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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

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Abstract
Homing and migration of bone marrow mesenchymal stem cells (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 in improving the homing and antifibrotic effect of BM-MSCs. In our study, Sprague-Dawley rats were randomly divided into six groups: Control group; Meloxicam group; Thioacetamide group; Thioacetamide + BM-MSCs group; Meloxicam + Thioacetamide group; Thioacetamide + Meloxicam + 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* BM-MSCs administration significantly improved histopathological changes and attenuated the elevated liver enzymes, lipid peroxidation marker malondialdehyde (MDA), inflammatory cytokine levels including tumor necrosis factor alpha (TNF-α), cyclooxygenase 2 enzyme (COX-2) enzyme, nuclear factor kappaB (NF-κB) and both hydroxyproline content and α-SMA were also reduced. Additionally, there was an increase in the activity of reduced glutathione (GSH) in liver tissue. Moreover, the PKH26 staining showed that meloxicam which induced the expression of claudin-4 resulted in more homing of BM-MSCs to the liver more than those without meloxicam 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 overexpression of claudin-4 by meloxicam.

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

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]. Thioacetamide (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].
Cyclooxygenase (COX)-2 has important roles in the process of inflammation [4, 5]. Also, COX-2 overexpression has been observed in different chronic inflammatory conditions such as rheumatoid arthritis, ulcerative colitis and Crohn's disease [6]. It is also worthy to mention that up-regulation of COX-2 has been observed in different inflammatory conditions related to the liver including Liver cirrhosis [7], hepatic fibrosis in chronic hepatitis C infection [8]. Meloxicam (Mel) belongs to the enolic acid group of non-steroidal anti-inflammatory drugs (NSAIDs). It is an oxicam derivative with preferential COX-2 inhibitory activity over COX-1 [9, 6]. Recent studies have shown that meloxicam has reduced liver fibrosis owing to its

ability to modulate oxidative stress and reduce levels of pro-inflammatory cytokines including TNF-α, interleukin-1 beta (IL-1β) and nitric oxide (NO) [10].

Bone Marrow-Mesenchymal stem cells (BM-MSCs) are a type of multipotent stem cells, which has properties of self-renewal and differentiation into various specialized Cell types [11]. 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 [12]. 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 [13].

There are at least 24 human Claudin family members that have been discovered [14]. Claudin play an important role in both formation and function of tight junctions (TJs). These tight junctions consist of cytoplasmic proteins, including zonula occludens (ZO) [15] and other three trans membrane proteins: occludin, junctional adhesion molecules (JAMs) and claudins (CLDNs) [16]. Claudins are seen in both epithelial and endothelial cells and this makes them distributed all over the body [17]. Also, the expression of claudins can be ubiquitous or cell type-specific [16].

Therefore, in this study, our aim is to investigate the possible hepatoprotective role of claudin-4 induction in improving the homing and migrating ability of BM-MSCs in thioacetamide induced liver fibrosis in rats.

2. Methods

2.1. Animals

Sixty Sprague-Dawley adult male rats were brought from the laboratory animal colony, Ministry of Health and Population (Helwan, Cairo, Egypt). The weight of the rats ranges from 170 to 200g and were kept in standard conditions including (22°C ± 2°C) room temperature and (60% ± 10%) 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). Claudin-4 antibody was purchased from Thermo Fisher Scientific Inc (Fremont, California). TNF-α was purchased from (Santa Cruz Biotechnology, Inc., Europe), COX-2 antibody was purchased from (Cayman Chemical, USA); NF-κB was purchased from (Santa Cruz Biotechnology, Inc., Europe). Determination of Alanine Transferase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP), Reduced Glutathione (GSH), Malondialdehyde (MDA) and Hydroxyproline was done using reagent kits purchased from Bio diagnostics Inc (Cairo, Egypt). Meloxicam was obtained as a yellow pure powder from the Egyptian Company for Chemicals and Pharmaceuticals (ADWIA).

2.3. Experimental design

After a period of adaptation, we randomly allocated 60 rats equally into 6 groups. Group I received saline 5ml/kg i.p three times weekly for 4 consecutive weeks and served as a negative control group (-ve). Group II received meloxicam i.p at 5mg/kg body weight (BW) three times weekly for 4 consecutive weeks [18]. Group III was challenged with TAA 200mg/kg (BW) i.p three times weekly for 4 consecutive weeks to induce liver damage [19]. Group IV were injected i.p. with TAA 200 mg/kg BW three times weekly for 4 consecutive weeks, and after 4 weeks will be treated with BM-MSCs (Single injection at a concentration of 1×10^6 cells/animal, via the tail vein) [20]. Group V will be injected by TAA 200 mg/kg BW i.p three times weekly for 4 consecutive weeks + Meloxicam 5mg/kg i.p 1hr before injecting the TAA. Group VI will be injected a combination of TAA 200 mg/kg BW i.p three times weekly for 4 consecutive weeks + meloxicam 5mg/kg 1hr before the injection of TAA and after the 4 weeks BM-MSCs (Single injection at a concentration of 1×10^6cells/animal, via the tail vein) will be given to the rats.

Rats were monitored carefully every day for any signs of toxicity throughout the experiment and body weight were recorded once weekly before the beginning of injections. At the end of the experiment, animals were euthanized by cervical decapitation and blood was collected from retro-orbital plexus and serum was separated by animal centrifugation at 2500 rpm for 15 minutes. The liver of each animal was extracted and then divided into 3 parts: the first part was formalin fixed, paraffin embedded, the second part was homogenized, and the third part was preserved at -70 °C.

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

The bone marrow of 6 week-old white albino male rats from the tibiae and femurs was harvested with Dulbecco's modified Eagle's medium (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 (Falcon) after centrifugation. First passage cultures are the resulting culture form centrifugation [21]**.** The adherent colonies of cells were trypsinized and counted on day 14.

2.5. Immunophenotype 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. Histopathological Examinations

2.6.1. Liver tissue examination with hematoxylin and eosin (H&E) stain

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 and Stevens [22]. Two different pathologists, blinded to the protocol, examined the stained liver sections.

2.6.2. Measurement of liver fibrosis

Tissue sections embedded in paraffin were stained with Masson's trichrome and microscopically examined using an image analyzer (Leica Qwin 550, Germany). Changes in histology were evaluated semiquantitatively by a pathologist unaware of the type of treatment.

2.6.3. Estimation of the number 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 [23].

2.6.4. Determination 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) [24]. 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.).

2.7. Serum biochemistry

2.7.1. Measurement of liver aspartate transaminase (AST), alanine aminotransferase (ALT), and Alkaline phosphatase (ALP)

Serum was carefully separated into clean dry Wassermann tubes using a Pasteur pipette and used to determine serum liver function tests; Aspartate transaminase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP) using the standard techniques [25, 26].

2.8. Biochemical measurements

2.8.1. Determination of liver hydroxyproline content

According to the method described by Sakaida *et al*. (1996) [27], 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 560nm, 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.

2.8.2. 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 hr 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 hr at room temperature. Protein bands on the membranes were developed by enhanced chemilumine scence (ECL) detection kit (Amersham, Piscataway, NJ, USA). β-Actin antibody (Abcam) was used to normalize between experiments.

2.8.3. Determination of lipid peroxidation (LPO) and reduced glutathione (GSH) levels

Malondialdehyde (MDA) is a thiobarbituric acid reactive substance measured by the method of Ohkawa *et al* [28]. MDA is a reflection of hepatic LPO. Also, liver content of GSH was determined by a colorimetric method using Ellman reagent and glutathione reductase (Moron *et al*., 1979) [29].

