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Research paper

Zinc loaded whey protein nanoparticles mitigate the oxidative stress and modulate antioxidative gene expression in testicular tissues in rats

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ABSTRACT

This study aimed to utilize whey protein nanoparticles (WPNPs) and zinc (Zn) loaded WPNPs to protect against carbon tetrachloride (CCl_4)-induced testicular damage in rats. Zn was loaded on WPNPs at three levels and Zn release was determined at different time intervals. Seventy male Wistar rats were divided into 7 groups and treated orally for 4 weeks as follows: the control group, CCl_4-treated group (0.5 ml/100 g b.w) twice a week, CCl_4 plus WPNPs-treated group (300 mg/kg b.w), CCl_4 plus Zn citrate-treated group (50 mg/kg b.w), and the groups treated with CCl_4 plus the three Zn-WPNPs formulations. Blood and testicular tissue samples were collected for different assays. Animals treated with CCl_4 showed a significant decrease in body weight and relative weight of the testis, testosterone level, antioxidant enzymes activity and mRNA expression of Nrf2, HO-1 and NQO1 and increased Malondialdehyde (MDA), nitric oxide (NO) and mRNA expression of Keep1 and induced histological changes in the testis. WPNPs alone, Zn alone or Zn loaded WPNPs at the three levels protect against CCl_4-induced testicular damage. These effects were more pronounced in the group received the fabrication with low Zn level. This study concluded that Zn-WPNPs could reduce the oxidative stress in the testicular tissue via different mechanisms mainly via the regulation of Nrf2-Keap1 antioxidative signaling pathway.

1. Introduction

Male sterility is an eminent clinical issue affecting more than 30% of men worldwide [1]. Several clinical disorders, including hypertension are almost occurred due to the over production of ROS and the decline of antioxidant capacity leading to the hypogonadism, androgens deficiency and the disorder of physiologically male sexual desire [2].

Carbon tetrachloride (CCl_4) is a widely used as an environmental toxicant model and well known to generate ROS, induce lipid peroxidation, damage to different organs, including the liver, kidneys, heart, brain and testis [3]. The mechanism by which CCl_4 induce its toxicity mainly due to the bio-activation into trichloromethyl peroxy radical ($^{\bullet}OOCCl_3$) and trichloromethyl radical ($^{\bullet}CCl_3$) by the cytochrome P450 [4].

Zinc (Zn) is an essential element affecting the majority of vital processes in the body, such as cell proliferation, immune function, the protection against oxidative stress [5]. Its role as antioxidant is mainly due to the maintaining of sufficient metallothionein levels as the essential component for Copper/Zn SOD synthesis [6]. It has a vital role in DNA repair, cell division and differentiation and neuroprotective effects [7]. Zn is found in several proteins in the cells and responsible for a variety of biological activities including development, growth and reproduction [8]. A relatively high concentration of Zn was found in the male reproductive organs and its deficiency lead to gonadotoxicity and affect the male fertility [9] and impairs the development of testis and the steroidogenesis [10].

Zn supplementation cures various diseases such as infection growth failure, wounds and skin diseases as well as cancer [11,12]. WHO recommended the use of water-soluble Zn compounds like zinc gluconate, zinc sulfate or zinc acetate as tablets or syrups for the treatment of infant diarrhea [13]. Zn citrate is a promising sensory characterizes high Zn content, water soluble, odorless and comparatively low cost [14]. Zn sulfate, Zn gluconate and Zn citrate are absorbed equally in human;

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List of abbreviations

Abbreviations	*Full form*
$^{\bullet}CCl_3$	Trichloromethyl radical
$^{\bullet}OOCCl_3$	Trichloromethyl peroxy radical
ARG	Arginine
CCl_4	Carbon tetrachloride
DNA	Deoxyribonucleic Acid
GLY	Glycine
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione -S- transferase
HIS	Histidine
HO-1	Heme oxygenase-1
HPLC	High-Performance Liquid Chromatography
Keap1	Kelch-like ECH-associated protein1
MDA	Malondialdehyde
METH	Methionine
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NO	nitric oxide
NQO1	NAD(P)H quinone dehydrogenase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SER	Serine
SOD	Superoxide Dismutase
STPP	Sodium tripolyphosphate
TEM	Transmission electron microscope
THR	threonine
VAL	Valinine
WP	Whey protein
WPNPS	Whey protein nanoparticles
ZN	Zinc
Zn-WPNPs	Zinc loaded whey protein nanoparticles

however, the absorption of ZnO is weak [15]. Zn citrate showed similar absorbability to Zn acetate, sulfate, oxide and gluconate [16] although an *in vitro* study revealed that the gluconate and oxide forms showed higher dialyzability compared to Zn citrate [17].

Although zinc oxide is cheap and has no side effects regarding the sensory perception, it has a low bioavailability due to its insolubility at the gastric pH [18]. The application of nanotechnology in the food industry is a promising tool to improve the bioavailability of nutraceutical compounds [19], particularly the poor soluble arise from their subcellular sizes [20]. Whey protein (WP) is widely used by the manufacturers of food due to its functional-values, health-promoting, nutritional and its antioxidant bioactivity [21–23] and is utilized as a food additive and a drug delivery for oral administration [19,24]. Metal ions can bind to the functional groups such as sulphydryl, carboxyl, imidazole, amino acids and peptide groups [25]. This study aimed to loading zinc citrate on whey protein nanoparticles (WPNPs) to promote the absorption of zinc and enhance its protection role against testicular oxidative damage in CCl_4-treated rats.

2. Materials and methods

2.1. Materials, chemicals and kits

Zinc citrate was purchased from El Nasr. Pharmaceutical Chemicals Co. (Cairo, Egypt). Whey protein isolate (92.6% proteins) was purchased from Davisco Foods International Inc. (MN, USA). Glutathione -S-transferase (GST), glutathione reductase (GR), glutathione (GSH) and nitric oxide (NO) kits were obtained from Eagle diagnostics (TX, USA). ^{TM}DNase and the removal reagent kit was purchased from Promega, Co. (WI, USA). Sodium tripolyphosphate (STPP) and RevertAid™ H Minus First Strand cDNA Synthesis Kits were purchased from Sigma Chemical Co. (St. Luis, Mo, USA). A radioimmunoassay (RIA) kit for testosterone was obtained from MyBioSource, Inc. (CA, USA) and malondialdehyde (MDA) kit purchased from Oxis Research™ Co. (PA, USA). TRIZOL reagent was purchased from InvitrogenTM (CA, USA). All chemicals used throughout the experiments were of the highest analytical grade available.

