
The induction of apoptosis and autophagy in rats bone Marrow mesenchymal stem cells following in vitro treatment with p-Nonylphenol

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Abstract

P-Nonylphenol (p-NP) as a non ionic surfactant is used in many industries such as pesticides, cosmetics, detergents and so on. Our previous study showed that the p-NP causes reduction in viability of the rats Bone Marrow Mesenchymal Stem cells (MSCs). The aim of the present study was to investigate the mechanism of the cell death due to p-NP exposure. The cells, after 3rd passage were treated with 100µM p-NP for a period of 36 hrs, then using TUNEL, caspase and comet assay as well as fluorescent dye like Hoechst, acridine orange and monodansylcadaverin (MDC) staining the mechanism of cell death was studied. The results of this study showed that the p-NP treated cells were TUNEL positive and also, activated caspase-3 enzyme in their cytoplasm was visualized. Hoechst staining showed chromatin condensation and nuclei breakage, at which the nuclear breakage was further confirmed by comet assay. In addition, staining the cells with acridine orange revealed that the cytoplasm of the treated cells contained numerous vesicles. Furthermore, staining of the cells with MDC showed the presence of the double layer autophagic vacuoles. In conclusion, the induced cell death due to p-NP toxicity was determined to be caspase dependent apoptosis as well as autophagy.

Keywords: Apoptosis; autophagy; caspase- 3; monodansylcadaverin; p-Nonylphenol

1. Introduction

p-nonylphenol (C₁₅H₂₄O) is a non ionic surfactant which has been used in many industries worldwide, such as adhesives, papers, textile manufacture and dyeing, paints, detergents, wetting agents, cosmetics and pesticides [1]. While p-nonylphenol (p-NP) shows some acute toxicity, it is also able to mimic important hormones, resulting in the disruption of several processes by interfering with the signals that control the overall physiology of the organism [2]. Because of its hydrophobic properties, p-NP is not water soluble and through the food chain it accumulates in animal tissues [3]. Most of the previous studies have focused on the toxicity of p-NP in developmental process and reproductive system. Little is known about the toxicological effect of p-NP on stem cells. Rat bone marrow mesenchymal stem cells (MSCs) with potential of self-renewal and differentiation [4] are one of the most important adult stem cells to differentiate into a variety of cell lines when needed through the lifespan. In our previous report

we showed that the p-NP caused the reduction of MSCs viability, time and dose dependency [5], but the underlying mechanism of the effects of p-NP on these cells was not clear. Although other investigators studied the toxicological effect of the p-NP on neural stem cells, human embryonic stem cells and in testicular Sertoli cells [6-9], to date no report has been published to reveal the mechanism of cell death in MSCs due to p-NP toxicity. Thus, the present study was designed to investigate the type of cell death and explore the possible mechanism of its signaling pathway in MSCs treated with p-NP.

2. Material and method

Chemical and reagents

p-NP was purchased from Acrose company (New Jersey, USA). The Dulbecco modified eagle medium (DMEM), penicillin-streptomycin, fetal bovine serum and trypsin-EDTA were purchased from Gibco company (Scotland). dimethylsulphoxide (DMSO), monodansylcadaverin (MDC), diaminobenzidine (DAB), Ethidium Bromide, acridine orange, propidium iodide and Hoechst were purchased from

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Sigma Company (Steinheim, Germany). Low melt agarose was used from (Fermentas Company, Iran). All other chemicals used in this research were analytical grade of highest purity and purchased from Merck Company.

