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**Mohamed Fathy El-Shafie**
a) Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt Nasr road, in front of Conference Hall, Nasr City, Cairo, Egypt
b) Department of Clinical pharmacy & pharmacy practice, Faculty of Pharmacy and Drug technology, Heliopolis University, Cairo, Egypt. 3 Belbeis – Cairo Desert Road, Cairo, Egypt

**Mohammed Sofian Hammad**
Department of Pharmacology and Toxicology, Faculty of Pharmacy, Modern Sciences and Arts University, Cairo, Egypt. 26 July Mehwar Road Intersection with Wahat Road, 6th of October, Cairo, Egypt

**Mona Mohammed El-Naa**
Associate Professor, Department of Pharmacology & Toxicology, Faculty of Pharmacy, Fayoum University, Egypt, Qesm Al Fayoum, Al Fayoum, Fayoum Governorate, Egypt

**Gouda Kamel Helal**
a) Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt Nasr road, in front of Conference Hall, Nasr City, Cairo, Egypt
b) Department of Clinical pharmacy& pharmacy practice, Faculty of Pharmacy and Drug technology, Heliopolis University, Cairo, Egypt, 3 Belbeis – Cairo Desert Road, Cairo, Egypt

**Correspondence**
**Mohammed Sofian Hammad**
Department of Pharmacology and Toxicology, Faculty of Pharmacy, Modern Sciences and Arts University, Cairo, Egypt. 26 July Mehwar Road Intersection with Wahat Road, 6th of October, Cairo, Egypt

# Claudin-4 induction could improve the homing and anti-fibrotic effect of bone marrow mesenchymal stem cells (BM-MSCs) in thioacetamide induced liver fibrosis in rats

**Mohamed Fathy El-Shafie, Mohammed Sofian Hammad, Mona Mohammed El-Naa and Gouda Kamel Helal**

**Abstract**
Homing and migration of BM-MSCs to a target tissue has been a major concern because, only few cells reach the target tissue and remain there after systemic administration. The current study aimed at investigating the potential hepatoprotective role of claudin-4 (CLDN-4) in improving the homing and anti-fibrotic effect of BM-MSCs. In our study, Sprague-Dawley rats were randomly divided into six groups: Control group; Forskolin (FSK) group; Thioacetamide (TAA) group; FSK+TAA group; TAA+BM-MSCs group; FSK+TAA+ BM-MSCs. Flowcytometry, Western Blot, hematoxylin and eosin (H&E), Masson's trichrome staining, immunohistochemical studies for the detection of α-Smooth muscle actin (α-SMA) and enzyme-linked immunosorbent assay (ELISA) were involved to measure different parameters. Results showed that *in vivo* combination of FSK added to BM-MSCs significantly improved histopathological changes and attenuated the elevated liver enzymes, hydroxyproline content, oxidative stress markers, inflammatory cytokine levels including tumor necrosis factor alpha (TNF-α), tumor growth factor beta (TGF-∝1), interleukin-1 beta (IL-∝1), cyclooxygenase 2 enzyme (COX-2) enzyme, transcription factor nuclear factor kappa B (NF-κB) and α-SMA were also reduced. Moreover, the PKH26 staining showed that FSK added to BM-MSCs resulted in more homing of BM-MSCs to the liver more than those without FSK addition. In conclusion, the present study demonstrated improved homing and anti-fibrotic effect of BM-MSCs, which is thought to be partly mediated by the modulation of CLDN-4.

**Keywords:** Liver fibrosis; thioacetamide; bone marrow mesenchymal stem cells; claudin-4; forskolin

## 1. Introduction
Liver fibrosis is characterized by excessive deposition of extracellular matrix (ECM) proteins as a result of liver injury due to different chronic liver diseases such as alcoholic or non-alcoholic steatohepatitis and viral hepatitis. Liver fibrosis in early phases could be reversible but if left untreated it could progressively lead to advanced fibrosis or cirrhosis and impaired liver function and subsequent morbidity and mortality [1]. Though liver transplantation is the most effective treatment for liver cirrhosis, the lack of suitable donors and the long waiting list makes the clinical application of this technique difficult to perform [2]. TAA has long been used in animal models of liver injury whether acute or chronic, due to its actions on protein synthesis and the modulation of DNA, RNA [3].
Forskolin is a diterpene derived from the roots and stems of the Indian plant Coleus forskohlii [4]. Notably, the natural compound forskolin has been used in traditional medicine for centuries, is considered reasonably priced, and its safety has previously been documented in modern medicine [5, 6]. It has been previously reported that forskolin exerted antioxidant, anti-inflammatory and anti-fibrotic effects [7].
BM-MSCs are a type of multipotent stem cells, which has properties of self- renewal and differentiation into various specialized cell types [8]. It has been also reported that BM-MSCs do have an anti-fibrotic effect through suppressing certain type of T- helper cells, IL-17 and their ability to migrate and differentiate into hepatocytes [9].

For this reason, homing and migration of BM-MSCs to a specific target tissue might not be easy as it seems because it has been shown that after systemic administration which is the most preferred route for BM-MSCs administration, only a few cells reach the target tissue and remain there [10].

There are at least 24 human claudin family members that have been discovered [11]. Claudins play an important role in both formation and function of tight junctions (TJs). These tight junctions consist of cytoplasmic proteins, including zonula occludens (ZO) [12] and other three trans membrane proteins: occludin, junctional adhesion molecules (JAMs) and claudins (CLDNs) [13]. CLDNs are seen in both epithelial and endothelial cells and this makes them distributed all over the body [14]. Also, the expression of claudins can be ubiquitous or cell type-specific [13]. Therefore, in this study, our aim is to investigate the possible hepatoprotective role of CLDN-4 in improving the homing and migratory abilities of BM-MSCs in TAA induced liver fibrosis in rats.

## 2. Methods

### 2.1 Animals

Sixty Sprague-Dawley adult male rats were brought from the animal house of the Nile Company (Cairo, Egypt). The weight of the rats ranges from 150 to 180 gm and were kept in standard conditions including (22°C ± 2°C) room temperature and (55% ± 3%) relative humidity with a 12-h light/dark cycle at the animal house, Faculty of Pharmacy, Al-Azhar University (Cairo, Egypt). The whole rats had free access to food, water ad libitum throughout the experiment. The animal experiments were performed in accordance with the protocol approved by Al-Azhar University Ethics Committee.

### 2.2 Chemicals

Thioacetamide was purchased from Sigma Chemical Company (St. Louis, U.S.A). Forskolin was obtained from Life extension Quality Supplements and Vitamins, Inc. (Ft. Lauderdale, Florida, USA) as capsules containing brownish powder. HPLC analysis was done to determine the average concentration of active ingredient. The capsule was opened to free the powder which is suspended in water. It was given to rats in a dose of 10 mg/kg BW, orally once daily for 4 weeks every day. It has a molecular weight of 410.5 gm/mol and molecular formula of $C_{22}H_{34}O_7$.

### 2.3 Experimental design

A total of 60 male adult Sprague-Dawley rats of 150-180 gm body weight were used. After acclimatization for two weeks, animals were randomly divided into 6 groups of ten rats each and treated for eight consecutive weeks as follows: Group I (Control): Rats were given distilled water (5 ml/kg BW, orally) once daily for 4 weeks and IP with normal saline in a dosing volume of (5 ml/kg BW) three times weekly for 4 consecutive weeks. After 4 weeks, rats were injected with normal saline (Single injection via the tail vein), these animals served as control group. Group II: Ten rats were given FSK (10 mg/ kg BW, orally) from day one for 4 weeks. The dose of FSK used in this experiment was based on the results of our preliminary study (data not shown). Group III: Ten rats were injected with TAA in a dose of (200mg/kg BW, IP), three times weekly for 4 consecutive weeks [15]. This group served as the liver fibrosis model. Group IV: Ten animals were challenged with a combination of FSK and TAA as mentioned before. FSK was administered one hour, ahead of TAA injection. Group V: Ten rats were injected IP with TAA (200 mg/kg BW), three times weekly for 4 consecutive weeks. After 4 weeks, rats were treated with BM-MSCs (Single injection at a concentration of $1\times10^6$ cells/200gm, animals, via the tail vein) [16]. Group VI: It was a replicate of group IV; rats received a combination of TAA and FSK. After 4 weeks animals were treated with BM-MSCs in a dose of ($1\times 10^6$ cells/1ml/200gm, rats, IV), given once. After 8 weeks, retro-orbital blood samples were withdrawn from the orbital plexus under anesthesia (thiopental sodium 30 mg/kg, IP), using heparinized micro-capillaries (Optilab, Berlin, Germany). Serum was separated by blood centrifugation at 4000 rpm for 10 min. at -4 ◦C (Heraeus Biofuge, Loughborough, UK). After terminal bleeding, animals were euthanized by cervical dislocation, and livers were dissected out, washed with normal saline and plotted dry on filter paper for biochemical analysis, histological and immunohistochemical examination.

