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Research

Protective Effect of Anar-5 Herbal Treatment on Experimental Chronic Atrophic Gastritis in Wistar Rats.

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Abstract

Background: Chronic gastritis is a slowly progressive disease. It includes atrophy of gastric mucosa, impairment of epithelial regeneration, formation of lymphoid tissue/germinal center, loss of gastric secretion and movement. The Mongolian traditional medicine used Anar-5 recipes for the treatment of stomachache, emesis, and improvement of gastric digestion. Based on these observations, we implemented a rat model of Anar-5 treatment in artificial chronic gastritis in order to elucidate its potential protective mechanisms scientifically.

Methods and material: We established an experimental rat model of chronic gastritis by use of ammonia water. We divided the rat cohorts in an Anar-5 – treated test group, an untreated cohort, and a control group. The untreated group was fed with 0.1% ammonia water and the treated group with both 0.1% ammonia water and administered Anar-5 100 mg/kg/day for 6 weeks. Gastric lesions were evaluated microscopically. The Prostaglandin E2 levels, cyclooxygenase COX-2 expression and the cellular proliferation marker Ki67 were in addition assessed.

Results: The Anar-5 cohort displayed with an increased thickness of the antrum mucosa, number ($p<0.05$) and regeneration zones of gastric mucous epithelial cells when compared to the results of the untreated cohort (693.1±63.8 µm versus 429.6±43.5 µm). The untreated cohort displayed with decreased PGE2 levels (14.8±0.62 ng/dl) when compared to those of the control group and Anar-5 cohort (19.5±1.22 ng/dl and 18.7+0.32 ng/dl protein, respectively). The Ki67- associated proliferation rate of the antrum mucosa was enhanced and measured 19.75% in the untreated cohort in comparison to a proliferation rate of 6.58% in the Anar-5 cohort.

Conclusion: The data of our rat experiment indicate that a contemporary application of Anar-5 herbs acts as a gastric mucosal protective agent. In addition it induces an overexpression of COX-2 and maintenance of the PGE2 level.

Keywords: Anar-5, Wistar rat model, ammonia water, experimental chronic atrophic gastritis.

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Introduction

Chronic atrophic gastritis is an age related, slowly progressive disease. It is induced by a broad variety of agents and displays with a nonspecific inflammation of the gastric mucosa [1]. It is characterized by diminished epithelial regeneration, atrophy of lymphoid tissue, and impairment of gastric motility functions and mucous secretion [2]. Evidence of the illness is microscopically noted by non specific inflammatory infiltrates, flattened epithelial surface, and reduced length of mucosal glands [1]. Gastroscopy reveals redness of the mucosa.
The disturbed balance between protective and damaging factors in chronic atrophic gastritis might proceed to stomach ulcer by the loss of acid base balance and mucosal bicarbonate deficiency in the stomach. Herein, the mucus of gastric mucosal layer constitutes a major role in pathogenesis of inflammation and ulcers because it defends the gastric epithelium and mucous membranes from hydrochloric acid. The glycoproteins of the mucin are not able by themselves to protect the mucous epithelium. They become protective only in an alkaline environment after transformation into a gel.
Digestive tract diseases are the second leading cause of death within the top five morbidity causes in Mongolia [2], and the incidence of chronic atrophic gastritis accounts to 12% of all digestive tract diseases [2].
These data indicate the increased demand to study the effects of and to develop low cost pharmacologic treatment with minimum side effects. Within such pharmaceutics herbal drugs might be able to lower the still high morbidity of digestive tract diseases in Mongolia. The search for appropriate agents became the main reason to conduct this study.
The Anar-5 herbal medicine has been used for early symptoms of any stomach pain, vomiting, disorders of digestion, stomach irritation and loss of appetite in Mongolian traditional medicine. We have tried to identify protective effects of Anar-5 herbal medicine for stomach in order to provide scientifically based description for potentially induced effects/mechanisms of this drug. Herein, we report the hypothesis, implementation, results and perspectives of a rat model to analyzing the effects of Anar-5 herbs in artificial chronic atrophic gastritis.

Material and Methods

The applied Anar-5 herbal drug was prepared by the traditional pharmaceutical factory of the Institute of Traditional Medicine and Technology of Mongolia. The herb formula Anar-5 corresponds to the composition of Deva-5 which is used in traditional medicine to treat acute infectious diseases [3-6]. Anar-5 is composed of five herbs: *Gentiana decumbens* L., *Momordica cochinchinensis* L., *Hypecoum erectum* L., *Polygonum bistorta* L., and *Terminalia chebula Retz*. The water extractions have been reported to promote anti-viral and anti-oxidative effects [3-6].

