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Research Article

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From Mesenchymal Stromal/Stem Cells to hepatocyte-like cells: Biochemical Evaluations

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KEY WORDS

Differentiation;
Hepatocyte;
Liver function,
Rats,
Mesenchymal stem cells

ABSTRACT

Objective: To investigate the ability of the liver extract to induce in vitro differentiation of rat adipose tissue derived mesenchymal stem cells into hepatocyte cells.

Methods: The experiment was involved 40 healthy Sprague Dawley male rats that were divided into 4 groups: group 1 was served as a normal control rats, group 2 was given intra peritoneal dose of CCl_4 served as positive group, group 3 was injected with CCl_4 and treated with intravenous injection of undifferentiated cells while group 4 was injected with CCl_4 and differentiated AD-MSCs Biochemical analysis was done including serum total bilirubin, albumin, ALT and AST. Liver catalase, Superoxidase dismutase (SOD) and Malondaialdehyde (MDA) levels were evaluated.

Results: After 21 days of in vitro differentiation of rat mesenchymal stem cells by using liver extract cells, it was observed a morphology changes: large nuclei and dark granular deposits within the cytoplasm emerged in the culture. By comparing with group 2 (+ve control); the liver function of rat group 4 was affected by injecting of differentiated MSCs by increasing albumin (p=0.001) and decreasing of ALT and AST concentrations (P= 0.0001 & 0.0001 respectively). Furthermore, there was a significant increase in the catalase and SOD levels (P=0.0001 & 0.0001 respectively), while a significant decrease in MDA was found (P=0.0001)

In conclusion: The liver extract media could differentiate AD-MSCs into hepatocytes like cells as well as biochemical activities of liver rat induced by CCl_4 have an ameliorations effect by injection of in vitro differentiated MSCs.

Introduction

Both liver function and kidney performance having a bidirectional communication, are reciprocally connected and affected by multiple factors: toxic agents, Schistosomiasis, Hepatitis C virus (HCV) …etc. **(Sharma *et al*., 2020).**

Liver fibrosis as a chronic hepatic injury is a fatal disease that can cause biochemical deviation e.g. insulin resistance, reduced albumin synthesis and reduced cholesterol synthesis **(Pradeep *et al*., 2019; Yuan *et al*., 2019)**. At present, transplantation is the only trust worthy treatment concerning end stage of hepatic fibrosis. However, there are many complications associated with transplantation like a shortage of organs and other transplantation complications, which urge researchers to find alternative therapeutic solution. Mesenchymal stem cells help to prevent the fibrotic lesions or enhances liver functions in experimental fibrosis models **(Fernández-Colino *et al*., 2019)**.

Stem cells (SCs) are undifferentiated mother cells that have the ability to transform into all cell types in the human body. They have two important characteristics: self-renewal and potency i.e. the ability to differentiate **(Guadix *et al*., 2017)**.

Among stem cells, mesenchymal stem cells (MSCs) are due to their immunomodulatory ability and capacity for differentiation into hepatocytes, tested in many preclinical and clinical studies as possible new therapeutic agents for the treatment of acute and chronic liver diseases including fibrosis. MSCs can alter immune response and regulate the proliferation, activation, and effector function of all immune cells **(Milosavljevic *et al*., 2018).**

Animal models and human studies show that MSCs derived from the bone marrow and other tissues when cultured under specific conditions in vitro can trans-differentiate into hepatocyte-like cells which can perform normal metabolic functions of liver cells **(Ghavamzadeh *et al*., 2018)**.

As hepatitis C is a global health problem. In worldwide, it is about 71.1 million individuals chronically infected, accounting for 1% (95% uncertainty interval: 0.8-1.1) of the population. HCV infection was projected to yield 750.210 PY of decompensated cirrhosis, 132.894 PY of hepatocellular carcinoma producing 127.821 deaths from cirrhosis and 117.556 deaths from HCC from 2009 to 2028 **(El-Ghitany, 2019; Roudot-Thoraval, 2021)**. The

completed experimental research using multiple infusion methods concurrently are only a few **(Yang *et al*., 2020)**.

