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In vivo Sub Acute Toxicity Studies of Zinc Oxide and Silver/Selenium Doped Zinc Oxide Nanoparticles after Oral Ingestion in Swiss Albino Mice

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The nanoparticles, such as ZnO, Ag doped ZnO ($Zn_{0.80}Ag_{0.20}O_{1-\delta}$) and Se doped ZnO ($Zn_{0.80}Se_{0.20}O_{1-\delta}$) were prepared by chemical synthesis route. In order to understand their sub-acute toxicity level in animal system, these nanoparticles were suspended in water and administered orally for 14 consecutive days to Swiss albino mice. At the end of 14th day, the animals were killed *via* cervical dislocation method. The examination of their serum and the organs, *viz.* liver and kidney was carried out. This comprises of resolving of serum biochemical levels and organ indices. A significant elevation of γ-GT, AST, ALT and LDH levels in mice when exposed with Ag/Se doped ZnO nanparticles in comparison with ZnO nanoparticles was found out. The results of enzymatic/non-enzymatic assays (protein, ROS, LPO, LDH, NO, GSH and GPx) inferred that the ZnO based nanoparticles treated mice group has shown higher levels of free radicals inside the cell and caused severe tissue damage. From AAS studies, the accumulation of zinc in the liver in mice was noticed. The result of western blotting analysis was also reported.

Keywords: ZnO nanoparticles, Oral ingestion, Swiss albino mice,Toxicity.

INTRODUCTION

Among the different nanoparticles being studied for variety of applications, zinc oxide (ZnO) nanoparticles are one among them which are mainly used in electronics and consumer applications. Since these nanoparticles have good UV absorption behaviour, they are recommended to be used in cosmetic and sunscreen applications also [1]. Since ZnO nanoparticles have tend to exhibit excellent antimicrobial properties, they used in packaging industry. They are also used as food additives, anticancer drugs, fungicides in agronomy and imaging material in biomedical fields [2]. Vimercati *et al.* [3] have investigated the toxicity level of ZnO in marine crustacean species, such as copepod *Tigriopus fulvus* and the amphypod *Corophium insidiosum.* They have finally concluded that the dissolution of zinc ions may be a valid reason for the toxicity effect of ZnO nanoparticles.

The toxicity effect of ZnO nanoparticles in human lung cells was explained by Sahu *et al.* [4]. They have found that substantial amount of lowering of GSH and enhancement in ROS levels because of germination of oxidative stress. They also concluded that oxidative stress-induced apoptosis might be the reason for the damaging of human lung cell. Cao *et al.* [5] studied the mechanism of cytotoxicity on HeLa cells using ZnO and lipid-coated ZnO with varied dimensions and weight %. Toxicity of mixture (nZnO/Zn^{2+}) was investigated on *Vibrio fischeri* by Chen *et al.* [6]. They proposed two models, *viz.* independent action (IA) and concentration addition (CA) to the level of toxicity. ZnO nanoparticles may directly enter water bodies and they can undergo physicochemical changes to become a potential toxic material. This was confirmed by verifying the toxicity level in *C. sorokiniana* by the investigation of gene expression involved in photosynthesis [7]. Adamcakova-Dodd *et al.* [8] reported that exposure of ZnO nanoparticles in mice resulted in the enhancement of macrophages in BAL fluid and a slight hike in the IL-12(p40) and MIP-1α, however no further toxicity was resulted. Liu *et al.* [9] have studied and reported the influence of ZnO nanoparticles in human nervous system.

The toxicity level of ZnO nanoparticles in embryonic/larval zebrafish was also studied and reported by Choi *et al.* [10]. The nanoparticles may affect the genes, resulting in pericardia edema in embryonic/larval growth levels. Rossner *et al.* [11] have recorded the variation in splice junction expression in genes associated with oxidative stress and inflammation.

ZnO nanoparticles have been used in biological sensors and photo-electronic devices [12]. The luminescent property of ZnO nanoparticles prepared by sol-gel method was studied by Chitradevi *et al.* [13]. ZnO doped with Ag and Se was also studied for variety of applications ranging from biotechnology to medical fields. The Se nanoparticles can exhibit good antimicrobial activity against *S. aureus* [14,15]. Majeet *et al.* [16] have synthesized Se doped ZnO nanoparticles using *Curcuma longa* extract. They have found that the oral ingestion of these particles resulted in slight changes in blood biochemistry and histopathology of important organs. It was also found that addition of metal impurities into ZnO nanopowder may be suitable for application in spintronic devices. The presence of silver in ZnO may damage human tissues [17].

However, the details on the toxicity effect of ZnO and Ag/Se doped ZnO nanoparticles in the health issues of animals are not yet investigated and published fully. Therefore, the present study has been commenced to investigate the sub-acute oral toxicity level of ZnO, Ag doped ZnO ($Zn_{0.80}Ag_{0.20}O_{1-\delta}$) and Se doped ZnO ($Zn_{0.80}Se_{0.20}O_{1-\delta}$) nanoparticles produced by chemical synthesis route. The oral ingestion of the above nanoparticles was carried out in male Swiss albino mice at different dosage levels. After 14 days, the animals were killed by dislocation method. The examination of their serum and their organs *viz.* liver and kidney was carried out. This contains finding of serum biochemical levels, organ indices and histopathological changes in a detailed manner. The obtained results were discussed in order to understand the sub-acute toxic behaviour of pure ZnO and doped ZnO based nanoparticles in animals like male Swiss albino mice.

EXPERIMENTAL

Synthesis of ZnO, Ag doped with ZnO and Se doped nanoparticles: ZnO, 20 mol% Ag doped ZnO ($Zn_{0.80}Ag_{0.20}O_{1-\delta}$) and Se doped ZnO ($Zn_{0.80}Se_{0.20}O_{1-\delta}$) nanoparticles were prepared as per the procedure reported earlier [18-20]. The prepared nanoparticles were used for sub acute toxicity examination in male Swiss albino mice without any further purification.

Histopathological examination

Animal care: The male Swiss albino mice of the age group of 11-12 weeks, weighing 25-30 g were purchased from Kerala Agricultural University, India and Tamilnadu Vertinery Animal Sciences University, Chennai, India. They were allowed to reside individually in poly propylene cages, maintained under normal laboratory conditions (temp.: 22-25 ºC, relative humidity 50-70% and 1:1 dark and light cycle) fed with normal food pellets and water *ad libitum.*

Animal ethics: As per the approval of University Animal Ethics Committee [vide approval number-IAEC/KU/BT/14/17 Dt. 11-10-2014], the experiments were conducted. The animals were maintained at University animal house, Department of Biotechnology, Karunya Institute of Technology and Sciences.

