Effect of tannic acid-templated mesoporous silica nanoparticles on iron-induced oxidative stress and liver toxicity in rats

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\textbf{A B S T R A C T}

The present study sought to investigate the effects of amino-functionalized tannic acid-templated mesoporous silica nanoparticles (TA-MS-NH\textsubscript{2} NPs) on giving rats protection against iron-induced liver toxicity. To this end, the TA-MS-NH\textsubscript{2} NPs were characterized using field-emission scanning electron microscope (FE-SEM), transmission electron microscopy (TEM), dynamic light scattering (DLS), and Fourier-transform infrared spectroscopy (FTIR). Moreover, 50 Wistar rats were randomly divided into one control group (group 1) and four experimental groups (groups 2-5) (n = 10), each of which received 100 mg/kg oral normal saline and FeSO\textsubscript{4}, respectively. Then, post-exposure hepatotoxicity and oxidative stress markers were measured in two intervals, i.e., after 4 and 24 h, followed by the measurement of the acute iron toxicity. Furthermore, hepatotoxicity markers, including the alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total antioxidant capacity (TAC), were measured via Ferri Reducing Antioxidant Power (FRAP) and 2,2,1-diphenyl-1-picrylhydrazyl (DPPH) assays. Also, malondialdehyde (MDA), total thiol groups, advanced oxidation protein products (AOPP), and nitrite/nitrate (NO\textsubscript{x}) levels were measured as oxidative stress markers in the serum samples. The results indicated that oral administration of iron significantly elevated the liver enzymes and altered the level of oxidative stress markers. It was also found that treatment with TA-MS-NH\textsubscript{2} NPs meaningfully protected against hepatotoxicity, decreased ALT, AST, ALP, and significantly improved oxidative stress markers by decreasing MDA, AOPP, and NO\textsubscript{x} levels and increasing TAC and thiol group contents, proving that TA-MS-NH\textsubscript{2} NPs could protect rats against iron-induced acute liver toxicity through their antioxidant features.

1. Introduction

Iron is known as a bio-element transition metal. However, inadequate or excessive intake of this essential micronutrient may cause physical pathological changes \cite{1}. Therefore, while low levels of iron (less than 20 mg/daily) are necessary for the human body, 20–60 mg/kg intake of iron can bring about moderate intoxication symptoms, and more than 60 mg/kg may lead to severe morbidity and mortality \cite{2}. As found by different studies, shock and liver failure are among the most common problems caused by iron intoxication \cite{3}.

In the United States, approximately 11,000 cases of iron exposures are documented among children under 5 years annually \cite{4}. However,
although the number of child deaths has currently been decreased, there is still high mortality caused by intentional or accidental iron ingestions, indicating significant toxicity of iron supplements [5].

Oxidative stress and the imbalance among the antioxidant defense are well known as the mechanism of iron overload toxicity, that accelerates the reactive oxygen species (ROS) production [6,7], that in turn may damage various cellular biomolecules such as carbohydrates, proteins, lipids, and polynucleotides, causing disease initiation and progression [9,10]. In this regard, the toxic effect of iron overload seems to be mainly associated with its participation in the Haber-Weiss and Fenton reactions that generate ROS species [8].

In contrast to iron absorption and recycling, there is no excretory mechanism for excess iron in mammalian bodies [11]. The most common treatments for excess iron include phlebotomy and erythrocytapheresis that are associated with rapid elimination of iron overload as hemoglobin and chelating drugs that bind selectively to iron and increase the removal of excess iron from the body [12]. Unfortunately, such treatments have multiple limitations and undesirable effects [12,13]. However, mesoporous silica nanoparticles (MS NPs) have recently received increasing attention in the nanomedicine field due to their unique intrinsic features [14,15]. As one of the most important classes of nanomaterials with ROS scavenging, MS NP has proved to have a protective effect on oxidative stress [16].

Many studies have so far been conducted on the preparation of MS NPs through neutral surfactants or non-surfactant templates such as tannic acid [17], tartaric acid [18], and boron oxide [19], out of which tannic acid has attracted more attention in recent years. Unlike expensive and toxic surfactants applied in traditional methods to prepare MS NPs, tannic acid is an inexpensive, non-surfactant molecule, eco-friendly, and effective antioxidant component used in preparing MS NPs [20].

