ASSESSMENT OF CURATIVE EFFECT OF USING STEM CELLS ON THE CONTROL OF PESTICIDE DAMAGING EFFECT ON LIVER

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ABSTRACT

Introduction: Liver failure is a growing health problem and one of the main causes of death worldwide. Regenerative medicine as stem cells therapy is a newly rapidly developing field in which diseased tissues are regenerated.

Aim: To evaluate the effectiveness of stem cell on liver degeneration induced by Chlorpyrifos as an organophosphorus insecticide widely used in agriculture in Egypt, and Comparing its efficacy with silymarin and colchicine as antifibrotic agents used in liver fibrosis.

Methods: Induction of liver degeneration and fibrosis by Chlorpyrifos (CPF) for 8 weeks to Swiss albino mice in randomly divided groups (B, C and D) and a control healthy group (A), each group composed of 15 mice. Group (C) was treated with oral silymarin and colchicine and group (D) was treated with intra-peritoneal injection of Mesenchymal stem cells (MSC) (10^{6} cells / mouse) after induction of liver fibrosis. After 4 weeks of treatment, all mice were sacrificed, blood was collected for chemical analysis (liver functions tests: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Albumin, Bilirubin). Histopathological evaluation and grading were assessed by H& E stain. **Results:** ALT, AST and Albumin were significantly improved in groups C and D. Bilirubin and liver tissue grading were significantly reduced in group D, with insignificant reduction in group C.

Conclusion: Treatment with MSCs may improve liver functions tests (ALT, AST, Albumin, and Bilirubin) and liver fibrosis. While, treatment with

colchicine and silymarin does not improve the liver fibrosis, nor improves total serum bilirubin, but may improve ALT, AST and Albumin.
Keywords: Chlorpyrifos, Colchicine, Silymarin, Mesenchymal Stem Cells

INTRODUCTION

Chlorpyrifos (*O, O*-diethyl 0-[3, 5, 6-trichloro-2-pyridinol phosphorothionate) is a broad-spectrum organophosphorus insecticide that widely used in agriculture and domestic pest control (Gralewicz *et al.,* 2002). Toxicity associated with Chlorpyrifos led to the restriction of some of its domestic uses by United State Environmental Protection Agency in 2011. Despite its restriction, Chlorpyrifos still remains one of the most widely used insecticides (Steenland *et al.,* 2000). Millions of people exposed to pesticides in the Eastern Mediterranean, an increasingly agricultural region. In Egypt, over 1 million children between the ages of 7 and 12 help with cotton pest management, exposing them to insecticides. Insecticides were the leading cause of deaths from poisonings. It is estimated that there are more than 15,000 tons of obsolete pesticide stocks in Africa, many of which are Persistent Organic Pollutants (POPs) as they do not break down in the environment and can enter the food chain through crops grown on contaminated sites and through fish from contaminated waters (WHO, 2016).

Chlorpyrifos (CPF) induce clinical, biochemical and histological alterations in exposed animals. It induce significant increase in the activities of ALT, AST and ALP (Alkaline Phosphatase) serum enzymes, and significant decrease in albumin level compared to the normal control rats (Heikal *et al.*, 2013; Orabi *et al.*, 2013).

Colchicine and silymarin are well known drugs in the Egyptian market used as antifibrotic agents. Silymarin is the extract of the milk thistle seeds, while colchicine extracted from plants of the genus Colchicum (autumn crocus), both used as hepatoprotective agents. Silymarin prevent hepatic fibrosis through suppression of inflammation and hypoxia in the hepatic fibrogenesis (Jeong *et al.,* 2005). The hepatoprotective effects of colchicine and silymarin were very similar in regard to the prevention of chronic liver damage (Favari and Pérez-Alvarez, 1997).

Federico *et al.,* 2017 in his review concluded that silymarin has three important activities: anti-inflammatory, antioxidant and proapoptotsis, which represent the "functional triad" that allows for antagonizing the onset and the progression of mechanisms of damage that are responsible for the progression of hepatitis to cirrhosis and Hepatocellular Carcinoma.

Mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) as an acceptable method for intervention of various pathological lesions have been extensively investigated in small animal models to treat acute and chronic liver injuries. Mechanisms of action are not clearly elucidated but may include their ability to differentiate into hepatocyte-like cells, to reduce inflammation, and enhance tissue repair at the site of injury (Wang *et al.,* 2016; Papanikolaou *et al.,* 2017).

By using specific culture conditions, some studies showed that MSCs undergo a phenotypic change; express genes typically expressed in hepatocytes, fulfill some metabolic functions similar to hepatocytes, and thus deduced that MSCs can transdifferentiate into hepatocyte-like cells in vitro (Wang *et al.,* 2016).

MATERIALS AND METHODS

Cross sectional Study on animal model for three months and ten days start at 28th of July 2016. Six weeks male Swiss albino mice weighted 33 ± 3 grams. Mice were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR), Egypt. The animals were randomly divided into four groups of 15 animals each. However, during the experiment 10 animals only were left in groups B, C, D due to the death induced by CPF.

1- **First group (A)** = Control, normal, non-diseased mice in a healthy environment and good nutritional status to be compared with the diseased and treated groups.

2- **Second group (B)** = Diseased mice, Chlorpyrifos oral administration (LD_{50} / 80th of 60 mg/ Kg Body Weight/ 3 times per week) for 8 weeks for induction of chronic liver disease (degeneration and fibrosis) according to **Sharma *et al*., 2013**.

3- **Third group (C)** = Treated with combination of oral silymarin (150 mg/ Kilogram body weigth) and colchicine (200 µg/ Kilogram body weight) three times per week for four weeks after induction of liver degeneration and fibrosis with chlorpyrifos.

4- **Fourth group (D)** = Treated with bone marrow derived mesenchymal stem cells (single intra-peritoneal injection of labeled BM-MSC 10^6 cells/ mouse intra-peritoneal) after induction of liver degeneration and fibrosis with chlorpyrifos.

- **Bone Marrow Derived Mesenchymal Stem cells preparation (BM-MSCs):** The femurs and tibias of six weeks old male Swiss albino mice were carefully dissected away from attached soft tissue, the ends of the bones were cut, and the bone marrow was aseptically flushed with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL). Nucleated cells were isolated and re-suspended in complete culture medium supplemented with 1% penicillin–streptomycin (GIBCO/BRL). All cells were incubated at 37 ^{o} C, in an atmosphere of 5% humidified CO_2 for 14 days. When cells grew to 80% confluency, they were harvested with 0.25% trypsin and 1 mmol/L ethylene-diamine-tetra-acetic acid (EDTA) (GIBCO/BRL) for 5 min at 37^{o}C. After centrifugation, cells were re-suspended with serum-supplemented medium and incubated in 50 cm culture flask (Falcon). The resulting cultures were referred to as first-passage cultures (Alhadlaq and Mao, 2004).
- **Labeling of BM-MSCs with PKH26 Fluorescent Dye:** BM-MSCs were labeled with PKH26 Fluorescent Dye (Sigma-Aldrich, Saint Louis, MO) to be detected easily in liver tissue by fluorescent microscope. Cells were first centrifuged, washed twice in serum-free medium and were pelleted and suspended in dye solution. Cells were injected intra-peritoneal into mice group D with 10^6 cells per mouse intra-peritoneal. After four weeks, liver tissues were examined with a fluorescence microscope to detect the cells (Abdel Aziz *et al.,* 2011).

After 4 weeks of treatment, animals were sacrificed

- **Collection of samples:**

1- Blood: were collected from retro-orbital venous plexus in vials containing heparin (10 IU/ml) and centrifuged at 1500 rpm for 15 minutes for plasma separation. Chemical analysis (ALT, AST, Albumin, and Bilirubin) was done by a colorimetric method for all animals groups and results were listed for statistical analysis.

2- Liver organs: At the time of sacrifice, anatomical dissections were done for all animal groups and liver tissue samples were collected and fixed in 10% neutral buffered formaldehyde for 24 hours. To stabilizes tissue proteins and prevent further changes.

