0.05), which means that there is a significant difference. NFkB Protein Concentration Distribution was shown in Fig.6 and Table1. After treatment. a further test was carried out, namely the posh hoc test, the following

Parameter	Unit	Result[1]
BDNF	pg/ml	126.60
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	15.40
IL-1β	pg/ml	2.64
IFN-γ	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

Fig. 5 UC MSC parameters

results were obtained based on the Treatment Preparation (Table 3)

It can be seen that groups C1, C2, C3, have effective results in reducing levels of NF-kβ at 24 and 48 hours after treatment compared to control, with p <0.05.

The most effective treatment group in reducing NF-kβ at 24th hours was group C2 (Mesenchymal

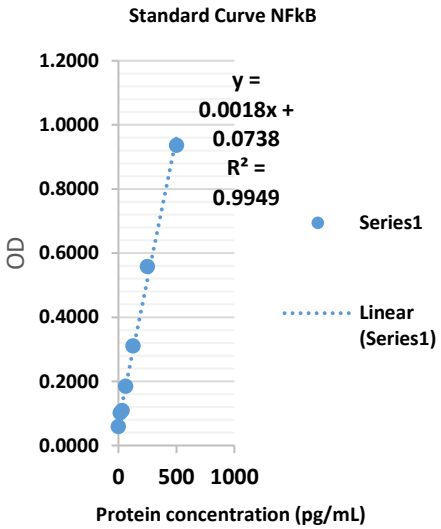


Fig. 6 NFkB Protein Concentration Distribution Standard Curve

Stem Cells 5x10^3 cells/well) with the lowest levels of NF-k, which was an average of 0.103 +0.008 (Fig.7) , but 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 reducing NF-kβ at 48th hours was group C1 (Silymarin 250ug/mL) with the lowest levels of NF-k, which was an average of 0.096 +0.006, and showed a significant difference with group C2 (p=0.009), but did not show a significant difference with the C3 group (p=0.687)

Effect of Mesenchymal Stem Cells and/or Silymarin on Caspase-3 on Pathogenesis of HepG2 Cell Line Fibrosis In Vitro

Table 1. NFkB Protein Concentration Distribution

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

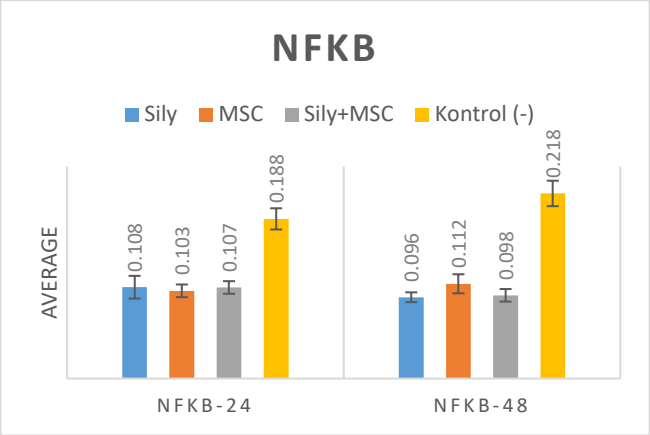


Fig. 7. Bar Diagram of NF- kβ Levels Based on Treatment Preparation

ANOVA Test

At 24th hours after treatment, the highest Caspase-3 levels were in the C3 treatment group (Mesenchymal Stem Cells + Silymarin), which was an average of 0.075 +0.007, and the lowest caspase-3 levels were in the Cb group (control without treatment). ie the average is 0.071 +0.007.

At 48th hours after treatment, the highest levels of Caspase-3 were in the C2 treatment group (Mesenchymal Stem Cells 5x10^3 cells/well), i.e. an average of 0.068 +0.004 nmol/ml, and the lowest

Table 2. NF-kβ Anova Test

Group	NF-kβ		
	N	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

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. One Way Anova Test; * significant α=5%

Table 3. NF-kβ Post Hoc Test

Group	NF-KB	
	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*

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. One Way Anova Test; * significant α=5%

levels of caspase-3 were found. In the C1 group (Silymarin 250ug/mL) the average was 0.060 +0.006.

Group	Caspase-3		
	N	24 hours[a] *(Mean±SD)*	48 hours[b] *(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*

Table 4. Caspase 3 One Way Anova Test

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. One Way Anova Test; * significant α=5%

The results of statistical tests at 24th hours (p = 0.458) which means that there is no significant difference in caspase-3 levels between various treatment preparations at 24 hours, while at 48 hours (p = 0.008) the p value <0.05 (Table 4.) This means that there is a significant difference in caspase-3 levels between various treatment preparations at 48th hours.

