Iranian Journal of Basic Medical Sciences

ijbms.mums.ac.ir

IJBMS

Biochemical and morphological changes in bone marrow mesenchymal stem cells induced by treatment of rats with p-Nonylphenol

Mohammad Hossein Abnosi [1]*, Elham Shojafar [1]

[1] Biology Department, Faculty of Sciences, Arak University, Arak, Iran

ARTICLEINFO

Article type:
Original article

Article history:
Received: Jul 1, 2014
Accepted: Dec 14, 2014

Keywords:
Apoptosis
Differentiation
Mesenchymal stem cells
Para-nonylphenol
Proliferation
Rats

ABSTRACT

Objective(s): In previous investigations, we have shown para-nonylphenol (p-NP) caused significant reduction of proliferation and differentiation of rat bone marrow mesenchymal stem cells (MSCs) *in vitro*. In this study, we first treat the rats with p-NP, then carried out the biochemical and morphological studies on MSCs.

Materials and Methods: Proliferation property of cells was evaluated with the help of MTT assay, trypan blue, population doubling number, and colony forming assay. Differentiation property was evaluated with quantitative alizarin red assay, measurement of alkaline phosphatase (ALP) activity as well as intracellular calcium content. In addition; morphological study, TUNEL test, activated caspase assay, and comet assay were performed to evaluate the mechanism of the cell death.

Results: The results showed significant reduction in the colony-forming-ability and population-doubling-number of extracted cells when compared to control ones. In addition, it was revealed that the p-NP treatment of rats caused significant reduction in nuclear diameter, cytoplasm shrinkage, and induction of caspase-dependent-apoptosis. Also there was significant reduction in ALP activity, intracellular calcium content, and intracellular matrix following osteogenic differentiation.

Conclusion: As MSCs are the cellular back up for bone remodeling and repair, we suggest more investigations to be conducted regarding the correlation between the increasing number of patients suffering from osteoporosis and p-NP toxicity. Also, we strongly recommend WHO and local health organization to prevent industries of using p-NP in formulation of industrial products which may cause changes in proliferation and differentiation properties of stem cells.

► *Please cite this paper as:*
Abnosi MH, Shojafar E. Treatment of rats with p-Nonylphenol caused biochemical and morphological changes in bone marrow mesenchymal stem cells. Iran J Basic Med Sci 2015; 18:317-324.

Introduction

Para-nonylphenol (p-NP), commonly used in many industries such as food container, leather, paper, cosmetics, and detergents has been considered as an environmental pollutant. P-NP, in today's life has been traced in many types of food, and was reported to be a source of water pollution (1-3). This chemical can find its way to the body of animals through food and water consumption (3, 4) and due to its lipophilic property, it accumulates in fat tissues, which may cause serious health problem (4-7). Previous *in vitro* investigations have revealed the significant reduction of cell viability due to induction of programmed cell death in the cells such as thymocytes, jurkets, neurons, adipocytes, cancer cells, embryonic stem cells, and sertoli cell (8-13).

Mesenchymal stem cells (MSCs) are considered as an important source of cellular backup for bone regeneration and repair, therefore if these cells are endangered, there might be high risks of diseases such as osteoporosis. In the previous investigation we have shown that the micromolar concentrations (0 to 250 µM) of p-NP in a dose and time dependent manner would cause significant reduction of viability of rats' bone marrow MSCs after 12, 24, 36, and 48 hr (14). In another study we showed that the treatment of MSCs with 100 µM of p-NP for 24 hr would result in chromatin condensation and nuclear breakage as well as cytoplasmic shrinkage and vacuolization. In the same study the cells showed positive comet test, positive TUNEL, and activated caspase 3 in their cytoplasm which all together was considered to be the signs of apoptosis (15). Another investigation in our lab with 0 to 5 µM of p-NP in a period of 21 days showed significant dose and time dependent reduction of viability and proliferation ability of MSCs. In the same study the induction of caspase dependent apoptosis due to a long treatment period of the MSCs with 0.5 and 2.5 µM of p-NP was shown (16). Furthermore it was revealed that the p-NP caused significant reduction of the osteogenic differentiation ability of the MSCs (17).

*Corresponding author: Mohammad Hossein Abnosi. Biology Department, Faculty of Sciences, Arak University, Arak, Iran. email: m-abnosi@araku.ac.ir

All the above investigations have been conducted *in vitro*, however MSCs are directly reachable *in vivo* only through blood circulation, and many biological barriers ameliorate and compensate the adverse effect of p-NP on MSCs when given orally. Therefore one question remained to be answered; if the animal is intoxicated via oral consumption, would the toxic effect of p-NP and the underlying mechanisms of toxicity be the same results obtained in the *in-vitro* situation? Thus in this study, the animals were treated orally with p-NP for a period of 3 months and then several biochemical and morphological investigations were conducted to answer the question.

