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PRECONDITIONING OF STEM CELLS WITH SILVER NANOPARTICLES CAN REDUCE LIVER FIBROSIS IN MICE

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Abstract
Liver fibrosis is the seven largest cause of death worldwide. Human life is very dangerously threatened by advanced liver fibrosis, which has a high morbidity and fatality rate. The only widely approved treatment option for those with severe liver fibrosis is an organ transplant. But due to shortage of donors, immune rejection and high cost of operation have made the liver transplantation process difficult. Therefore, there is a dire need to treat the danger liver disease on time. The aim of this study was to enhance the efficacy of stem cells by preconditioning with biosynthesized silver nanoparticles for the reduction of liver fibrosis. Silver Nanoparticles were extracted from *Citrullus Colocynthis leaves and* characterized by UV-vis, FTIR, XRD, SEM, and TEM. liver injury was induced in Balb/C mice by four weeks of injection with CCl_4. Mice were divided into three groups , Normal, CCl_4 control and preconditioning stem cells with selenium nanoparticles treated group. Biochemical such as ALT and Bilirubin, Histopathological of H & E staining and molecular at gene expression levels results showed that preconditioning stem cells with silver nanoparticles is a novel idea treat liver diseases, as these cells have strong hepatoprotective ability to reduce live fibrosis in mice.

Keywords: Silver, Nanoparticles, Mice, Liver, Plant, Fibrosis

Introduction
According to Higashi et al., (2017), liver fibrosis is characterized as a wound-healing process which is caused by nonalcoholic fatty liver disease (NAFLD), alcoholic, hepatitis B or C viral infection, as well as autoimmunity and genetic illnesses, and long-time hepatic injury. The initial stages of liver fibrosis are advantageous since they assist the liver in repairing its damaged tissues and regaining its full mass following various acute injuries. The liver fibrosis, however, will eventually proceed to cirrhosis of the liver and even hepatocellular carcinoma (HCC) if the injury is ongoing and long-lasting. Extracellular matrix (ECM), collagen I, and collagen III collect excessively and cause scarring throughout liver fibrosis as a result of an imbalance between fibrogenesis and fibrinolysis (Li and Tuo, 2021). A particularly well-studied model of the general inflammation-fibrosis-

progression/resolution pathological continuum is represented by liver fibrosis, the common end pathway of almost every persistent inflammatory liver damage. Because persistent liver disease is becoming more prevalent and because of cirrhosis, it is currently considering the fifth greatest cause of mortalities in the UK. Research interest in hepatic fibrosis is continuing to increase. As a paradigm for the general characteristics of this pathological process, liver fibrosis is at the forefront of research on ECM and ECM turnover in clinical pathology (Iredale et al., 2013).

Although various drugs are available to help sufferers rebuild their liver's function, hardly any treatments are helpful at repairing the preexisting development of myofibroblasts and ECM. The most common therapy for end stage liver diseases now is a transplanted liver, according to estimates. However, the lack of donor organs and rejection by the immune system restrict transplantation. MSCs therapy has become a different type of treatment for hepatic disorders. The combined effect of many elements aimed at minimizing extreme injury to tissues with the goal of enhancing regeneration is attributed for the positive effects of MSCs. According to Aithal et al., (2019), these factors are thought to lower hepatocyte death, boost regrowth, reverse liver fibrosis, and improve liver functionality. The possible effect of MSCs on initial dysplastic alterations associated with liver fibrosis, the potential for homing of transplanted MSCs, and several other concerns are still up for debate. The first is whether these possibilities continue with the long-term existence of the causal agent. Another barrier to the clinical applications of MSCs based therapeutics is the incomplete understanding of the molecular pathways underlying MSCs trafficking to sites of injury. Therefore, developing new tools to monitor MSCs behavior is still a top research priority that would help MSC-based therapy work better (Rustad et al., 2012). In several disorders comprising cardiovascular disease in animal models, tissues comprising cord blood, placenta, skeletal muscle, and skin stem cells also been demonstrated to have the power for regeneration (Ii et al., 2011).

Recently, multipotent MSCs have demonstrated promise for tissue regeneration in human disease. MSCs are a rare heterogeneity subgroup of pluripotent stromal stem cells that can be derived from a variety of mature organs and bone marrow that possess the capacity to give rise development to cells of multiple lineages. Therefore, MSCs are considered one of the best cells sources for regenerative medicine. MSCs have been shown to have positive impacts in tissue or organs repair and regeneration in numerous studies. After culture expansion and in vivo administrations, MSCs synergistically upregulating anti-inflammatory and pro-survival proteins while downregulating pro-inflammatory cytokines, MSCs can be administered to wounded tissue and control the inflammatory response (Cho et al., 2012).