2.2. Determination of amino acids and preparation of whey protein nanoparticles (WPNPs)

The determination of amino acids in WPI was carried out by HPLC according to the method described by Hassan et al. [26]. WPNPs were prepared by the pH-cycling method previously described by Giroux et al. [27].

2.3. Characterization of zinc loaded whey protein nanoparticles

Zinc citrate loaded WPNPs was carried out according to the method described by Hassan et al. [26] at three concentrations: 7 mM/g (low dose; LD), 14 mM/g (medium dose; MD) and 28 mM/g (high dose; HD). The z-average diameter and size distribution of WPNPs alone or Zn-WPNPs at the three levels were carried out using Nano ZS/ZEN3600 Zetasizer (Malvern Instruments Ltd., UK) with a He/Ne laser ($\lambda = 633$ nm), refractive index 1.35 and scattering angle 90° scattering optics. The samples were prepared for TEM according to the method described by Moslehishad and Ezzatpanah [28]. The grid was air dried and examined by TEM using a JEOL JEM-1400 with an accelerating voltage of 100 kV at a magnification of 200,000 x.

2.4. In vitro zinc release

In vitro release of zinc loaded whey protein nanoparticles was determined by using a dissolution apparatus (Sotax-CH-4123 AllSCWIL/Basel Switzerland). The simulated gastric fluid was prepared according to Shao et al. [29]. In brief, 0.2 g NaCl and 0.32 g pepsin were dissolved in 70 ml Milli-Q water, and the pH was adjusted to 1.2 using 1 M HCl. The solution was placed in a 100 ml volumetric flask and diluted with water to a volume. The *in vitro* release of zinc-loaded WPNPs was evaluated according to Akbas et al. [30] with some modifications. Briefly, 10 mg of freeze-dried samples were added to 5 ml simulated gastric fluid. The mixture was incubated at 37 °C in 10 ml glass tube in a shaking water bath. Two ml of the reactant were centrifuged at 1300 rpm and 25 °C and the released of zinc in the supernatant fluid was taken at interval time ranged from 5 to 60 min then after 2 hs. Zinc was measured by atomic absorption flame spectrophotometer (Analytik Jena, Germany) computed with Aspect CS 2.1.1.0 software.

2.5. Experimental animals

Seventy sexually mature male Sprague-Dawley rats (3 months old, 120–130 g) were purchased from the National Organization of Drug Control and Research (NODCR). The animals were maintained on a standard lab diet purchased from Meladco Feed Co., (Cairo, Egypt) in filter-top polycarbonate cages in a room free from any chemical contamination under standard condition of illumination with a 12 h

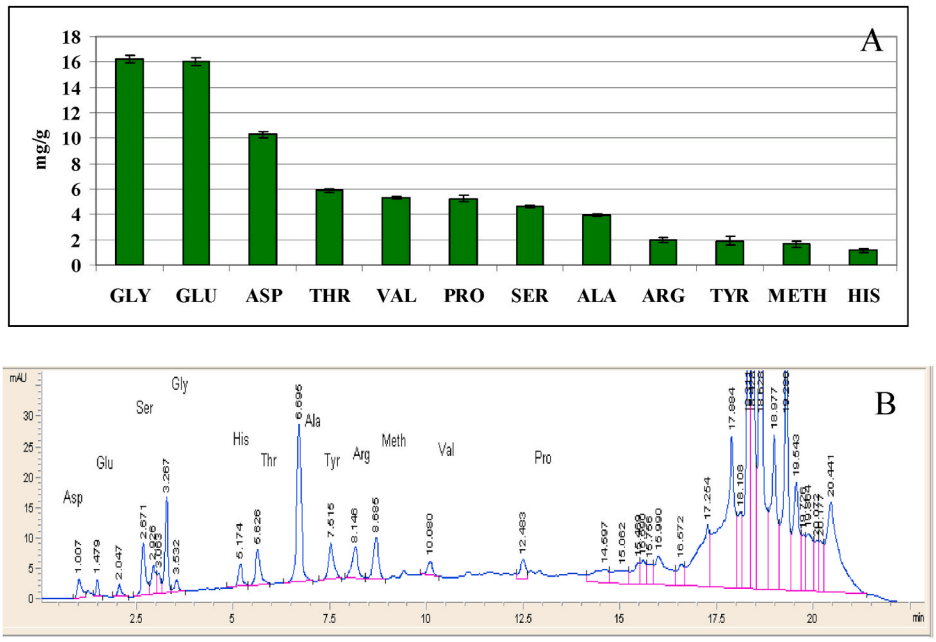

Fig. 1. Amino acids content **(A)** and HPLC chromatogram for amino acid in WPI **(B)**.

dark/light cycle, 25 ± 1^{O}C and humidity (50 ± 5%) at the Animal House Lab., NODCR, Giza, Egypt. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre and the National Institutes of Health (NIH publication 86–23 revised 1985).

2.6. Experimental design

After 1 week acclimatization period, the animals were divided into 7 groups (10 rats/group) and treated orally for four weeks included: the control group; the group treated with CCl_4 (3.3%) in corn oil (0.5 ml/100 g b.w) twice a week; the group treated with WPNP (300 mg/kg b.w.) plus CCl_4; the group treated with Zn citrate (50 mg/kg b/.w) plus CCl_4 and the groups treated with 50 mg/kg b.w of Zn1-WPNPs (7 mg/g), Zn2-WPNPs (14 mg/g) or Zn3-WPNPs (28 mg/g) plus CCl_4. Body weight was recorded in the first and last day. On day 28, all animals were fasted for 12 h and then blood samples were collected from the retro-orbital venous plexus under diethyl ether anesthesia. The serum was separated from all samples using cooling centrifugation and stored at −20 ^{O}C until analysis for testosterone determination by radioimmunoassay (RIA) according to Chen et al. [31]. After the collections of blood samples all animals were sacrificed by cervical dislocation and samples of the two testes were collected, weighted. The testes index was calculated as relative weight as follows: left testes weight/body weight × 100. Then three testis samples from each animal within different treatment groups were used for the determination of biochemical parameters, cytogenetic analysis and histological examination. One sample from each animal was dissected, weighed and homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate and was used for the determination of MDA and NO then it was further diluted to give 2% and 0.5% dilution for the determination of testicular GR, GST and GSH activities [32]. Another sample from each animal was used for the determination of the molecular genetic analyses. The third testis sample from each animal was fixed in 10% neutral formalin and paraffin embedded. Sections (5 μm thickness) were stained with hematoxylin and eosin (Hx & E) for the histological examination [33].