2.9. Determination of Claudin-4 (CLDN-4) in rat liver tissue

Determination of Claudin-4 (CLDN-4) was performed using commercial ELISA kit (Thermo Fisher Scientific Inc (Fremont, California) according to the manufacturer's instructions. CLDN-4 specific antibody was used to precoate the microtitre plate provided with the kit. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to CLDN-4. Next, avidin conjugated to Horseradish peroxidase (HRP) is added to each micro plate well and incubated. After TMB substrate solution is added, only those wells that contain CLDN-4, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometric ally at a wavelength of 450nm ± 10nm. The concentration of CLDN-4 in the samples is then determined by comparing the optical density (O.D.) of the samples to the standard curve.

2.10. Statistics

All data are presented as mean ± standard deviation (SD) [30]. Group differences were analyzed using 1-way analysis of variance (ANOVA) followed by Tukey test as a post-ANOVA multiple comparison tests on raw data (Sigma Stat version 3; SPSS Inc, Chicago, Illinois). 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 95, and CD105 cells were determined using flowcytometry. CD95 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 (Fig. 1).

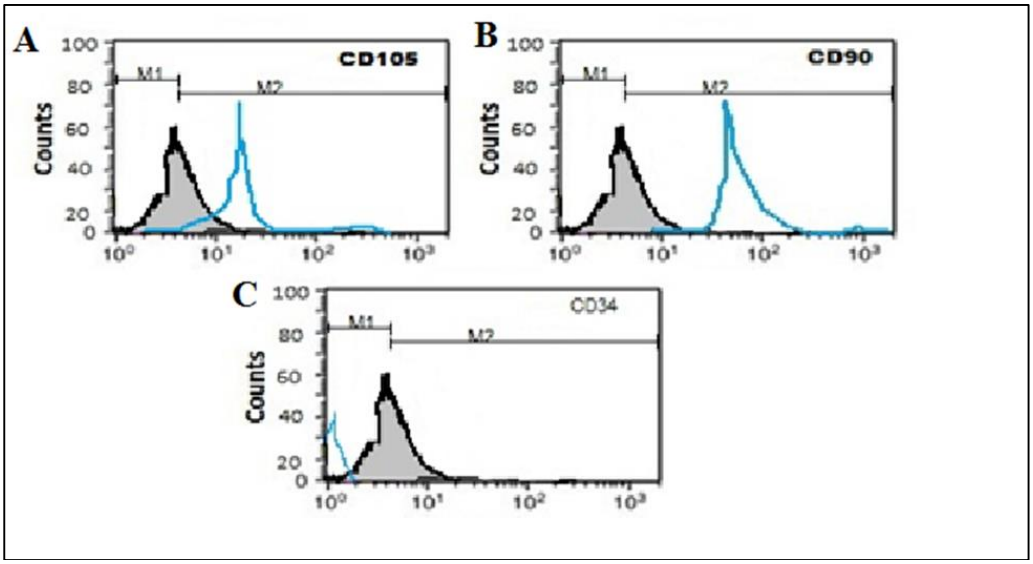


Fig 1: Immunophenotype of BM-MSCs cells were examined by flow cytometry. BM-MSCs cells were negative for the hematopoietic marker (CD34) (C), while strongly positive for mesenchymal stem cell specific markers including CD105 and CD90 (A) and (B) respectively. The blue histograms represent antibody labeled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

3.2. Detection of labeled BM-MSCs using PKH26 red Dye by fluorescence microscope indicates that Claudin-4 overexpression by Meloxicam improved homing of BM-MSCs to liver tissue.

It is seen that the addition of meloxicam to BM-MSCs in thioacetamide injected rats improved homing to the liver tissues which is thought to be mediated by the induction of claudin-4 by meloxicam (Fig. 2 Right), and this can be seen by an increase in the density of PKH26 red dye in the added meloxicam group compared to BM-MSCs without meloxicam addition (Fig. 2 Left).

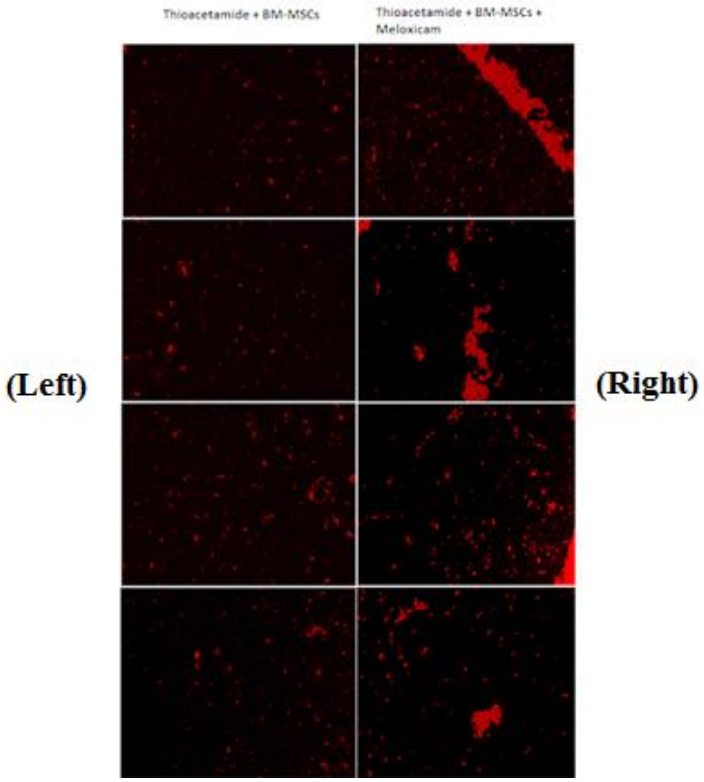


Fig 2: Represent the homing of BM-MSCs by the PKH26 red dye under florescence microscope, this figure shows a comparison between Thioacetamide + BM-MSCs group (Left) and combination between Thioacetamide + BM-MSCs + Meloxicam (Right), and it clearly indicates that the addition of Meloxicam and induction of claudin-4 has improved the homing of BM-MSCs to the liver and consequently improving overall pathological changes in liver tissue which will be seen later as improvement in histopathological examination and reduction in liver enzymes and inflammatory cytokines.

3.3. Histopathological Examination (H&E and Masson's trichrome stain)

Liver lobules of control sections showing average central vein, average portal tracts with average portal veins, and average hepatocytes arranged in single-cell cords with average intervening blood sinusoids (Fig. 3A). They are also negative for Masson's trichrome stain (Fig. 4A). Also, the Meloxicam only group had no alterations in histopathology and show average central vein, average portal tracts with average portal veins and also average hepatocytes arranged in single-cell cords with scattered karyomegaly (Fig. 3B). They are also negative for Masson's trichrome stain like control sections (Fig. 4B). In contrast, thioacetamide injected sections for 4 weeks show significant alterations in histopathology such as markedly dilated central vein with detached epithelial lining, expanded portal tracts with dilated portal veins, karyomegaly, bi-nucleation, vacuolar degeneration and marked apoptosis (Fig. 3C). For Masson's trichrome stain it shows massive fibrosis detected along all examined fields especially around portal tracts and central vein (Fig. 4C). TAA + BM-MSCs had a significant improvement compared to thioacetamide group and it shows mildly dilated central veins with congested blood sinusoids, average portal tract with average portal vein, hepatocytes showed karyomegaly and scattered apoptotic hepatocytes with mild inflammatory cellular infiltrate and minimal affection of hepatic parenchyma than thioacetamide group (Fig. 3D). Masson's stain shows areas of fibrosis detected over the fields examined but less than thioacetamide group (Fig. 4D). TAA+Meloxicam also showed better results than thioacetamide group showing a mildly dilated central vein with congested blood sinusoids, and hepatocytes showed mild steatosis, with few scattered apoptotic hepatocytes (Fig. 3E). Masson's stain shows short fibrous band extending from a central vein (Fig. 4E). Combination of TAA + Meloxicam + BM-MSCs resulted in a preserved liver architecture and mild inflammatory cellular infiltrate around central vein and portal tracts besides average portal tract with mildly dilated portal veins, average bile ducts (BD), average hepatic artery and average hepatocytes (Fig. 3F). Masson's stain in this group shows mild fibrosis better than TAA+ MSCs and TAA+Meloxicam group as well (Fig. 4F).

