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Original Article

Effects of Hydrogen Peroxide Oxidative Stress on the Pattern of Pro-apoptotic and Anti-apoptotic Genes Expression During PC12 Cells Differentiation

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Background and Aims: In neurodegenerative disorders,oxidative stress mediated by reactive oxygen species is strongly associated with increased neuronal damages which can lead to apoptosis. Pro-apoptotic and anti-apoptotic gene expressions are changed during the cell differentiation that affect cell viability and differentiation. Therefore, this study was conducted to determine the effects of hydrogen peroxide-induced oxidative stress on the apoptotic cell death in the differentiated rat pheochromocytoma (PC12) cells.

Materials and Methods: Semi-differentiated PC12 cells were treated with 400 µM hydrogen peroxide (H2O2). Characteristic morphological changes as apoptotic index were evaluated by DAPI staining. MTT assay were applied in order to evaluate the cells survival as well as cell activity.Pro-apoptotic and anti-apoptotic gene expressions were estimated by real time-PCR.

Results: The obtained data indicated that PC12 cell survival rate decreased H2O2 treated condition during the differentiation. Moreover, H2O2 was proved to increase apoptotic genes expressions including caspase 6 as well as PIN1 and to decrease anti-apoptotic genes including SIRT1 as well as SIRT7.

Conclusion: The findings of the present study revealed that H2O2-induced oxidative stress can retard the differentiation of PC12 cell in the form of neural-like cells through the apoptotic gene expression. On the other hand, although the PIN1 acts as an apoptotic gene, this study illustrated that this gene expression can get increased during the differentiation under oxidative stress conditions.

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Introduction

Oxidative stressplays a key roleinapoptosisof neural cells [1] which leads to cell damage in a variety of neurodegenerative diseases, including Alzheimer's disease and Parkinson's [2].Various types of chemical and physiological oxidative stress inducers are available that can cause apoptotic cell death [1-3]. Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion and hydroxyl radicals can result in oxidative stress [2,4]. These mediators are produced in the normal metabolic and inappropriate processes consuming the molecular oxygen. ROS can attack proteins, membrane lipids and deoxynucleic acid that canlead to their malfunction and entirety ROS through apoptosis leading to cell death[2]. Apoptosis or programmed cell death is a regulated process involving molecular agents that canlead to the cell death [5]. $H_2O_2$is produced during the Redox process involvingcellular signaling cascades [3]. H_2O_2-induced apoptosisis associated with changes in apoptosis and anti-apoptosis proteins [4]. Caspase-6 belongs to the cysteine-aspartic acid protease family. As a matter of fact, continuous activity of caspasesplays a central role in the apoptosis induction. Caspase-6 can break down proteins involving in the chromatin condensation and nuclear shrinkage,leading to the apoptosis beginning [6]. Peptidyl-prolylcis-transisomerase NIMA-interacting1 (PIN1) can be mentioned as a member of the peptidyl-prolylisomerase enzyme family being disabled by its phosphorylatedresidues. Oxidative stress can reduce the level of PIN1 phosphorylation in the neurons and increase their activity [7]. PIN 1 protein can induce apoptosis via strengthening the expression of proapoptotic proteins such as P53 (proapoptotic protein). PIN1 augments P53-induced mitochondrial damage and induces apoptosis by releasing cytochrome C through the mitochondria [9]. Silent mating-type information regulation 2 homolog (SIRT) 1 works as nicotinamide adenine dinucleotide-dependent histone deacetylase. SIRTuins involve a group of enzymes that lead to the displacement of their substrates between the nucleus and cytoplasm through their acetylation. SIRTuin family proteins play a key role in the physiological regulation including regulation of gene transcription, metabolism, growth, cancer, circadian rhythms and aging [9]. SIRT1 deacetylates containing both histone and non-histone proteins are involved in the cell growth, apoptosis, cell senescence and tumor production [10]. As a matter of fact, increased levels of SIRT1 can protect the cells against reactive oxygen species-induced DNA damage and reduce apoptotic death in vitro [11]. SIRT7 can be regarded as another member of the SIRTuin family that is less studied, and can decrease P53 activity as well as DNA damage. Therefore, it causes resistance to apoptosis and improves cell survival under genomic stress conditions. It should be noted that SIRT7 deficiency induces apoptosis [12]. In truth, the apoptotic effects of caspase-6 and PIN1 as well as anti-apoptotic effects of

SIRT1 and SIRT7 were reported in the literature, though role of these genes in the differentiation rate of PC12 cells under oxidative stress has not yet been studied. Since PC12 cells are considered as neural progenitors and these genes play a crucial role in their differentiation and growth, the present study intended to evaluate their expression under oxidative stress conditions during early stages of PC12 cells differentiation into neural like cells.

