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Expression and Analysis of EPOR after Radiation Injury of Salivary Glands in Rats

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Abstract

Objective: To study the expression of erythropoietin receptor (EPOR) after radiation injury of the submandibular gland in rats, and to provide theoretical basis for further exploring the dysfunction of salivary glands.

Methods: 30 Wistar rats were divided into 2 groups: the control group and the experimental group. A large dose of 15Gy radiation was used to irradiate the head and neck in rats, the control group did not receive radiation. Paraffin sections were made from the submandibular glands of rats in the control and experimental groups on the third days, thirtieth days and sixtieth days, respectively. HE staining, the expression of erythropoietin receptor protein was observed by immunohistochemical staining. The average optical density values were calculated according to Imagepro plus 6.0 for quantitative analysis.

Results: 1) The expression of EPOR in the submandibular glands of rats in the experimental group increased significantly after radiation, and was higher than the control group at the same time. 2) The expression of EPOR in the control group increased with age. 3) EPOR was found to be expressed in the cytoplasm and cytoplasm of glandular ducts, but not to be found in acinar cells.

Conclusion: The expression of EPOR increased after radiation injury of salivary glands in rats.

Keywords: EPOR; Rats; Submandibular gland; Radiation injury

Introduction

Radiation therapy, as one of the main treatment methods for head and neck malignant tumor, has achieved extremely remarkable curative effect in clinic. The salivary glands are usually located in the superficial layer of tumor tissue or covering the surface of the tumor, and often in the radiation field. In addition, the salivary glands, especially the parotid gland containing serous acinus and the submandibular gland are sensitive to radiation. Therefore, radiation damage of the salivary glands is a common complication of radiation therapy in the head and neck. Clinically, patients often suffer from dry mouth, oral mucositis, ulcer and osteoradionecrosis, etc. [1,2]. Scholars and experts at home and abroad have done a lot of research and exploration on the mechanism of radiation injury of salivary glands. Some scholars have found that [3] EPO has protective effects on radiation damage of salivary glands. While EPO works, it requires binding with its receptor EPOR to form a homodimer that makes EPOR related tyrosine kinase JAK-2 autophosphorylation, and then lead to phosphorylation of multiple signaling pathways downstream, and thus play a protective role. In addition, it is thought that [4] EPOR was expressed only in early erythroid cells. However, many studies in recent years found that EPOR is expressed in many non-hematopoietic tissues and cells, such as endothelial cells, cardiomyocytes, smooth muscle cells and hortega cells, astrocytes and brain tissues. However, there is no report on the expression of EPOR in rat submandibular gland at present. In this paper, we established the rat submandibular gland radiation injury model to study the expression of EPOR, and to verify the presence of EPOR expression in rat submandibular glands and to analyze EPOR expression changes after radioactive injury, which provides a theoretical basis for further exploration of radiation-induced salivary gland dysfunction.

Materials and Methods

Animals and reagents

30 male wistar rats, weighing 180 g to 220 g, were purchased at the laboratory animal center of Jilin University, and they were familiar with the environment and free diet a week before the experiment. Fixed deep X-ray machine (Precision X-Ray company, USA), Olympus BX51T microscopy (Olympus, Japan), natrium citricum pH=6.0 (Boster Biological Technology co. ltd), PBS buffer pH=7.2-7.6 (Boster Biological Technology co. ltd). pAb anti-EPO Receptor (NSP1-19388, NOVUS) 3% hydrogen peroxide deionized water, polymer auxiliary agent, Poly Peroxidase-anti-Rabbit IgG (GBI company, USA), DAB Color Development Kit (Boster Biological Technology CoLtd), HE staining, immunohistochemical staining reagent was provided by immunohistochemical Laboratory.

