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BENEFICIAL IMPACTS OF N-3 POLYUNSATURATED FATTY ACIDS ON GOLD NANOPARTICLES -INDUCED LIVER INJURY BY ACTIVATING PPARγ AND NRF2/HO-1 SIGNALING PATHWAYS

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ABSTRACT

Gold nanoparticles (AuNPs) have many biomedical applications. However, the toxic impacts of these nanomaterials, due to their nanosize, surface area, and much particle number, on human health are still unclear. The present study was undertaken to explore the adverse toxic impacts of AuNPs (about 20nm) on the livers of experimental animals and the potential protective and therapeutic roles of N-3 polyunsaturated fatty acids (N-3 PUFA). Forty male adult rats were partitioned into four groups, G I normal animals; G II rats were injected with a suspension of AuNPs (20µg/Kg b.w.) for six days; GIII rats were injected with AuNPs and co -administered orally with N-3 PUFA (100 mg/Kg b. w.) for six successive days; G IV: rats were ingested orally with N-3 PUFA for 6 successive days, followed by injection with AuNPs daily for 6 days. The results showed that either protective or therapeutic administration of N-3 PUFA to AuNPs intoxicated rats, significantly decreased the increases in hepatic nitric oxide (NO) and malondialdehyde (MDA). N-3 PUFA also aggravated the elevation in the expressions of hepatic transcription factors, nuclear factor erythroid 2-related factor 2 (Nrf2) and hemeoxygenase-1 (HO-1) and significantly up-modulated the depletion in the hepatic expression of peroxisome proliferator-activated receptors-γ (PPAR γ). In addition, the present data revealed that protective or therapeutic treatment of AuNPs intoxicated rats with N-3 PUFA, markedly ameliorated the alterations in the serum hepatic function markers, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total and direct bilirubin and albumin. The current biochemical investigations were documented by histopathological observation. Conclusion, the present results may support the use of N-3 PUFA as a protective or a therapeutic agent against liver damage caused by AuNPs toxicity.

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Introduction

Nanotechnology is now considered as a future domain for treatment and diagnosis of many ailments [1]. However, the toxicity of many materials with nano-scale has been documented due to their tiny physical dimensions [2]. A report has revealed that exposure to nanoscale particles can cause inflammatory and cytotoxic impacts in comparison to larger scale particles [3]. It has shown that nano-scale particles can strongly react with biological biomolecules and have a deleterious efficacy because of their shape, nano-scale size, composition and huge particle number [2,4-5]. Gold nanoparticles (AuNPs) have attracted multiples of scientific and technological interest. This is because they are chemically stable, easily synthesized, as well as having unique optical features. AuNPs have been used in many therapeutic applications, including chemical sensing, biological imaging, drug and gene delivery, and cancer treatment. Although their benefits in clinical applications, the toxic effects of AuNPs have been reported [5]. It has been found that injection of AuNPs in animals caused pathological symptoms, including exhaustion, lack of appetite and weight and eventually death after 21 days, suggesting that AuNPs can accumulate in different animal organs including liver and damage them [2,6]. Some authors reported that liver is the most target organ of AuNPs toxicity [7-8]. The uptake of AuNPs by the liver is higher than other organs and can accumulate and exert their toxic impacts on this organ [6]. Also, some authors have found that AuNPs can stimulate the activation of

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Kupffer cells (KCs) in the liver, confirming the toxic potential of AuNPs on liver [2]. AuNPs have the ability to induce oxidative stress, increase in cellular membrane lipid peroxidation and significantly affect the gene expression in rat livers [6, 9].

Attempts to discover therapeutic drugs which are effective for mitigating liver injuries induced by AuNPs are considered very urgent in clinical conditions. Without effective treatments of liver toxicity at an early stage, an irreversible liver damage may be caused by hepatotoxicity. Therefore, it is important to find drugs that can counteract the adverse impacts of AuNPs to protect hepatocytes from damage.