### 2.4 Isolation and propagation of (BM-MSCs) from rats

The bone marrow of 6 week-old white Sprague-Dawley male rats from the tibiae and femurs was harvested with (DMEM, GIBCO/BRL) with 10% fetal bovine serum (GIBCO/BRL). Isolation of nucleated cells was done with density gradient [Ficoll/ Paque (Pharmacia)] and suspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). As the primary culture or by the formation of large colonies; in 5% humidified CO2 for 12-14 days the cells were incubated at 37°C. When (80-90% confluence) of large colonies is obtained, cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL) for 5 min at 37°C. Again; cells were resuspended with serum supplemented medium and incubated in 50 $cm^2$ culture flasks after centrifugation. First passage cultures are the resulting culture form centrifugation [21]. The adherent colonies of cells were trypsinized and counted on day 14 [17].

### 2.5 Immunophenotypes of BM-MSCs

Injected BM-MSCs were examined by flowcytometry. Cells were negative for the hematopoietic marker (CD34), while strongly positive for mesenchymal stem cell-specific markers including CD105 and CD90.

### 2.6 Homing of labeled BM-MSCs

According to the manufacturer's recommendations; BM-MSCs (undifferentiated) were labeled with PKH26 red dye (Sigma, Saint Louis, Missouri, USA). Method of administration was injecting the cells into rat tail vein. After 4 weeks. Liver sections with undifferentiated BM-MSCs were examined with a fluorescence microscope to detect the number of cells stained with PKH26 [18].

### 2.7 Histopathological Examinations

Paraffin-embedded specimens were cut into sections of 4 to 6 µ thickness and stained with hematoxylin and eosin (H&E) according to the method of Bancroft & Gamble [19]. Changes in histology were evaluated semi quantitatively by a pathologist unaware of the type of treatment.

### 2.8 Measurement of liver fibrosis
Tissue sections embedded in paraffin were stained with Masson's trichrome and microscopically examined. Two different pathologists, blinded to the protocol, examined the stained liver sections [20].

### 2.9 Assessment of liver alpha-smooth muscle actin (α-SMA)
Determination of α-SMA expression on paraffin sections of liver tissue of control, diseased, and different treated groups was detected by immunohistochemical studies using avidin-biotin-peroxidase (DAB, Sigma Chemical Co.) according to the method described by Hsu *et al*., 1981 [21]. Liver sections were treated with a monoclonal antibody for α-SMA (Dako Corp, Carpenteria, CA) and required reagents for the avidin-biotin-peroxidase (Vactastain ABC peroxidase kit, Vector Laboratories) were done by detecting antigen-antibody complex. Expression of α-SMA marker was visualized by the chromagen 3, 3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.).

### 2.10 Assessment of liver function tests
Liver Serum was carefully separated into clean dry Wassermann tubes using a Pasteur pipette and used to determine serum aspartate transaminase (AST), alanine aminotransferase (ALT), and Alkaline phosphatase (ALP) enzymes; using the standard techniques [22, 23].

### 2.11 Biochemical measurements
According to the method described by (Woasner, 1961) [24], hydroxyproline content measured reflects the determination of liver tissue collagen content. Briefly, liver samples were homogenized and hydrolyzed in 6 M HCl at 99°C for 15 min. chloramines T was added to a final concentration of 2.5 mM after decreasing the sample temperature to room temperature. 410 Mm para-dimethyl-amino-benzaldehyde was added to the mixture and incubated for 30 min at 60 °C. Using spectrophotometer at an absorbance of 550 nm, the concentration of hydroxyproline content in each sample was determined. The standard curve was generated from the known quantities of hydroxyproline. Each liver sample was measured in triplicate, and the mean value of hydroxyproline was used for analysis. The results were expressed as micrograms per gram of wet tissue.

### 2.12 Western blot assays
Liver tissues were homogenized using a Tissuelyser II (QIAGEN GmbH, Haan, Germany) provided with a tissue protein extraction reagent (T-PER; Pierce, Rockford, IL, USA). Centrifugation of the lysates was done at 13.000 rpm for 15 min at 4°C, and the supernatants of protein concentrations were measured using protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Electrophoreses of thirty micrograms of each liver protein on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE); then the Electrophoresed part was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). 5% skim milk in Tris-Buffered Saline (TBS) containing 0.1% Tween-20 for 1 hour at room temperature was used to block the membranes. After that; membranes were incubated with primary antibodies at 4 °C overnight. The primary antibodies were as follows: TNF-α (Santa Cruz Biotechnology, Inc., Europe), COX-2 (Cayman Chemical, USA), NF-κB (Santa Cruz Biotechnology, Inc., Europe). Secondary antibodies conjugated-Horseradish peroxidases (HRP) were incubated for 1 hour at room temperature. β-Actin antibody (Abcam) was used to normalize between experiments.

### 2.13 Hepatic Oxidative stress markers
Malondialdehyde (MDA) is a thiobarbituric acid reactive substance measured by the method of (Uchiyama and Mihara, 1978) [25]. MDA is a reflection of hepatic lipid peroxidation. Also, liver content of GSH was determined by a colorimetric method using Ellman reagent and glutathione reductase (Ellman, 1959) [26]. Liver catalase activity (CAT) and liver superoxide dismutase (SOD) were assessed using the methods of (Claiborne, 1985) and (Marklund, 1985) respectively [27, 28].

### 2.14 ElISA for TGF- β1, IL- 1β and CLDN-4
Determination of CLDN-4 was performed using commercial ELISA kit (Cloud-clone Corp, USA) according to the manufacturer's instructions. Also, TGF- β1 and IL- 1β (R&D Systems, Minneapolis, MN, USA) and (Cell Biolabs, San Diego, USA) using sandwich ELISA kits were used according to the manufacturer's instructions.

### 2.15 Statistics
All data are presented as mean ± standard error (SEM) [29]. Group differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey test as a post-ANOVA multiple comparison test on raw data using GraphPad Prism (ISI® software, USA, version 5). The differences were accepted as statistically significant when *the P* value was less than 0.05.

## 3. Results
### 3.1 Immunophenotypes and characterization of BM-MSCs
The Immunophenotypes for CD34, CD 90, and CD105 cells were determined using flowcytometry. CD90 or CD105 (which are positive markers of BM-MSCs) were expressed in more than 98% of the cells. However, BM-MSCs cells were negative for the hematopoietic marker CD34 (which is a negative marker of BM-MSCs) was expressed in less than 1% of the cells (Figure 1).

