

RESEARCH ARTICLE

Excess iron ion reduction in a thalassemia model using silver nanoparticles modified by the tannin fraction of *Myrtus communis* extract

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ABSTRACT

Objective(s): Nowadays, iron ions intoxication is the center of attention of interest in the management and treatment of thalassemia and different sorts of anemia associated with regular blood transfusions. Due to the major side effects of current drugs, they should be replaced with safer alternatives. Thus, in this study, functionalized hybrid silver nanoparticles, as an emerging perspective, were investigated for absorbing excess iron ions and their removal in an animal thalassemia model.

Methods: The silver nanoparticles were green-synthesized using the *Myrtus communis* leaf methanolic extract (MC-AgNPs). The produced hybrid nano-Sorbents based on hydrolyzable tannin matrix loaded with silver nanoparticles were delivered to test for chelating iron in the blood of thalassemia mouse model with iron excess. MC-AgNPs and desferal were injected intraperitoneally four times a week for one month in mice with excess iron load. Total iron and serum Fe³⁺ content was assessed under a plasma-atomic spectrometry microscopy and a Fe³⁺ ion measuring kit, severally. The level of liver enzymes was assessed by an automated analysis. Also, hepatic enzyme levels were appraised by using an auto-analyzer based the corresponding kits. Morphological transformations of the liver were assessed through Prussian blue staining method.

Results: Compared with iron overload mice, the serum iron content of mice treated with MC-AgNPs was significantly reduced. MC-AgNPs revealed satisfying effectiveness to chelate excess iron in mice.

Conclusions: This method could be considered as a competitive option for lowering the level of excess iron in vivo.

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INTRODUCTION

There are more than one human disorder prerequisites prompted with the aid of opportunistic deposition of poisonous iron ions ensuing in one-of-a-kind pathologies and even death. Fe^{3+} intoxication is the center of attention of one of a kind interest in view that it might likewise be manageable to fix through chelation in the body. For example, thalassemia is first and foremost, and different kinds of anemia are stipulations dealt with with normal blood transfusions. These generally end result in Fe^{3+} overload, followed by oxidative stress and deterioration of endocrine organs, liver and even intelligence. The ideal treatment for these prerequisites is the use of iron chelator. Iron chelation therapy has been used for a long time as the first choice of care for patients experiencing thalassemia and other types of anemia. The contemporary iron chelating agents for managing and treating thalassemia and different transfusion based anemias consist of desferal (DFO), deferiprone (L1), and deferasirox (ICL-670). Although these therapeutic agents are incredibly effective, they have different effects and necessitate their exchange with safer options [1].

Meanwhile, nanotechnology-based products can help reduce the effective dose of therapeutic compounds and mitigate their toxicity in order to achieve biological treatment standards [2]. Metal nanoparticles such as silver nanoparticles (AgNPs) have been studied thanks to their unique behavior, high adsorption capacity, great (surface-area / volume), higher degree of dispersion, surface modifiability, and comparatively low cost [3]. These nanoparticles usually contain zero metals to absorb heavy metal ions from contaminated water [2]. Recently, AgNPs have been proposed as a promising technological know-how and very fantastic desire for doing away with unique metal ions such as As(V), Cd(II), Hg(II), Cr(VI), Ni(II), Pb(II), Co(II), Al(III), and Fe(III) in aqueous options [2-10]. Hence, we decided to use the above method to absorb excess iron ions and remove them under in vivo conditions in an animal thalassemia model. Note that the field of absorbing excess iron ions under in vivo conditions using functionalized hybrid nanoparticles is an emerging region.

In general, bare metal nanoparticles suffer inherent instability of nanoparticles within the optimal size range and the tendency to form agglomerates. They are also chemically highly reactive and easily oxidized in air [11, 12]. All these

