

Evaluation of genotoxicity and cytotoxicity induced by different molecular weights of polyethylenimine/DNA nanoparticles

Leila GHOLAMI¹, Hamid Reza SADEGHNIA², Majid DARROUDI^{3,4}, Reza KAZEMI OSKUEE^{1,4,*}

¹Targeted Drug Delivery Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

²Pharmacological Research Center of Medicinal Plants, Department of Pharmacology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

³Nuclear Medicine Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

⁴Department of Medical Biotechnology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Received: 21.09.2013 • Accepted: 28.01.2014 • Published Online: 14.04.2014 • Printed: 12.05.2014

Abstract: Nonviral gene delivery systems are considered to be a safe alternative to the viral system used in gene therapy. Although polyethylenimine (PEI) is among the most promising gene-carrier candidates for efficient nonviral gene delivery, safety concerns regarding its toxicity remain challenging. The aim of this study was to evaluate the less considered aspects of toxicity including genotoxicity effects of branched PEI with different molecular weights (1800 Da, 25 kDa, 750 kDa). Neuro2A mammalian cells were cultured in DMEM with 10% FBS and exposed to PEI/DNA complexes at different polymer/DNA ratios (known as the C/P ratio). To evaluate metabolic activities and genotoxicity, the treated cells were subjected to MTT and comet assays, respectively. Intracellular reactive oxygen species were also determined by using the fluorescent dye dichlorofluorescein diacetate. Concentration with relation to toxicity, genotoxicity, and ROS production was observed in Neuro2A cells treated with PEI/DNA nanoparticles. The difference among toxicities induced by different molecular weights of PEI at higher C/P ratios was significant, whereas no significant toxicity effect was induced by PEI at C/P ratios of 1 and lower. We found concentration-dependent genotoxicity and a direct correlation between nanoparticle concentrations and DNA damage, which cannot be ignored and should be considered for the design of new generations of PEI derivatives with enhanced delivery properties and reduced toxicity.

Key words: Polyethylenimine, toxicity, genotoxicity, polyplex, reactive oxygen species, Neuro2A murine neuroblastoma cells

1. Introduction

Gene therapy can be an optimized strategy for the treatment of many diseases that are currently considered incurable. The major aim of gene therapy is the introduction of normal genes into cells that contain defective genes to reconstitute a missing protein product. Gene therapy is used to correct a deficient phenotype so that sufficient amounts of a normal gene product are synthesized to improve a genetic disorder (Godbey and Mikos, 2001; Lungwitz et al., 2005). All gene therapy strategies depend on getting the gene or genetic material into the target cells (Yalçınkaya et al., 2013). Generally, 2 different types of vectors have been used in gene therapy: viral vectors and nonviral vectors. Although viral vectors are the most practical vectors, their potential immunogenicity and oncogenicity have limited their applications in human gene therapy (Lee, 2007). Compared to viral vectors, the distinguishing characteristics of nonviral vectors, such as easy preparation or modification and relatively safety qualities, have attracted great interest

(Li and Huang, 2000). Cationic polymers and liposomes are the most studied nonviral vectors, with cationic polymers reported with significant differences because of their unique characteristics and potential to generate nanoparticles with DNA for gene delivery (Nakayama et al., 2005; Kafil and Omid, 2011). Polyethylenimine (PEI) is one of the best-known cationic polymers, which has been widely used for nonviral transfection in vitro and in vivo (Ulasov et al., 2011). PEI is a cationic synthetic polymer available in linear and branched forms, and in a vast range of molecular weights, from <1000 Da up to 1.6×10^6 Da (Godbey and Mikos, 2001; Neu et al., 2005; Oskuee et al., 2009). Branched PEI is considered as the gold-standard transfection agent in experimental gene therapy, with protonable primary, secondary, and tertiary amines (ratio of 1:2:1) giving the polymer the ability to act as an effective buffering system. Those properties are expected to be mainly responsible for the 'osmotic burst'-driven endosomal escape mechanism of the formulation,