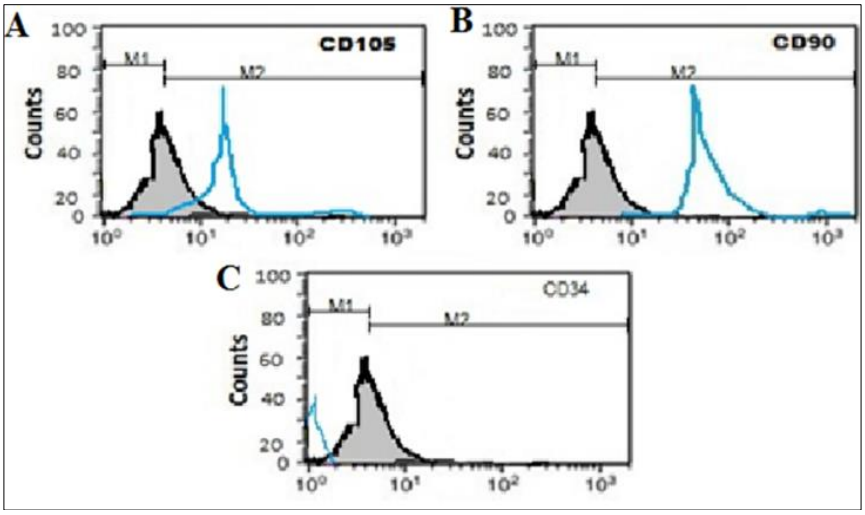



**Fig 1:** Immunophenotype of BM-MSCs cells were examined by flowcytometry. BM-MSCs cells were negative for the hematopoietic marker (CD34) (C), while strongly positive for mesenchymal stem cell specific markers including CD105 and CD90 (A) and (B) respectively. The blue histograms represent antibody labeled cells while the gray histogram shows the profile of the isotype control

### 3.2 Confirmation and assessment of the movement and migratory abilities of BM-MSCs-labeled with PKH26 fluorescent dye to the liver tissues using fluorescent microscope with and without FSK

Fluorescence microscopy for the slides prepared from liver sections after deparaffinization with unstained sections and red fluorescent spots (BM-MSCs labeled with PKH26 fluorescent dye) were displayed in the treated groups, confirming the movement of these cells to the liver tissues (Figure 2A). On the other hand, the administration of FSK to BM-MSCs in TAA injected rats improved homing to the liver tissues, and this can be seen by an increase in the density of PKH26 red dye in the added FSK group compared to BM-MSCs without FSK addition (Figure 2B).

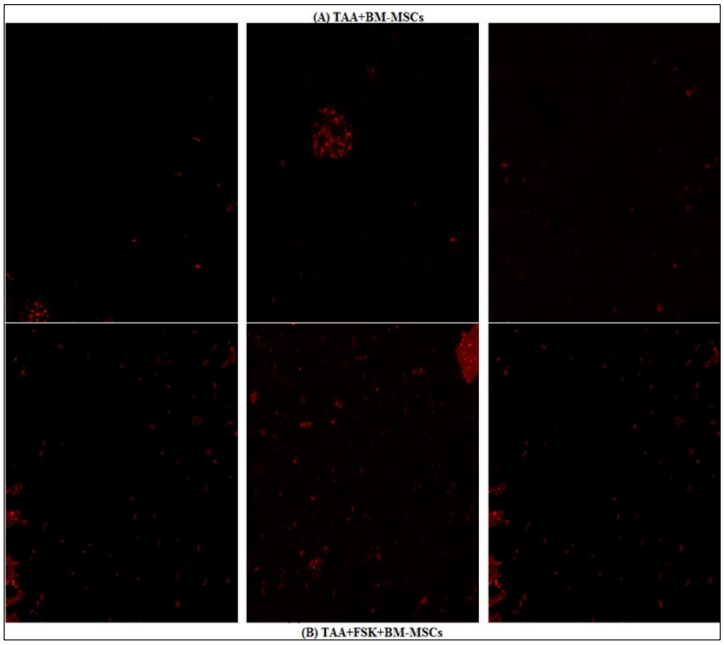



**Fig 2:** Photomicrograph of fluorescent microscopy for the liver sections of BM-MSCs treated rats. (A) Variable sized red fluorescent spots show homing of the cells in the liver tissue confirming the arrival of the labeled stem cells to the target tissue. (B) Variable sized red fluorescent spots show more homing of the BM-MSCs cells to the liver tissue by the addition of FSK. PKH26 red dye, magnifications: (×200)

### 3.3 FSK and/or BM-MSCs ameliorated histopathological deterioration by TAA

As shown in (Figure 3A), liver sections obtained from control group have average portal tract with average portal veins, average central vein and average hepatocytes. The FSK only group have average central vein, portal tracts with average portal veins and average hepatocytes (Figure 3B). In contrast, TAA rats have markedly dilated central vein (CV) with detached epithelial lining (black arrow) and expanded portal tract with dilated portal vein (PV) (Figure 3C). FSK+TAA group have mildly dilated central vein and average portal tract with mildly dilated portal vein (black arrow) (Figure 3D). TAA+BM-MSCs group have dilated central vein, hepatocytes with mild steatosis (black arrow) (Figure 3E). Combination of FSK+TAA+BM-MSCs show average portal tract with mildly dilated portal veins (black arrow) and average central vein (blue arrow) and average hepatocyte (red arrow) (Figure 3F).

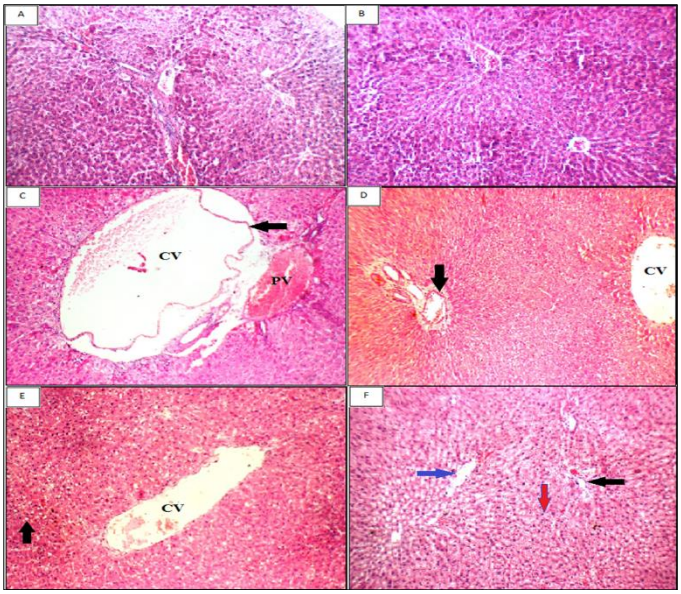



**Fig 3:** Histology of liver samples of control, FSK treated group, TAA-injected rats, FSK+TAA, TAA+BM-MSCs and combination of FSK+TAA+BM-MSCs. (A) Control group: average central vein, average portal tracts and average hepatocytes.; (B) FSK only group: average central vein and average hepatocytes. (C) TAA-injected group: markedly dialated CV with detached epithelial lining and expanded portal tract with dilated portal vein.; (D) FSK+TAA group: mildly dilated central vein and average portal tract with mildly dilated portal vein.; (E) TAA+BM-MSCs treated group: dilated central vein with scattered apoptotic hepatocytes and hepatocytes with steatosis. (F) FSK+TAA+BM-MSCs group: preserved liver architecture with average hepatocytes. H&E staining, magnifications: (×200).

### 3.4 FSK and/or BM-MSCs mitigated TAA induced hepatic fibrogenesis

Normal liver tissues have minimal collagen deposition Figure (4A). FSK only treated rats have virtually the same tissue architecture and collagen profile as in control animals Figure (4B). In contrast, TAA sections for 4 weeks showed thick fibrous band extending from central vein to another central vein (black arrow) Figure (4C). FSK+TAA group showed thin fibrosis band and decrease in collagen deposition Figure (4D). TAA+BM-MSCs group showed a thin fibrous band in areas examined Figure (4E). Combination of FSK+TAA+BM-MSCs showed a preserved liver architecture in the examined fields and average collagen distribution in portal tract and in peri-portal area Figure (4F).