Some Egyptian studies investigated many toxic agents affecting liver and kidney. They showed the serious results of local polluted drinking water on the functions of both liver and kidney. Liver cirrhosis cases were related to iron-contaminated drinking water **(Mandour, 2012)**. Industrial progress has a poisonous nature. Carbone tetra-chloride (CCl_4) is one of the most potent environmental contaminants. Humans are exposing to CCl_4 via oral, inhalation and dermal routes. CCl_4 intoxication is associated with high free radical production in several organs, including the liver and kidney **(Elsawy *et al*., 2019)**.

Consequently, we conducted this study to contribute to the studies related to liver and kidney diseases. Our objective was to evaluate MSCs could treat cirrhosis and end-stage liver disease as well as improving of kidney functions.

Materials and Methods

Animals

Healthy Sprague Dawley male rats weighing 150-200g were obtained from Animal House Department of Urology and Nephrology Center, Mansoura University, Egypt. The rats were maintained under standard laboratory were fed on a basal diet for adaptation and supplied with water, *ad libitum*. They were divided into 4 experimental groups: 10 animals for each group. **Group 1** were served as a normal control group, **group 2** were given intra peritoneal dose of carbon tetrachloride (CCl_4) in olive oil (1ml/Kg) twice a week served as a diseased positive group **(Kazarlis *et al*., 2001)**, **group 3** were injected with CCl_4 as in group 2 and then they were treated with intravenous injection of 3×10^6 cells of undifferentiated cells and **group 4** were injected with CCl_4 as in group 2 and then they were given an intravenous injection of differentiated AD-MSCs ($3x10^6$ cells per rat).

Isolation of mesenchymal stem cells from rat adipose tissue

Under the sterile biological safety cabinet (class II A2), adipose tissue sample was excised from rat abdominal cavity and washed with PBS then transferred to sterile petri dish.

The adipose tissue was minced in sterile HBSS containing 0.075% collagenase type I then mixed together and filtered with 0.2 µm syringe filter (Thermo Scientific). Next, the digestion of the adipose tissue was carried out into shaking water bath at 37^oC for 45 min.

At the end of digestion period, 10 ml of DMEM supplemented with 10% FBS was added. The digested tissue was filtered with 70 µm nylon mesh cell strainer to remove tissue debris then centrifuged at 1800 rpm for 5 minutes. The supernatant was discarded and the pellet was suspended in 10 ml out growth medium (DMEM) and cultured in 25 cm^2 tissue culture flask. The cells were cultured at density of 1 x 105/cm^2 in 5% CO_2 incubator at 37 °C. After 3 days of culture, the non-adherent cells were discarded **(Gabr *et al*., 2014).**

Culturing of Adipose Mesenchymal Stem Cells

Stem cells (SCs) secrete proteins which enable these cells to attach to the surface of tissue culture polystyrene flasks through establishing a robust bridge between the cell and the surface. Thus, these proteins must be fractured to draw the adherent cells from the culture flask. The enzyme, trypsin, can break such proteins at specific protein. Prepared Trypsin solution contains primarily EDTA that attracts some metal ions which prevent the efficient activity of trypsin. This was performed according to **Soleimani and Nadri, 2009.**

The old media was removed by aspiration using sterile pipette then the cultured cells were washed by 10 ml PBS. The PBS was left on the cells for about 30 seconds to remove any traces of complete media, and then the PBS was removed. After that, 10 ml of 0.05% trypsin dissolved in 0.02% EDTA was added and the flasks were checked under inverted microscope (Olympus corporation 1X71, Shinjuku, Tokyo, Japan) till cells were separated from each other but they were still attached to the flask. At this stage, the shape of the cells changed from spindle to spherical.

Adipogenesis differentiation

All animals were handled in accordance with the guidelines established by our Institutional Ethical Committee. One-month-old healthy Swiss mice were used to acquire liver tissue. The liver tissues were washed twice or three times in PBS with 1 antibiotic-antimycotic (Sigma-Aldrich, MO). Tissues were sliced in 5 ml of PBS mixed with an RNAse inhibitor cocktail (PBSi) at a 1:200 dilution ratio (Sigma-Aldrich, MO). Cut tissue was placed in a cryo-tube (Corning, NY). Before adding 15ml PBSi and homogenizing with a sonicator, the sliced tissue was ground with liquid nitrogen. The supernatant was recovered and kept at 20°C after centrifuging at 13,000 rpm for 10 minutes. SDS-PAGE and the Bradford technique were used to

assess the quality and amount of total proteins isolated from tissues **(Nhung *et al.*, 2015)**.