Based on the ANOVA test, it was known that there was a significant difference in CASPASE-3 levels between various treatment preparations at the 24th and 48th hours with p <0.05, then a further test was carried out, namely the posh hoc test, the following results were obtained

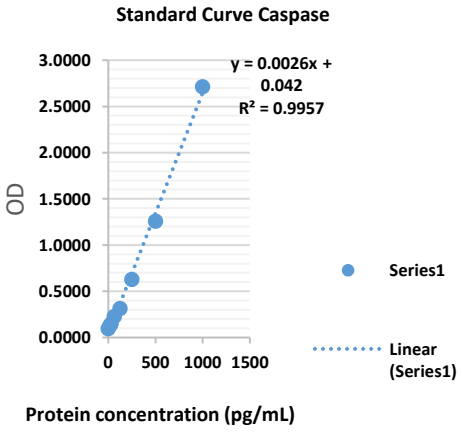


Figure 8. Caspase 3 Protein Concentration Distribution Standard Curve

It can be seen that the levels of caspase-3 groups C1 (p = 0.964), C2 (p = 0.372) C3 (p = 0.185) had results that were not significantly different from the control at 24th hours after treatment (Table 6). Caspase3 Protein Concentration Distribution was shown in Fig.8 and Table 5.

At 48th hours, group C1 (p=0.053), group C2 (p=0.072) and group C3 (p=0.095) did not show a significant difference with the control, but group C1 (p=0.021) showed a significant difference with the control group. Group C2, where group C2 had higher levels of caspase 3 than C1 (Fig. 9)

Effect of Mesenchymal Stem Cells and/or Silymarin on Apoptosis on Pathogenesis of HepG2 Cell Line Fibrosis In Vitro

ANOVA Test

At 24th hours after treatment, the lowest Apoptosis level was in the C1 treatment group (Silymarin 250ug/mL), which was an average of 3699.86 +1741.16, and the highest Apoptosis level was in the Cb group (control without treatment) which was an average of 2763000.00 +961994.11. At 48th hours after treatment, the lowest Apoptosis level was in the C1 treatment group (Silymarin 250ug/mL), which was an average of 4476.86 +750.17, and the highest Apoptosis level was in the Cb group (control without treatment), which was an average of 1631028.57 +517758 (Table 7).

Standard Curve	OD 1	OD 2	Average
0	0,091	0,099	0,0951
15,625	0,115	0,141	0,1281
31,25	0,145	0,147	0,1462
62,5	0,238	0,216	0,2267
125	0,317	0,312	0,3145
250	0,545	0,712	0,6284
500	1,219	1,300	1,2591
1000	2,813	2,616	2,7145

Table 5. Caspase 3 Protein Concentration Distribution

Table 6. Post Hoc Test Caspase-3 Variables Based on Treatment

Group	CASPASE-3	
	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

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. One Way Anova Test; * significant α=5%

Figure 9. Bar Diagram of Caspase-3 Levels Based on Treatment PReparation

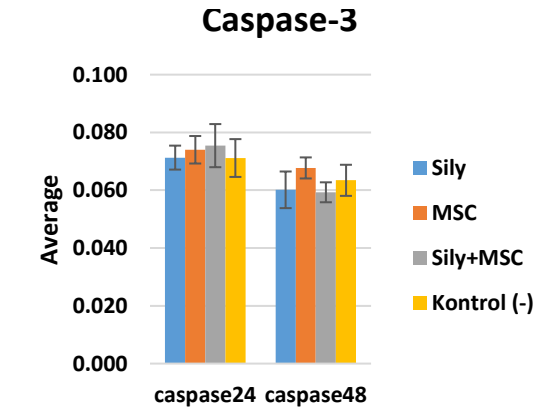


Table 7. ANOVA Test for Apoptotic Variables Based On Treatment Preparations

Group	N	Apoptosis 24 hours[b] *(Mean±SD)*	Apoptosis 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
p-value		<0,001*	<0,001*

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. One Way Anova Test; * significant α=5%