- **Histopathological analysis:**

Conventional tissue processing was proceeded. The blocks of wax were sectioned by the microtome into thin sections (5 μ) then stained with Haematoxylin and eosin stain (H&E). Scoring was estimated to every group according to Ishak *et al.*, 1995.

RESULTS

- **Biochemical parameters:** Results and significance of the effect of chlorpyrifos exposure and treatment on liver functions profile of albino mice are shown in Tables (1&2).

Table (1): The relations of ALT, AST, Albumin and Bilirubin of the groups A, B, C and D:

Groups	No.	ALT $\tilde{X}$ ±SD	AST $\tilde{X}$ ±SD	Albumin $\tilde{X}$ ±SD	Bilirubin $\tilde{X}$ ±SD
Group A	15	18.1±1.6	27.1 ±2.4	3.8 ± 0.3	0.7± 0.2
Group B	10	67.7±4.1	100.2±6.1	2.6 ± 0.2	1.9± 0.3
Group C	10	43.2±3.7	64.9 ±5.3	2.9 ± 0.2	1.7± 0.1
Group D	10	36.3±2.5	55.0 ±3.8	3.2 ± 0.1	1.1± 0.1

Group A = Control
Group B = Chlorpyrifos exposure (CPF) only.
Group C = Chlorpyrifos exposure and treated with Antifibrotic drugs (Colchicine and Silymarin)
Group D = Chlorpyrifos exposure and treated with Mesenchymal stem cell (MSC)
S.D = Stander deviation
$\tilde{X}$ = mean
ALT = Alanine aminotransferase enzyme
AST = Aspartate aminotransferase enzyme

Table (2): Multiple Statistical Comparisons between all groups A, B, C and D by ANOVA tests:

Groups	Grouping	P value of ALT	P value of AST	P value of Albumin	P value of Bilirubin
Group A	Group B	0.001*	0.001*	0.001*	0.001*
	Group C	0.001*	0.001*	0.001*	0.001*
	Group D	0.001*	0.001*	0.001*	0.001*
Group B	Group A	0.001*	0.001*	0.001*	0.001*
	Group C	0.001*	0.001*	0.002*	0.086
	Group D	0.001*	0.001*	0.001*	0.001*
Group C	Group A	0.001*	0.001*	0.001*	0.001*
	Group B	0.001*	0.001*	0.002*	0.086
	Group D	0.001*	0.001*	0.002*	0.001*
Group D	Group A	0.001*	0.001*	0.001*	0.001*
	Group B	0.001*	0.001*	0.001*	0.001*
	Group C	0.001*	0.001*	0.002*	0.001*

*Significant

The daily oral administration of chlorpyrifos led to significant elevation in the level of ALT, AST and Bilirubin with decreased albumin level. While in treated group C the enzymes (ALT &AST) were lesser than group B but around triple the Control group, and in MSCs treated group (group D) the enzymes was significant lesser than group B and C. ANOVA tests were significant between all groups and $P < 0.001$ (Table 2).

Albumin in group C was very low to control group, while in MSC treated group it was nearly normal, close to control group. ANOVA tests were significant between all groups and $P < 0.001$(Table 2).

In group C, Bilirubin was significant high to control group, while in MSCs treated group it was lower than group B and C. ANOVA test were significant between all groups and $P < 0.001$. Except, the relations between groups B and C were insignificant (Table 2).

These deduce that, treatment with mesenchymal stem cells therapy improves liver functions tests (ALT, AST, Albumin, and Bilirubin). While, treatment with colchicine and silymarin does not improve total serum bilirubin, but improves ALT, AST and Albumin.

- **Pathological evaluation:** Eight weeks of oral chlorpyrifos (group B) induced liver degeneration and fibrosis. Showed necrosis and vacuolar degeneration (yellow arrow) of zone 3 cells with pyknotic nuclei, fatty changes varies from droplets to diffuse involvement of liver cells with displacement of the nuclei, bile duct proliferation, marked inflammatory activity in portal zone, and appearance of congested blood vessels (black arrow) with moderate fibrosis and loss of hepatic architecture (Figure 2).