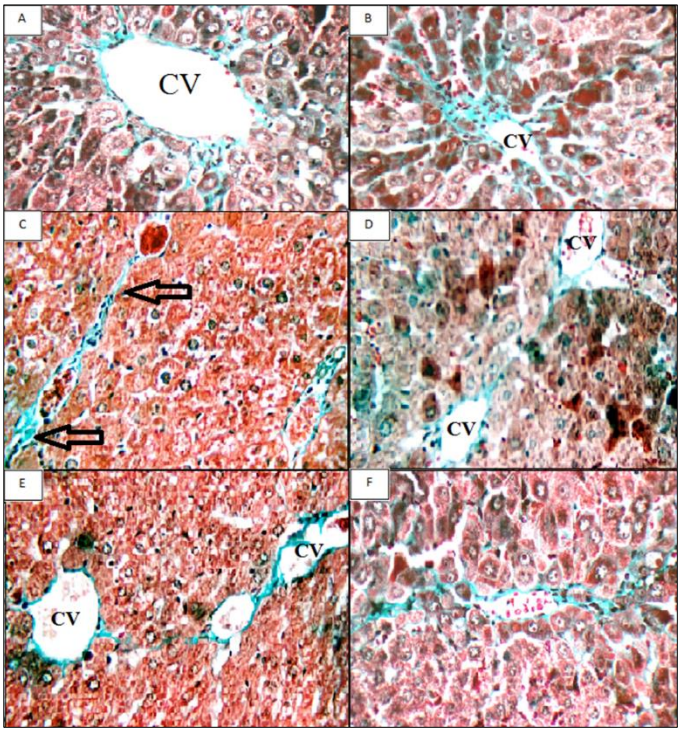



**Fig 4:** Masson's Trichrome stain of liver samples of control, FSK treated group, TAA-injected rats, FSK+TAA, TAA+BM-MSCs and combination of FSK+TAA+BM-MSCs. (A) Control group: normal liver tissues with minimal collagen deposition.; (B) FSK only group: virtually the same tissue architecture and collagen profile as in control animals. (C) TAA-injected group: thick fibrosis detected along all examined fields especially from central vein to another one.; (D) FSK+TAA group: showed mild fibrosis and decrease in collagen deposition.; (E) TAA+BM-MSCs treated group: thin fibrous tissues from central vein to another. (F) FSK+TAA+BM-MSCs group: preserved liver architecture with normal collagen deposition. Masson-Trichrome staining, magnifications: (×400)

### 3.5 FSK and/or BM-MSCs alleviated TAA induced liver expression of α-SMA

Microscopic examination of various sections of the liver of control rats showed faint normal expression of α-SMA around the portal vein in the portal areas and around the central vein (Figure 5A). Also, livers of FSK administrated rats showed normal faint positive expression of α-SMA around the central veins and around the portal vein in the portal areas (Figure 5B). In contrast, examination of livers injected with TAA showed strong immunopositivity of α-SMA in the portal areas and along the portal to portal fibrous bands (arrow) as well as in scattered hepatocytes and spindle shape activated stellate cells (Figure 5C). Livers of TAA administrated rats treated with FSK showed marked retraction of hepatic fibrosis and decreased immunopositivity of α-SMA and its limitation to the portal areas (arrow) and in scattered activated cells among the parenchyma (Figure 5D). TAA+BM-MSCs sections showed marked retraction of fibrosis with scattered α-SMA positive cells in between the hepatic cells (arrow) and in few hepatocytes (Figure 5E). The combined use of BM-MSCs and FSK in TAA rats showed few scattered α-SMA reactive cells (Figure 5F).

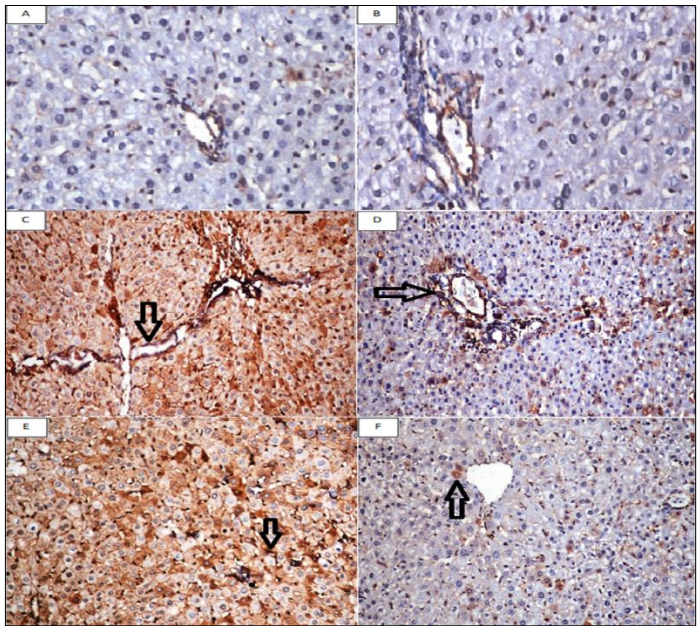
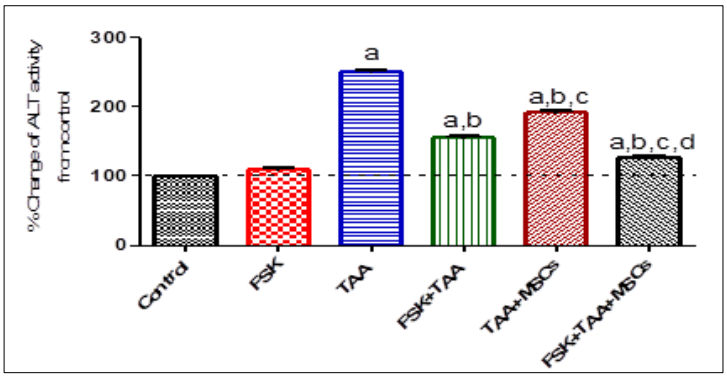



**Fig 5:** Immunohistochemistry of α-SMA of control, FSK only group, TAA-injected rats, FSK+TAA, TAA+BM-MSCs, and combination of FSK+TAA+BM-MSCs. (A) Control group: faint normal expression of α-SMA.; (B) FSK group: normal faint positive expression of α-SMA.; (C) TAA group: strong positive expression of α-SMA in the portal area along the proliferated fibrous tissue, along the extended septa.; (D) FSK+TAA group: marked retraction of hepatic fibrosis and decreased immunopositivity of α-SMA and its limitation to the portal areas.; (E) TAA+BM-MSCs group: marked retraction of fibrosis with scattered α-SMA positive cells in between the hepatic cells and in few hepatocytes.; (F) FSK+TAA+BM-MSCs group: absence of fibrous bands and apparently few α-SMA immnuopositive cells scattered along the parenchymal cells. Avidin-biotin peroxidase staining, magnifications: (×200).

### 3.6 FSK and/or BM-MSCs amended TAA-induced hepatic damage

No significant changes were observed in serum ALT, AST and ALP in the FSK only treated group when compared with the values from the control group (Figure 6). The administration of TAA significantly increased serum levels of ALT, AST, and ALP activity compared to the normal control rats. The FSK+TAA and TAA+BM-MSCs resulted in a significant decrease in ALT, AST and ALP activity when compared to the TAA-injected rats. The addition of FSK to BM-MSCs resulted in a significant decrease in ALT, AST and ALP compared to TAA-injected rats (Figure 6).



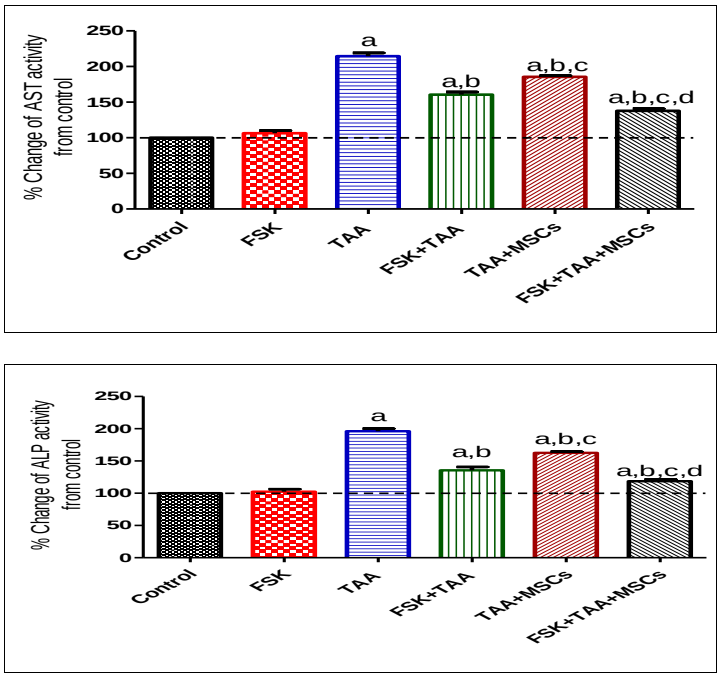



**Fig 6:** Effect of FSK and/or BM-MSCs on serum liver enzymes ALT, AST and ALP. Data are presented as mean ± standard error (SEM) of 10 animals/group; a, b, c and d, indicates significant difference from control, TAA, FSK+TAA, and TAA+BM-MSCs, respectively, at P ≤ .05 using Tukey test as post ANOVA test. ANOVA indicates analysis of variance