2.7. Molecular genetics analyses

2.7.1. RNA extraction and cDNA synthesis

Total RNA (ribonucleic acid) was extracted from testis tissue samples using TRIzol® Reagent (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and concentration of RNA were measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNase-free DNase kit (Promega) was used to remove any DNA contamination from extracted RNA. DNase-treated RNA was reverse-transcribed into first-strand cDNA (complementary DNA) using a HiSenScript TM cDNA kit (Intronbio, Korea) in a 20 μl reaction containing 1000 ng total RNA according to the method described previously [26].

2.7.2. Real-time PCR analysis

The expression of phase II detoxifying/antioxidant enzymes like nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), NAD(P)H quinone dehydrogenase 1(NQO1) and Kelch-like ECH-associated protein1 (Keap1) genes as well as Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as internal control), in all groups was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The primer sequences and their condition are shown in Table (1). RT-qPCR was carried out on Stratagene Mx3005P Real-Time PCR System (Agilent Technologies) in a 20 μL reaction volume using, 1 μl cDNA, 10 μM of forward and reverse primers, 10 μL TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics) and DNAse-free water. All samples were amplified in a minimum of triplicates. Amplification was performed with a 15 min denaturation step at 95 °C, followed by 40 cycles of 95 °C for 15 Sec, 58–60 °C for 30 Sec and 72 °C for 30 Sec. To assess amplification specificity, melting curve analysis was performed. Relative gene expression levels normalized to GAPDH were calculated using the $2^{-\Delta\Delta Ct}$ method [34] following the end of each reaction to confirm specific.

2.8. Statistical analysis

All data for biochemical parameters were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio. All statements of significance were

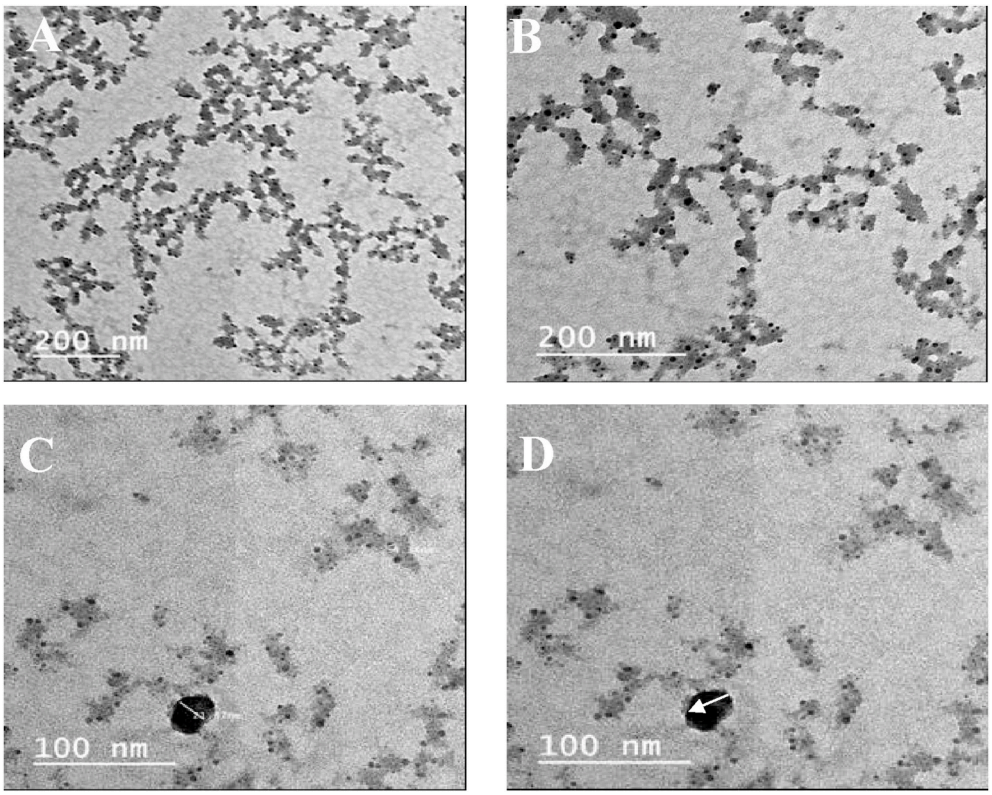

Fig. 2. TEM images for **(A)** WPNPs, **(B)** Zn1-WPNPs, **(C)** Zn2-WPNPs and **(D)** Zn3-WP-NPs.

based on probability of $P \leq 0.05$.

3. Results and discussion

The HPLC analysis showed WPI contained 12 amino acids varied in their concentrations and glycine was the higher followed by glutamic acid then aspartic acid; however, histidine and methionine were the lowest amino acids (Fig. 1a and b). Despite no available data on the amino acids in WPNPs, a previous report showed that WPI contains threonine, aspratate and alanine in high concentrations and glutamate, proline and phenylalanine were found in low concentrations [35]. In a previous work, we reported that Zn-WPNPs at the low level contain the same amino acids of WPNPs [26]. However, several amino acids included METH, GLY, SER, HIS, ARG, VAL and THR were completely disappeared by increasing Zn ratio [26]. Zn level in the three formulas was 14.3%, 32.2% and 45.3%, in Zn1-WPNPs, Zn2-WPNPs and Zn3-WPNPs, respectively. These levels were in the range recommended by UNICEF and WHO [36] for human consumption.

