EFFECTS OF TRADITIONAL CHINESE MEDICINE WEI-WEI-KANG-GRANULE ON THE EXPRESSION OF EGFR AND NF-KB IN CHRONIC ATROPHIC GASTRITIS RATS

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Wei-Wei-Kang-Granule(WWKG) is a traditional Chinese medicine (TCM) preparation for the treatment of chronic atrophic gastritis (CAG). We examined the pathologic change and the effects of Wei-Wei-Kang-Granule (WWKG) on the expression of EGFR (epidermal growth factor receptors) and NF-κB (nuclear transcription factor KappaB) in rats with chronic atrophic gastritis (CAG), and evaluated the possible mechanisms. Ninety rats were randomly divided into control group and four experimental groups. CAG rat models were induced by repeated stimulating experiments in the experimental groups. After modeled rats were intragastrically injected (i.g.) with WWKG at 6000mg/kg (large dose WWKG group), WWKG at 3000mg/kg (small dose WWKG group), San-Jiu-Wei-Tai-Granule(SJWTG) at 1600mg/kg(SJWTG group), and normal saline(0.9%)at 20ml/kg (model group and control group), respectively, once a day for 30 days. After 30 days, all rats were sacrificed and samples were taken from the sinus ventriculi and body of stomach. The gastric specimens were prepared for microscopic view with hematoxylin and eosin (H-E). The immunohistochemistry method was used to observe the expression of protein of EGFR and NF-κB in gastric tissue. The data were analyzed in pre- and post-treatment by computer image automatic analysis system. Immunohistochemistry detection showed that the average optical density of EGFR and NF-κB in antrum was lower in large and small dose WWKG groups than the model group ($P<0.01$). CAG in rats was related with the damage of barrier in gastric mucosa and the misbalance of cell proliferation and apoptosis. One of the mechanisms is perhaps to reduce the expressing of EGFR and NF-κB in gastric mucosa.

Key words: Chronic atrophic gastritis(CAG), EGFR,NF-κB**Introduction**

In 1998 gastrointestinal pathologists reached a consensus on the definition of chronic atrophic gastritis (CAG), which was described as programmed loss of gastric gland and/or replacement by intestinal glands in gastric mucosa. The imbalance in the cellular proliferation and apoptosis of gastric epithelial cells are considered to be attributable to the change of genetic events in CAG. Inhibition of apoptosis or over-proliferation could lead to mutant cells accumulation and the development of gastric neoplasm. Multiple factors are involved in this progression, including change of human environment, gene inheritance and medical intervention pathways.

At present, CAG is treated in clinic using antiacid, spasmolytic, mucosa and protectant. In recent years, the doctors of TCM pay more emphasis on the study of the pathogenesis of CAG and made certain progress. TCM preparations from the natural have been accepted by more and more patients with gastric diseases because TCM have double-deck function of the treatment and the recuperation, and show fewer side effects. But, to date, the research of traditional Chinese

Herbs treating the disease is still kept at the initial stage at home and abroad. Our laboratory has investigated CAG since 1999. Clinical studies for decades have confirmed that WWKG is more effective on chronic atrophic gastritis than stomach enzyme, and do not show significant side effect. So, WWKG is very suitable for treating chronic atrophic gastritis, and it has been applied for many years in hospital affiliated to Binzhou Medical College. Our previous clinical study showed that the total effective rate of WWKG for CAG was 96.43% (Zhao et al., 2008). Our previous experimental study showed that WWKG could significantly improve the atrophy status of the gastric mucosa, and show statistical difference by comparing with positive control group ($P < 0.01$). The number and average optical density of G cells and D cells in the gastric mucosa were significantly increased following the treatment ($P < 0.01$).

In this study, we aimed at studying the effects of WWKG on the expression of EGFR and NF- κ B in rats with chronic atrophic gastritis (CAG), and exploring the possible mechanisms.