Grouping and processing

The rats were equally divided into two groups: The control group (not irradiated) and the experimental group (irradiated). The rats were successfully anesthetized by intraperitoneal injection of 10% chloral hydrate, and then placed in a self-made lead box at a dorsal decubitus. Only the 1.5 cm region below the inferior border of the mandible was exposed to the irradiated field, other areas were covered with a 2.0 mm thick plate. The radiation was sent by a fixed deep X-ray machine, electric current 12.38 mA voltage 299.8 KV filter board 2 mm Al, dose 15Gy [5], dosage rate 2Gy/min, target skin distance 60 cm, the control group received only anesthesia without irradiation (Tables 1-3).

Histological observations

Rats were killed by dislocation on three days, thirty days and sixty days after operation, the bilateral submandibular glands were taken after the perfusion fixation. Continued fixing 48 h, gradient alcohol dehydration, dimethyl benzene replacement, paraffin embedding

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Control (d)	Average IOD
3d	0.206 ± 0.008
30d	0.243 ± 1.005
60d	0.248 ± 0.007
ANOVA: F=57.210, p<0.001	

Table 1: Expression of EPOR in rat sub-mandibular glands of control.

Radiation (D)	Average IOD
3D	0.244 ± 0.005
30D	0.264 ± 0.008
60D	0.298 ± 0.007
ANOVA: F=81.014, p<0.001	

Table 2: Expression of EPOR in rat sub-mandibular glands of radiation.

S. No	3d	30d	60d	3D	30D	60D
Sum (IOD)						
1	77729.938	84466.211	122203.350	87852.273	121778.590	103913.500
2	77825.852	87852.287	100329.230	98486.328	114105.780	98834.633
3	71945.094	105814.850	101481.610	106096.160	106096.160	117989.060
4	81169.820	97965.289	9894.188	80025.656	90549.961	98648.430
5	95163.594	83793.859	88198.102	104420.880	101141.670	114347.960
Sum (Area)						
1	382467	366153	482357	368292	475408	333442
2	383402	368292	419486	404965	409040	338434
3	363804	454439	387895	401131	401131	410026
4	391741	404474	397862	337767	348176	327566
5	435537	444544	362419	423091	390740	375380

Table 3: Sum (IOD) and Sum (Area) of each group.

and making wax blocks, section, thickness 3 μm, HE staining, dimethyl-benzene dewaxing, gradient alcohol removal of benzene, hematoxylin staining nucleus, washed with water, hydrochloric acid and alcoholflushing, added ammonia to blue, eosin staining, gradient alcohol dehydration, dimethyl- benzene was added to become transparent, microscopic observation.

EPOR expression analysis

Immunohistochemical staining was performed on sections of each group by specific pAb, anti-EPO receptor antibodies. I, II dimethyl-benzene 15 ml each, I, II absolute ethyl alcohol, 10 ml each. Febrile antigen restoration (0.01 mmol/L natrium citricum buffer, pH=6.0) PBS washed, 3% hydrogen peroxide, goat serum sealing solution, dropped pAb anti-EPO Receptor, antibody dilution multiple 1:200, incubated overnight at 4°C, PBS washed, dropped polymer auxiliary agent, poly peroxidase-anti-Rabbit IgG, DAB color development, the reaction time was controlled under microscopically. Hematoxylin re-staining slightly, hydrochloric acid and alcohol differentiation, added ammonia to blue, 5 high-power field of view (X400) was randomly selected from each section to observe the positive expression of EPOR.

Average optical density calculation

Immuno-histochemical staining sections of each group were observed under Olympus BX51T microscope, 5 high-power field of view (X400) were randomly selected from each section. ImagePro Plus6.0 image analysis software (Media Cybernetics, USA) was used to quantitatively analyze the expression of EPOR protein, took Sum (IOD)/Sum (Area) as the average optical density value Average (IOD) (Tables 1-3 and Figures 5-7).

Statistical analysis

Data analysis was carried out with SPSS 24 software, the measurement data was expressed by the mean ± standard deviation (x ± s). One-way ANOVA and two independent sample t-test were used to compare the mean between groups. p<0.001, the difference was statistically significant.

