10 Nickel Oxide Nanoparticles Induced Transcriptomic Alterations in HEPG2 Cells

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Abstract

Nickel oxide nanoparticles (NiO-NPs) are increasingly used and concerns have been raised on its toxicity. Although a few studies have reported the toxicity of NiO-NPs, a comprehensive understanding of NiO-NPs toxicity in human cells is still lagging. In this study, we integrated transcriptomic approach and genotoxic evidence to depict the mechanism of NiO-NPs toxicity in human hepatocellular carcinoma (HepG2) cells. DNA damage analysis was done using comet assay, which showed 26-fold greater tail moment in HepG2 cells at the highest concentration of 100 μg/ml. Flow cytometric analysis showed concentration dependent enhancement in intracellular reactive oxygen species (ROS). Real-time PCR analysis of apoptotic (p53, bax, bcl2) and oxidative stress (SOD1) genes showed transcriptional upregulation. Transcriptome analysis using qPCR array showed

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over expression of mRNA transcripts related to six different cellular pathways. Our data unequivocally suggests that NiO-NPs induces oxidative stress, DNA damage, apoptosis and transcriptome alterations in HepG2 cells.

Keywords
Nanotoxicity · Apoptosis · DNA damage · Oxidative stress · Transcriptome · Nickel Oxide Nanoparticles · NiO

10.1 Introduction

In recent years' nanotechnology has exhibited exponential growth in various sectors to accomplish market commodities with higher prospective applications [1]. At least thousand consumer products are available which contains nanoparticles (NPs), ranging from everyday household items to medical diagnostic tools, imaging, drug delivery and aerospace engineering [2, 3]. Compared to the bulk counterpart, the small size and large specific surface area of NPs endow them with high chemical reactivity and intrinsic toxicity. Such unique physiochemical properties of NPs draw global attention of scientists and environmental watchdogs to keep concern about NPs potential risks and adverse effect on human health [4]. NPs find route to human body via skin penetration, ingestion, inhalation or injection and interact with cellular organelles for longer time period [5]. Consequently, NPs have been found to effortlessly interact with cells and organs by various mechanisms [6]. Since methodologies for exposure assessment are non-consistent, the toxicological research on NPs is still lagging. Therefore, in order to plug the gap between development and toxicity of NPs, a major effort is needed to study the effects of exposure to NPs.

In this study, we have selected nickel oxide nanoparticles (NiO-NPs) owing to its increasing application in ceramic material, catalysts, electronic component and biosensors [7–9]. Despite its wide use, NiO-NPs has raised concerns about its adverse effects on the environment and human health. NiO-NPs generated from welding fumes during the coastal region developments were considered as a potential nano-pollution source in coastal seawaters (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans). Direct aerial emission of NiO-NPs has the tendency to pollute surface waters through leakages, spills and indirect storm-water runoff from land [10]. The metallic Ni-NPs has been recently used to catalyze the reversible hydration of CO_2 to carbonic acid, which is holding extreme importance in CO_2 capture technologies and mineralization processes. These advantages led to its utilization to point flue sources like air-conditions outlets on top of building or power plants [11, 12]. Being the 24th most abundant element in the Earth crust, nickel compounds ($NiSO_4$, NiO, nickel hydroxides and crystalline nickel) are well known as an environmental pollutant and classified as carcinogenic agents to humans (Group 1) by the International Agency for Research on Cancer (IARC) [13]. The in vivo studies on NiO-NPs have been mostly focused on pulmonary pathology. Female Wistar rats intratracheally instilled with NiO-NPs exhibited a significant increase in the bronchiolar alveolar lavage fluid (BLAF), activation of IL-1β, IFN-Y, MIP-2 and histological changes [14]. A short-term exposure of rats to 500 cm^2/ml of NiO-NPs induced polymorphonuclear neutrophils in the BALF [15]. Inhalation exposure of rats with NiO-NPs in nebulizer chamber exhibited biopersistance of NPs in lungs and inflammatory responses [16]. Long term intratracheally instillation of NiO-NPs in rats exhibited increased vacuolization in alveolar macrophages and CINC-1 concentrations [17]. Wistar rats instilled with NiO-NPs after 4 days of exposure showed eosinophilic and neutrophilic inflammation, along with release of eotaxin and cellular disintegration by the release of Ni ions [18]. Female Wistar rats exposed by pharyngeal route to NiO-NPs showed enhanced proinflammatory cytokines, LDH, lymphocytes, polymorphonuclear leukocytes in BALF [19]. DNA