***In vivo* sub-acute toxicity studies:** The body weight of the animal was measured at the beginning and a day before the killing of the animal. The nanoparticles were dispersed in distilled water and given to the animals orally once at dose levels (per group) of 0 (control), 175, 500, 1000 and 2000 mg/kg body weight (b.w.) as per OECD guidelines (425) for analyzing acute oral toxicity chemical for the duration of 14 days. The test sample was made just before administration to the animal. The dosage level was kept as 8 mL/kg b.w. for all the animals. The animals were checked two times per day during the course of investigation. During the investigation, the general look, body position and the behavioural pattern of the animals were monitored. Other problems, such as diarrhea, salivation, convulsion, skin symptoms and lethargy were also checked. At the end of 14th day, the rats were killed *via* cervical dislocation method. Their livers and kidneys were separated, weighed and examined macroscopically for any lesions and/or abnormalities. Both the organs were maintained in formalin (10% solution) for histopathological studies. This study was done to understand the toxicity level of nanomaterials in animals by a continuous oral addition.

Biochemical assays in serum: The serum was collected by centrifuging process of the whole blood at 3,000 rpm for 20 min. The serum biochemical levels including gamma glutymal transferase (γ-GT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were assayed by a diagnostics kit (manufactured by Agappe, Kerala, India). The standards were run before measuring each assay.

Enzymatic and non-enzymatic antioxidant assay: After sacrificing of animals, their liver and kidney tissues were purified with cold salt water. The tissues were censored into pieces and homogenized with three volumes (w/v) of the specific buffer using a Polytron PT (1600E) with a Teflon pestle and centrifuged at 12,000 rpm for 20 min at 4 ºC. The supernatant liquid was taken to estimate the following biochemical parameters, *viz.* protein content, reactive oxygen species (ROS), lipid peroxidation (LPO), lactate dehydrogenase (LDH), nitric oxide (NO), reduced glutathione (GSH) and glutathione per-oxidase (GP_X). The above biochemical parameters were estimated as per standard procedures reported in the literature [21,22].

Atomic absorption analysis of Zn in the tissues of mice: After treatment, the tissues were taken from mice by survical dislocation process. The tissues were processed in HNO_3 throughout the night. The following day, 5 mL of conc. HNO_3 and $HClO_4$ mixture (6:1) was mixed in every sample and heat treated at 80-90 ºC until the solutions became colourless and clear. The solutions were further diluted with 25 mL of 1% HNO_3. This solution was used to check with atomic absorption spectrometer especially to find out the presence of zinc, if any in the tissues.

Western blotting analysis: The liver and kidney tissues of mice were standardized with ice cold RIPA lysis buffer (50 mM Tris-HCl, w/v, pH 7.4, 150 mM NaCl, w/v, 1% NP40 w/v, 0.25%, w/v sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), w/v and 1x protease inhibitor cocktail,

Sigma-Aldrich). The homogenate was centrifuged at 12,000 rpm for 20 min in 4 ºC, then the supernatant was taken and the total protein content was analyzed using the Bradford method (Sigma-Aldrich, USA) and bovine serum albumin (BSA) was practiced as the standard (1 µg/µL). Protein sample (40 µg) was equally loaded in every lane separated by 10% w/v, SDS–PAGE gel electrophoresis and changed into a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked using 5% fat free BSA and incubated throughout the night with primary antibodies against rat anti-Hsp40 (2:2000), anti-Hsp70 (2:2000), rat anti-Hsp90 (2:2000). The proteins were examined using horseradish peroxidase-conjugated anti-rat secondary antibodies (1:2500) and visualized by 3,3′-diaminobenzidine (DAB) staining kit. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was considered as the internal control to ensure equal sample loading. Densitometric experiments of immunoblots were quantified using image analysis software (NIH) [23].

RESULTS AND DISCUSSION

Serum biochemical studies: The serum biochemical results obtained on control mice and ZnO, Ag doped ZnO and Se doped ZnO treated mice are shown in Table-1. The level of enzymes such as γ-glutamyltransferase (γ-GT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) was analyzed to check the proper functioning of liver. The biochemical results revealed that the mice treated with 500 mg/kg resulted in moderate enhancement in the levels of γ-GT, AST, ALT and LDH in the serum. This implies that ZnO, Ag doped ZnO and Se doped ZnO nanoparticles might have induced hepatic injury in animals. The enzymes such as ALT, AST, LDH and γ-GT elevated in the serum either due to liver cell inflammation or due to subsequent necrosis [24].

An initial methodology in finding out liver damage is an easy blood analysis to find out the presence of certain liver enzymes in the blood. The activity of these enzymes is generally recommended to appraise the liver function. Under normal circumstances, these enzymes reside within the cells of the liver. Among various enzymes present in the liver of mice, ALT and AST were found to be more sensitive. These enzymes were generally present within liver cells. If the liver gets damaged, the liver cells may throw the enzymes into blood. The serum biochemical parameters can be convenient in the diagnosis of toxicity because they shall easily be carried out in most analytical laboratories [25].

γ-Glutamyl transferase (γ-GT): The γ-GT levels in the blood are usually high when the liver is injured. This experiment is often carried out with other testing methods that find out liver enzymes if there is a possibility of liver damage. The enzyme γ-GT is found in higher concentrations in the cytoplasm. This cytosolic enzyme is pushed into the circulation as a result of hepatocellular damage and is regularly used in the assessment of liver function [26].

From the biochemical results shown in Table-1, it was found that γ-GT level significantly increased for ZnO, Ag doped ZnO and Sn doped ZnO treated groups as 26.50 ± 0.95, 50.56 ± 1.54 and 33.22 ± 0.72, respectively compared with control group (15.51 ± 0.33). The results of the present investigation, are also in line with the earlier reports in the mice model after oral exposure of ZnO nanoparticles (600 mg/kg dosage levels) which resulted in significant increase γ-GT levels compared with control group [27]. The mice treated with 500 mg dosage of herbicide (glyphosate) group resulted in significant elevation in the γ-GT levels from 634 ± 37 (control group) to 680 ± 38 (treated group) [28]. Further, it was reported that zinc-alumina-levodopa nanocomposite treated with mice resulted in the significant elevation in γ-GT levels [29]. This test has been carried out often done with other tests in order to understand the function of liver enzymes, which in turn reveals if there is any damage in the liver.