3.1. Characterization of Zn-WPNPs

TEM images of WPNPs alone or the three formulations of Zn-WPNPs revealed a semi spherical shape for Zn-WP-NPs. Zn appear as dark area in the images and the shape of Zn-WPNPs did not change were stable during the TEM analysis which indicated the strong binding between Zn and WPNPs. The average particle size of WPNPs and the three formulations of Zn-WPNPs were 98, 144, 197 and 230 nm, respectively (Fig. 2a,b,c and d). The ratio of Zn in the three formulas was 14.3%, 32.2% and 45.3%, respectively. These results indicated that the incorporation of Zn to WPNPs did not change the shape of particles but it only affects the particles size and their zeta potential. Previously, Gülseren et al. [37] synthesized WPNPs incorporated with Zn in the average daily dose required for the healthy adults. However, the particles size reported herein was a bit higher compared to the particles size of Gülseren et al. [38] who refer to the different method of preparation since metal ions able to bind to sulphydryl and peptides in the protein [25]. Additionally, WPNPs alone or Zn-WPNPs at the three doses showed a negative zeta potential value of −95, −114, −85 and −79, respectively which is similar to those reported in our previous work [26].

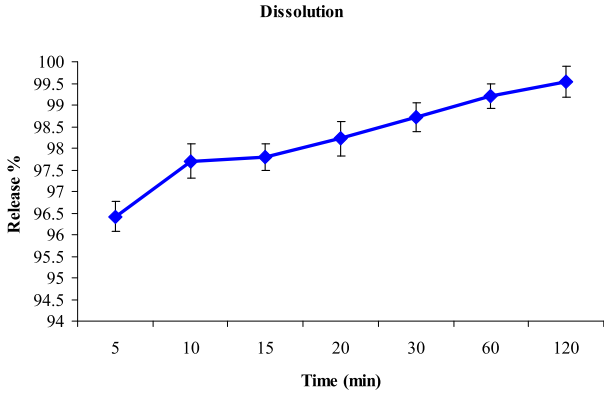

Fig. 3. Zinc release profile in gastric fluid from zinc loaded WPNPs (means ± SD).

3.2. In vitro zinc release

The data presented in Fig (3) showed the recoveries of zinc concentration at different time intervals (5, 10, 15, 20, 25, 30, 60 min and 2 h). The obtained results from 6 replicates showed that the release rate was quite high. After 5 min the release was more than 96% of the analysis and it was 97.80, 99.72, 99.20% and 99.53% after 5, 10, 15, 30, 60 min and 2 h, respectively. A high recovery of Zn was obtained after 5 min suggested that the formulas of Zn-WPNPs are more active for the stability and the duration of Zn release up to 2 h. It can be extracted that

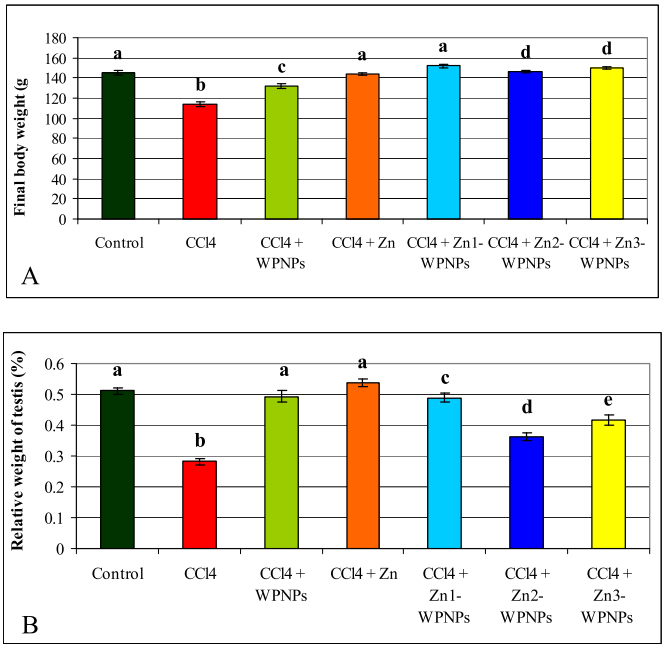

Fig. 4. Effect of WPNPs, Zn or Zn loaded WPNPs on final body weight **(A)** and relative weight of the testis **(B)**.
Within each column, means superscript with different letters (a, b, c, …) are significantly different ($P \leq 0.05$).

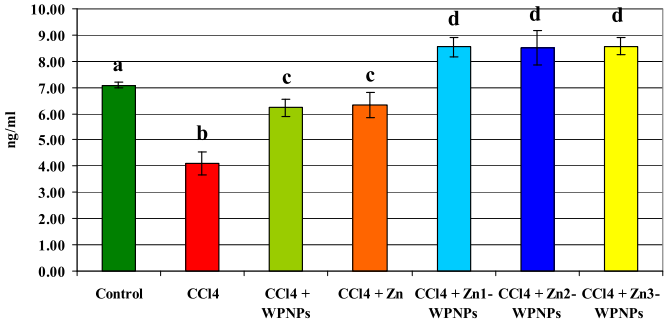

Fig. 5. Effect of WPNPs, Zn and Zn loaded WPNPs on testosterone level in rats treated with CCl_4
Within each column, means superscript with different letters (a, b, c, …) are significantly different ($P \leq 0.05$).

Table 2
Effect of WPNPs, Zn and Zn loaded WPNPs on oxidative stress markers and antioxidant enzyme activities in testes of rats treated with CCl4.