Bone marrow isolation and culture

Rats were deeply anesthetized with diethyl ether and sacrificed, then under sterile condition their femora and tibiae were removed surgically. Using flushed out technique the bone marrow content were extracted in a falcon tube containing 3ml of Dulbecco modified eagle medium (DMEM) supplemented with 15 % FBS and penicillin/streptomycin. The content of the tube was centrifuged at 2500rpm for 5min at room temperature and then the pellets of the cells were homogenized with 1ml fresh culture media and transferred to a culture flask. After 24 hrs, unattached cells were washed off the flask with PBS⁺ (containing Mg⁺⁺ and Ca⁺⁺ chloride) and adherent fibroblast-like cells were allowed to grow for 10-14 days, with every three days of culture media replacement. Cells were passaged at 90% confluence by trypsinization using trypsin-EDTA and reseeded at a density of 10⁵ cells in plastic flask up to 3rd passage [5].

Exposure to p-NP

Stock solution of p-NP was prepared in DMSO; the final concentration of DMSO in culture medium was below 0.01% which, at this concentration, DMSO do not affect the normal cell growth. Cells exposure to 100 μM of p-NP was performed after 24h when ensuring the cells were attached to the plastic plate [7].

Morphological study

The attached MSCs in a 24-well plate were treated with 100 μM of p-NP in culture media for 36h. Chromatin staining was performed using Hoechst to detect nuclear morphology then propidium iodide was used along with Hoechst to counter stain as well as to differentiate live cells from dead ones under the fluorescence microscope (Olympus, IX70) [3]. In addition, morphology of the treated cells cytoplasm was investigated using light microscope as well as under fluorescence microscope (Olympus, IX70) with the help of acridine orange (1mg/ml), where acidic vesicular organelles can be stained [10].

Comet assay

DNA breakage was observed using single-cell gel

electrophoresis (comet assay) as described by Lynn et.al [11] with some modification. Briefly, MSCs after 36 hrs of p-NP treatment were harvested and embedded in 1% low melt agarose gel at a density of 1×10⁶ cells/ ml, and spread on a microscopic slide previously coated with normal melting agarose. The slides were immersed in ice-cold lysis buffer (10mM Tris-HCl, 2.5M NaCl, 100mM Na₂EDTA, 1% sodium N-lauryl sarcosinate, PH 10) for 1h at 4°C. Cellular DNA was denatured in electrophoresis buffer (300mM NaOH and 1 mM Na₂EDTA) for 20 minutes at room temperature then electrophoresis was performed for 20 minutes at 25 constant voltages. All of the procedure was carried out under indirect light, then the slides were washed in distilled water and renatured in 0.4M Tris-HCl (pH7.5). The slides were stained with ethidium bromide (2μg/ml) and examined under the fluorescence microscope (Olympus, IX70).

TUNEL assay

Apoptotic cells in the treated samples were end-labeled in situ by TUNEL (Terminal deoxy nucleotidyl transferase-mediated dUTP nick-end labeling) staining using "In situ Cell Death Detection Kit- POD" (Roche, Germany, LOT: 13965100) according to the manufacturer's instructions. In brief, attached cells on the 24 well plates were fixed with 4% paraformaldehyde in PBS⁻ buffer (freshly prepared) for 1 hr at 25°C. The slides were then washed with PBS⁻ and endogenous peroxidase was blocked by incubating the slide in a blocking solution containing 3% H₂O₂ in methanol for 10 minutes at 25°C. Slides were rinsed with PBS⁻ two times, for two minutes each time and incubated in permeabilization solution (TritonX-100 and sodium citrate in water, freshly prepared) on 4°C for two minutes. 50μl TUNEL Reaction Mixture (50μl Enzyme solution and 450μl label solution, freshly prepared) was added to the samples then covered with a layer of parafilm and incubated for 60 minutes at 37°C under humid conditions with protection from light. Negative control was prepared by incubating the sample with only 50 μl of label Solution, without the enzyme terminal transferase. After washing three times in PBS, the slides were incubated with 50 μl of anti-fluorescein antibody conjugated with horseradish peroxidase (POD) for 30 minutes at 37°C in a humidified chamber and finally treated with DAB in a solution in the presence of H₂O₂ for 30 minutes in the dark. After washing the slides with PBS, they were observed by light microscope under 40X magnification.

