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Effects of alpha-particle irradiation on cell injury and the expressions of insulin sensitive-related genes in 3T3-L1 preadipocyte

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ABSTRACT

Exposure of cells to densely ionizing radiation can initiate a series of molecular and cellular events, which result in the gain and loss of functions in critical regulatory genes and cellular damage. In the present study, alpha-particle radiation-induced insulin resistance and toxic injury to 3T3-L1 preadipocyte were observed, and the mechanism underlying the action was investigated. 3T3-L1 preadipocytes were irradiated by different doses of alpha-particle at 0 Gy , 0.3 Gy , 0.6 Gy , 1.0 Gy , 1.5 Gy , 2.0 Gy , and 4.0 Gy . Cloning efficiency was investigated to evaluate the cellular toxic effect of alpha-particle irradiation on cellular viability. Cytokinesis-block micronucleus technique (CB-MNT) and single-cell comet assay COMET assay ÿ were used to observe alpha-particle irradiation-induced DNA damage. Expression of caveolin-1, IRS-1 (insulin receptor substrate-1) and GLUT-4 (glucose transporter-4) were analyzed by western blot and Reverse-transcription PCR (RT-PCR). Alpha-particle irradiation inhibited cloning efficiency, and increased micronucleus cells and the DNA damage in 3T3-L cells in a dose-dependent manner. Alpha-particle irradiation decreased the mRNA expression of GLUT-4 and IRS-1, and inhibited the protein expression of caveolin-1. The present results indicate that alpha-particle irradiation induced toxic injury and insulin resistance in 3T3-L1 preadipocyte, which might be associated with inhibition of GLUT-4 and IRS-1 expression by down-regulating caveolin-1.

KEYWORDS

Alpha-particle;
3T3-L1 cell;
Irradiated injury;
GLUT-4;
IRS-1.

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INTRODUCTION

Radon, a colourless, odourless and tasteless ubiquitous noble gas in the environment, is one of primary harmful radiation sources for humans, and decays in a cascade of daughters by releasing the high energy alpha-particles[1,2]. If this gas is inhaled, deposited radon and radon daughters will enter blood from the lung, and affect peripheral tissue. Radon has high solubility in fat, so radon may concentrate in adipocytes[3,4]. Adipose tissue sensitive to insulin has been involved in insulin-stimulated glucose transportation and glucose metabolism. In the processes of insulin secretion and signal transduction, there are two important factors, insulin receptor substrate-1 (IRS-1) and glucose transporter-4 (GLUT-4), locating in caveolae. Caveolin-1, a integral membrane protein, is a principal structural component of caveolae. Glucose uptake mediated by GLUT-4 is the rate-limiting step for glucose utilization in peripheral tissues[5]. IRS-1 is a major intracellular substrate of insulin signal transduction[6]. The reduction of caveolin-1 inhibites GLUT-4 translocation and IRS-1 expression[7,8], which decrease peripheral glucose utilization and increase insulin resistance. However, the effect of alpha-particles on the adipocyte is unclear.

In this study, we used alpha- particle to irradiate 3T3-L1 preadipocyte. alpha-particle irrradiation induced toxic injury to 3T3-L1 preadipocyte, and inhibited IRS-1 and GLUT-4 expression by down-regulating caveolin-1 in 3T3-L1 preadipocyte.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from GIBCO/BRL. The antibodies anti-caveolin-1 and anti-β-actin were purchased from Santa Cruz Biotechnology.

Cell Culture

3T3-L1 cells (a cell line of preadipocyte) were cultured with DMEM medium supplemented with 50 unit/ml penicillin, 50 μg/ml streptomycin, and 10% FBS, in a cell culture incubator containing 5% CO_2.