Materials and Methods

Materials

Wistar rats (male, 8-week-old, 200 ± 20 g) were purchased from Yantai Green Leaf Pharmaceutical Co. Ltd (Yantai, China). Sanjiu Weitai granule was bought from 999 Co. Ltd. (Xian, China) (Z44020705). NaSA was ordered from National Pharmaceutical Group Chemical Reagent Co., Ltd (Dalian, China) (T20070226). EGFR polyclonal-antibody of rabbit anti-rat (Santa Cruz Company, USA), NF- κ B p65 monoclonal-antibody of rabbit anti-rat and SP kit were offered by Beijing Zhongshan Biotechnology Co., Ltd (Beijing, China).

Preparation of WWKG

WWKG consists of 5 different plant species and 1 animal substance. The composition and active ingredients are shown in Table 1. All of these formulations were provided and prepared by 999 Modern Chinese Medicine Co. Ltd. (999 Co. Ltd., Shenzhen, China), and carefully authenticated by doctor Xiling Sun, Pharmaceutical Preparation Section, 999 Co. Ltd. Voucher specimens (listed in Table 1) were deposited at the Herbarium of 999 Co. Ltd. After drying, the formulations were mixed in proportion and then macerated for 1h at room temperature with distilled water. After that, the whole mixture was decocted twice for 1h. The filtrate was mixed and condensed and then dried by vacuum-drier at 60°C . The granule was stored at 4°C .

Experimental animals

Ninety Wistar rats were housed in an air-conditioned animal room with 12h light/dark cycle, temperature of $22 \pm 2^{\circ}\text{C}$ and humidity of $50 \pm 10\%$. Rats were provided with a laboratory diet and water for a week before the experiment. After a week, the rats were randomly divided into control group ($n=18$) and experimental group ($n=72$). The 72 experimental group rats were used to make CAG model based on the literature (Zhang et al., 2001). In the course of making the model, two rats died. After making the model, we randomly sacrificed 6 rats, and collected their stomach samples. The model was determined by routine pathological examination. In accordance with table of random digit, the remaining 64 animals after modeling were randomly divided into four experimental groups: model group ($n=16$), SJWTG group ($n=16$), small dose WWKG group ($n=16$) and large dose WWKG group ($n=16$). The control group animals ($n=18$) were freely given clean tap water and fed with standard rat diet. Two of them were sacrificed 6 weeks later, compared with model group. After six weeks, all the rats were fed with the standard rats ration, and raised under the same environment and conditions, free access to water. Body weight was measured to determine the dose in week 1. The rats were treated by intragastric administration (i.g.) with WWKG at 6000mg/kg (large dose WWKG group), WWKG at 3000mg/kg (small dose WWKG group), SJWTG at

1600mg/kg (SJWGTG group), and normal saline (0.9%) at 20ml/kg (model group and control group), respectively, once a day for 30 days. All the rats were sacrificed after treated with 30 days.

Tissue and specimen preparation

After sacrifice of animal, the belly was opened immediately, cadaver was ligature, the whole stomach was taken off. The sinus ventriculi and body of stomach had been fixed in 4% paraformaldehyde for more than 12 h, following by flushed with water for 24 h. The sample was dehydrated using 50%, 75%, 95%, and 100% alcohol, respectively. After made transparent by xylene, the sample was soaked and embedded in paraffin. The section was continuously cut into 3 pieces with 5 μ m of thickness using ultramicrotome LKB8800 (LKB-Produkter AB, Sweden). One section was dyed using the conventional hematoxylin and eosin for pathobiological examination. The other two sections were immunohistochemically stained for EGFR and NF- κ B.

Microscopic view of gastric antrum

Histological change in gastric antrum was assessed by the diagnostic criteria of gastritis in Houston in 1996 (Dixon et al., 1996). The mean number of infiltrated inflammatory cells was calculated in each of 10 microscopic fields of antrum mucosa and ranked into 7 scales (0, 0.5, 1, 1.5, 2, 2.5 and 3 scales respectively).