Immunochemical staining of activated caspase 3

Detection of cleaved caspase 3, a key executioner of apoptosis, was performed by using a SignalStain IHC detection kit (Chemicon, Germany, LOT# 0605029945). Cells were seeded onto a 12-well plate and after 36 hrs were fixed with 4% paraformaldehyde and the procedure was performed according to the manufacturer instructions. Briefly, to prevent nonspecific binding, the cells were immersed in a blocking solution for 1 h at 25°C and then the cells were first incubated with a pre-diluted primary antibody at 4°C overnight and rinsed for 15 minutes with PBS. The cells were incubated with biotinylated secondary antibody for 30 minutes at room temperature, and rinsed for a further 15 minutes with PBS. The cells were then stained and counterstained according to the manufacturer's protocol with DAB and hematoxylin respectively. The cells were observed under a light microscope under 40X magnification equipped with a digital camera. Cells exhibiting the brown cytoplasmic stain were considered positive for activated caspase-3 [12].

Monodansylcadaverine staining

Autophagic vacuoles were detected with monodansylcadaverine (MDC) [13]. The treated cells were washed with PBS to remove the culture medium and then incubated with 0.05 mM MDC (prepared in hot methanol) at room temperature for 1 hr. After incubation, the cells were washed three times with PBS and immediately analyzed by fluorescence microscopy (Olympus IX70) under 40X magnification.

3. Results

Morphological changes

Light microscopy analysis of the untreated cells showed the cells in monolayer and in typical shape as well as attached to the bottom of the flask (Fig. 1a), whereas the treated cells were observed to be detached and round in shape with vacuoles in the cytoplasm (Fig. 1b). Using Hoechst fluorescent dye it was shown that 100 µM of p-NP caused chromatin condensation and nuclear breakage as compared to control. In addition, with the help of propidium iodide as counter stain, an elevated number of cell deaths were observed (Figs. 1c and d).

Autophagic characteristics

Using acridine orange and MDC, the morphology of the cytoplasm and the nature of the vacuoles were investigated. Fluorescence microscopy of

treated cells revealed a punctuate distribution of enhanced fluorescent dye in the cell cytoplasm stained with MDC (Fig.1h) and acridine orange (Fig. f), whereas the control cells (Figs.1g and e) stained with these fluorescent dyes showed no vacuoles.

Apoptosis detection of MSCs

In agreement with the result of chromatin staining, which showed chromatin condensation and breakage, TUNEL assay also showed a clear increase in the number of TUNEL-positive cells in treated group (Fig. 2b) as compared to control group (Fig. 2-a). In addition, immunochemical staining of the cells showed that the caspase-3 was activated in the cytoplasm of the treated MSCs (Fig. 2c) as compared to the control one (Fig. 2d).

Comet assay

The comet assay (single cell gel electrophoresis) showed that the DNA of the cell treated with 100 µM of p-NP was fragmented and under electrophoretic force migrated to form a tail behind the nuclei which appeared as a comet (Fig. 3b) [11] as compared with control cells (Fig. 3a).

4. Discussion

Many reports have classified p-NP as hazardous to the health of human [8] and animals [9]. But the effects of this chemical on cell death and the related underlying mechanisms are not fully understood [7]. It was reported that p-NP was able to trigger apoptosis in human embryonic stem cells via Fas/ Fas ligand pathway [7-9], and could induce apoptosis in thymocyte via caspase-3 activation and mitochondrial depolarization [3, 9, 14]. In a previous study we observed that the p-NP was toxic to rat Bone Marrow Mesenchymal Stem Cells [5] and concentration of 100 µM of p-NP at 36h was enough to induce highly significant reduction ($p < 0.001$) of viability.