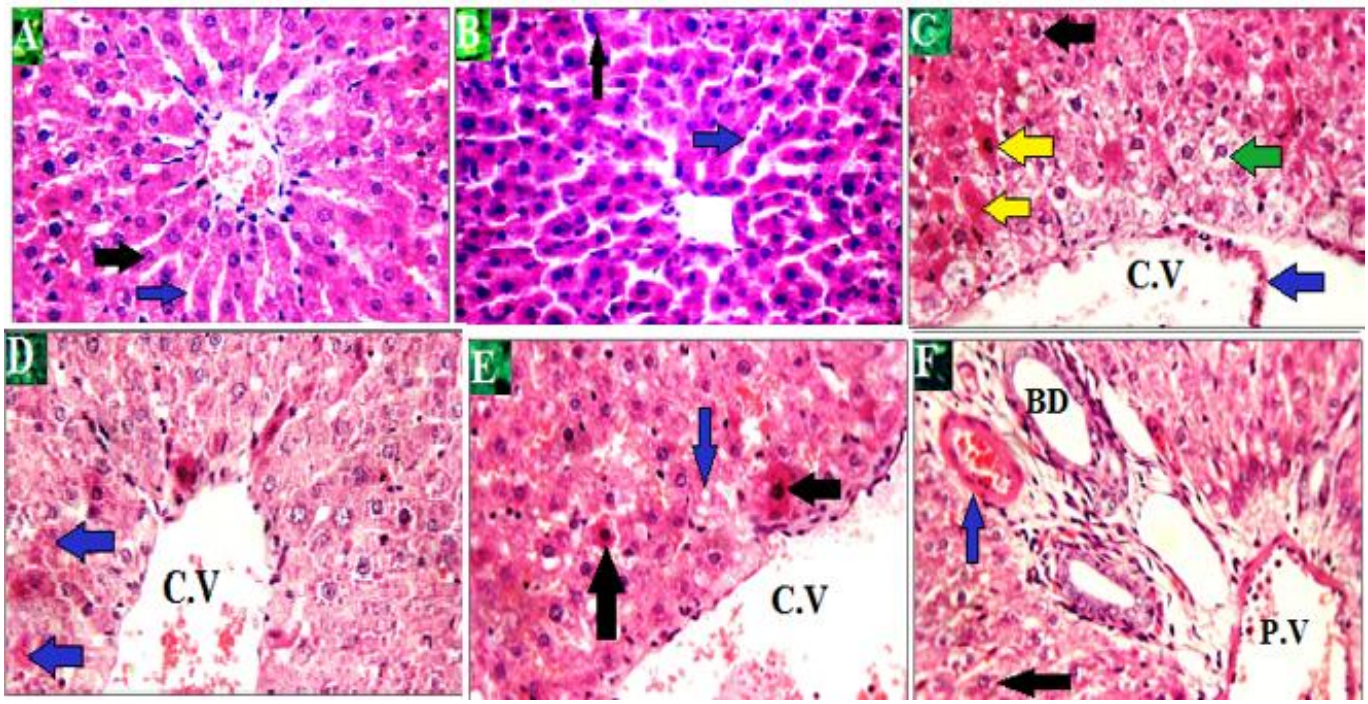


Fig 3: This figure represent H&E micrographs from liver tissues sampled from rats treated with;(A) Saline (Control), (B) Meloxicam (Mel), (C) Thioacetamide (TAA), (D) TAA+BM-MSCs, (E) TAA+Meloxicam, (F) TAA+ Meloxicam+ BM-MSCs. (A) Control sections showing average central vein, average portal tracts with average portal veins, and average hepatocytes arranged in single-cell cords (blue arrow) with average intervening blood sinusoids (black arrow). (B) Meloxicam sections also didn't show significant alterations from the control sections, and it also shows average central vein, average portal tracts with average portal veins and average hepatocytes arranged in single-cell cords (blue arrow) with scattered karyomegally (black arrow). (C) TAA injection for 4 weeks induced significant alterations characterized by markedly dilated central vein with detached epithelial lining, expanded portal tracts with dilated portal veins, karyomegally (black arrow), bi-nucleation, vacuolar degeneration (green arrow), marked apoptosis (yellow arrows). (D) TAA sections treated with BM-MSCs are showing enhanced histopathology compared to thioacetamide group and they show mildly dilated central veins with congested blood sinusoids, average portal tract with average portal vein, hepatocytes showed karyomegally and scattered apoptotic hepatocytes with mild inflammatory cellular infiltrate and minimal affection of hepatic parenchyma than thioacetamide group (blue arrow). (E) TAA+Meloxicam group showing mildly dilated central vein with congested blood sinusoids, and hepatocytes showed mild steatosis, (blue arrow) with few scattered apoptotic hepatocytes (black arrow) (F) Combination of TAA+ BM-MSCs + Meloxicam resulted in a preserved liver architecture and mild inflammatory cellular infiltrate around central vein and portal tracts besides average portal tract with mildly dilated portal veins, average bile ducts (BD), average hepatic artery (blue arrow) and average hepatocytes (black arrow).

