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Original Research Article

Quercetin Suppresses Apoptotic Genes Expression and DNA Fragmentation Induced by Nano-rich Diesel Exhaust Particles in the Fetal Brain

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Abstract

Nano-rich diesel exhaust particles (NRDEPs) are an extensive range of toxic metals existing generally on environmental system and associated with nervous system disorders, including autism spectrum and induce apoptosis through oxidative stress. Our aim was to evaluate the potential effects of NRDEPs on the brain tissue as apoptosis inducer agent via oxidative stress in hand and the quercetin as powerful anti-oxidant, anti-apoptotic on other hand. Forty pregnant (from the 5th to the 20th day of gestation) albino rats were randomly divided into control group which received normal saline and quercetin group which received 100 mg/kg quercetin. The 3rd one received 20 mg/kg of NRDEPs and the 4th received 100 mg/kg quercetin two hours prior to NRDEPs exposure. The dams were anaesthetized by the end of gestation and fetal brain was removed for immunohistochemistry, biochemical parameters estimation, comet assay and real time polymerase chain reaction (RT-PCR). Our results showed that maternally co-treatment with quercetin diminished NRDEPs-induced oxidative stress (reduced MDA level, increased GSH level and enhanced both GST and PON activities). Moreover, quercetin also down regulated the expression of Bax and caspase-3 and reduced DNA fragmentation. These outcomes indicate that quercetin has a suppressive role against oxidative stress and apoptosis induced by nano-rich diesel exhaust particles in rat fetal brain.

Keywords: Diesel exhaust, Nanoparticles, Quercetin, Apoptosis, Fetus.

Abbreviations

Bax, Bcl-2–associated X protein; Bcl-2,B-cell lymphoma 2; Bcl-XL, B-cell lymphoma-extra large; CNTs, carbon nanotubes; DE, diesel exhaust; NP, nanoparticles; NRDEPs, nano-rich diesel exhaust particles; PAHs, Polycyclic Aromatic Hydrocarbons; PM, Particulate Matter; ROS, Reactive oxygen species.

INTRODUCTION

Diesel exhaust contains a wide range of organic and inorganic compounds that have been described as particulate matter air pollutants (Kagawa, 2002). As diesel fuel undergoes combustion in engines, it produces

particles known as nano-rich diesel exhaust particles (NRDEPs) which composes of some different chemicals with different sizes and physical proprieties such as polycyclic aromatic hydrocarbons, toxic metals, diesel derived nanoparticles (NPs), carbon nanotubes (CNTs) and carbon nanofibers (Jung et al., 2013).

NRDEPs are very toxic because of their minute size, large reactive surface area and modified quantum effects (Buzea et al., 2007). Once inhaled, NRDEPs can cross the cellular barriers, transfer through the blood circulation and finally accumulate in secondary organs as brain, liver, heart and spleen (Semmler-Behnke et al., 2014).

Previous studies have shown that the trophoblastic cells and fetal capillaries relative volume decreased after maternal exposure to NRDEPs as they have ability to pass through the placenta as well as reach the blood stream of embryo (Valentino et al., 2016; Veras et al., 2008). NRDEPs can affect the fetal central nervous system development which leads to pathological abnormalities of fetal brain and various neurological disorders (Sugamata et al., 2006).

Recently, the potential relationship between oxidative stress and many diseasespathophysiologyin human and animal has been getting an inordinate deal of attention. Oxidative stress induced by nanoparticles has been known as a source of developmental toxicity (Sun et al., 2013). NRDEPs induce oxidative stress by generation of hydroxyl and oxygen free radicals along with reduction in the cellular antioxidant defenses system (Han et al., 2001; Ross and Kasum, 2002). NRDEPs is also reported to generate reactive oxygen species and induce the programmed cell death (Hiura et al., 2000).

Neuroprotective approaches support the supplementation of a valuable, highly efficient and low-cost of natural antioxidants which maintains the balance between oxidant and cellular antioxidant in turn protect the central nerves system from oxidative stress-related diseases, neural degeneration and apoptosis. Quercetin is a bioactive antioxidant plant that has an extensive range of health benefits and distributes over a large number of fruits, vegetables and medical herbs (Slikkkerm et al., 1999). Quercetin also has various protective and ameliorative functions including, metal chelator, free radical's scavenger so it may prevent oxidative stress, cell injury and apoptosis (Bu et al., 2012).