limit their practical application. In this regard, application of bio-polymers (e.g. poly phenolic compounds) significantly increases the usability of nanoparticles through the possibility that nanoparticle-attached bio-polymers functional groups give it [13, 14]. Polyphenolic compounds, while stabilizing metal nanoparticles, can create complexes with available metal ions in aqueous media and can then reduce these ions at their surface and thus remove them from aqueous media [15]. In recent years, nanoparticles composed of metal nanoparticles encapsulated in organic molecules have been examined extensively because of their improved physical and chemical properties, obtained through combining the attractive functionalities of both components [10]. Biosynthesis is a better alternative to prevalent chemical methods because of biocompatibility, cost-effectiveness, and relatively longer life-time. Different materials are classified as biologic for nanoparticle synthesis, including plant extract and enzymes of microorganisms. Among them, plant extracts have received much attention owing to their bio-renewability, the nature of their phytochemical compounds, and ability to provide sustainable solutions for large-scale production [16]. Among the various biopolymers used, hydrolyzable tannins, a classic sort of polyphenolic compound, blended ellagitannins / gallotannin consisting of ellagic acid / gallic acid esters of glucose [17], are considered as a potentially very good antioxidant for preparing and stabilizing silver nanoparticles. Tannins have shown a stronger antioxidant activity than flavonoids and phenolic acids [18]. For example, tannic acid (as a representative of hydrolyzable tannins) can quench the generation of hydroxyl radical from the reaction of fenton, by creating complex with iron ions [19]. Thus, tannin-rich plant extracts may be able to control the toxicity of Fenton reaction.

In this paper, we used the *Myrtus communis* methanolic leaf extract (MCLE), which was the hydrolyzable tannins of the main metabolites present in the plant extract (~80%) for biosynthesis of silver nanoparticles [20, 21]. In addition, *Myrtus communis* has a wide range of pharmacological and healing results, including antioxidant, anti-diabetic, antimicrobial, anti-cancer and liver protection, used in traditional medicine worldwide [22]. The use of the plant has been considered not only for the synthesis of silver nanoparticles but also for preventive oxidative stress-related disorders caused by nanoparticles.

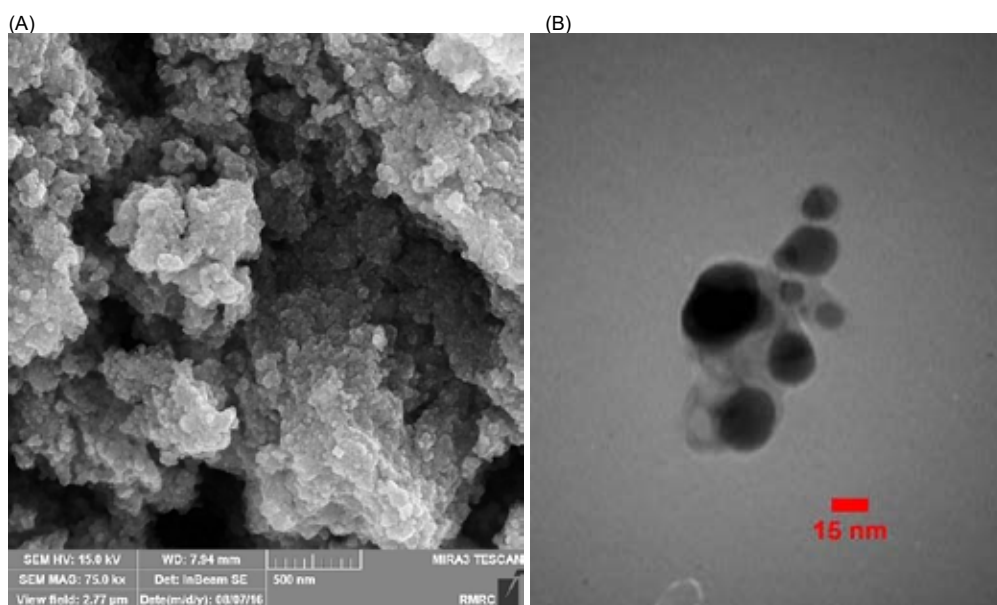


Fig. 1. The SEM (A) and (B) TEM images of *Myrtus communis* leaf extract-capped AgNPs.

Nowadays, there has been growing interest in using herbal products, which are safer than chemical synthetic products. Accordingly, the study about of biological synthesized AgNPs is of unique significance as they commonly include giant quantities of biologically active compounds. Due to the high tannin contents in the surface coating of nanoparticles and the subsequent good antioxidant activity of the nanoparticles, these functionalized hybrid nanomaterials based on nano sized zero valente Ag entrapped in tannin matrix were introduced as a test to chelate iron in vivo. In our opinion, the current find out about is the first to check out the capacity of *Myrtus communis*-synthesized silver nanoparticles (MC-AgNPs) to chelate excess iron in a mouse model of iron overload.