Induction by liver extract

The cytotoxicity of the liver extract was assessed using media containing liver extract (6µg/ml) in a total period of 21 days. ADSCs were subcultured at tissue culture polystyrene flasks, with the media replaced every 24 hours. ADSC differentiation was promoted by a medium containing mouse liver extract. ADSCs were grown at 5×10^5 cells/cm^2 in 10 µg/ml fibronectin-coated culture flasks in liver extract media. The liver extract medium was made consisting of DMEM/F12 media supplemented with 10% FBS, 1 antibiotic-mycotic (Sigma-Aldrich, MO), and 6 µg/ml liver extract. Cells were grown in 5% CO_2 at 37°C. Every three days, the medium was changed. After 4, 10, 14 and 21 days, cell morphology was examined. After 14 and 21 days, cells were harvested for further study. As a negative control, ADSCs were grown in basic medium **(Nhung *et al.*, 2015)**.

Transplantation of isolated ADMSCs in SD rats

Rats of groups (3), (4) were used as cells recipient. Injected via tail vein, the recipient rats were anesthetized with pentobarbital (50 mg/ml) at a dose of 0.1 ml/ 100 gm of body weight. Group (3) was transplanted with ADMSCs (3X 10^6 per rat) body weight in a volume of 0.2 ml PBS which injected via tail vein of by 26-gouge needle and group (4) rats were transplanted with differentiated ADMSCs. The transplanted rats were then returned to the metabolic cages for further daily monitoring excess food and water were allowed *ad-libitum*.

Biochemical analysis

Liver function tests:

Serum levels of total bilirubin, albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to manufacturer's instruction on Architect plus c4000 auto-analyzer system (Abbott, Abbott Park, USA). Total bilirubin and albumin were determined using diazonium and bromocresol green methods, respectively, whereas ALT and AST were estimated using kinetic methods (NADH without pyrodixal-5-phosphate) [CAT. No. AL 10 31 (45) and AS 10 61 (45), Bio-Diagnostic Company, Giza, Egypt].

CAT, SOD, MDA Levels

Spectrophophotometric kits (Bio-diagnostic, Giza, Egypt) were used to assess oxidative parameters (MDA, CAT and SOD) in rat liver tissues. According to manufacture instructions,

the parameters were done in which the concentrations were reported per tissue mass unit [CAT. No. CA 25 17, SD 25 21 and MD 25 29 Bio-Diagnostic Company, Giza, Egypt].

Results

Microscopic investigation of rat mesenchymal stem cells

Figure (1) shows the morphology of the undifferentiated rat mesenchymal stem cells. The cells obtained from culture before differentiation (day 0). Cells were examined every 3 days, by the inverted microscope (100 to 400×) for assessment of the cell morphology (fibroblastiod appearance), and observed the viability of cells.

Figure 2 shows the morphology of the differentiated mesenchymal stem cells after 14 days of treatment using liver extract cells. Cells with large nuclei and dark granular deposits within the cytoplasm emerged in the culture. At the end of the second stage a homogeneous population of differentiated cells was attained as shown in Fig. (3) after 21 days of differentiated mesenchymal stem cells after culturing under liver extract conditions and further matured in the presence of 2% FBS.

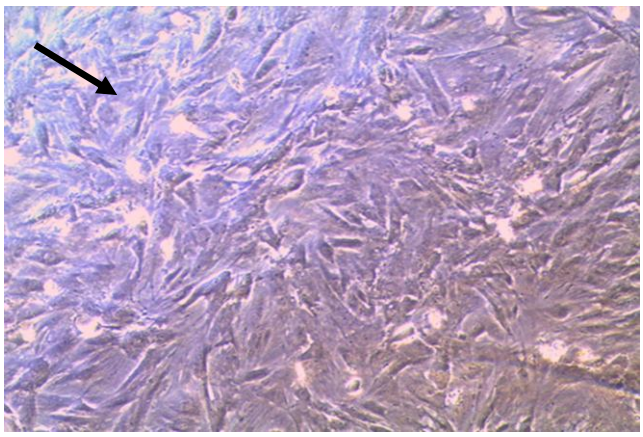

Fig. (1): Undifferentiated rat mesenchymal stem cells by inverted microscope.