* Correspondence: oskueekr@mums.ac.ir

making it possible for the nucleic acid to be released from the endosome without degradation (Funhoff et al., 2003; Oskuee et al., 2009). Although PEI is one of the most successful gene carriers, toxicity concerns are an obstacle for in vivo use of this vector, remaining a challenging issue in gene delivery studies (Fischer et al., 1999). Different studies have reported increasing toxicity up to necrosis levels induced by high-molecular-weight PEI, while low-molecular-weight PEI has been reported to be less toxic or nontoxic, but its low transfection efficacy is a major problem (Fischer et al., 2003; Funhoff et al., 2003; Godbey et al., 2000). Since there are few studies that have reported the mechanism of PEI toxicity and its relation to polymer molecular weight, the aim of this article was to gain a better understanding of the genotoxicity and cytotoxicity induced by different molecular weights of branched PEI in order to provide more data about the relation between PEI structure and its toxicity, which can be used in future projects regarding less toxic and more efficient nonviral vectors.

2. Materials and methods

2.1. Materials

Branched PEI with average molecular weights of about 75 kDa, 25 kDa and 1800 Da was purchased from PolyScience Inc. (Niles, IL, USA). N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Munich, Germany). Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were purchased from GIBCO (Gaithersburg, MD, USA). Low-melting-point agarose and regular agarose were obtained from Fermentas (Sankt Leon-Rot, Germany). Tris, NaOH, HCl, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), and Triton X-100 were purchased from Merck (Darmstadt, Germany).

2.2. Amplification and purification of plasmid DNA

Plasmid DNA (pDNA) encoding *Renilla* luciferase (pRL-CMV; Promega, Madison, WI, USA) was transformed into *Escherichia coli* bacterial strain DH5 and amplified in selective Luria-Bertani medium. The pDNA was then purified by using an endotoxin-free QIAGEN Mega Plasmid Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and the purity of pRL-CMV was certified by measuring the A260/A280 ratio.

2.3. Preparation of PEI/pDNA complexes

PEI/pDNA complexes were prepared in HBG (20 mM HEPES, 5.2% glucose, pH 7.2) at various weight ratios in the range of 0.5:1 to 8:1 (w/w). Different amounts of PEI were diluted and added to the pDNA solution; the resulting solution was mixed by pipetting and left at room temperature for 20 min before use.

2.4. Cell line and cell culture

Neuro2A murine neuroblastoma cells (ATCC CCL-131, Manassas, VA, USA) were grown in DMEM (1 g/L glucose, 2 mM glutamine) supplemented with 10% FBS, streptomycin at 100 µg/mL, and penicillin at 100 U/mL. All cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

2.5. Cell viability assay

Evaluation of metabolic activity was performed by using MTT assay (Mosmann, 1983). Briefly Neuro2A cells were seeded at a density of 1×10^4 cells per well in 96-well plates and incubated for 24 h before the procedure. Thereafter, the cells were treated with varying types of PEI/pDNA complexes (20 µL in HBG per well) in the presence of 10% FBS, resulting in a concentration of 200 ng pDNA per well. After further incubation at 24 and 48 h, 10 µL of the MTT solution (5 mg/mL in PBS buffer) was added to each well and the cells were further incubated for 1 h at 37 °C. The medium that contained unreacted dye was discarded and 100 µL of DMSO was added to dissolve the formazan crystal formed by living cells. The optical absorbance was measured at 590 nm (reference wavelength: 630 nm) by microplate reader (Stat Fax 2100, Awareness Technology, Palm City, FL, USA) and cell viability was expressed as percentage relative to untreated control cells. Values of metabolic activities are presented as means \pm SD of triplicates.

2.6. Determination of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) generation was measured using a ROS-sensitive dye, H2DCF-DA. H2DCF-DA passively enters the cell, converts to dichlorofluorescein diacetate, reacts with ROS, and forms the fluorescent product, dichlorofluorescein (DCF). Briefly, the cells were seeded at a density of 10^4 cells/well in 96-well microassay plates and were incubated for 24 h before the procedure. Polyplexes were prepared and added to the wells with the same concentration used for the MTT assay. At 4 and 24 h after treatment, cells were incubated with 20 µM H2DCF-DA for 1 h. Afterwards, the fluorescence intensity was measured at 480 nm excitation and 530 nm emission with a fluorescence microplate reader (Victor X5, PerkinElmer, Waltham, MA, USA).

