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X-ray irradiation has positive effects for the recovery of peripheral nerve injury maybe through the vascular smooth muscle contraction signaling pathway

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ABSTRACT

Introduction: It is well known that moderate to high doses of ionizing radiation have a toxic effect on the organism. However, there are few experimental studies on the mechanisms of LDR ionizing radiation on nerve regeneration after peripheral nerve injury.
Methods: We established the rats' peripheral nerve injury model via repaired Peripheral nerve injury nerve, *vascular endothelial growth factor a* and *Growth associated protein-43* were detected from different treatment groups. We performed transcriptome sequencing focusing on investigating the differentially expressed genes and gene functions between the control group and 1 Gy group. Sequencing was done by using high-throughput RNA-sequencing (RNA-seq) technologies.
Results: The results showed the 1 Gy group to be the most effective promoting repair. RNA-sequencing identified 619 differently expressed genes between control and treated groups. A Gene Ontology analysis of the differentially expressed genes revealed enrichment in the functional pathways. Among them, candidate genes associated with nerve repair were identified.
Discussion: Pathways involved in cell-substrate adhesion, vascular smooth muscle contraction and cell adhesion molecule signaling may be involved in recovery from peripheral nerve injury.

1. Introduction

Ionizing radiation is ubiquitous and affects virtually everyone, all of us are exposed to low doses of radiation(LDR) from cosmic rays, soil radioactivity and diverse man-made electronic equipment (Nickoloff and Alderson, 2001). LDR has applications in a variety of medical diagnostics and clinical cures (Dilworth et al., 2013). In medical diagnostics such as CT scanning, 30–70 m Gy are used in head scan series and 20–50 m Gy in an abdominal series (Brix et al., 2009). In the clinical cure area, LDR is found in conventional radiotherapy regimes (Mechlenburg et al., 2009). It is used to treat cancer in large tissue volumes using a low dose exposure. Recently clinical data has shown that LDR has applications in some peripheral nerve injuries and other diseases by helping with recovery (Mohammed et al., 2007). In the rats, has some promoting effects on the recovery of the reproductive system. This means that the LDR maybe a better way to cure or be a useful auxiliary cure in some clinical diseases.

High dose radiation generates cytotoxic, cytogenetic and inheritable effects that can cause severe scarring (Betlazar et al., 2006). The Differences between high dose radiation and low dose radiation has been documented with effects in areas such as immune-stimulatory, antioxidative and anti-inflammatory functions (Rodel et al., 2008). Previous studies also show that multiple exposures to LDR at 25 m Gy significantly suppresses diabetes-induced systemic and renal inflammatory responses and oxidative damage (Shao et al., 2014). This results in prevention of renal dysfunction and fibrosis. This shows that LDR can have positive effects in the protection and recovery from some diseases.

Vascular endothelial growth factor a (*VEGFA*), a vascular endothelial cell-specific heparin-binding growth factor, induces

Abbreviations: LDR, low doses of radiation; *VEGF*, vascular endothelial growth factor; *GAP-43*, growth associated protein-43; PNI, spinal cord injuries; DEG, differentially expressed genes; GO, gene ontology; CAMs, cell adhesion molecules; KEGG, Kyoto Encyclopedia of Genes and Genomes

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angiogenesis in vivo (Lin et al., 2017). It is secreted by certain tumor cells, through the vascular endothelium with the corresponding receptor binding it to promote endothelial cell proliferation, while increasing the vascular permeability of endothelial cells migration (Mu et al., 2017). Growth associated protein-43 (*GAP-43*), also known as neuromodulin, is an axon membrane protein that is involved in neuronal extracellular growth and synaptic development and neuronal regeneration (Park et al., 2016). It is expressed at a high levels during neuronal development and regeneration and can mediate axonal extension and change the cell's morphology (Zhang et al., 2016). As an intracellular signal, it can greatly enhance G protein-coupled receptor transport. *VEGFA* and *GAP-43* play an important role in axonal regeneration and synaptic plasticity after nerve injury.

Medical workers have been constantly studying ways to promote peripheral nerve regeneration. It is well known that moderate to high doses of ionizing radiation have a toxic effect on the organism. High doses of ionizing radiation often cause peripheral nerve injury and disease, while LDR radiation can accelerate the proliferation of a variety of cells, producing an excitatory effect (Waksman et al., 2013; Deng et al., 2013; Howell et al., 2012). However, there are few experimental studies on the mechanisms of LDR ionizing radiation on nerve regeneration after peripheral nerve injury. In this study, we used X-ray irradiation to see if it could promote repair in peripheral nerve injury (PNI). *VEGFA* and *GAP-43* were detected from different treatment groups. The results showed the 1 Gy group to be the most effective promoting repair. Transcriptome deep sequencing was used to study the mechanism of these processes. Identified 619 differently expressed genes between control and treated groups. Pathways involved in cell-substrate adhesion, vascular smooth muscle contraction and cell adhesion molecule signaling may be involved in recovery from peripheral nerve injury.