Although embryonic organs are more sensitive to toxic materials than adults and the data about the suppressive role of quercetin against apoptosis induced by NRDEPs in fetal brain is limited, so evaluating the safety of nanoparticles is very important at this critical period of pregnancy. Consequently, the present aim was to divulge the association between oxidative stress and NRDEPs-induced apoptosis on the brain of albino rat fetuses in hand and the suppressive role of quercetin on the other hand.

MATERIALS AND METHODS

Chemicals used

The NRDEPs were obtained from the Egyptian Transport Network. Quercetin (2,3′,4′,5,7-pentahydroxyflavone), other chemicals and substrates were obtained from Sigma Chemical Company (U.S.A.).

Experimental design

Forty pregnant albino rats (*Rattusnorvegicus*) were randomly divided into four experimental groups as follows: control group (G1): dams received normal saline, quercetin group (G2): received 100 mg/kg quercetin, NRDEPs group (G3): received 20 mg/kg of NRDEPs with intra-tracheal instillation, quercetin + NRDEPs group (G4):received 100 mg/ kg quercetin two hours prior to the intra-tracheal instillation of 20 mg/kg NRDEPs. The experimental study was done from the 5^{th} to the 20^{th} day of gestation, and this protocol was approved by The Institutional Animal Care and Use Committee (IACUC) at Cairo University (CU.1.S.74.17).

Specimen

By the end of gestation, the dams were anaesthetized and brain specimens were removed immediately from the fetus, washed, dried and fixed by formalin for immunohistochemistry.Other fatal brain tissues were kept frozen at -80°C for biochemical analysis, comet assay and real-time polymerase chain reaction (Real-Time PCR).

Biochemical analysis

Preparation of the tissue fraction

Brain tissues were homogenized in pre cold saline, then the homogenate was centrifuged and the resultant supernatantwas used for MDA and GSH levels measurement as well as PON and GST activities assessment. The protein concentration was measured calorimetrically by the method adopted by (Bradford 1976) with bovine serum albumin as standard using Spectronic Helios Alpha UV-Vis (England).

Determination of paraoxonase (PON) activity

The activity of paraoxonase was determined spectrophotometrically and p-nitrophenyl acetate was employed as a substrate, as previously described (Gan et al., 1991). The enzyme activity was measured by the

Table 1. Oligonucleotide primers used in SYBR Green *RT-PCR*

Gene	Primer sequence (5'-3')	Accession Number	Reference
ß-actin	*F*-TCCTCCTGAGCGCAAGTACTCT *R*-GCTCAGTAACAGTCCGCCTAGAA	V01217	(Banni et al., 2010)
BAX	*F*-CACCAGCTCTGAACAGATCATGA *R*-TCAGCCCATCTTCTTCCAGATGGT	RRU49729	(Kinouchi 2003)
Caspase-3	*F*-AGTTGGACCCACCTTGTGAG *R*-AGTCTGCAGCTCCTCCACAT	NM_012922.2.298	(Shi et al., 2009)

increase in the developed yellow colour through p-nitrophenol released.

Determination of glutathione-S-transferase (GST) activity

The activity of GST was assayed spectrophotometrically following a previously published method (Habig et al., 1974). This method based on measure the yellow colour developed as a result of the conjugation of 1-chloro-2, 4-dinitrobezene (CDNB) with reduced glutathione (Habdous et al., 2002).

Determination of reduced glutathione (GSH) content

Reduced glutathione content was determined as previously described (Ellman 1959). The method is based on the determination of a yellow color that develops when [5, 5 dithiol- bis (2-dinitrobenzoic acid)] (DTNB) is added to sulphydryl compounds, the developed color was measured spectrophotometrically at 412 nm.

Determination of lipid peroxidation level

The method is based on the determination of MDA as an end product of lipid peroxidation, which can react with thiobarbituric acid in acidic medium to yield a pink colored of trimethine complex exhibiting an absorption maximum at 532 nm (Yoshioka et al., 1979).