Radon irradiation

3T3-L1 cells were seeded at a density of 3×10^5 cells in 2.0μm mylar embedding, 2.5μm-thick, special dishes for radiant apparatus of alpha particles. Cells were irradiated when they growed up to 95–98% confluent. Cells were exposed to alpha-particles from a collimated $^{238}PuO_2$ (the radiological apparatus in the Academy of Military Medical Sciences) at a dose rate of 0.3Gy/min[9]. The total activity of ^{238}PuO2 radioactive source is 1.85×10^8 *Bq*, and the average energy of alpha-particles is 5.34 *Mev*. The cells were incubated in the radiological apparatus for different time so at to get different radioactive dose. Control cells, referred to as external control, were sham-manipulated and handled in parallel with test cells.

Cell survival assay

After irradiated by different doses of alpha particles including 0 Gy00.3 Gy00.6 Gy01.0 Gy01.5 Gy02.0 Gy and 4.0 Gy, Cells were trypsinized from the special dishes and suspended in DMEM medium. 1 ml diluted single-cell suspensions with known numbers of cells was added to each general dishes (internal diameter 60mm). Each dishes was pre-seeded with 250 cells irradiated by alpha particles below 1.5 Gy, or 500 cells irradiated by alpha particles beyond 1.0 Gy. The cells were incubated in a 37°C incubator for about 7 days and stained with crystal violet in 0.125% methanol. Colonies containing more than 50 cells were counted. The clone-forming efficiency (CFE) was calculated by dividing the number of colonies per dish by the number of cells originally seeded. The surviving fraction (SF) was calculated as the ratio of irradiated CFE to control CFE.

Cytokinesis-block micronucleus assay

3T3-L1 cells were cultured for 6 hours after irradiation. Cytochalasin B (Cyt-B, from Sigma Co.) was added at the concentration of 3 μg/ml to arrest cytokinesis. After 48 hours, the cells were centrifuged, resuspended in 75 mM KCl, and centrifuged again, and finally fixed with Carnoy's solution (3:1 of methanol and glacial acetic acid). The fixation was repeated twice. Then, The fixed cells were dropped on clean slides and allowed to dry. For each groups, four slides were prepared. Each slides were stained with 10% giemsa for 15min. 1×10^3 3T3-L1 cells were counted to deter-

mine the percentage of cells with micronuclei.

Single-cell comet assay

3T3-L1 cells were cultured for 0.5 hour after irradiation, and viability assayed by the single cell gel electrophoresis test. 30-40μl cell suspension with the density of 3×10^7 was mixed with 100 μl of 0.65% low melting point agarose at 37°C, and 60μl was immediately spreaded onto slides pre-coated with 1% normal melting point agarose. Coverslips were added and the slides were allowed to gel at 4°C for 10 minutes. The coverslips were gently removed and the slides were then immersed in freshly prepared lysing solution consisting of 2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% sodium lauryl sarcosine, 1% Triton X-100 and 10% DMSO (pH 8.5) at 4°C for 2 hours. The slides, which were protected from light, were left to 0.5% TBE solution for 3 hours to neutralize. Subsequently, the slides were positioned at the anode end, and left in the electrophoresis buffer of 0.5% TBE. The electrophoresis was ran at 25 V in an ice bath. 20 minutes later, the slides were stained with 50 μl of 1.25μg/L propidine iodide solution, and briefly rinsed with distilled water, and covered with a coverslip. Images was immediately captured at 400× magnification, using a fluorescence microscope (Nikon) with a 400 nm excitation filter and a 590 nm barrier filter. The comet image was considered to be from a injured cell when it presented a cloudy appearance, or a very small head and a balloon-like tail. The length of the comets (head + tail) was measured using the comet image analysis system (www.casp.of.pl). The parameters for quantifying DNA damage included Olive Tail Moment (OTM in arbitrary units), %Tail DNA and Tail length (migration of the DNA from nucleus in micrometre). Data were collected for Tail length (micrometre) and % Tail DNA to compute OTM by software using formula OTM = Tail length × % Tail DNA/100. Frequency distribution of cells with different OTM values (OTM d" 2.5, 5, 7.5 and e"10) was computed and plotted. The personal bias in data collection was avoided using traditional random sampling method where first 50 specimens were sampled randomly from three slides of each treatment group.