A total of 35 male Wistar rats weighing 250-280g was included in this study and hosted in the Experimental Animal Center of Institute of Traditional Medicine and Technology of Mongolia, Ulan Bator). They were fed ad libitum at controlled temperature (20±20 C°) humidity (55-65%), and 12-hour light/dark cycle.

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This study was conducted according to Guideline for Medical Ethics, (2007). It follows the designated referral to basic principles of ethics that are listed in the Guideline for Ethics of Biomedicine and the Guideline for Biomedical Research. It has been approved by International Committee for Medical Organizations of Biomedicine.

Our chronic atrophic gastritis rat model was established according to the method described by (Takao Noguchi et al. 2007) [7]. A total 35 rats was selected for the experiment. The rats were divided into 3 groups.
1 - *Control cohort*: 5 normal rats allowed to have free access a tap of distilled water and eat ad libitum (n=5).
2 - *Untreated cohort* (0.1% ammonia): 15 rats were allowed to have free access to a tap of 0.1% ammonia water and eat ad libitum (n=15).
3 - *Anar-5 cohort:* 15 rats were allowed to have free access to a tap of 0.1% ammonia water and eat ad libitum. In addition, they were administered to Anar-5 100 mg/kg/day.
The whole experiment lasted for 6 weeks. The histopathological examinations of the rat stomachs were conducted at the end of the study.

Histological Evaluation

Tissue and specimen preparation:
Animals were anesthetized with ketamine hydrochloride (90 mg/kg) through an injection to their abdomen and the gastric specimens were taken from the greater curvature of the stomach. The excised specimens were fixed with 10% neutral buffered formalin for 24 hours, followed by dehydration and paraffin embedding.

Microscopic evaluation:

Tissue cuts of 0.2 µm thickness were prepared, deparaffinized, rehydrated, and stained with hematoxylin-eosin (HE), Alcian Blue, and the *Periodic acid-Schiff* reaction (PAS) for histological examination. The tissue cuts were microscopically analyzed by objectives (*10, *20, *40 (Olympus BX 51). The thickness of the mucosal glandular layer (µm) was measured and the atrophy index calculated in percent (%).

Defining PGE2 in gastric tissue:

The removed stomachs were cleaned with PBS solution. 100 mg of the gastric mucosa were put into 1ml (1xPBS-1µm EDTA+triton x100) solution and homogenized by an ultrasonic homogenizer (JY 88-IIN).

The homogenates were centrifuged (14000 spin/minute for 10 minutes) and the supernatants were collected. The ELISA ChroMate 4300 Immunoassay Kit (rat PGE2 ELISA kit: Shanghai MLBIOBiotechnology Co.Ltd) served for the measurement of the PGE2 supernatant levels.

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Immunohistochemistry Protocol of Ki67 and COX2:

The ABC (Avidin Biotin Complex, Diaminobencidine) visualization protocol (OptiView DAB IHC Detection kit Ventana Benchmark, Germany) was applied to visualize the nuclear protein (Ki67-Mib1) in the epithelial cells of gastric glands and the inflammatory mediator (COX2) (rabbit monoclonal antibody against COX-2, dilution 1:200; Ventana Benchmark, Germany).

The immunoreactivity was graded by an intensity score (0–4: 0, negative staining; 1, weakly staining; 2, moderate staining; 3, intensive staining; 4 out of balance staining).

The interactive 'ImageJ v 2.0.0' software served for evaluating and grading the staining intensity.

Statistic analysis:

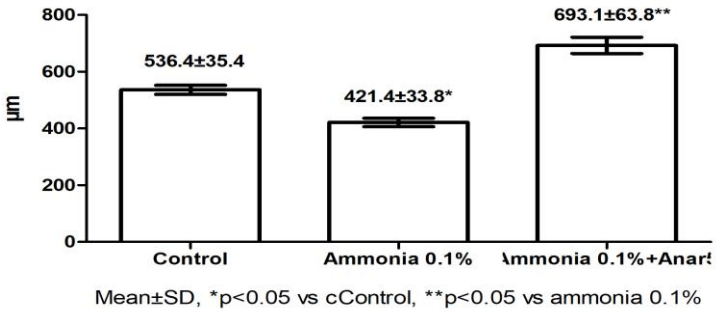
The SPSS 20.0 package was used to statistical analysis. Derived figures were constructed the GraphPad Prism v. 5.0. package.