Immunohistochemistry

Paraffin embedded brain tissues were cut into five µm sections to detect caspase-3 and Bax proteins. Rabbit caspase-3 and Bax antibodies were used as a primary antibody. Formerly biotinylated secondary antibody was added, and then horse-radish peroxidase conjugated with streptavidin. As streptavidin has a high affinity toward biotin, it binds to the site where primary antibody coated the background, after adding a chromatogen (DAB) a reddish color appears (Sati et al., 2010), a light microscope with a digital camera were used for images capture.

Comet assay

The comet assay was applied under alkaline conditions using ordinary microscope slides as previously described by (Collins 2004; Singh et al., 1988). The brain tissues of fetuses were minced in Hank´s balanced salt solution, (HBSS) pH 6.5 (Ca^{++} and Mg^{++} free) with 20 mM EDTA and 10% DMSO). Minced brain tissues were collected and centrifuged at 2,000 rpm at 4 C for 10 min. cell pellet was suspended in PBS-CMF. The slides (were prepared one day before) were pre-coated with 200 µL of 1.5% normal melting point agarose and dried up at room temperature. Then a mixture of 10 µL of cell suspension and 75 µL of 0.5% low melting point agarose was added. The covers were gently slid off and third layer (80 µL of 1.0% low melting point agarose) was added to the slides. The covers were replaced and the slides were allowed to harden for 3–5 minutes and the slides were slowly immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mMTrizma base with pH 10. Freshly 1% Triton X-100 and 10% DMSO)and refrigerated for one hour. The slides were gently removed from lysing solution and rinsed carefully to remove detergents and salts. The slides were placed close together on horizontal gel electrophoresis (25 V and 300 mA for 40 min; JSB-30, USA). After the electrophoresis, slides were gently washed three times using neutralization buffer. Then 70 % ethanol was used for fixing the slides for 10 min and dried up at room temperature. Then the slides were stained with aqueous ethidium bromide solution. Fluorescence microscope (Lx 400, Labomed, USA) was used for DNA fragmentation detection. The degree of DNA damage was determined by v1.5 of Comet Score.

Expression of apoptosis genes determination

RNA was extracted from fetal brain according to RNeasy Mini Kit instructions (Catalogue no.74104). RNA was then reverse transcribed using (Revert Aid Reverse Transcriptase Thermo Fisher, Catalog number: K1622) according to manufacturer's instructions. Primers were obtained from (Metabion, Germany) and the sequences of these primers obtainable in Table 1. Real-time quantification was performed in the Stratagene MX3005P instrument using the Quantitect SYBR green PCR kit (Cat. No. 204141). The reactions were performed in a

Table 2. Effects of NRDEPs and co-treatment with quercetin on MDA and GSH levels, GST and PON activities in fetal brain tissues

	MDA (nmol/g tissue)	GSH (µM/g tissue)	GST (µM/min/mg protein)	PON (µM/min/mg protein)
G1	10.02 ± 0.6	20.16 ± 0.67	524.55±20.49	123.9±3.93
G2	9.688 ± 0.44[c,d]	21.87 ± 0.84[c,d]	517.49±10.08[c]	125.02±2.73[c,d]
G3	21.68 ± 0.82[a,b,d]	15.15 ± 0.72[a,b,d]	372.02±8.59[a,b,d]	89.56±3.32[a,b,d]
G4	15.7 ± 0.55[a,b,c]	18.61 ± 0.57[b,c]	476.19±16.07[c]	109.01± 5.02[a,b,c]