RT-PCR

Total RNA was extracted from the cells using trizol reagent (Gibco BRL) according to the manufacturer's protocol. 3 μg of total RNA were used for reverse transcription in a total volume of 20μL with the superscript preamplification system (Promega, Madison, MI). Aliquots of 2μl cDNA were subsequently amplified in a total volume of 25μL using the GeneAmp PCR kit (Promega) following conditions recommended by the manufacturer. The sense and antisense primer for IRS-1 were 5'- GTG GCT TCT ATT GAG GAA TA -3' and 5'- ACC TTG GCA ATG AGT AGT AA -3' (524bp); The sense and antisense primer for GLUT-4 were 5'- GAT TCT GCT GCC CTT CTG TC -3' and 5'- ATT GGA CGC TCT CTC TCC AA -3' (432bp); the sense and antisense primers for actin that used as an internal control were 5'- ATG ATA TCG CCG CGC TCG TCG TC -3' and 5'- CGC GGT TGG CCT TGG GGT TCAG -3' (120bp). The cycling conditions were as follows: 95°C for 5min, followed by 28 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s, and a final extension of 72°C for 10min. PCR products were separated in 1.5 % agarose gel. These data were acquired with Alpha Imager 2200 software.

Western blotting analysis

3T3-L1 cells were cultured for 4 hour after irradiation and collected as before[10]. Total protein was extracted from cell lysates using 1× lysis buffer. Lysates were centrifuged for 10 min at 15,000 rpm at 4°C. The supernatant was collected and protein concentration was determined by bicinchoninic acid (BCA) protein assay kit. 50μg of protein was seperated by 12% SDS-PAGE, and proteins were then transferred to polyvinylidene difluoride membrane (PVDF) from SDS-PAGE as described previously[10]. The membrane was blocked with a commercial blocking buffer (Life Technologies Inc) at room temperature for 2hr. The blots were incubated at room temperature with the caveolin-1 or β-actin antibodies with dilution rate of 1:1000 for 1hr, and followed by incubation with secondary antibody (horseradish peroxidase-conjugated) for 1 hr. The presence of target proteins was revealed by a chemiluminescent assay (Amersham-Pharmacia Biotech).

Statistical analysis

The values are expressed as the mean ± SE. Statistical analysis of the data was performed using student's t test and ANOVA as appropriate. Values with $P < 0.05$

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were considered as statistical significance.

RESULTS

Alpha-particle irradiation inhibited 3T3-L1 Cellular viability

3T3-L1 cells were irradiated by alpha-particle with different doses (0Gy00.3 Gy00.6 Gy01.0 Gy01.5 Gy02.0 Gy and 4.0 Gy) in vitro. With the dose increasing, the CFE decreased. The CFE in control group is 21.75±0.92%. The CFE in the groups of 0.6Gy and 4.0Gy respectively were 15.64±0.71% and 5.79±0.27% (Figure 1A). When the irradiation dose reached 1.7Gy, the cell surviving fraction decreased to 50% according to cell-survival curve (Figure 1B).