As an effective natural antioxidant component, tannic acid has widely been researched in recent years [21]. While the antioxidant activity of tannic acid was previously proved to be associated with its ability to form a complex with Fe (II) and prevent the Fenton reaction [22], recent studies have shown the protective effect of tannic acid against free-radical formation and ROS scavenging [22–24]. Moreover, although some studies have suggested the protective effect of TA-MS-NH$_2$ NPs [25], its positive effect on iron-induced hepatotoxicity has not been established. Therefore, the present study sought to synthesize TA-MS-NH$_2$ NPs, determine their unique properties, and examine the antioxidant capacity of TA-MS-NH$_2$ NPs against iron-induced hepatotoxicity in rats.
2. Material and methods

2.1. Materials

Tetraethyl orthosilicate (TEOS), 3-Aminopropyltriethoxysilane (APTES), sodium acetate trihydrate, acetic acid (C$_2$H$_4$O$_2$), 2,4,6-tripyr- idyl-s-triazine (TPTZ), and tannic acid powder were purchased from Sigma-Aldrich (USA). Iron (II) sulfate heptahydrate (FeSO$_4$.7H$_2$O), ammonia solution (32 %), methanol (99.9 %), and ethanol (96 %) were purchased from MERCK. Then, the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and Alkaline Phosphatase (ALP) were measured via the enzymatic method by Pars Azmoon (Tehran, Iran) kits. Moreover, the Ferric Reducing Antioxidant Power (FRAP), 2,2,1-diphenyl-1-picrylhydrazyl (DPPH), Thiobarbituric acid reactive substances (TBARS), thiol groups, and Advanced Oxidation Protein Products (AOPP) assay kits were obtained from Zantox (Birjand, Iran). Finally, the total nitric oxide kit was purchased from ZellBio GmbH (Germany).

2.2. Preparation of TA-MS-NH$_2$ NPs

The nanoparticle synthesis was conducted based on the method presented by Jiang and colleagues [26,27]. To synthesize TA-MS-NH$_2$ NPs, 408 mg tannic acid was dissolved in 300 mL ethanol and stirred for about 5 min, and then the 150 mL ammonia solution was added under stirring. After 30 min, APTES (360 μL) was mixed with 1.8 mL of TEOS and ethanol, which was then, added dropwise to the tannic acid solution in an inert atmosphere. After vigorous stirring for 2 h, the gray precipitate was separated by centrifugation and washed with methanol solution. Then, these products were washed 5 times with water/methanol solutions to remove the free tannic acid. Finally, the precipitates were dried in a vacuum desiccator at ambient temperature.

2.3. Characterisation methods

2.3.1. Particle size and zeta potential measurement

Hydrodynamic size, polydispersity index (PDI), and zeta potential of the synthesized TA-MS-NH$_2$ NPs were characterized through dynamic light scattering (DLS) measurements using a Zetasizer Nano ZS (Malvern Instruments, UK). Before measurements, all samples were suspended in deionized (DI) water and sonicated for 10 min. The size distribution curves were reported in the intensity mode.

2.3.2. Morphology determination

The shape, size, and structure of the synthesized TA-MS-NH$_2$ NPs were analyzed using Field Emission Scanning Electron Microscope (FE-SEM) and transmission electron microscopy (TEM) (Zeiss, EM10C, Germany).

2.3.3. Fourier-transform infrared (FT-IR) spectroscopy measurements

TA-MS-NH$_2$ NPs’ FT-IR spectrum was obtained using KBr-pressed disk in the 4000–450 cm$^{-1}$ range (Perkin–Elmer, USA).
2.4. Experimental design

Fifty male Wistar rats (200–250 g body weight) were provided from the Animal Laboratory for Experimental Medicine Research Center, Birjand University of Medical Sciences. The rats were housed on a 12 h alternating light-dark cycle at a constant temperature (22 ± 1 °C) with free access to pellets and fresh water. This study was accepted by the ethical committee of Birjand University of Medical Sciences (ID: IR.iums.REC.1398.352).

