Life Sciences 181 (2017) 62–69

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

# Nicotinamide induces mitochondrial-mediated apoptosis through oxidative stress in human cervical cancer HeLa cells

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ARTICLE INFO

*Article history:*
Received 13 March 2017
Received in revised form 19 May 2017
Accepted 3 June 2017
Available online 4 June 2017

*Keywords:*
Nicotinamide
Oxidative stress
Mitochondrial apoptotic pathway
Cervical cancer

ABSTRACT

*Aims:* Nicotinamide participates in energy metabolism and influences cellular redox status and modulates multiple pathways related with both cellular survival and death. Recent studies have shown that it induced proliferation inhibition and apoptosis in many cancer cells. However, little is known about the effects of nicotinamide on human cervical cancer cells. We aimed to evaluate the effects of the indicated concentrations nicotinamide on cell proliferation, apoptosis and redox-related parameters in HeLa cells and investigated the apoptotic mechanism.

*Materials and methods:* After the treatment of the indicated concentrations nicotinamide, HeLa cell proliferation was evaluated by the CCK-8 assay and the production of ROS (reactive oxygen species) was measured using 2′,7′-Dichlorofluorescin diacetate. The apoptotic effect was confirmed by observing the cellular and nuclear morphologies with fluorescence microscope and apoptotic rate of HeLa cell apoptosis was measured by flow cytometry using Annexin-V method. Moreover, we examined the mitochondrial membrane potential by JC-1 method and measured the expression of apoptosis related genes using qRT-PCR and immunoblotting.

*Key findings:* Nicotinamide restrained the HeLa cell proliferation and significantly increased the accumulation of ROS and depletion of GSH at relatively high concentrations. Furthermore, nicotinamide promoted HeLa cell apoptosis via the intrinsic mitochondrial apoptotic pathway.

*Significance:* Our study revealed that nicotinamide induced the apoptosis through oxidative stress and intrinsic mitochondrial apoptotic pathways in HeLa cell. The results emerge that nicotinamide may be an inexpensive, safe and promising therapeutic agent or a neoadjuvant chemotherapy for cervical cancer patients, as well useful to find new drugs for cervical cancer therapy.



## 1. Introduction

Cervical cancer is the second most commonly diagnosed cancer and the third leading cause of cancer death among females [1]. Currently, surgery, radiotherapy and cisplatin-based chemotherapy are the primary methods for treating cervical cancer [2]. However, drug resistance of cervical cancer leading to the low efficacy of the clinical therapeutics and the high cost of the treatment became the major cause of the high incidence and the high mortality rate in developing parts of the world. Hence, it is urgent to find the safe, effective, and affordable treatment for cervical cancer. Human cervical cancer HeLa cell line, derived from cervical cancer cells taken on February from a patient named Henrietta Lacks who died of her cancer on October in 1951 [3], was selected as the experimental model in the present study.

Nicotinamide, the amide form of niacin (vitamin B3), is the necessary nutrient that provided by dietary source and supplement [4]. It also has been used to treat with pellagra clinically for a long time and lack of reported side effects [5]. Nicotinamide is a precursor of the coenzyme nicotinamide adenine dinucleotide ($NAD^+$), participating in the cellular energy metabolism in the mitochondrial electron transport chain [6]. In addition, nicotinamide also is essential for the synthesis of nicotinamide adenine dinucleotide phosphate ($NADP^+$) [7], which is involved in the synthesis of fatty acids and cell survival under oxidative stress condition [8]. And the reduced pyridine nucleotides, NAD(P)H, depending upon an adequate supply of $NAD(P)^+$ [8], also play a pivotal role in the regulation of cellular redox status.

So far, various studies have demonstrated that nicotinamide could modify redox balance in vivo and in vitro, from human fibroblasts [9] to mouse preadipocyte cells [10]. Additionally, emerging evidences reported that nicotinamide might be a potential candidate as anticancer agent or in combination with other chemotherapeutics in several cancer cell models, including pancreatic cancer [11], chronic lymphocytic leukemia [12] and prostate carcinoma [13], breast cancers [14].

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http://dx.doi.org/10.1016/j.lfs.2017.06.003
0024-3205/

Furthermore, as a traditional component of accelerated radiotherapy with carbogen and nicotinamide (ARCON), nicotinamide has been extensively studied for its radiosensitising properties, which is a therapeutic strategy used in head, neck and bladder cancer [15]. Thus, treatment with nicotinamide in cervical cancer cells would be expected to disrupt homeostasis of redox status.

However, to the best of our knowledge, few data are available on the effects of nicotinamide on human cervical cancer cells. Therefore, our present study investigated the in vitro effect of nicotinamide on cell proliferation, apoptosis, production of ROS and redox-related parameters in human cervical cancer HeLa cells to explore its potential mechanism. And further identified the underlying pathway of induction of apoptosis. We found that nicotinamide effectively eliminated HeLa cells through oxidative stress-induced pathway. We expect our study can provide more evidences for nicotinamide to be a safe and inexpensive potential anticancer agent or for combined therapy for cervical cancer patients.

## 2. Material and methods

### 2.1. Cell culture and reagents

HeLa cells, the human cervical carcinoma cell line [3], were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) according to the instruction provided by the American Type Culture Collection, supplemented with 10% fetal calf serum (FBS, GEMINI) and 1% penicillin-streptomycin (Gibco) at 37 °C and 5% $CO_2$ in a humidified incubator. Nicotinamide, *N*-acetylcysteine (NAC) and all other chemicals were purchased from Sigma Aldrich. All sterile plastic materials for cell culture were purchased from Corning Incorporated.