Parameter Groups	GR (μmol/g)	GST (mu/g)	GSH (mg/g)	MDA (nmol/g)	NO (μnol/g)
Control	6.84 ± 6.88^{a}	13.0 ± 0.85^{a}	285.1 ± 13.23^{a}	396.84 ± 6.88^{a}	113.0 ± 0.85^{a}
CCl_4	3.16 ± 9.27^{b}	5.33 ± 0.33^{b}	152.4 ± 1.17^{b}	650.16 ± 9.27^{b}	225.33 ± 0.33^{b}
CCl_4 + WPNPs	4.63 ± 10.17^{c}	14.0 ± 1.53^{a}	363.93 ± 24.71^{c}	428.63 ± 10.17^{c}	134.0 ± 1.53^{c}
CCl_4 + Zn	4.79 ± 3.56^{c}	14.33 ± 1.20^{a}	393.97 ± 26.02^{d}	406.79 ± 3.56^{d}	126.33 ± 1.20^{d}
CCl_4+Zn1-WPNPs	6.49 ± 1.28^{a}	16.0 ± 0.88^{c}	368.07 ± 10.40^{c}	304.49 ± 1.28^{e}	116.33 ± 0.88^{a}
CCl_4 + Zn2-WPNPs	5.49 ± 4.08^{d}	14.73 ± 0.33^{a}	353.73 ± 8.50^{e}	304.49 ± 4.08^{f}	113.33 ± 0.33^{a}
CCl_4+Zn3-WPNPs	5.27 ± 2.50^{d}	15.73 ± 0.58^{c}	325.20 ± 12.14^{f}	311.27 ± 2.50^{g}	106.0 ± 0.58^{e}

Data are presented as mean ± SE.
Within each column, means superscript with different letters (a, b, c, …) are significantly different ($P \leq 0.05$).

the WPNPs is a good carrier and could act as a reservoir for zinc and confirmed that WPNPs can be used to encapsulate, protect and enhance the release profile of micronutrients. The results indicated that WPNPs have a high ability to bind Zn which may be due to the interaction between Zn ion with a positive charge and the negatively charged amino acids cysteine and histidine [38]. The obtained data are agreed with Gülseren et al. [39] who reported that the encapsulation capacity of WPNPs for zinc retained was high and remained stable for 30 days at 22 °C.

3.3. *In vivo results*

The protective role of Zn alone WPNPs alone or Zn loaded WPNPs at three levels against CCl_4 impairs testicular function was evaluated in rats. The selective doses of CCl_4, Zn, WPNPs or Zn loaded WPNPs were based on our previous work [26]. The current results also revealed that the final body weight (Fig. 4A) and the relative weight of the testis (Fig. 4B) were significantly decreased in the group treated with CCl_4. WPNPs alone, Zn alone or Zn loaded WPNPs at the three levels ameliorated the toxicity of CCl_4 and could protect against its adverse change in the weights. The improvement in final body weight was more pronounced in the group treated with CCl_4 plus the high level of Zn (28 mM/g) loaded WPNPs. However, the improvement in the relative weight of the testis was clearer in the group treated with CCl_4 plus Zn.

The data presented in Fig. (5) revealed that CCl_4 administration

Table 1
Gene bank accession numbers, primer sequences and conditions of the genes investigated by real-time RT-PCR.

Gene	Sequence(5′→3′)	Product size(bp)	40 PCR cycles
Nrf2 (NM_031789)	F: 5′ TTTGGAGGCAAGACATAG 3′ R: 5′ TGGGCAACCTGGGAGTA 3′	253	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s
HO-1 (NM_012580)	F: 5′ TTCACCTTCCCGAGCAT 3′ R: 5′ GCCTCTTCTGTCACCCTGT 3′	110	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s
NQO1 (NM_017000)	F: 5′ CCATTCCAGCCGACAAC 3′ R: 5′ AGCCGTGGCAGAACTATC 3′	199	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s
Keap1 (NM_057152.1)	F: 5′ CTGCATCCACCACAGCAGCGT 3′ R: 5 GTGCAGCACACAGACCCCGGC 3′	250	95 °C for 30 s 58 °C for 30 s 72 °C for 30 s
GAPDH (NM_001289726.1)	F: 5′ AACTTTGGCATTGTGGAAGG 3′ R: 5′ ACACATTGGGGGTAGGAACA 3′	223	95 °C for 15 s 63 °C for 30 s 72 °C for 30 s

Nrf2: NF-E2 related factor 2; HO-1: heme oxygenase-1; Keap1: Kelch-like ECH-associated protein1; NQO1: quinone oxidoreductase 1 and GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

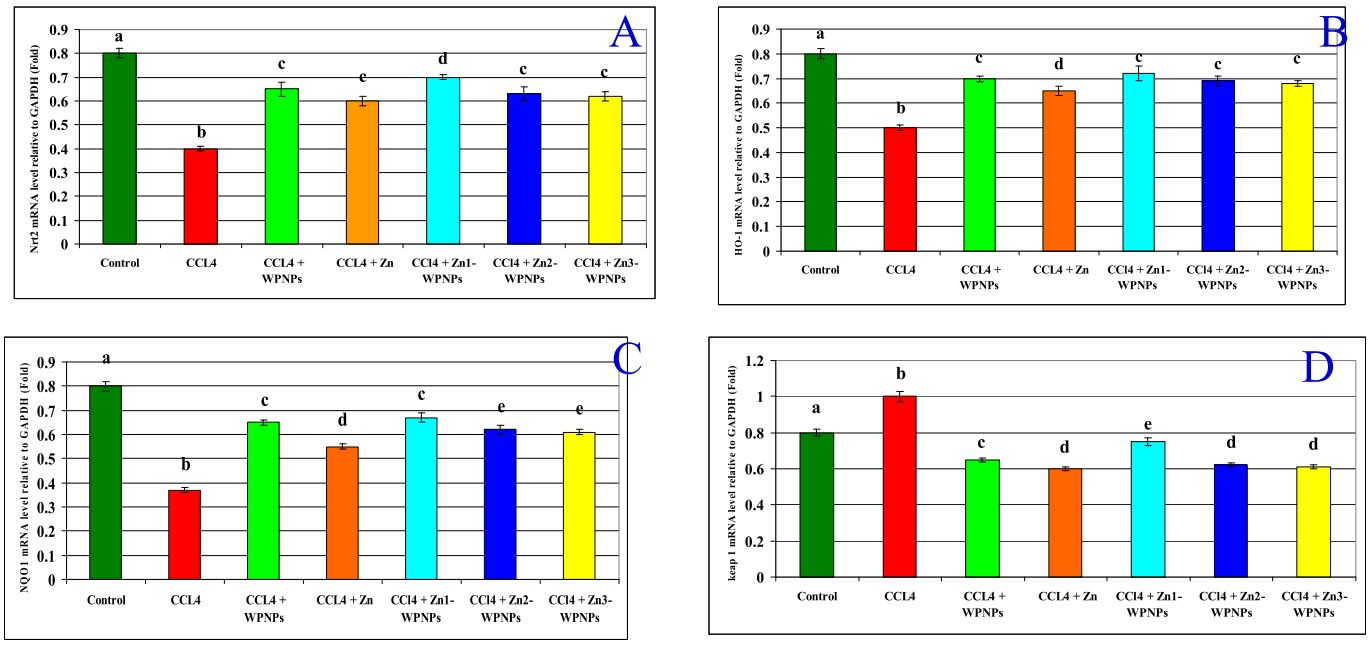

Fig. 6. Effect of WPNPs, Zn and Zn loaded WPNPs on the detoxifying/antioxidant enzymes mRNA gene expression in the testicular tissue of rats treated with CCl_4 Within each column, means superscript with different letters (a, b, c, …) are significantly different ($P \leq 0.05$).