Results

Thickness of gastric mucosa

The thickness of the gastric mucosa was significantly decreased (429.6±43.5 µm) in the untreated cohort when compared with the control cohort (536.4±35.4 µm). This result is in agreement with the induction of an artificial chronic atrophic gastritis via the application of 0.1 % ammonia.

Figure 1. Thickness of rat gastric mucosa



The thickness of untreated gastric mucosa (ammonia 0.1%) is significantly decreased and that of the Anar-5 (ammonia + Anar-5) significantly increased in comparison to mucosa of the control cohort ($p<0.05$).

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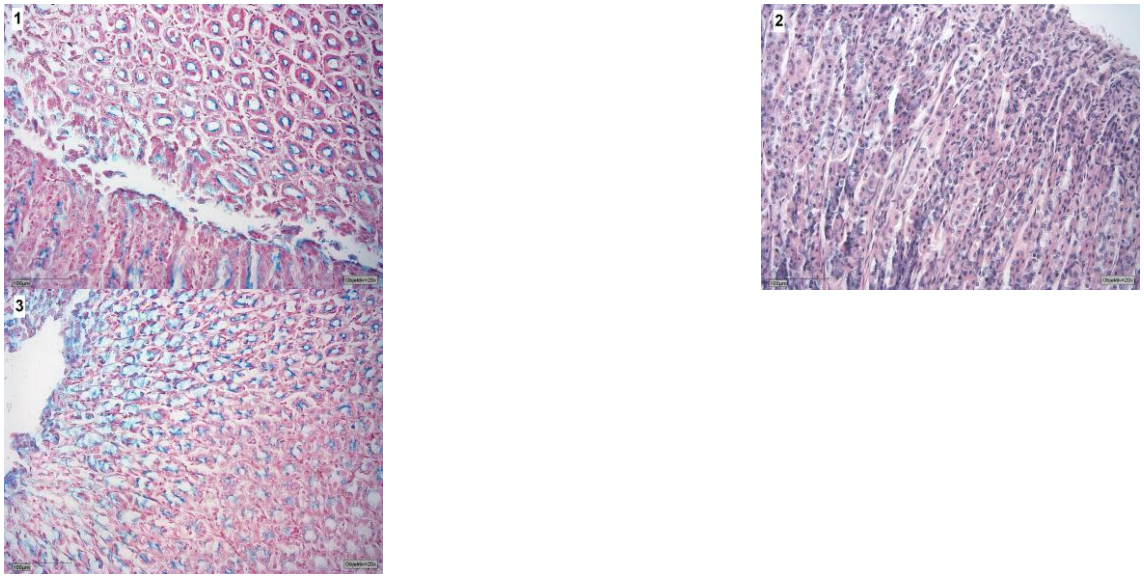
The number of epithelial cells in the superficial layer of gastric mucosa was decreased and that of the degenerated cells increased after six weeks of ammonia 01% application. The Anar-5 cohort displays with the contrary findings (Figure 2). The application of ammonia 0.1 % induces degenerative and dystrophic changes of epithelial cells in the gastric mucosa, which are marked by the blue color of the Alcian – Blue/PAS stain (Figure 2).

Figure 2. Epithelial cells of the gastric mucosa



1-control cohort, 2- untreated cohort, 3- Anar-5 cohort. The presence of mucus and goblet cells is indicated by the blue color (x20, Alcian Blue stain).

Some preservation of goblet cells and mucus remains at the bottom of the mucosa in the untreated and Anar-5 cohort.

Changes of the PGE2/COX-2 system

The potential protective effect of Anar-5 was studied and the involvement of the PGE2/COX-2 system was investigated. COX-2 is usually expressed at very low levels in the normal gastric mucosa (Figure 3). The COX-2 expression was decreased in the gastric mucosa of the untreated cohort. It was increased and diffusely expressed in the Anar-5 cohort (Figure 3).