damage and low expression of HO-1 and Nrf2 proteins were observed in Male Sprague Dawley rats when intratracheally instilled with Ni-NPs for two weeks. In addition, the animals showed alterations in the normal morphology of lungs, liver and kidneys [20]. Ultrafine-size particles and NiO-NPs of nickel compounds have greater bioavailability and toxicity as compared to its fine-size nickel compounds [21]. We have recently reported that NiO-NPs induces liver toxicity, cytogenetic anomalies and apoptosis via p53, MAPK, caspase 8 and 3 signalling in rats [22]. Another recent study in the same line expressed NiO-NPs genotoxicity, chromosomal aberrations, DNA damage in lymphocytes, liver and kidney of female rats [23]. Zebrafish exposed to NiO-NPs for longer time showed higher bioaccumulation and toxicity [24]. It is well documented and established that solubilization of Ni^{2+} from NiO-NPs plays vital role in inducing toxicity in animal, invertebrate, cell line and plant [18, 25–27].

Concerning the in vitro studies, recent reports suggest NiO-NPs as neurotoxic in SH-SY5Y neuroblastoma cells and cytotoxic for human breast carcinoma cells (MCF7) [28, 29]. NiO-NPs induced *HIF-1α* transcription factor followed by upregulation of its target NRDG1 (Cap43) in human lung epithelial (H460) cells [21]. In the same line, NiO-NPs induced oxidative stress and cytotoxicity in human alveolar epithelial cells (A549) has also been reported [30]. HepG2 cells exposed to NiO-NPs resulted in cytotoxicity and apoptosis responses via reactive oxygen species (ROS) generation, which is likely to be mediated through bax/bcl-2 pathway [31].

Despite these facts, a systematic interpretation on the underlying mechanism of NiO-NPs induced hepatotoxicity is scarce. In this context, NiO-NPs has been reported to induce cytotoxicity and apoptotic cell death in HepG2 cells via bax/blc2 pathway [31]. However, authors did not explain the vital queries on HepG2 transcriptomic profile. To decipher these unattended queries, we have provided a concrete evidence on hepatotoxicity under in vitro condition. Primary human hepatocytes have been considered as gold standard model for xenobiotic metabolism and cytotoxicity studies [32]. However, the complexity in isolation procedures, short life-span, inter-individual variability, cost effectiveness and rare availability of fresh human liver samples, constitute serious limitations for the use of aforesaid in vitro systems in screening [33]. Such constrains were run-over by immortalized liver-derived cell lines, owing to their unlimited availability and phenotypic stability. A first alternative is the widely used HepG2 cells, as these cells are highly differentiated and display many of the genotypic features of normal liver cells [34]. HepG2 can be used to screen the cytotoxic potential of new chemical entities at the lead generation phase and imitate the normal metabolic pathway in vivo [35, 36]. In this study, we have selected HepG2 cells as a model system for studying the hepatotoxic effects of NiO-NPs.

Consequently, the current study was aimed to evaluate molecular mechanism of NiO-NPs in vitro toxicity in HepG2 cells by the measurement of (i) intracellular ROS generation (ii) DNA damage (iii) transcriptional activation of array of genes related to human stress and toxicity pathways.

10.2 Materials and Methods

10.2.1 NiO-NPs Characterization

NiO-NPs (Cat. No. 637130) was purchased from sigma chemical company (St. Louis, MO, USA). A stock of NiO-NPs (1 mg/ml) was prepared in MQ water and sonicated for 20 min at 40 W. TEM analysis of NiO-NPs were done by dropping the stock solution on copper grids and subjected to microscopic analysis at 200 KeV (JEM-2100 F, JEOL, Japan).

10.2.2 Cell Culture and NiO-NPs Exposure

Human liver hepatocellular carcinoma (HepG2) cells were cultured in DMEM with 10% FBS, antibiotic-antimycotic solution and incubated at 37 °C with 5% CO_2. HepG2 cells were seeded in 96 and 6-well plates and allowed to attach with

the surface for 24 h prior to NiO-NPs treatment. Before each experiment, the ultra sonicated NiO-NPs (25, 50 and 100 μg/ml) solutions added to cell culture and grown for 24 h. Control groups were not added with NiO-NPs.

10.2.3 In Vitro DNA Damage Analysis by Comet Assay

The HepG2 cells exposed for 3 h with 25, 50 and 100 μg/ml of NiO-NPs were detached and centrifuged at 3000 rpm for 3 min to collect the pellets. The cells (4×10^4) from untreated and treated groups were suspended in 100 μl of Ca^{++} Mg^{++} free PBS and mixed with 100 μl of 1% low melting point agarose (LMA). The cell suspension (80 μl) was then layered on one-third frosted slides, pre-coated with normal melting agarose (NMA) (1% in PBS) and kept at 4 °C for 10 min. After gelling, a layer of 90 μl of LMA (0.5% in PBS) was added. After the solidification of agarose on slides, all of them were kept in lysis solution for overnight, followed by unwinding and electrophoresis at 24 V (300 mA) for 20 min. Cells were stained with ethidium bromide (20 μg/ml) and DNA damage were scored under fluorescence microscope.