Results

Histological observation

Figure 1 shows the submandibular gland granular convoluted tubule (GCT) of rats in A, B and C groups were obvious and large, the lumen was homogenized and stained, and were composed of simple columnar epithelium. The acinus of the submandibular gland in the rat were plump, with clear structure fit closely each other. The submandibular glands of the rats in Figure 2A group showed atrophy of acinar cells at various degrees after a single exposure of 15Gy, the acinar cell atrophy in Figure 2B group was more obvious, vacuoles appeared at the top, and the number of vacuoles increased obviously. The acinar cell's nucleus in Figure 2C group showed deep staining and partly lost normal acinar structure. Granular curved tubes and striated tubes were markedly reduced, eosinophilic staining substance could be seen in the lumen, acinar cells and serous duct cells showed obvious degenerative changes, but without obvious inflammatory cell infiltration.

Immunohistochemical staining showed that the acinar nuclei of the control group 3D (Figure 3A) were dyed blue, no positive expression of cytoplasm, the expression of EPOR protein was found only in GCT and the cytoplasm of stria duct cell in 30d (Figure 3B) and 60d (Figure 3C) group, light yellow, a little of which the coloring of 60d group (Figure 3C) was stronger than that of 30d group (Figure 3B), the positive expression of protein in the 30d group (Figure 3B) was more obvious than that in 3D (Figure 3A). In experimental group: 3D (Figure 4A), 30D (Figure 4B) and 60D (Figure 4C), the yellow stain could be seen in GCT of submandibular gland, stria duct and the cytoplasm and membrane of the excretory duct in rats, which were the positive expression of EPOR after irradiation, while the acinar nuclei were blue and the cytoplasm was colorless, the expression of EPOR has not been found, a large number of EPOR could be seen in GCT and stria duct in 60D group (Figure 4C). The cytoplasm was dark yellow, partly cell membrane was brown, with strongest positive expression; compared with the group 3D (Figure 4A), 30D (Figure 4B) group showed a marked increase in yellow staining and was expressed in the duct of the submandibular gland. Compared with the group 30D (Figure 4B), the expression of EPOR in 60D (Figure 4C) group was mostly in the cell membrane of the duct. The staining was deeper and the range was larger than that of 3D (Figure 4A) group.

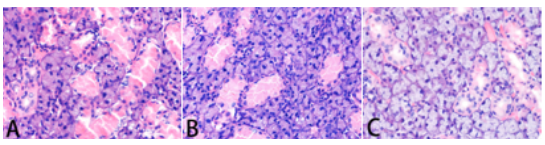

Figure 1: Histology sections of rat sub-mandibular glands of control (×400) (A) Control group 3D. (B) Control group 30D. (C) Control group 60D.

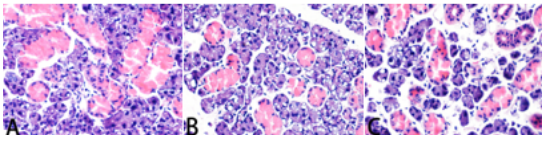

Figure 2: Histology sections of rat sub-mandibular glands of radiation (×400) (A) Radiation group 3D. (B) Radiation group 30D. (C) Radiation group 60D.

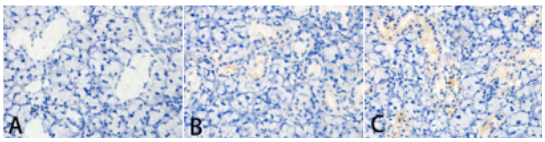

Figure 3: Immuno-histochemical expression of EPOR in rat submandibular glands of control (×400) (A) Control group 3D. (B) Control group 30D. (C) Control group 60D.

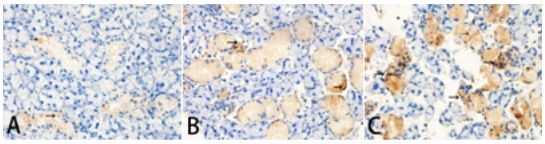

Figure 4: Immuno-histochemical expression of EPOR in rat submandibular glands of radiation (×400). (A) Radiation group 3D. (B) Radiation group 30D. (C) Radiation group 60D.