Materials and Methods

Animal treatments and MSCs isolation

Wistar rats were divided in two groups, namely treated (N=6) and control (N=6) and were kept in the animal house of Arak University under standard condition of food, water, and temperature. The treated group received 300 mg kg^{-1} per day of p-NP dissolved in sesame oil for three months, whereas the control group was treated only with same amount of the oil. After the treatment period the rats were anesthetized using diethyl ether and euthanized according to the laboratory animal protocol approved by Arak University. Then under sterile condition their femora and tibias were removed surgically and using flashed out technique the bone marrow content were extracted in 3 ml of Dulbecco modified Eagle medium (DMEM) supplemented with 15% FBS and penicillin/ streptomycin. The bone marrow content was centrifuged at 2500 rpm for 5 min at room temperature and pellet of the cells were homogenized with 1 ml fresh culture media and transferred in a culture flask. After 24 hr, unattached cells were washed off the flask with phosphate-buffered saline (PBS^{+}) containing Mg^{++} and Ca^{++} 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 (Trypsin/EDTA solution; sigma) and reseeded at a density of 10^5 in another plastic flask (18). The time required for cell to reach the passage (in course of days) and the number of cells (using hemocytometer chamber) in each passage were noted down.

Quantification of Proliferation ability

To quantify the proliferation ability of the cells after 3rd passage, the colony forming assay and the population doubling number were performed. To carry out colony forming assay, 1×10^4 cells extracted from treated and control rats were separately seeded in 3 cm^2 sterile dishes. Cells were allowed to grow for 14 days, with every three days of culture media replacement. After 14 days crystal violet staining (0.5 g crystal violet in 100 ml methanol solution) was performed and the number and diameter (µm) of the colonies were estimated using light microscope equipped with graticule eyepiece.

To estimate the population doubling number, 1×10^4 cells extracted from treated and control rats were separately seeded in 3 cm^2 sterile dishes. Cells were allowed to grow for 5 days, with every three days of culture media replacement, then the cells were washed twice with PBS, harvested with trypsin-EDTA, and the number of the cells was counted using hemocytometer chamber. The population doubling of the cells was calculated using the equation $PDN = \log N/N_0 \times 3.31$; where N_0 is the initial number of the cells seeded and N is the number of the cells harvested after 5 days (19).

Cell viability assays

The viability test on MSCs extract from treated and control rats were carried out after 3rd passage using (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT colorimetric test and trypan blue staining assay. Performing MTT assay, the cells were cultured in ELISA microplate in equal density and after 24, 48, and 72 hr the plates were washed with PBS followed by addition of FBS depleted fresh media to the plates. Then 10 µl of MTT solution (5 mg/ml in PBS) was added to each wells and plate was incubated for 4 hr in an incubator. After incubation period, to extract crystals of formazan, 100 µl of DMSO was added to each well and the plate was incubated for 30 min. The extracted solutions were transferred to another well and absorbance was taken at 505 nm using an automated microplate reader (SCO diagnostic, Germany). Standard graph was plotted and linear formula $Y=0.016X+0.037$ with $R^2=0.996$ was used to estimate the number of the live cells in the MSCs extracted from p-NP treated and control groups (20). In the above mentioned formula Y is the absorption and X is the cell number.

In trypan blue staining assay the MSCs extract from p-NP treated and control rats were also harvested after time interval of 24, 48, and 72 hr and washed with PBS, then the cells were stained with trypan blue solution (0.4 g/100 ml in PBS) for 2 min in 37 °C. The total number of the cells and number of the live (transparent) and dead (blue in color) cells were estimated using hemocytometer chamber and the percentage of the live cell were reported.

Morphology

The MSCs after the 3rd passage were cultured in a 24-well plate for 24 hr then the nuclear morphology of the cells was studied using Hoechst 33342 (1 mg/ml in PBS) after 5 min of incubation in the dark. The diameter (µm) of the cells was also measured with Motic Image software (Micro optical group company, version 1.2). Hoechst is a fluorescent dye which penetrates the cells through the intact plasma membrane and stains the DNA where the changes in nuclear morphology such as chromatin condensation and fragmentation can be investigated (19). Co-staining

the cells with Hoechst and propidium iodide (1 mg/ml in PBS) for 5 min at room temperature was used to discriminate between dead and live cells. Propidium iodide is also a fluorescent nucleic acid binding dye which cannot penetrate the membrane of viable cells but readily enters the cells through the impaired cell membrane (19). The morphology of the cell cytoplasm was investigated using acridine orange solution 1 mg/ml in PBS which stains the nucleus green and the cytoplasm orange. The stained cells were washed twice with PBS, examined, and immediately photographed under an inverted fluorescence microscope (Olympus, IX70) equipped with a camera (DP72) using 40^X magnification.

Single cell gel electrophoresis

DNA breakage was observed using single-cell gel electrophoresis (comet assay) as described by Lynn *et al* (21) with some modification. Briefly, after 3rd passage the MSCs extracted from p-NP treated and control rats were harvested and embedded in 1% low melt agarose (Fermentas company) gel at a density of 1×10^6 cells/ml, and spread on a microscopic slide previously coated with normal melting point agarose. The slides were immersed in ice cold lysis buffer (10 mM Tris-HCl, 2.5 M NaCl, 100 mM Na2EDTA, and 1% sodium N-lauryl sarcosinate, pH 10) for 1 hr at 4ºC. Cellular DNA was denatured in electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA) for 20 min at room temperature then electrophoresis was performed for 20 min at constant voltage (25 V). All the procedure was carried out under indirect light and then the slides were washed in distilled water and renatured in 0.4 M Tris-HCl (pH 7.5). The slides were stained with ethidium bromide (2 µg/ml) and examined under the fluorescence microscope (Olympus, BX51) equipped with camera (DP71). The broken DNA under the influence of electrical field forms a tail like comet at which it could be observed behind the nuclei of the cells.