induced a significant decrease in testosterone level compared to the control group. Co treatment with CCl_4 plus WPNPs or Zn resulted in a significant improvement in testosterone level compared to CCl_4-treated group. Moreover, no significant difference was observed in testosterone level between these two groups. On the other hand, the administration of CCl_4 plus Zn loaded WPNPs at the three tested doses induced more improvement in testosterone level with no significant difference between these groups. Furthermore, testosterone level in these groups was higher than the control group. The data presented in Table (2) showed that CCl_4 induced oxidative damage in the testis as indicated by the increase in testicular MDA and NO. WPNPs alone, Zn alone or Zn loaded WPNPs at the three tested levels succeeded to counteract the oxidative damage of CCl_4 in testicular tissue. Moreover, Zn alone was more effective than WPNPs alone and Zn loaded WPNPs induced further protection; however, the high dose of Zn loaded WPNPs was more effective compared to the other formulas. These results confirmed the earlier findings which showed that CCl_4 induce oxidative damage to several organs including the testis. *CCl_4 is well known as environmental toxicant and the basis of its toxicity lies in the free radical generation and the induction of oxidative stress via its biotransformation into trichloromethyl radical ($CCl_3^{\bullet}$) by the hepatic Cyt P-450* [40–42]. *These radicals react with the polyunsaturated fatty acids leading to the formation of covalent adducts with lipids and microsomal proteins* [43] resulting in the lipid peroxidation, damage of *cell membranes and the injury of testis* [44]. *The increase level of NO also indicated the oxidative stress generated by CCl_4. Although NO radicals play a principal role in the inflammatory response; however, their toxicity appears only after the reaction with O2•- radicals and the formation of highly reactive peroxynitrite, which attack the biomolecules such as nucleic acids, proteins and lipids* [45].

The alteration of antioxidant status in the testis of rats in different treatment groups is presented in Table (2) and showed that CCl_4 administration decreased GR, GST and GSH. Animals treated with CCl_4 plus WPNPs or Zn showed a significant improvement in the antioxidant parameters tested. Moreover, animals treated with CCl_4 plus Zn loaded WPNPs showed pronounced improvement in the testicular antioxidant and the improvement was correlated with the Zn level since the high level of Zn loaded WPNPs showed the highest improvement of the antioxidant status. The decrease in antioxidant enzymes activity GR, GST and GSH in animals treated with CCl_4 indicated the protein inactivation by ROS since oxidative damage leads to the depletion of protein function [46]. The oxidative damage in enzymes and the protein structure plays an important role in the pathophysiology of several diseases [47]. Actually, the decrease of antioxidant enzymes in the testicular tissue in CCl_4-treated animals was reported previously [48] and indicated the decrease in the bioavailability of GSH due to the enhancement of lipid peroxidation and/or the inactivation of the antioxidant enzymes during oxidative stress and the accumulation of superoxide anion O_2 [49]. CCl_4 induced a significant decrease in serum testosterone level, which may be directly through the degeneration of Leydig cells due to the excessive oxidative stress [48] or indirectly through the stimulation of P450, which catalyses the production of estrogen from androgen [50,51] as was reported by several authors [52, 53].

The effect of Zn loaded WPNPs against oxidative stress in rats treated with CCl_4 was further investigated via the evaluation of the detoxifying/antioxidant enzymes Nrf2 (Fig. 6A), HO-1 (Fig. 6B), NQO1 (Fig. 6C) and Keap1 (Fig. 6D) gene expression levels that involved in testis remodeling. The results revealed that the administration of CCl_4 induced a down regulation of Nrf2, HO-1 and NQO1 accompanied with the up-regulation of Keap1 mRNA expression compared to the control group. Co-treatment with CCl_4 plus WPNPs, Zn or Zn loaded WPNPs at the three tested levels induced a significant improvement in the mRNA expression of the tested genes. It is worthy to mention that animals treated with Zn1-WPNPs showed the best improvement in the expression of Nrf2, NQO1 and Keap-1 mRNA. However, no significant difference was observed in Ho-1 mRNA expression between the groups treated with Zn, WPNPs alone or the medium or high level of Zn loaded WPNPs although these treatments significantly attenuated the increase in Keap-1mRNA levels compared to CCl_4-treated group. Nrf2 is confined in cytosol via the interacting with the specific repressor Keap1 [54]. During the oxidative stress, the Nrf2 dissociates from Keap1 and moves to the nucleus to regulate the expression of different antioxidant-related genes such as HO-1. Hence, Nrf2 is a critical transcription factor for the protection against the excessive oxidative stress [55] and the target genes [56]. It is well documented that the Nrf2-ARE signaling is a key pathway for the cellular antioxidant and the antioxidant enzyme and the enzymes responsible for phase II detoxification are regulated via this signaling pathway to eliminate the harmful ROS [57]. Activation of Nrf2-ARE