By conveying genes into stem cells, improving stem cell preservation, enabling the pro-angiogenic action of stem cells, and simulating the extracellular setting, NPs may help in overcoming some of the obstacles and augment benefits of cell therapy (Sun et al., 2020). The purpose of this research is to investigate the protective effect of preconditioning stem cells with green synthesized AgNPs on CCl_4 injured liver injury. The current study is different from previous studies in many respects. First, in this study AgNPs were synthesized via green approach using medicinal plant extract as a reducing agent; *C. colocynthis*. Furthermore, this plant has many biological properties, such as antioxidative, antibacterial, anti-cancerous, anti-inflammatory, anti-microbial and antidiabetic. Therefore, the results indicate that preconditioning stem cells therapeutic effect on CCl_4-induced hepatic injured mice is more significant as compared to their monotherapy.

2. Materials and Methods

2.1 Synthesis and characterization of NPs

Citrullus colocynthis whole plant was collected from district Charsadda. The district of Charsadda is located in the Khyber Pakhtunkhwa Province of Pakistan. To remove any mud and dust, plant was washed with tap water, followed by distilled water and then shad dried completely at room temperature for 1 to 2 weeks. After shade drying, the plant was crushed into fine powder via grinder and were brought to the laboratory in polythene bags. This plant is considered one of the precious assets in the medicinal plant market due to its medicinal properties. Due to their medicinal importance, this plant is highly desired to be used for green synthesis of NPs. For *C. colocynthis*

extract preparation, add 5gm of finely cut plant in a beaker containing 100 ml double distilled water and boiled for 20 min at 95 ˚C in water bath. The extract was filtered using Whatman filter paper no. 1 after being cooled to room temperature. The filtered extract was either used to create nanoparticles or sealed up and kept at 4 ˚C for additional utilization.

2.2 Synthesis and characterization of AgNPs

To synthesize AgNPs, first we need to prepare aqueous solution of $AgNO_3$. Briefly, 100ml aliquot of 10 mM $AgNO_3$ aqueous solution were prepared by adding $AgNO_3$ in separate 1000 ml conical flasks and then poured deionized H_2O. The solution was mixed by vigorous shaking until the $AgNO_3$ was dissolved completely and then made the volume upto 100ml. To synthesis AgNPs, aqueous solution of $AgNO_3$ (10 mM) were mixed with fresh plant extract of *C. colocynthis* at a ratio of 9:1. To get the highest production of NPs, the pH of the mixture that was used was adjusted through the addition of a few droplets of HCl or NaOH.

In an innova 43 orbital shaker (New Brunswick Scientific, USA) the resulting mixtures were stirred at 250 rpm at 60 ˚C for three hours, or until their color changed in order to create AgNPs. The solutions turned dark brown, showing the synthesis of AgNPs. Formation of AgNPs was also confirmed by UV-Visible spectroscopy. All stages of the experiment were implemented in three replicates. The pellet resulting by centrifugation from reaction mixture was washed with deionized water and dried at 60 ˚C in an oven. The synthesized NPs were studied with the help of UV–Vis spectrophotometry (UV), fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), and high-resolution transmission electron microscopy (HRTEM).

The size, crystal structure, elemental makeup, and a number of other physical features of nanoparticles have all been described using a variety of methodologies. Physical characteristics can frequently be assessed using a variety of methods. Following nanoparticle creation, the size, crystal structure, and chemical makeup of the NPs are also carefully examined. The choosing of the best appropriate method is made more difficult by the various advantages and disadvantages of each methodology; frequently, a combinatorial characterization approach is required. The notion of the approach being used, the data it can provide, or the materials it is intended for are used to categorize various characterization techniques. We outline the primary methods and how they serve the NPs characterization concepts (Khan et al., 2023).

2.3 Experimental animals

Female Balb/C albino mice 6 weeks old, weighting 25–35gm were purchased from Veterinary Research Institute (VRI). They were kept in their own polypropylene cages throughout the course of the study. The animals were kept in a monitored environment with a 12-hour cycle of light and darkness at standard temperature. The mice were feed with free access to standard laboratory chow and tap water ad libitum. All efforts were made to minimize animal suffering. All procedures were conducted according to the guidelines for the care and use of laboratory animals approved by our institutional ethical committee.