TUNEL assay

MSCs extract from p-NP treated and control rats after 3rd passage were cultured in 12 well-plates for 24 hr and then were fixed using 4% paraformaldehyde in PBS for 1 hr at room temperature. The nuclear DNA fragmentation was detected by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method using the *in situ* cell death detection kit (Roche, Germany, Catalog #: 11684817910) according to company instruction and the brown nuclei were visualized under the light microscopy in 20X magnification.

Activated caspase-3 examination

MSCs extract from p-NP treated and control rats after 3rd passage were seeded at a density of 1×10^5 cells/ml in a 12 well-plate separately. Then activated caspase-3 enzyme was examined using a fluorogenic substrate assay kit (Chemicon, Germany, Catalog #: APT403) according to company instruction. To visualize the activated caspase-3 under the light microscope equipped with camera, the cells were stained and counterstained according to the manufacturer's protocol with DAB (3, 3'-diaminobenzidine) and hematoxylin, then the microphotograph of the cells were taken in 20X magnification.

Osteogenic induction and estimation of osteogenic property

Mineralization was induced on confluent mono-layers of cells with the addition of DMEM containing 15% (v/v) FBS, streptomycin-penicillin, and osteogenic supplements (1 mM sodium glycerophosphate, 50 µg/ml L-ascorbate, and 10^{-8} M dexamethasone); all the chemicals were purchased from Sigma-Aldrich company. Culture flasks were incubated at 37˚C with 5% CO_2 and their medium was changed every 3 days for 21 days. To estimate the osteogenic property; quantification of mineralization, ALP activity and matrix calcium concentration were measured.

Quantification of mineralization

Extracted MSCs from treated and control rats after 3rd passage were cultured in 6-well plates and treated with osteogenic media for 21 days. After 21 days the cells were washed with PBS then fixed with 10% (v/v) formaldehyde (Sigma-Aldrich) at room temperature for 15 min. The cells were then washed twice with an excess of dH_2O and 1 ml of 40 mM alizarin red solution (ARS) (pH 4.1) was added to each well. The plates were then incubated at room temperature for 20 min with gentle shaking. The excess of dye was poured off and the plates were washed 4 times with dH_2O and then the photographs were taken. To quantify the level of absorbed alizarin red, 800 µl of 10% acetic acid (v/v) was added to each well, and the plate was incubated at room temperature for 30 min with gentle shaking. Then, the loosely attached cells were scraped from the plate with a cell scraper and transferred to a 1.5 ml microcentrifuge tube. After vortexing for 30 sec, the slurry was overlaid with 500 µl mineral oil (Sigma-Aldrich), heated at 85˚C for 10 min, and then kept on ice for 5 min. The slurry was then centrifuged at 13500 rpm for 15 min and 500 µl of the supernatant was transferred to a new microcentrifuge tube and 200 µl of 10% ammonium hydroxide (v/v) was added to neutralize the acid. Aliquots of the supernatant (100 µl) was read in triplicate at 405 nm in a microplate reader (SCO diagnostic, Germany) and quantified against standard graph (22). In order to prepare alizarin red standards graph, working ARS (40 mM) was diluted 20 times with a mixture of 5:2 of 10% acetic acid and 10% ammonium to give a concentration of 2000 µM. Then, using serial

Table 1. Effect of *para*-nonylphenol on the time (days) required for the cells extracted from the control and treated rats to reach the next passage

	Control group	Treated group
Passage 1 (primary ulture)	14*± 0.57	19± 1.70
Passage 2	4*± 0.60	6± 1.50
Passage 3	8*± 0.60	11± 0.57
Passage 4	9± 1.50	Nil
Passage 5	11± 0.60	Nil

Values are mean±SD. * significantly different in row (t-test, *P*-value <0.05), Nil: the cell of the treated groups after the passage 3 did not reach the confluency, therefore no time was recorded

dilution, standard solution of 2000 to 31.3 µM was prepared and the absorption was taken at 405 nm using a microplate reader. The concentration of the unknown samples was calculated using linear formula Y= 0.179X+0.094 with R2= 0.997 where Y is the absorbance and X is the concentration (mM) of alizarin red.