N-3 polyunsaturated fatty acids (N-3 PUFA, α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid) are main fatty acids that should be ingested as a part of the diet because they cannot synthesize endogenously [10]. N-3 PUFA have a key role in the cellular membrane composition, so affecting cell liquidity and biochemical signaling of the cell membranes. N-3 PUFA have many other effects, including anti-oxidative stress, anti-inflammatory, antioxidants and hepatoprotective activities [11]. N-3 PUFA have antioxidant beneficial effect against tumor and cardiovascular illness [12]. N-3 PUFA act as a natural ligand for certain nuclear receptors known as peroxisome proliferator-activated receptors-γ (PPAR γ) that affect gene expression. PPAR γ is a nuclear receptor that functions as a ligand-activated transcription factor. N-3 PUFA are ligands for PPARγ, which have been reported to exhibit beneficial effects through the stimulation of these receptors [13]. PPARγ can regulate gene expressions by joining to DNA sequence elements, termed PPAR response elements (PPREs) which regulate many genes which are included in lipid metabolism, cell division and differentiation, and inflammatory reactions [14]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is another transcription factor that has essential impact in protecting liver against inflammation and oxidative injury via promoting antioxidant protein expression [15]. So this factor has a key role in protecting liver against oxidative stress [15]. Hemeoxygenase-1 (HO-1) is a microsomal enzyme which has an essential role in heme degradation. The expression of this enzyme is induced in response to oxidative stress in different tissues [16]. The enzyme has a key role in catabolizing heme into biliverdin and bilirubin which have potential antioxidant activities through scavenging peroxyl radical [16]. This enzyme is considered another factor that can protect the liver tissue against oxidative stress [16].

To the best of our knowledge, no previous reports have demonstrated the toxic impact of AuNPs on the expression of hepatic PPAR γ, Nrf2 and HO-1 which have the important roles in protecting liver against oxidative damage. Also, no previous studies have shown the hepatoprotective effects of N-3 PUFA against the toxic effect of AuNPs induced alterations in these markers.

The aim of the present study was to explore the adverse toxic impacts of AuNPs on oxidative stress markers (MDA and NO) as well as on the expression of hepato-protective markers, Nrf2, HO-1 and PPAR γ in rat livers. The study also was extended to investigate the potential impact of N-3 PUFA in attenuating Au NPs hepatotoxicity by modulating these markers

Materials and Methods

Chemicals

AuNPs were gotten from Sigma–Aldrich company (USA**).** N-3 PUFA were bought from General Nutrition Center (GNC) in Saudi Arabia.

Preparation and Characterization of AuNPs

AuNPs were synthesized by the reduction of gold salts (hydrogen chloroauric acid, $HAuCl_4.\ 3H_2O$) in the presence of suitable stabilizing agent to counteract nano- particle agglomeration. In brief, 100 ml of aqueous solution of HAuCl4. $3H_2O$ (1 mM) were boiled and solution of trisodium citrate (10ml, 38.8mM) was added up to the solution become wine red. The morphology and mean particle size of the prepared NPs were evaluated utilizing transmission electron microscopy. The zeta potential was evaluated by dynamic light scattering (DLS), utilizing nano zetasizer particle analyzer [17-18].

Animals and treatment

Forty adult male Wistar albino rats (150-170 g) were utilized in this study. The animals were gotten from Laboratory Animal Production, King Fahd Research Centre, King Abdulaziz University. Animals were housed under controlled conditions (23-25 ºC, humidity 50-65%, 12 h dark/light cycles). Animals were provided by standard rat pellet food and water *ad libitum*. Rat handling was performed in accordance to the roles of the King Abdul-Aziz University, Faculty of Science. The animals were left for seven days for adaptation and then classified into four groups, each of ten rats as follows:

Group I: Normal rats were injected intraperitoneally with normal saline only.

Group II: Rats were injected intraperitoneally with a suspension of AuNPs (20 µg/kg) for 6 successive days [7].