10.2.4 ROS Measurements in HepG2 Cells

After the specified treatment, cells were trypsinized, pelleted and washed twice with cold PBS, followed by the resupension of cells in 500 μl PBS (Ca^{++} and Mg^{++} free) containing 5 μM of dichloro-dihydro-fluorescein diacetate (DCFH-DA) dye. All cells were incubated for 60 min at 37 °C in dark followed by washing and the fluorescence were recorded upon excitation at 488 nm at FL1 Log channel through 525 nm band-pass filter on Beckman Coulter flow cytometer (Coulter Epics XL/Xl-MCL, USA). Qualitative analysis of ROS in NiO-NPs treated cells were also done by staining the HepG2 cells with 5 μM of DCFH-DA for 60 min at 37 °C in CO_2 incubator. Fluorescence images were captured on microscope equipped with fluorescent lamp (Nikon Eclipse 80i, Japan).

10.2.5 RT² Profiler PCR Array Analysis

PCR array experiments were done with HepG2 cells exposed for 24 h with NiO-NPs (100 μg/ml). In brief, total RNA was isolated using the commercially available kit (RNeasy Mini Kit, Cat. No. 74106, Qiagen, USA), purification was done using iPrep™ PureLink™ kit (Invitrogen, USA) by Invitrogen® automated system. Purity of total RNA was verified by use of a Nanodrop 8000 spectrophotometer (Thermo Scientific, USA). The first-strand cDNA synthesis was performed with 1 μg of total RNA and 100 ng of oligo-p(dT)12-18 primer and MLV reverse transcriptase (GE Health Care, UK). Changes in the relative gene expression of 84 genes responsible for human stress and toxicity pathway were quantified using 96-well format of RT² Profiler™ PCR Array (Cat. No. PAHS-003 A, SABiosciences Corporation, Frederick, MD). cDNA equivalent to 1 μg of total RNA was used for each array. The arrays were run on Roche® LightCycler® 480 (Roche Diagnostics, Rotkreuz, Switzerland) following the recommended cycling programs. Online software from SABiosciences Corporation, Frederick, MD, was used to analyze the expression data. NiO-NPs expression results were normalized to the average Ct value of five housekeeping genes (*B2M*, *HPRT1*, *RPL13A*, *GAPDH* and *ACTB*) and expressed with respect to the untreated control. RT-PCR array data were evaluated from at least three independent experiments and the resultant $^{\Delta}$Ct values were combined to calculate the average fold regulation values. Genes that were significantly different for NiO-NPs versus control were determined by Students t-test ($p < 0.05$) by comparing the $^{\Delta}$Ct values for the triplicate trials for each test sample with the $^{\Delta}$Ct values for the control. Then PCR array data were validated by measuring the mRNA expression of some selected genes (*P53*, *BAX*, *BCL2*, *SOD1*) using real-time PCR analysis (Table 10.1).

Table 10.1 Primers of candidate genes for qPCR array-qPCR validation

Gene symbol	Sense primer	Antisense primer
P53	CCCAGCCAAAGAAGAAACCA	TTCCAAGGCCTCATTCAGCT
Bax	TGCTTCAGGGTTTCATCCAG	GGCGGCAATCATCCTCTG
Bcl2	AGGAAGTGAACATTTCGGTGAC	GCTCAGTTCCAGGACCAGGC
SOD1	AGGGCATCATCAATTTCGAG	TGCCTCTCTTCATCCTTTGG
GAPDH	CCACTCCTCCACC TTTGAC	ACCCTGTTGCTGTAGCCA

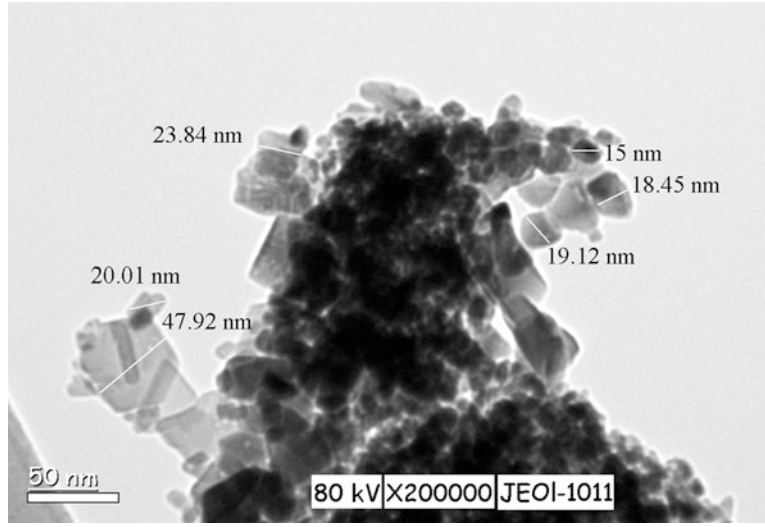

Fig. 10.1 Depicts the particle characterization of NiO-NPs by TEM at 200000× magnification