Materials and Methods

Cell culture and viability

PC12 cells (Pasteur Institute, Tehran, Iran) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% of fetal bovine serum (FBS), 100 U/ml penicillin and 10 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The medium was changed every other day. Regarding the experiments, the cells were seed in a 96-well plate (1×10^4 cells /well), in which they were allowed to grow. PC12 cells were cultured and treated with retinoic acid (1µl/ml) as well as free serum medium in order to differentiate morphologically into the neural cells. After 5 days, the PC12 cells were converted into the new differentiated cells that were morphologically similar to neurons with some neurites. For induction of apoptosis, the medium was treated by 400 µm of H_2O_2[13], and the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (sigma Aldrich) was utilized in order to determine the cell viability. Briefly, 24h after the treatment, the medium was removed and replaced with a medium (150 µl/well) containing 1% FBS. Then, 10 µl of a 5 mg/ml MTT solution in Phosphate-buffered saline was added to each well. After 3h at 37°C, the cell supernatants were discarded, and the MTT crystals (formazan) were generated by the mitochondrial dehydrogenase activity of live cells which were dissolved in 200µl/well of dimethyl sulfoxide (Sigma Aldrich). The absorbency of the specimens was evaluated at a wave-length of 560 nm with 690 nm as a reference wave-length. The range of MTT alternation in H_2O_2 treated cells is represented as a percent of the control values (100%).

DAPI staining assay

In order to detect the nuclear fragmentation, the fluorescent dye, DAPI, was used to distinguish the apoptotic cells from PC12 cells. The differentiated PC12 cells were incubated at 37°C with 400µM of H2O2 for 24h and then were stained via the DNA-specific fluorochrome dye, DAPI (1 µg/ml) for 20 min. After staining, the cells were washed with PBS and fixed with 4% of paraformaldehyde. The plates were observed under an inverted fluorescence microscope (Nikon eclipse Ti-u Japan) and some photos were taken from different fields of the cells [4] (Fig.1).