In the present study, staining the cell nuclei with Hoechst fluorescent dye showed chromatin condensation and DNA breakage, which might be considered as an indication of apoptosis [15]. Our data demonstrated caspase-3 activation and the presence of activated caspase-3 fragments in response to p-NP [16]. Cleaved caspase-3 is an effector caspase and activates the DNA fragmenting factors, which degenerate chromatin [17]. Additionally, there were many more TUNEL positive cells in the p-NP treated cells than in the untreated ones. Based on these results, we concluded that p-NP had the ability to stimulate the apoptosis [18]. Furthermore, the comet assay analysis of MSCs showed a substantial increase in DNA damage in the p-NP treated cells compared to the control [19]. The combined findings confirm that

p-NP induced apoptosis via a caspase-dependent mechanism in Bone Marrow Mesenchymal Stem cells.

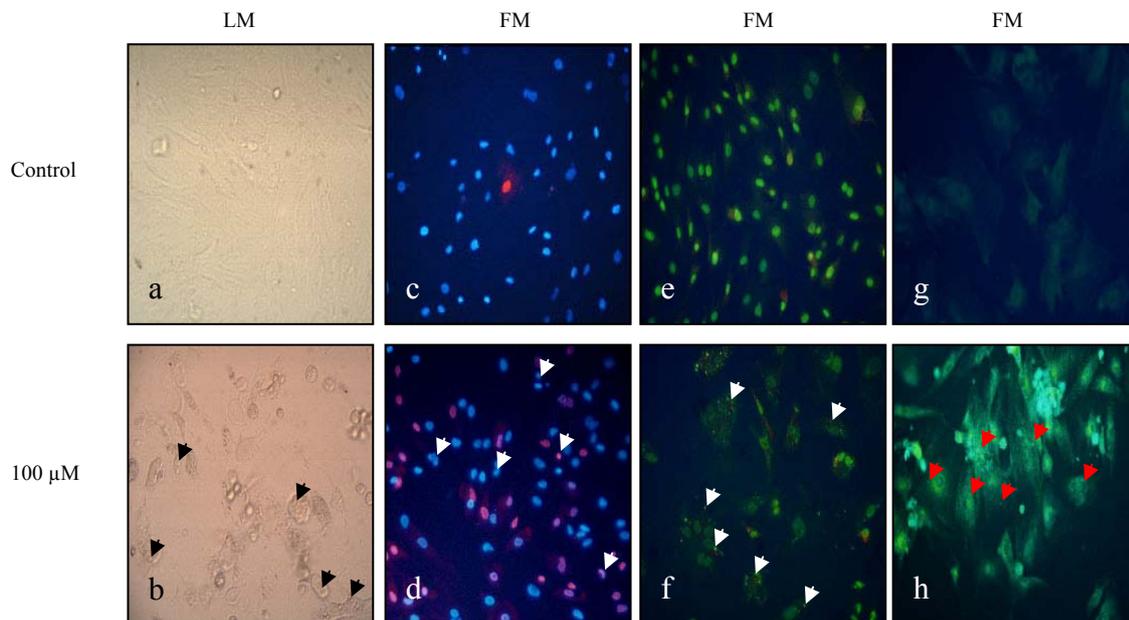


Fig. 1. The cells after 36 hrs treatment with Para-Nonylphenol. (a and b) Light microscopic (LM) images of the control and treated cells in which extensive cytoplasmic vacuolization was observed in treated MSCs (arrows). (c and d) control and treated cells stained with Hoechst and propidium iodide, chromatin condensation and nuclear breakage, as well as an elevated number of death cells can be observed (arrows). (e and f) control and treated cells stained with acridine orange, at which acidic vacuoles were visualized in treated cells. (g and h) control and treated cells were labeled with MDC where the nature of the vacuoles was confirmed to be autophagic (40X magnification).