2.4 Isolation and culturing of bone marrow

Female Balb/C albino mice were killed by cervical dislocation and then transferred to a new plate after being soaked in a 100-mm cell culture dish containing 70% (v/v) ethanol. The skin is subsequently entirely removed off the forelimbs and hind limbs by pushing in the direction of the claw's cutting side. Carefully, remove the muscles, ligaments, and tendons from the tibia and femur bones using micro-dissecting scissors and a scalpel. By cutting at the joints, tibias and femurs were dissected and then placed in a new dish with complete culture media (CCM) containing DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin. To prevent contamination, the soft tissues are totally separated from the bones.

The bones were washed with PBS twice and then transferred to a fresh culture dish with complete medium in a biosafety cabinet. Forceps are used to hold the bone while the two ends are cut off right

below the marrow cavity using micro dissecting scissors. The marrow cavities were repeatedly flushed with 5 ml sterile syringes containing CCM, in order to get sufficient marrow cells.
Through 100 µm cells strainer bone marrow suspension was passed and then centrifuged at 1200rpm for 10min at room temperature (RT). Cells pellet were resuspended in CCM in 25 cm^2 culture flask and incubated in 5% CO_2 at 37 ˚C, according to protocol described by Soleimani and Nadri, (2009). On third day the culture medium was aspirate, washed with PBS to remove debris, dead cells and erythrocytes and add new media and incubate again. The cells were observed daily under phase contrast microscope. Media were refreshed twice a week until the cell confluency reached to 80-90%. After confluency, media were removed, washed with PBS twice, and add 0.25% trypsin for 2-5 minutes at 37 ˚C and then observed through microscope to detached the cells completely. The detached cells were resuspended in a 75 cm^2 cell culture flask at a split ratio of 1:3 and considered as 1^{st} passage. BM-MSCs at passage 3 (P3) were resuspended at 1×10^6 cells/ml in CCM for transplantation.

2.5 Experimental groups

The mice will be equally divided into following groups (each group includes 5 mice): Group I (Normal group): Mice received olive oil (vehicle for CCl_4) intraperitoneally (i.p) at a dose of 1 ml/kg body weight twice/week for six weeks. Group II (CCl_4-treated group): This group were injected CCl_4 olive oil (1:1, v/v) intraperitoneally, twice weekly for six weeks at a dose of 1 ml/kg body weight. Group III (Preconditioning Stem cells with AgNPs treated group): Group II mice were treated with a Preconditioning stem cell according to the protocol used by Amin et al., 2017. CCl_4 treatment will be maintained continued to all groups except normal group throughout experiment. Mice of all experimental groups were sacrificed after six weeks to evaluate the extent of hepatic damage caused by CCl_4 and therapeutic effect of preconditioning MSCs.

2.6 Liver function test

For serum assays, after scarification of experimental model, blood sample were collected from cardiac puncture and centrifuged at 8000 rpm for 10ments to isolate serum for biochemical analysis to determine liver functionality. The level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin were measured in blood serum by using UV-vis spectrophotometer UV-2550 (Shimadzu, Kyoto, Japan).

2.7 Histopathological study

In the various groups, autopsy samples from each mouse were collected and fixed in 10% formalin saline for 24 hours. After washing with tap water, dehydration was induced using a series of dilutions of alcohol (30%, 50%, 70%, 90%, and absolute). Samples were cleaned in xylene before being heated to 56 degrees for 24 hours and then embedded in paraffin. Using a microtome, paraffin wax tissue blocks were cut into sections that were 4 microns thick. The tissue sections were placed on glass slides, deparaffinized, and stained with hematoxylin and eosin (H&E) stain before being examined under a light microscope (Shalby et al., 2017).

2.8 Quantitative RT-PCR (qRT-PCR) analysis

Following the manufacturer's instructions, total RNA was extracted from the homogenized liver of each experimental group of mice using the TRIzol reagent (Life Technologies, Inc.). Thermo Fisher Scientific Inc.'s NanoDrop 2000 UV-Vis Spectrophotometer was used to detect absorbance at 260 nm, which is a common method of measuring total RNA content. Utilizing the 260/280 nm absorbance ratio, the purity was calculated. The RT reagent kit was used to perform reverse transcription using 1 g of total RNA in accordance with the manufacturer's instructions. On a Light Cycler 480 II real-time PCR instrument (Roche, Switzerland), quantitative RT-PCR (RT-qPCR) analysis using SYBR Green PCR Super Mix (Bio Rad, USA) was carried out under the following conditions: 95 °C for 5 minutes, followed by 30 cycles of 95 °C for 45 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds, and final extension was at 72 °C for 10 minutes. The relative expression of

the target genes was assessed using the comparative CT technique (Ct value). The levels of mRNA expression were adjusted to β-actin. Gene mRNA expression levels in the livers of Bulb/C mice were compared to those in the vehicle control group following various treatments. An increase of 1.5 folds or more was deemed biologically significant.