The rats were randomly divided into 5 groups (n = 10 rats per group) as follows:

- **Group 1**: Control (normal saline)
- **Group 2**: Acute iron toxicity induced by FeSO₄ (100 mg/kg body weight), was evaluated after 4 h. (FeSO₄-4 h)
- **Group 3**: Acute iron toxicity induced by FeSO₄ (100 mg/kg body weight), was treated by TA-MS-NH₂ NPs (1 g/kg) after 30 min and evaluated after 4 h. (TA-MS-NH₂ NPs treated-4 h)
- **Group 4**: Acute iron toxicity induced by FeSO₄ (100 mg/kg body weight), and it was evaluated after 24 h. (FeSO₄-24 h)
- **Group 5**: Acute iron toxicity induced by FeSO₄ (100 mg/kg body weight), treated by TA-MS-NH₂ NPs (1 g/kg) after 30 min and evaluated after 24 h. (TA-MS-NH₂ NPs treated-24 h)

Eventually, the rats’ serum samples were collected after 4 or 24 h to evaluate hepatotoxicity and oxidative stress markers.

2.5. Serum biochemical measurement

The levels of serum liver enzymes (AST, ALT, and ALP) were measured by a Chemistry Analyzer (Tokyo Boeki Prestige 24i) using commercially available kits (Pars Azmoon, Tehran, Iran).

2.6. Measurement of antioxidant parameters

2.6.1. Total antioxidant activity (TAC) measurement

**FRAP assay.** The FRAP assay, first introduced by Benzie et al. [28] as a direct procedure for assessing the TAC, assesses the antioxidant capacity of samples in reducing Fe³⁺ (ferric ion) to Fe²⁺ (ferrous ion) [29]. Thus, the TPTZ working solution was prepared in this study by mixing 25 mL reaction buffer, 2.5 mL Fe³⁺ ion reaction, and 2.5 mL TPTZ solution. Using Zantox kits (Birjand, Iran), 250 μL freshly-prepared TPTZ working solution was added to 10 μL of samples in microplate wells, and it was kept at 37 °C. After 15 min, the absorbance was recorded at a wavelength of 593 nm. The results were expressed as μmol/L.

**DPPH assay.** The scavenging capacity of DPPH radical was measured by using Zantox kits (Birjand, Iran) and the slightly modified...
protocol introduced by Brand-Williams et al. [30]. Briefly, 250 μL DPPH in ethanol solution was mixed with 5 μL serum samples and 5 μL standard solutions in microplate wells. The solution was shaken well and placed in a dark environment at room temperature. The absorbance rate was recorded after 15 min at a wavelength of 517 nm using a microplate reader (Epoch). Moreover, DPPH values were calculated from standard curves using Trolox as a standard, the results of which were presented as μmol Trolox equiv/L.

2.6.2. Malondialdehyde (MDA) measurement

As a lipid peroxidation marker (LPO), the MDA serum levels were measured via Zantox kits (Birjand, Iran) using the thiobarbituric acid reactive substances (TBARS) method [31]. In short, 100 μL of the samples and standard solutions was added to the tubes. Then, 1000 μL of TBARS reagent and 10 μL of Butylated hydroxytoluene (BHT) solution were also added. The solution was incubated at 96 °C for 20 min and placed in an ice bath for another 10 min. After that, 1100 μL of n-butanol was added and centrifuged at 2000 rpm. Also, the supernatant was used directly to determine MDA, and finally, the absorbance rate was measured at 532 nm. Furthermore, 1,1,3,3 Tetramethoxypropane was used as standard, and the results were presented in μmol/L.

2.6.3. Thiol groups measurement

To measure the concentration of thiol groups, the researchers used Zantox kits (Birjand, Iran) and the Ellman’s reagent (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB) [32]. Briefly, 200 μL of reaction buffer was added to 10 μL of the samples and standard solutions. The absorbance rate was recorded at a wavelength of 412 nm (sample blank absorbance) and, then, 10 μL of DTNB reagent was added to the solution (sample absorbance). Moreover, reduced glutathione was used as a standard. Also, to determine the total thiol concentration (μmol/L), sample blank absorbance was subtracted from the sample absorbance, and, then, the thiol group concentration rate was calculated based on the standard curve. The results were presented in μmol/L.