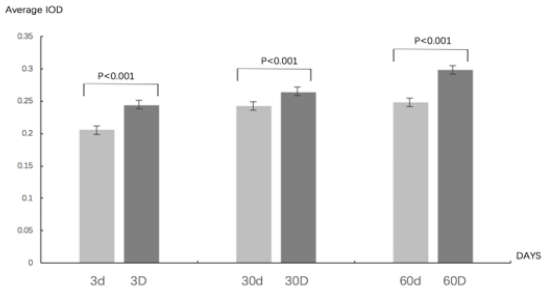

Figure 5: Average IOD of each group. Independent test: (1) 3d/3D t=-9.007, p<0.001; (2) 30d/30D t=-4.977, p<0.001; (3) 60d/60D t= -11.294, p<0.001.

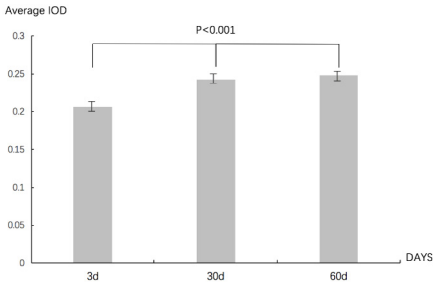

Figure 6: Average IOD of control group.

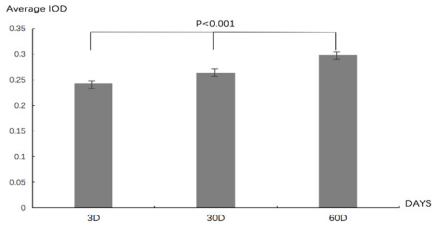

Figure 7: Average IOD of radiation group.®

Discussion

Human salivary gland is composed of parotid gland, submandibular gland, sublingual gland and many small salivary glands. The amount of saliva secreted by normal adults is about 1000 ml-1500 ml per day, pH = 6.5-7.4, of which 70% is secreted by submandibular gland, 25% is secreted by parotid gland, 5% is secreted by sublingual gland. At present, radiation therapy, as one of the main treatment methods for head and neck malignant tumor, has achieved very remarkable curative effect in clinic. However, the acinar cells of salivary glands are highly differentiated cells and are sensitive to ionizing radiation. Scholars and experts at home and abroad have done a lot of research and exploration on the mechanism of radiation injury of salivary glands. Seyed et al. [6,7] reported that ionizing radiation produced by radiotherapy can produce reactive oxygen species (ROS) and other toxic substances that affect transcription of DNA and RNA, leading to cell dysfunction and death. In addition, radiation can not only kill cancer cells in tumor tissues, but also cause inevitable damage to normal tissues, which seriously affects the therapeutic effect and quality of life of patients. In addition, Pérès Elodie et al. [8] showed that EPOR silencing on glioma cells increases the toxicity of TMZ and X-rays in GBM cells mainly through the enhancement of senescence leading to the induction of mitotic catastrophe.

In recent years, many studies have found that the function of EPO depends on the mechanism of EPO-EPOR signal transduction mechanism [9]. The theory holds that the combination of EPO and EPOR can promote the cross-linking of EPOR to form homodimer, resulting in the auto-phosphorylation of EPOR related tyrosine kinase JAK-2 and phosphorylation of multiple signaling pathways downstream. The major signaling pathways are RAS mitogen activated protein kinase (RAS/MAPK) [10], phosphatidylinositol kinase-3 (PI3-K) [10], signal transcription and transduction activating factor 5 (STAT5) [11], and nuclear factor -KB (NF-KB) [11]. The major biological roles of those signaling pathways include:

1. Promote the proliferation, differentiation and maturation of erythroid progenitor cells and block the programmed cell death of erythroid progenitors [12];

2. Antioxidation, stabilizing the function of erythrocyte membrane, improve the lipid fluidity and protein conformation of erythrocyte membrane, promote the activity of ATP of membrane sodium ion and potassium ion, and maintain normal osmotic pressure inside and outside membrane; promote ATP enzyme activity of membrane Na^+ and K^+, and maintain normal osmotic pressure inside and outside the membrane [13];

3. Affect the survival of nerve cells directly, neurotrophic effects are also demonstrated in nerve cell cultures and regulate nerve regeneration [14];

4. They are involved in anti-apoptosis, anti-hypoxia, promoting the formation of new blood vessels, regulating immune function and coordinating the effects of related cytokines, supplying nutrients to tissue cells influencing the sensitivity of chemo-therapy etc. [15].