Table 8. Post Hoc Test Apoptotic Variables Based On Treatment Preparations

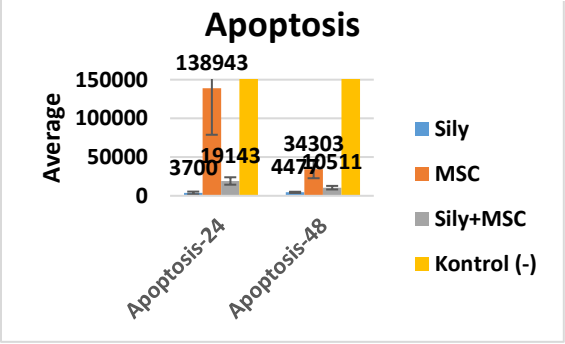
Group	Apoptosis	
	24 hours[b] (p-value)	48 hours[a] (p-value)
Cb *vs* C1	0.002*	<0.001*
Cb *vs* C2	0.002*	<0.001*
Cb *vs* C3	0.002*	<0.001*
C1 *vs* C2	0.002*	0.831
C1 *vs* C3	0.002*	0.966
C2 *vs* C3	0.002*	0.865

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. One Way Anova Test; * significant α=5%

The results of statistical tests at the 24th hour (p=<0.001) and 48th hour (p=<0.001) got p<0.05. Which means that there is a significant difference in Apoptosis levels between various treatment preparations at 24th and 48th hours after treatment

Based on the simultaneous difference test, it was found that there was a significant difference in the level of apoptosis between the various treatment preparations at the 24th and 48th hours with a p value <0.05, then a further test was carried out, namely the posh hoc test, the following results were obtained.

Fig. 10. Bar Diagram of Apoptosis Levels Based on Treatment PReparations



It can be seen that groups C1, C2, and C3 have results that have an effect on reducing Apoptosis levels at the 24th and 48th hours after treatment compared to the control, with p<0.05 (Table 8).

The most influential treatment group in reducing Apoptosis at 24th hours was group C1 (Silymarin 250ug/mL) with the lowest level of Apoptosis, which

was an average of 3699.86 +1741.16 (Fig.10), and showed a
significant difference with group C2 (p=0.002) and group C3. (p=0.002)

The most influential treatment group in reducing Apoptosis at 48th hours was group C1 (Silymarin 250ug/mL) with the lowest Apoptosis levels, namely an average of 4476.86 +750.17, but did not show a significant difference with group C2 (p = 0.831) and group C3 (p=0.966)

Effect of Mesenchymal Stem Cells and/or Silymarin on Necrosis on Pathogenesis of HepG2 Cell Line Fibrosis In Vitro

ANOVA Test

At 24th hours after treatment, the lowest necrosis rate was in the C2 treatment group (Mesenchymal Stem Cells 5x10^3 cells/well), which was an average of 1,596 +0.134, and the highest necrosis rate was in the C3 group (Mesenchymal Stem Cells + Silymarin) which is an average of 15,078 +0.857 (Table 9.).

Group	N	Necrosis	
		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*

Table 9. ANOVA test Necrosis Variables Based on Treatment Preparations

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. *Post Hoc LSD Mann Whitney Test* significant α=5%

At 48th hours after treatment, the lowest necrosis rate was in the C2 treatment group (Mesenchymal Stem Cells 5x10^3 cells/well), which was an average of 2,066 +0.369, and the highest necrosis rate was in the C3 group (Mesenchymal Stem Cells + Silymarin) which is an average of 15,066 +0.931 (Fig.11).

The results of statistical tests at the 24th hour (p=<0.001) and 48th hour (p=<0.001) got p<0.05. Which means that there is a significant difference in the levels of necrosis between the various treatment preparations at the 24th and 48th hours after treatment.

Based on the simultaneous difference test, it was found that there was a significant difference in the level of necrosis between the various treatment preparations at the 24th and 48th hours with p <0.05, then a further test was carried out, namely the posh hoc test, the following results were obtained.

Group	Necrosis	
	24 hours[b] (p-value)	48 hours[a] (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 Necrosis Variables Based on Treatment Preparations

It can be seen that at the 24th hour group C3 (p=0.002) had results that had an effect on increasing the level of necrosis, and group C2 (p=0.002) had results that had an effect on reducing levels of necrosis at the 24th hour after treatment compared to the control, with a p value <0.05. At 48 hours, groups C1 (p=<0.001) and C3 (p=<0.001) had results that had an effect on increasing levels of necrosis, and group C2 (p=<0.001) had results that had an effect on decreasing levels of necrosis at 48 hours after treatment. Compared with the control with a p value <0.05 (Table 10).