Immunohistochemistry detection

The expression of EGFR and NF- κ B in gastric tissue were detected with the SP method according to the kit's instructions. EGFR polyclonal-antibody of rabbit anti-rat at a titer of 1: 100, NF- κ B p65 monoclonal-antibody of rabbit anti-rat at a titer of 1: 120, both are used to replace a PBS as negative resistance. Slides were examined under microscope in the same high magnification (10 $\times$ 40), 5 gastric mucosa in each immunohistochemical slices were randomly analyzed using computer image automatic analysis system. The average optical density values of soma were measured, respectively. The result was represented using the average.

Statistical analysis

All data were put into statistical software package (Version 17.0, SPSS Inc., Chicago, IL, USA). Data were expressed as mean $\pm$ standard deviation (SD). The differences between groups were performed by one-way analysis of variance, with statistical significance indicated by a value of $P < 0.05$.

Results

Pathological findings of gastric tissue

The glossy gastric mucosa folds were flat or disappeared, with pale appearance and thin mucin in the model group rats. The gastric wall elasticity was decreased. Light microscope showed that irregular arrangement and reduction layer of gastric gland, while increasing thickness of muscularis mucosa in the model group rats, as shown in Figure 1b.

With the extension of treatment time, the epithelial cells were relatively complete relatively of small dose WWKG group and large dose WWKG group (see Figs. 1c.) with normal cellular morphology, less irregular gland arrangement. Myometrial became thicker and thicker and the thickness was fairly uniform. The surface of mucosa wasn't defluviated and the gland atrophy was unobvious. The cell infiltration in antrum and the intrinsic film between the junction of stomach and

Table 1: Composition and active ingredients of Wei-wei Kang granule

Components	Voucher specimens number	Part used	Amount used(g)
<i>Panax quinquefolium</i> L	0904015	Root	20
<i>Atractylodes macrocephala</i> Koidz	0908066	Root	15
<i>Paeonia lactiflora</i> Pall	0907026	Root	15
<i>Corydalis yanhusuo</i> W.T.Wang	0906015	Root	10
<i>Panax notoginseng</i> (Burk.)F.H.Chen	0905032	Root	10
<i>Ursus arctos</i> Linnaeus	0907024	dried bile	5

Figure 1: Morphological and pathologic changes of gastric glands and epithelial cells in rats.

(a) control group; (b) model group; (c) small dose WWKG group (HE×100) .

Figure 2: Gastric mucosa under SEM observation.

(a) control group; (b) model group; (c) large dose WWKG group (Bar=2um) .

Figure 3: Immuno-stain of proteins of EGFR($\times 400$).

(a) control group; (b) model group; (c) large dose WWKG group

Figure 4: Immuno-stain of proteins of NF-kB($\times 400$).

(a) control group; (b) model group; (c) large dose WWKG group.

Figure 5: Effect of WWKG on average optical density of EGFR and NF-kB.

Data are expressed as mean $\pm$ S.D.; ^{##}P<0.01 significantly different from control group; ^{**}P<0.01 significantly different from model group; ^{ΔΔ}P<0.01, ^ΔP<0.05 significantly different from SJWTG group. (1 Control group; 2 Model. Group; 3 Large dose WWKG group; 4 Small dose WWKG group; 5 SJWTG group)

gastric reduced significantly. Natural membrane oedema and vascular dilatation improved in contrast to model group.

Under SEM observation, the gastric mucosa of the rats in the model group was diffused erosion or ulceration and the mucin thickness was decreased (Figs.2b). The scale of inflammation in antrum was 0.81 ± 0.26 in large dose WWKG group, compared with 1.22 ± 0.26 in the model group, and was remarkably decreasing ($P<0.05$).

Detection of expression of proteins of EGFR and NF-kB

Immunohistochemistry detection showed EGFR protein expression in the basal and bilateral membrane, and the cytoplasm in CAG gastric tissue (Figure 3a~3c). The positive material of NF-kB p65 protein was brown, which was mainly located in cytoplasm of the gland epidermis. The express of NF-kB in model group was more than the other groups and nucleus can be seen even coloring by chance. The express of NF-kB in large dose WWKG group obviously decreased. (Figs.4a~4c). The comparison of the average optical density of EGFR and NF-kB in all groups referred to Figs.5.