10.3 Results

10.3.1 NiO-NPs Characterization

The size and morphology of NiO-NPs were measured by transmission electron microscopy (TEM). In TEM analysis, NiO-NPs appeared as an aggregate showing crystallite's spheres. The particles size of NiO-NPs analyzed from six TEM images were determined to be 24.05 ± 2.9 nm (Fig. 10.1).

10.3.2 DNA Damage in HepG2 Cells

HepG2 cells exposed to NiO-NPs for 3 h resulted in DNA damage. The representative comet image from NiO-NPs (100 µg/ml) treatment clearly demonstrates the broken DNA liberated from the comet head (Fig. 10.2). NiO-NPs at 25, 50 and 100 µg/ml induced significant 25.1, 25.3 and 26.7-fold higher Olive tail moment (OTM) parameter of comet assay *vis-à-vis* the control showed a background of 0.28 ± 0.05 OTM (Table 10.2). The advantage of comet assay is that it is capable of analysing population of cells with various degrees of DNA damage. Nevertheless, the differences in distribution of DNA damage exist in the cell population. Variation in distribution of DNA damage by NiO-NPs exposure in-terms of frequency is shown in Fig. 10.2.

10.3.3 Quantitative and Qualitative Analysis of Intracellular ROS

A concentration dependent increase in the intracellular ROS generation in HepG2 cells, as evident by the shift of DCF peaks in treated groups (Fig. 10.3a). Compared to the 100% DCF fluorescence in control, cells treated with 25, 50 and 100 µg/ml of NiO-NPs showed significant 134%,

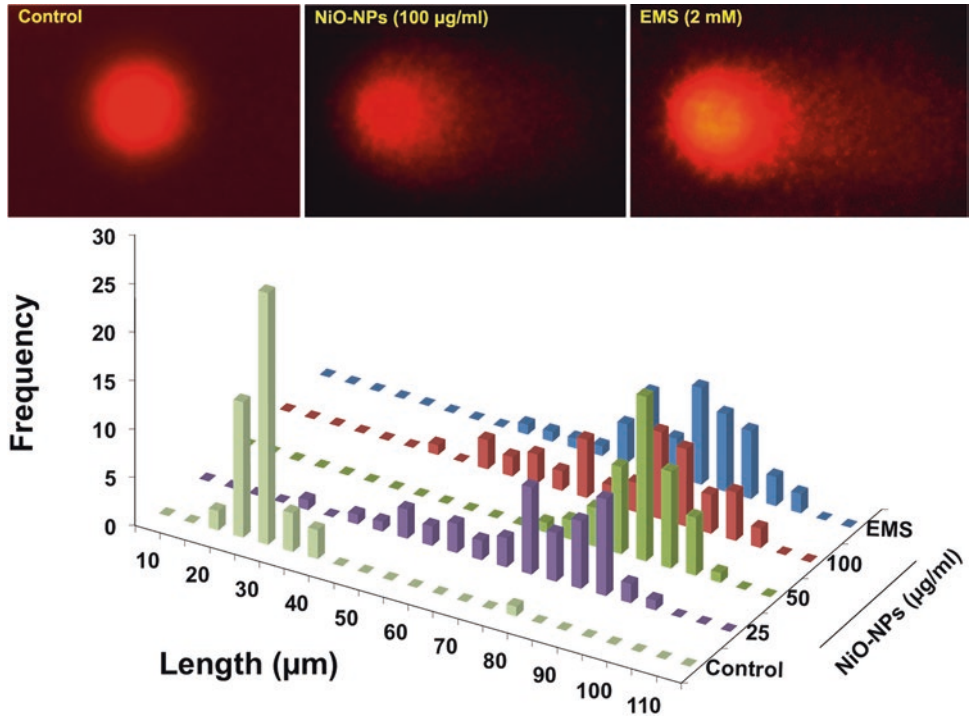

Fig. 10.2 Photomicrographs showing DNA strand breaks analysis by comet assay in NiO-NPs treated HepG2 cells. Histograms showing frequency distribution of DNA damage in HepG2 cells treated with varying concentrations of NiO-NPs for 3 h

Table 10.2 NiO-NPs induced DNA damage in HepG2 cells analyzed using different parameters of alkaline comet assay