Group III: Rats were injected with AuNPs (20nm, 20 µg/Kg) and co –administered with N-3PUFA (100 mg/kg) daily for 6 days [19].

Group IV: Rats were supplemented orally with N-3 PUFA (100 mg/kg) daily for 6 days, followed by injection with AuNPs (20 µg/kg) for 6 successive days.

After the experimental duration, rats were starved for about 12 hours. Blood specimens were gathered in tubes for clotting and serum separation. The tubes were centrifuged at 2000 g for 15 min and the isolated rat sera were utilized for estimating of some biochemical markers. The animals were then scarified by decapitation and the livers were collected, washed with cold saline and utilized for biochemical and histopathological studies.

Biochemical analysis

Serum analysis

Serum ALT, AST, ALP, albumin, T-bilirubin and D- bilirubin were measured as biomarkers of liver injury utilizing an automated analyzer

Liver tissue analysis

Nitrite (as an indicator of NO production) [20] and MDA [21] levels were assayed as markers of oxidative stress. HO-1 and Nrf2 were estimated utilizing rat enzyme-linked immunosorbent assay (ELISA) Kits (MyBioSource, Canada), depending on the instructions provided by the manufacturer. PPAR-γ was measured using rat ELISA kits (LifeSpan Biosciences, USA) following the instructions supplied by the manufacturer.

Histopathological studies

Specimens of liver were immediately fixed in 10%formalin, treated with ascending concentration of ethanol and then cleared in xylol and embedded in paraffin blocks. The blocks of liver Specimen were then sectioned (3-5 µm). The sections were stained with Haematoxylin and Eosin (H&E) for light microscopic examination.

Statistical analysis

The mean values of different markers were analyzed utilizing analysis of variance (ANOVA). Values were considered statistically significant at $p \leq 0.05$.

Results

Characterization of prepared AuNPs

The mean hydrodynamic diameter and zeta potential of AuNPs were 19.620 ± 2.54 and -36.5 ± 3.5 mv respectively. TEM showed that AuNPs were spherical in shape with particle size of 24.4 ± 5.5 nm (Figure 1).

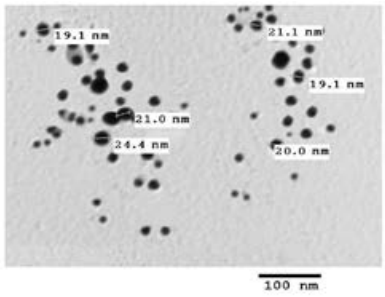

Fig 1 : Characterization of AuNPs by TEM (Mag. 13000x)

Protective and therapeutic effects of N-3 PUFA on liver damage biomarkers in AuNPs intoxicated rats

The protective and therapeutic impacts of N-3 PUFA on the concentrations of oxidative stress indices in rat groups intoxicated with AuNPs are shown in Figures 2 & 3 respectively. The data illustrated that administration of AuNPs to rats (group 2), significantly elevated the hepatic oxidative stress biomarkers, MDA (an index of oxidation of lipid) and NO (a marker of nitrosative stress) with relation to control rats (group 1, $P \leq 0.001$). Oral prophylactic (group 3) or therapeutic (group 4) ingestion of N-3 PUFA to AuNPs intoxicated rats, significantly down-modulated the hepatic elevation in the oxidative markers (MDA and NO) compared with untreated AuNPs rats ($P \leq 0.001$).

The results also showed that injection of AuNPs to rats, markedly up-regulated the expression of hepatic HO-1 and the transcription factor, Nrf2, compared with the control rats (Figures 4 & 5 respectively). Prophylactic or therapeutic treatment of AuNPs intoxicated rats with N-3 PUFA, significantly aggravated the expression of both HO-1 and Nrf2 proteins compared with AuNPs intoxicated rats ($P \leq 0.001$).

