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# Effect of umbilical cord mesenchymal stem cells with/without silymarin on apoptosis, immunomodulation, proliferation, and necrosis of HepG2 cells

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ABSTRACT

**Background:** Liver cirrhosis and liver cancer are the main problems of liver disease, and liver
cirrhosis is the 11th leading cause of death worldwide. Fibrosis is a progressive process of liver disease before cirrhosis. Currently, stem cells are designed to treat various conditions, including chronic liver disease. Stem cells can inhibit the progression of fibrosis based on their immunomodulation, anti-apoptotic and proliferative effects.
**Methods:** This study was an *in vitro* experimental study. Mesenchymal stem cells (MSC)
were taken from the umbilical cord of newborns. This study used the HepG2 cell line because it has the characteristics of hepatocyte cells. Cell lines were divided into four groups[RJ1] . The examination was performed on Nuclear factor kappa B (NF-κB)[RJ2] , caspase-3, apoptosis, necrosis, and proliferation.
**Results:** Mesenchymal stem cells have the effect of reducing NF-κB levels, increasing caspase-3
levels, reducing necrosis events, and increasing proliferation in HepG2 cells. Combining mesenchymal stem cells with silymarin can increase caspase-3 levels and reduce the incidence of necrosis in HepG2 cells.
**Conclusions:** Mesenchymal stem cells can regulate immunomodulation, increase proliferation, reduce
necrosis, and regulate apoptosis of HepG2 cells.

## MSC Isolation and Culture

1 MSCs were obtained from the healthy donor's umbilical cord and processed within 48 hours after cesarean section. Umbilical cords were delivered in a sterilized bottle with buffer saline. Under the biosafety cabinet, cords were washed with phosphate buffer saline (PBS) to remove any contaminating blood. Vessels were removed, cut the cord into small pieces, and placed in a culture dish in Growth Medium. The growth medium consists of DMEM supplemented with 5% Human Plate and antibiotics (penicillin 100 µg/mL, streptomycin 10 µg/mL, amphotericin B 250 µg/mL). The culture was maintained at 37°C and 5% humidified CO2. Growth mediums were changed every 3-4 days. When colonies of fibroblast-like cells appeared and reached 80% confluency, cells were harvested using TrypLe Express and re-plated in suitable tissue culture dishes for further expansion.

## HepG2 and UC-MSC Culture

2 1. HepG2 cells and UC-MSC were cultured in 100 mm Petri dishes using DMEM + 5% HPL growth medium for 5 days until they reached >80% confluency.
2. When confluent, cells were harvested by adding 3 ml of TrypLe solution and incubated at 37 oC; 5% CO2 for 7 minutes.
3. Gently tap the petri dish to release the cells completely.
4. Neutralize TrypLe with growth medium (1:1).
5. Collect the entire solution in the petri dish into a conical tube.
6. Centrifuge the sample for 5 minutes at 500g to obtain cell suspension.
7. Discard the supernatant and top the cells with 1 ml PBS for cell count.

## Proliferation Test

3 1. A 100 µL HepG2 cell suspension was grown in 96-well plates at 5 x 103 cells/well density.
2. Incubate the cells at 37 oC; 5% CO2 for 24 hours.
3. Add 1mM Oleic Acid at 100 µL in each well to induce cell fibrosis and incubate the cells for 24 hours at 37oC; 5% CO2.
4. 100 µL of test and control solutions were put into the wells. The test solutions used were:
- Control group, which was not given any treatment (Cb)
- Silymarin group, which was given silymarin (Sigma-Aldrich, SCM152) with 250 mcg/ml solution (C1)
- UC-MSC group with a 1:1 ratio in DMEM growth medium (C2)
- UC-MSC group in silymarin carrier solution (C3).
5. Incubate the cells at 37oC; 5% CO2 with incubation periods of 24 hours and 48 hours.
6. Discard the test and control solutions and add 10 µL MTT reagent with a 5 mg/mL concentration into each well.
7. Cells were re-incubated in an incubator at 37oC; 5% CO2 for 4 hours.
8. Add 100 µL MTT reagent solubilization solution into each well.

9. Absorbance was measured at 450 nm wavelength using an ELISA reader.

## Apoptosis and Necrosis Assays

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1. A 100 µL HepG2 cell suspension was grown in white 96-well plates at 5 x 103 cells/well density.
2. Incubate the cells at 37oC; 5% CO2 for 24 hours.
3. Add 1mM Oleic Acid for 100 µL in each well to induce cell fibrosis and incubate the cells for 24 hours at 37oC; 5% CO2.
4. 100 µL of test and control solutions were put into the wells. The test solutions used were:
   - Control group, which was not given any treatment (Cb)
   - Silymarin group, which was given silymarin (Sigma-Aldrich, SCM152) with 250 mcg/ml solution (C1)
   - UC-MSC group with a 1:1 ratio in DMEM growth medium (C2)
   - UC-MSC group in silymarin carrier solution (C3).
5. Incubate the cells at 37oC; 5% CO2 with incubation periods of 24 hours and 48 hours.
6. Discard the test and control solutions and add an amount of RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay solution 100µL to each well.
7. Luminescence and fluorescence were measured at 500-550 nm intensity using a GloMax microplate reader.

## NF-kB and Caspase Test

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1. A 500 µL HepG2 cell suspension was grown in 96-well plates at 5 x 104 cells/well density.
2. Incubate the cells at 37oC; 5% CO2 for 24 hours.
3. Add 1mM Oleic Acid as much as 500 µL in each well to trigger cell fibrosis and incubate the cells for 24 hours at 37oC; 5% CO2.
4. 500 µL of test and control solutions were put into the wells. The test solutions used were:
   - Control group, which was not given any treatment (Cb)
   - Silymarin group, which was given silymarin (Sigma-Aldrich, SCM152) with 250 mcg/ml solution (C1)
   - UC-MSC group with a 1:1 ratio in DMEM growth medium (C2)
   - UC-MSC group in silymarin carrier solution (C3).
5. Incubate the cells at 37oC; 5% CO2 with incubation periods of 24 hours and 48 hours.
6. Addition of 100µl 1X Cell Extraction Buffer PTR and remove cells using mini cell scrapper.
7. Cells were collected in a 1.5 ml microcentrifuge tube.
8. A total of 50µl cells were then plated in each well of white 96-well plates.
9. At the same time, Lyophilized NFkB p65 Control Lysate and Cleaved Caspase-3 (Asp175) lyophilized standards were prepared by stepwise dilution.
10. A 50µl standard was added to the first 2 columns of the 96-well plate.
11. Add 50µl Antibody Cocktail for NFkB and Caspase for each sample and standard.
12. Cover the 96-well plates with a seal and incubate for 1 hour on a plate shaker and at room

temperature.
13. Wash each well with Wash Buffer 3 times.
14. Add 100µl TMB Development Solution and incubate for 30 minutes in the dark for 1 hour on a plate shaker.
15. We add 100µl Stop Solution to each well and shake the plate to level the solution with a plate shaker.
16. The absorbance was reading at 450nm wavelength.