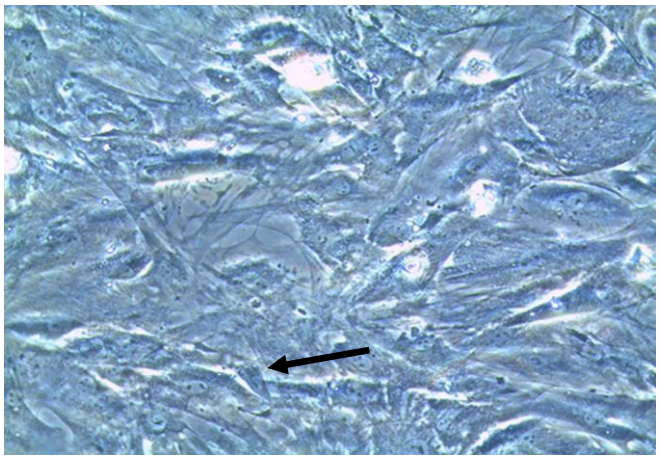

Fig. (2): Differentiated mesenchymal stem cells after 14 days of treatment.

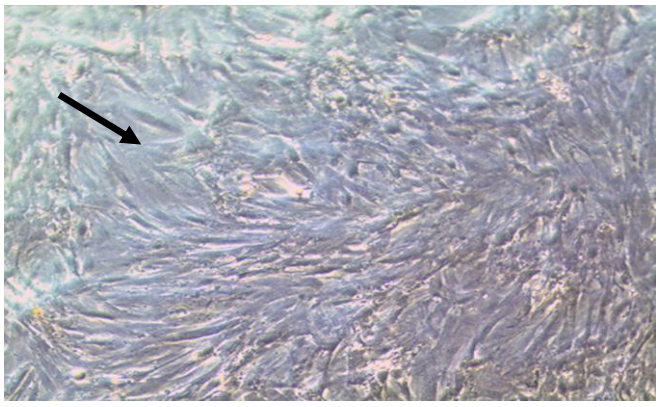

Fig. (3): The differentiated mesenchymal stem cells after 21 days of in vitro differentiation under inverted microscope.

Liver function tests

Table (1): liver function in studied groups

Parameters	Normal	+ve control	Undifferentiated	differentiated	P value
T. Bil. (mg/dl)	**0.14±0.06[a]**	**0.19±0.05[a,b]**	**0.24±0.1 [b]**	**0.18±0.09 [a,b]**	**0.083**
Albumin (mg/dl)	**3.67±0.42 [a]**	**5.5±0.55 [b]**	**4.39±0.56 [c]**	**4.04±0.65 [a,c]**	**0.0001**
ALT (U/l)	**62.66±11.23[a]**	**147.55±28.90[b]**	**119.22±17.13[c]**	**68.55±16.62 [a]**	**0.0001**
AST (U/l)	**100.0±23.7 a**	**182.2±40.16 b**	**136.0±22.14 c**	**74.22±7.12 d**	**0.0001**

Different letters mean significant at $P \leq 0.05$ and sample letters mean insignificant at $P > 0.05$

Liver enzymes (ALT and AST) levels were elevated associated with loss of hepatic function in CCl_4 treated animals (group 2) as compared to group 1 (Table 1). In addition, group 2 has a substantial rise in T. Bil as compared to group 1 while albumin was significantly lower than in healthy control rats of group 1 (Table 1). Differentiated MSCs treatment was enhancement to improve all these markers of liver damage.

Antioxidant and lipid peroxidation

Table (2): Antioxidant and lipid peroxidation in studied groups

Parameters	Normal	+ve control	undifferentiated	differentiated	p
Catalase	**5.78±0.41[a]**	**7.09±0.32[b]**	**6.10±0.19 [a]**	**4.20±0.41 [c]**	**0.001**
SOD	**0.78±0.03 [a]**	**0.46±0.05 [b]**	**0.69±0.03 [c]**	**1.35±0.14 [d]**	**0.0001**
MDA	**11.33±0.51[a]**	**17.61±0.67[b]**	**15.87±0.29[c]**	**12.71±0.27[d]**	**0.0001**

Rat SOD activity and catalase levels were revealed to be impacted by CCl_4 and by injecting of differentiated MSCs treatments. Differentiated MSCs treatments produced statistically significant increase in antioxidant levels, while CCl_4 produced a significant decrease in there levels. Treatment with differentiated MSCs treatments greatly reduced these declines. CCl_4, on the other hand, caused much higher MDA levels than group 4.