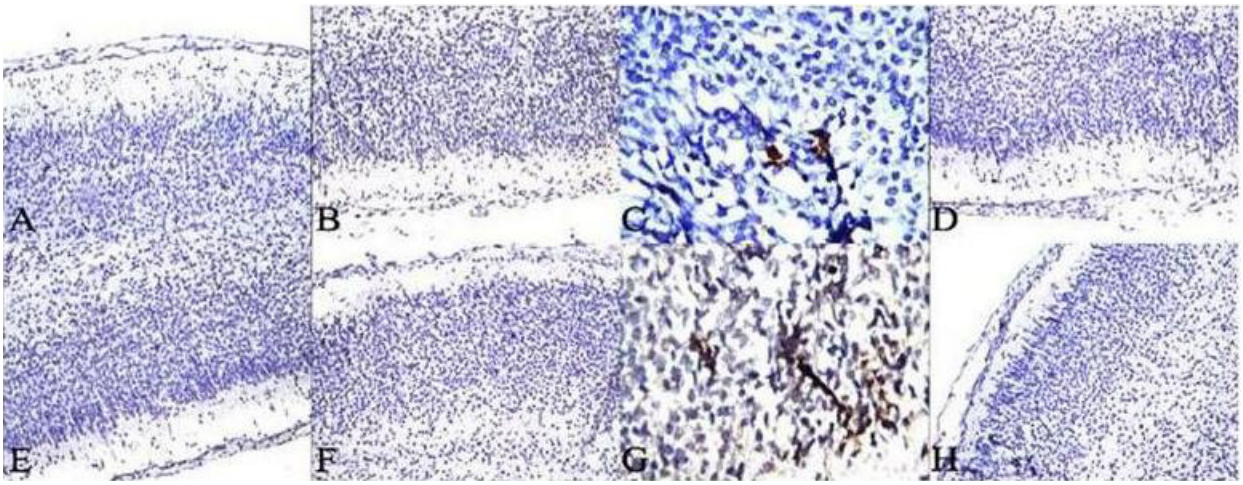

Figure 1. A photomicrograph of caspase-3 (top) and Bax (bottom) immunohistochemical staining of fetal brain sections on the 20th day of gestation; (A,E) control group; (B,F) quercetin group; (C,G) NRDEPs group; (D,H) NRDEPs + quercetin group.(Magnification ×40)

25 µl volume mix containing; 12.5 µl 2x SYBR Green PCR Master Mix, 1 µl primers, 2 µlcDNA, and 8.5 µl of RNase Free Water. Dissociation stage was preformed after the amplification to verify the specificity of the PCR products.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Differences between groups were achieved by the analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc analysis. Significance was set at probability P ≤ 0.05 using IBM SPSS version 22 software package (SPSS, IBM, Chicago, IL, USA).

RESULTS

Malondialdehyde (MDA) level

The biochemical results presented in this study revealed that fetal brain MDA level was significantly increased (P < 0.05) in both NRDEPs and NRDEPs+ quercetin groups as compared with control one on the other hand the co-treatment with quercetin significantly ameliorated the fetal brain MDA level (Table 2).

Antioxidant system of fetal brain

Maternally intoxication with 20 mg/kg NRDEPs pointedly disrupted the antioxidant system of fetal brain as GSH level as well as GST and PON activities were significantly decreased (P < 0.05) as compared with control. Inside out, the co-treatment with quercetin exhibited a significant increase in GSH level (P<0.05) and enhanced the GST and PON activities as compared with the NRDEPs group (Table 2).

Each value represents the mean and SME. One-way analysis of variance (ANOVA) followed by Turkey's honestly significant difference (HSD) test, the p ≤ 0.05 level was set as statistically significant different; (a) significant compared to control, (b) significant compared to quercetin group, (c) significant compared to NRDEPs group, (d) significant compared to NRDEPs +quercetin group.

Results of Caspase-3 and Bax Transcripts Using Real-Time RT-PCR

The Bax and caspase-3 genes expression exhibited significantly up-regulated (P < 0.05) by 22.4 and 28.7 folds respectively, among NRDEPs group and by 2.7 and 3.3 folds respectively, among NRDEPs + quercetin group