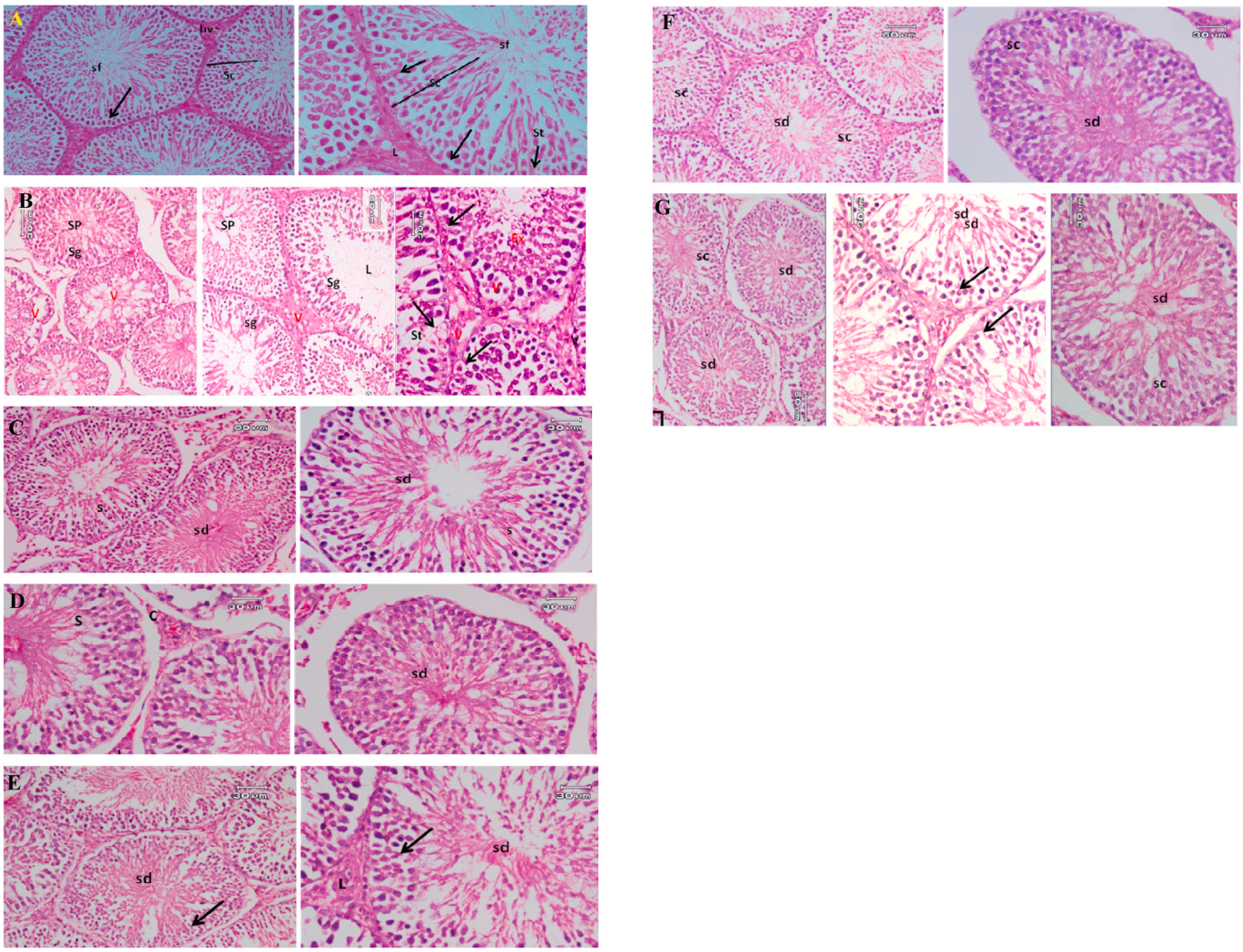

Fig. 7. Low and high power of photomicrographs of transverse section (T.S) of testis of:
A: control group showing normal seminiferous tubules with rounded and regular outline. The interstitial spaces showed Ledig cells (L) and normal capillaries (b.v). Notice the whorly appearance of sperm flagella (sf), normal spermatogenic cells (sc) formed of layers of different stages of spermatogenesis, spermatogonia cells (st), spermatocytes, spermatid (St), sertoli cell (St; small arrow) appears pyramidial and resting on the basement membrane. Notice Ledig cells in the interstitial spaces (L),
B: Testis of rats treated with CCl_4 showing disturbance and disappearance of seminiferous tubules spermatogenic series (Sg) with vacuolar degeneration and pyknotic nuclei, some tubules have enlarged lumen without sperm (L) vacuolation and exfoliation cells (EX). Enlarged interstitium with vacuolaion and aggregation of leydig's cells,
C: Testis of rats treated with CCl_4 plus WPNPs showing intact epithelium of seminiferous tubules and nearly complete spermatogenic germ cell series, spermatogonia cells, spermatocytes and spermatid (sd) with whorly appearance of sperm flagella. Few tubules show separation of spermatogenic cells from tubule basement membrane and disorganization,
D: Testis of rats treated with CCl_4 plus Zn showing restoration of the normal structure in most of seminiferous tubules and interstitium congested blood vessels. Notice, the lumen fill with sperms flagella,
E: Testis of rats treated with CCl_4 plus Zn1-WPNPs showing rearrangement of seminiferous tubules spermatogenic series, sertoli cells (arrow) and lumen filled with considerable amount of sperm (Sd). The interstitium with normal leydig's cells also seen,
F: Testis of rats treated with CCl_4 plus Zn2-WPNPs showing intact seminiferous tubules with normal spermatozoa in the lumen (Sd), complete spermatogenic germ cell series (sc) and interstitial Ledig's cells,
G: Testis of rats treated with CCl_4 plus Zn3-WPNPs showing considerable improvement in epithelium of seminiferous tubules, complete spermatogenic germ cell series, spermatogonia cells, spermatocytes, spermatid (sd) and the Sertoli cells with whorly appearance of sperm flagella. The interstitial spaces show acidophilic exudates and congested blood vessels.

signaling pathway induces the transcription of several protective genes, including HO-1 and GSH to overcome the oxidative damage [58]. On the other hand, Keep1 undergoes oxidation as a response to oxidative signals and this leads to Nrf2 stabilization [59]. Activation of Nrf2 through the binding with ARE promote the downstream gene expression mainly HO-1 which is considered the strongest antioxidant to improve cell survival [60].