### 3.7 FSK and/or BM-MSCs decreased liver hydroxyproline content in TAA liver injury

Administration of FSK produced a non-significant increase in liver hydroxyproline content compared to normal control rats. Treatment with TAA caused a substantial increase in liver hydroxyproline content amounted to about 566% compared to control level (Figure 7). The FSK+TAA and TAA+BM-MSCs resulted in a significant decrease in liver hydroxyproline content compared to TAA-injected rats. The combination group significantly decreased the levels of hydroxyproline content compared to TAA-injected rats (Figure 7).

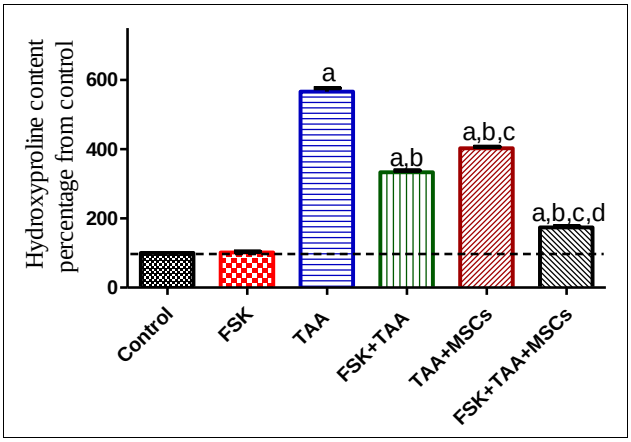



**Fig 7:** Effect of FSK and/or BM-MSCs on liver hydroxyproline content. Data are presented as mean ± standard error (SEM) of 10 animals/group; a, b, c and d, indicates significant difference from control, TAA, FSK+TAA, and TAA+BM-MSCs, respectively, at P ≤ .05 using Tukey test as post ANOVA test

### 3.8 FSK and/or BM-MSCs attenuated liver TAA-induced oxidative stress

GSH levels, liver CAT activity and liver SOD activity were markedly decreased while levels of liver TBARs were significantly increased in the TAA group, compared with normal control (Figure 8, 9). Surprisingly, FSK and or BM-MSCs significantly elevated GSH levels, liver CAT activity and liver SOD activity and also markedly decreased liver TBARs thus protecting against TAA induced oxidative hepatic damage. Besides, rats given FSK only did not show any significant change in levels of GSH, liver CAT activity, liver SOD activity and liver TBARs levels when compared with control values (Figure 8, 9).

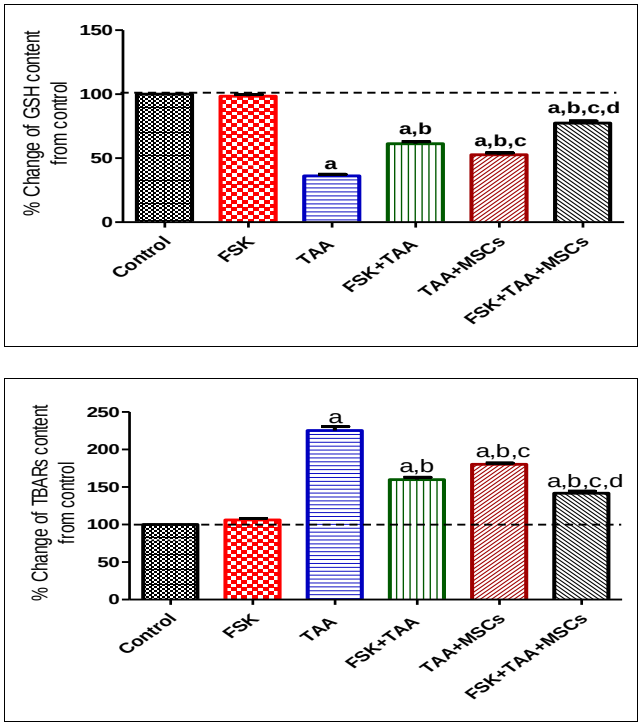
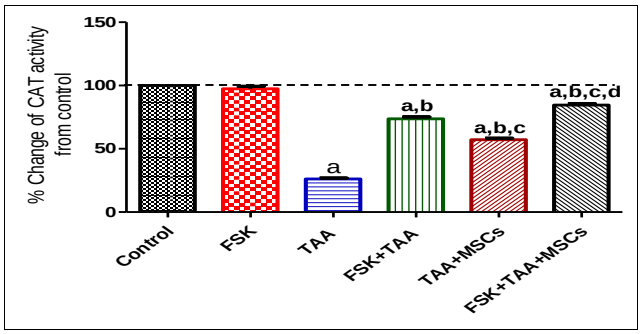



**Fig 8:** Effect of FSK and/or BM-MSCs on hepatic GSH and TBARs contents of TAA-injected rats. Data are presented as mean ± standard error (SEM) of 10 animals/group; a, b, c and d, indicates significant difference from control, TAA, FSK+TAA, and TAA+BM-MSCs, respectively, at P ≤ .05 using Tukey test as post ANOVA test.



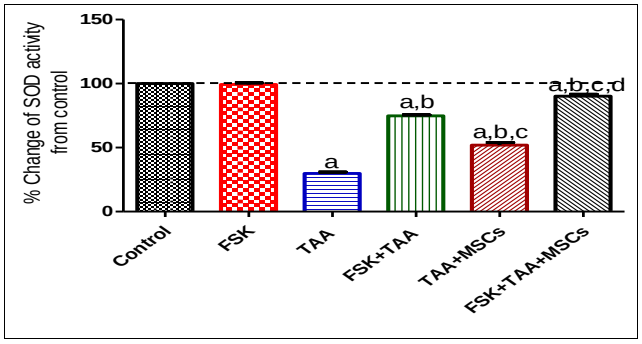
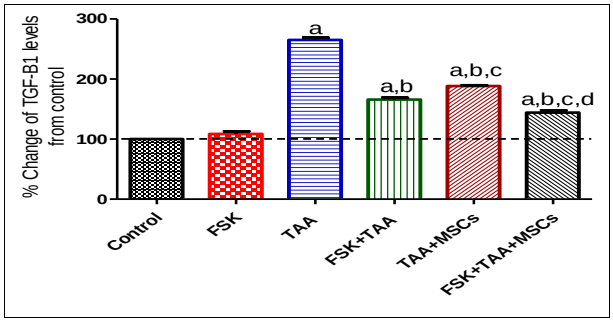




**Fig 9:** Effect of FSK and/or BM-MSCs on hepatic CAT and SOD activities of TAA-injected rats. Data are presented as mean ± standard error (SEM) of 10 animals/group; a, b, c and d, indicates significant difference from control, TAA, FSK+TAA, and TAA+BM-MSCs, respectively, at P ≤ .05 using Tukey test as post ANOVA test.

### 3.9 FSK and/or BM-MSCs attenuated TGF-□1 and IL-1□ levels in TAA-injected liver rats

Rats given FSK only had a non-significant change in liver TGF-□1 and IL-□1 when compared to normal control rats (Figure 10). Administration of TAA caused a substantial increase in liver content of TGF-□1 and IL-□1 compared to control values. The FSK+TAA and TAA+BM-MSCs resulted in a significant decrease in liver TGF-□1 and IL-□1 in comparison with the TAA-injected rats. The combination group significantly decreased liver TGF-□1 and IL-□1 compared to TAA-intoxicated rats (Figure 10).