2.7. Assessment of DNA damage by comet assay

The cells were seeded at a density of 10^6 cells/well in 12-well microassay plate and were incubated for 24 h before the procedure. Polyplexes were added to the cells and, after incubation at 37 °C for 24 h, DNA damage studies were carried using the comet assay method with some modifications (Hosseinzadeh et al., 2008). Slides were prepared in duplicates per concentration and immersed in cold lysis solution at pH 10. This solution consisted of 2.5 M NaCl, 100 mM Na2EDTA, 10 mM Trizma base, 1% Triton X-100, and 10% DMSO; it was kept at 4 °C for 60 min. After lysis, the DNA was allowed to unwind in the electrophoresis

buffer (300 mM NaOH and 1 mM Na₂EDTA, pH 13.5) for 30 min. This was followed by electrophoresis at a constant voltage of 23 V and 300 mA at 4 °C. Slides were neutralized in 0.4 M Tris (pH 7.5) for 5 min and rinsed in water, with each experiment repeated twice.

2.8. Scoring the comet assay slides

The slides were stained with EtBr (20 µg/mL) and photographed using a fluorescent microscope (Nikon, Kyoto, Japan) equipped with appropriate filters (520–550 nm) and a barrier filter of 580 nm at 400× magnification. Scoring was done by image analysis software (CASP Software), reporting the percentage of DNA in the tail (% tail DNA), which gave a clear indication of the extent of DNA damage (Kumaravel and Jha, 2006). Images of 100 cells per concentration were analyzed for different polymers. The median values of each concentration with respect to the comet parameter were calculated by CASP.

2.9. Statistical analyses

All quantitative data were analyzed. The results expressed as mean ± SD. Statistical significance was determined by 2-way analysis of variance. P-values of less than 0.05 were considered to be statistically significant.

3. Result

3.1. MTT assay

In the MTT assay, Neuro2A cells were exposed to different ratios of PEI/DNA complexes for 24 and 48 h. For all 3 molecular weights of PEI, the metabolic activity decreased in a ratio-dependent manner, indicating that metabolic activity started to decrease from C/P 2 and that maximal decrease was observed at C/P 8. We used 2 incubation

times to understand the effect of time on toxicity, but the difference between these results was not significant (Figure 1).

Lower C/P ratios (0.5 and 1) were tried as well in both incubation times, but no cytotoxicity was detected. The cytotoxicity of PEI complexes of 1800 Da was less than that of the others, and the difference between cytotoxicity of PEI complexes of 25 and 750 kDa was significant at C/P 6.

3.2. Determination of intracellular ROS

To evaluate the effects of different molecular weights of PEI on ROS generation, we used the fluorescent dye dichlorofluorescein diacetate. ROS concentrations were elevated compared to a control group in Neuro2A cells following exposure to polyplexes. With all 3 molecular weights of PEI complexed with DNA, the production of ROS was increased, having the maximal increase in ROS levels at C/P ratios of 6, 8, and 8 for PEI of 25 kDa, 750 kDa, and 1800 Da, respectively, at 4 h (Figure 2).

3.3. Comet assay

To assess the degree of possible DNA fragmentation within treated cells, the widely used single cell electrophoresis (comet assay) was utilized and % tail DNA was considered as an indicator of DNA damage. Our results indicated that by increasing the PEI/DNA complex ratio, the amount of damage increased significantly, and the highest damage was observed at C/P 6 for all 3 molecular weights (Figures 3A–3D and 4).