MATERIALS AND METHODS

Materials used

In this study, materials with high purity analytical class were used that did not need to be refined for use. Silver nitrate and sodium hydroxide were acquired from Merck (Germany). For calibration, standard iron solutions were purchased from the German company Merck as iron storage solutions in nitric acid (0.2% w/v). The required deionized distilled water is supplied by Millipore SAS 67120 (France). The human liver cancer (HepG2) was obtained from Pasture Institute, Iran. *Biological synthesis of the Myrtus communis-capped*

Ag⁰ nanoparticles

Silver nanoparticles were synthesized in the same way as in our previous work [13]. Briefly, after preparing the extract solution (50 mL, 4×10^{-3} gL⁻¹), the pH was elevated to 10 and the resulting clear extract was added drop wise into a AgNO₃ solution (50 mL, 40mM) under the temperature of 65°C (200 rpm) away from light. The color change of AgNO₃ solution to yellowish brown to deep brown indicated the formation of AgNPs. To purify the resulting AgNPs, after centrifugation repeatedly (20,000×g, 20 minutes), the pellets were washed four times in (deionized water / ethanol) and purified. The decontaminated pellets were re-dispersed in normal saline for further studies. The bio-synthesized MC-AgNPs were biphasic in nature (based on the XRD pattern), and spherical in structure with a nearly uniform size distribution (based on the SEM results).

The core of the nanoparticles was coated with a transparent organic layer which was the same as tannin molecules (based on the TEM image), (Fig. 1). The hydrodynamic diameter of MC-AgNPs measured via the dynamic light scattering technique was 142 nm, core (Nano Ag⁰) was 30 nm (TEM) and shell (Tannin biomolecules) was 112 nm, corresponding to the effective size of nanoparticles in drug delivery (50-200 nm) [16, 23, 24]. The zeta potential of the MC-AgNPs was -45.0mV [13].

In vivo Study design

The biochemical and histopathological studies were leading on males of white NMRI rats mice (20–25 g). All manipulations with animals were carried out in accordance with Mazandaran Medical Sciences University's Ethical Committee Guideline of working with Laboratory Animal (approval no. 911, 2012). The mice were divided into 9 experimental groups of 6, with intraperitoneal injection: group 1— control (Normal saline); group 2— iron overloaded (100 mg/kg/day iron dextran); group 3— iron-overloaded + DFO (1 mg/kg/day); group 4— iron-overloaded + MC-AgNPs (50 mg/kg/day); 5— iron-overloaded + MC-AgNPs (100 mg/kg/day); 6— iron overload + MCLC (50 mg/kg/day); 7—iron-overloaded + MCLC (100 mg/kg/day); 8—MC-AgNPs (100 mg/kg/day); 9—MCLC (100 mg/kg/day). To produce excess iron, injections were given for 4 consecutive weeks, five days a week, and then discontinued for one month to balance the excess iron balance. This is while a group of mice received normal saline in the same way. In the third month, mice with iron-overloaded were randomly divided into eight groups. And the treatment with DFO, nanoparticles and plant extracts was done for a month as an intraperitoneal injection. After the end of the twelfth week, the mice were euthanized with ether and a blood sample was taken. Serum samples were separated to measure iron content, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels. The liver was taken out and stored in 10% formalin buffer for histopathological studies [25].

Analysis of the total iron content in mice serum

The total iron was assessed via microwave plasma-atomic emission spectroscopy of model Agilent 4100, MP-AES (USA). Iron standard stock solutions for the calibration of the apparatus were prepared in the structure of iron inventory options in 0.2% (w/v) nitric acid from the German business enterprise Merck. The mice serum samples were tested for their iron immediately after making ready 1:50 dilution with ultrapure water [25]. Iron was once reported as $\mu\text{g dL}^{-1}$.