2.6.4. Measurement of the Advanced Oxidation Protein Products (AOPPs)

AOPP concentration was measured by spectrophotometric methods [33]. The required samples were prepared as follows: Diluted sample (40 μL) was added to each well of a 96-well plate, and the standards of chloramine T (190 μL; 1.25, 2.5, 5, 10, 20, and 40 μM) were added to the plate. Then, 10 μL potassium iodide-KI (1.16 M) and 160 μL N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1 %) were added. Moreover, the absorbance of the reaction mixture was measured at a wavelength of 540 nm (OD540) using a spectrophotometer (Epoch). AOPP levels were reported in μmol/L as chloramine-T equivalents.

2.6.5. Total nitrite and nitrate (NOx) measurement

Serum NOx concentrations were measured by ZellBio GmbH (Germany) and the Griess reaction [34]. In brief, 300 μL serum samples and 10 μL sulfanilic acid were centrifuged for 10 min at 3000–4000 rpm. Then, the supernatants were used to determine NOx. Next, 100 μL of supernatant and 100 μL of standards were added to micro wells and which was followed by the addition of 100 μL Griess Reagent [50 μL sulfanilamide (2%) and 50 μL N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1 %)]. Moreover, the absorbance rate was measured after 30 min at a wavelength of 540 nm using the microplate reader (Epoch). Serum NOx level was determined based on the established linear standard curve using sodium nitrate as a standard whose result was presented in μmol/L.

2.7. Statistical analysis

The collected data were analyzed both the SPSS 16.0 software (SPSS
All statistical analyses were examined via one-way analysis of variance (ANOVA) and Tukey’s tests. P-values < 0.05 were regarded as statistically significant. The data were presented as mean ± standard deviation.

3. Results

3.1. Characterization of TA-MS-NH$_2$ NPs

The TA-MS-NH$_2$ NPs morphological features, size, and shape were examined using FE-SEM and TEM images (Fig. 1A, B). The FE-SEM image showed monodispersed TA-MS-NH$_2$ NPs with a spherical shape, and TEM images indicated the formation of spherical porous nanoparticles, suggesting that TA-MS-NH$_2$ NPs are of 140–180 nm size with rough surfaces. Moreover, DLS results showed that TA-MS-NH$_2$ NPs had an average size of 189.46 ± 1.46 nm and a PDI of 0.039 (Fig. 1C).

Additionally, the zeta potential of TA-MS-NH$_2$ NPs was +23.46 ± 1.72 mV. Also, FT-IR measurement was carried out to show the typical peaks of silica in TA-MS-NH$_2$ NPs (Fig. 2). Then, the FT-IR peaks at 3430 cm$^{-1}$ were assigned to the stretching vibration of Si–OH and adsorbed water, and the peaks at 1638 cm$^{-1}$ were assigned to bending vibration of water. It was also found that the peaks at 803 cm$^{-1}$ and 468 cm$^{-1}$ corresponded to the symmetric stretching and bending vibration of Si–O–Si, the peaks at 1122 cm$^{-1}$ corresponded to asymmetric stretching of Si-O-Si, and the peaks at 950 cm$^{-1}$ corresponded to symmetric stretching of Si–OH groups [35].

3.2. Effects of TA-MS-NH$_2$ NPs on serum enzyme levels

Administering iron in rats significantly increased serum hepatocellular injury biomarker levels (AST, ALT, and ALP). As shown in Fig. 3, while AST and ALT increased at time, the rise of the ALP level was delayed. Moreover, the activity of ALP level in the FeSO$_4$–24 h group was significantly increased compared to the FeSO$_4$-4 h group. On the other hand, although oral administration of TA-MS-NH$_2$ NPs decreased the levels of serum marker of liver enzymes, it was not significant for the ALP after 4 h.