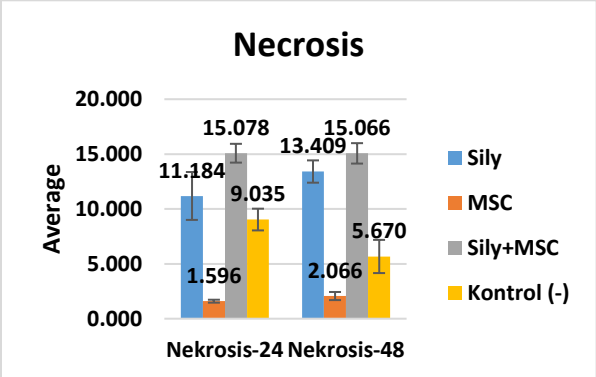


Fig. 11 Bar Diagram of Necrosis Levels Based on Treatment Preparations

Effect of Mesenchymal Stem Cells and/or Silymarin on Proliferation in Pathogenesis of HepG2 Cell Line Fibrosis In Vitro

ANOVA Test

At 24th hours after treatment, the lowest proliferation rate was in the C1 (Silymarin 250ug/mL) treatment group, which was an average of 0.187 +0.039, and the highest proliferation rate was in the C2 (Mesenchymal Stem Cell) group, which was an average of 0.777 +0.123 (Table 11). At 48th hours after treatment, the lowest proliferative level was in the treatment group C1 (Silymarin 250ug/mL), which was an average of 0.232 +0.075, and the highest proliferative level was in the control group (control without treatment) which was an average of 1.084. +0.031 (Fig.12).

Table 11. ANOVA Test Proliferation Variables Based on Treatment PReparations

Group	Proliferation		
	N	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 ±0.040	0.290 ±0.048
Cb -	7	0.657 ±0.196	1.084 ±0.031
p-value		<0,001*	<0,001*

C1: Silymarin 250ug/mL. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. Kruskal Wallis Test; * significant α=5%

The results of statistical tests at the 24th hour (p=<0.001) and 48th hour (p=<0.001) got p<0.05. Which means that there is a significant difference in the rate of proliferation between various treatment preparations at the 24th and 48th hours after treatment.

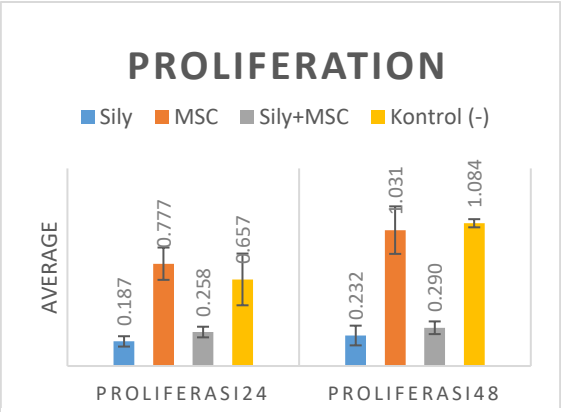


Fig. 12 Bar Diagram of Proliferation Rates Based on Treatment Preparations

Based on the simultaneous difference test, it was found that there was a significant difference in the levels of proliferation between the various treatment preparations at the 24th and 48th hours with a p value <0.05, then a further test was carried out, namely the posh hoc test, the following results were obtained.

Table 12. Post Hoc) Proliferation Variables Based On Treatment Preparations

Group	Proliferation	
	24 hours (p-value)	48 hours (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. **C2:** Mesenchymal Stem Cells 5x10^3 cell/well. C3: Mesenchymal Stem Cells + Silymarin. Cb: Control without intervention. *Post Hoc LSD Mann Whitney Post Hoc Test* significant α=5%

It can be seen that at 24th hours groups C1 (p=0.002), C2 (p=0.338) and C3 (p=0.002) had results that had an effect on increasing proliferation compared to the control group with p<0.05 and group C2 (p = 0.338) has the most significant result. At 48th hours, groups C1 (p = 0.002), C2 (p = 0.337) and C3 (p = 0.002) had results that had an effect on increasing Proliferation levels compared to the control group with p < 0.05, and group C2 (p = 0.337) has the most significant result (Table 12).

Discussion

The purpose of assessing the stage and rate of fibrosis (fibroblasts) is to estimating response to therapy, provide therapy as needed. 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, and estimate the time of occurrence of liver cirrhosis. This is necessary to anticipate patient care[6].

Several things may cause fibroblasts not to occur are liver parenchyma tissue that is damaged either when a biopsy is performed or the time interval for cell culture is performed, the media used for growth is not suitable, the emergence of growth complications (infection) and unsuitable growth reagents for cell culture growth

Isolation of Umbillical Cord-Mesenchymal Stem Cell culture showed fibroblast growth, 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 adhered to plastic under normal/standard culture conditions. Second, expressing positive CD105, CD73, and CD90 and does not express (negatively) CD11b, CD14, CD34, CD45, CD79a and HLADR.