The protective and therapeutic impacts of N-3 PUFA on the level of the transcription factor PPAR-γ in animals intoxicated with AuNPs is illustrated in Figure 6. The result demonstrated that injection of AuNPs to rats, significantly down modulated the expression of hepatic PPARγ with respect to control rats ($P \leq 0.001$). Prophylactic or therapeutic ingestion of N-3 PUFA to AuNPs intoxicated rats, significantly up-regulated the expression of PPARγ versus AuNPs untreated rats ($P \leq 0.001$).

The protective and therapeutic impacts of N-3 PUFA on the levels of serum liver function indices are demonstrated in Table 1. The result showed that marked increases in ALT, AST and ALP, total and direct bilirubin with a concomitant depletion in albumin concentration in AuNPs injected rats compared with control ones ($P \leq 0.001$). Administration of N-3 PUFA to AuNPs injected rats, significantly reduced the increases in the serum liver function markers (ALT, AST ALP, total and direct bilirubin) and modulated the decrease in albumin level versus AuNPs intoxicated rats ($P \leq 0.001$).

Histopathological observation

The results of the biochemical investigation were documented by the histomorphological examination of liver tissues (Figure 7). This examination demonstrated that rats injected with AuNPs showed congestion of central blood vessels, hepatocyte vacuolation, massive infiltration of inflammatory immune cells and hepatocellular necrosis (Figure 7 b). Liver sections of AuNPs injected rats and received protective (Figure 7 c) or therapeutic (Figure 7 d) N-3 PUFA treatment , showed more or less normal liver architecture.

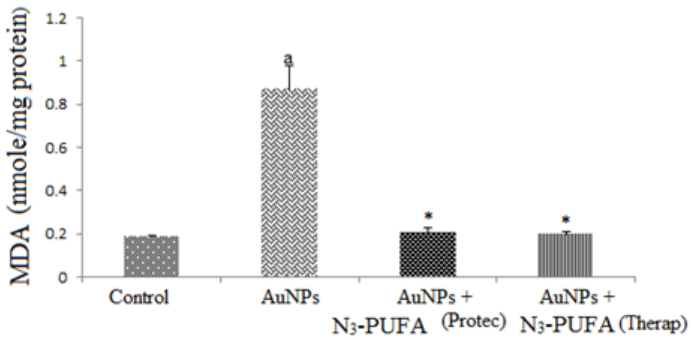

Fig 2 : Protective (Protec) and therapeutic (Therap) effect of N3 –PUFA on hepatic MDA content in AuNPs intoxicated rats. Data are presented as mean ± SD of 10 rats, $^{a}P \leq 0.001$ compared with control group (G1), $^{*}P \leq 0.001$ compared with AuNPs intoxicated group

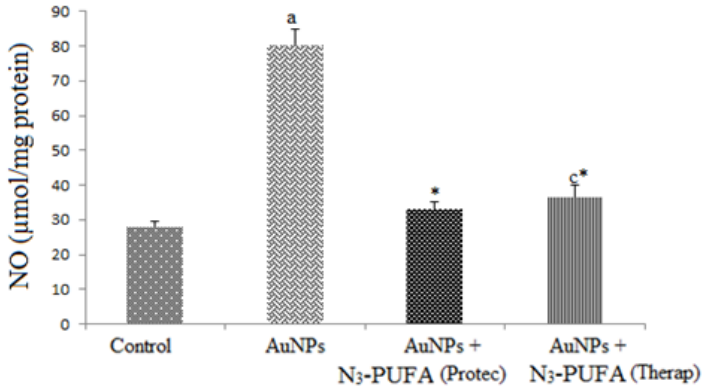

Fig 3 : Protective (Protec) and therapeutic (Therap) effect of N3 –PUFA on hepatic NO content in AuNPs intoxicated rats. Data are presented as mean ± SD of 10 rats, $^{a}P \leq 0.001$, $^{c}P \leq 0.05$ compared with control group, $^{*}P \leq 0.001$ compared with AuNPs intoxicated group