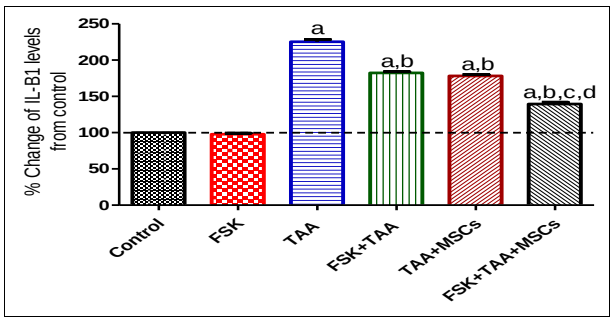




**Fig 10:** Effect of FSK and/or BM-MSCs on liver TGF-□1 and IL-□1. Data are presented as mean ± standard error (SEM) of 10 animals/group; a, b, c and d, indicates significant difference from control, TAA, FSK+TAA, and TAA+BM-MSCs, respectively, at P ≤ .05 using Tukey test as post ANOVA test

### 3.10 FSK and/or BM-MSCs alleviated the levels of liver inflammatory markers in TAA-injected liver rats

No significant changes were observed in liver content of NF-κB, COX-2 and TNF-α in the FSK only treated group when compared with the values from the control group (Figure 11). The administration of TAA significantly increased hepatic content of NF-κB, COX-2 and TNF-α when compared with normal control rats. The FSK+TAA and TAA+BM-MSCs resulted in a significant decrease in liver content of NF-κB, COX-2 and TNF-α compared with TAA-injected rats. The combination group resulted in a significant decrease in liver content of NF-κB, COX-2 and TNF-α compared to TAA-intoxicated rats (Figure 11).

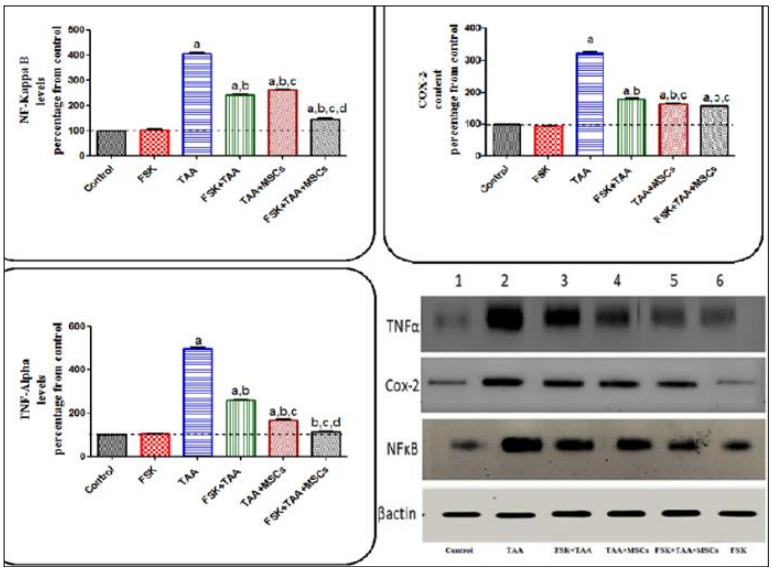



**Fig 11:** Effect of FSK and/or BM-MSCs on hepatic inflammatory mediators NF-κB, COX-2 and TNF-α. Data are presented as mean ± standard error (SEM) of 10 animals/group; a, b, c and d, indicates significant difference from control, TAA, FSK+TAA, and TAA+BM-MSCs, respectively, at P ≤ .05 using Tukey test as post ANOVA test. Scanning densitometer quantitative Western Blot of TNF-α, COX-2 and NF-κB versus β-actin in the following studied groups: 1-Control, 2-TAA, 3-FSK+TAA, 4-TAA+BM-MSCs, 5-FSK+TAA+BM-MSCs and 6-FSK only group

### 3.11 FSK and/or BM-MSCs restored the levels of liver CLDN-4 in TAA-injected liver rats

The FSK only group non-significantly decreased liver CLDN-4 content in comparison to the control rats. TAA group significantly reduced the liver CLDN-4 content when compared to the control animals (Figure 12). The FSK+TAA and TAA+BM-MSCs resulted in a significant increase in liver CLDN-4 content in comparison to the TAA-intoxicated rats. The combination group significantly increased the levels of CLDN-4 content when compared to TAA-injected rats (Figure 12).

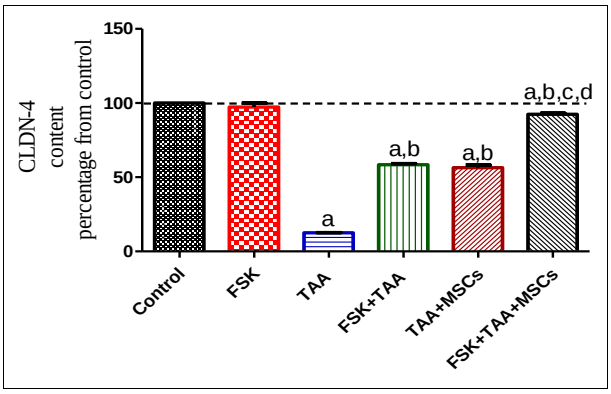



**Fig 12:** Effect of FSK and/or BM-MSCs on liver CLDN-4 levels. Data are presented as mean ± standard error (SEM) of 10 animals/group; a, b, c and d, indicates significant difference from control, TAA, FSK+TAA, and TAA+BM-MSCs, respectively, at P ≤ .05 using Tukey test as post ANOVA test

## 4. Discussion

Liver fibrosis is the abnormal accumulation of extracellular matrix proteins, such as collagen, that occurs in the majority of chronic liver diseases. Cirrhosis, liver failure, and portal hypertension are all complications of advanced liver fibrosis, which frequently necessitates liver transplantation which is the most effective treatment option especially in liver cirrhosis. Several recent studies have focused on finding newer strategies for the treatment of liver fibrosis [30, 31]. MSCs have been found to be a promising treatment option for end-stage liver disease in a variety of animal models due to their ability to differentiate into hepatocytes and move to injured sites [32]. MSCs can be administered systemically [intravenous (IV) or intra-arterial (IA) injection] or locally [intracoronary (IC) injection or direct injection into the tissue of interest] when used in clinical applications. The most commonly used route is intravenous injection because it is the least invasive, infusions can be easily repeated, and cells will stay close to the oxygen and nutrient-rich vasculature after migration into the targeted site. However, after intravenous injection, cells were found to be trapped in the lungs, which may impair efficient homing to the targeted tissue. Thus, recent studies are directed into finding new strategies for better homing of MSCs [33]. CLDNs function as paracelluar tight junction channels (PTJC), which have many of the same biophysical properties as traditional ion channels, such as ion charge selectivity, permeability dependence on ion concentration, and competing for permeative molecule movement. It has been discovered that CLDNs interact with each other to establish channels that allow cations and anions to pass through [34]. Disruption of tight junctions barrier including CLDNS and ZO have been implicated in several diseases such as liver fibrosis and the restoration of these tight junctions could possibly ameliorate the advancement of liver fibrosis [35]. Forskolin, a natural product traditionally used in ancient medicine as well as in modern medicine. It has been shown to have antioxidant, anti-inflammatory and antifibrotic actions [7]. Thus, the aim of this study is to investigate the possible role of CLDN-4 modulation as an effective strategy to improve homing and migratory abilities of BM-MSCs and also to investigate the potential antifibrotic effect of FSK and or BM-MSCs in ameliorating TAA-induced liver injury in rats.