Groups	Olive tail moment (arbitrary unit)	Tail length (µm)	Tail intensity (%)
Control	0.28 ± 0.05	27.65 ± 1.87	2.63 ± 0.04
EMS (1 mM)	6.42 ± 0.32**	81.45 ± 3.88**	30.53 ± 1.24**
NiO-NPs (µg/ml)			
25	7.05 ± 0.43**	72.71 ± 2.61**	33.12 ± 1.11**
50	7.11 ± 0.23**	77.43 ± 4.35**	43.13 ± 1.09**
100	7.48 ± 0.46**	76.88 ± 3.09**	42.64 ± 2.43**

Data represent the mean±S.D. of three independent experiments done in duplicate
EMS ethyl methanesulphonate
**$p < 0.01$ vs. control

150% and 143% ($p < 0.01$) increase in ROS generation (Fig. 10.3a, Inset). Fluorescence images further validated the flow cytometric data by showing an enhanced level of DCF fluorescence in the NiO-NPs treated cells (Fig. 10.3b).

10.3.4 qPCR Array of HepG2 Cells

HepG2 cells treated with NiO-NPs (100 µg/ml) for 24 h exhibited differential expression of genes in the RT^2 profiler PCR array. The corresponding heat map suggested strong oxidative or metabolic stress, growth arrest and senescence, apoptosis signalling, proliferation and carcinogenesis, and activation of proinflammatory responses upon NiO-NPs exposure (Fig. 10.4). *CYP2E1* gene in oxidative or metabolic stress group has exhibited a maximum of 3.4-fold up-regulation. Considerable number of genes in this pathway was up-regulated, and 1.4, 1.5 and 1.1-fold of maximum up-regulation has been recorded for *HMOX1*, *SOD2* and *SOD1* genes. Among the set of seven genes responsible for growth arrest and senescence, *GDF15*, *DDIT3*, *GADD45A*, *MDM2* and *P53* genes have exhibited 4.6, 2.4, 1.6, 1.2 and 1.2-fold up-regulation. *TNFSF10*, *TNFRSF1A*, *CASP8* and *NFKB1A* genes in apoptosis signalling group showed maximum up-