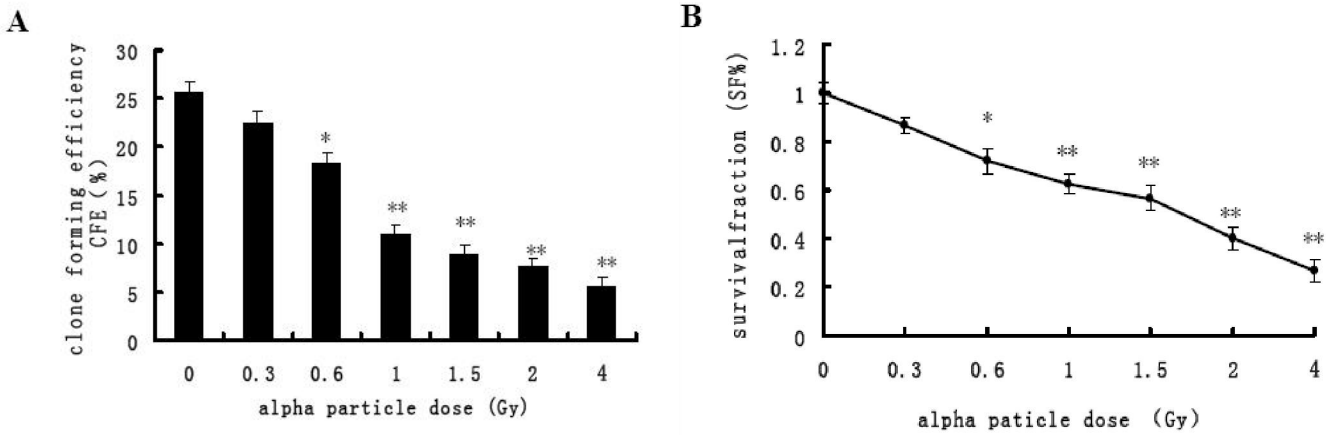

Figure 1 : The irradiation of alpha-particle inhibited 3T3-L1 Cellular survivability. A : The effect of alpha-particle irradiation on cellular CFE. After irradiated by different doses of alpha particles including 0 Gy00.3 Gy00.6 Gy01.0 Gy01.5 Gy02.0 Gy and 4.0 Gy, 3T3-L1 cells were trypsinized from the special dishes and pre-seeded with 250 cells irradiated below 1.5 Gy, or 500 cells irradiated beyond 1.0 Gy in general dishes each group separately. Cells were sequentially incubated for 7 days, and the CFE was calculated. B: The effect of alpha-particle on surviving fraction of 3T3-L1 cells. (n=3, mean±SD,*P<0.05,P<0.01, *vs* control group).**

Alpha-particle irradiation increased micronucleus cells

Dual-core cells and tri-core cells were formed when Cyt-B (3μg/ml) was added to arrest cytokinesis for 48 hours (Figure 2A 1-2). The alpha-particle-induced micronuclei were observed at 0.6, 1.0, 1.5, 2.0 and 4.0Gy (Figure 2A 3-4). The rate of micronucleus cells increased in a dose-dependent manner. When the dose reached 4.0Gy, the micronucleus cells formed by induction of alpha-particle were approximately 7.2 times more than that of their spontaneous control (0 Gy) (