3.3. Effects of TA-MS-NH$_2$ NPs on TAC levels

TAC results achieved by applying FRAP and DPPH methods on analyzed samples are reported in Fig. 4A and B. Accordingly, oral administration of FeSO$_4$ led to a time-dependent reduction in FRAP level compared to the control group. On the other hand, 24 h after the administration of TA-MS-NH$_2$ NPs, the FRAP level was statistically increased (605.7 ± 41.79 μM Fe(II)/L) compared to what obtained for the FeSO$_4$-24 h group (392.1 ± 71.56 μM Fe(II)/L). Moreover, there was no significant difference between TA-MS-NH$_2$ NPs treated-24 h and the control group, indicating these nanoparticles’ ability to reduce Fe$^{3+}$ to Fe$^{2+}$. It was also found that DPPH level was significantly decreased in FeSO$_4$ groups after 4 and 24 h (93.45 ± 57.38 and 58.94 ± 29.56 μM Trolox equiv/L, respectively) compared to what experienced by the control group (298.4 ± 78.68 μM Trolox equiv/L).

As shown in Fig. 4A, TA-MS-NH$_2$ NPs showed significant DPPH radical scavenging properties. Accordingly, DPPH level was statistically increased in 4 h-treated and 24 h-treated TA-MS-NH$_2$ groups (260 ± 69.01 and 241.4 ± 7913 μM Trolox equiv/L, respectively) in comparison with FeSO$_4$-4 h and FeSO$_4$-24 h groups, respectively. Furthermore, no significant difference was found between the DPPH levels of 4 h-treated and 24 h-treated TA-MS-NH$_2$ NPs and the control group, suggesting these nanoparticles’ ability to neutralize free radicals.

3.4. Effects of TA-MS-NH$_2$ NPs on MDA level

The level of MDA, which is the final product of LPO in the liver tissues, is presented in Fig. 5. The findings of this study also confirmed a significant increase in MDA levels found via TBARS in FeSO$_4$-4 h and FeSO$_4$-24 h groups (0.9775 ± 0.0378 and 1.630 ± 0.3156 μM/L, respectively) in comparison with the control group (0.6266 ± 0.0689 μM). Following exposure of nanoparticles for 4 h, no significant reduction of MDA levels was observed compared with the control group. However, after 24 h, MDA levels were significantly decreased in TA-MS-NH$_2$ NPs treated-24 h group (0.9198 ± 0.2431 μM) compared with the FeSO$_4$-24 h group (Fig. 5).

3.5. Effect of TA-MS-NH$_2$ NPs on thiol level

The level of thiol groups (-SH) was significantly decreased in FeSO$_4$-4 h and FeSO$_4$-24 h groups (273.8 ± 189.7 and 85.81 ± 52.18 μM/L, respectively) compared with the control group (p < 0.05) (484.8 ± 211.6 μM/L). In contrast, 24 h after the administration of TA-MS-NH$_2$ NPs, the level of thiol groups was significantly increased in the TA-MS-NH$_2$ NPs treated-24 h group (436.2 ± 48.48 and 495 ± 174 μM/L) compared with the FeSO$_4$-24 h group. Also, after 24 h, no significant difference was observed in thiol levels between TA-MS-NH$_2$ NPs treated-24 h group and the control groups, indicating the ability of these nanoparticles to increase the level of serum thiol to normal level after 24 h (Fig. 6).

3.6. Effects of TA-MS-NH$_2$ NPs on AOPP level

As shown in Fig. 7, after oral administration of FeSO$_4$ for 4 and 24 h, the level of serum AOPP was significantly higher in FeSO$_4$-4 h and FeSO$_4$-24 h rats (27.10 ± 12.02 and 38.00 ± 17.85 μmol chloramine T equiv/L, respectively) compared with what observed in the control group (7.628 ± 5.348 μmol chloramine T equiv/L). While there were no statistical differences between the TA-MS-NH$_2$ NPs treated-4 h and the control group, suggesting these nanoparticles’ ability to neutralize free radicals.