Aspartate aminotransferase (AST): AST transfers the amino group from aspartate to α-ketoglutarate as a result aspartate is converted to oxaloacetate and α-ketoglutarate is converted to glutamate. This is a reversible reaction and requires a co-enzyme PLP (pyridoxyl phosphate).

$$\text{L-Aspartate} + \alpha\text{-ketoglutarate} \underset{\text{PLP}}{\overset{\text{AST}}{\rightleftharpoons}} \text{Oxaloacetate} + \text{L-glutamate} \quad (1)$$

It is present in all tissues of the body, inclusive of RBCs. Its amount is generally more in cardiac muscle and liver, intermediate in skeletal muscle and kidney and much lesser in other tissues. In normal serum, the transaminases are present at a low concentration, but when tissue cells containing larger amount of this enzyme is injured or killed, the enzyme diffuses into the blood stream, where a temporary increase of this enzyme activity occurs [30].

TABLE-1
SERUM BIOCHEMICAL PARAMETERS OBTAINED IN MICE AFTER ORAL EXPOSURE OF ZnO, Ag DOPED ZnO AND Se DOPED ZnO NANOPARTICLES FOR 14 CONSECUTIVE DAYS

Parameter	Results obtained from mice control	Results obtained from mice introduced with ZnO nanoparticles	Results obtained from mice introduced with Ag doped ZnO nanoparticles	Results obtained from mice introduced with Se doped ZnO nanoparticles
	(a)	(b)	(c)	(d)
γ-GT (U/L)	15.51 ± 0.33	26.50 ± 0.95	50.56 ± 1.54	33.22 ± 0.72
AST (U/L)	87.15 ± 5.34	213.37 ± 10.81	360.73 ± 10.64	257.87 ± 11.30
ALT (U/L)	43.43 ± 2.80	87.25 ± 6.36	154.41 ± 10.81	111.68 ± 5.28
LDH (U/L)	1456.05 ± 72.37	1855.2 ± 60.76	2528.33 ± 86.71	2054.51 ± 44.70

Values represent mean ± S.D of six animals (n = 6), [b]group b *vs.* group a (**$p < 0.001$), [c]group c *vs.* group a (**$p < 0.01$), [d]group d *vs.* group a (**$p < 0.01$). The entire group showing more significant when compared to control groups.

The results demonstrated that ZnO, Ag doped ZnO and Se doped ZnO treated mice show significant elevation of AST matched with control mice (Table-1). It was reported that zinc-alumina-levodopa nanocomposite nanoparticles treated mice resulted in significant elevation of AST levels in the treated group (152 ± 30) compared with control group (114 ± 11) [27]. Further, it was reported that Na_2SeO_3 nanoparticles treated mice resulted in significant elevation of AST levels in the treated group [31]. From present results, it is inferred that an enhancement level of AST is the main reasons for damaging in liver dysfunction in treated mice.

Alanine aminotransferase (ALT): ALT catalyzes the transfer of amino group from alanine to α-ketoglutarate and is converted to glutamate.

$$\text{L-Alanine} + \alpha\text{-ketoglutarate} \underset{\text{PLP}}{\overset{\text{ALT}}{\rightleftharpoons}} \text{Pyruvate} + \text{L-glutamate} \quad (2)$$

Generally, the concentration of ALT will not be really as high as that of aspartate transaminase. It may present in slightly more in liver, but will be lesser in cardiac and skeletal muscles [32]. ALT is the most frequency relied biomarker of hepatotoxicity. Enhanced amount of this enzyme is sent out during liver damage [32]. From the biochemical results on ALT, it was found that ZnO, Ag doped ZnO and Se doped ZnO nanoparticles (500 mg) treated mice groups have resulted in significant increase in the ALT enzyme when checked with control mice (Table-1). It was reported that the mice exposed orally to ZnO nanoparticles at a dosage of 300 mg showed significantly higher level of ALT compared with control mice [33]. The level of ALT was significantly increased in mice serum after administered with either low or high repeated doses of ZnO nanoparticles for 5 consecutive days [34]. Present results are in line with the reported results, which clearly show the significant damage found in liver of treated mice.

Lactate dehydrogenase (LDH): LDH is a hydrogen transfer enzyme that promotes the oxidation of L-lactate to pyruvate with the mediation of NAD^+ as hydrogen receptor.

$$\text{L-lactate} + \text{NAD} \underset{\text{pH (8.8 - 9.8)}}{\overset{\text{LDH pH (7.4 - 7.5)}}{\rightleftharpoons}} \text{Pyruvate} + \text{NADH} + \text{H}^+ \quad (3)$$

It is localized in the cytoplasm of the cells and this is sent out into the serum when the cells are damaged. LDH enzyme is found in higher concentrations in the cytoplasm. This cytosolic enzyme is released into the cytoplasm as a result of hepatocellular damage and regularly used in the assessment of liver function [35].

The LDH results revealed that the mice treated with 500 mg dosage of ZnO, Ag doped ZnO and Se doped ZnO nanoparticles resulted in moderate enhancement in the LDH levels in comparison with control group (Table-1), which infer that the liver of mice is damaged. It was reported that ZnO nanoparticles treated mice resulted in higher level of lactate dehydrogenase in serum with dosage of 400 mg/kg body weight [36]. Similarly, the mice treated with 2000 mg dosage of iron oxide nanoparticles resulted in the increase level of LDH from 6.45 ± 0.35 (control group) to 9.47 ± 0.35 (treated group) [37].

From the serum biochemical results, it was confirmed that the exposure to ZnO based nanoparticles can induce significant increase in γ-GT, AST, ALT and LDH levels compared to the control group, resulting that these particles can cause a liver dysfunction. Further, necrosis, hepatocyte swelling and congestion might result in animals due to the damage in the liver cells, such as LDH. Therefore, a high amount of these enzymes present in liver indicates the destruction of liver cells.

Enzymatic and non-enzymatic antioxidant assay: The enzymatic and non-enzymatic antioxidant assay obtained on liver and kidney samples of mice after oral exposure of 500 mg dosage with ZnO, Ag doped ZnO and Se doped nanoparticles is presented in Tables 2 and 3.

Protein: From the results, it was confirmed that the total protein content in both liver and kidney of mice has significantly reduced after the oral exposure of ZnO, Ag doped ZnO and Se doped nanoparticles for 14 days. This result suggested that the estimation of total protein in the body is useful in differentiating between normal and damaged liver function as the majority of plasma protein like albumins and globulins are formed in the liver. Total protein is often lowered slightly but the albumin to globulin ratio may decrease severely during hepatocellular injury [38].