2.9 Statistical analysis

Software package SPSS version 20.0 was used for all statistical analyses. Results was expressed as mean ±SD. Statistical difference was performed using Student's t-test or one-way analysis of variance (ANOVA), taking $p < 0.05$ as statistically significant.

3. Results

3.0 Green synthesized AgNPs and their characterization

3.1 Citrullus colocynthis plant extract

Citrullus colocynthis has numerous biological importance, such as antioxidative, antibacterial, anti-cancerous, anti-inflammatory, anti-microbial and antidiabetic. After boiling in deionized water and filtering through Whatman paper, the aqueous extract of *C. colocynthis* was dark greenish in color.

3.2. MSCs culture

Mouse bone marrow derived MSCs were cultured in DMEM medium supplemented with 10% FBS and 1% Penicillin/streptomycin. The cells used in this study adhered to plastic culture plate and were homogeneously distributed. The isolated cells' morphology was examined, and it showed that they exhibited uniform elongated structures as we reported previously (Shams et al., 2015).

3.3 Biochemical study of liver enzymes

To evaluate hepatic functionality, serum ALT, AST, ALP, and total bilirubin level were measured in all experimental group of mice. As shown in figure 3.1, serum level of all these enzymes were highly elevated in CCl_4 treated group as compared to normal group. Preconditioning MSCs with AgNPs transplantation reduced the serum levels of ALT, AST, ALP, and total bilirubin in treated group. The overall biochemical results show significant control in mean value of serum biomarker in preconditioning treated group toward normal which show restoration in liver function.

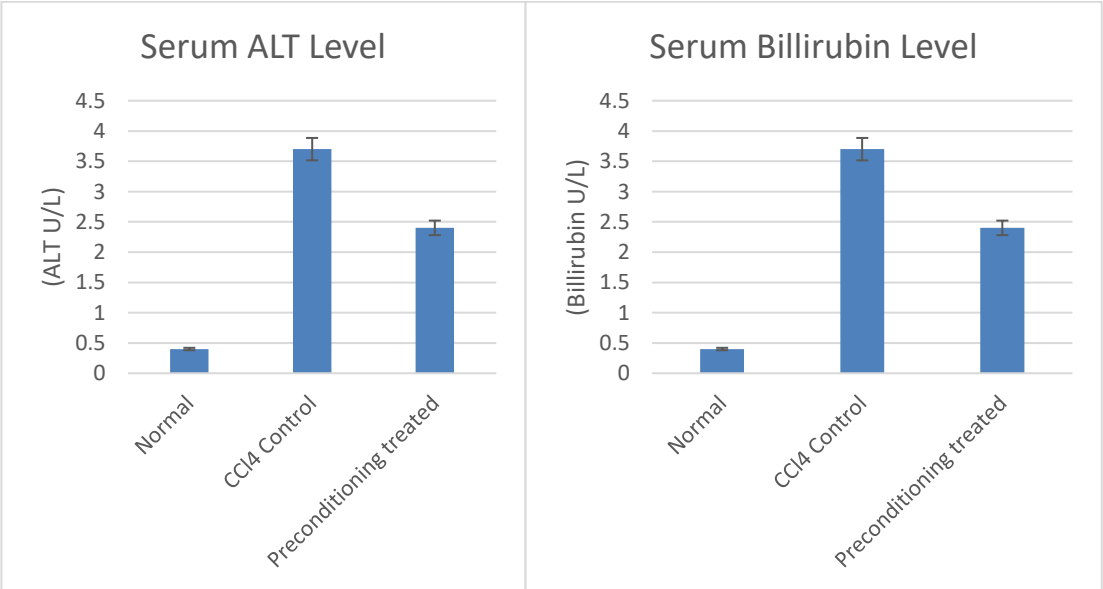


Figure 3.1. Represent the effect of preconditioning MSCs on the means value of serum (a) ALT; (b) total bilirubin level in CCl_4 treated group. The restorative effect of preconditioning MSCs with AgNPs on serum ALT and Billirubin toward normal is more significant in CCl_4 intoxicated mice as

compared to CCl4 control group. The error bar presented as mean ± SD which are statistically significant with p value < 0.05

3.4 Histopathological examination

Histopathological observation of H&E stained liver sections of normal liver showed normal liver architecture with normal appearance of hepatocytes (normal size and shape), no inflammatory cell reaction and the central vein appear to be normal size. After receiving CCl_4, the liver had an unorganized hepatic architecture with a severely enlarged central vein, an inflammatory cell reactivity, and a lack of hepatocyte arrangement. On transplantation of preconditioning MSCs, hepatic architecture were improved and of most of the hepatocytes were restored in liver section of MSCs treated group (Figure 3.2).