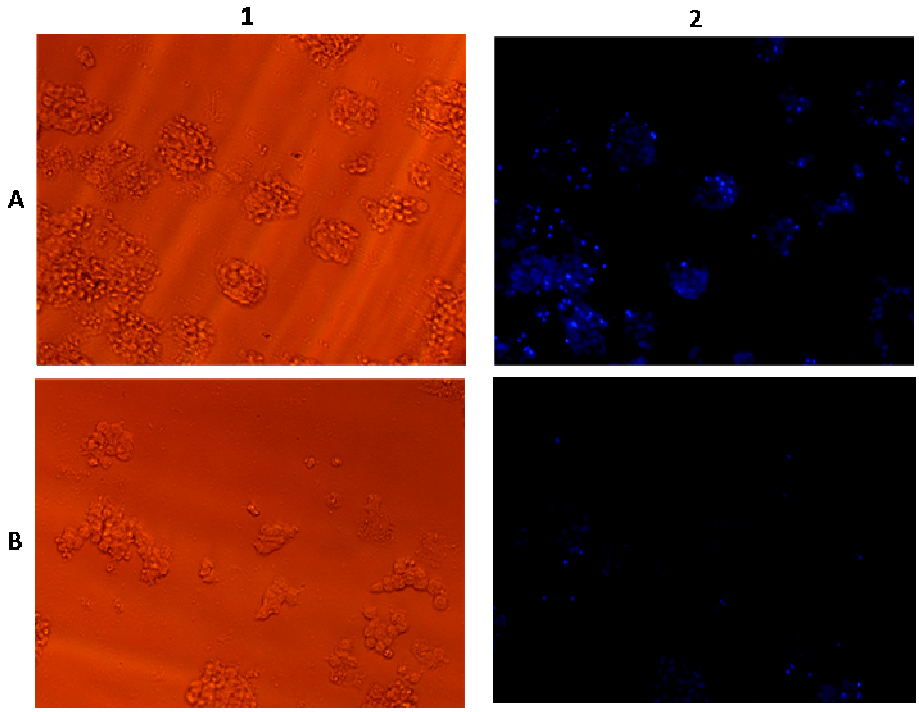

Fig. 1. Apoptotic effect of H_2O_2 on morphology of differentiated PC12 cells. Chromatin condensation and nuclear fragmentation were analyzed by fluorescence microscopy utilizing the DNA binding fluorescent dye DAPI that illustrates apoptotic cells. (A-1) showed H_2O_2–treated PC12 cells by the normal microscopy. (A-2) illustrated H_2O_2–treated PC12 cells by the fluorescence microscopy. (B-1) indicated non-treated PC12 cells by the normal microscopy. (B-2) showed non-treated PC12 cells by the fluorescence microscopy

Real time PCR

The total RNA was extracted according to RNA purification kit (Jena Bioscience, Germany) directions. The extracted RNA was then reverse-transcribed into single stranded cDNA synthesis using MMLV Reverse Transcriptase and Oligo (dT)15 Primers according to the manufacturer's instructions (Vivantis, Malaysia) at 65°C for 5 min., 25°C for 10 min, 50°C for 60 min., and 70 °C for 10 min and then samples were chilled on the ice. Regarding gene expression analysis, relative quantitative PCR (qPCR) was performed using SYBR-Green-based protocols in Rotorgen (Qiagen) system and software (Qiagen, Australia). The primers were designed using Allele identification software version 7.5 (Primer Digital Ltd). The studied genes consisted of caspase6, SIRT1, SIRT7, pin1 and beta-actin, as housekeeping genes. Toward the augmentation reactance, the qPCR SYBR Master Mix was applied (Qiagen SYBR Green PCR Master Mix). The oligonucleotide primers (TakapoZist Company, Tehran, Iran) sequences are demonstrated in Table 1, and the qPCR conditions were set as follows: 10 min. at 95°C, followed by 35 cycles at 95°C for 15 seconds, 52°C for 1 min. The expression levels of the target genes in each sample were calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$ composition) since as in standardized to the Ct value of the beta-action. The experiments were carried out according to the

guidelines of animal ethics committee of Shahid Sadoughi Medical University.

Table 1. The name of genes, their ID in Gen bank and their designed primer sequences

Gene	Primer sequences	Size	Gene ID
cas6-F	5'- CACACATTTCCCTTCTACAC-3'	154	NM_001271984.1
cas6-R	5'- GATTTCTTTAGCCCTTTCCC-3'		
sir7-F	5'- GCAAAGCAGACACAATCC-3'	185	NM_001107073
sir7-R	5'- CCGCATTACATCATCACATT-3'		
sir1-F	5'- ATGAAGTATGACAAAGATGAAGT-3'	142	XM_006256146
sir1-R	5'- GTAGATGAGGCAGAGGTT-3'		
pin1-F	5'- CAGGAGAGGAGGACTTTG-3'	193	NM_001106701
pin1-R	5'- GTGCGTAGGATGATATGGA-3'		
Act b-F	5'- CTGTGCTATGTTGCCCTA-3'	103	NM_031144
Act b-R	5'- TAGTGATGACCTGACCGT-3'		

Statistical analysis

The data analysis among groups was performed using unpaired t student test via Prism 5 software. P-values less than 0.05 were considered statistically significant, and the data were presented as mean± standard error of mean (SEM).

Results

The mean absorbance (560/690) in the H_2O_2-treated cells significantly (17.9±0.49) decreased compared to non-treated cells (24.84±0.30) (P<0.0001) (Fig.2A). DAPI staining was performed in order to estimate the apoptotic rate. As it is shown in fig.1, the nuclei from normal cells revealed an approximately similar staining with no clues of chromatin condensation. In contrast, H_2O_2-treated cells indicated a staining pattern illustrating chromatin fragmentation. The study results demonstrated the mean percentage of apoptotic cells was significantly increased in H_2O_2-treated cells (70±3.077) compared to non-treated cells (4±1.128) (P<0.0001) (Fig.2B).