Inc., Chicago, IL, USA) and GraphPad Prism version 6 (San Diego, CA). All statistical analyses were examined via one-way analysis of variance (ANOVA) and Tukey’s tests. P-values < 0.05 were regarded as statistically significant. The data were presented as mean ± standard deviation.
significant differences in serum AOPP levels in the TA-MS-NH2 NPs treated-4 h group, the serum AOPP levels were significantly decreased (15.66 ± 0.65 μmol choline T equiv/L) in the TA-MS-NH2 NPs treated-24 h group. Moreover, no significant difference was found in AOPP levels between the TA-MS-NH2 NPs treated-24 h group and the control group.

3.7. Effects of TA-MS-NH2 NPs on NOx levels

Fig. 8 shows the serum NOx levels in different groups. Compared with the control group (29.04 ± 8.31 μmol/L), the serum NOx levels were found to be higher in FeSO₄-4 h and FeSO₄-24 h groups (62.47 ± 14.01 and 72.02 ± 6.13 μmol/L, respectively). The results also showed that TA-MS-NH2 NPs significantly decreased the NOx levels in TA-MS-NH2 NPs treated-4 h and TA-MS-NH2 NPs treated-24 h groups compared with what occurred in FeSO₄-4 h and FeSO₄-24 h groups (45.97 ± 5.549 and 40.31 ± 6.086 μmol/L, respectively). There was no significant difference in NOx levels between the TA-MS-NH2 NPs treated-24 h group and the control group, indicating the particles’ ability to reduce the level of serum NOx in normal level after 24 h.

4. Discussion

As found by many studies, oxidative stress is associated with the pathophysiology of many diseases. According to the findings of different studies, as iron regulate the formation of damaging oxygen radicals and oxidative injury [36,37], its level has a profound effect on various diseases. Moreover, enjoying a supreme capacity to capture heavy metals, TA-MS NPs are appropriate candidates for reducing oxidative stress [38]. Therefore, this study sought to synthesize and characterize the MS NPs using tannic acid as a natural non-surfactant molecule. To this end, before the mention, the size of TA-MS-NH₂ NPs was determined through FE-SEM, TEM, and DLS methods.

The FE-SEM and TEM images showed that TA-MS-NH₂ NPs were monodisperse spherical-like particles. The distribution of TA-MS-NH₂ NPs’ size, which was measured by DLS, revealed a hydrodynamic diameter. Also, the TA-MS-NH₂ NPs’ average size was found to be 189.466 nm with a narrow size distribution. To appraise TA-MS-NH₂ NPs as the potential antioxidant agent, we evaluated the protective role of TA-MS-NH₂ NPs against the oxidative stress changes in iron-induced hepatotoxicity in the serum samples collected from the rat’s blood.

According to the study’s findings, TA-MS-NH₂ NPs reduced iron overload-induced oxidative stress, which is referred to as an imbalance between free radicals and antioxidants and can be measured via oxidative stress-related parameters [39].

The liver is the main human body’s storage organ for iron and the excess iron contents involved in liver diseases [40,41]. This study found that the serum levels of AST and ALT were significantly higher in FeSO₄-24 h groups than in the control group and that the ALP level was increased with delay time, which is probably due to the fact that ALP level usually increases late in bile duct damage, which is associated with reduced thiol levels. It was also found that the serum levels of AST and ALT were significantly higher in FeSO₄-4 h and FeSO₄-24 h groups compared with what occurred in FeSO₄-4 h and FeSO₄-24 h groups (50.549 ± 5.549 and 40.31 ± 6.086 μmol/L, respectively). There was no significant difference in NOx levels between the TA-MS-NH2 NPs treated-24 h group and the control group, indicating the particles’ ability to reduce the level of serum NOx in normal level after 24 h.

5. Conclusion

According to the study’s results, it can be concluded that the protective effect of TA-MS-NH₂ NPs against hepatic injury in iron-treated rats is possibly related to their antioxidant features. TA-MS-NH₂ NPs could be an effective and promising treatment for iron overload. This study clearly showed that TA-MS-NH₂ NPs had hepatoprotective application in preventing or minimizing iron-induced liver damage.

Conflict of Interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

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