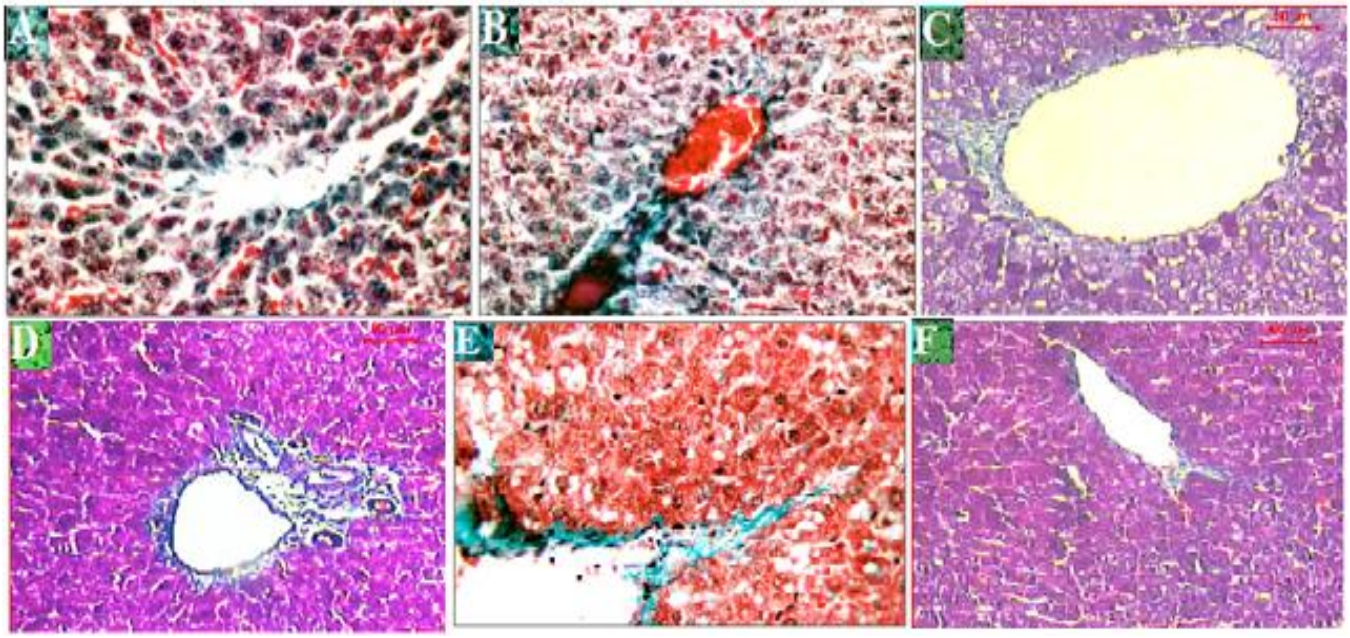


Fig 4: This figure represents Masson's trichrome stain of liver tissues collected from rats treated with;(A) Saline (Control), (B) Meloxicam (Mel), (C) Thioacetamide (TAA), (D) TAA+BM-MSCs, (E) TAA+Meloxicam, (F) TAA + Meloxicam + BM-MSCs. (A) Control liver sections are negative for the Masson's trichrome stain. (B) Meloxicam liver sections are also negative for the Masson's trichrome stain. (C) TAA injection for 4 weeks shows Massive fibrosis detected along all examined fields especially around portal tracts and central vein. In addition, disturbed liver architecture with areas of necrosis, cytoplasmic vaculation and inflammatory cellular infiltrate. (D) TAA sections treated with BM-MSCs are showing areas of fibrosis detected all over fields examined but less than TAA group. Also, extended fibrosis to the sinusoids was also detected. Also, Mild inflammatory cellular infiltrate, minimal affection of hepatic parenchyma than thioacetamide group. (E) TAA+Meloxicam group showing short fibrous band extending from central vein. (F) Combination of TAA+BM-MSCs+Meloxicam shows mild fibrosis and mild inflammatory cellular infiltrate around central vein and portal tracts better than TAA+ BM-MSCs and TAA+Mel group as well, while parenchymal affection is the highest in TAA+Meloxicam+MSCs group.

3.4. Immunohistochemistry results of liver alpha-smooth muscle actin (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 (Fig. 5A)**.** Also, livers of meloxicam administrated rats showed normal faint positive expression of α-SMA around the central veins and around the portal vein in the portal areas (Fig. 5B). In contrast, examination of livers injected with TAA showed strong positive expression of α-SMA in the portal area along the proliferated fibrous tissue, along the extended septa, in the surrounding hepatic cells, and in activated stellate cells. Strong immunopositivity of α-SMA was also noticed along the portal to portal fibrous bands and in scattered hepatocytes and spindle shape activated stellate cells. Generally, widespread immune-reactivity of α-SMA was observed in the activated spindle stellate cells, in the hepatic cells and along the fibrous bands (Fig. 5C). Regarding the treated groups, it was observed that the combined use of BM-MSCs and meloxicam had the highest decrement effect of α-SMA expression followed by the sole use of BM-MSCs+TAA and finally the use of meloxicam+TAA. In regards to livers of TAA administrated rats treated with BM-MSCs showing marked retraction of hepatic fibrosis and decreased immune-positivity of α-SMA and its limitation to the portal areas and in scattered activated cells among the parenchyma as well as its faint expression in the cells surrounding the portal tracts (Fig. 5D). Livers of TAA administrated rats treated with meloxicam showed marked retraction of fibrosis with scattered α-SMA positive cells in between the hepatic cells and in few hepatocytes (Fig. 5E). Concerning, livers of TAA administrated rats treated with combined BM-MSCs and meloxicam revealed; absence of fibrous bands and apparently few α-SMA immunopositive cells scattered along the parenchymal cells (Fig. 5F).

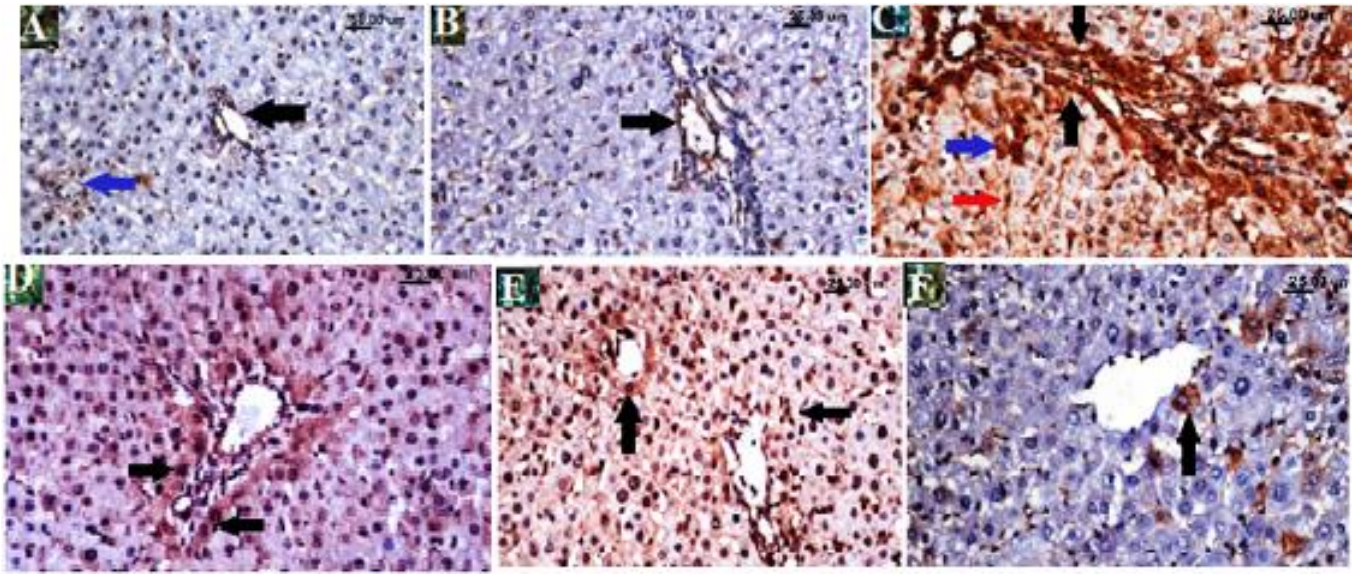
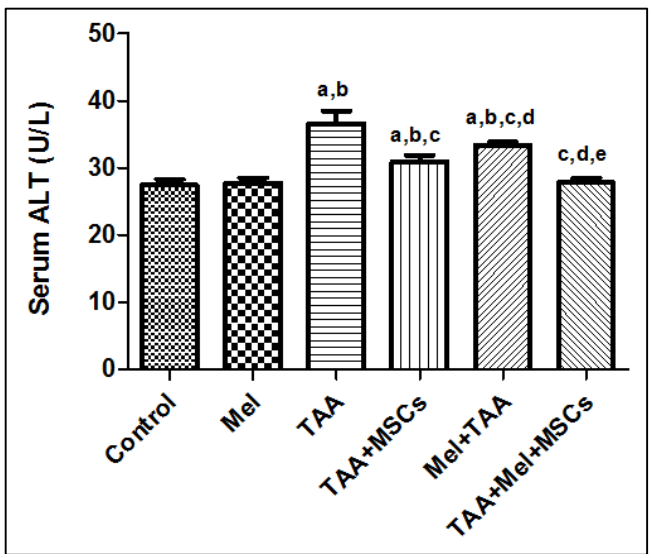


Fig 5: This figure shows the results of immunohistochemistry of α-SMA. (A) Liver of control rat showing faint normal expression of α-SMA around the portal vein in the portal areas (black arrow) and around the central vein (blue arrow). (B) Meloxicam sections showed normal faint positive expression of α-SMA around the portal vein (black arrow) in the portal area. (C) Thioacetamide sections showing strong positive expression of α-SMA in the portal area along the proliferated fibrous tissue, along the extended septa (black arrows), in the surrounding hepatic cells (blue arrow) and in activated stellate cells (red arrow). (D) Thioacetamide+BM-MSCs group showing marked decrease in α-SMA immune reactive cells, with only faint expression in the cells surrounding the portal tracts (black arrow). (E) Thioacetamide+Meloxicam sections showing immune-reactive α-SMA cells (black arrows) around the portal areas with marked retraction of fibrous proliferation. (F) Thioacetamide+Meloxicam+MSCs showing absence of fibrous bands and apparently few α-SMA immnuopositive cells (black arrow) scattered along the parenchymal cells.