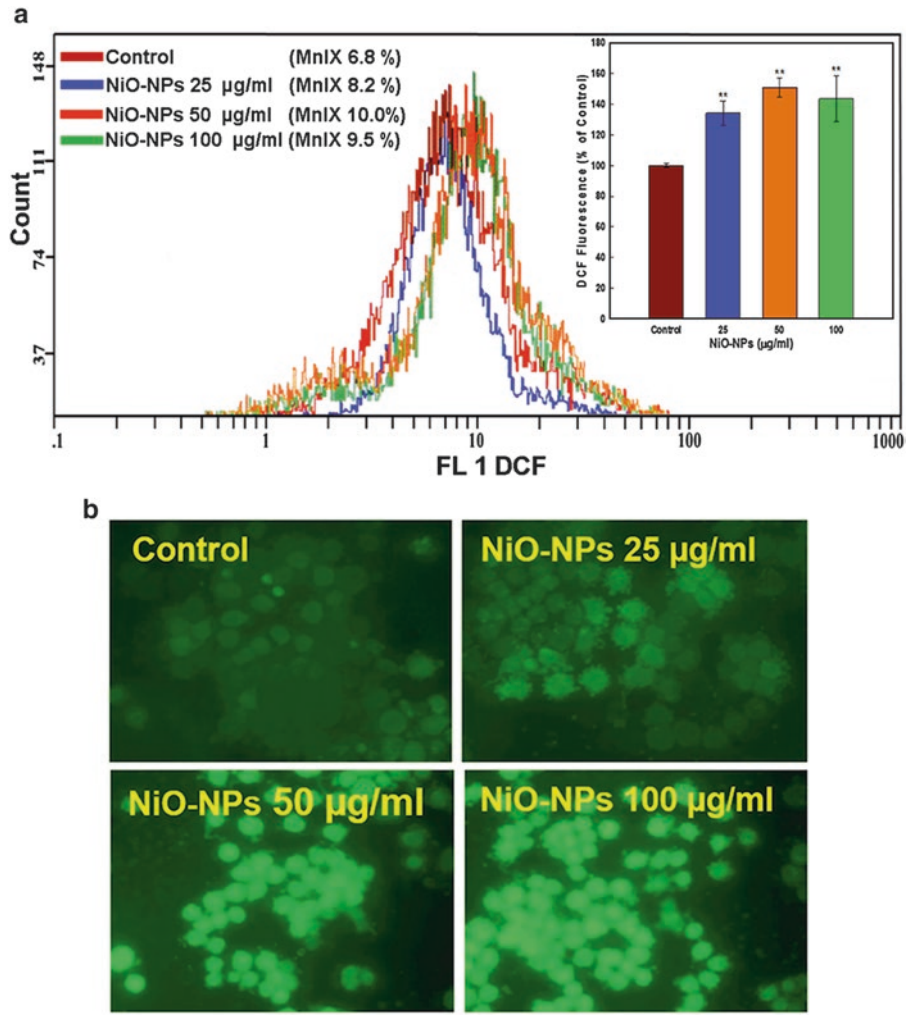

Fig. 10.3 (**a**) Fluorescence enhancement of DCF indicating ROS production with increasing NiO-NPs concentrations in HepG2 cells analyzed by flow cytometry. Each histogram in inset represents the values of mean±SD of three independent experiments done in triplicate wells (**p < 0.01 vs. control). (**b**) Fluorescence microscopic images of treated cells showing an enhancement in green fluorescence of DCF in treated cells

regulation of 1.8, 1.1, 1.4 and 1.0-fold, while the *BCL2L1* expression was down-regulated to 1.1-fold. Within the proliferation and carcinogenesis pathway *EGR1* showed 1.2-fold up-regulation. Among the proinflammatory genes, *NOS2* was maximally up-regulated to 2.3-fold. *HSPA6* gene in heat shock group, showed up-regulation of 2.5-fold. qPCR array data validation was done by measuring the expression of selective genes (*P53*, *BAX*, *BCL2* and *SOD1*) by real-time PCR. The expressional analysis also showed 1.0, 1.2, and 1.1-fold up-regulation of *P53*, *BAX* and *SOD1*. *BCL2* was found under expressed to 1.2-fold (Fig. 10.5).

10.4 Discussion

An integrated approach was used to identify toxicity mechanism induced by NiO-NPs. In this study, low, medium and high (25, 50 and 100 µg/ml) doses of NiO-NPs has been chosen to expose the human liver cells. Lowest concentration was chosen with the aim to imitate the potential