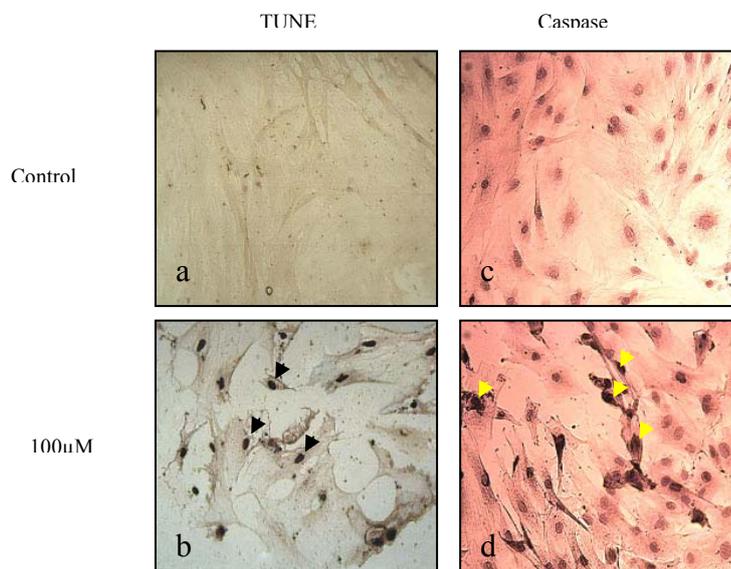


Fig. 2. (a and b) immunostaining of the control and treated cells using TUNEL assay, the deep brown nuclei of the treated cells confirmed the breakage of the chromatin in the treated cells (c and d) control and treated cells were immunostained for activated caspase, arrows show activated caspase in the cytoplasm of the treated cells. (40X magnification).

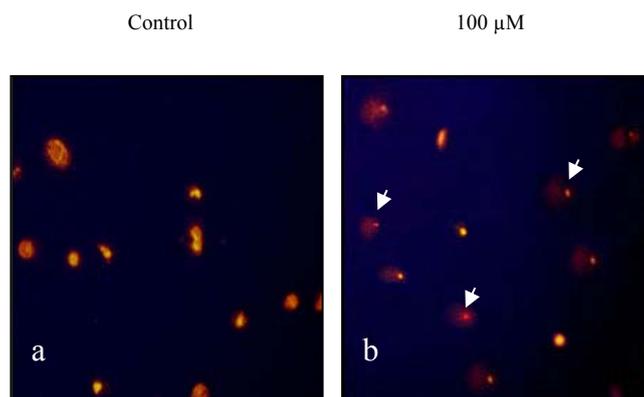


Fig. 3. Comet assay (single cell gel electrophoresis) of the control and treated cells after exposure to p-NP for 36 hrs (a) no DNA break was observed in control, (b) The DNA is fragmented and a tail can be observed due to DNA breakage in the treated cells(20X magnification).

Our data also suggested that p-NP induced autophagic character of cells, including cytoplasmic vacuolization under light microscopy. In addition, MDC and acridine orange staining confirm the presence of such autophagic vacuoles in the cytoplasm of the treated cells. Like other lysosomal compartments, autophagic vacuoles possess an acidic pH which is generated by a V- type H^+ -ATPase [20], thus one of the most frequently used methods for following autophagic character is staining the vacuoles with acidotropic dyes such as MDC and acridine orange [21].

The sarcoplasmic reticulum Ca^{+2} -ATPase is thought to be a specific site of nonylphenol action in animal cells [2]. Stress that affects the ER is widely known to induce apoptosis through the intrinsic pathway. ER stress is also a particularly efficient stimulus of autophagy that specifically affects the ER [22]. Autophagy and apoptosis share many common inducers, thus the type of initiating stimulus might determine which process will dominate [22].

In this report, the cellular and molecular mechanisms of programmed cell death induced by p-NP in rat bone marrow mesenchymal Stem Cells were examined. In conclusion, our finding showed that p-NP can induce both caspase dependent apoptosis and autophagic cell death, where in other research work carried out on p-NP no indication of autophagic mechanism has been pointed out, therefore this is the first report to reveal the occurrence of apoptosis and autophagy together in rat bone marrow mesenchymal Stem Cells due to p-NP toxicity.

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