While liver sections from control group (group A) revealed normal hepatocytes arranged in parallel plates radiating from the central vein with large bright and vesicular nuclei, no inflammatory cells in the peri-portal areas and normal liver sinusoids (Figure 1).

Treatment with colchicine and silymarin (group C) as a standard therapy did not show any reduction in the fibrosis, and there were ballooning hepatocytes with vacuolar degeneration and pyknotic nuclei (yellow arrow), moderate portal inflammatory activity (black arrow) and loss of architecture (Figure 3).

Treatment with BM-MSCs (group D) was markedly reduced in the extent of portal cellular infiltration (black arrow) and reduced congestion with improving of hepatocytes degeneration and restoration of liver architecture (yellow arrow) (Figure 4).

- **Ishak scoring for these groups in H& E Stain was as follows:**

Group A (HAI 0, F 0) indicated no inflammatory reaction with no fibrosis.

Group B (HAI 15, F 3) indicated sever inflammatory reactions with moderate fibrosis.

Group C (HAI 11, F 3) indicated moderate inflammatory reactions with moderate fibrosis.

Group D (HAI 6, F 1) indicated mild inflammatory reactions with mild fibrosis.

These deduce that, treatment with bone marrow derived mesenchymal stem cells therapy reduce liver fibrosis and improves liver functions tests (ALT, AST, Albumin, and Bilirubin). While, treatment with colchicine and

silymarin do not improve the liver fibrosis, nor improve the total serum bilirubin, but improve ALT, AST and Albumin.

DISSCUSSION

In this study, the regenerative effect of BM-MSCs on hepatic degeneration and fibrosis induced by chronic organophosphorus poisoning (Chlorpyrifos) was investigated and compared its effects with known antifibrotic drugs (colchicine + silymarin).

- **The major findings of our study are:**

1- Intra-peritoneal administration of **BM-MSCs** (10^6 cells/ mouse) recovered liver functions, architectural changes and fibrous tissue degradation by increasing liver regeneration.

2- **Colchicine and Silymarin** recovered liver functions (ALT, AST, and Albumin), but not recovered bilirubin level nor liver fibrosis. This deduce that, colchicine and silymarin had regenerative effects on hepatocytes themselves with decreasing inflammatory reaction in portal tracts with no effect on collagen deposition in liver neither cholestasis.

Biochemical evaluation due to long term exposure to CPF (group B) led to significant elevation of serum liver enzymes (ALT, AST) indicate degeneration of hepatocytes, with significant elevation of serum bilirubin level which indicate cholestatic liver disease due to fibrosis of portal tracts with obstructions of bile flow from chronic toxicity, and significant reduction of serum albumin level due to loss of synthetic function of liver tissue in comparison to control group (A). These results were in consistence with previous reports by Heikal *et al.,* 2013 and Orabi *et al.,* 2013.

Through H&E staining in CPF group, histological evaluation was performed, showed necrosis and vacuolar degeneration with loss of liver architecture and appearance of congested blood vessels with moderate fibrosis around portal tracts. Ishak scoring was (HAI 15, F3) indicated sever inflammatory reactions with moderate fibrosis. These confirm chronic toxicity of CPF effects on liver tissue. This finding agreed with Sharma *et al.,* 2013.

Treating liver degeneration and fibrosis by BM-MSCs was evaluated after four weeks of BM-MSCs transplantation (group D) to make sure homing took place as previously reported (Yu *et al.,* 2012). We detected PKH26-labeled BM-MSCs in the injured liver tissue of recipient mice. Biochemical evaluation led to significant reduction of serum liver enzymes (ALT, AST) indicate regeneration of hepatocytes, with significant reduction of serum bilirubin level which indicate improvement of the cholestatic liver disease (reduction of fibrosis of portal tracts), and significant elevation of serum albumin level due to improvement of synthetic function of liver tissue in comparison to CPF group (B). These results suggest a possible therapeutic role of BM-MSCs in fibrotic liver injury, this consistent with previous studies of severe hepatic and renal damage (Quintanilha *et al.,* 2013; Li *et al.,* 2014). Histological evaluation in this group (D) indicated significant improvement of hepatic fibrosis compared to the untreated CPF group. There was markedly reduction in the extent of portal cellular infiltration and reduced congestion with improving of hepatocytes degeneration and restoration of liver architecture. There was a clear histological variability of severity within fibrosis and inflammatory activities classified by the Ishak scoring system as