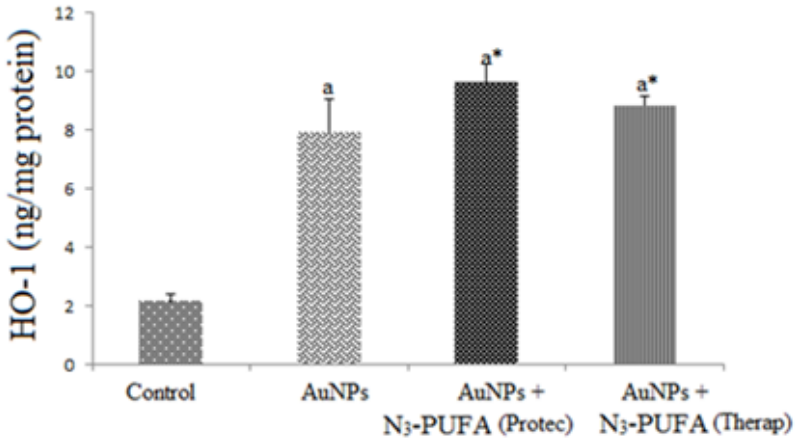

Fig 4 : Protective (Protec) and therapeutic (Therap) effect of N3 –PUFA on the level of hepatic HO-1 in AuNPs intoxicated rats. Data are presented as mean ± SD of 10 rats, $^{a}P \leq 0.001$ compared with control group (G1), $^{*}P \leq 0.001$ compared with AuNPs intoxicated group

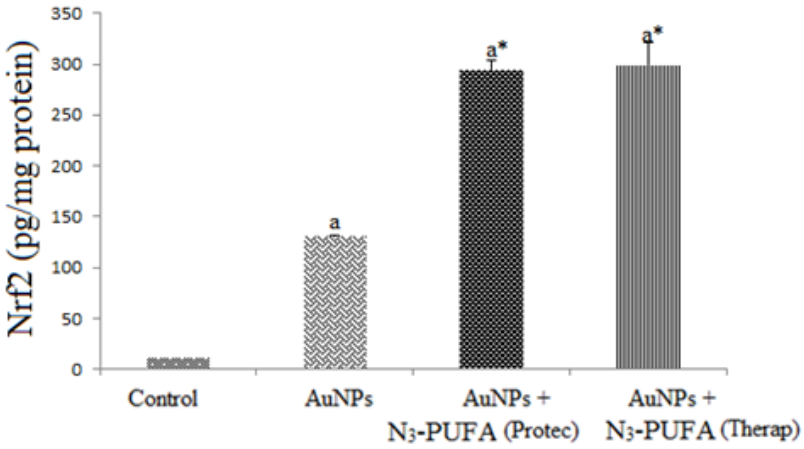

Fig 5 : Protective (Protec) and therapeutic (Therap) effect of N3 –PUFA on hepatic Nrf2 in AuNPs intoxicated rats. Data are presented as mean ± SD of 10 rats, $^{a}P \leq 0.001$ compared with control group (G1), $^{*}P \leq 0.001$ compared with AuNPs intoxicated group

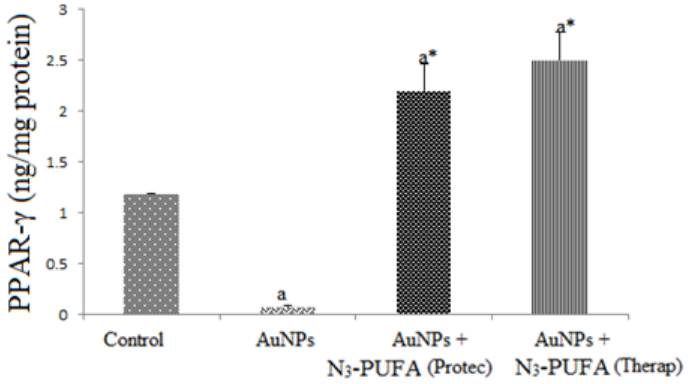

Fig 6 : Protective (Protec) and therapeutic (Therap) effect of N3 –PUFA on hepatic PPAR-γ in AuNPs intoxicated rats. Data are presented as mean ± SD of 10 rats, $^{a}P \leq 0.001$ compared with control group (G1), $^{*}P \leq 0.001$ compared with AuNPs intoxicated group

