The Apoptosis And Regeneration Of The Animal Model Of Obstructive Atrophy Parotid Gland

To explore the regeneration and recovery of parotid gland after parotid duct ligation and recanalization in rat. Methods: Wistar rats' parotid duct was ligated for 7 days (group A), 14 days (group B), 21 days (group C) and then released. HE staining?Immunohistochemical and Tunel fluorescence detection were used to observe apoptotic and regeneration changes of parotid specimens which were obtained at 60 and 90 days after releasing the duct. Results: Day0, acinar cells atrophy and decrease while ductal cell proliferation and ductal ectasia. The expression of PCNA and P53 and apoptotic ratio were higher than normal control group. They increased gradually and reached the highest value in A 3?B5,and then decreased gradually to the normal control group in A14?B21. The gland tissue structure returned to normal,but it didn't occur in group C. Conclusion: After release the ligated duct, the gland can be return to normal within the appropriate time, otherwise, it cannot be fully recovered or completely restored.

Atrophy of the parotid gland is characterized by acinar atrophy, which causes dysfunction of salivary gland secretion, thus induces inflammation of oral mucosa, affects talking, swallowing, and tasting, etc[1]. In severe cases, the disease may cause organic damage such as rickets, periodontal diseases and so on[2,3], affect the daily life and socialization of patients and reduce the quality of life seriously. How to promote the recovery of atrophic glandular tissue is one of the focuses of oral medical research[4,5]. In this study, we used the rat parotid regeneration after atrophy model induced by recanalization of the main duct of the parotid gland, to study the mechanism of parenchymal cell regeneration after gland atrophy, which will provide a theoretical basis for clinical treatment of salivary gland atrophic diseases.

Method

Main reagents and instruments

Primary antibody: PCNA monoclonal antibody (ab92552, Abcam, UK), P53 polyclonal antibody (ab131442, Abcam, UK); hematoxylin and eosin (HE) staining kit (Shanghai Nuolun Biomedical Technology Co., Ltd., China); one-step Tunel cell apoptosis test kit (green FITC label fluorescence detection method, universal type) was purchased from Jiangsu Kaiji Biotechnology Co., Ltd.; Fluorescent Mounting Medium DAPI was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, China.; confocal laser microscope (SP5II, Leica, Germany)

Animal

A total of 204 adult male Wistar rats with weight of approximately (200±20) g were purchased from Jinan Pengyue Experimental Animal Breeding Company. We selected 204 numbers from 1 to 204, and then the 204 numbers were randomly arranged by the random number generator in SPSS23.0. The first 198 numbers listed in ascending order were assigned to experimental group, and the remaining 6 numbers listed in ascending order were assigned to control group. This number is the sequence number of the rats' number. In this way, the rats were randomly divided into experimental group (n=198) and control group (n=6). The right main parotid duct of

the experimental group was ligated. After ligation for 7 days (group A), 14 days (group B), and 21 days (group C), the parotid duct was released. Rats were sacrificed at 1, 3, 5, 7, 10, 14, 21, 28, 60, and 90 days after recanalization to obtain fresh parotid gland. Specimens were fixed in 4% paraformaldehyde solution to prepare paraffin blocks. In control group, the main duct of the parotid gland was only dissected and isolated, and then the wound was stitched up. All operations throughout the study comply with the ARRIVE guidelines. The animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Binzhou Medical University, with the approval of the Institutional Animal Care and Use Committee.

HE staining

HE staining was performed according to the standard procedures in HE staining kit. The paraffin sections with 4 ?m thick were prepared. The paraffin sections were stained with hematoxylin for 6 minutes, and differentiated with 1% hydrochloric acid in ethanol for 12 seconds. After that, the paraffin sections were stained with scott tapwater/bluing for 15 minutes, and stained with Eosin 30 seconds, and then paraffin sections were placed under a microscope for observation and photographing.

Immunohistochemical staining

After a 4 ?m thick paraffin section was prepared, a two-step immunohistochemistry procedure was proceeded. Then the primary antibody (1:100) was added, overnight at 4 °C, DAB was developed, and the negative control was used PBS instead of primary antibody. After the film was mounted, the staining results were observed under a microscope, and five non-overlapping fields were taken for each film to calculate the average gray value of PCNA and P53.

Tunel cell apoptosis detection

A 4 ?m thick paraffin section was dewaxed and dehydrated according to the one-step TUNEL apoptosis detection kit. The TdT enzyme reaction solution and the fluorescent labeling solution were made when they were using, and the reaction was avoided light at 37 ° C in the dark. The section was mounted by anti-fluorescence quenching capsule DAPI and photographed by a laser confocal microscope.