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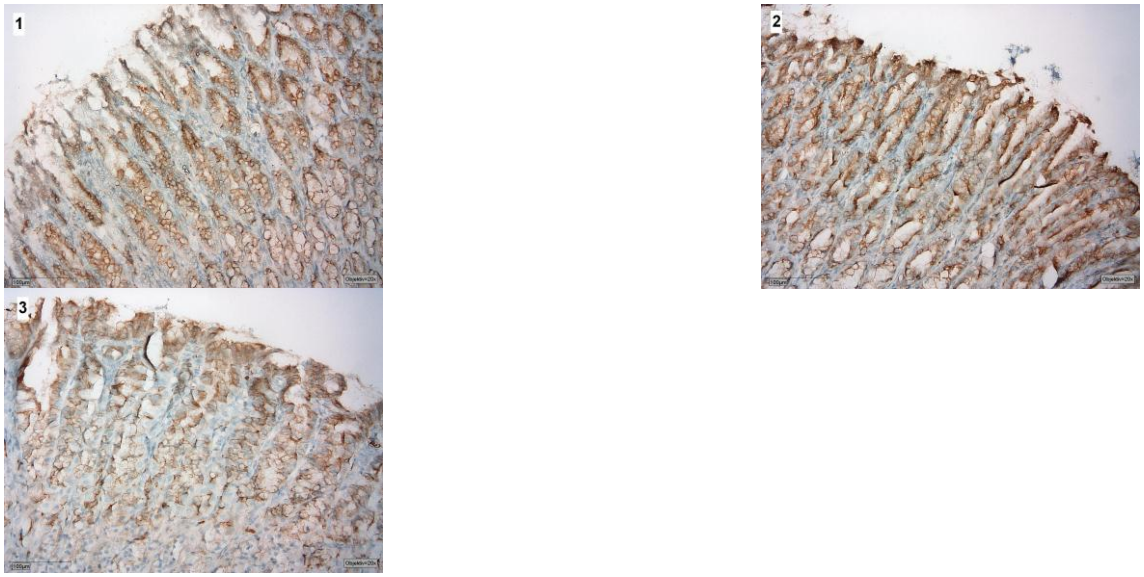
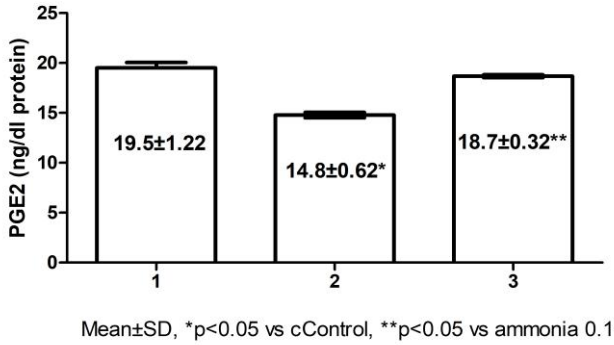
Figure 3. Expression of COX-2.



1- Normal expression of COX-2 (control cohort); 2- increased expression of COX-2 (Anar-5 cohort); 3- decreased expression of COX-2 (untreated cohort) (x20, ABC –DAB visualization)

The PGE2 levels of the gastric mucosa were measured by ELISA essays, and compared between the 3 study cohorts. PGE2 was decreased (14.8±0.62 ng/dl) in the untreated cohort when compared to the control and Anar-5 cohort. No significant differences were observed between the control and Anar-5 cohort (Figure 4).

Figure 4. ELISA essays of PGE2 levels



Mean±SD, *p<0.05 vs cControl, **p<0.05 vs ammonia 0.1%

1- normal level (Control cohort); 2- decreased level (untreated cohort (p<0.05); 3- close to normal level (Anar-5 cohort).

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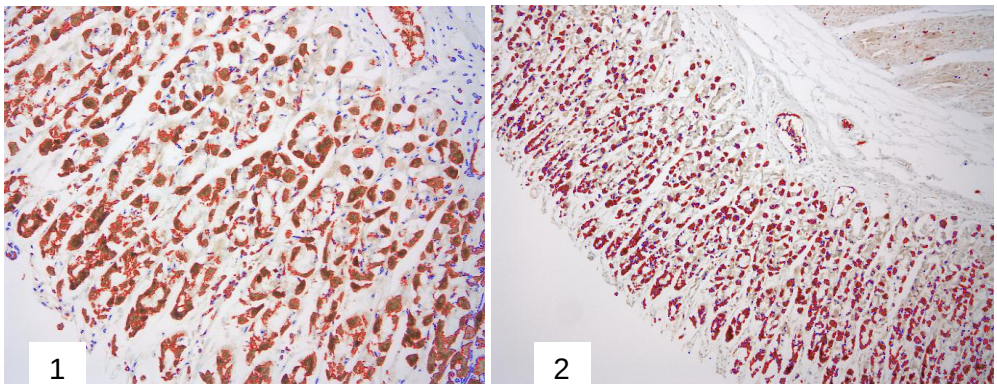
Epithelial cell regeneration

The epithelial regeneration was investigated by measuring the proliferation associated Ki67 rate.