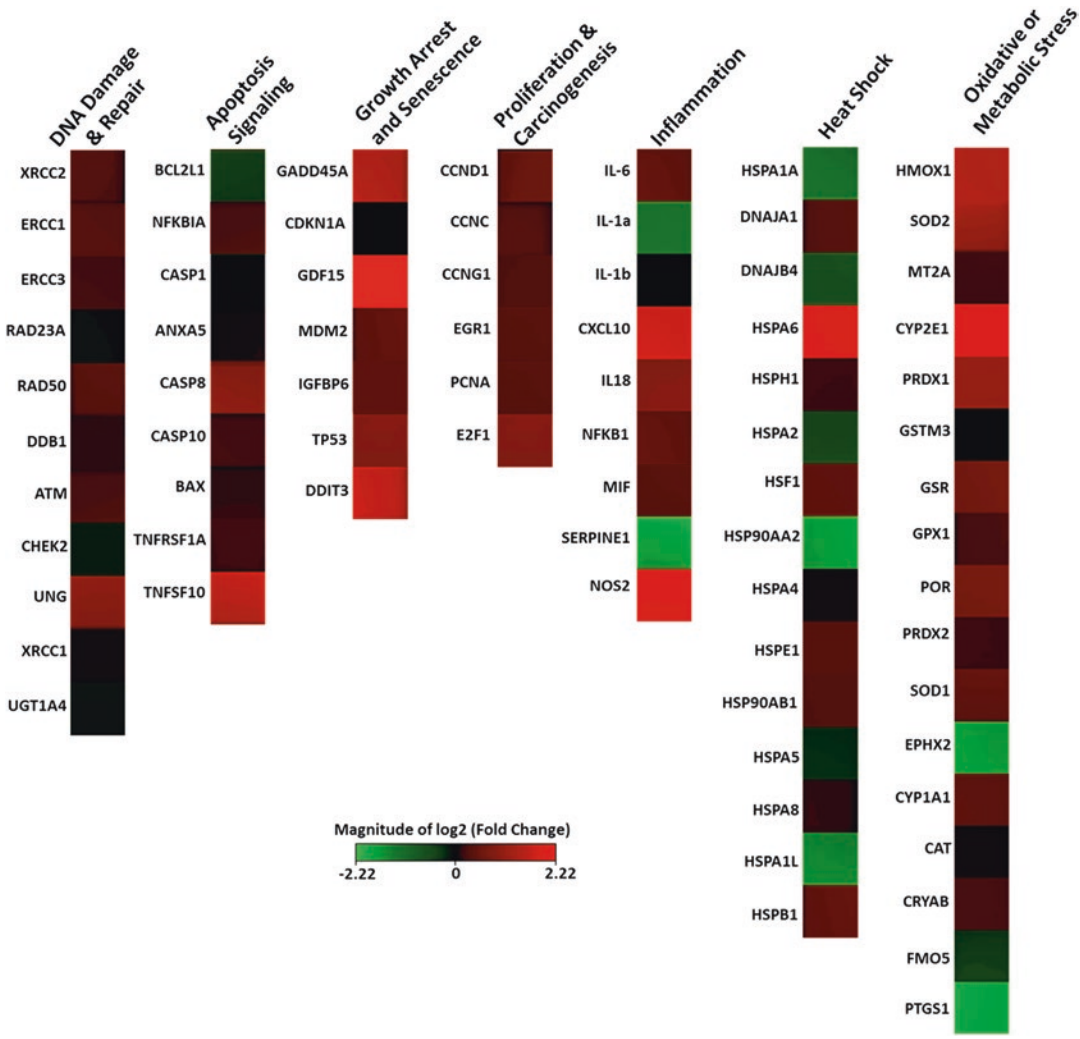

Fig. 10.4 Effect of NiO-NPs on oxidative stress and toxicity pathway genes in HepG2 cells. Heat map showing the relative gene expression of different genes responsible for human stress and toxicity pathway in NiO-NPs (100 μg/ml) treated HepG2 cells after 24 h of exposure

human exposure, on the other hand highest concentration was selected to reflect toxicological effects upon accidental exposure of NiO-NPs. In this line an enhanced level of ROS has been observed in NiO-NPs treated cells. These results corroborate with enhanced ROS level in NiO-NPs treated HepG2 and A549 cells [31, 37]. We suggest oxidative stress in HepG2 cells. The current study demonstrates that NiO-NPs can induce DNA damage after short exposure of 3 h, and corresponds with previous reports on DNA damage in HepG2 and WISH cells exposed to NiO, TiO_2 and $ZnFe_2O_3$-NPs [31, 38, 39]. The appearance of comet tail with NiO-NPs exposure unequivocally suggest the impairment of DNA repair machinery. The enhancement in intracellular ROS and DNA damage data are in agreement with our recent report on NiO-NPs induced liver toxicity in rats [22]. Ni^{2+} is involved in ROS generation and accounted for inducing high level of damage via direct oxidative damage by H_2O_2 production [40]. Hence, the elevated toxicity and damage in our study could also be an additive oxidative action of Ni^{2+} released from NiO-NPs.

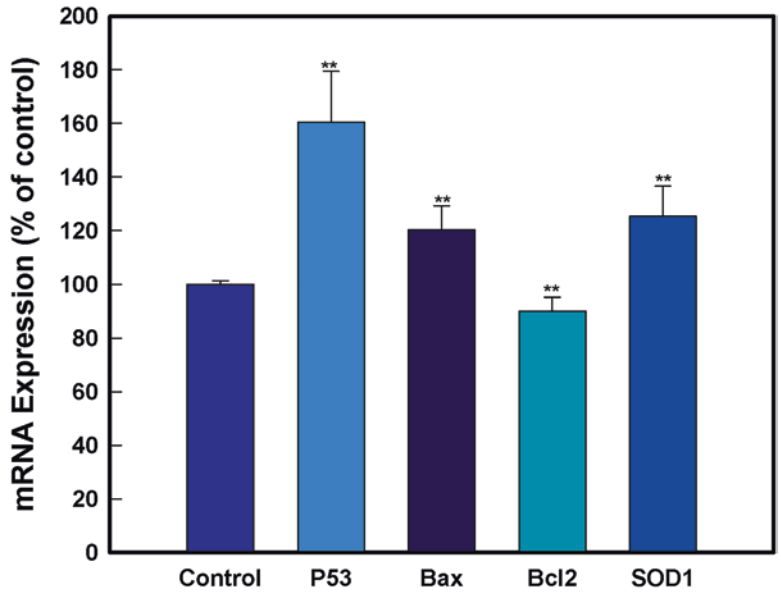

Fig. 10.5 Transcriptional activation of apoptotic and oxidative stress genes in NiO-NPs treated HepG2 cells. Transcript levels were determined by real-time quantitative PCR. $^{**}p < 0.01$ using one-way ANOVA (Dunnett's multiple comparison test) significantly different when compared to control

PCR array revealed that NiO-NPs treatment resulted in the up-regulation of genes related to different pathways. We found that *TNFSF10*, *TNFRSF1A*, *CASP8* and *NFKBIA* genes in apoptosis signalling pathways were up-regulated. *TNFSF10* and *TNFRSF1A* belong to the tumor necrosis factor receptor (*TNFR*) family and their up-regulation has been suggested to induce cell death [41]. Up-regulation of *CASP8* expression has been linked to execute the apoptotic signaling mainly through extrinsic pathway [42]. Activation of the above genes strongly suggests the participation of death receptor-mediated *TNFR* family members to induce apoptosis via intrinsic as well as extrinsic pathways. *TNFR* genes can act through an autocrine pathway to induce cell growth arrest and apoptosis through *NFKB* activation [43]. Therefore, the *NFKB* pathway and related genes could also be an important molecular mechanism by which NiO-NPs induces apoptosis in HepG2 cells.