Statistical analysis

The experimental data was analyzed using SPSS23.0, and all values were expressed as mean ± standard deviation (mean ± SD). Differences within the groups were compared by one-way analysis of variance (after LSD correction for multiple testing), and Independent sample t-test was used for comparison between groups. P

Result

Histological result

After ligation of the parotid gland main duct for 7 days (group A), the acinar atrophied and ductal dilatated. Compared with the normal tissue, the number of the acinar/catheter ratio and

glandular lobules volume were decreased. On the third day after recanalization, the number of acinus increased significantly, and the acinus mainly included immature acinus and residual acinus. On the 10th day after recanalization, no significant different in the proportion of acinar/catheter number was found when compared with the normal group. On the 14th day after recanalization, the parotid gland tissue was almost restored to normal. For group B, after ligation of the parotid gland main duct for 14 days, a large number of duct dilated, but the number of acinar was significantly reduced. On the fifth day after recanalization, dilated duct was significantly narrowed, and the acinar cells proliferated significantly. On the 14th day after recanalization, the acinar cells filled the gap between the fistula and the striate tube, but the connection between the acinar cells was loose. On the 21st day after recanalization, the ratio of acinar to catheter was appropriate, and the glandular lobular structure is tight and similar to normal glandular tissue structure. When the main duct of the parotid gland is ligated for 21 days (group C), the proliferation and expansion of the duct are more obvious, and the number of acinar cells reduced. The remaining acinus can only be found at the edge of the glandular lobule. As the recanalization time prolonged, the phenomenon of recovery of parotid gland tissue in groups A and B were not found. Some parotid gland tissues were even gradually replaced by connective tissues such as fat. (Figure 1)

Immunohistochemical staining

The expression of protein PCNA and P53 in the parotid gland is shown in Figure 2 (PCNA protein is mainly expressed in the acinar nucleus of the salivary gland, and P53 protein is mainly expressed in the acinar cytoplasm of the salivary gland). 7 days after the ligation of parotid main duct, the main duct expanded and proliferated, meanwhile, the acinar cells showed some degree of atrophy, and the positive expression rate of PCNA and P53 reduced. On the third day after recanalization, the acinar cells proliferated in a large amount, and the PCNA expression in the nucleus of the acinar cells were increased. The apoptosis of the catheter increased gradually, and the expression of P53 was observed. On the 10th day, PCNA and P53 expression tended to be stable. On the 14th day, the immune index of the recanalization group was the same as that of the normal control group. After 14 days of main duct ligation, P53 expression was observed in the duct and acinus cells, while the PCNA expression was mainly in ductal cells. On the 5th day after recanalization, immature acinar and residual acinar cells proliferated, and PCNA was highly expressed, ductal cells were apoptosis, and P53 was positively expressed. On the 14th day after recanalization, the expression of PCNA and P53 tended to be stable. On the 21st day after recanalization, the expression of PCNA and P53 was not different from that of the normal control group. After 21 days of main duct ligation, PCNA was highly expressed in the ductal cells, P53-positive expression was observed in fibrous connective tissue. There was no obvious curve change in the expression of PCNA and P53 with the recanalization time. It was observed that 90 days after recanalization, no atrophic parotid gland recovered to normal.

Tunel and calculation of apoptotic rate

In the normal parotid gland, there are few numbers of apoptotic cells (green fluorescent signal markers). After catheter ligation, apoptotic cells increased significantly, which were mainly found in the acinar region. Besides, with the time extension of catheter ligation, the number of apoptotic cells increased (P

Discussion

Chronic obstructive parotid disease is a relatively common chronic inflammation of the parotid gland, and the most causes of the disease are ductal stones, stenosis of catheter, and tumor constriction[6]. Due to the suppression or stoppage of saliva flow and the retrograde infection of pathogenic bacteria in the oral cavity, the glands often swell and pus repeatedly. For the treatment of severe chronic obstructive parotid diseases, it is generally preferred to remove the glands through surgery[7,8]. However, after removal of the gland, complications such as dry mouth might affect the daily life of the patient seriously. There is no widely accepted treatment that could reverse glandular damage and the disease could not be cured right now.