Oxidative stress markers: The oxygen stress makers studied in this research work were ROS, LPO, LDH and NO. Large amount of ROS could be formed even when only small

TABLE-2
ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT ASSAYS ANALYZED IN LIVER SAMPLE AFTER EXPOSURE OF ZnO, Ag DOPED ZnO AND Se DOPED ZnO NANOPARTICLES IN MICE FOR 14 CONSECUTIVE DAYS

Parameter	Results obtained from mice control (a)	Results obtained from mice treated with ZnO nanoparticles (b)	Results obtained from mice treated with Ag doped ZnO nanoparticles (c)	Results obtained from mice introduced with Se doped ZnO nanoparticles (d)
Protein (mg/g)	201.46 ± 4.82	132.33 ± 3.19	114.01 ± 3.34	133.7 ± 5.03
ROS (%)	124.42 ± 3.72	237.2 ± 11.26	342.04 ± 11.57	239.36 ± 9.49
LPO (μM/g tissue)	4.50 ± 0.56	14.41 ± 1.01	20.62 ± 1.21	16.92 ± 0.83
LDH (IU/L)	230.50 ± 3.48	385.26 ± 3.44	471.11 ± 15.31	422.73 ± 7.85
NO (μM/g tissue)	14.31 ± 0.92	23.21 ± 1.32	33.56 ± 1.68*	27.42 ± 1.33
GSH (mg/mL)	7.83 ± 0.54	3.28 ± 0.42	1.36 ± 0.22	3.25 ± 0.15
GP_x (U/mg protein)	0.51 ± 0.55	0.12 ± 0.01	0.04 ± 0.01	0.09 ± 0.01

Values represent mean ± S.E of six animals (n = 6), [b]group b *vs.* group a (**$p < 0.01$), [c]group c *vs.* group a (**$p < 0.01$), group d *vs.* group a (**$p < 0.01$). All the Group is showing more significant when compared to control groups.

TABLE-3
ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT ASSAY ANALYZED IN KIDNEY SAMPLE AFTER EXPOSURE OF ZnO, Ag DOPED ZnO AND Se DOPED ZnO NANOPARTICLES IN MICE FOR 14 CONSECUTIVE DAYS

Parameter	Results obtained from mice control (a)	Results obtained from mice treated with ZnO nanoparticles (b)	Results obtained from mice treated with Ag doped ZnO nanoparticles (c)	Results obtained from mice introduced with Se doped ZnO nanoparticles (d)
Protein (mg/g)	127.30 ± 4.40	62.18 ± 3.03	41.75 ± 2.64	62.65 ± 3.03
ROS (%)	185.06 ± 3.31	247.33 ± 10.20	311.55 ± 3.13	265.55 ± 7.19
LPO (μM/g tissue)	2.83 ± 0.36	7.95 ± 0.49	14.87 ± 0.78	8.72 ± 0.54
LDH (IU/L)	86.12 ± 3.87	121.31 ± 2.47	236.25 ± 6.89	211.20 ± 7.68
NO (μM/g tissue)	11.13 ± 1.18	25.05 ± 1.23	38.90 ± 1.62	37.45 ± 1.20
GSH (mg/mL)	3.70 ± 0.33	1.60 ± 0.11	0.53 ± 0.06	1.74 ± 0.06
GP_x(U/mg protein)	0.41 ± 0.03	0.14 ± 0.01	0.075 ± 0.01	0.17 ± 0.01

Values represent mean ± S.E of six animals (n = 6), [b]group b *vs*. group a (**$p < 0.01$), [c]group c *vs*. group a (**$p < 0.01$), group d *vs*. group a (**$p < 0.01$). The entire group is showing more significant when compared to control groups.

amount of ZnO, Ag doped ZnO and Se doped ZnO nanoparticles are incorporated in to the cells. Upon entering the cell, particles may bring intracellular oxidative stress by disturbing the balance between oxidant-antioxidant processes. Excessive oxidative stress may also change proteins, lipids and protein and nucleic acid, which further encourages the antioxidants defense system or even leads to cell death. The small sized and large surface area of nanoparticles may be the main reason attributed for ROS over generation in mice found in present study. Excessive formation of ROS can be a crucial role in the induction and progression of several diseases in organs of animals including liver [39]. A significant elevation of ROS in the liver tissue was found out in a group treated with ZnO based nanoparticles prepared by chemical method. Present results indicated that the liver ROS levels are elevated in the mice treated with 500 mg dosage of ZnO nanoparticles (237.2 ± 11.26) compared with control mice (124.42 ± 3.72). The ROS levels in kidney were enhanced promptly after oral exposure of ZnO nanoparticles in mice from 185.06 ± 3.31 (control mice) to 247.33 ± 10.20 (treated mice), which is similar to the result obtained in liver tissues. A significant elevation of ROS in the liver and tissues was observed in group treated with Ag and Se doped ZnO nanoparticles (Tables 2 and 3). This may be due to the phagocytosis of nanoparticles by erythrocytes which in turn activate a membrane bound to NADPH oxidase leading to increase in the ROS level [40].

Biomarkers are helpful tools in toxicological studies, because their responses integrate spatial and temporal variations in environment, modulating the exposure of organisms to contaminants. Lipid peroxidation (LPO) is reported as a non-specific biochemical marker. Malodialdehyde (MDA) is an index of lipid peroxidation damages, which can crosslink cell membrane phospholipids [41]. The significant increase of LPO levels in the liver of mice treated with 500 mg dosage of ZnO nanoparticles (14.41 ± 1.01) compared with control mice (4.50 ± 0.56) was found in present study. Similarly, LPO level also was significantly increased in the kidney of mice treated with 500 mg dosage levels after 14 consecutive days (7.95 ± 0.49) when compared with control mice (2.83 ± 0.36). Also, LPO level was considerably enhanced in both liver and kidney of mice treated Ag and Se doped ZnO nanoparticles. It was reported in the literature that LPO level has increased after exposure of ZnO nanoparticles from 196.32% to 231.58% [42].

Lactate dehydrogenase (LDH) helps in energy production. Elevated levels of this enzyme are released from damaged cells in many areas of the body, including the liver. It also helps in detecting hepatocellular necrosis [43]. Enormous level of enhancement of LDH in both liver and kidney of mice treated with 500 mg dosage of ZnO, Ag and Se doped ZnO nanoparticles was found in this study (Tables 2 and 3). Similar studies were reported with the mice treated with ZnO nanoparticles *via* inhalation method for 14 days in which LDH levels changed from 63 ± 18 (control group) to (93 ± 23) for treated group [44].