Discussion

In the experimental study of liver diseases, CCl_4 is one of the most often utilised hepatotoxins **(Alajez *et al.*, 2018; Al-Essawy *et al.*, 2020).** In our experiment, we investigated the efficacy of undifferentiated MSCs and differentiated MSCs on the treatment of liver fibrosis induced by CCl_4 as well as the biochemical, histochemical, and molecular changes in a fibrotic liver model in which forty rats were divided into four equal groups as follows: negative control group, positive diseased group, undifferentiated MSCs group, and differentiated MSCs group. MSCs were extracted from adipose tissues around the rat testes using an enzymatic technique. The separated MSCs were diverse throughout the first few days of culture. MSCs with fibroblast form were distinguished from other cells after 5-6 days by their capacity to adhere to the polystyrene tissue culture flask and their spindle shape, as illustrated in Fig. (1). The cells were subcultured into many passages at 70-80 percent confluence after twelve days, as illustrated in Fig. (2). These findings are consistent with Azandeh *et al.*, 2012. and Salehinejad *et al.*, 2012 who confirmed that enzymatic isolation of MSCs is an excellent and the most quick approach to separate MSCs within 24-48hr **(Azandeh *et al.*, 2012; Yu *et al.*, 2015)**. The liver damage model revealed that rats were intraperitoneally injected with CCl_4 in olive oil (1ml/Kg of body weight) twice a week for 4 weeks, resulting in liver injury and parallelism between biochemical and antioxidant parameters. This is in line with the findings of **Theocharis *et al.*, 2001 and Lee *et al.*, 2006**, who showed that CCl_4 can cause liver fibrosis **(Theocharis *et al.*, 2001; Abdelaziz and Ali, 2014)**. Because of its active metabolite and trichloromethyl radicals, CCl_4 is extremely hepatotoxic. These activated radicals bind covalently to macromolecules and cause peroxidative destruction of the endoplasmic reticulum membrane's polyunsaturated fatty acids. This results in the creation of lipid peroxides, which cause pathological alterations such as decreased protein synthesis, decreased antioxidant activity like SOD and CAT, increased free radical activity like MDA, and higher levels of blood liver enzymes like ALT, AST, GGT, and ALP **(Abdelaziz and Ali, 2014)**. According to Ujah *et al.*, 2013, CCl_4 can impair the process of cellular energy production and increase the permeability of the hepatocyte membrane, causing the cytosolic isoenzymes of ALT and AST to flow into sinusoids and then peripheral circulation. Additionally, the

permeability of the mitochondrial membrane increases, resulting in the release of mitochondrial isoenzymes **(Ujah *et al.*, 2013).** As shown in Table 1, our biochemical tests revealed a significant rise in blood levels of liver enzymes (Bil., ALB, AST and ALT) in the diseased group compared to the normal control group. In addition, the results of antioxidant activity and free radicals measurements in the current study confirmed that CCl_4 can increase the activity of MDA and CAT can significantly decrease the activity of SOD in the homogenates of diseased group, as shown in Table 2, indicating that the liver tissue was damaged, in agreement with **(Jayakumar *et al.*, 2006; Lee *et al.*, 2006)** who discovered that CCl_4 has a significant oxidative impact on liver tissues, as evidenced by significant increases in MDA and CAT, as well as a decrease in SOD activity, resulting in the failure of cellular antioxidant enzymes to protect CCl_4-injected rats from excessive hepatocyte oxidative stress **(Jayakumar *et al.*, 2006; Lee *et al.*, 2006)**. MSCs transplantation demonstrated a significant increase in antioxidant and free radical activities, demonstrating the potential of these cells to overcome the oxidative stress caused by CCl_4 and minimize liver damage in the treated groups, as noted by **(Nasir *et al.*, 2022)**. When compared to the sick group, the biochemical analysis results indicated the efficacy of AD-MSCs and differentiated AD-MSCs in improving the clinical signs of liver fibrosis in these groups. The blood level of liver enzymes improved to near normal levels, as reported in Table 2. They also reveal that there was a significant drop in CAT and MDA activity and an increase in SOD activity in the homogenates of the hepatic tissues of these groups as compared to the sick group. There is ongoing debate over the best pathway for MSC transplantation. In this study, the dependability of several transplantation methods was examined; tail vein transplantation was shown to be more successful than portal vein transplantation **(Kim *et al.*, 2013)**. According to recent findings, AD-MSCs are expandable and easily accessible. Tail vein transplantation has the potential to increase microcirculation and reduce liver fibrosis. More research is needed to fully understand the differentiated AD-MSCs mechanism of fibrosis reduction and to clinically evaluate the AD-MSCs therapeutic potential. As a result, there is a lot of excitement about finding new medications for liver fibrosis without side effects, where the field of