The submandibular gland of the rat is composed of the secretory portion and the duct. The secretory portion is a mixed acinus dominated by serous acini, the duct portion includes excretory duct, stria duct, granular convoluted tubules (GCT) and intercalated duct. In the experiment, though HE observation, whether it is 3d, 30d or 60d group, the submandibular gland granular convoluted tubule (GCT) of rats in control group were obvious and large, the acinus of submandibular gland was plump, with clear structure, fit closely each other (Table 1). However, in the experimental group, the acinar cells of the 3D group were atrophied after 15Gy irradiation. The acinar cell atrophy in 30D and 60D groups were more obvious, vacuoles appeared at the top, the number of vacuoles increased obviously, cell arrangement disorder, the nuclei was stained deeply, GCT and striated tubes were markedly

reduced, a series of degenerative changes caused by radiation damage appeared.

Immunohistochemical staining showed that there was no positive expression of EPOR in the control group 3D, light yellow positive expressing structures were seen only in GCT and stria ducts, which may be due to the age changes is related to EPOR expression. One-way ANOVA showed $p<0.001$, the difference was significant. In the experimental group 3D, 30D and 60D, yellow staining was visible in the GCT, stria ducts, excretory ducts cytoplasm and membranes of the submandibular glands in the rat, while the acinar nucleus was blue, and the expression of EPOR was not found in cytoplasm and membrane. The reason may be related to the functional changes in the amount of saliva and the osmotic pressure after exposure to sodium, potassium, and saliva. In group 60D, EPOR had the highest average optical density and the strongest positive expression. Compared with the 30D group, the expression of 60D group was mostly on the cell membrane of the duct, which may be associated with EPO-EPOR binding and activation of signaling pathways to prevent apoptosis and to respond to radiation damage. Compared with group 3D, the expression of EPOR in 30D group increased significantly, and the positive expression of EPOR increased significantly after irradiation, the average optical density of each group was measured. The positive expression of EPOR protein increased gradually in experimental group 3D, 30D and 60D. $p<0.001$, the difference was statistically significant (Table 2).

It is thought that EPOR was expressed only in early erythroid cells [4]. However, many studies in recent years found that EPOR is expressed in many non-hematopoietic tissues and cells, such as endothelial cells, cardiomyocytes, smooth muscle cells and hortega cells, astrocytes and brain tissues. In this study, EPOR was found to be expressed positively in the submandibular gland of rats. Erythropoietin receptor (EPOR) is a single stranded transmembrane protein composed of 508 amino acids. Nucleotide sequence data show that the amino acid sequence of human EPOR is 81.6% homology to that in rat. The structure can be divided into 3 parts, including the extracellular portion, the transmembrane region, and the intracellular region. There are 226 amino acids in the extracellular membrane, and the N-terminal is connected with a signal peptide composed of 24 amino acids. The transmembrane region consists of 22 hydrophobic amino acids [16]. However, there are few reports about the EPOR.

Conclusion

In this study, the expression of EPOR was found in the salivary duct of rats by HE and IHC staining, and more expression was found in the cell membrane. No expression was found in the secretion of submandibular glands; meanwhile, the expression of EPOR is more and more by the prolongation of radioactive injury. However, the study on the changes of EPOR and the damaged glandular cells in salivary glands remains to be further studied. To sum up, the phenomenon of the experimental results is analyzed in this article, but further studies such as molecular pathways need more experiments to confirm.

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