The hepatic nitric oxide (NO) level was increased significantly in liver of mice treated with ZnO nanoparticles after 14 consecutive days of oral exposure from 14.31 ± 0.92 (control group) to 23.21 ± 1.32 (treated group) [45]. It was found that the mice treated with 500 mg dosage levels resulted significant increase in the NO level in the kidney samples (25.05 ± 1.23) compared with control mice (11.13 ± 1.18). Similar results were found for the liver and kidney of mice treated with Ag and Se doped ZnO nanoparticles (Tables 2 and 3).

Enzymatic assay (GSH and GSH-Px): The GSH levels in liver of mice exposed with ZnO nanoparticles were decreased from 7.83 ± 0.54 (control group) to 3.28 ± 0.42 (treated group). Similar studies were carried out by the researchers in mice by treating with ZnO nanocomposite (ZnO/ascorbyl palmitate nanocomposite) and reported that GSH level decreased from 147.66 ± 7.44 (control group) and 138.00 ± 8.71 (treated group) [46]. Similarly, the GSH levels were reduced in the mice treated with Ag and Se doped ZnO in current study.

Glutathione peroxidase (GSH-Px) results suggested that the mice treated with 500 mg dosage of ZnO nanoparticles resulted in decrease in the treated group (0.12 ± 0.01) compared with control group (0.51 ± 0.55) in the liver sample. The GSH-Px results suggested that mice treated with 500 mg dosage of Ag doped ZnO nanoparticles resulted in decrease in the treated group (0.14 ± 0.01) compared with control group (0.41 ± 0.03) in the kidney sample. Generally, for all the samples, the reduction in GSH-Px level was noticed. The variations in the enzymatic/non-enzymatic assays in the treated sample may be due to the presence of ZnO, Ag doped ZnO and Se doped

ZnO nanoparticles in the liver and kidney tissues of the mice sample as reported [47].

AAS analysis: The significant enhancement in the zinc content was found in the liver of mice treated with 500 mg/kg ZnO nanoparticles (1.85 ± 0.10 ppm) after 14 days as compared to the control (0.53 ± 0.02 ppm). The Zn content was increased in the liver of mice treated with 500 mg/kg Ag doped ZnO nanoparticles (1.28 ± 0.13 ppm) after 14 days as compared to the control (0.53 ± 0.02 ppm). Further, Zn content was found in the liver of mice treated with 500 mg/kg Se doped ZnO nanoparticles (1.48 ± 0.16 ppm) after14 days as compared to the control (0.53 ± 0.02 ppm). Zinc is required in small quantities by the body, however higher levels are toxic which may introduce apoptosis or sometimes necrosis. These results suggested that the accumulated zinc in the liver may lead to cellular injury after sub-acute oral exposure of ZnO, Ag and Se doped ZnO nanoparticles [35].

Western blotting analysis

ZnO treated mice: Western blot is a method that is highly recommended tool for protein detection as it allows the user to quantify the protein expression as well. The family of heat shock proteins (Hsps) was initially analyzed as a highly conserved battery of genes whose expression could be induced by heat shock. Heat shock proteins play a prominent role in many of the cell's most basic processes [48]. Heat shock proteins (Hsps) of liver expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiments showing protein levels in control and ZnO treated mice are shown in Fig. 1a. The histogram showing (Hsp40, Hsp70 and Hsp90) levels obtained in the liver of mice after oral ingestion of ZnO nanoparticles is shown in Fig. 1b. Heat shock proteins of kidney expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiments showing protein levels in control and ZnO treated mice are shown in Fig. 2a. The histogram showing (Hsp40, Hsp70 and HspSP90) levels obtained in kidney of mice after oral ingestion of ZnO nanoparticles is shown in Fig. 2b.

Many heat shock proteins function together in co-chaperone complexes, such as Hsp70/Hsp40 (bacterial DnaK/DnaJ) that along-with GrpE acts as an ATP-regulated shuttle complex for newly prepared proteins. Heat shock proteins serve as the molecular sensor to protect the liver from various xenobiotic insults. In the present study, we tested the changes in the protein expression level of Hsp40, Hsp70 and Hsp90 in liver of control and experimental groups (Fig. 1b). A significant decreased expression of Hsp40 was observed in the liver of ZnO treated mice when compared to control. Besides, an increased protein expression of Hsp90 and Hsp70 was found in the liver of ZnO treated mice when compared to control [49].

In present study, the changes in the protein expression levels of Hsp40, Hsp70 and Hsp90 in the kidney of control and experimental groups (Fig. 2b) were tested. An increased protein expression of renal Hsp40, Hsp70 and Hsp90 were found in the ZnO nanoparticles treated mice when compared to control. From the studies, it was found that the Hsp protein levels varied because of the occurence of ZnO nanoparticles in the organs of liver and kidney of mice and in turn which may be due to the injured organs.

Silver doped ZnO treated mice: Heat shock proteins (Hsps) of liver expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiments showing protein levels in control and Ag doped ZnO treated mice are shown in Fig. 3a. The histogram showing (Hsp40, Hsp70 and Hsp90) levels obtained in the liver of mice after oral ingestion of ZnO nanoparticles is shown in Fig. 3b. Heat shock proteins of kidney expression (Hsp40, Hsp70 and Hsp90) levels and the corresponding western blotting experiments showing protein levels in control and Ag doped ZnO treated mice are shown in Fig. 4a. A histogram showing (Hsp40, Hsp70 and HspSP90) levels