regenerative medicine is rapidly developing and preparing for novel helpful mediations through cell treatments and tissue building methodologies that can reshape the biomedical field. Finally, our findings show that AD-MSCs, when differentiated into hepatocytes, have the potential to repair fibrotic tissue in the liver. Furthermore, the activity of antioxidant enzymes and DNA content are all significantly negatively connected with hepatic fibrogenesis in toxicated rats, indicating that MSCs are a viable therapeutic.

Conclusion

The liver extract media could induce AD-MSCs to differentiate into hepatocytes like cells, in vitro. By injecting of these differentiated MSCs, the biochemical activities of rat livers as well as SOD activity and catalase levels have an ameliorations effect in CCl_4 induced rat group.

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Conflict of Interest statement

The authors declare no conflicts of interest.

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إستنباط خلايا كبدية شبيه من الخلايا الجذعية الوسيطة : تقييم الحيوية الكيميائية للخلايا

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الهدف من البحث : إجراء تجربة معملية لدراسة قدرة المستخلص الكبدى على إحداث تمايز للخلايا الجذعية الأولية المشتقة من الأنسجة الدهنية لفئران التجارب.

الجزء العملي: أجريت التجربة التالية علي أربعون من ذكور فئران التجارب السليمة بدنياً و التي قد تم تقسيمها إلى أربعة مجموعات متساوية بحيث أن المجموعة الاولي كانت بمثابة نموذج للفئران الطبيعية و المجموعة الثانية تم إعطائها جرعة من رابع كلوريد الكربون داخل التجويف البطني كمجموعة مرضية أما المجموعة الثالثة تم حقنها برابع كلوريد الكربون مع الحقن الوريدي بالخلايا الجزعية الأولية بينما تم حقن المجموعة الرابعة برابع كلوريد الكربون مع الحقن الوريدي بالخلايا الجزعية المتمايزة معملياً.

تم إجراء التحاليل الكيميائية الدقيقة لقياس مستوى البيليروبين والزلال و لأنزيمين من الوظائف الكبدية وهما الألانين أمينوترانسفيراز و الأسبارتات أمينوترانسفيراز. وأيضاً قد تم قياس بعض مستويات مضادات الأكسدة الحيوية (الكاتاليز و مالونديألديهيد و سوبرأوكسيد ديسمويتيز).

النتائج النهائية : قد إستمرت التجربة لمدة إحدى وعشرون يوماً معمليا للخلايا الجذعية أضيفت خلالها المواد المحفزة لتمايز الخلايا الجذعية باستخدام المستخلص الكبدى وبنهاية التجربة شوهد مجهرياً حدوث تغيرات واضحة في شكل الخلايا الجذعية فقد ظهرت للخلايا نوى كبيرة ورواسب حبيبية داكنة داخل سيتوبلازم الخلايا بالمقارنة بالمجموعة الثانية؛ تأثرت وظيفة الكبد للمجموعة الرابعة بحقن الخلايا الجزعية الميزينشيمية المتمايزة عن طريق زيادة الألبومين ($p = 0.001$) وخفض تركيزات الألانين أمينوترانسفيراز و الأسبارتات أمينوترانسفيراز ($P = 0.0001$ & 0.0001) على التوالي. علاوة على ذلك ، كانت هناك زيادة ذات دلالة في مستويات الكاتاليز و سوبرأوكسيد ديسمويتيز ($P = 0.0001$ & 0.0001) على التوالي، بينما كان هناك انخفاض ذو دلالة في المالونديألديهيد ($P = 0.0001$)

في الختام: يمكن لوسائط استخراج الكبد أن تميز الخلايا الجزعية الميزينشيمية إلى خلايا كبدية شبيه وكذلك الأنشطة الكيميائية الحيوية لفئران الكبد التي يسببها رابع كلوريد الكربون لها تأثير تحسين عن طريق حقن الخلايا الجذعية الميزينشيمية المتباينة في المختبر.