Normal parotid cells are branched-type, highly differentiated terminal cells. Although the ability of differentiation is weak, the salivary gland adult stem cells (SGASC) have the ability of self-repair. It has been demonstrated that SGASC is mainly distributed in large drainage tubes [9], and the uneven distribution determines the speed and extent of recovery after parotid tissue injury. Under normal conditions, the parotid gland tissue maintains a balance between apoptosis and proliferation, and the stem cells keep in a relatively static state. Only when the injury occurs, the signal molecules in the body are widely active, and then the stem cells are activated to repair the body damage. PCNA is a nuclear protein synthesized and expressed only in proliferating cells[10,11]. It is divided into two types including soluble and insoluble protein, and the change in the expression level of insoluble PCNA is consistent with DNA synthesis. Liver regeneration experiments induced by partial hepatectomy in rats have confirmed that PCNA can be used as an accurate indicator for analyzing cell proliferation tests and is an effective parameter for analyzing tissue regeneration [12]. With the development of molecular medicine, PCNA may become a molecular target for follow-up, prognosis, and treatment of cancer patients. Bin Xiang et al [13] have confirmed that after radiation injury of the submandibular gland, phenylephrine activates ?1 receptor, which can improve cell proliferation effectively and inhibit cell apoptosis. The study provides another new target for the treatment of salivary gland diseases.

Apoptosis could be found in many biological phenomena, in contrast to the mitotic proliferation, and plays a very important role in the regulation of cell colony. Overexpression of P53 and Fasl is a hallmark of apoptosis activity [14]. MDM2 (murine double minute gene 2) could bind to P53 gene promoter and then inhibit P53 transcription, down-regulate MDM2 expression, and increase P53 expression. PGE2 (prostaglandin E2) induces cell apoptosis by increasing P53 gene transcriptional activity and Fasl expression. P53 can also induce Fasl expression to activate the exogenous apoptotic pathway to stimulate apoptosis. The increased expression of P53 could up-regulate Bax expression and down-regulate Bcl-2 expression to promote apoptosis.

It was observed in our study that the ligation of parotid main duct can induce parotid atrophy. which has been reported in the study of Zuo et al. [15]. Gland atrophy begins at the center of the glandular lobe and gradually spreads to the periphery. The expression of P53 increase more with the ligation time, the more atrophy of parotid gland is induced. It may be due to the continuous increase of pressure in the catheter after the ligation of parotid main duct. After recanalization, glandular atrophy can be reversed within a certain period of time. in the groups of 7 days and 14 days after recanalization of the parotid main duct, the glandular gradual recovery was observed, and the expression of PCNA in the acinus gradually increased, thus the

proliferation of acinar cells promoted, and the dilated duct gradually decreased and recovered. And the glandular lobular arrangement returned to normal from the disorder, the acinar/catheter ratio gradually turned to be the same as the control group, and the recovery time in the group of 7 days after the ligation of parotid main duct was shorter and sooner than that of the 14-day group. 21 days after the ligation of parotid main duct and recanalization, the number of vesicles is very small, and the dilated ducts occupy almost all of the glandular lobules. No obvious and regular recovery of the glandular lobules was observed with the recanalization time prolonged, and even the parotid gland is replaced by adipose tissue and fibrous connective tissue in some groups.

The model of regeneration after submandibular gland injury showed that cells in the proliferative site of the parotid gland have the same characteristics as stem/progenitor cell [16]. In our experiment, group A and group B can achieve parotid regeneration, while group C fails, it was probably because group A and group B have shorter ligation time, and there are enough stem cells to differentiate and proliferate in parotid gland to compensate for apoptosis. In group C, the ligation time is longer, and most or even all of the stem cells in the gland are destroyed, and it was unable to differentiate into new cells, thus leading to atrophy of the gland. In conclusion, after the ligation of parotid main duct, the expression level of P53 is increased, which can promote the atrophy of the gland. After the catheter is recanalized, the PCNA expression is increased, which promotes the regeneration of atrophic acinar cells. PCNA and P53 expression can be used as molecular basis for evaluating the timing of treatment of chronic atrophic mumps, and it may also be used as an indicator for evaluating the feasibility and effectiveness of parotid regeneration surgery. The study by S. Takahashi et al. [17] also confirmed that after atrophy of the gland, the duct cells proliferated and differentiated to form new cells to compensate for the acinus that disappeared due to apoptosis. The rate of regeneration after atrophy of the parotid gland is faster. In addition to the differentiation of stem cells, there are self-proliferating and dividing supplements of remaining acinar cells. In addition, it was shown that the model of animal parotid regeneration is similar to the process of salivary gland embryo development [18], especially secreted proteins, showed no difference between the two models. parotid gland tissue can recover after induction of apoptosis in animal studies provides hope for the treatment of parotid injury in human beings.

In summary, the parotid gland can regenerate after the ligation of parotid main duct. The recanalization mechanism of the parotid gland tissue provides a theoretical basis for the clinical treatment of parotid atrophic disease, and it may develop into the study focus of tissue regeneration in the future[19]. However, if the time of the ligation of parotid main duct exceeds the minimum time for tissue stem cell differentiation, the tissue will not be regenerated. The realization of tissue regeneration and the promotion of tissue regeneration are the focus of the next step. And other treatment methods such as stem cell transplantation [20] and tissue engineering technology [21] need to be considered.