4. Discussion

Cationic polymers including PEI exhibit interesting properties for the delivery of nucleic acids. Generally,

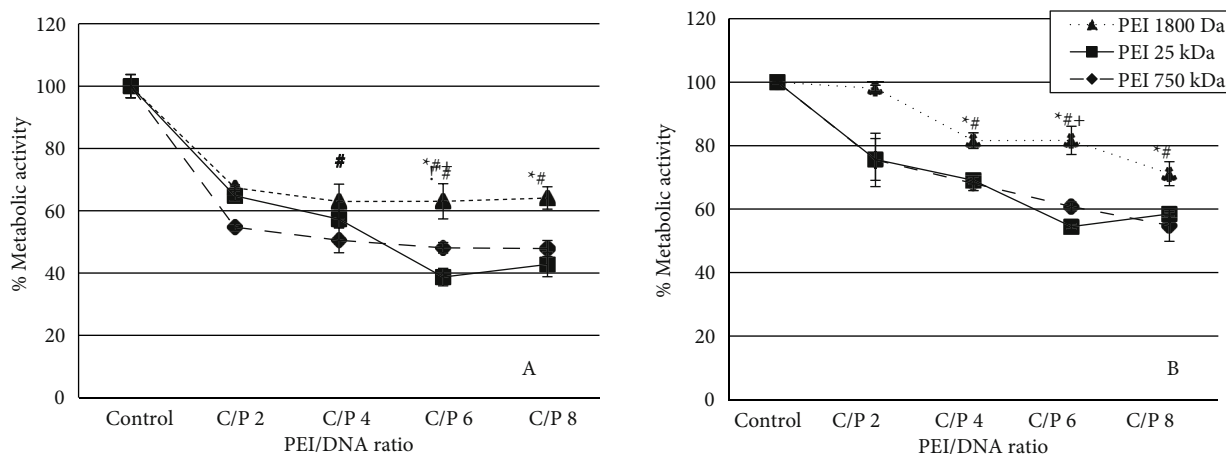


Figure 1. Cytotoxicity of polyplexes formed with polyethylenimine and 200 ng of pRL-CMV plasmid DNA. Neuro2A cells were incubated with polyplexes formed from PEI of 1800 Da, 25 kDa, and 750 kDa in HBG over the indicated range of polycation to DNA C/P (w/w) ratios for 24 h (A) and 48 h (B). Cytotoxicity was assayed by the MTT method and expressed as the percentages of cell metabolic activity by setting untransfected cells as 100%. Metabolic activity is presented as mean values ± SD of triplicates. *: P < 0.001 for PEI 1800 Da compared to PEI 25 kDa, #: P < 0.001 for PEI 1800 Da compared to PEI 750 kDa, +: P < 0.001 for PEI 25 kDa compared to PEI 750 kDa.