Discussion

The results of our study showed that WWKG showed significant effect on CAG rats. Compared with the model

group, the pathology detection and electron microscopy were significantly different in therapeutic efficacy. It suggested WWKG can treat chronic atrophic gastritis by improving pathological changes. The average optical density of EGFR and NF- κ B in large and small dose WWKG groups was lower than the model group. There was no significant difference between large dose WWKG group and small dose WWKG group, indicating that the effect of WWKG has little relation with dosage. EGFR was rarely detected because of not being exposed in intact epithelial membrane. When damage of gastric barrier and perfusion rate in the epithelial membrane increased, the level of the expression of EGFR was elevated. Brzozowski et al. (1996) reported that repeated stimulation with ammonia in rats could result in adaptive cytoprotection and cell proliferation. The EGFR-mRNA expression was high in the surface mucosal layer but low in the deep and muscle layers of the stomach (Ichikawa et al., 2000). Abnormal expression of EGFR has been identified as a molecular marker of dysphasia and malignant growth in gastric epithelial cells, and had close relationship with carcinogenesis (Wang et al., 2002; Kopp et al., 2002). Our study showed that there was high expression of EGFR protein in atrophic gastritis in rats, which indicated high trends of carcinogenesis in CAG. EGFR would combine with EGF and took effect in the pathway of the oncogene expression, breaking the normal self-regulation of the cell cycle, and resulting in the development of gastric cancer.

Nuclear factor κ B (NF- κ B) is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, tumorigenesis, inflammation, and so on. In its inactive form, NF- κ B is sequestered in the cytoplasm, bound by members of the I κ B family of inhibitor proteins. The activation of NF- κ B can lead to a lot of over-expression of inflammation related factors, causing significant inflammation. The study discovers recently that NF- κ B has important role in the development of tumour. It can regulate cells' generation, differentiation, apoptosis and malignant transformation. Loss of control of its activity is concerned with tumorigenesis in mammal (Sweeney C et al., 2004, Tai DI et al., 2000). Evidences indicate that the persistent activation of NF- κ B can be a sign of entity tumor, such as colon carcinoma, pancreatic cancer, breast cancer and so on. (Lin A et al., 2003, Yu LL et al., 2004, Ross JS et al., 2004, Oya M et al., 2003, Ghosh S et al., 2002, Sovak MA et al., 1997, Sweeney C et al., 2004).

The present study suggested that CAG in rats was related with the damage of barrier in gastric mucosa and the misbalance of cell proliferation and apoptosis. WWKG can decrease the express of EGFR and NF- κ B in gastric mucosa, which is the perhaps mechanisms. CAG was recognized to be closely related with development of gastric cancer and listed as precancerous lesions in this meeting (Genta, 1998). According to Correa (1992)'s cascade of gastric carcinogenesis, gastric cancer was believed to develop from a multistep progression from CAG, intestinal metaplasia (IM), dysplasia (DYS) and subsequently to intestinal-type adenocarcinoma, though the definite mechanisms have not been completely identified. The positive rate of EGFR and NF- κ B are high in gastric precancerous lesions, showing that the protein involves in the progress of gastric precancerous lesions, which is a earlier period event in stomach cancer. The express of EGFR and NF- κ B can be marker in detecting early cancerization. The results of experiments indicate that WWKG can prevent the gastric precancerous lesions.

The present study has several limitations. Firstly, this research was a temporary experiment, which was difficult to reproduce pathogenesis of human completely. Secondly, the limited sample size may in part contribute to the lack of significant findings. Finally, this study was an in vivo rat study, and caution should be exercised when applying these results to the clinic. Therefore, further studies are needed to examine the effect of WWKG on EGFR and NF- κ B.

In conclusion, WWKG had significant effect on CAG rats. The mechanism was associated with EGFR and NF- κ B. Further studies are needed to examine how WWKG regulate the function of EGFR and NF- κ B.

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