NiO-NPs treatment resulted in the up-regulation of *EGR1*, *MDM2*, *GADD45A* and *DDIT3* genes. Although the induction of *EGR1*, a family of zinc finger transcription factors, is directly linked with oxidative stress per se, other condition like mitochondrial dysfunction contributes well in its up-regulation [44]. We have found in vivo mitochondrial dysfunction in rats exposed to NiO-NPs for 7 and 14 days [22]. Therefore, the observed mitochondrial dysfunction and oxidative stress data strongly substantiate the likelihood that NiO-NPs may function as an initiator to increase the expression of *EGR1* in treated HepG2 cells. Up-regulation of *DDIT3* and *GADD45A* transcripts can be correlated with the fact that under stressed condition *EGR1* is known to initiate *DDIT3* and *GADD45* family genes by binding to 5′-flanking regions [45]. The oxidative stress related genes (*SOD1*, *SOD2*, *GPX1* and *HMOX1*) were found up-regulated after NiO-NPs exposure. In view of the higher ROS generation by NiO-NPs, we suggest that cytoplasmic (*SOD*1), mitochondrial (*SOD2*), glutathione system (*GPX1*) and *HMOX1* might have involved in scavenging the free radicals and cytoprotection. However, the excessive oxidative stress was beyond the attenuation capacity of these enzymes to subtle the DNA damage in treated cells. Up-regulation of above genes corresponds with increased expression of *SOD, GPX*, and *HMOX1* in human cells, when treated with ZnO-NPs and polyphenolic compounds [46, 47]. *NOS2* is a hallmark of inflammatory response and its up-regulation is governed by oxidative stress, metals and lipopolysaccharides [48]. *NOS2* expression

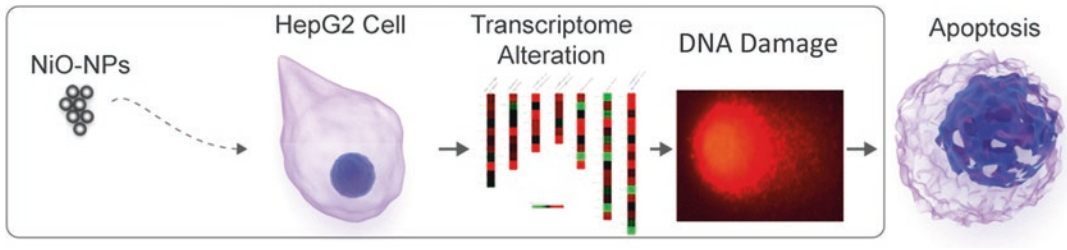

Fig. 10.6 Scheme showing NiO-NPs induced transcriptomic alterations and DNA damage leading to cell death in HepG2 cells

is in accordance with our previous work on $ZnFe_2O_4$-NPs, exhibiting its induction in WISH cells [38]. *GDF15* overexpression corresponds well with *p53-GDF15* link, and points towards its important role during inflammatory responses after NiO-NPs treatment [49]. Within the set of heat shock genes, *HSPA6* was found highly up-regulated in NiO-NPs treated cells. Heat shock proteins (HSP) are highly conserved class of stress response proteins, which work as molecular chaperons to correct the protein conformation under stress condition to maintain cellular homeostasis and protect the cells from apoptotic cell death [50]. Nonetheless, the DNA damage in NiO-NPs treated HepG2 cells supports the view that HSP fails to intervene the apoptotic process, as depicted in the image (Fig. 10.6).

10.5 Conclusion

We conclude that NiO-NPs have the potential to alter the transcriptome of HepG2 cells. We observed that NiO-NPs generated ROS and these free radicals induce heavy oxidative stress, which has affected the cell survival and promoted DNA. Transcriptional analysis of PCR array revealed overall up-regulation of different pathway genes, suggesting a pleiotropic effect of NiO-NPs to induced HepG2 cell death. The analysis of transcriptome was helpful to reveal potential molecular mechanism underlying NiO-NPs induced effects on HepG2 cells. The observed toxicity in HepG2, corresponds well with our recent study on rat's showing hepatotoxicity. Hence, NiO-NPs widespread application should be given meticulous attention for potential adverse biological effects.

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