Alkaline phosphatase activity

Extracted MSCs from p-NP treated and control rats after 3rd passage were cultured in 6-well dishes and treated with osteogenic media for 21 days. The ALP activity of the cells was determined by p-nitrophenyl-phosphate (pNPP) hydrolysis method, using the ALP assay kit (Darman Kave, Iran) after 21 days; the activity of ALP was determined in the cells of passage 3. The cells were washed 3 times with PBS and homogenized in lysis buffer (0.25 M Tris-HCl, Triton X-100, pH 7.5) (22) and the samples were centrifuged at 12000 rpm for 10 min at 4˚C. The supernatant was kept in -20˚C for the analysis of ALP activity and protein content. The total protein content of each sample was determined according to Bradford, using bovine serum albumin (BSA) as the standard benchmark. ALP activity was determined in protein lysate based on equal amount of protein using pNPP as substrate according to the manufacturer's instructions. Absorbance was measured at 410 nm using spectrophotometer (T80+ PG instrument Ltd, England) and then ALP activity was determined from a pNPP standard curve.

Matrix calcium concentration

Passage 3 cells in 6-well plates including the MSCs from treated and control rats were treated with osteogenic media for 21 days. The cells were first washed twice with PBS and then their calcium content was extracted in 50 µl of 0.5 N HCl for 24 hr (17). The amount of calcium was determined using commercial kit (Darman Kave, Iran) and the developed color was measured at 575 nm using spectrophotometer (T80+ PG instrument Ltd, England).

Table 3. Effect of *para*-nonylphenol on proliferation ability of the cells extracted from treated and control rats based on population doubling number, number of the colonies (number of colony /cm^2) and diameter of the colonies (mm)

	Control group	Treated group
Population doubling number after 5 days	1.15*±0.05	0.77±0.02
Colony number /cm^2 after 14 days	27.33*±1.50	6.66±0.57
Colony diameter of MSCs after 14 days(mm)	1.65*±0.05	1.44±0.03

Values are mean±SD. * significantly different in row (T-test, *P*-value <0.05)

Statistical analysis

Analysis of the data was performed using t-test by SPSS. Results were shown as mean±SD and *P*-value <0.05 was accepted as the minimum level of significance.

Results

Time required for the cells to reach the next passage

The time (days) required for the cells extracted from the treated rats to reach the next passage was significantly longer (*P*-value <0.05) than the time for cells extracted from control group of rats. In addition, the cells extracted from treated rats could not proliferate and grow enough to form monolayer of the cells at the bottom of the culture flask beyond 3rd passage. Thus no time was recorded for the 4th and 5th passages for cells in treated group compared to the cells extracted from control rats (Table 1). Therefore the passage numbers which the cells extracted from p-NP treated rats could tolerate was reduced as compared to the cells extracted from control rats.

Effect of p-NP on cell viability

Cell viability determination using MTT colorimetric assay showed that, as the time passed, the viability of the cells extracted from treated rats was significantly reduced (*P*-value <0.05) compared to the control group of cells. In addition the viability of the cells extracted from treated rats after 24, 48, and 72 hr defers significantly (*P*-value <0.05) from each other (Table 2). Also these results were confirmed by cell viability determination using trypan blue staining method (Table 2).

Table 2. Effect of *para*-nonylphenol on viability of MSCs extracted from treated and control rats, based on MTT colorimetric and trypan blue staining assay

Time(hr)	Averaged number of alive cells (×1000) with MTT assay		Averaged percentage of alive cells with trypan blue staining assay	
	Treated group	Control group	Treated group	Control group
24	14.62^{c}±0.78	18.76^{ab}±0.80	94.33^{ca}±0.57	95.33^{a}±0.57
48	9.31^{d}±0.35	18.54^{a}±0.67	80.00^{d}±1.00	96.66^{a}±0.20
72	5.67^{e}±0.20	19.24^{b}±0.16	18.00^{e}±1.00	84.66^{b}±0.11

Values are mean±SD. Means with the same letter code do not differ significantly from each other (T-test, *P*<0.05)