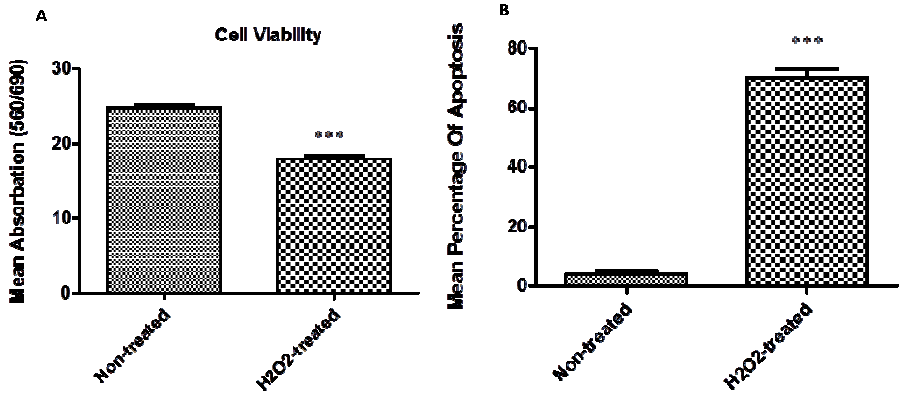

Fig. 2. Quantification of MTT assay & DAPI staining: (A) MTT assay for cells viability after 24h (B) DAPI staining for indicating apoptotic cells rate after 24h
*** P<0.001

Real time-PCR assay revealed that SIRT1 expression significantly decreased in the H_2O_2-treated cells (0.0236±0.0011) in comparison with non-treated cells (1±0.057) (P<0.001) (Fig.3A). Moreover, the study data illustrated significant down expression of SIRT7 in the H_2O_2-treated cells (0.00711±0.0007) in comparison with non-treated cells (1±0.057) (P<0.001) (Fig.3B). As figure3C demonstrates, pin1 expression was exceptionally augmented in the H_2O_2-treated cells (9.694± 2.816) compared to non-treated cells (1±0.17) (P<0.05). It was also noted that caspase-6 significantly increased in the H_2O_2-treated cells (1.806± 0.044) as compared to non-treated cells (1±0.057) (P<0.001) (Fig.3D).