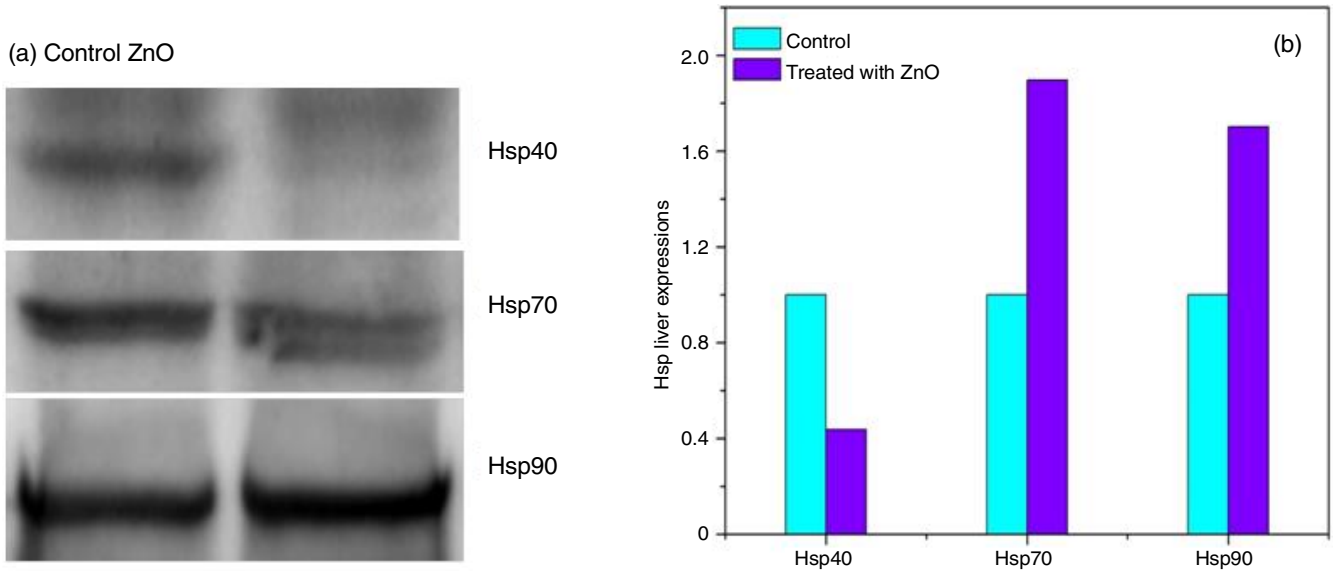

Fig. 1. (a) Heat shock proteins (Hsps) of liver expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiments showing protein levels in control and ZnO treated mice, (b) Histogram showing (Hsp40,Hsp70 and Hsp90) levels obtained in the liver of mice after oral ingestion of ZnO nanoparticles

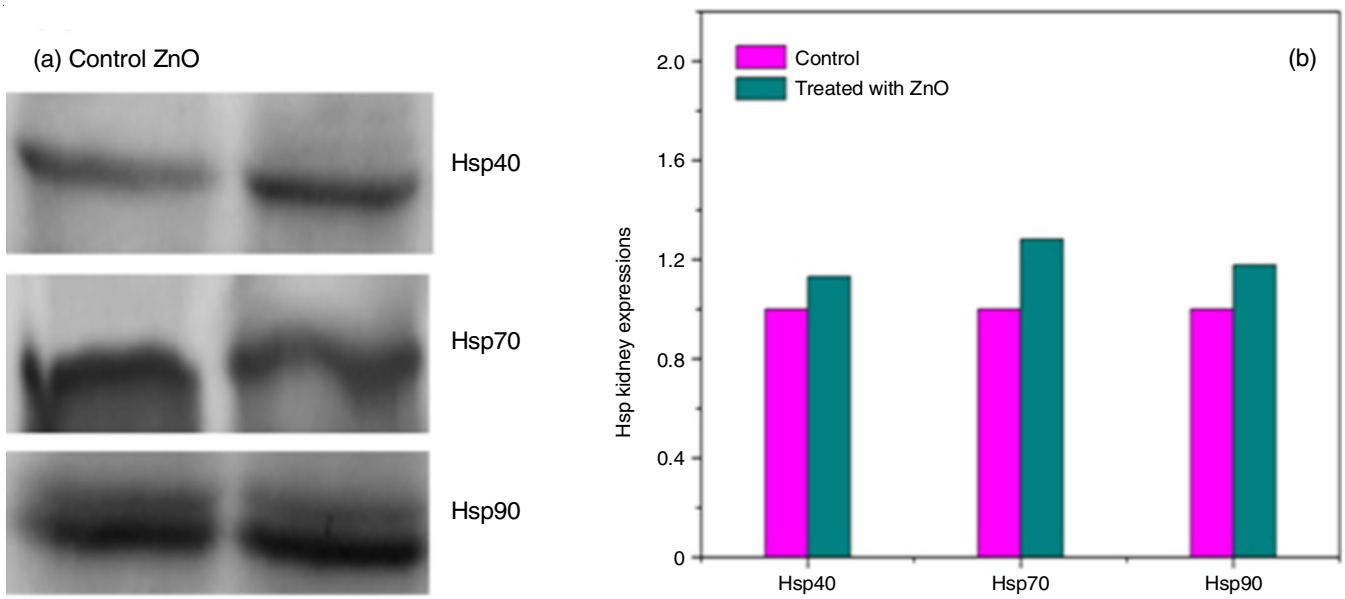

Fig. 2. (a) Heat shock proteins (Hsps) of kidney expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiment showing protein levels in control and ZnO treated mice, (b) Histogram showing (Hsp40, Hsp70 and Hsp90) levels obtained in the kidney of mice after oral ingestion of ZnO nanoparticles