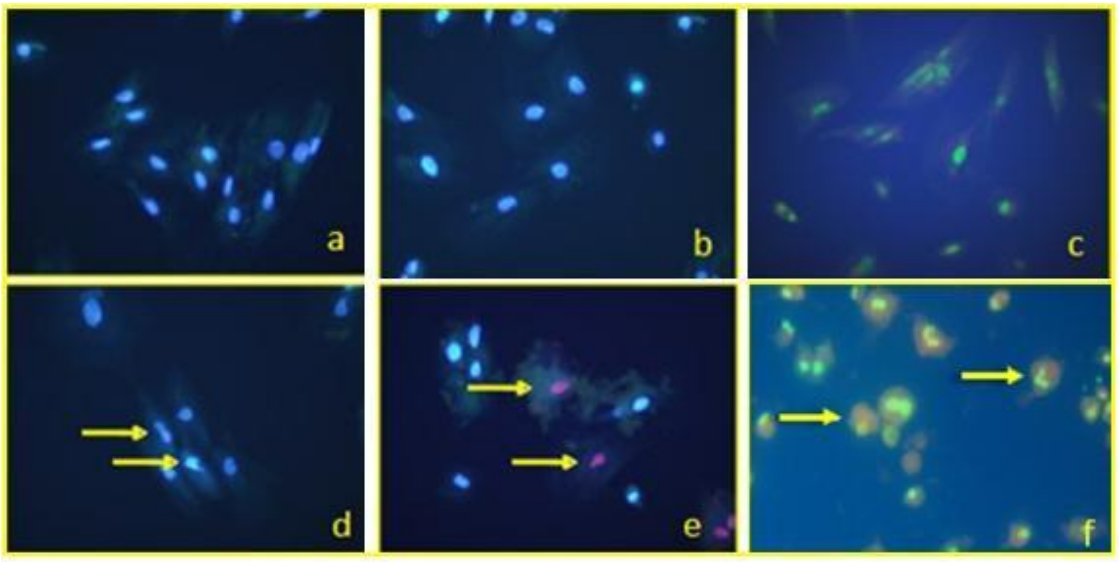

Figure 1. Morphology of the cells a) cells extracted from control rats stained with Hoechst, nuclei appeared large with no nuclear breaking b) co-staining of the same cells with Hoechst and propidium iodide, cell nucleus are stained in blue c) cells extracted from control rats stained with acridine orange, typical morphology of the mesenchymal cell cytoplasm was visualized d) MSCs extracted from p-NP treated rats stained with Hoechst, nuclei appear small as compared with control with nuclear breakage e) co-staining of MSCs extracted from p-NP treated rats with Hoechst and propidium iodide, some of the nucleus (shown by arrows) are stained in red which indicate the cell membrane damage f) MSCs extracted from p-NP treated rats stained with acridin orange, cell cytoplasm shrinkage and roundness was observed. (20X magnification)

Quantification of Proliferation ability

The cells extracted from the treated rats showed significant reduction (*P*-value <0.05) of number and diameter of the colonies (Table 3) compared to the cells extracted from the control rats. Population doubling number of the cells extracted from the treated rats also showed a significant reduction (*P*-value <0.05) compared to the cells extracted from the control rats (Table 3).

Morphology

Morphological study of the nuclei of mesenchymal stem cells extracted from the treated rats showed chromatin condensation and nuclear breakage (Figure 1-a & d) as well as significant reduction (*P*-value <0.05) in nuclei diameter (mm) (9.4±0.45) when compared to the diameter (mm) of the nuclei of the cells extracted from the control rats (12.2±0.20). In addition, with the help of propidium iodide as counter stain, an elevated number of dead cells were monitored (Figure 1-e) in treated group when compared to the control (Figure 1- b). It was also noticed that p-NP treatment of the rats caused remarkable changes in the morphology of cytoplasm (Figure 1-f) such as cell roundness, cytoplasm destruction, and in some cells complete disappearance of cytoplasm content as well as aggregation of cytoplasmic debris when compared to the control (Figure 1-c).

Estimation of osteogenic property

Data analysis of alizarin red quantification assay showed that the mineralization of cells extracted from treated rats under influence of osteogenic differentiation media was reduced significantly (*P*-value <0.05) when compared to cells extracted from control group (Table 4). Matrix calcium concentration of the cells also decreased significantly (*P*-value <0.05) as compared to control ones (Table 4). In addition, significant decrease (*P*-value <0.05) of the activity of ALP enzyme was also observed in the cells extracted from treated group of rats as compared with control ones (Table 4).

Apoptosis detection

Apoptosis detection of the cells extracted from treated and control rats, was studied using single cell gel electrophoresis (comet assay), TUNEL test, and caspase-3 activity estimation. In agreement with the result of chromatin staining, which showed chromatin condensation and breakage, TUNEL assay also revealed a clear increase in the number of TUNEL-positive cells in the culture dishes containing cells extracted from treated group of rats (Figure 2-b) as compared to control group (Figure 2-a). In addition, immunochemical staining of the cells showed that the caspase-3 was activated in the cytoplasm of the cells extracted from treated rats (Figure 3-b) as compared

Table 4. Effect of *para*-nonylphenol on osteogenic property of the cells extracted from treated and control rats based on quantity of mineralization (mM), alkaline phosphatase activity (U/l) and matrix calcium concentration (mg/dl)

	Control group	Treated group
Quantity of mineralization	44.66±5.50	20.15±4.5
Alkaline phosphatase activity after 21 days	63.13*±12.33	24.78±5.57
Calcium concentration after 21 days	29.21*±3.10	11.97±1.20

Values are mean±SD. * significantly different in row (t-test, *P*-value <0.05)

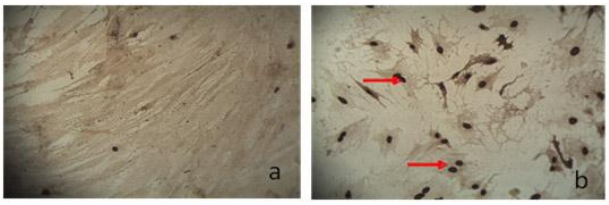

Figure2. TUNEL assay of cells extracted from control and p-NP treated rats a) control cells, no brown color nucleus is observed b) MSCs extracted from p-NP treated rats, most of the cell nuclei were stained brown which indicated the chromatin breakage in the cell nuclei (20X magnification)

to the control one (Figure 3-a). The single cell gel electrophoresis also showed that the DNA of the cells extracted from treated rats was fragmented and under electrophoretic force migrated to form a tail behind the nuclei (Figure 4-b) when compared to cells extracted from control rats (Figure 4-a).