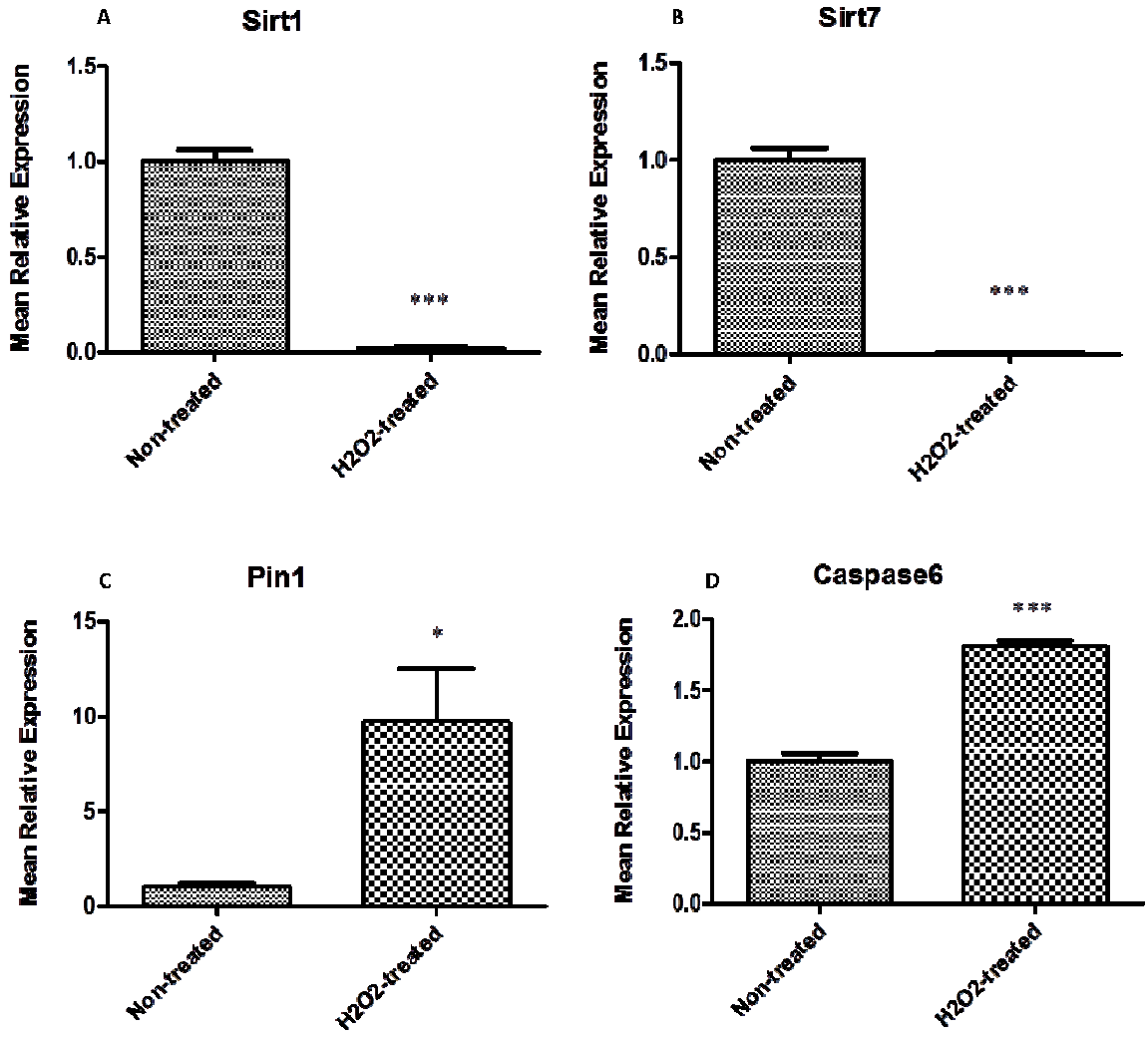

Fig. 3. Gene expression in differentiated PC12 cells after 24 hr. (A) Quantitative data of SIRT1 expression. (B) Quantitative data of SIRT7 expression. (C) Quantitative data of Pin1 expression. (D) Quantitative data of Caspase-6 expression.

* P<0.05 , *** P<0.001

Discussion

In this study, the PC12 cells were cultured, which underwent differentiation into neural-like cells in the presence of retinoic acid. During ongoing differentiation, cells in the initial phases of transformation received a single oxidative stress by H_2O_2. Apoptosis-induced changes entail cell rounding, protrusions development (pseudopods), cell volume reduction, chromatin concentration (pyknosis), nuclear fragmentation (karyorrhexis) [12]. The previous studies have proposed that when PC12 cells are exposed to H_2O_2-induced stress, some cells often show new morphology including plasma membrane changes. Furthermore, DNA fragmentations have been reported following the stress of hydrogen peroxide in PC12 cells. At higher doses of H_2O_2, most cells demonstrated necrotic cell death consisting of cell membrane perforation signs and the cell surface bubble [14]. In the current study, the results of DAPI staining confirmed that when PC12 cells were treated with H_2O_2 (400 µmol), the percentage of apoptotic cells significantly increased compared to the untreated cells. In addition, the results of MTT assay revealed that H_2O_2 can reduce the survival rate of PC12 cells. As it was mentioned, these findings are in line with the results of the related previous studies. Many studies were conducted on PC12 cells and other cell lines in regard with the H_2O_2 effects of the expression of apoptotic and anti-apoptotic cell death pathway. Xie et al. proposed that $H_2O_2$with concentrations ≥50 µm leads to an increase in MnSOD (manganese superoxide dismutase)gene and apoptosis in the spiral ganglion cells [15]. One study demonstrated that H_2O_2 at a dose of 120 µmol/L decreases SIRT1 gene expression protein, cell survival, though it increases caspase3 expression in the PC12 cells [16]. Caspase-6 has been widely expressed in the brain and peripheral tissues. Apoptotic cell death is involved in neurodegenerative diseases, including Huntington's disease, Alzheimer's, Parkinson's and stroke. The caspase-6 is emerged as a major player in the degeneration as well as nerve death which is activated early in the disease process [17]. The results of the present study indicated that H_2O_2 stress significantly increases caspase-6 gene expression in comparison with the untreated cells. PIN1 coordinates the activities of P53 family members, which is involved to control the P53 accumulation and apoptotic function in the cells exposed to genotoxic stress [18]. PIN1 is regarded as a key regulator of the P53-induced apoptosis in the neurodegenerative diseases. Within neurons, PIN1 is located in the mitochondrial membrane and provides the possibility of its direct action on other metabolic and apoptotic regulators that are in the mitochondria [19]. PIN1 strengthens P53-induced mitochondrial damage and initiates apoptosis by the release of cytochrome C from mitochondria [8]. The results of the present study demonstrated that H_2O_2 significantly increased PIN 1 gene expression as compared to the untreated cells. Studies have shown that an increase in SIRT1 protects the cells against