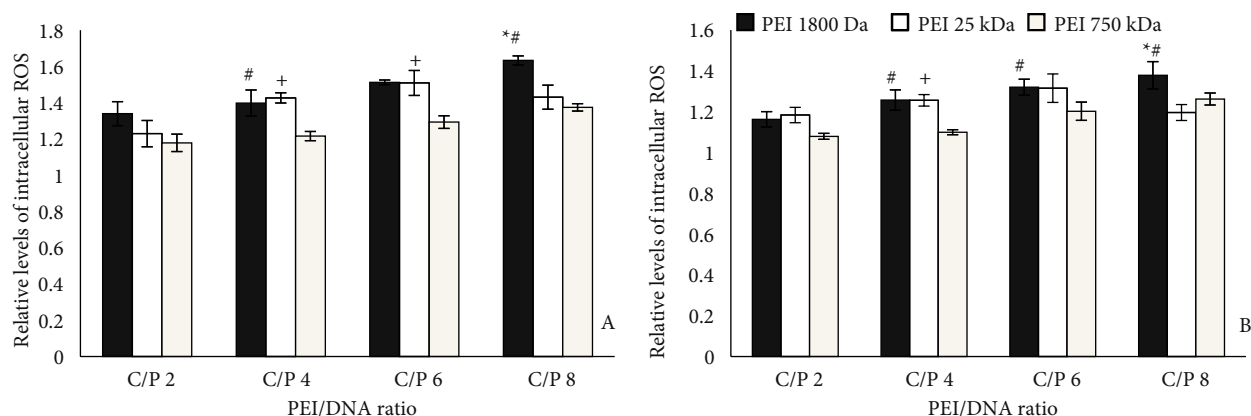


Figure 2. Effect of polyplexes on intracellular ROS level in Neuro2A cells. Neuro2A cells were incubated with polyplexes formed from PEI of 1800 Da, 25 kDa, and 750 kDa in HBG over the indicated range of polycation to DNA C/P (w/w) ratios for 4 h (A) and 24 h (B). The ROS level was measured by the fluorescence intensity of DCF. Data are presented as mean values \pm SD of triplicates. *: $P < 0.001$ for PEI 1800 Da compared to PEI 25 kDa, #: $P < 0.001$ for PEI 1800 Da compared to PEI 750 kDa, +: $P < 0.001$ for PEI 25 kDa compared to PEI 750 kDa.

the following steps are involved in PEI-mediated DNA delivery: DNA condensation and complexation, uptake, endocytosis, escape from the endosome, intracellular release, nuclear entry, and release. PEI has shown promising potential to overcome the major barriers of DNA delivery such as low uptake across the plasma membrane, inadequate release of DNA with limited stability, and lack of nuclear targeting. However, toxicity remains one of the challenges of this vector. There are many targets and assays that can be used to give better insight to different aspects of toxicity (Ulasov, 2011; Fischer et al., 2003; Oskuee et al., 2009), while little is known about genotoxicity and intracellular ROS generation effects of PEI.

In this study we examined the metabolic activity, genotoxicity, and ROS generation effects of branched PEI (with 3 molecular weight of 1800 Da, 25 kDa, and 750 kDa) within Neuro2A cells using MTT, comet, and H2DCF-DA assays, respectively.

The MTT assay showed the effect of PEI concentration on metabolic activity. For all molecular weights, through the increase in polymer concentration (C/P ratio), toxicity was increased, and no significant difference was observed between the 2 times of incubation.

As previously reported, due to excessive accumulation of cationic polymer on the outer cell membrane, toxicity increases with higher molecular weights of cationic polymer, which mostly leads to necrosis (Fischer et al., 1999).

The positive charge surface is one of the factors that makes PEI not only bind to the negatively charged DNA but also to many other biological materials such as cell membrane phospholipids, cell membrane proteins, and blood proteins (Fischer et al., 1999). Molecular weight and the resulting charge density of the polycation are the

main factors for the interaction with the cell membrane and the damage (Fischer et al., 2003). The cytotoxicity of PEI also depends on factors such as molecular weight, the degree of branching, the particle size, and zeta potential. Branched PEI, compared to linear PEI, was reported to be less toxic, while the complex formation with DNA reduces its toxicity significantly (Kafil and Omidi, 2011; Kunath et al., 2003).

Another study reported that PEI, compared to poly-L-lysine and other cationic polymers, creates more toxicity. In this study, the toxicity was a function of time and concentration. One of the most commonly tried approaches to reduce toxicity is the preparation of modified PEI by reducing the surface charge of the polymer (Nimesh et al., 2007). Other structural modifications (Malaekheh-Nikouei et al., 2009; Oskuee et al., 2009) and the targeting of ligand conjugation (Dehshahri et al., 2012a, 2012b) were also tried.

There is much evidence suggesting the role of ROS in oxidative stress in various cells and disorders. Since ROS are ubiquitous in many cellular processes, production of excess ROS can stimulate oxidative stress, which affects many of the normal cell processes (Kermanizadeh et al., 2012). Detection of intracellular ROS is a precise analytical way to determine early cellular toxicities, because the extent of intracellular ROS has been reported to be associated with apoptosis activities (Lee et al., 2011). In our study, the cells were exposed to different ratios of PEI/DNA complexes for 4 and 24 h. The ROS products increased in a ratio-dependent manner, a pattern similar to metabolic activities. No significant differences between PEI of 1800 Da and 25 kDa were observed, but, interestingly, the levels of ROS in cells that were treated with PEI of 750 kDa were less than with other PEIs. There is a possibility that due