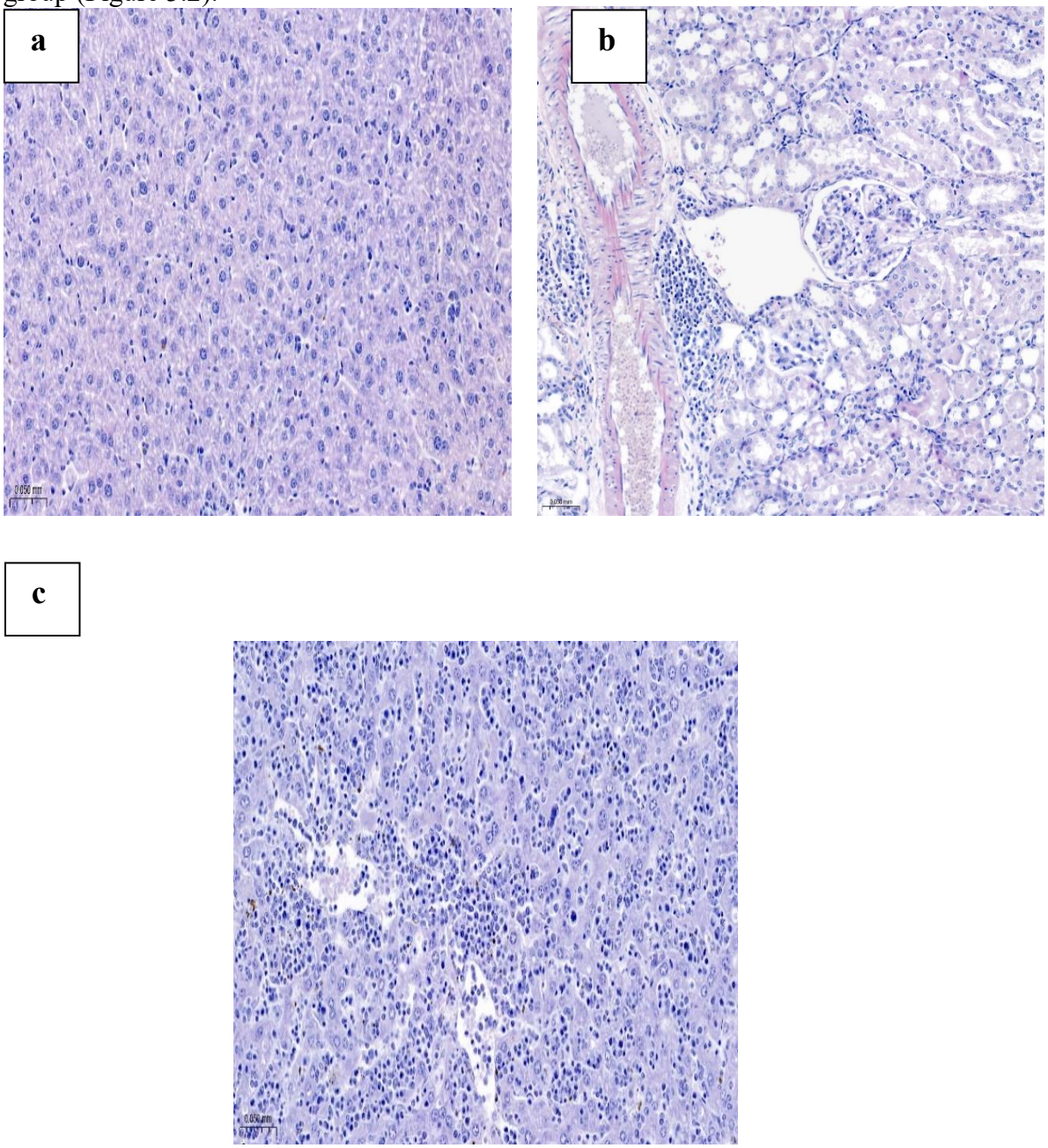
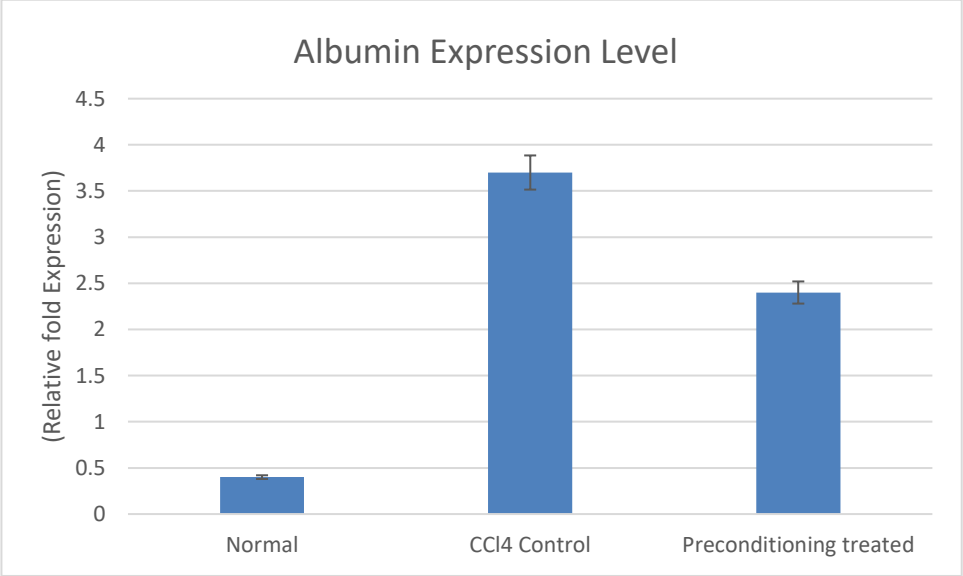