3.5. Effect of Meloxicam addition on Serum Biochemical Parameters

The serum levels of liver functions (ALT, AST, and ALP) are presented in (Fig. 6). In TAA injected group, the serum levels of ALT, AST, and ALP significantly, $P< .05$, increased to 36.60±1.82, 44.15±2.10, 78.24±2.17, respectively, compared to negative control values of 27.45±0.85, 31.70±3.36, 61.25±2.98, respectively. Treatment of TAA injected rats with BM-MSCs after 4 weeks significantly, $P< .05$, decreased the TAA-induced elevation of these marker levels to 30.93±0.95, 35.83±1.48, 70.96±0.61, respectively. Also compared to TAA group, TAA+Meloxicam significantly, $P< .05$ lowered the marker levels to 33.38±0.53, 36.55±1.42, 71.98±1.97, respectively. Combination treatment with TAA+BM-MSCs+Meloxicam significantly, $P< .05$, had the most reduction in liver markers to 27.84±0.61, 32.68±0.94, 65.68±0.87, respectively compared to TAA group. Moreover, combination treatment with TAA+BM-MSCs+Meloxicam significantly $P< .05$, lowered ALT, ALP levels but non significantly lowered AST levels compared to TAA+BM-MSCs and TAA+Mel.



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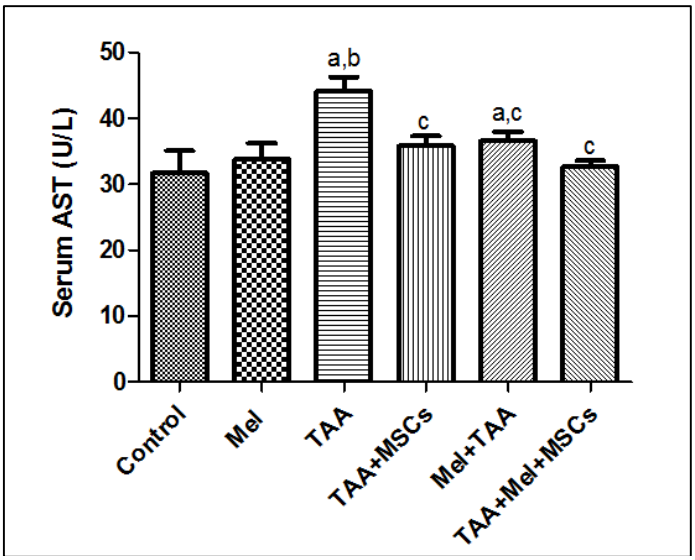

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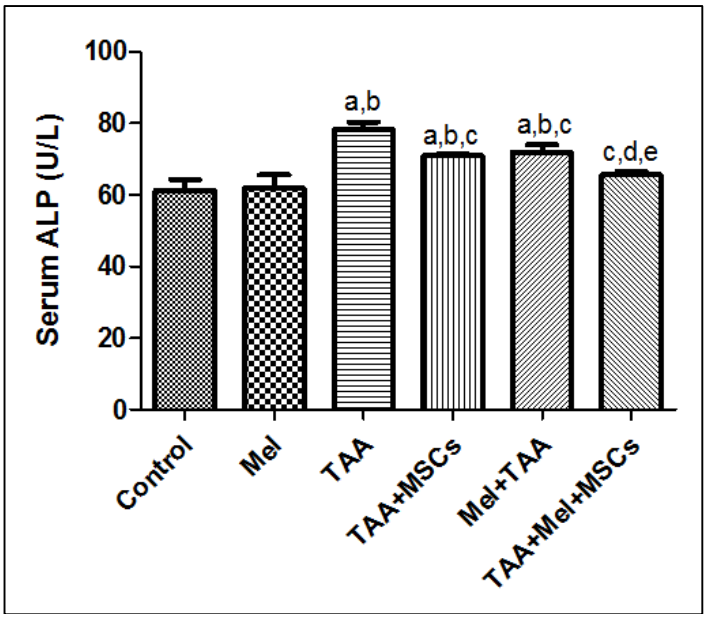


C.

Fig 6: Effect of meloxicam (Mel) with or without BM-MSCs on serum liver enzymes after 4 weeks of treatment. Addition of meloxicam to BM-MSCs significantly abrogated thioacetamide (TAA)-induced liver enzymes close to normal; more than TAA+MSCs and TAA+Mel as manifested in figures; (A) serum Alanine aminotransferase (ALT), (B) serum aspartylaminotransferase (AST), and (C) serum alkaline phosphatase (ALP). Data were presented as mean ± standard deviation (SD) of 10 animals/group; a, b, c, d, or e indicates significant difference from control, meloxicam, thioacetamide, TAA+MSCs, or Mel+TAA, respectively, at $P \leq .05$ using Tukey test as post ANOVA test. ANOVA indicates analysis of variance.

3.6. Effect of Meloxicam addition on hepatic Hydroxyproline content

Hepatic contents of hydroxyproline significantly elevated after TAA injection to 83.85±0.62 compared to control values 15.30±0.25. Meloxicam (Mel) alone non-significantly lowered hepatic hydroxyproline level to 13.80±0.78 compared to control group. Treatment with BM-MSCs significantly abrogated TAA-induced elevation of hydroxyproline content in liver tissues to 29.38±1.62. Mel treatment without BM-MSCs also significantly abrogated TAA-induced elevation of hydroxyproline content in liver tissues to 49.65±1.83. Meloxicam (Mel) addition to BM-MSCs significantly lowered the levels of hydroxyproline content to 20.08±2.15 more than TAA+MSCs and TAA+Mel (Fig. 7).

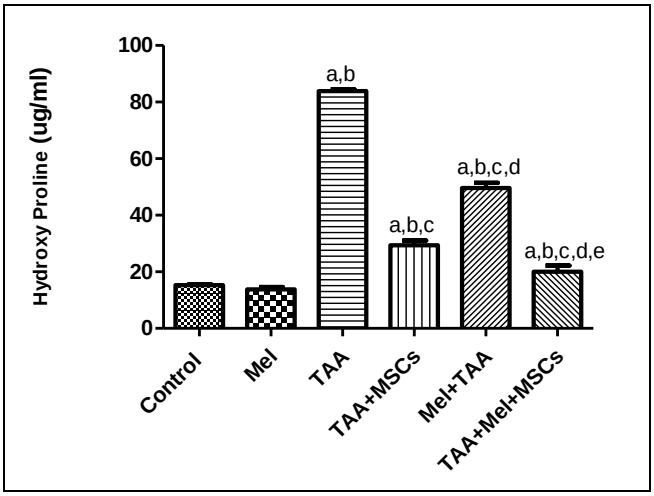
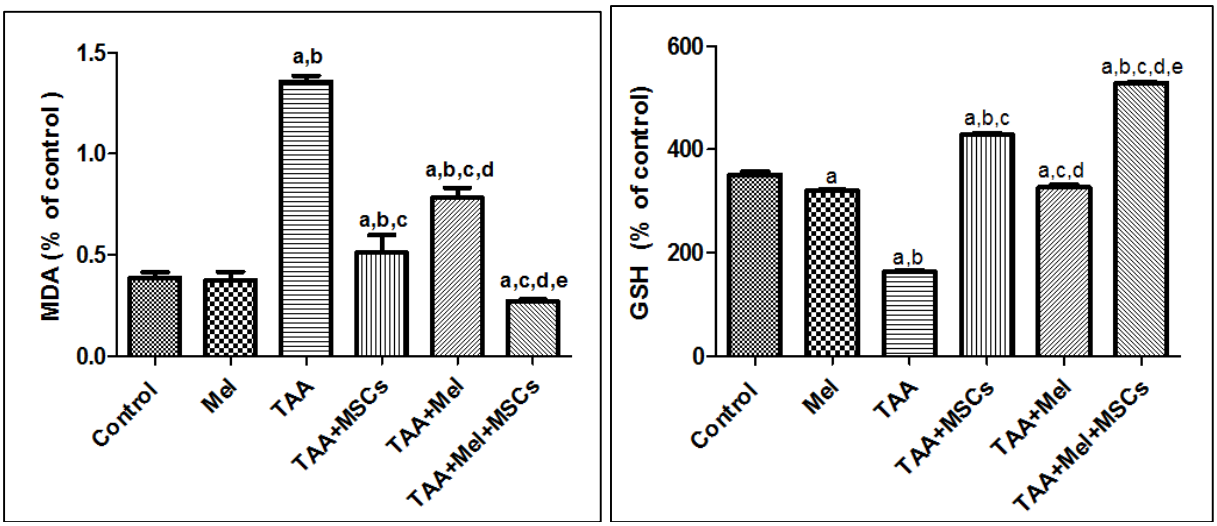