### 2.2. Cell proliferation assay

Cell proliferation was assessed by Cell Counting Kit-8 (CCK-8, Dojindo, Molecular Technologies, Japan) as the manufacturer's instruction. Briefly, after treatment with the indicated concentrations and time points, cells were incubated with 50 μl of CCK-8 solution for 2 h. Optical density (450 nm) was measured using a microplate reader (Sunrise Tecan). The growth curves were calculated as the mean OD values of each group at the different time points.

### 2.3. Measurement of intracellular ROS

Intracellular ROS was measured using 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Molecular Probes). Because the groups of 0.25 and 0.5 mg/ml did not induce apparent effect, thus these two groups were excluded. Cells were plated in six-well plates and incubated for 24 h to allow exponential growth. After pretreatment with 5 mM NAC for 1 h and treatment with the indicated concentrations of nicotinamide for 24 h, the cells were trypsinised and incubated with 10 mM DCFH-DA for 20 min at 37 °C in the dark. Then the cells were washed twice with PBS and analyzed the mean fluorescence intensity immediately using flow cytometry (CytoFLEX).

### 2.4. Measurement of lipid peroxidation and GSH

Intracellular lipid peroxidation and GSH were measured using the Lipid Peroxidation Malondialdehyde (MDA) assay kit and the Reduced glutathione assay kit (Nanjing Jiancheng, China), respectively. MDA is the biomarker of plasma membrane lipid peroxidation [16]. Briefly, after pretreatment with 5 mM NAC and treatment with the indicated concentrations of nicotinamide for 24 h, the cells were harvested and then homogenized and sonicated in RIPA buffer on ice. Cells lysates were centrifuged at 12,000 g for 15 min at 4 °C to collect the supernatant, then measured their concentrations and subjected to MDA assay and GSH assay as described in the manufacturer's instructions. Thereafter, the levels of MDA and GSH were detected using microplate readers at 532 nm and 405 nm, respectively.

### 2.5. Morphologic analysis of HeLa cells

After treatment with the indicated concentrations of nicotinamide for 24 h, cells were washed with PBS, examined directly or stained with 4′,6-diamidino-2-phenylindole, (DAPI, Sigma) for 5 min at 37 °C and then imaged on fluorescence microscope at 200× (Axio Observer Z1, Carl Zeiss) in order for the cellular morphology and nuclear morphology determination.

### 2.6. Cell apoptosis assay

Annexin V-FITC/PI Apoptosis Detection Kit (BD biosciences, USA) was used to evaluate cellular apoptosis. After treatment with the indicated concentrations of nicotinamide for 24 h in the absence or presence of pretreatment of 5 mM NAC for 1 h, the cells were trypsinized, washed, collected and resuspended in 200 μl binding buffer, labeled with 5 μl Annexin V-FITC and 5 μl PI for 15 min in the dark at room temperature. Then 300 μl binding buffer was added to each sample and the cells were evaluated by flow cytometry (CytoFLEX, Beckman Coulter).

### 2.7. Measurement of mitochondrial membrane potential ($\Delta\psi m$)

$\Delta\psi m$ was measured using the mitochondrial membrane potential assay kit with JC-1 (Beyotime, China). HeLa cells were seeded at a density of 5 × 10^5 per well in six-well plates and incubated for 24 h. After pretreatment with NAC and treatment with the indicated concentrations of nicotinamide for 24 h, the cells were trypsinised, washed and incubated with 0.5 ml JC-1 working solution at 37 °C for 20 min. Subsequently, removed the staining solution at 600 g centrifugation for 3 min, washed the cells twice with buffer. Then the cells were resuspended in 0.3 ml buffer and analyzed using the flow cytometry (CytoFLEX).

### 2.8. Identification of the apoptotic pathways of HeLa cells induced by nicotinamide with western blotting assay

To make sure whether the apoptotic effect of HeLa cells induced by nicotinamide is mediated through activation of the intrinsic or extrinsic apoptotic pathways, HeLa cells were seeded in six-well plates, incubated for 24 h and treated with nicotinamide at the indicated concentrations for 24 h. Then total protein was extracted using the RIPA Lysis Buffer Kit (Beyotime, China). The protein concentration was measured using a BCA Kit (Beyotime, China) and equal amounts of protein was separated by a 12% SDS-polyacrylamide gel and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Then the membranes were blocked by 5% non-fat milk at room temperature for 2 h and incubated with various primary antibodies against caspase-3 (Abcam, ab32351), caspase-9 (ab185719), caspase-8 (ab32379) and GAPDH (Jetway Biotech, E021060). After washing three times with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Jetway, E030120) for 1 h at room temperature. The immunoblots were visualized by the Enhanced Chemiluminescence method using ChemiDoc Touch Imaging System (Bio-Rad). Densitometric analyses were done on scanned the membranes by using the public domain ImageJ software.

### 2.9. Identification of redox-related genes by quantitative real-time PCR (qRT-PCR)

To evaluate the expression of redox- and apoptosis-related genes, HeLa cells were seeded in six-well plates, incubated for 24 h and treated with nicotinamide at the indicated concentrations for 24 h. Total RNA was isolated from the treated cells using RNAprep pure Cell Kit (Tiangen Biotech) and then was reverse transcribed to cDNA using the ReverTra