Mesenchymal stem cell phenotypes are not necessarily the same in all types of cancer. So it is necessary to identify specific biomarkers including cell surface markers to determine the prognosis and survival of patients.

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 because the dose used is too small, the materials and reagents used are damaged, the time used to evaluate is too short, and differentiation effect of Mesen-chymal Stem Cell

Results obtained first, nuclear factor kappa Beta (NF-kβ). Statistically, Umbilical Cord-Mesenchymal Stem Cells and/or Silymarin gave significant suppression to the control NF-kβ, but when compared with each research variable, there is no significant difference. The most effective treatment group in reducing NF-kβ at 24^{th} hours was group C2 (Mesenchymal Stem Cells 5x10^3 cells/well) with the lowest levels of NF-kβ, which was an average of 0.103 +0.008, but 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 reducing NF-kβ at 48^{th} hours was group C1 (Silymarin 250ug/mL) with the lowest levels of NF-kβ, which was an average of 0.096 +0.006, and showed a significant difference with group C2 (p=0.009), but did not show a significant difference with the C3 group (p=0.687) (In accordance with the theory of Umbilical Cord-Mesenchymal Stem Cells and/or silymarin significantly suppressed NF-kβ)

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 as well as between study variables. This can occur because the mechanism of caspase inhibition in the pathogenesis of fibrosis can occur due to several 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 can activate casepase-3. Its role is carried out by mitochondria which are activated by tBid, Bcl-2 Bcl-X which is activated by casepase-8 and casepase-10. Mitochondria also activate apaf-1 and then activate casepase-9 to activate casepase-3 in its role in influencing caspase3 apoptosis, also known as apoptotic executor[7].

Third, there is apoptosis. The effect of Umbilical Cord-Mesenchymal Stem Cells and/or silymarin was shown to significantly suppress apoptosis at either 24^{th} or 48^{th} hours compared to controls. For each treatment variable for 24^{th} hours, there was a strong suppression of silymarin variables compared to Umbilical Cord-Mesenchymal Stem Cells and the combination (Umbilical Cord-Mesenchymal Stem Cells + silymarin) significantly different. However, the suppression between variables did not differ at 48^{th} hours (according to the theory that mesenchymal stem cells and/or silymarin had a significant role in suppressing apoptosis). Apoptosis is directly influenced 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 has a dual effect in terms of proliferation and apoptotic effects[5].

The last result is necrosis. Umbilical Cord-Mesenchymal Stem Cells gave a stronger suppressive effect than the control at 24^{th} hours. Meanwhile, silymarin and Umbilical Cord-Mesenchymal Stem Cell + silymarin did not have a suppressive effect of necrosis compared to controls (according to the research objective, the effect of Umbilical Cord-Mesenchymal Stem Cell was to inhibit necrosis). 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 fibers leading to cirrhosis.

Several journals suggest using silymarin as early as possible, because the longer you use silymarin the less the effect will be, and toxic effects have been reported (Ebrahimi *et al.*, 2016). 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)[8].

Umbilical cord- mesenchymal stem cells increased proliferation strongly 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 growth factor ligand molecules with receptor tyrosine kinases, which then activates the embryonic signal transduction pathway and/or MAPK which ends with the activation of various proliferative genes[9]. Silymarin did not stimulate proliferative growth compared to control because of its weak proliferative effect and toxic effect[5].

There are still many limitations 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, isolation of cultured cells from liver biopsy tissue did not grow sufficiently. There are several possibilities for failure of fibroblast growth, including tissues used, the reagents used, there needs to be a suitable medium for growth, the need for 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 using elisa method, and flow cytometry, and short assessment time

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-k, apoptosis, necrosis activity, and increasing proliferation. This highlighted potential benefit of mesenchymal stem cells against liver fibrosis.

Declarations

Acknowledgments

None

Author Contribution

Kusnanto Paulus wrote the main manuscript text and Purwanto Bambang supported manuscript theory, Wasita Brian prepared the laboratory analysis, and Widyaningsih Vitri carried out the statistical analysis.

Consent for Publication

This manuscript, as submitted or its essence in another version, is not under consideration for publication elsewhere and will not be published elsewhere while under review by BMC Gastroenterology. All authors have made substantive contributions to the study, and all authors endorse the data and conclusions. All authors have no commercial association or sources of support that might pose a conflict of interest.

Competing Interest

The authors declare no conflict of interest.

Data Availability Statement

We have attached the data as a file with the submission

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Ethics approval

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