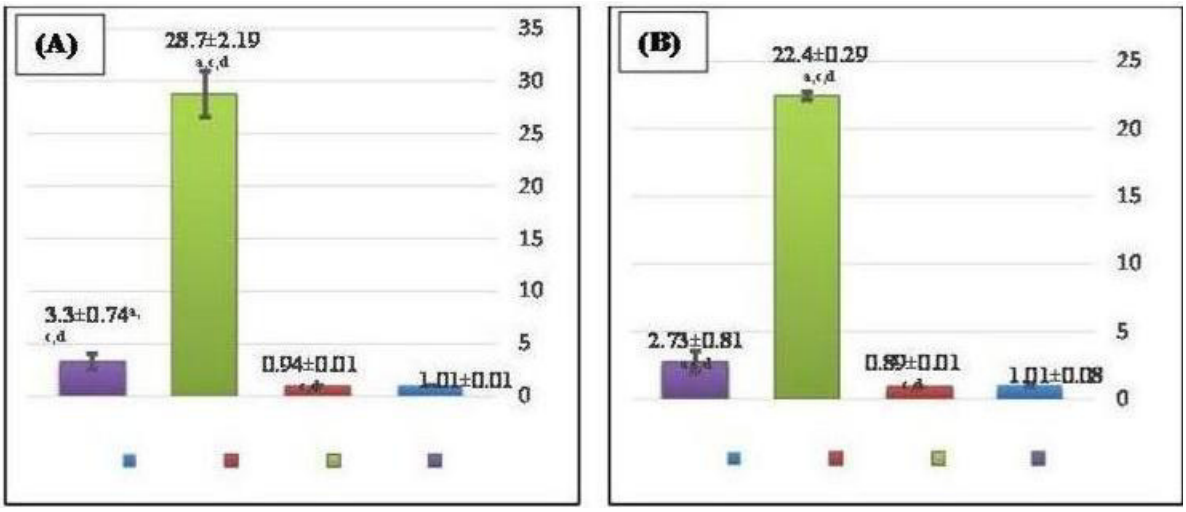

Figure 2. Fold changes of caspase-3 and Bax genes expression in brain of albino rat fetuses maternally exposed to the NRDEPs and co-treatment with quercetin; (A) Bax gene and (B) caspase-3 gene.

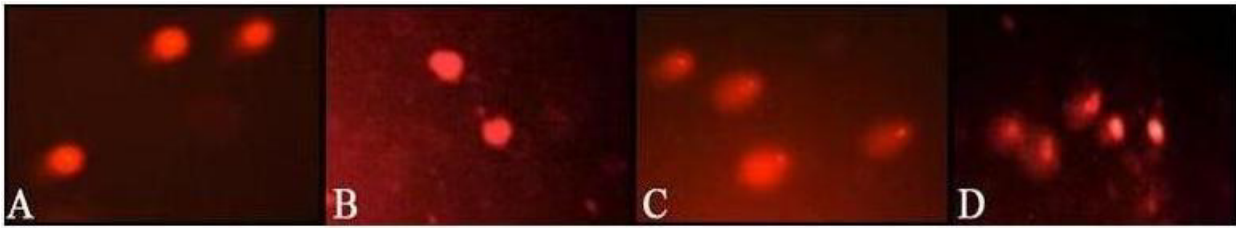

Figure 3. A photomicrograph of comet assay in fetal brain (A) control group; (B) quercetin group; (C) NRDEPs group; (D) NRDEPs + quercetin group.

Table 2. Effects of NRDEPs and co-treatment with quercetin on comet parameters in fetal brain tissues

	Head DNA (%)	Tail DNA (%)	Tail Length (µm)	Tail Moment (AU)
G1	91.65 ±1.78	8.35 ±1.67	3.13 ±0.025	0.535 ±0.036
G2	90.45 ±1.94^{c,d}	9.55 ±1.92^{c,d}	3.46 ±0.22^{c,d}	0.578 ±0.015^{c,d}
G3	77.68 ±1.76^{a,b,d}	22.32 ±1.75^{a,b,d}	5.23 ±0.25^{a,b,d}	1.35 ±0.177^{a,b,d}
G4	82.7 ±1.19^{a,b,c}	17.3 ±1.19^{a,b,c}	3.58 ±0.06^{a,b,c}	0.7 ±0.072^{a,b,c}

as both compared with control. However, non-significant down regulation ($P < 0.05$) was observed between quercetin and control groups. Inside out, the co-treatment with quercetin revealed an efficient down-regulation of both Bax and caspase-3 gene expression in fetal brain tissues, shown in (Fig. 2A and 2B).