Fig 7: Represents the levels of hepatic hydroxyproline content. Thioacetamide (TAA) significantly elevated the hepatic hydroxyproline content of liver after 4 weeks. Meloxicam (Mel) alone non-significantly lowered hepatic hydroxyproline level compared to control group. Treatment with BM-MSCs resulted in a significant reduction in hydroxyproline levels more than TAA and TAA+Mel group. Interestingly, molexicam addition to BM-MSCs significantly lowered the hydroxyproline content more than the other groups TAA, TAA+MSCs and TAA+Mel respectively. Data were presented as mean ± standard deviation (SD) of 10 animals/group; a, b, c, d, and e indicates significant difference from control, meloxicam thioacetamide, TAA+MSCs, TAA+Mel, respectively, at $P \leq .05$ using Tukey test as post ANOVA test. ANOVA indicates analysis of variance.

3.7. Effect of Meloxicam addition on (lipid peroxidation) LPO and reduced glutathione (GSH) Levels

TAA caused a substantial elevation in liver MDA content to 1.35±0.02 with concomitant depletion in GSH content 162.7±3.0 compared to the negative control group 0.38±0.02, 351.3±6.1, respectively (Fig. 8). Administration of Meloxicam alone showed a non-significant decrease in liver MDA content to 0.37±0.04 (Fig. 8A), While resulted in a significant decrease in GSH content to 320±2.16 compared to negative control group (Fig. 8B). Surprisingly, treatment with BM-MSCs significantly lowered levels of MDA to 0.51±0.08 compared to TAA group. Interestingly, BM-MSCs resulted in a significant increase in GSH levels to 428.5±3.87 compared to TAA group, Control, Meloxicam alone and TAA+Mel 162.7±3.0, 351.3±6.11, 320±2.16, 326.5±5.44 respectively (Fig. 8B). Also, treatment of TAA with meloxicam resulted in a significant reduction in MDA levels to 0.78±0.04 compared to TAA group but not lower than BM-MSCs+TAA (Fig. 8A). Again, addition of meloxicam to BM-MSCs resulted in the most significant reduction of MDA levels to 0.27±0.01 more than control, TAA, TAA+MSCs, and TAA+Mel groups respectively (Fig. 8A). Surprisingly too, the addition of meloxicam to BM-MSCs increased significantly the levels of GSH to 529±2.16 more than any other group (Fig. 8B).