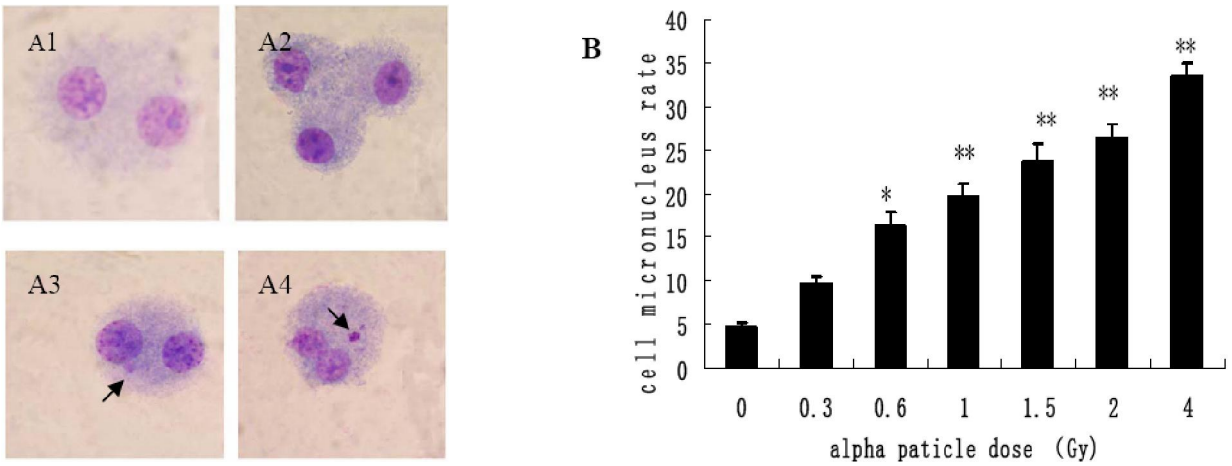

Figure 2 : Alpha-particle irradiation increased the rate of micronucleus cells A: Representative images of micronucleus cells (400×); A1: dual-core cell; A2: tri-core cell; A3-4: micronucleus cell (the arrows show the micronuclei). B: the cell micronucleus rate analysed by cytokinesis-block micronucleus assay. (n=4, *P<0.05, **P<0.01, vs control group).

Figure 2B).

Alpha-particle irradiation promoted DNA damage

To explore the effect of alpha-particles on DNA damage, we observed comet cells by single-cell gel electrophoresis assay. With the increase in radiation dose, the tail of the cells singnificantly became longer (Figure 3). Comet assay parameters such as tail moment (TM), olive TM (OTM), were significantly higher in irradiation groups than that in control group, and increased in a dose-dependent manner (TABLE 1).

The effect of alpha-particle irradiation on the RNA expression of GLUT-4 and IRS-1 in 3T3-L1 cells

3T3-L1 cells were continuously cultured for 4 hour after irradiation, and then total RNA was extracted from these cells. The mRNA expression of GLUT-4 was decreased after alpha-particle irradiation in a dose-dependent manner (Figure 4). The mRNA expression of IRS-1 has the same trend as GLUT-4 (Figure 5).

The effect of alpha-particle irradiation on the expression of caveolin-1 in 3T3-L1 cells

The caceolin-1 expression was decreased by alpha-particle irradiation in a dose-dependent manner in 3T3-L1 cells. When the radiation dose exceeded 0.6 Gy, caveolin-1 expression significantly decreased as compared to control group (Figure 6).