Immunohistochemistry

The brain tissues of the NRDEPs group revealed a positive reaction for active caspase-3 (Fig.1 C)and Bax (Fig.1 G) in the neurons as compared with control (Fig.1 A and E) and quercetin (Fig.1 B and F and) groups that showed negative reaction for caspase-3 and Bax. Quercetin co-treatment attenuated the up-regulation of caspase-3 (Fig.1 D) and Bax (Fig.1 H).

Fetal brain DNA damage

In this study, comet assay demonstrated the genotoxicity of NRDEPs as evidenced by elevation of all estimated parameters of DNA damage (% DNA in tail, % DNA in head, Tail length and Tail moment) in fetal brain tissues compared to control values. On the other hand the co-treatment with quercetin significantly minimized the DNA damage induced by NRDEPs as shown in (tables 3 and Fig. 3).

Each value represents the mean and SME. One-way analysis of variance (ANOVA) followed by Turkey's honestly significant difference (HSD) test, the $p \leq 0.05$ level was set as statistically significant different. (a) significant compared to control, (b) significant compared to quercetin group, (c) significant compared to NRDEPs group, (d) significant compared to

NRDEPs + quercetin group.

DISCUSSION

Nano-rich diesel exhaust particles are worldwide relevant air pollutants that exert a fierce human and animal health effect (Dockery et al., 1993), so our study was conducted to evaluate the suppressive role of quercetin against apoptosis induced by nano-rich diesel exhaust particles in albino rat fetal brain.

NRDEPs induce oxidative stress by (I) transition element-based nanoparticles such as CNT (II) free radicals present on surfaces of particles such as quartz and carbonaceous particles, (III) redox-active sites resulting from functionalization of nanoparticles as quinones capable of generating ROS (Knaapen et al., 2004; Li et al., 2010) (IV) NRDEPs chemical composition such as transition metals (Al, Fe, Mn, Cu, Pb, Zn, Cr, V, Ba and Ni) as well as PAH and their derivatives (Andersson et al., 1998). The distinctive physicochemical structures of nanoparticles, as their nanosize with large reactive surface area are responsible for generating ROS and oxidative injury (Auffan et al., 2009). Beside their direct or indirect damaging effects on cellular lipids, proteins, and DNA, an increased production of ROS triggers programmed cell death.

Surely, NRDEPs pass through the placenta, then taken by the fetuses and entered fetal circulation and reach the brain after passing through the not fully developed fetal blood brain barrier which lead to induce oxidative stress and apoptosis (Hougaard et al., 2008; Sugamata et al., 2006).

Elevated levels of MDA in the current study, revealed a damaging effects on cell membrane structure and functions by the theory of lipid peroxidation. Oxidative stress provoked by free radicals such as H_2O_2 is stated to be a vital mechanism of many infections and is currently evaluated to be one of the main reasons leading to the adverse health effects induced by NRDEPs. ROS production and changes in cell cycle metabolism contributing to oxidative stress in NRDEPs exposed cells (Cachon et al., 2014).

Quercetin showed an effective antioxidant property that is most often attributed to its hydroxyl groups, which relates with its electron donating capability (Tu et al., 2015). It is supposed that quercetin prevent the free radical chain reaction progression through the mechanism of free radicals trapping at the interface of the membranes (Ross and Kasum 2002).

According to our results, co-treatment with quercetin after 2 hours of exposure to NRDEPs attenuated lipid peroxidation due to its ability to inhabit lipid peroxidation (Bakheet 2011), quercetin is an effective ions chelator and can chelate transition metal ions present in NRDEPs that may responsible for increasing oxidative stress (Rice-Evans et al., 1996).

The drop in GSH level may due to scavenging of NRDEPs – induced free radicals production, suppression GSH synthesis and/or restriction of intracellular reduction of oxidized GSH (Mohan et al., 2013).