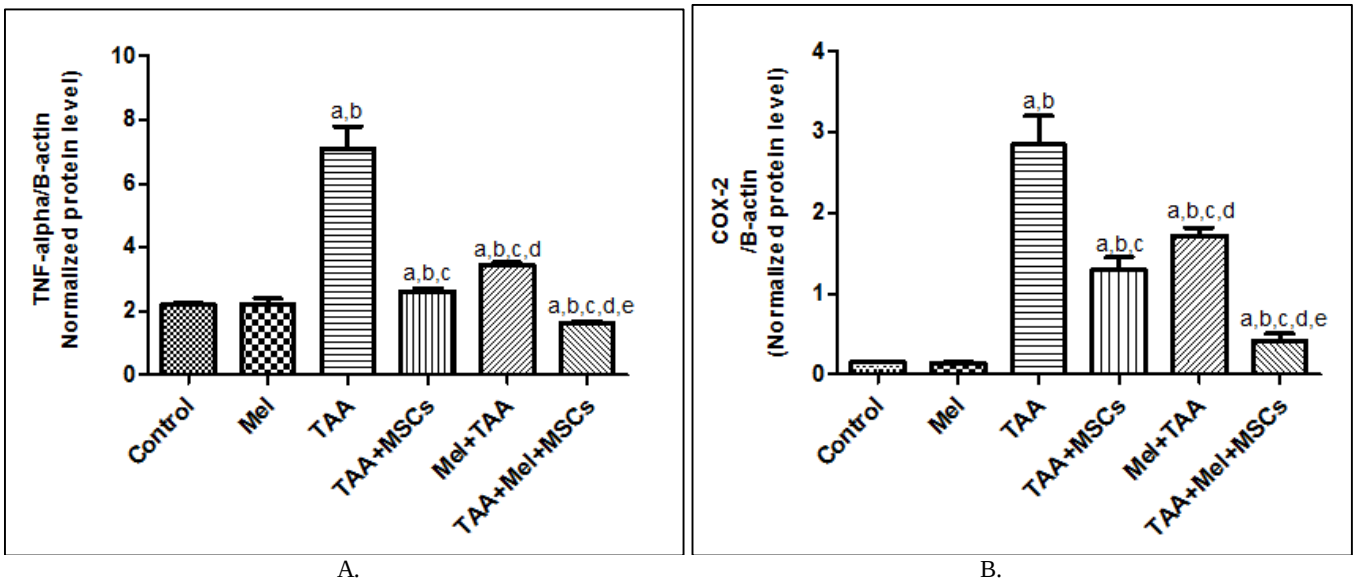


A. **B.**

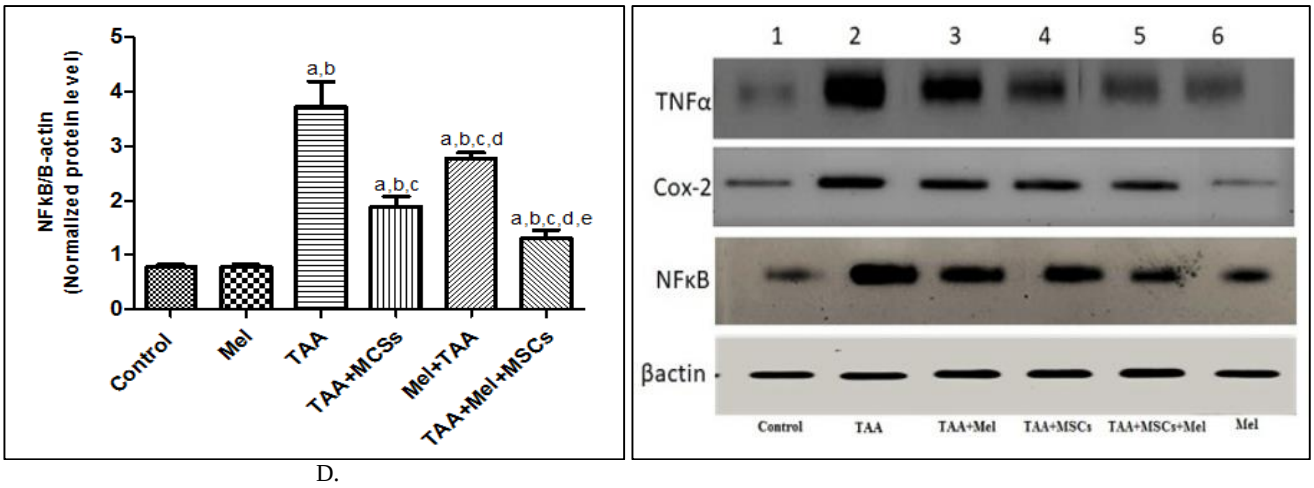
Fig 8: Effect of Thioacetamide (TAA), Meloxicam (Mel), and BM-MSCs with or without meloxicam addition on liver content of Malondialdehyde (MDA) and reduced glutathione (GSH) level in rats. Meloxicam addition to BM-MSCs resulted in a significant reduction in (A) Malondialdehyde (MDA) content compared to control, TAA, TAA+MSCs and TAA+Mel groups respectively; and the most significant increase in (B) reduced glutathione (GSH) content compared to all other groups. MDA was taken as an indicator for lipid peroxidation. Data are plotted as percentage of corresponding control value. Data were presented as mean ± standard deviation (SD) of 10 animals/group; a, b, c, d, and e indicates significant difference from control, meloxicam thioacetamide, TAA+MSCs, TAA+Mel, respectively, at $P \leq .05$ using Tukey test as post ANOVA test. ANOVA indicates analysis of variance.

3.8. Effect of Meloxicam addition on inflammatory mediators levels in rat liver tissues

In TAA injected rats there were significant increases in hepatic levels of TNF-α, COX-2 enzyme, NF-κB to 7.0±0.71, 2.84±0.36, 3.72±0.45 compared to normal control group 2.19±0.07, 0.14±0.008, 0.77±0.04 respectively. Treated groups with BM-MSCs had significantly decreased hepatic levels of the above markers to 2.62±0.09, 1.29±0.15, 1.88±0.19 compared to TAA and TAA+Mel group. Additionally, meloxicam added to TAA reduced the inflammatory mediator levels to 3.46±0.05, 1.72±0.09, 2.76±0.11 respectively compared to TAA group. Surprisingly, meloxicam addition to BM-MSCs significantly lowered the levels of TNF-α, COX-2 enzyme, NF-κB to 1.63±0.05, 0.40±0.09, 1.29±0.16 compared to TAA, TAA+BM-MSCs, and TAA+Mel respectively (Fig. 9).



A. B.



D.

Fig 9: TAA significantly, $P \leq .05$ increased hepatic levels of TNF-α (A), COX-2 (B), NF-Kb (C) compared to normal control group. Treated groups with BM-MSCs significantly decreased hepatic levels of the above markers to compared to TAA and TAA+Mel group. Additionally, meloxicam added to TAA reduced the inflammatory mediator levels compared to TAA group. Surprisingly, meloxicam addition to BM-MSCs significantly lowered the levels of TNF-α, COX-2, NF-κB compared to TAA, TAA+BM-MSCs and TAA+Mel (Figure 9). Data were presented as mean ± standard deviation (SD) of 10 animals/group; a, b, c, d, and e indicates significant difference from control, meloxicam thioacetamide, TAA+MSCs, TAA+Mel, respectively, at $P \leq .05$ using Tukey test as post ANOVA test. ANOVA indicates analysis of variance. In addition, scanning densitometer quantitative western blot of TNF-α, COX-2 enzyme and NF-Kb versus β-Actin in the following studied groups: (1) Control, (2) Thioacetamide (TAA), (3) TAA+Mel, (4) TAA+BM-MSCs, (5)TAA+Mel+BM-MSCs, (6) Mel.

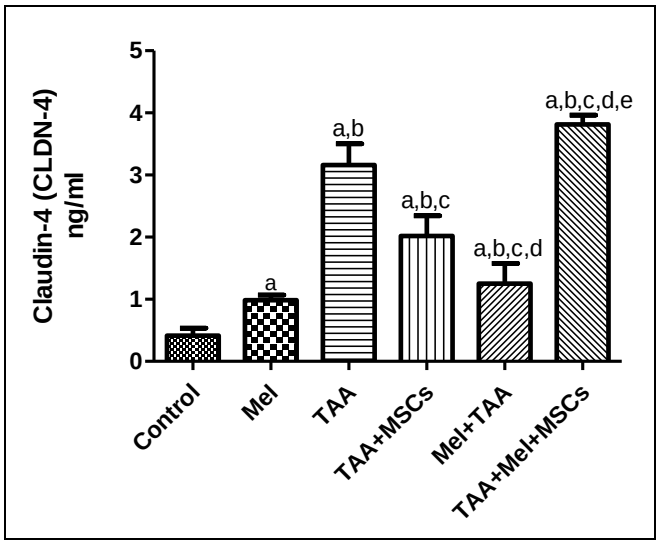


Fig 10: this figure represent the effect of meloxicam with or without addition to BM-MSCs on Claudin-4 expression. Meloxicam alone significantly resulted in over expression of levels of claudin-4 compared to control group. In TAA injected rats the expression of claudin-4 was significantly increased compared to normal control group. BM-MSCs treated rats significantly reduced the expression of claudin-4 compared to TAA group. This significant reduction of claudin-4 expression is more in TAA rats treated with meloxicam compared to TAA group and TAA+BM-MSCs. Surprisingly, when meloxicam is added to BM-MSCs the expression of claudin-4 significantly increased again compared to all previous groups control, meloxicam, TAA, TAA+BM-MSCs and TAA+Mel respectively (Figure 10).

3.9. Effect of Meloxicam addition on Claudin-4 expression

This figure represents the expression levels of Claudin-4 using ELISA kit. As shown in (Fig. 10), meloxicam significantly increased the expression levels of Claudin-4 to 0.98±0.08 compared to control group 0.41±0.11. In TAA injected rats the expression of claudin-4 was significantly increased to 3.16±0.33 compared to normal control. BM-MSCs treated rats significantly reduced the expression of claudin-4 to 2.02±0.32 compared to TAA group. This significant reduction of claudin-4 expression is more in TAA rats treated with meloxicam to 1.25±0.32 compared to TAA group and TAA+BM-MSCs. Surprisingly, when meloxicam is added to BM-MSCs the expression of claudin-4 significantly increased again to 3.81±0.15 compared to all previous groups control, meloxicam, TAA, TAA+BM-MSCs, and TAA+Mel respectively (Fig. 10).

4. Discussion

In our present study, the TAA injected rats, resulted in increased levels of COX-2 and other inflammatory mediators which could indicate the possible strong inflammatory response reported in histopathological findings. This overproduction of COX-2 could have led to the generation of reactive oxygen species (ROS) which are related to oxidative stress [31]. These ROS could explain the cellular damage found in liver cells along with detected LPO. The generation of COX-2-mediated ROS could also explain the disruption of liver cells and eventually the leakage of ALT, AST into the blood from dead hepatocytes and possible disruption of hepatic functions manifested by increased levels of ALP. Injecting BM-MSCs significantly ameliorated liver fibrosis induced by TAA, and this is seen by the significant reduction in serum liver enzymes such as ALT, AST, and ALP (Fig. 6). In addition, proposed mechanisms by which BM-MSCs led to the recovery from liver fibrosis are by lowering both hepatic hydroxyproline content and α-SMA expression, both of these markers reflect the degree of fibrosis present in liver tissue which was significantly reduced compared to TAA injected rats, this also could explain why BM-MSCs exert anti-fibrotic effects as previously reported in the literature [32]. Moreover, 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 (Fig. 2). Moreover, treating rats with BM-MSCs resulted in a significant reduction in MDA, an LPO marker and a significant increase in reduced GSH (GSH)

content and this also reflects the recovery of liver tissue against fibrosis by a possible reduction in oxidative stress. Additionally, BM-MSCs led to significant reduction in hepatic inflammatory mediators including TNF-*α,* COX-2enzyme, and nuclear factor kappa B(NF-κB) which might explain the further reduction in liver fibrosis (Fig. 9).