HAI 15, F 3 in group B and HAI 6, F 1 in group D. These results were in consistence with previous studies (Gaia *et al.*, 2006; Gordon *et al.*, 2006).

Biochemical evaluation of group C (treated with colchicine and silymarin) resulted in significant reduction of serum liver enzymes (ALT, AST) indicated regeneration of hepatocytes, and significant elevation of serum albumin level due to improvement of synthetic function of liver tissue with insignificant reduction of serum bilirubin level which indicated the presence of the fibrous tissue around portal tracts without degradation in comparison to CPF group (B). This finding was consistent with Láng *et al.*, 1990. But, they did not consistent with Fried *et al.*, 2012. Histopathological evaluation of this group resulted in insignificant reduction in the fibrosis, and there were ballooning hepatocytes with vacuolar degeneration with decreased portal inflammatory activity and loss of architecture. Ishak scoring for this group was (HAI 11, F 3) indicated moderate inflammatory reactions with moderate fibrosis. These results suggest that there was a role of colchicine and silymarin in liver inflammation, but not in liver fibrosis and cholestatic liver disease. These results were in agreement with Muriel *et al.*, 2005. But, they did not consistence with previous report of Morazzoni and Bombardelli, 1995.

CONCLUSION

The use of BM- MSCs as cell therapy improving liver functions and fibrosis in chronic liver disease. But treatment with Colchicine and Silymarin had insignificant antifibrotic effect on liver fibrosis and bilirubin, with

significant improvement of liver enzymes (ALT, AST) and synthesis (Albumin).

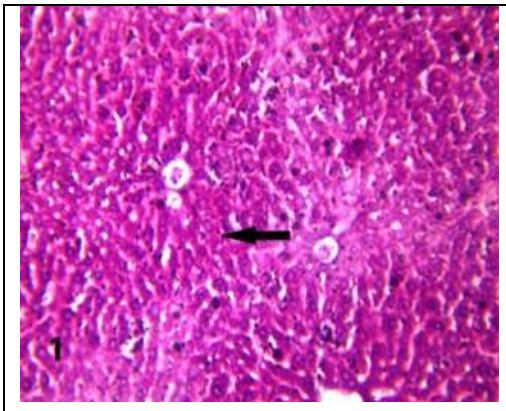

Figure (1): H & E stain of liver tissue Group A showing normal liver architecture (black arrow) with no inflammatory cells nor fibrosis, at X 200

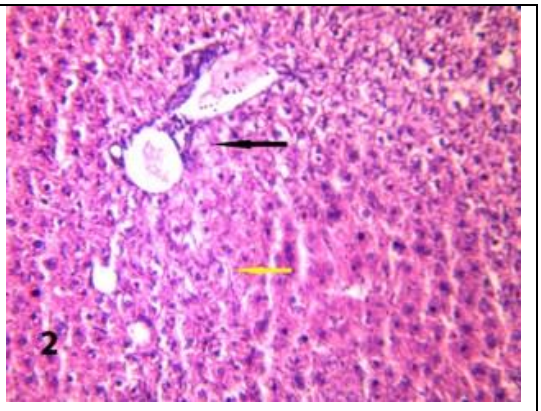

Figure (2): H & E stain of liver tissue Group B showing vacuolar necrosis and degeneration and pyknotic nuclei (yellow arrow), congested blood vessel (black arrow), at X 200