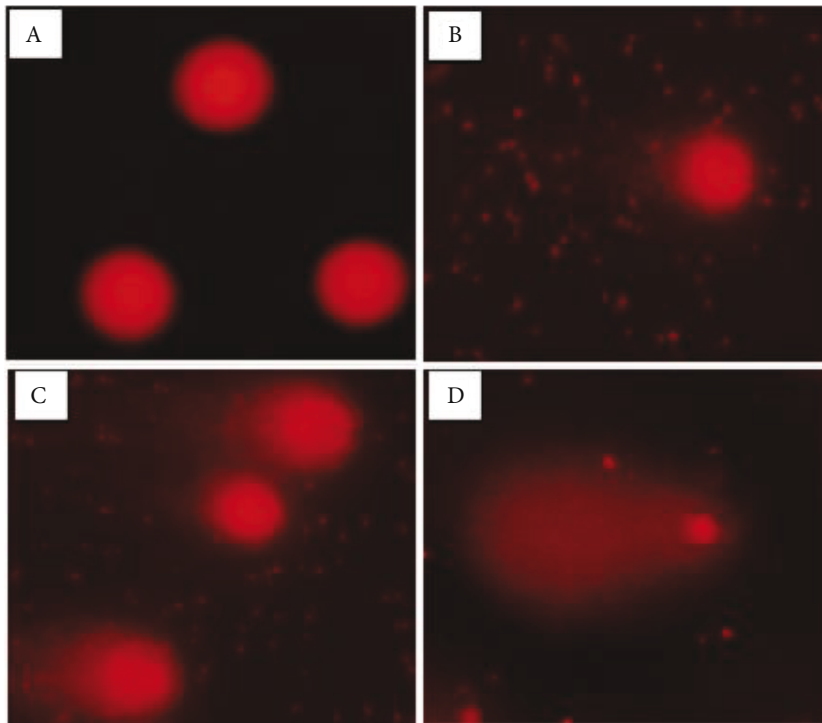


Figure 3. Representative comet assay images of Neuro2A cells treated with different weight ratios of 25 kDa PEI/pDNA (C/P) are demonstrated for untreated (A), C/P 2-treated (B), C/P 4-treated (C), and C/P 6-treated (D) Neuro2A cells.

to high amine content of PEI of 750 kDa, the PEI itself acts as a free radical-quenching agent, but more analysis is needed to prove this (Degli Esposti, 2002).

Increased ROS levels play a predominant role in the production of neuronal damage by apoptosis (Hunter and Moghimi, 2010; Kim et al., 2010; Çiftçi et al., 2013; Darroudi et al., 2013). PEI is known as an apoptotic agent (Moghimi et al., 2005; Beyerle et al., 2009), while the present ROS values also confirmed that apoptosis occurred.

ROS production increases the rate of lipid peroxidation at both cellular and organelle levels, which leads to apoptosis; mitochondrial injuries with increase in ROS level are particularly more evident than in other organelles (Hunter, 2006; Parhamifar et al., 2010; Lee et al., 2011). Some studies reported the reduction of ROS levels by the modification of PEI, which was also associated with reduced toxicity (Beyerle et al., 2010b; Dey et al., 2011). Lee et al. (2011) demonstrated that the intrinsic apoptotic pathway (mitochondrial signaling), which was induced by PEI of 25 kDa after 24 h of treatment, caused intracellular stress and mitochondrial alterations, which finally leads to apoptotic cell death at higher doses (Beyerle et al., 2010a).

Another aim of this study was to evaluate the DNA damage (genotoxicity) induced by different ratios of PEI/DNA complexes. Single cell gel (SCGE) electrophoresis (comet assay) values revealed an increase in the extent

of DNA damage and genetic instability caused by higher ratios of PEI/DNA complexes (Choi et al., 2010). Through SCGE and by the help of DNA tail type analysis, it is possible to predict the kind of damage (Collins, 2004;