Analysis of the Fe^{3+} ion content in mice serum

The quantity of serum Fe^{3+} ions used to be measured via an Iron Assay kit-Ferene organized via the Iranian organization ZiestChem Diagnostics. The iron connected to transferrin in mice serum

samples in the acidic surroundings is generally launched to structure Fe^{3+} and is transformed to Fe^{2+} via reducing agent. The complex (Ferene- Fe^{2+}) creates a pink set. The colour depth is relative to the quantity of iron present in the sample which can be estimated at 600 nm. Fe^{3+} content material was once represented as $\mu\text{g/dL}$.

Assessment of liver enzymes in mice serum

Blood serum biochemical analysis (AST, ALT and ALP) was performed on an autoanalyzer of model BT3000 PLUS (Biotechnica, Rome, Italy) with commercial colorimetric assay kits (Pars Azmon, Iran) by photometric approach in accordance to the International Federation of Clinical Chemistry (IFCC) and Laboratory Medicine and standard method of the German Society of Clinical Chemistry (DGKC). The foundation of the work of these kits is as follows: Amino transferases AST and ALT reduce the absorbance of NADH by catalyzing the switch of an amino group from L-aspartate to 2-oxoglutarate using the enzyme malate dehydrogenase. The decreased absorbance of NADH ($\lambda_{\text{max}} = 340 \text{ nm}$) is proportional to increased amino transferases activity of AST and ALT. P-nitrophenyl phosphate absorbance ($\lambda_{\text{max}} = 405 \text{ nm}$) is increased by diphosphorylation by the ALP. An increase in solution absorption indicates an increase in ALP activity [26, 27].

Histopathology of the mice liver

Histological examination was performed at "Avicenna" hospital (Sari, Iran). At the give up of the trial period, the animals were slaughtered under anesthesia. The liver was immediately isolated and washed with normal saline, and part of the tissue was promptly fixed at 10% formalin. The formalin-fixed tissues were implanted in paraffin wax blocks and parts of it were cut to a thickness of 5 mm. After placing the paraffin wax blocks on the surface of the clean glass slides and fixing the sample on the surface of the slides, staining with Prussian blue was performed [28]. The stained slides were examined by using light microscopy (Lx 400, USA).

Statistical analysis

A Prism software (GraphPad, version 8.0.2 (263), USA) was used to analyze the data obtained from the experiments. Using Ordinary one-way variance analysis, significant was assessed and statistical comparison between groups was performed using Bartlett's test. All of the records

were stated as the mean \pm standard deviation. Expressing $P < 0.05$ in statistical analysis of all data obtained from this study confirms the significance of the difference.

RESULTS AND DISCUSSION

Changes in the serum total iron content of iron-overloaded mice after exposure to MC-AgNPs

The highest iron content measured in the iron-overload group by means of MP-AES was $3745.8 \pm 200.2 \text{ mg dL}^{-1}$, which was significantly different ($P < 0.0001$) from the control group (normal saline receiving group). In the normal saline group, the iron content was $921 \pm 92.4 \text{ mg dL}^{-1}$. The iron-overloaded mice exposed with MC-AgNPs at both doses of 50 and 100 mg kg^{-1} revealed a significant diminution ($P < 0.0001$) in serum iron compared to the iron overloaded mice group (Fig. 2A). A significant difference ($P < 0.0001$) was found

between the iron-overloaded mice exposed with MC-AgNPs at both doses of 50 and 100 mg kg^{-1} and the group exposed with MCLE at a dose of 100 mg kg^{-1} . Fig. 2 indicates that the diminution of excess iron, more than the plant extract influenced by the nano sized zero valent Ag encapsulated in the extract shell. Furthermore, no difference ($P > 0.05$) was found between the group exposed to only MC-AgNPs and the normal saline group.

Changes in the serum levels of Fe^{3+} ions of iron-overloaded mice after exposure to MC-AgNPs

The maximum iron content ($731.0 \pm 44.32 \text{ mg dL}^{-1}$) was observed in the over-iron group through Fe^{3+} ion level measuring via an Iron Assay kit-Ferene, which was significantly different from the normal saline group ($P < 0.0001$) compared to the normal saline group ($213.6 \pm 29.8 \text{ mg dL}^{-1}$), (Fig. 2B). The Fe^{3+} ion content in over-iron mice