Table 1. Protective (Protec) and therapeutic (Therap) impacts of N-3 PUFA on the levels of the serum liver function markers in AuNPs treated rat groups

Parameters	Control	AuNPs	AuNPs + N-3 PUFA (Protec)	AuNPs + N-3 PUFA (Therap)
ALT	15.92± 1.43	60.50±3.41 ^{a}	34.53±0.76 $^{a*\$}$	41.55±0.42^{a*}
AST	9.81±0.62	13.59±0.32 ^{a}	9.82±0.63*	11.4±0.29 ^{b*}
ALP	47.7±4.63	127.7±4.06 ^{a}	85.45±2.8 $^{a*\$}$	54.18±2.13^{b*}
Albumin	5.18±0.18	1.88±0.06 ^{a}	3.59±0.8 ^{b*}	3.52±1.20 ^{b*}
T-Bilirubin	0.45±0.05	1.47±0.04 ^{a}	0.36±0.03 ^{b*}	0.37±0.013 ^{b*}
D-Bilirubin	0.33±0.06	1.11±0.009 ^{a}	0.32±0.06*	0.29±0.009*

Data are presented as mean ± SD of 10 rats, $^{a}P \leq 0.001$, $^{b}P \leq 0.05$ compared with control group, $^{*}P \leq 0.001$, compared with AuNPs group, $^{\$}P \leq 0.05$ compared with N-3 PUFA (Therap).

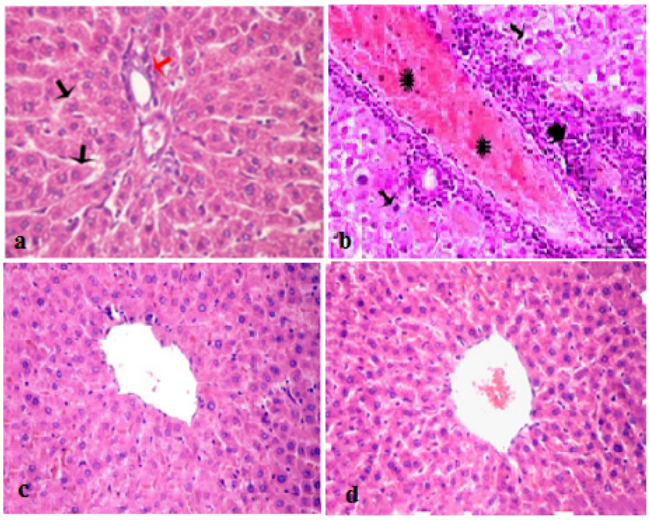

Fig 7: Light micrographs, showing the protective and therapeutic effects of N3 –PUFA on hepatic histomorphological pictures in rats exposed to AuNPs toxicity. (a) Liver section of control rat, showing normal hepatocytes (black arrow) and a prominent central vein (red arrow). (b) Liver section of rat intoxicated with AuNPs, showing congested blood vessels (*), massive infiltration of inflammatory cells (thick arrow) and hepatocellular necrosis (thin arrow). (c&d) Liver sections of AuNPs intoxicated rats and the protective (c) and the therapeutic (d) treatment with N3 –PUFA , showing normal liver architecture (H&EX400).

Discussion

Increased utilization of AuNPs in many biological and biomedical applications, have led to the risk of exposure to these particles. The objective of this investigation was to study the toxic impacts of Au NPs on rat livers and the potential protective and therapeutic roles of N-3 polyunsaturated fatty acids (N-3 PUFA).