Discussion

In this study, the time to reach the next passage was defined as the time period when the bottom of the culture flask was covered with the monolayer of the cells in course of days. The present data showed that the time needed to reach the next passage was increased significantly for the cell extracted from the treated rats compared to the cells extracted from the control ones. Also the proliferation of the cells could not be continued after 3rd passage and therefore the number of the passages was reduced to 3 as compared to passage number of the cells extracted from control rat which was 5. In a recent study Choo and *et al* showed that the oxidative stress can induce pre-mature senescence in MSCs derived from Wharton's Jelly (23), since p-NP is able to induce oxidative stress (7). Therefore it might be one of the reasons which reduce the ability of the MSCs extracted from p-NP treated rats to undergo lesser number of passages. As stated before, in our previous study we showed that the *in vitro* treatment of the MSCs by p-NP causes significant reduction of viability and proliferation ability in short and long treatments with high and low doses of p-NP (14-16). Present study also showed that *in vivo* treatment of the rats with 300 mg/kg/day of p-NP for a period of 3 months also have the same impact on viability and proliferation property of the extracted cells. Therefore the reduction of proliferation ability and viability might be another reason for significant increase of the time required to reach the passage and the reduction of passage number. The reduction of viability could be due to many reasons: 1) the loss of the cell membrane integrity 2) intracellular organelle damage 3) nuclear and chromatin damage 4) induction of gene expression 5) mutation in the receptor or ion gate genes (14, 17).

To pinpoint the mechanism of cell death, in the present study we conducted morphological investigations, which showed that 1) the p-NP treatment affects the cell membrane integrity based on entry of propidium iodide to the cell; as this florescent dye cannot cross the intact membrane 2) chromatin condensation and nuclear breakage which might be due to activation of certain nucleases 3) cytoplasmic shrinkage and destruction which might be due to inhibition of cytoskeletal rearrangement and assembly. It was postulated that the main reason of the above mentioned damages might be apoptosis; TUNEL test, comet assay, and visualization of activated caspase-3 were run to investigate the possibility of apoptosis. The TUNEL test proved that there is chromatin breakages due to activation of certain nucleases which are able to cut the double stranded of DNA. These nucleases are normally activated when the cell undergo apoptosis. In addition, the single cell gel electrophoresis or comet assay (which large pieces of DNA form a tail like structure behind the remaining of the cell nuclei) confirmed the chromatin breakage in an electrical field. The chromatin breakage could have happened due to necrosis or non- programmed cell death, so the presence of activated caspase-3 as an affecter caspase was visualized to confirm the induction of apoptosis. Based on these results, the induction of apoptosis can be considered as the main mechanism of the cell viability reduction and decrease of passage number due to p-NP treatment. Previous *in vitro* studies on MSCs also indicated that the chemicals such as sodium arsenite (18, 19), cadmium chloride (24), and p-NP (14, 15) can cause morphological changes as well as induction of caspase-dependent apoptosis similar to what happened in the present study.

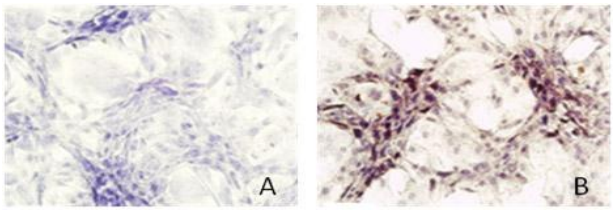

Figure 3. Activated caspase-3 assay of the cells extracted from control and p-NP treated rats. A) Control cells co-stained with DAB and hematoxylin, no sign of brown color in cytoplasm was detected. B) MSCs extracted from p-NP treated rats co-stained with DAB and hematoxylin; most of the cell cytoplasm showed brown in color to indicate the presence of the activated caspase- 3. (20X magnification)

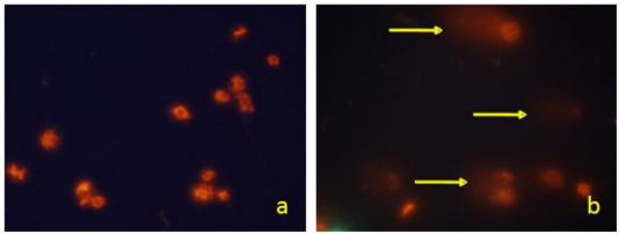

Figure 4. Comet assay (single cell gel electrophoresis) of the MSCs extracted from control and p-NP treated rats. (a) No DNA break was observed in control, (b) The DNA is fragmented and a tail can be observed due to DNA breakage in MSCs extracted from p-NP treated rats' cells (20X magnification)