TAA has been widely used in experimental models of liver fibrosis in rats. TAA is broken down by flavin-containing monooxygenase (FMOs) systems and cytochrome P450 (Cytp 450). TAA is converted to thioacetamide S-oxide (TAASO), a toxic reactive intermediate; the oxidation process causes oxidative stress in hepatic cells, which eventually leads to centrilobular necrosis and liver injury [36]. In our experimental model, TAA did cause hepatic injury evidenced by the elevation in serum ALT, AST and ALP. FSK+TAA and TAA+BM-MSCs significantly attenuated the elevated liver enzymes, indicating improved functional status of hepatocytes, shown by the H&E staining. Surprisingly, the combination group of FSK+TAA+BM-MSCs resulted in a significant decrease in serum liver enzymes as well as improvement in histopathology and preserved liver architecture more than FSK+TAA and TAA+BM-MSCs (Figure 3, 6).

Liver fibrosis of any underlying cause is characterized by an abnormal accumulation of ECM components, particularly collagen. TAA injection resulted in liver fibrosis and collagen deposition, as demonstrated by Masson's trichrome staining of collagen fibers and increased liver hydroxyproline content, a marker of collagen deposition. The current study shows that FSK and or BM-MSCs has effective antifibrotic activity and inhibits TAA induced fibrogenesis. This was supported by a significant decrease in liver hydroxyproline levels (Figure 7), which accompanied with a decrease in collagen deposition as measured by Masson's trichrome stain (Figure 4). The combination treatment had the most decrement in the collagen content. Moreover, it is worth to mention that the PKH26 dye under fluorescence microscope showed that labeled BM-MSCs reached the liver successfully; which might explain the recovery of liver cells from fibrosis by the potential differentiation into mature hepatocytes by the delivered MSCs (Figure 2A). In trying to further support the idea that FSK addition to BM-MSCs is an effective combination, the PHK26 red dye showed that FSK addition to BM-MSCs resulted in enhanced homing of BM-MSCs and possibly better anti-fibrotic effect (Figure 2B), and this is supported by the evidence that the combination resulted in a decrease in serum liver markers, fibrosis markers and improved histopathological examination.

The main pathophysiological event in liver fibrosis is the activation of Hepatic stellate cells (HSCs) and their trans differentiation to myofibroblasts. Activated HSCs are the primary fibrogenic cells in injured liver and are distinguished by increased proliferation, ECM overproduction, and de novo expression of α-SMA, an indicator for activated HSCs [37]. As a result, antifibrotic strategies that target activated HSCs are being developed as a proper risk mitigation and treatment of liver fibrosis. The significant reduction in α-SMA expression confirmed that FSK and or BM-MSCs have antifibrotic effect, evidenced

by inhibiting HSC activation and the subsequent fibrogenic process. Also, the combination group was the most effective in decreasing the expression of α-SMA than when compared to other treatment groups (Figure 5).

The toxicity of TAA contributes to oxidative stress by producing free radicals. This was supported by the discovery that TAA administration substantially increased lipid peroxides, as evidenced by increased TBARs levels in our model, as well as a significant decrease in liver GSH levels, CAT activity and SOD activity [38]. In line with previous research, our findings show that FSK and or BM-MSCs must have potent antioxidant activity. As evidenced by decreased levels of TBARs, and increased levels of GSH levels, CAT activity and SOD activity (Figure 8, 9).

TAA-induced liver injury increases the expression of proinflammatory cytokines such as TNF- α, TGF-□1 and IL- 1β, as well as transcription factors like NF-κB and proinflammatory enzymes like COX-2 [39, 40]. In our study, the FSK+TAA and TAA+BM-MSCs significantly reduced the expression of NF-κB, COX-2 and TNF-α when compared to TAA only group (Figure 11). In addition the FSK+TAA and TAA+BM-MSCs also markedly lowered the expression of TGF-□1 and IL- 1β in comparison to TAA-only group (Figure 10). The combination of FSK+TAA+BM-MSCs significantly lowered the expression of the previous inflammatory mediators compared to the TAA-intoxicated rats. CLDNs are a large family of integral membrane proteins that are required for the formation of tight junctions. CLDN-4, in particular, has been shown to protect against lung injury and liver injury [41, 42]. Tight junctions and adherent junctions are critical componen ts of intestinal epithelial barrier integrity, physically and fun ctionally protecting the body from bacteria and toxic substan ces in the intestinal lumen.

Disruption of these barriers is commonly linked to a variety of diseases. Increased data have recently confirmed the dysf unction of the intestinal epithelial barrier not only in various cirrhotic animal models, but also in patients with chronic li ver diseases and cirrhosis [43, 44]. Tight junction molecules (occludin, claudin-4, and ZO-1) or adherens junction molecule (E-cadherin) were greatly decreased in the cirrhotic rats [42]. Our experiment is in accordance with the previous research, in that TAA significantly reduced the levels of CLDN-4 when compared to the normal control values (Figure 12). The FSK+TAA and TAA+BM-MSCs significantly elevated the levels of CLDN-4 in comparison with TAA only group. Previous research has shown that injecting BM-MSCs resulted in restoration of CLDN-4 in lung injury [45]. Also, the restoration of the tight junctions (occludin, CLDN-4, and ZO-1) by celecoxib greatly alleviated liver injury in rats. Our results support the previous results; BM-MSCs has significantly elevated the levels of CLDN-4 compared with TAA injected rats, also FSK administration in TAA rats resulted in a significant elevation in CLDN-4 levels. Surprisingly, the combination group has significantly restored the levels of CLDN-4 compared to other treatment groups (Figure 12).

## 5. Conclusion

Claudin-4 modulation could improve the homing and migratory abilities and also the antifibrotic effects of BM-MSCs in TAA liver injury in rats. Also, to investigate the potential antifibrotic effect of FSK and or BM-MSCs in ameliorating TAA-induced liver injury in rats.

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## 7. References

1. Ebrahimi H, Naderian M, Sohrabpour A. New Concepts on Pathogenesis and Diagnosis of Liver Fibrosis; A Review Article. Middle East Journal of Digestive Diseases. 2016;8(3):166-178. DOI: 10.15171/mejdd.2016.29
2. Neuberger J. An update on liver transplantation: A critical review. Journal of Autoimmunity. 2016;66:51-59. DOI: 10.1016/j.jaut.2015.08.021
3. Akhtar T, Sheikh N. An overview of thioacetamide-induced hepatotoxicity. Toxin Reviews. 2013;32(3):43-46. DOI: 10.3109/15569543.2013.805144.
4. Kanne H, Prasanna V, Burte N, Gujjula R. Extraction and elemental analysis of Coleus forskohlii extract. Pharmacognosy Research. 2015;7(3):237. DOI: 10.4103/0974-8490.157966
5. Ammon H, Müller A. Forskolin: From an Ayurvedic Remedy to a Modern Agent. Planta Medica. 1985;51(06):473-477. DOI: 10.1055/s-2007-969566
6. Godard M, Johnson B, Richmond S. Body Composition and Hormonal Adaptations Associated with Forskolin Consumption in Overweight and Obese Men. Obesity Research. 2005;13(8):1335-1343. DOI: 10.1038/oby.2005.162
7. El-Agroudy N, El-Naga R, El-Razeq R, El-Demerdash E. Forskolin, a hedgehog signalling inhibitor, attenuates carbon tetrachloride-induced liver fibrosis in rats. British Journal of Pharmacology. 2016;173(22):3248-3260. DOI: 10.1111/bph.13611
8. Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, *et al*. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science. 1999;284(5411):143-147. DOI: 10.1126/science.284.5411.143
9. Milosavljevic N, Gazdic M, Simovic Markovic B, Arsenijevic A, Nurkovic J, Dolicanin Z, *et al*. Mesenchymal stem cells attenuate liver fibrosis by suppressing Th17 cells - an experimental study. Transplant International. 2017;31(1):102-115. DOI: 10.1111/tri.13023
10. Sudulaguntla A. Stem Cells: Cultivation and Routes of Administration. Current Trends In Biomedical Engineering & Amp; Biosciences, 2017, 2(1). DOI: 10.19080/ctbeb.2017.02.555579
11. Krause G, Winkler L, Mueller S, Haseloff R, Piontek J, Blasig I. Structure and function of claudins. *Biochimica Et Biophysica* Acta (BBA) – Biomembranes. 2008;1778(3):631-645. https://doi.org/10.1016/j.bbamem.2007.10.018
12. Mitic L, Anderson J. Molecular architecture of tight junctions. Annual Review of Physiology. 1998;60(1):121-142. https://doi.org/10.1146/annurev.physiol.60.1.121