The decrease in GST activity might be owing to GST participated in detoxifying NRDEPs to non-toxic metabolites or by rapidly binding and very slowly turning over these xenobiotics (Jebur et al., 2014; Lonare et al., 2014). Our results suggest that NRDEPs or its metabolites, other than free radicals, were responsible for the reduction in PON activity which may act as scavenger for these radicals. NRDEPs contain a variety of reactive components that are highly reactive towards thiols forming sulfhydryl-ether linkage (Nishio and Watanabe 1997), and/or able to connect with Zn^{2+} ion in the active center of PON enzyme and it can diminish the PON activity (Demir 2011). Otherwise NRDEPs might have negative effects on PON activities or on their 3-dimensional structures that result in some changes in the metabolisms (CEBECİ et al., 2014).

The improved GSH, GST and PON activities propose that quercetin mediated the modulation of cellular antioxidant levels. In agree with several previous studies which reported that quercetin can elevate GSH, GST and PON as well as reduce lipid peroxidation levels (Attia 2010; Kapiszewska et al., 2007; Papiez et al., 2008).

Our immunohistochemistry and RT-PCR results showed that the expression level of Bax and caspase-3 in fetal brain were significantly up-regulated after maternally intoxicated with NRDEPs.

We suggested that the NRDEPs may produce free radical and induce oxidative stress as result from inhibition of the enzymatic and nonenzymatic defenses systems. If this suggestion is valid, NRDEPs may induce apoptosis via mitochondrial intrinsic pathway through activation of caspase-9 which turn on activation of caspase-3 that triggers the rapid DNA fragmentation.

In fact, there is a very strong relation between high oxidative stress and apoptosis. Apoptosis has two pathways: the intrinsic and the extrinsic pathways. The intrinsic pathway implicates mitochondrial perturbation causing cytotoxicity (Danial and Korsmeyer 2004; Marnett 1999; Sun et al., 1999).

The Bcl-2 family consists of proteins that have anti-apoptotic (e.g., Bcl-2 and Bcl-XL) and a pro-apoptotic (e.g., BH3 and Bax) members, which exerts an important role in the controlling of intrinsic mitochondrial pathway of programmed cell death (Desagher et al., 1999). High Bax mRNA expression levels lead to formation of homo- or heterodimers by its ability to interacting with other Bcl-2 members resulted in permeabilization of the outer membrane of mitochondrial (Szabo and Zoratti 2014) which causes disturbance in its transmembrane potential (Kroemer et al., 2007) This lead to release of cytochrome c from mitochondria into the cytoplasm and formation of a complex known as apoptosome (Lü et al., 2003).

Apoptosome can activate caspase-9 that sequentially activates the effector caspase-3 (Johnson and Jarvis 2004). Caspase-3 is responsible for destruction of the specific proteins that lead to initiates apoptosis by releasing caspase-activated deoxyribonuclease which triggers rapid DNA fragmentation (Han et al., 2011) as described in our study by comet assay.

It was also reported that NRDEP induced oxidative stress via production of reactive oxygen species which leads to mitochondrial toxicity or reduce mitochondrial activity, induce mitochondrial O_2 – generation (Hiura et al., 2000; Tseng et al., 2015), which are highly reactive with DNA and induce both apoptosis and DNA fragmentation (Gustafsson and Gottlieb, 2007; Shen et al., 2013).

In the present study, quercetin co-treatment effectively down-regulated the high expression of caspase-3 and Bax, which they are often revealing the degree of apoptosis in fetal brain after maternally exposure to NRDEPs.

Quercetin has antiapoptotic function, which has effectively suppressed of Bax-dependent apoptosis and inhibited of several proapoptotic transcription factors along with up-regulation of anti-apoptotic member Bcl-XL (Attia 2010; Bu et al., 2012).

CONCLUSION

On conclusion, nano-rich diesel exhaust particles induce up-regulation of Bax and caspase-3 level expression leading to trigger the intrinsic mitochondrial apoptosis pathway through oxidative stress in addition to DNA fragmentation. Nevertheless the potent antioxidant quercetin has ameliorative roles against NRDEPs induced oxidative stress along with enhancing cellular antioxidant defense mechanism and inhibiting Bax and caspase-3 level expression so, it can alleviate apoptosis and DNA fragmentation.

Conflict of interest

The authors declare that they have no competing of interest.

Ethics approval and consent to participate

See MATERIALS AND METHODS.

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