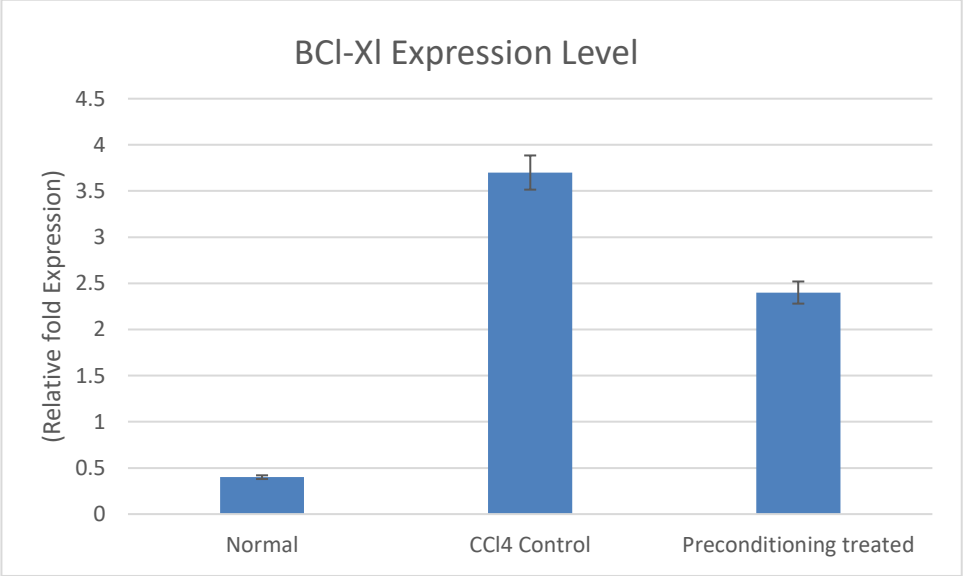
Figure 3.2. Effect of Preconditioning MSCs with AgNPs on histopathological changes in CCl_4 injured mice liver, stained with H&E; (**a**) normal liver shows normal appearance of hepatocytes and normal size of central vein ((H&E, 10×). (**b)** CCl_4 treated liver section shows severe enlargement of central vein and also lack of hepatocyte configuration (10×). (**c**) Preconditioning MSCs with AgNPs liver section shows significant reduction in hepatocyte alteration and hepatic lesion, normal size and shape of most hepatocyte (40×). The size measurement of scale bar line is 500 µm.