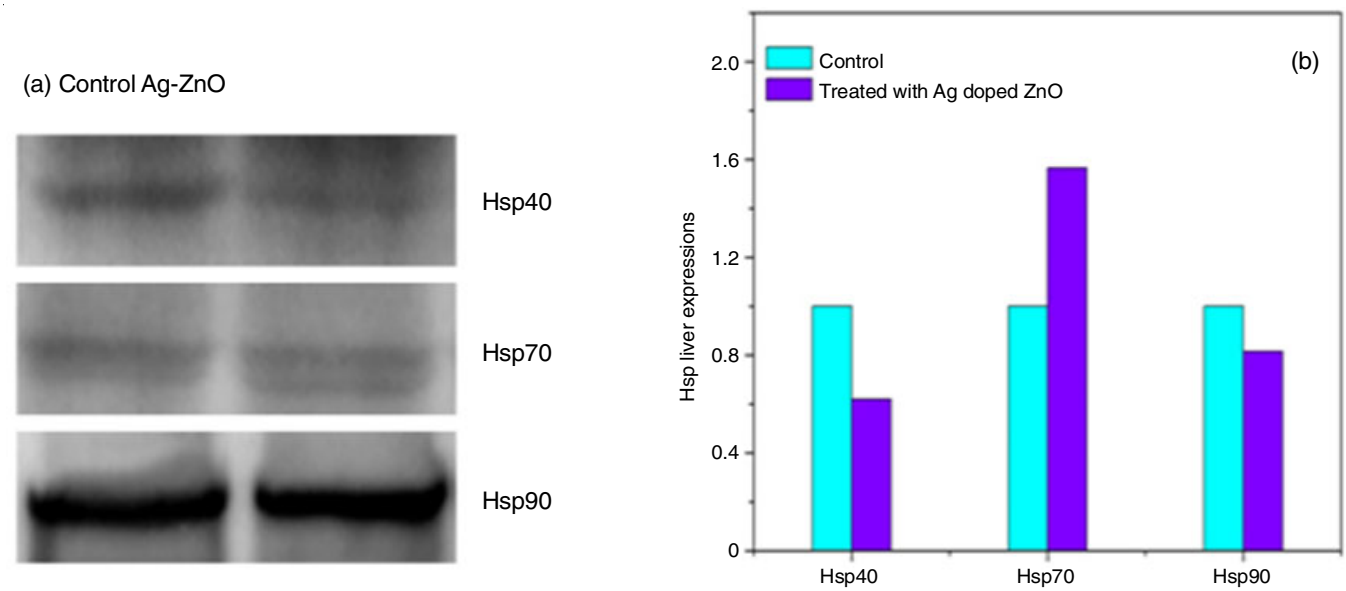

Fig. 3. (a) Heat shock proteins liver expression (Hsp40, Hsp70 and Hsp90 levels and corresponding western blotting experiment showing protein levels in control and Ag doped ZnO treated liver of mice, (b) Histogram showing (Hsp40, Hsp70 and Hsp90) levels obtained in the liver of mice after oral ingestion of Ag doped ZnO nanoparticles

obtained in the kidney of mice after oral ingestion of ZnO nanoparticles is shown in Fig. 4b. In present study, the changes in the protein expression levels of Hsp40, Hsp70 and Hsp90 in liver of control and experimental groups (Fig. 3b) were analyzed. From the Hsp liver expression results, the levels of Hsp40 and Hsp90 were decreased, when compared to control and the level of Hsp70 was increased with respect to control. Similarly, the changes in the protein expression levels of Hsp40, Hsp70 and Hsp90 in the kidney of control and experimental groups were also studied (Fig. 4b). In this case, Hsp40 levels decreased when compared with control and Hsp70 and Hsp90 levels increased when compared with control. The above study suggested that the presence of Ag doped ZnO nanoparticles may be the reason for the damage caused in the liver and kidney protein levels of mice.

Se doped ZnO treated mice: Heat shock proteins (Hsps) of liver expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiments showing protein levels in control and Se doped ZnO treated mice are shown in Fig. 5a. A histogram (Fig. 5b) shows that the Hsp40, Hsp70 and Hsp90 levels obtained in the liver of mice after oral ingestion of Se doped ZnO nanoparticles. Heat shock proteins (HSPs) of kidney expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiments showing protein

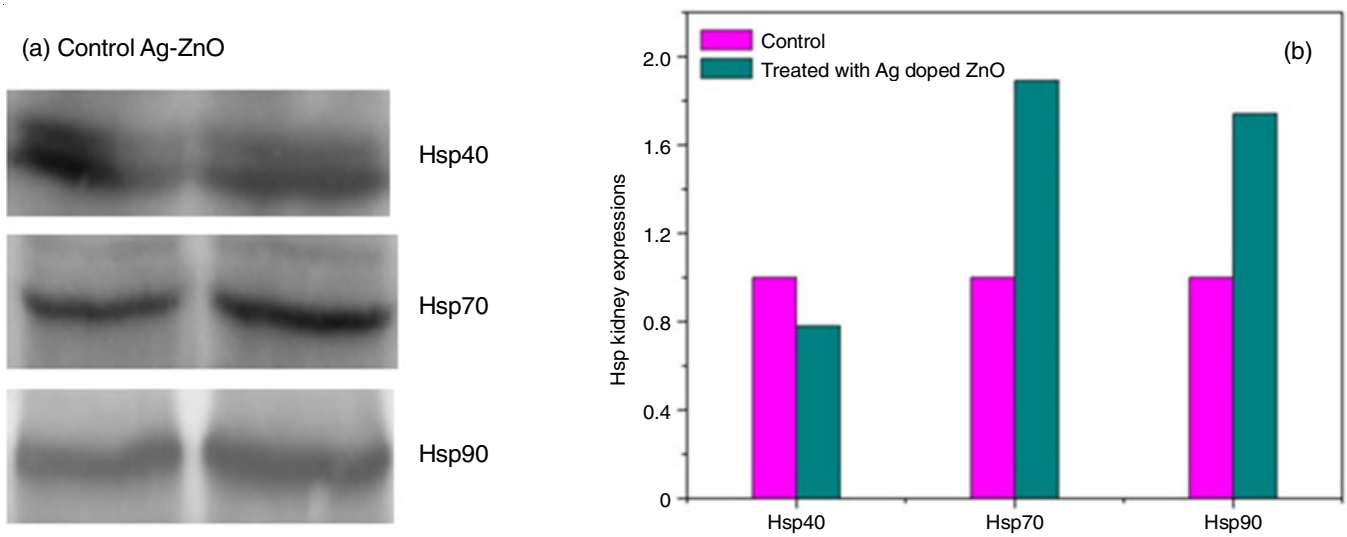

Fig. 4. (a) Heat shock proteins (Hsps) kidney expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiment showing protein levels in control and Ag doped ZnO treated kidney of mice, (b) Histogram showing (Hsp40, Hsp70 and Hsp90) levels obtained in the kidney of mice after oral ingestion of Ag doped ZnO nanoparticles