The histological examination of testis of control group (Fig. 7A) showed normal seminiferous tubules with rounded and regular outline, Leydig's cells and normal capillaries were found in the interstitial spaces. The same sections showed the appearance of sperm flagella and normal spermatogenic cells formed of layers in different stages of spermatogenesis, spermatogonia, spermatocytes and spermatids. Sertoli cells were also appeared pyramidal and resting on the basement membrane. The testis sections of rats treated with CCl_4 (Fig. 7B) showed disturbance and disappearance of seminiferous tubules spermatogenic series with vacuolar degeneration and pyknotic nuclei, some tubules have enlarged lumen without sperm, vacuolation and exfoliation cells

along with enlarged interstitium with vacuolaion and aggregation of Leydig's cells. The examination of testis sections of rats treated with CCl_4 plus WPNPs (Fig. 7C) showed intact epithelium of the seminiferous tubules and nearly complete spermatogenic germ cell series, spermatogonia cells, spermatocytes and spermatids with whorly appearance of sperm flagella. Few tubules showed separation of spermatogenic cells from tubule basement membrane and disorganization. Animals treated with CCl_4 plus Zn alone (Fig. 7D) showed restoration of the normal structure in most of seminiferous tubules and interstitium congested blood vessels. The testis sections of rats treated with CCl_4 plus Zn1-WPNPs (Fig. 7E) showed rearrangement of seminiferous tubules spermatogenic series and Sertoli cells. The lumen filled appeared with considerable amount of sperm and normal Leydig's cells were seen in the interstitium. Moreover, the testis of animals treated with CCl_4 plus Zn2-WPNPs (Fig. 7F) showed intact seminiferous tubules with normal spermatozoa in the lumen and complete spermatogenic germ cell series and interstitial Ledig's cells. The animals treated with Zn3-WPNPs (Fig. 7G) showed considerable improvement in epithelium of seminiferous tubules and complete spermatogenic germ cell series, spermatogonia cells, spermatocytes, spermatid and the Sertoli cells with whorly appearance of sperm flagella. The interstitial spaces also showed acidophilic exudates and congested blood vessels. Similar observations were reported by El-Faras et al. [52] who found a marked degeneration of the spermatogenic layers and interlobular hemorrhage. Al-Olayan et al. [48] reported a prominent inflammation, complete swallowing of seminiferous tubules and degenerated germ cells in the testis or rats treated with CCl_4. Moreover, Rahmouni et al. [53] reported that CCl_4 induced disorganization and atrophy in seminiferous tubules.

Zn is the essential trace element affected the majority of different vital processes. It affects the cell proliferation, the immune system and protect against free radicals generation and oxidative stress via its role in the antioxidant activation [5]. In male reproductive organs, Zn is found in relatively high concentrations [61] and responsible for the regulation of several physiological activities including apoptosis and cell proliferation [62]. Zn is playing a critical role in the conservation of germ cells and spermatogenesis development [56]. Its deficiency leads to apoptosis to germ cells due to high oxidative stress [62]; however, excess Zn also has adverse long-term effects on the epigenome [63]. In the current study, WPNPs, Zn or Zn loaded WPNPs at the three tested levels showed a protective role against CCl_4-induced testicular oxidative stress. Animals treated with CCl_4 plus WPNPs, Zn or Zn-WPNPs showed a significant improvement in body weight, relative weight of testis, testosterone level along with a significant decrease in testicular MDA, NO and a significant increase in GR, GST and GSH. Moreover, a significant improvement was observed in the histological picture and the concerned genes expression mainly Nrf2, HO-1, NQO1 and Keap1 which are responsible for detoxifying/antioxidant enzymes. WP is widely used in the food industry due to its health-promoting and functional-values activities [22]. The protective role of WP is mainly due to its higher content of amino acids and sulfhydryl compounds which enhance the production of GSH, the main tool for the protection against oxidants [64]. Moreover, the synthesis of WPNPs enhanced the antioxidant capacity since these particles can encounter various cell types since the size of 100 nm make these particles able to enter the cell [65]. However, the protective effect of Zn is attributed to its role to protect the biological structures against free radicals through the maintenance of an adequate metallothioneins level and it is an essential for SOD synthesis [66]. Zn also protects thiol groups and prevents their interaction with other chemical and the formation of disulfide which may lead to the loss of enzyme activity [67]. Moreover, it prevents peroxidation of lipid in microsome membranes and mitochondria and stabilizes the structure of cell membrane and prevents the osmotic fragility of the erythrocyte membranes [68].

Taken together, both WPNPs and Zn are well known as protective agents against CCl_4-induced testicular damage. Interestingly, the improvement in testicular tissue in the groups treated with CCl_4 plus Zn loaded WPNPs indicated a synergistic effect of both agents. Nevertheless, the group treated with Zn1-WPNPs was better than those treated with Zn2-WPNPs and Zn3-WPNPs. This can be explained by the amount of amino acids in these formulas since some amino acids were absent by increasing the Zn level. For instance, METH, THR, HIS, VAL and ARG were absent in the formula Zn2-WPNPs and METH, GLY, SER, HIS, ARG, VAL and THR were absent in the formula Zn3-WPNPs. The disappearance of these amino acids in the two formulas reflects their antioxidant potential and their protective role against testicular oxidative damage [69].

4. Conclusion

WPNPs with average particle size of 98 nm can be synthesized. Zn loaded WPNPs at levels of 14.3%, 32.2% and 45.3% increased the particles size to 144, 197 and 230 nm, respectively. Zn release from the fabrication was 96% after 5 min and reached 99.53% after 2 h CCl_4 impairs testicular tissues, decreased testosterone level, the antioxidant enzymes activity and down-regulates mRNA expression of mRNA of Nrf2, HO-1 and NQO1, increased MDA, NO and up-regulated mRNA expression of Keep1. CCl_4 also induced histological changes in the testis. WPNPs alone, Zn alone or Zn loaded WPNPs at the three levels showed protective effects against CCl_4-induced testicular damage. These effects were more pronounced in the group received the fabrication with low Zn level. It could be concluded that Zn-WPNPs could reduce the oxidative stress in the testicular tissue via different mechanisms mainly via the up-regulation of Nrf2-Keap1 antioxidative signaling pathway.

CRediT author statement

This work was carried out in collaboration between all authors. Authors MA Hassan, AA El-Nekeety, SH Abdel-Azeim and NS Hassan carried out the experimental work and shared in writing the first draft of the manuscript. Author I Jaswir and HM Salleh managed the literature searches and performed the statistical analysis and shared in writing the first draft of the manuscript. Authors MA Abdel-Wahhab wrote the protocol, managed the project, managed the analyses of the study and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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