Viability and proliferation ability of the MSCs extracted from bone marrow of the p-NP treated rats were not the only characters of MSCs to be affected by p-NP. The differentiation property of MSCs was also investigated by estimating the level of mineralization with quantitative alizarin red assay (QAR) after incubating them with osteogenic media for 21 days. Analysis of the data revealed that the matrix mineralization of the cells extracted from p-NP treated rats was reduced significantly. The differentiation of MSCs to osteoblasts by ostogenic induction needs expression of several genes such as ALP, osteopontin, and osteocalcin (25). The ALP activity starts about the day 4 and increases to reach its maxima till the day 7 and continues to the day 14 of osteogenic development in the *in vitro* (26). In the present study the activity of the ALP as the phenotype of the gene expression showed a significant reduction in the MSCs extracted from p-NP treated rats. In addition, calcium concentration of differentiated cells was reduced significantly as compared with cell extracted from control rats. Calcium is one of the main mineral of the osteoblast cell matrix which participates in the formation of hydroxyapatite crystals besides phosphate and collagen (27). Imbalance of the ALP activity and calcium concentration would cause the improper osteogenic differentiation of the MSCs. Our previous *in vitro* studies also showed the same effect on differentiation property of MSCs due to p-NP toxicity (17). Therefore it is strongly concluded that the p-NP affects the proliferation ability of MSCs in any situation. However, in addition to ALP activity and calcium content, there are many other reasons such as metabolic imbalance due to inhibition of enzymes activity or reduction of gene expression responsible for production of cytoskeletons (5,28), that should be considered for reduction of proliferation ability of the MSCs.

In conclusion, we can say that there are no differences in mechanism of p-NP toxicity *in vivo* or *in vitro*. However in the *in vivo* situation one would expect versatile and complex systems such as detoxification roles played by kidney, liver, spleen, and other organs to ameliorate or reduce the effect of such chemicals. But the present study showed that even treatment of the rats with one third or one fourth of LD_{50} dose of p-NP for a period of 3 months would bring about an accountable damage to proliferation and differentiation property of the bone marrow MSCss. P-NP is a lipophilic agent which can accumulate in the fat tissues and remain for long period of time in the same tissue (29). Lipophilic agents can travel via blood in accompany with other biomolecules such as albumin, therefore p-NP might be blood born to the bone marrow which is rich in fat (30). Then it can accumulate in course of time and bring about the same results which have been observed in this research work. We strongly recommend more precaution to be taken with the toxicity of p-NP as it has been used in many industries products such as cosmetics. The application of cosmetics in today's life has been increased even in teenagers. The use of this chemical for long time can bring about its accumulation in the fat portion of the skin and can cause serious damage to the skin stem cells which may cause wrinkling and premature ageing of the skin. We strongly recommend the WHO and local health organization to prevent industries to use p-NP in formulation of industrial products and conduct more investigations on toxicity of p-NP specially with respect to osteoporosis and the health of skin stem cell lineage.

Acknowledgment

Here we cordially acknowledge the authorities of the Arak University which financially help us to be able to carry out this research work.

References

1. Canadian Environmental Protection Act. Priority substances list assessment report: Nonylphenol and its Ethoxylates. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/psl2-lsp2/nonylphenol/index-eng.php.

2. Institute for health and consumer protection. European Union Risk Assessment Report: 4-nonylphenol (branched) and nonylphenol. (http://europa.eu.int), CAS Nos: 84852-15-3 and 25154-52-3, volume 10. 2002. http://www.bfr.bund.de/cm/343/4_nonylphenol_und_nonylphenol.pdf

3. United States Environmental Protection Agency. Office of Water: Ambient aquatic life water quality criteria for nonylphenol – Draft. Available from: www.epa.gov/ waterscience/criteria/nonylphenol

4. Gong Y, Wu J, Huang Y, Shen S, Han X. Nonylphenol induced apoptosis in rat testicular Sertoli cells via endoplasmic reticulum stress. Toxicol Lett 2009; 186:84-95.

5. Latendresse JR, Newbold RR, Weis CC, Delclos KB. Polycystic kidney disease induced in f1 sprague-dawley rats fed para-nonylphenol in a soy-free, casein-containing diet. Toxicol Sci 2001; 62:140-147.

6. Daidoji T, Inoue H, Kato S, Yokota H. Glucuronidation and excretion of nonylphenol in perfused rat liver. Drug Metabol Dispos 2003; 31:993-998.

7. Soleimani Mehranjani M, Noorafshan A, Hamta A, Momeni HR, Abnosi MH, Mahmoodi M, *et al*. Effects of vitamin E on ovarian tissue of rats following treatment with p-nonylphenol: A stereological study. IJRM 2010; 8:1-9.

8. Masuno H, Okamoto S, Iwanami J, Honda K, Shiosaka T, Kidani T, *et al*. Effect of 4-Nonylphenol on cell proliferation and adipocyte formation in cultures of fully differentiated 3T3-L1 cells. Toxicol Sci 2003; 75:314–320.

9. Yao G, Yang L, Huc Y, Liang J, Liang J, Hou Y. Nonylphenol-induced thymocyte apoptosis involved caspase-3 activation and mitochondrial depolarization. Mol Immunol 2006; 43:915–926.