beta-amyloid-induced reactive oxygen species production as well as DNA damage and reduces apoptotic death in vitro conditions. P53 is regarded as one of the important proteins affected by SIRT1 that is a proapoptotic protein. P53 deacetylation by SIRT1 reduces P53 stability, inhibits apoptosis and as a result, improves the cell survival [11]. SIRT1 in the stressful situations (such as metabolic and oxidative stress, and hypoxia) are associated with the pathology of many diseases, including diabetes mellitus, cardiovascular diseases, neurodegenerative disorders and kidney diseases. It inhibits the cells apoptosis and promotes the cells survival in these diseases [20, 21]. The findings of the current study demonstrated that H_2O_2 significantly decreases SIRT1 gene expression. SIRT7 involves a positive regulator of RNA polymerase I(pol1) required for cell survival in the mammals. SIRT7 reduction and its catalytic activity inhibition lead to a decrease in pol1, ribosomal DNA (rDNA) relation, and pol1 transcription. In fact, SIRT7 reduction halts cell proliferation and causes apoptosis [22]. One study showed that lack of SIRT7 will be hyperacetylate P53invivo.The results demonstrated that SIRT7 causes resistance to doxorubicin-induced apoptosis. Ultimately, it was concluded that SIRT7 decreases DNA damage as well as P53 response and it improves cell survival under the genomic stress conditions [12]. The present study findings illustrated that H_2O_2 significantly decreases the expression of SIRT7. On the other hand, studies have shown that SIRT1 can be expressed in the cytoplasm in the PC12 cells. Cytoplasmic SIRT1 improves NGF-induced neurite growth in the PC12 cells [23]. Other studies have demonstrated that SIRT1 can be observed in the cytoplasm of adult and fetus neural progenitor cells. SIRT1, as a factor enhancing growth, could strengthen neuronal cell differentiation [24]. Neural differentiation is reduced by inhibiting SIRT1 [25]. A study has proposed that a decreased level of Pin1 suppresses neural differentiation in the neural progenitor cells and over expression of Pin1 has strengthened differentiation through beta-catenin (Inducer of neural progenitor cells differentiation) activity. Pin1 levels increase in neuronal differentiation invitro [26]. Nerve growth factor (NGF) can lead to overexpression of Pin1 in the differentiated PC12 cells [27]. In another study, an increase in the levels of Pin1 was observed after neuronal differentiation of the SY5Y cells with retinoic acidor NGF as well as the differentiation of NT2 cells into hNT nerve cells [28].The experimental results of this study indicated that under conditions of oxidative stress caused by hydrogen peroxide in PC12 cells in the first stage of differentiation, the SIRT1 gene expression dropped sharply and almost was inhibited. Moreover, Pin1 gene expression was significantly increased. This study results showed that Pin1 and SIRT1 both are involved in the differentiation and growth, though Pin1 was more important to accelerate differentiation in terms of oxidative stress. Therefore, it seems that SIRT1 normally acts as a growth inducer and differentiation, and

Pin1 is increased under the conditions of high oxidative stress in the cells demonstrating a cell differentiation. Neural differentiation can be influenced by oxidative stress and hydrogen peroxide can generally reduce PC12 cell differentiation. In the present study, apoptotic and anti-apoptotic genes were investigated, involved at the early stages of PC12 cells differentiation into neuron-like cells. In the event that previous studies have shown that the role of genes in different stages of differentiation may vary in the apoptosis and growth. Neuritogenesis has three phases consisting of neurite initiation, neurite maintenance and elongation [29]. So limit the study of genes involved in growth and apoptosis only in the initial phase(the growth ofneurites), which is better for us evaluate each of these factors in the next phase of differentiation(neurite elongation and maturation).

Conclusion

The study findings revealed that H_2O_2 induces apoptosis in PC12 cells through increasing apoptotic genes such as PIN1 and caspase6 as well as reducing anti-apoptotic genes such as SIRT1 and SIRT7. On the other hand, although PIN1 acts as an apoptotic gene, this study demonstrated that the expression of this gene is increased during the differentiation under oxidative stress conditions.

Conflict of Interest

The authors declare no conflict of interests in this work.

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