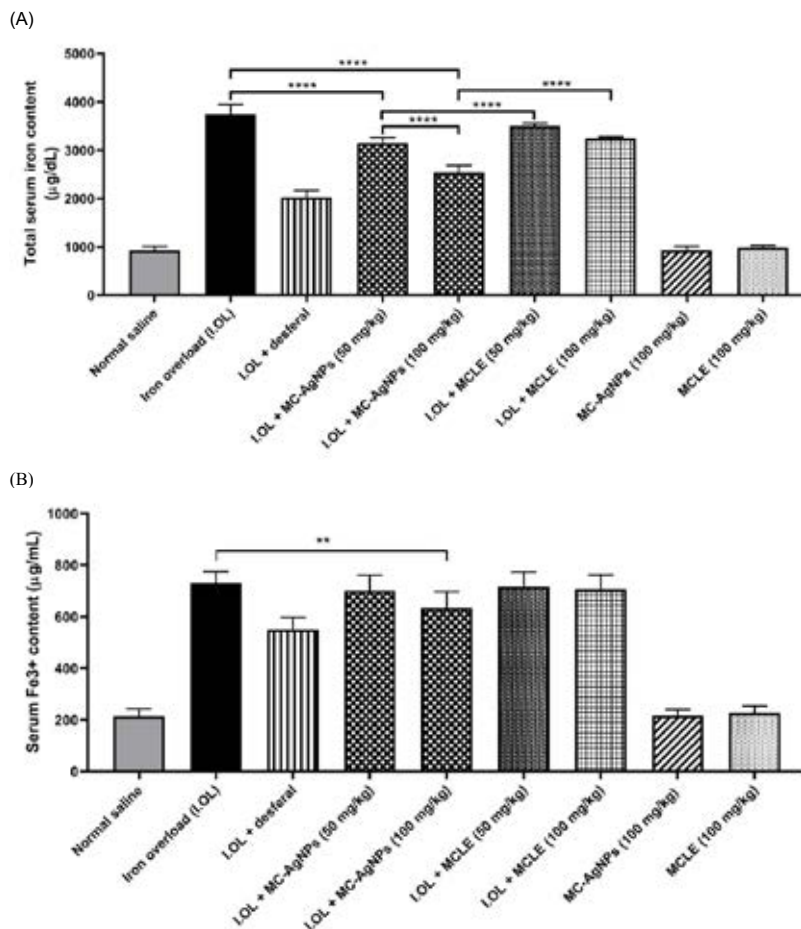


Fig. 2. Evaluation of the serum total iron content (A) and Fe^{3+} ion content (B) in iron-overloaded mice after treatment with *Myrtus communis*-capped AgNPs. All contents are represented as mean \pm SD. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****).

treated with MC-AgNPs was significantly reduced compared to the untreated group. The Fe^{3+} content in the groups received 50 and 100 mg / kg of MC-AgNPs was estimated to be 701.3 ± 60.0 and 635.4 ± 61.8 mg dL^{-1} , respectively (Fig. 2B). Once more, the iron-overloaded mice that were exposed

to MC-AgNPs on the dose of 100 mg kg^{-1} which was significantly reduced ($P < 0.01$) in serum Fe^{3+} compared with the mice too much iron group. Likewise, it was found that exposure with the nanoparticles in mice too much iron did not reduce Fe^{3+} compared to the group receiving normal saline.