10. Michelangeli F, Ogunbayo OA, Wootton LL, Lai PF, Al-Mousa F, Harris RM, *et al.* Endocrine disrupting alkylphenols: Structural requirements for their adverse effects on Ca2+pumps, Ca2+ homeostasis & Sertoli TM4 cell viability. Chemico-Biol Interact 2008; 176:220–226.
11. Vivacqua A, Recchia AG, Fasanella G, Gabriele S, Carpino A, Rago V, *et al.* The food contaminants bisphenol a and 4-nonylphenol act as agonists for estrogen receptor in MCF7 breast cancer cells. Endocrine 2003; 22:275–284.
12. Yao G, Ling L, Luan J, Ye D, Zhu P. Nonylphenol induces apoptosis of Jurkat cells by a caspase-8 dependent mechanism. Int Immuno pharmacol 2007; 7:444–453.
13. Kudo C, Wada K, Masuda T, Yonemura T, Shibuya A, Fujimoto Y, *et al*. Nonylphenol induces the death of neural stem cells due to activation of the caspase cascade and regulation of the cell cycle. J Neurochem 2004; 88:1416–1423.
14. Abnosi MH, Mehranjani MS, Momeni HR, Najafabadi MM, Barati M, Shojafar E. The effect of *para*-nonylphenol on the viability and morphology of rat bone marrow mesenchymal stem cells. Arak Med Univ J 2011; 14:1-11.
15. Abnosi MH, Solemani Mehranjani M, Momeni HR, Mahdiyeh Najafabadi M, Barati M, Shojafar E. The induction of apoptosis and autophagy in rats bone Marrow mesenchymal stem cells following *in vitro* treatment with p-Nonylphenol. Iran J Sci Technol Trans A-Sci 2012; 36:239-244.
16. Abnosi MH, Soleimani Mehranjani M, Shariatzadeh SMA, Mahdiyeh Najafabadi M, Dehdehi L. The effect of long term treatment of lowest effective dose of para-nonylphenol on viability, morphology and proliferation of rat bone marrow mesenchymal stem cells. Physiol Pharmacol 2011; 15:308-317.
17. Abnosi MH, Soleimani Mehranjani M, Shariatzadeh MA, Dehdehi L. Para-nonylphenol impairs osteogenic differentiation of rat bone marrow mesenchymal stem cells by influencing the osteoblasts mineralization. Iran J Basic Med Sci 2012; 15:1131-1139.
18. Abnosi MH, Soleimani Mehranjani M, Momeni HR, Shojafar E, Barati M. Induction of apoptosis in the rat bone marrow mesenchymal stem cells following sodium arsenite treatment with the dose lesser than that used for treatment of malignant patient. Iran J Basic Med Sci 2012; 15:900-906.
19. Abnosi MH, Jafari Yazdi Z. Low dose and long term toxicity of sodium arsenite caused caspase dependent apoptosis based on morphology and biochemical character. Cell J 2012; 14:161-170.
20. Abnosi MH, Soleimani Mehranjani M, Momeni HR, Mahdiyeh Najafabadi M, Shojafar E, Barati M. Effect of sodium arsenite on viability, morphology and protein profile of the rat bone marrow mesenchymal stem cells. J Hamedan Med Uni 2010; 17:24-32.
21. Lynn S, Gurr JR, Lai HT, Jan KY. NADH oxidase activation is involved in arsenite-indused oxidative DNAdamage in human vascular smooth muscle cells. Circ Res 2000; 86:514-519.
22. Abnosi MH, Jafari Yazdi Z. Sodium arsenite caused mineralization impairment in rat bonemarrow mesenchymal stem cells differentiating to osteoblasts. Iranian J Toxicol 2012; 6:577-587.
23. Choo KB, Tai L, Hymavathee KS, Wong CY, Nhi Nguyen PN, Huang CJ, *et al*. Oxidative stress-induced premature senescence in wharton's jelly-derived mesenchymal stem cells. Int J Med Sci 2014; 11:1201-1207.
24. Abnosi MH, Soleimani H. Cadmium affects viability of bone marrow mesenchymal stem cells through membrane impairment, intracellular calcium elevation and dna breakage. Indian J Med Sci 2010; 64:177-186.
25. Weinreb M, Shinar D, Rodan GA. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. J Bone Min Res 1990; 5:831–842.
26. Sila-asna M, Buniyaratvej A, Maeda S, Kitaguchi H, Buniyaratvej N. Osteoblast differentiation and bone formation gene expression in strontium-inducing bone marrowmesenchymal stem cell. Kobe J Med Sci 2007; 53:25-35.
27. Boskey AL. Mineralization of Bones and Teeth. Elements 2007; 3:387–393.
28. Robertson LS, McCormick SD. The effect of nonylphenol on gene expression in Atlantic salmon smolts. Aqua Toxicol 2012; 122–123:36– 43.
29. Rhind SM, Kyle CE, Telfer G, Duff E, Smith A. Alkyl phenols and diethylhexyl phthalate in tissues of sheep grazing pastures fertilized with sewage sludge or inorganic fertilizer. Environ Health Perspect 2005; 113:447–453.
30. Deschamps-Labat L, Péhourcq F, Jagou M, Bannwarth B. Relationship between lipophilicity and binding to human serum albumin of arylpropionic acid non-steroidal anti-inflammatory drugs. J Pharma Biomed Anal 1997; 16:223-229.