13. Ono Y, Hiratsuka Y, Murata M, Takasawa A, Fukuda R, Nojima M, *et al*. Claudins-4 and -7 might be valuable markers to distinguish hepatocellular carcinoma from cholangiocarcinoma. Virchows Archiv. 2016;469(4):417-426. https://doi.org/10.1007/s00428-016-1984-z
14. Van Itallie C, Anderson J. Claudins and epithelial paracellular transport. Annual Review of Physiology. 2006;68(1):403-429. https://doi.org/10.1146/annurev.physiol.68.040104.131404
15. Said M. Hepatoprotective Effect of Eplerenone, A Selective Mineralocorticoid Receptor Antagonist, Against Thioacetamide Induced Liver Injury in Rats. American Journal of Biomedical Sciences. 2016, 114-122. https://doi.org/10.5099/aj160200114
16. He X, He X, Lian L, Wu X, Lan P. Systemic Infusion of Bone Marrow-Derived Mesenchymal Stem Cells for Treatment of Experimental Colitis in Mice. Digestive Diseases and Sciences. 2012;57(12):3136-3144. https://doi.org/10.1007/s10620-012-2290-5
17. Alhadlaq A, Mao J. Mesenchymal Stem Cells: Isolation and Therapeutics. Stem Cells and Development. 2004;13(4):436-448. https://doi.org/10.1089/scd.2004.13.436
18. Ude C, Shamsul B, Ng M, Chen H, Norhamdan M, Aminuddin B, *et al*. Bone marrow and adipose stem cells can be tracked with PKH26 until post staining passage 6 in *in vitro* and *in vivo*. Tissue and cell. 2012;44(3):156-163. https://doi.org/10.1016/j.tice.2012.02.001
19. Bancroft JD, Gamble M. Theory and practice of histological techniques, Elsevier health sciences, 2008, 725.
20. Masson P. Some histological methods. Trichrome stainings and their preliminary technique. J Tech. Meth. 1929;12:75-90. Retrieved 12 December 2021, from.
21. Hsu S, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. Journal of Histochemistry & Amp; Cytochemistry. 1981;29(4):577-580. https://doi.org/10.1177/29.4.6166661
22. Reitman S, Frankel S. A Colorimetric Method for the Determination of Serum Glutamic Oxalacetic and Glutamic Pyruvic Transaminases. American Journal of Clinical Pathology. 1957;28(1):56-63. https://doi.org/10.1093/ajcp/28.1.56
23. Belfield A, Goldberg D. Revised Assay for Serum Phenyl Phosphatase Activity Using 4- Amino - Antipyrine. Enzyme. 1971;12(5):561-573. DOI: 10.1159/000459586
24. Woessner J. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of Biochemistry and Biophysics. 1961;93(2):440-447. https://doi.org/10.1016/0003-9861(61)90291-0
25. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical Biochemistry. 1978;86(1):271-278. https://doi.org/10.1016/0003-2697(78)90342-1
26. Ellman G. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics. 1959;82(1):70-77. https://doi.org/10.1016/0003-9861(59)90090-6
27. Claiborne A. Catalase activity. In: Greenwald, R.A. (ed.) Handbook of methods of oxygen radical research. CRC Press, Boca Raton, Florida, 1985, 283-284.
28. Marklund S. Product of extracellular-superoxide dismutase catalysis. FEBS Letters. 1985;184(2):237-239. https://doi.org/10.1016/0014-5793(85)80613-x
29. Calinski T, Steel R, Torrie J. Principles and Procedures of Statistics: A Biometrical Approach. Biometrics. 1981;37(4):859. https://doi.org/10.2307/2530180
30. Bataller R, Brenner D. Liver fibrosis. Journal of Clinical Investigation. 2005;115(2):209-218. https://doi.org/10.1172/jci24282
31. Koyama Y, Xu J, Liu X, Brenner D. New Developments on the Treatment of Liver Fibrosis. Digestive Diseases. 2016;34(5):589-596. https://doi.org/10.1159/000445269
32. Zhou W. Pathogenesis of liver cirrhosis. World Journal of Gastroenterology. 2014;20(23):7312. https://doi.org/10.3748/wjg.v20.i23.7312
33. Becker A, Riet I. Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy? World Journal of Stem Cells. 2016;8(3):73. https://doi.org/10.4252/wjsc.v8.i3.73
34. Tang V, Goodenough D. Paracellular Ion Channel at the Tight Junction. Biophysical Journal. 2003;84(3):1660-1673. https://doi.org/10.1016/s0006-3495(03)74975-3
35. Gao J, Wen S, Tong H, Wang C, Yang W, Tang S, *et al*. Inhibition of cyclooxygenase-2 alleviates liver cirrhosis via improvement of the dysfunctional gut-liver axis in rats. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2016;310(11):G962-G972. https://doi.org/10.1152/ajpgi.00428.2015
36. Wallace M, Hamesch K, Lunova M, Kim Y, Weiskirchen R, Strnad P, *et al*. Standard Operating Procedures in Experimental Liver Research: Thioacetamide model in mice and rats. Laboratory Animals. 2015;49(1):21-29. https://doi.org/10.1177/0023677215573040
37. Friedman S. Molecular Regulation of Hepatic Fibrosis, an Integrated Cellular Response to Tissue Injury. Journal of Biological Chemistry. 2000;275(4):2247-2250. https://doi.org/10.1074/jbc.275.4.2247
38. Shaker M, Salem H, Shiha G, Ibrahim T. Nilotinib counteracts thioacetamide-induced hepatic oxidative stress and attenuates liver fibrosis progression. Fundamental & Amp; Clinical Pharmacology. 2011;25(2):248-257. https://doi.org/10.1111/j.1472-8206.2010.00824.x
39. Akbay A, Ĉinar K, Uzunalimoglu Ö, Eranil S, Yurdaydin C, Bozkaya H, *et al*. Serum cytotoxin and oxidant stress markers in N-acetylcysteine treated thioacetamide hepatotoxicity of rats. Human &Amp; Experimental Toxicology. 1999;18(11):669-676. https://doi.org/10.1191/096032799678839518
40. Verbeke L, Mannaerts I, Schierwagen R, Govaere O, Klein S, Vander Elst I, *et al*. FXR agonist obeticholic acid reduces hepatic inflammation and fibrosis in a rat model of toxic cirrhosis. Scientific Reports, 2016, 6(1). https://doi.org/10.1038/srep33453.

41. Mitchell L, Overgaard C, Ward C, Margulies S, Koval M. Differential effects of claudin-3 and claudin-4 on alveolar epithelial barrier function. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2011;301(1):L40-L49. https://doi.org/10.1152/ajplung.00299.2010
42. Gao J, Wen S, Tong H, Wang C, Yang W, Tang S, *et al*. Inhibition of cyclooxygenase-2 alleviates liver cirrhosis via improvement of the dysfunctional gut-liver axis in rats. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2016;310(11):G962-G972. https://doi.org/10.1152/ajpgi.00428.2015
43. Goode E, Warburton R, Gelson W, Watson A. Activated Intestinal Macrophages in Patients with Cirrhosis Release NO and IL-6 that May Disrupt Intestinal Barrier Function. Gastroenterology. 2013;145(6):1481-1484. https://doi.org/10.1053/j.gastro.2013.10.031
44. Hartmann P, Haimerl M, Mazagova M, Brenner D, Schnabl B. Toll-Like Receptor 2–Mediated Intestinal Injury and Enteric Tumor Necrosis Factor Receptor I Contribute to Liver Fibrosis in Mice. Gastroenterology. 2012;143(5):1330-1340.e1. https://doi.org/10.1053/j.gastro.2012.07.099
45. Zheng Y, Cai W, Zhou S, Xu L, Jiang C. Protective effect of bone marrow derived mesenchymal stem cells in lipopolysaccharide-induced acute lung injury mediated by claudin-4 in a rat model. American journal of translational research. 2016;8(9):3769-3779.