3.5 RT-qPCR analysis

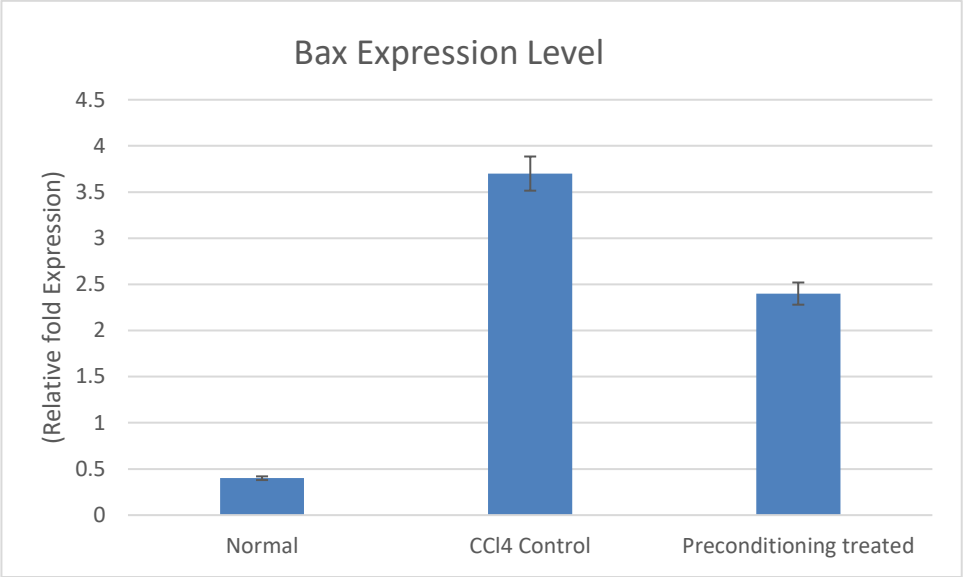
The effect of preconditioning MSCs with NAgPs on the expression levels of hepatic, apoptotic, and antiapoptotic markers were analyzed in CCl_4 injured mice with quantitative real time PCR. Predictably, CCl_4 induced a significant increase of mRNA expression of fibrotic markers such as Bax, and those expression levels of hepatic (Albumin) and antiapoptotic (Bcl-xl) markers were significantly reduced as compared to normal group. The expression levels of hepatic and antiapoptotic marker were upregulated in of preconditioning MSCs with NAgPs treated group than those of CCl_4 treated group as shown in (Figure 3.3). RT-qPCR results of genes expression were analyzed using the $2^{-\Delta\Delta CT}$ method. Data were expressed as mean fold change ± SEM.



(a)



(b)



(c)

Figure 3.3. Hepatocyte-specific gene expression as detected by real-time PCR: CCl_4 intoxicated mice livers treated with Preconditioning MSCs with AgNPs were analyzed for expression of mice specific ALB, Bcl-xl, and BAX mRNA. The expression of **(a)** Alb and **(b)** Bcl-xl expression was increased in Preconditioning MSCs with AgNPs treated group compared to CCl_4 treated group. Conversely**, (c)** Bax expression were significantly higher in CCl_4 treated group and down-regulated Preconditioning MSCs with AgNPs treated group. Results are presented in triplicate. Statistical analysis was assessed using one-way ANOVA. * $p < 0.05$ for Preconditioning MSCs with AgNPs treated group vs. CCl_4.

4. Discussion

In the present study preconditioning stem cells with AgNPs against oxidative damage induced by CCl_4 in an attempt to establish a strategy for observing the hepatoprotective effect of NPs. Depending on the concentration used and the exposure time, it has been reported in vitro that liver damage caused by CCl_4 by two different ways: an indirect effect through the production of free radicals or a direct solvent effect of the molecule itself (Chen et al., 2017). The hepatoprotective properties of preconditioning stem cells with AgNPs were because of its antioxidant activities. AgNPs antioxidant capacity is assessed using the differents tests. It was required to prove their nontoxicity before examining the hepatoprotective activity of AgNPs at varied doses. According to Torres-González et al. (2016), definition of toxicity as a >60% drop in cell viability compared with normal cells, these doses were not cytotoxic.

Liver is the main organ responsible for the biotransformation and subsequent detoxification of xenobiotics, enzymes important for biotransformation are critical. Liver disorders, particularly those brought by viruses or medications, are major health issues that demand quick treatment and remedies with minimum adverse effects. Zhang, et al, reported that CCl_4 is a well-known hepatotoxin used in animal models to test liver injury. The major reasons behind CCl_4-induced hepatopathies are lipid peroxidation, decreased antioxidant enzyme activity, and the generation of free radicals (Zhang et al., 2019). In our experimental study we developed CCl_4-induced liver injury mice model to investigate therapeutic effects of BMSCs synergistically with AgNPs.