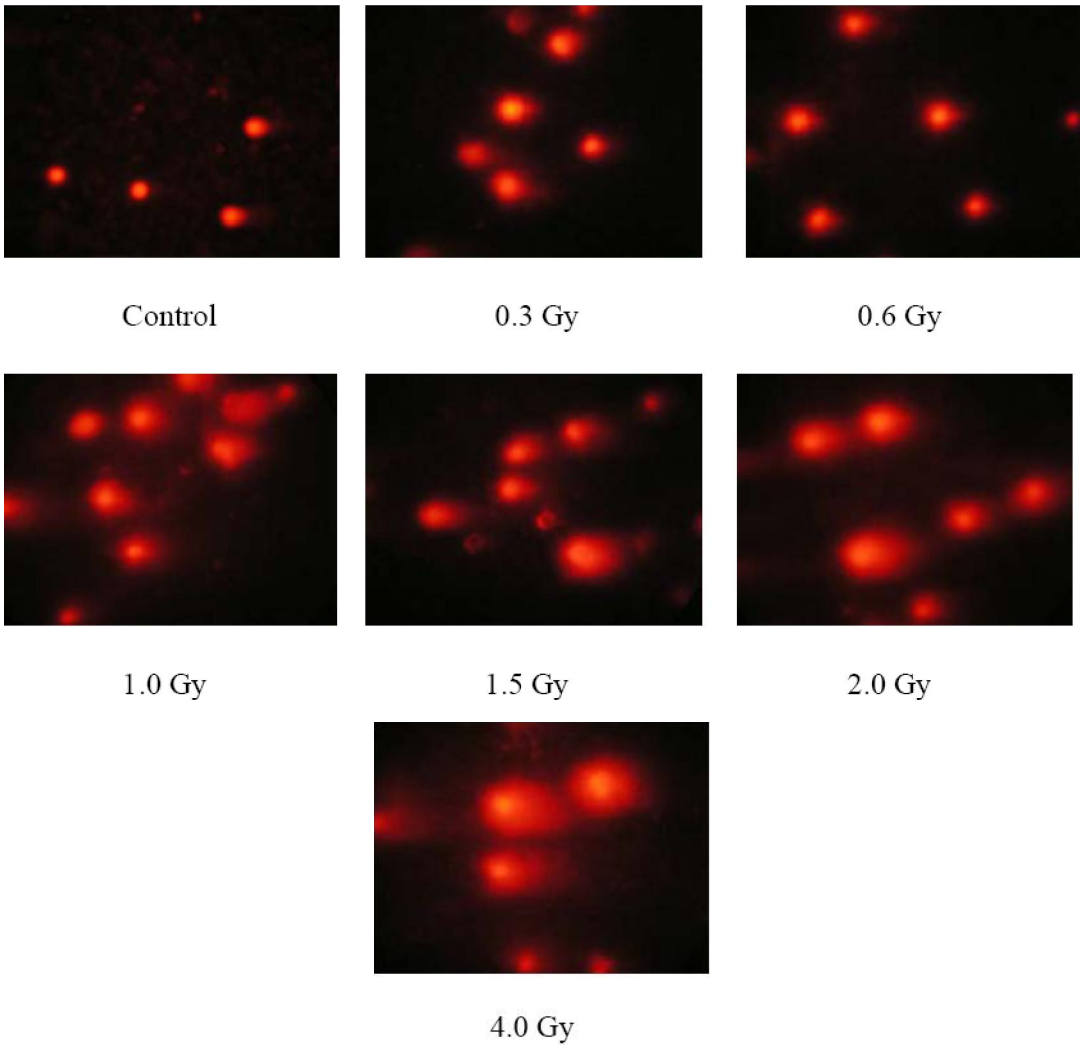

Figure 3 : Representative images of single-cell gel electrophoresis in different dose of alpha-particle radiation (400×). 3T3-L1 cells were cultured for 0.5 hour after different doses of alpha particles irradiation (0 Gy00.3 Gy00.6 Gy01.0 Gy01.5 Gy02.0 Gy and 4.0 Gy), viability assayed by the single cell gel electrophoresis test. The comet image was considered to be from a injured cell when it presented a cloudy appearance, or a very small head and a balloon-like tail. Images from 150 cells (50 from each replicate slide) were analyzed and data presented as mean±SE.

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TABLE 1 : The results of single 3T3-L1 cell gel electrophoresis after irradiated ($\bar{x} \pm s$)

Dose(Gy)	Cell	Tail length (arbitrary unit)	Tail DNA (%)	Tail moment(mM)	Olive Tail moment
0	50	6.70±0.03	6.97±0.08	0.38±0.02	0.23±0.04
0.3	50	17.93±0.04*	7.80±0.07	0.55±0.03	1.88±0.08
0.6	50	30.23±0.02*	14.09±0.08*	1.83±0.04*	4.84±0.09*
1.0	50	46.23±0.04*	19.76±0.08*	3.53±0.05*	7.32±0.08*
1.5	50	60.66±0.05**	23.17±0.04**	5.23±0.04**	9.65±0.06**
2.0	50	77.94±0.04**	26.18±0.08**	7.93±0.02**	12.73±0.09**
4.0	50	89.12±0.06**	29.68±0.06**	16.18±0.03**	21.23±0.05**

(n=3, * *P*<0.05, **P<0.01, *vs* control group)