In the present study, the addition of meloxicam to BM-MSCs significantly reduced both serum ALT and ALP levels compared to TAA+Meloxicam and TAA+BM-MSCs and also led to significant reduction in AST serum levels compared to TAA group (Fig. 6). In addition to this, meloxicam added to BM-MSCs resulted in a significant reduction in fibrosis markers such as hydroxyproline content (Fig. 7) and α-SMA (Fig. 5F) more than the previous groups TAA, TAA+BM-MSCs, TAA+Meloxicam respectively. Our results also demonstrated enhanced histopathological examination especially in Masson's trichrome staining (Fig. 4F); it clearly indicates that the fibrosis in TAA+BM-MSCs+Meloxicam is the least compared to TAA, TAA+BM-MSCs, TAA+Meloxicam groups respectively. Moreover, the addition of meloxicam to BM-MSCs affected the levels of inflammatory mediators TNF-α, COX-2 enzyme, nuclear factor kappaB (NF-κB) respectively. Meloxicam addition to BM-MSCs led to the most significant reduction in all of these hepatic inflammatory mediators more than the rest of the groups TAA, TAA+BM-MSCs, TAA+Meloxicam respectively. These results obtained by the addition of meloxicam to BM-MSCs can be attributed to several proposed mechanisms such as: both meloxicam and BM-MSCs exert anti-fibrotic effects along with the ability of the delivered BM-MSCs to differentiate into mature hepatocytes as part of their mechanism of action [10, 32] thus both of them could have a synergistic relationship with each other. In trying to further support the idea that meloxicam addition to BM-MSCs is an effective combination, the PHK26 red dye showed that meloxicam addition to BM-MSCs resulted in enhanced homing of BM-MSCs and possibly better anti-fibrotic effect (Fig. 2), and this is supported by the evidence that the combination resulted in a decrease in serum liver markers, fibrosis markers, histopathological examination and other inflammatory mediators as mentioned above. Interestingly, this enhanced homing by meloxicam is clearly better than the homing of TAA+BM-MSCs alone (Fig. 2).

In our study, we wanted to see whether the over-expression of claudin-4 would enhance homing and anti-fibrotic effects of BM-MSCs. Thus, we assumed that if we used meloxicam, it would also induce the expression of Claudin-4 through increasing the intracellular calcium concentration same as indomethacin and celecoxib as conducted in a research by the American Association for cancer research in 2005 by Mima *et al*. [33]. To our knowledge, this is the first study by far which show the effect of meloxicam on claudin-4 expression. Surprisingly, in this study, meloxicam significantly increased the overexpression of claudin-4, which was measured by an ELISA kit (Fig. 10), and this induction might be related to the increase in intracellular calcium concentration.

Since Claudins constitute a part of the tight junctions along with other junctional proteins, the transmigratory cups formed by BM-MSCs must pass the inter-endothelial cell-cell connections formed by adherens (Vascular endothelial cadherin, VE-cadherin), gap (connexins), and tight junctions (occludin, claudin, and junctional adhesion molecules) in order to reach the injured site [34, 35]. In our study we assumed that claudin-4 overexpression could enhance the homing and migration properties of mesenchymal stem cells since it could be constituting a part of the tight junctions essential for BM-MSCs homing and transmigration, our assumption is based on evidence that claudin-4 overexpression enhanced the protective role of BM-MSCs in lipopolysaccharide (LPS)-induced Acute lung injury [36]. In addition, induction of claudin-4 inhibited cell migration in AGS cells [33]. Furthermore, it has been reported that BM-MSCs trigger tight junction disassembly in human brain microvessel endothelial cells (BMEC) monolayers which further support the assumption that an interaction between claudins specifically claudin-4 and BM-MSCs might exist [37]. Therefore, in our experiment, meloxicam alone significantly induced claudin-4 expression as mentioned previously; it is also noticed that TAA injection resulted in a significant increase in claudin-4 expression. However, it is crucial to consider that any changes in the expression level of a certain claudin or complement of claudins during a given inflammatory response cannot be only attributed to a change in barrier properties but can also serve as protective response to host defense. An example of such idea is the alteration in the expression levels of claudin-2. Claduin-2 expression is restricted to proliferative colonic crypt base epithelial cells but during the inflammation of the mucosa, an upregulation in the expression levels of claudin-2 is noticed beyond the crypt-base proliferative cells in the colon [38]. Therefore; the overexpression of claudin-4 by TAA could reflect a protective response to host defense same as claudin-2.

Interestingly, injection of BM-MSCs in TAA rats resulted in a significant decrease in the expression of claudin-4. This down-regulation might be due to the concept that BM-MSCs transmigration triggers tight junction disassembly [37]. Similarly, meloxicam added to TAA also reduced claudin-4 expression. Surprisingly, when meloxicam is added to BM-MSCs, claudin-4 expression significantly increased compared to other groups TAA, TAA+MSCs, TAA+Meloxicam respectively. This down-regulation and overexpression of claudin-4 might be due to the re-establishment of claudin-4 as part of the tight junctions after BM-MSCs has completed its transmigration, this assumption is based on the idea that in a vitro model of blood-brain barrier Claudin-5 moved out of the junction at the point of transmigration event but was rapidly re-established in the endothelial junction as soon as trans-epithelial migration (TEM) was completed [39]. Though the exact underlying mechanisms which could explain the role of claudins specifically claudin-4 and it's over-expression and down-regulation during diseased and treatment phases remain to be determined and require further investigations; in our study the addition of meloxicam to BM-MSCs resulted in better homing and possibly better anti-fibrotic effects which is reflected in all measured parameters including serum liver markers, fibrosis markers and histopathological examination which demonstrated that this combination had the least degree of fibrosis compared with other groups, it is also suggested that this improvement in the mentioned results could be partly mediated by claudin-4, since claudin-4 might be playing a role in enhancing the transmigration ability of BM-MSCs to the liver tissues, and this could mean more delivery of BM-MSCs to the site of inflammation and better differentiation capacity into mature

hepatocytes and finally improvement in overall status of liver tissue. The underlying mechanisms behind the role of claudins specifically claudin-4 in enhancing the homing and migration of BM-MSCs and the different expression patterns according to the stage of disease and treatment need further investigations in order to fully understand the expression patterns of claudins as a member of other tight junction molecules. In summary, meloxicam addition to BM-MSCs synergistically improved homing and the potential therapeutic efficacy of BM-MSCs. Also, this improved efficacy might be due to the induction of claudin-4 and this over-expression of claudin-4 obtained by meloxicam addition could be playing a role in improving the homing of BM-MSCs and hence the therapeutic efficacy. This idea is supported by a reduction in serum liver functions, inflammatory mediators, fibrosis markers, and histopathological examinations. Moreover, combination therapy could overcome obstacles seen in sole administration of BM-MSCs alone including inadequate homing of the cells and the number of BM-MSCs injected, and this can lead eventually to the reduction of cost.

5. Conclusion

Meloxicam, improved homing and antifibrotic effect of Bone marrow mesenchymal stem cells in thioacetamide induced liver fibrosis in rats partly mediated by induction of claudin-4.

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