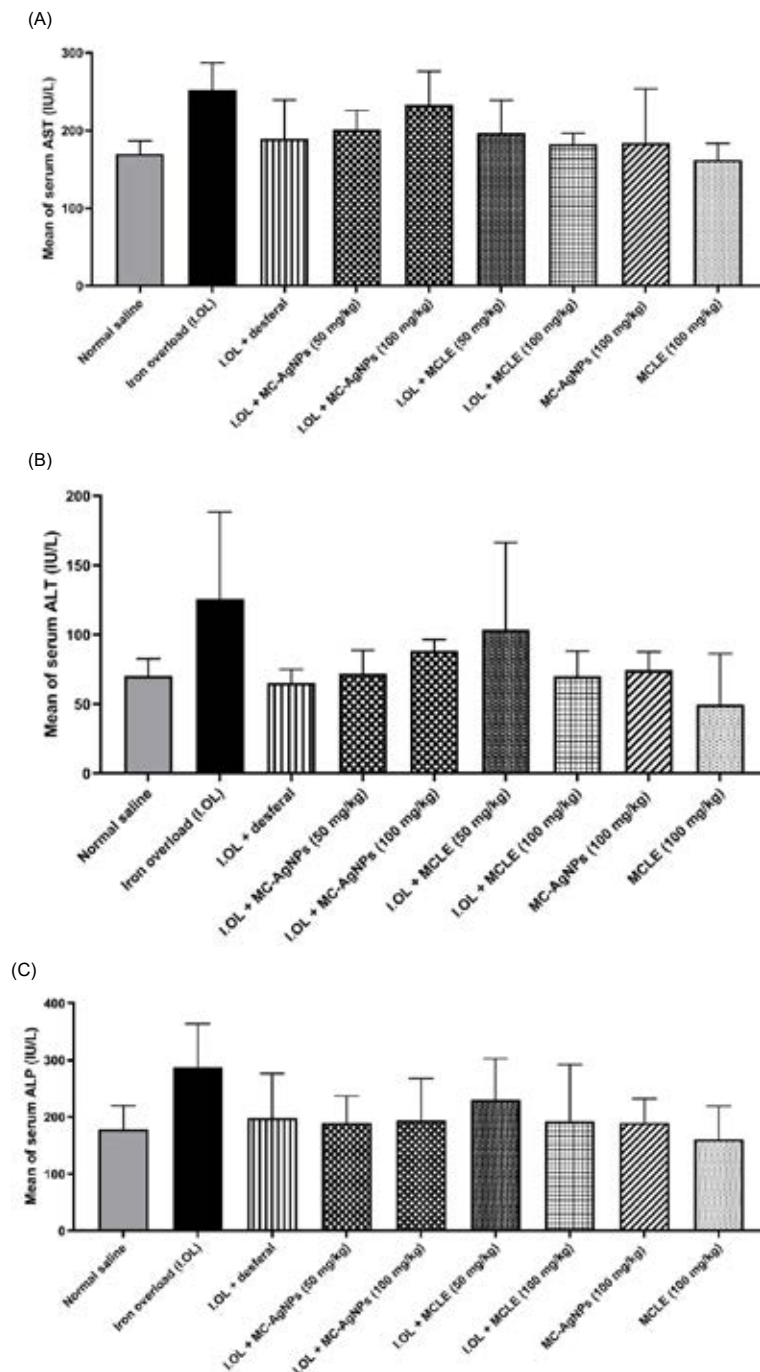


Fig. 3. Effect of *Myrtus communis*-coated Ag nanoparticles on serum content of AST (A), ALT (B) and ALP (C) in mice (n = 6) and (means \pm standard deviation, $p < 0.05$).

Subchronic (one month) toxicity study

In this part, the practical inhibition of the influence of MC-AgNPs on the hepatotoxicity of over-iron deposition in mice has been investigated primarily on the content of liver enzymes. In addition, the protective results of nanoparticles in the liver of over-iron mice have been reported, as evidenced by a reduction in tissue inflammation and iron deposition in histopathological examination compared to control mice.

Change in the serum levels of liver-function enzymes after treatment with MC-AgNPs

Liver-function enzymes can point out liver impairment, specially AST, which is greater unique to mouse liver. Rezaei and cooperators pronounced that serum ALT and AST degrees have been immediately concerned in iron poisonousness [29]. Alterations in liver characteristic have been appraised by using the degrees of these enzymes (Fig. 3A–C), elevated in over-iron mice comparative to the normal saline group (AST; $P = 0.0184$, ALP; $P = 0.1308$) and ALT; $P = 0.1498$). In over-iron mice, compared with the untreated group, MC-AgNP treatment was able to reduce the amount of AST, ALT and ALP according to the dose ($P > 0.05$). At the identical time, AST, ALT and ALP degrees of DFO-exposed mice had been additionally lessened, in contrast to those of the over-iron mice ($P > 0.05$). Overall, exposure of over-iron mice to the MC-AgNPs radically weakened the hepatic impairment (the content of liver enzymes). In summary, it was found that ALP content in the MC-AgNPs-treated group was lesser than in the DFO-exposed mice

and also closer to its control mice content.