Oxidative stress is known to have a pivotal impact in the pathogenesis of many organs in response to AuNPS toxicity [22]. The present study revealed that injection of rats with AuNPS, significantly increases the hepatic oxidative stress biomarkers, MDA (an index of lipid peroxidation) and NO (a marker of nitrosative stress) in comparable to control ones . Some authors have reported that liver is the most sensitive target organ for AuNPs toxicity [7]. Similarly, previous studies have demonstrated that exposure of experimental animals to AuNPs can induce oxidative stress in different organs including liver represented by increased lipid peroxidation and stimulation of reactive oxygen species (ROS) and nitrogen species (RNS) production with a concomitant decreased antioxidant defense system [7,22-24]. It has demonstrated that accumulation of NPs in the tissue induces stimulation of inflammatory immune cells, leading to generation of high amount of RNS, including NO, within the tissues [25]. Animal studies have showed that high concentration of $NO^{\bullet}$ causes pro-apoptotic cell death, leading to liver damage and dysfunction [26]. The toxic impact of NO• is accelerated by binding with $O2^{\cdot-}$ to give more reactive peroxynitrite (ONOO$\bar{}$), which has the ability to oxidize cellular components, causing lipid peroxidation, DNA damage, protein modification and finally cell death [27]. $ONOO^-$ can bind to the proteins of complex I in mitochondrial electron transport chain and thereby suppressing its activity [28]. Prophylactic or therapeutic administration of N-3 PUFA to AuNPs intoxicated rats, significantly down-modulated the hepatic elevation in the oxidative markers (MDA and NO) compared with untreated AuNPs rats. Similarly, a previous study revealed that N-3 PUFA have a beneficial impact in reducing lipid peroxidation in oxidative stress experimental animal model [29].

The current study revealed that injection of AuNPs to rats, markedly up-regulated the expression of hepatic HO-1and Nrf2 compared with control rats. Induction of HO-1 and Nrf2 may consider other markers of oxidative stress induced in rats under the effect of AuNPs. This result is coped with Lai *et al*. [30] who demonstrated that AuNPs induces the expression of HO-1 and Nrf2 in human endothelial cells. HO-1 has a cytoprotective impact against oxidative stress by enhancing the disintegration of pro-oxidant free heme to the radical neutralizing bile pigments, biliverdin and bilirubin [31]. Thus, HO-1 has an important role in detoxification of heme, thereby attenuating free heme accumulation and preventing the oxidative stress induced by Fe^{3+} [31]. Also, Nrf2 is an important regulatory transcription factor that protects tissues against oxidative tissue damage by regulating intracellular redox homeostasis and activating phase II antioxidants, including HO-1 [15,32]. In addition, Nrf2 can translocate from cytosol to nucleus, causing overexpression of the antioxidant defense molecules [33]. It has been found that induction of HO-1 by AuNPs is depending on the activation of the transcription factor Nrf2 [30]. These authors demonstrated that AuNPs can promote the expression of HO-1 by inducing the expression and translocation of Nrf2 protein to the nucleus[30]. Our results suggest that hepatic expression of HO-1 and Nrf2, may consider a protective survival response against

the severe hepatic toxicity of AuNPs. Prophylactic or therapeutic intake of N-3 PUFA to AuNPs intoxicated rats, significantly aggravated the expression of both HO-1 and Nrf2 protein compared with untreated AuNPs. This result suggests that the anti-oxidative impacts of N-3 PUFA against AuNPs-induced hepatotoxicity may be mediated by activation of Nrf2 and overexpression of HO-1and this may considered as one of promising therapeutic strategies to alleviate AuNPs induced hepatotoxicity.