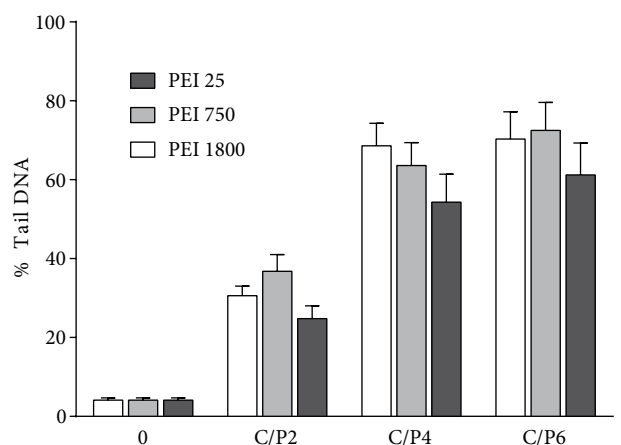


Figure 4. Single cell gel electrophoresis (comet assay) for detection of DNA damage induced by polyethylenimine within Neuro2A cells. Cells were incubated with polyplexes consisting of pRL-CMV complexed with branched 1800 Da/25 KDa/750 KDa PEI at different C/P ratios for 24 h. DNA damage was expressed as % tail DNA.

Braga et al., 2006), explaining why this assay is so widely used for the study of DNA damage in cells (Singh et al., 1988; McArt et al., 2010). There was a general trend toward increased DNA damage in treated cells compared to the control group. Increased genotoxicity was observed at C/P ratios of 2, 4, and 6 in 3 types of polymers. We also tested lower C/P ratios such as C/P 0.5 and 1 for all 3 polymers but no damage was observed, and at C/P 8, the cells faded and only a red halo was seen.

It seems that at higher ratios of PEI/DNA complexes, the type of damage changes from apoptosis to necrosis. Figure 3D illustrates the typical diffuse migration of damaged DNA around each of the single cells during electrophoresis, in the shape of an astronomical comet. An extreme apoptotic tail was observed at C/P 6 and images at higher ratios showed necrotic cells. Some studies have reported that PEI is an apoptotic agent, and this cationic polymer can elevate the levels of apoptotic markers, such as caspase-3, caspase-9, and cytochrome c (Moghimi et al., 2005; Hunter, 2006).

To our knowledge, PEI's genotoxic effects have not been evaluated before, and this study is among the limited studies that consider cationic polymers as genotoxic materials.

Among the studies done on other cationic polymers, Omidi et al. (2005a) investigated the genotoxic effects of 2 types of cationic lipids, Lipofect and Oligofectamine, on A549 and A431 cell lines. Results indicated the extent of the damage to cells, but no significant difference was reported between damage induced by cationic lipids and the control group. They concluded that changes in gene expression by cationic lipids was not associated with an increase in DNA damage. In another similar study by Omidi et al. (2005b), the effects of 2 types of polypropylenimine dendrimers on gene expression and the extent of damage were assessed. They mentioned that although some extent of DNA damage was observable in treated cells compared to the untreated control cells, this was not statistically significant. They

concluded that some of the observed gene expression changes might be an early response to potential DNA damage and apoptosis. Comparative studies on genotoxicity of linear and branched PEI of 25 kDa also showed the same results (Kafil and Omidi, 2011).

Choi et al. (2010), in a study on comparison of toxicity of 2 types of cationic polymer polyamidoamine and PEI in Jurkat T-cells, reported significant and dose-dependent genotoxicity effects for both polymers. They concluded that both polymers induce single-strand DNA breaks and may have an effect on chromosomal damages at lower doses. It is likely that at higher doses of both polymers, the DNA damage mechanism transforms to the necrotic pathway.

In conclusion, the general toxicity of cationic polymers could be different and dependent on many factors such as the types of cationic polymers, molecular weight, and branching degree considering the cell line used in the experiment, which may have impacts on the results. Most studies conceded that genotoxic effect of cationic polymers cannot be ignored. Due to the genotoxicity effects observed with concentrations of PEI that are routinely used for transfection of nucleic acids, those effects should be considered in the design of new generations of PEI derivatives with enhanced delivery properties and reduced toxicity. As our study showed, the toxicity induced by higher-molecular-weight PEIs is negligible at lower concentrations; therefore, one approach to suitable vectors could be modifying these polymers instead of the current trend of using lower-molecular-weight polymers.

Acknowledgments

We would like to thank the Vice Chancellor of Mashhad University of Medical Science for providing financial support to carry out this project. This study was a part of the MSc thesis of L Gholami.

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