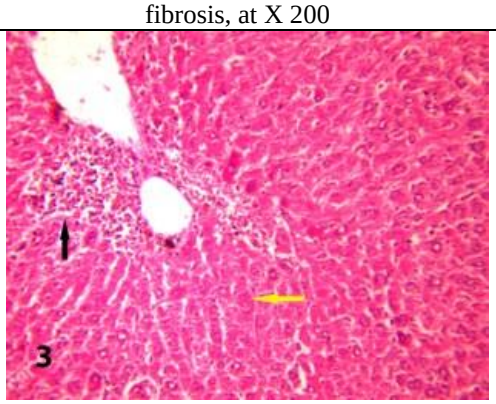

Figure (3): H & E stain of liver tissue Group C showing ballooning hepatocyte with vacuolar degeneration and pyknotic nuclei (yellow arrow), moderate portal inflammatory activity (black arrow), at **X** 200

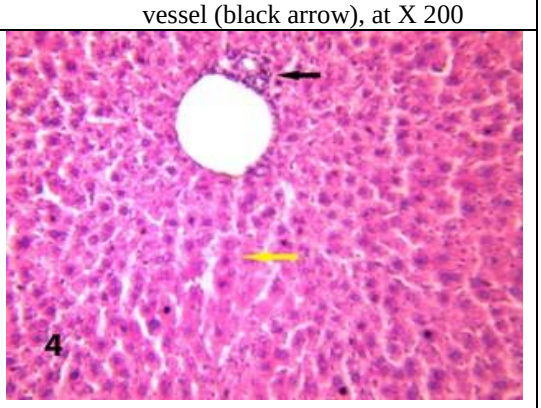

Figure (4): H & E stain of liver tissue Group D showing mild portal cellular infiltration (black arrow), healthy hepatocytes with large bright and vesicular nuclei (yellow arrow), at X 200

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تقييم التأثير المعالج لاستخدام الخلايا الجذعية على التحكم فى التأثير المدمر للمبيدات على الكبد

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المستخلص

مقدمة: الفشل الكبدى مشكلة صحية متزايدة وواحد من الأسباب الرئيسية للوفاة فى العالم. فمن اساليب العلاج الجديدة العلاج بالخلايا الجذعية وهو يعيد انشاء الخلايا المريضة.
الهدف من الدراسة: تقييم فعالية الخلايا الجذعية في علاج الضمور الكبدى الناجم عن الكلوربيريفوس وهو من المبيدات الفسفورية الذى يستخدم على نطاق واسع في الزراعة في مصر، ومقارنة فعاليته مع السيليمارين والكولشيسين المستخدمان في علاج التليف الكبدى.
خطوات العمل: استقراء ضمور وتليف الكبد بالكلوربيريفوس لفئران ألبينو سويسرية في مجموعات مقسمة عشوائياً (ب، ج، د) ومجموعة مراقبة صحية(أ). تعالج مجموعة (ج) بالسيليمارين والكولشيسين ومجموعة (د) تعالج بالحقن البريتوني للخلايا الجذعية. وبعد ٤ أسابيع من العلاج، يتم جمع الدم للتحليل الكيميائي (وظائف الكبد)، والاعضاء للتقييم النسيجي له.
النتائج: لقد تحسنت جميع وظائف الكبد تحسنا كبيرا فى المجموعة (ج ، د)، ما عدا البيليروبين فى المجموعة (ج) لم يتحسن. ولقد تحسن التليف الكبدى فى المجموعة (د) ولم يتحسن فى المجموعة (ج).
الاستنتاج: العلاج بالخلايا الجذعية يقلل التليف الكبدى ويحسن جميع وظائف الكبد لذلك الخلايا الجذعية تصلح لعلاج امراض الكبد المزمنة. بينما العلاج بالكولشيسين والسيليمارين لا يحسن التليف الكبدى، ولا يحسن البيليروبين، ولكن يحسن باقى وظائف الكبد لذلك العلاج بالكولشيسين والسيليمارين لا يصلح لعلاج التليف الكبدى، وانما يصلح لعلاج الالتهاب الطفيف بالكبد.
كلمات دلالية :الكلوربيريفوس، الكولشيسين، السيليمارين، الخلايا الجذعية