Study reported that CCl_4 is converted to a number of metabolites such as radicals like CCl_3. They causing various hepatic disorder like cirrhosis and hepatic carcinoma (Khan et al., 2012). Its exposure to rats induces liver injuries due to free radicals generation. It also causes increase in ALT and AST to plasma and prominent alteration in lipid profile (Chang et al., 2009). The changed liver function as shown by blood ALT and AST enzymes, serum albumin, and total serum bilirubin further reflected the CCl_4-induced liver damages (Wang et al., 2016). Nanotechnology is developed as a combination of biology, chemistry, engineering, and medicine. Nanoparticles have proven useful for the

development of novel treatments and early detection of tumors (Cai et al., 2008). It has been reported previously that SeNPs have strong antioxidant and antidiabetic properties in gestational diabetic rat models (Hassan et al, 2021). As compared to the results from the normal group, the current study showed increase serum ALT, AST, ALP, and total bilirubin level CCl_4 treated mice that represents important indicators of CCl_4-induced hepatic injury. The information showed that MSCs treatment might reduce ALT, AST, ALP, and total bilirubin levels caused by CCl_4. As seen in figure 3.1, preconditioning stem cells with AgNPs treatment also significantly reduced the activities of ALT and total bilirubin as compared to CCl_4 treated mic. These findings support the findings of Dkhil et al., who reported that administering AgNPs has a protective effect against acetaminophen-induced liver damage and modulates the liver enzymes (ALT, AST, and ALP). (Dkhil et al., 2016)].

Histopathological examination of acetaminophen injured liver treated with Nano-Se 10-20 nm displayed normal size and shape of liver cells along with the normal appearance of nucleolus and nuclear membrane, demonstrated that Nano-Se protected the hepatocytes from acetaminophen induced pathological lesions (Zhang et al., 2019). To investigate the therapeutic effects of BMSCs on the CCl_4-induced liver fibrosis model histopathological analysis were performed in vivo. Histological analysis of the CCl_4 treatment group revealed several manifestations of hepatic lobular affection, including detached endothelial lining of the central vein, clogged blood sinusoids, and clogged portal vein. Our result finding suggested that preconditioning of MSCs with AgNPs reduce the histopathological impairment induced by CCl_4 and significantly enhanced normal hepatocyte morphology. Comparatively to the other groups, preconditioning MSCs treated group's H&E stained sections revealed almost normal organization of hepatic lobules with dilated sinusoids and dilated portal vein (Figure 3.2). In a study reported that intravenous administration of MSCs provides the most effective treatment to prevent fibrosis as compared to intraperitoneal or intrahepatic administration (Zhao et al., 2012).

At the molecular level, CCl_4 exposure increased the BAX activity, indicating an elevation of apoptotic events in CCl_4-induced liver injury. However, treatment of preconditioning of MSCs with AgNPs, was shown to reduce the activity of BAX to the levels comparable to CCl_4 treated cells (Figure 3.3 c). Therefore, the molecular level upregulation of hepatic expression that was observed may be viewed as the key factor in the therapeutic actions of preconditioning MSCs with AgNPs against CCl_4-induced liver injury.

Conclusion

The aim of this research was to prepare and characterize AgNPs as a new potential cytoprotective (antioxidant) agent to reduce oxidative stress in vitro and improve the hepatoprotective effects of MSCs against CCl_4-induced liver injury. In vivo results showed that therapy of preconditioning of MSCs with AgNPs restored normal oxidative stress caused by CCl_4 and reduced tissue damage. This study was the first to validate the reduction in liver injury in mice and found that AgNPs exerted antifibrotic effects by enhancing the antioxidant effect of MSCs and inhibiting ROS produced caused by CCl_4. Collectively, the present study provides substantial evidence that specific dose of AgNPs is a good antioxidant and prevents oxidative stress caused by CCl_4. In vivo results confirmed that preconditioning therapy of MSCs with specific dose of AgNPs may act as strong hepatoprotective and preventing oxidative stress to restore CCl_4-induced liver injury.

Institutional Review Board Statement: The present study was approved by the Institutional Review Board of Abdul Wali Khan University Mardan, Pakistan.

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Conflict of Interest: All the authors declare that they have no conflict of interest.

Data Availability Statement: All the data is contained within the manuscript.

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