2. Materials and methods

2.1. Preparation of samples

Male SD rats 100 (Suzhou University Animal Experimental Center), weighing between around 200–250 g were used. All rats were bred in groups under specific-pathogen-free conditions and had free access to diet and tap water. All of the animal experiments were approved by the Ethics Committee on Animal Experiments of the Soochow University. Rats were anesthetized with 3.6% chloral hydrate (1 ml/100 g body weight). After PNI nerve exposure, PNI nerves were transected at the midpoint between the PNI nerve pelvic outlet and the tibiofibular nerve bifurcation at the midpoint of the PNI nerve, and then operated on under a microscope 9–0 with a microsurgical in-situ suture used on the epineurium. The whole process was done under sterile conditions, nerve stitching operations all by the same surgeon and other experiments were completed with the surgeon and his assistance.

The 100 rats were randomly divided into five groups (20 each group): irradiated groups (4 Gy, 2 Gy, 1 Gy and 0.5 Gy) and control group (0 Gy). Peripheral nerve injury was caused by physical methods described in previous articles, post operation irradiation was used as treatment. The experimental groups were irradiated with different intensities (4 Gy, 2 Gy, 1 Gy and 0.5 Gy) according to the experimental arrangement, and the control group was not irradiated. X-ray irradiation was performed using X-ray irradiation generated by a medical linear accelerator (Siemens Primus, Siemens Medical Systems, Concord, CA, USA). After surgery 24-h rats in the irradiation group were anesthetized via the abdominal cavity the left side of the PNI nerve was exposed, and the other parts of body protected by lead. The distance between the irradiation source and rat skin was 100 cm after 72 h of irradiation the Spinal cord was sampled for the next experiment.

2.2. RNA extraction

Total RNA was obtained using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Briefly, one gram of tissue sample was added to liquid nitrogen to obtain a fine powder and evenly mixed with4 ml of preheated (65 °C) extraction buffer (2%CTAB, 2% PVP, 0.1 M Tris-HCl, 2.0 M NaCl, 25 m MEDTA, 2% beta-mercapto ethanol, pH 8.0). Then the mixture was incubated for 6 min at 65 °C and shaken 4 times during this process. After a short time cooling, 3 ml isopropanol was added to the mixture. After 2 min being vortexed, the mixture was centrifuged at 13,000*g* for 20 min at 4 °C. RQ1 DNase was added to the extraction to remove DNA. Smartspecplus (BioRad) was used to measure the absorbance at 260/280 nm (A260/A280) to qualify and quantify the collected RNA. Lastly, the integrity of the extracted RNA was further detected using 1.5% agarose gel electrophoresis.

2.3. Real-time PCR validation

Total RNA was extracted from independent biological replicates using and RNA extraction kit according to the manufacturer's protocol. 10 μg of total RNA was subjected to DNaseI treatment with 1 U DNaseI (NEB, USA). The reaction was carried out at 37 °C for 10 min followed by heat inactivation at 65 °C for 10 min. 2.5 μg of DNase-treated RNA was used for cDNA synthesis with reverse transcriptase (BioRad, USA) in accordance with the manufacturer's protocol. The expression of *Gapdh* gene was detected to be stable in the transcriptome database, and it was used as the control in qRT-PCR. Primers were designed for selected transcripts from the transcriptome database and real time PCR was performed by SYBR green I master mix (Roche, GmBH) on CFX-Connect™ Real time system (BioRad, USA). Relative expression of the transcripts was calculated using the △△Ct method. The primer was shows in the Supplemental Table 1.

2.4. Western blotting

VEGF and *GAP-43* proteins were examined by western blotting. Specifically, sample was treated using RIPA Lysis Buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM protease inhibitor phenyl methane sulfonyl fluoride and centrifuged at 13,000*g* for 10 min. The supernatant was extracted and re-suspended in 50 μl of SDS-PAGE buffer, boiled for 5 min, and then 20 μl was loaded onto a 12% polyacrylamide gel. Proteins were transferred onto a polyvinylidene fluoride membrane via electrophoresis at 60 V for 4 h using a Bio-Rad transfer system (Bio-Rad, Hercules, CA, USA). The membrane was saturated for 2 h with sealing fluid at room temperature and probed with suitable antibodies diluted 1:10.000 in saturation buffer. The membrane was incubated for 2 h with horseradish peroxidase-labelled secondary antibodies (Sigma). Then diluted 1:20.000 in saturation buffer, and signals were detected using the enhanced chemiluminescence system.

2.5. RNA-seq library construction and sequencing

For RNA-seq, one rat from the 1GY exposure group and one from the control group was used, 20ìg of total RNA was collected for the RNA-seq library preparation. The collected mRNAs were purified and concentrated using oligo(dT)-conjugated magnetic beads (Invitrogen) before library preparation. The purified mRNAs were randomly cut into fragments by the fragmentation buffer. MRNA was used as a template, with six-base random primers (random hexamers) used to synthesize the first strand of cDNA. dNTPs, RNase H and DNA polymerase I were then added to the buffer, to synthesize the second strand cDNA. Lastly, we used the AMPure XP beads to purify the synthesized cDNA. The purified double-stranded cDNA was performed with end repair and A-tailing. The AMPure XP beads were used to select the size of fragments.