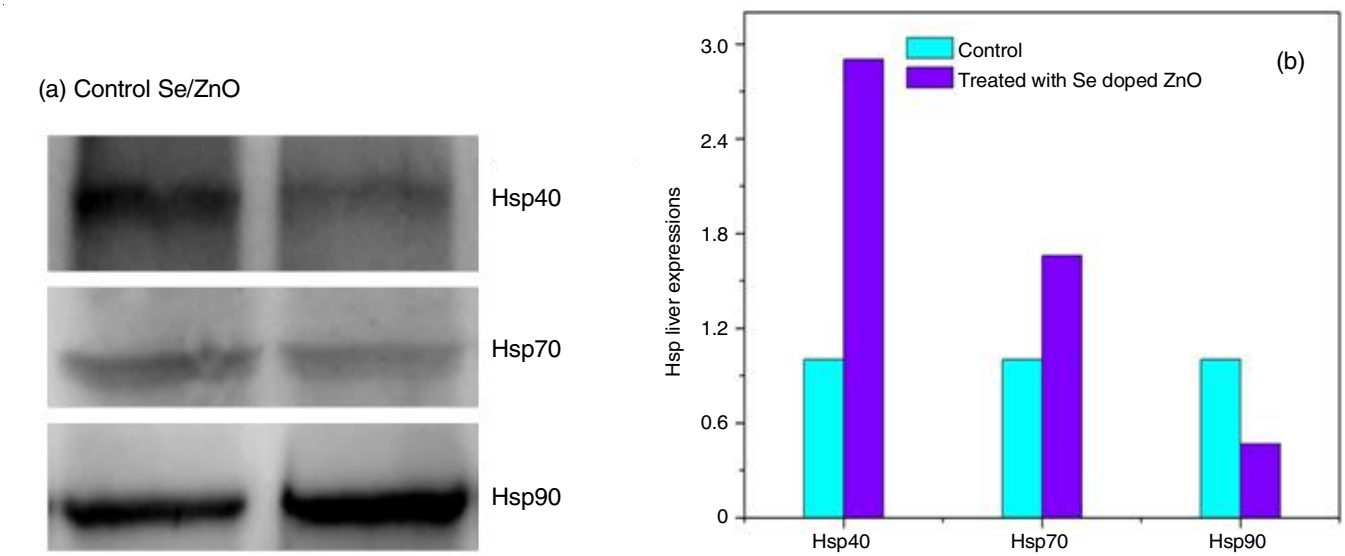

Fig. 5. (a) Heat shock proteins (Hsps) of liver expressions (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiments showing protein levels in control and Se doped ZnO treated liver of mice, (b) Histogram showing (Hsp40, Hsp70 and Hsp90) levels obtained in the liver of mice after oral ingestion of Se doped ZnO nanoparticles

levels in control and Se doped ZnO treated mice are shown in Fig. 6a. Another histogram (Fig. 6b) shows that the Hsp40, Hsp70 and HspSP90) levels obtained in the kidney of mice after oral ingestion of Se doped ZnO nanoparticles. In present study, the changes in the protein expression levels of Hsp40, Hsp70 and Hsp90 in liver of control and experimental groups (Fig. 5b) were tested. From the heat shock protein liver expression results, the levels of Hsp40 and Hsp70 were increased when compared to the control and the decreased level of Hsp90 was observed when compared to control. Further, the changes in the protein expression levels of Hsp40, Hsp70 and Hsp90 in the kidney of control and experimental groups (Fig. 6b) were also studied . In this case, the expressions of Hsp40, Hsp70 and Hsp90 levels increased in the Se doped ZnO treated group when compared with control. The above study suggested that the presence of ZnO as well as Ag and Se doped ZnO nanoparticles has altered the functions of liver and kidney and also caused severe damage in the organs.

Conclusion

Three nanoparticles *viz*. ZnO, Ag doped ZnO ($Zn_{0.80}Ag_{0.20}O_{1-\delta}$) and Se doped ZnO ($Zn_{0.80}Se_{0.20}O_{1-\delta}$) were prepared by simple wet chemical route. In order to study their sub-acute toxicity level in animal model, these nanoparticles were suspended in water and administered orally for 14 consecutive days to Swiss albino mice. At the end of 14th day, the animals were sacrificed *via* cervical dislocation. Serum biochemical studies predicted that the oral exposure of ZnO based nanoparticles caused the liver injury in mice. The enzymatic and non-enzymatic assays studied in the liver and kidney tissues

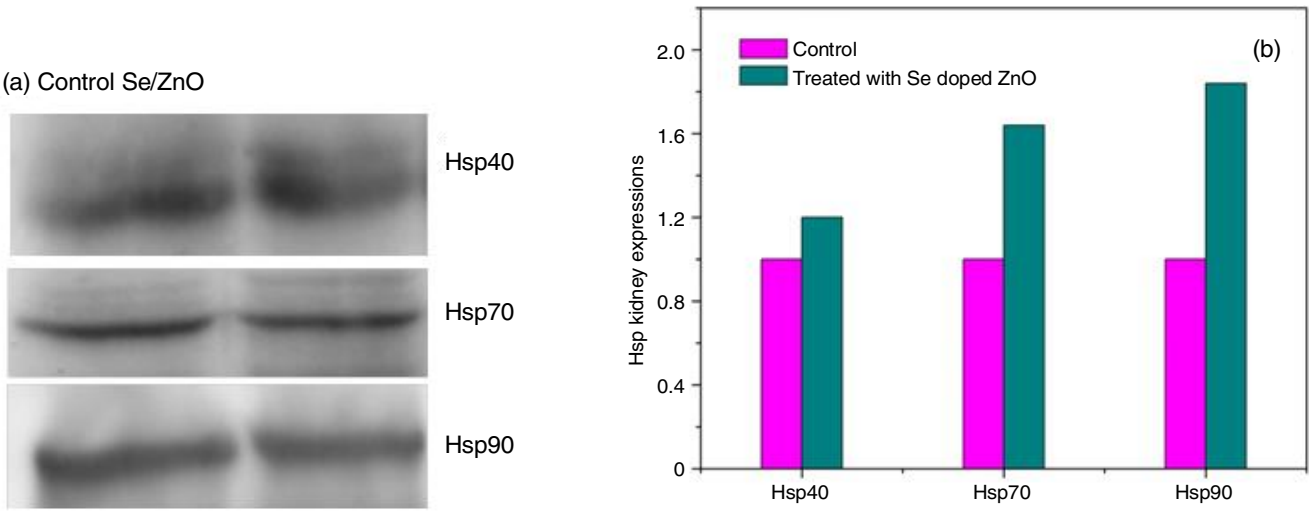

Fig. 6. (a) Heat shock proteins (Hsps) of the kidney expression (Hsp40, Hsp70 and Hsp90) levels and corresponding western blotting experiment showing protein levels in control and Se doped ZnO treated kidney of mice, (b) Histogram showing (Hsp40, Hsp70 and Hsp90) levels obtained in the kidney of mice after oral ingestion of Se doped ZnO nanoparticles

showed a significant enhancement in the levels of ROS, LPO, LDH and NO and declining in the levels of GSH, GPx and protein. Heat shock proteins levels varied in the treated liver and kidney tissues because of the damage. The *in vivo* sub-acute studies carried out with ZnO based nanoparticles in Swiss albino mice revealed that the organs such as liver and kidney are moderately injured after 14 consecutive days of continuous oral ingestion of nanoparticles.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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