Histopathological evaluation of the mice liver via Prussian blue staining

The liver is extra inclined to excessive iron impairment than organs. In conclusion, we utilized liver tissue to display excessive iron harm and the conceivable inhibitory outcomes of MC-AgNPs. Procedure of staining with Prussian blue was applied for pathological adjustments of the relevant tissue. Blue to black iron sediments are observable with the arrow sign in the cytoplasm of liver cells in over-iron mice with the arrow sign (Fig. 4B). After treatment with MC-AgNPs, the amount of iron sediments in the liver tissue decreased. The extra iron sedimentation in the cells helps manufacturing of reactive free radicals, as a substantial way for liver damage [30]. Because MC-AgNPs have correctly demonstrated a properly free radical and iron neutralizer undertaking [13], we imagined that they may additionally be a promising therapy for over-iron and hepatic harm brought on in mice. For this reason, in the current study, we investigated the potential of MC-AgNPs iron chelate as a talented compound. Consequences we have tested that MC-AgNP may want to limit the serum iron degrees. The MC-AgNPs have been additionally successful in lowering the iron agglomeration in liver tissue, main to diminished tissue hurt. The nanoparticles had been in a position to mitigate tissue inflammation. Overall, MC-AgNPs have been determined to be a high-quality material to avert or minimize the unsafe influences of the immoderate iron in animal. The

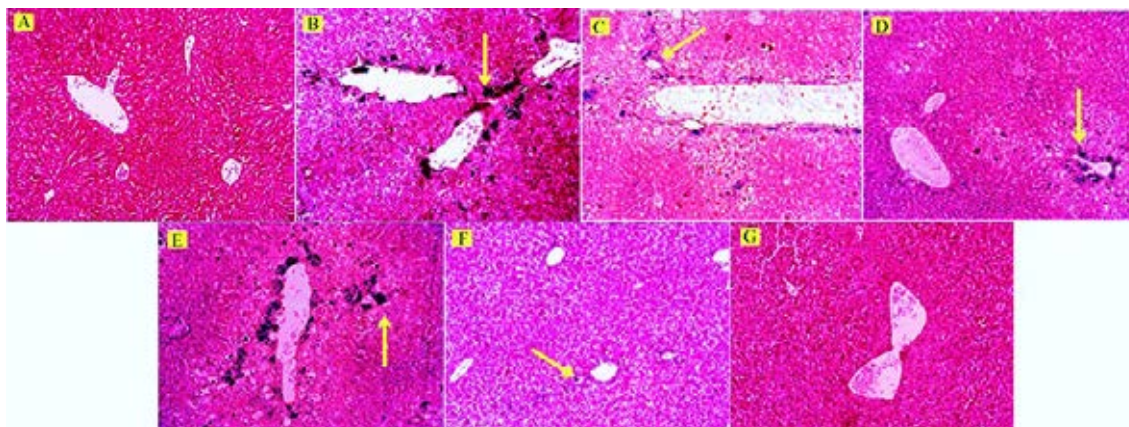


Fig. 4. The micrographs of histopathology of mice liver tissues (Prussian blue staining, $\times 400$). Group: A. Normal saline, B. iron-overloaded, C. iron-overload + DFO, D. iron-overload + MC-AgNPs, E. iron-overload + MCLE, F. MC-AgNPs and G. MCLE. The iron sediment revealed by the arrow.

protecting outcome of MC-AgNPs on the liver of mice with excess iron may be due to the antioxidant properties of the biomolecules attached to the nanoparticles [13].

CONCLUSION

An innovative and promising hybrid nanomaterial (nAg⁰ + biomolecule) is introduced to reduce excess iron ions in a thalassemia model for the first time. MC-AgNPs in the form of a colloidal solution demonstrates acceptable power for chelating of excess iron and improve pathological changes in iron overload mice, hypothetically providing an alternative creative approach for the treatment of diseases associated with excess iron. According to the results, the decent iron chelating ability of the MC-AgNPs, can be related to both the decent sorption specification of the Ag core as well as the activity of functional groups with negative charge in the biomolecules adhered to the nanoparticle surface. The results suggest a new drug program from MC-AgNP, which requires further investigation.

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CONFLICT OF INTEREST

Potential conflicts of interest were not disclosed.

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