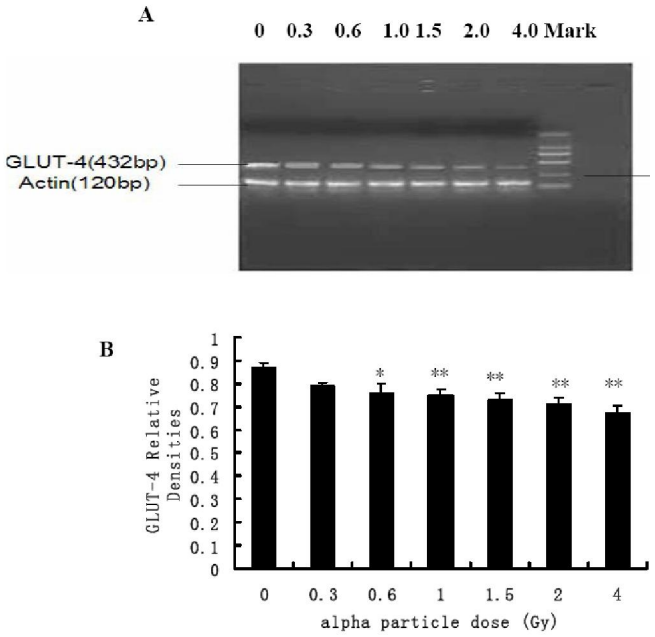

Figure 4 : Effect of alpha-particle on expression of GLUT-4 mRNA by RT-PCR. A: RT-PCR of GLUT-4 mRNA expression. B: Quantitative data of GLUT-4 expression, results were normalized to actin. Data are the mean ± SE of three independent experiments.,*P<0.05 and **P<0.01 as compared with control group.

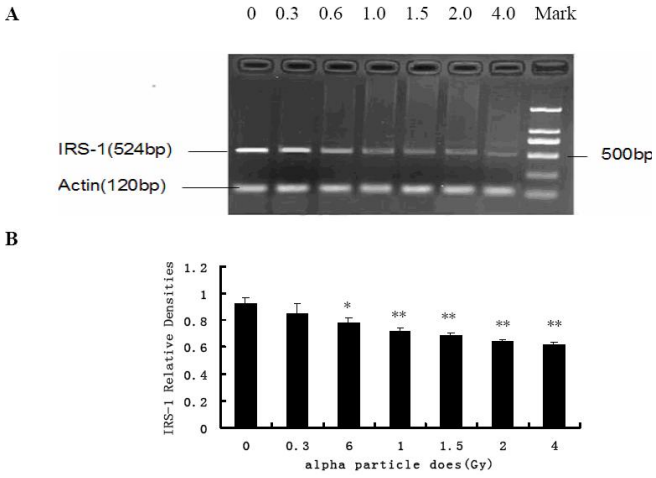

Figure 5 : Effect of alpha-particle on expression of IRS-1 mRNA by RT-PCR. A: RT-PCR of IRS-1 mRNA expression. B: Quantitative data of IRS-1 expression, results were normalized to actin. Data are the mean ± SE of three independent experiments.,*P<0.05 and **P<0.01 as compared with control group.

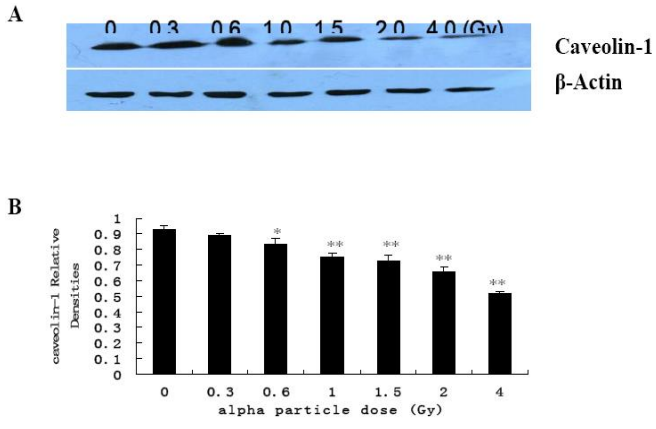

Figure 6 : The effect of alpha-particle irraditaion on expression of caveolin-1. A: Representative western blot data showing the effects of alpha-particle on caveolin-1 proteins in 3T3-L1 cells. B: Quantitative data of the alpha-particle effect on caveolin-1 expression. (n=3, *P<0.05,P<0.01ÿvs control group)**

DISSCUSSION

The cell membrane, nucleus or cytoplasm, as well as DNA and other biological macromolecules were injuried after cells were exposured to chronic low-dose or high-dose radiation, which finally led to cell death or apoptosis[1]. In this study, we found that after cells were exposured to alpha-particles, cells survival rate were dose-dependently reduced.