In addition, the current study demonstrated that injection of AuNPs to rats, markedly down modulated the expression of hepatic PPARγ compared with control animals. The decreased PPARγ expression in livers of AuNPs intoxicated rats may imply a pathophysiological response to the severity of hepatiotoxic influence of AuNPs, suggesting an alteration in the expression of PPAR-γ protein caused by AuNPs toxicity. Prophylactic or therapeutic administration of N-3 PUFA to AuNPs intoxicated rats, markedly up-regulated the expression of PPAR-γ. N-3 PUFA, natural ligands (agonist) for PPAR γ, have a beneficial impact in the down-regulation of inflammatory reactions caused liver damage through stimulation of these receptors [13-14]. Evidence has suggested that activation of PPARγ by their agonists can modulate hepatotoxicity [34]. In addition, it has reported that PPAR γ agonists could repress liver fibrosis by suppression liver stellate cell division and inflammation, suggesting that PPAR γ can counteract liver toxicity [34]. Our result may indicate that the anti-hepatotoxic impact of N-3 PUFA is PPARγ dependent.

The levels of serum liver enzymes (ALT, AST and ALP), total and direct bilirubin as well as albumin are utilized as diagnostic indices of liver tissue injury [35]. In AuNPs injected rats, it was observed that, significant increases in the serum liver function markers, namely AST ALT, ALP, total and direct bilirubin compared with control rats. These results are coped with a previous study has reported that the levels of these liver function markers are significantly increased in experimental animals treated with AuNPs [36]. The elevation of serum liver enzymes is a sign of hepatic cellular leakage due to dysfunction of hepatocyte membranes in response to AuNPs hepatotoxicity. ALP is excreted with bile by the liver, increasing in serum ALP and total and direct bilirubin in rats under the effect of AuNPs toxicity is an index of failing of liver to excrete bilirubin [35]. Administration of N-3 PUFA to AuNPs injected rats, significantly reduced the increases in the serum liver function markers compared with AuNPs untreated rats, indicating to their hepatoprotective potential impact. This result may indicate that N-3 PUFA can restore the function ability of the damaged hepatic plasma membranes in response to AuNPs toxicity. Depletion of the elevated serum ALP and bilirubin in AuNPs rats treated with N-3 PUFA may imply that N-3 PUFA can ameliorate the biliary dysfunction in rats under the effect of AuNPs toxicity. The hepatoprotective impact of N-3 PUF has been documented [11]. Depletion in the serum albumin in AuNPs injected rats, is another index of hepatic abnormality caused by AuNPs toxicity. Our result may give a clue that metabolic alteration and/or modification in hepatic protein in response to AuNPs. Previous investigation has reported that tissue proteins may be influenced by the accumulation of free radicals, causing the generation of carbonyl protein derivatives. These free radicals can cause peptide breaking and oxidative alteration of amino acid side chains [37]. Treatment with N-3 PUF markedly elevated the serum content of albumin, implying the capability of N-3 PUF with their antioxidant properties, to mitigate AuNPs induced free radicals' generation, which have the major role in oxidative alteration of protein [11]. The present hepatic damage induced in rats under the effect of AuNPs toxicity is documented by hepatic histopathology. Livers of rats injected with AuNPs showed cyto-morphological changes, including congestion of central blood vessels, hepatocyte vacuolation, massive infiltration of inflammatory immune cells and hepatocellular necrosis. The adverse histocytological alterations in rat livers in response to AuNPs toxicity have been previously documented by Abdelhalim and Jarrar [38] who have suggested that the hepatic histological changes may result from interaction of AuNPs with hepatic cellular components, causing oxidative stress and inflammation.

Conclusion

The current investigation has illustrated that exposure of rats to AuNPs can induce hepatotoxicity as shown by elevation in oxidative stress markers, alteration in HO-1, Nrf2 and PPARγ, and alteration in the serum liver function markers as well as histopathological changes of liver tissue. Prophylactic or therapeutic treatment with N-3 PUF ameliorated AuNPs induced liver damage through modulating the alteration in the studied parameters as well as in histomorphological pictures of rat livers. The beneficial impact of N-3 PUF may be via activating Nrf2/HO-1 and PPAR-γ signaling pathways. So, this study may support the use of N-3 PUF as a promising hepatoprotective drug against liver damage induced by NPs toxicity.

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