Micronucleus, a kind of small nucleus in the cytoplasm independent from the main nuclear, forms in the

cytoplasm when the damage of outside factors on cells lead to chromosome loss or breakage[11]. Micronucleus is mainly induced by two types of factors: mutagen, that can cut genomic DNA into chromosome fragments which are excluded from the nucleus and form the micronucleus during subsequent cell division; and spindle fibers inhibitor, that can inhibit the spindle formation or disrupt the formative spindle fibers, and delay the chromosome split to form micronuclei. Cytokinesis block micronucleus test (CB-MNT) was sensitive to analyse radial damage. Alpha-particle irradiation can increase the micronucleu production. Hecht SS[12-14] found that radon induced cell micronuclei production. Our studies found that alpha-particle irradiation increased the number of micronuclei in 3T3-L1 cells, which may be ralated to the decrease of DNA repair capacity.

COMET assay also known as Single-cell gel electrophoresis assay (SCGEA), is a rapid detection method of DNA damage in mammalian cells[15]. COMET assay have been applicable to detect DNA damage induced by mutagen or ray, and monitor the genetic damage of body by environmental pollution. Hellman et al[16] found that DNA damage significantly increased in peripheral blood lymphocytes on the residents who lived in the room filled with radon beyond 200 Bq/m^3 levels. We detected that alpha-particle irradiation induced DNA damage in 3T3-L1 preadipocyte in a dose-dependent manner. 3T3-L1 preadipocyte is sensitive to insulin[17], so we studied the effects of alpha-particles on the expression of insulin sensitivity-related genes.

Glucose transporter 4 (GlUT-4) is an important molecule in the signal transduction of insulin secretion. GLUT-4 exists in insulin-sensitive skeletal muscle cells, heart muscle cells, and fat cells[18]. GLUT-4 is activated by insulin receptor substrate-1 (IRS-1), and continually activates the downstream signaling molecules such as phosphatidyl inositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK). This signaling pathway is involved in cell metabolism, growth, differentiation[19,20]. IRS-1 was a key intermediate molecular in insulin regulation[21]. IRS-1 induces the translocation of GLUT-4 to cell surface, and promotes glucose uptake and glycogen synthesis. The IRS-1/GLUT-4 pathway plays an important role in insulin-mediated glucose and lipid metabolism[22]. Our results showed that alpha-particle irradiation decreased the mRNA expression of GLUT-4 and IRS-1 in 3T3-L1 preadipocyte in a dose-dependent manner. It suggests that alpha-particle irradiation regulated insulin sensitivity by inhibiting the expressions of key molecule in insulin signaling.

It have been reported that IRS-1and GLUT-4 locate in caveolae with caveolin-1. Caveolin-1, a main structural component of caveolae, is an important regulatory factor in insulin signaling. The structural integrity of caveolae is necessary for GlUT-4 translocation[23]. The reduction of caveolin-1 may destroy the structure of caveolae and inhibit the GlUT-4 translocation, and ultimately affect the glucose transport. We found that alpha-particle irradiation decreased the expression of caveolin-1 in 3T3-L1 preadipocyte in a dose-dependent manner. It implies that alpha-particle irradiation may regulate insulin signaling by inhibiting the expression of caveolin-1.

Insulin sensitivity is related to many diseases such as hypertension, coronary heart disease, obesity and stroke. The studies on the relationship between alpha-particle irradiation and insulin sensitivity provides a new way to prevention and cure of many diseases aggravated by environmental pollution.

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