Figure5. The amount of Ki57 marker shown in ImageJ program



1-Untreated cohort; Clear visualization of cell proliferation in the gastric mucosa in ammonia group. Also there was loss of nucleus: cytoplasm ratio, presence of overcrowded nuclei and mitotic changes. 2-Anar-5 cohort; 2- Clear visualization of cell proliferation in the gastric mucosa in Anar-5 experimental group. (magnification x40; visualized by Image J v. 2.0.0)

Discussion

Chronic atrophic gastritis and gastric inflammation are considered to predispose of cancer and malignant precursors [1]. Therefore, appropriate preventive interventions will reduce the risk of cancer and, in addition, assist to detect early cancer stages and appropriate treatment [1].

International researchers have implemented experimental animal models and created pathogenic techniques to induce gastritis – like lesions and to test potential curative medical and pharmacologic treatments [7].
Drinking ammonia water increases level of gastric saline and creates an alkaline environment in the stomach. It neutralizes the hydrochloric acid and induces atrophic cellular changes in mucosa, which will be followed by a severe damage of the gastric mucosa (ulcer) and occurrence of abnormal cells [7].

We have administered 0.1% concentration of ammonia water in rats and established a model of chronic gastric inflammation in order to investigate the effects of Anar-5 herbs that are used by Mongolian traditional medicine in treating gastric diseases. The basic observations of our study are supported by the microscopic tissue examinations and measurements of the thickness and

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atrophy, the PGE2 levels, cellular proliferation in three different study cohorts. These include 1-control cohort (5 rats); 2- untreated ammonia cohort (15 rats), and 3-Antar-5 cohort (15 rats).

In our study, different laboratory techniques such as semi-quantitative image measurements, immunohistochemistry, and ELISA essays have been applied to measure the extent of atrophy, inflammatory and proliferative changes in the gastric mucosa.

In the literature, the urease-ammonia (NH_4OH) system has been proposed to play a major role in the pathogenesis of the helicobacter pylori-associated gastritis [8]. The authors report, that an individual application of ammonia is accompanied by prostaglandin inhibition and the decrease in gastric blood flow (GBF) of approximately 30% of the normal value [9].

In our study, a remarkable reduction of epithelial gastric cells and their secretion was observed in the untreated cohort after 6 weeks ($p<0.01\%$). These findings are in agreement with the results of a Japanese study [7]. The authors report that an administration of 0.1% Ammonia water induces a chronic gastric inflammation after 6 weeks. The artificial inflammation is characterized by the atrophy of the gastric mucosa and the degeneration of mucosal cells [7].

An intensive increase of inflammatory cells was noted within all layers of the mucosa as well as a significant decrease of mucosal thickness in the untreated cohort (see figure 3). Similar results have been reported by Chinese scientists who established chronic gastritis model with ethanol [10, 11, 12].

A potential association of the epidermal growth factor (EGF) and PGE2 with the development of artificial chronic atrophic gastritis was also investigated. In our study, the EGF plasma concentration of the untreated cohort was significantly elevated and that of PGE2 (2.24±0.83 µg/L, 73.7±6.7 µg/L) diminished when compared to those of the Aman-5 cohort (0.61±0.28 µg/L and 125.1±41.3 µg/L respectively) [12].

Similar data report Wei Zhao, Feng Zhu et al (2009) of their pathogenic model of chronic atrophic gastritis induced by ethanol [13]. They used gastric malondoaldehyde, myeloperoxidase, PGE2, and COX2 markers in trying to analyze the protective effect of DIDS (4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid). The gastric mucosa of untreated rats stained poorly for COX2 markers and displayed with an increased COX2 presence in ethanol group. COX2 reaction was also enhanced in the cohort of administered DIDS solution. The PGE2 serum concentration was positively related to the presence of COX2 [13]. Our results weak staining of COX-2, reduced PGE2 levels, and increased cell proliferation in the gastric mucosa of the untreated cohort are in complete agreement with the data reported from the alcohol model [13] confirm the validity of our pathogenic model.

Furthermore, a remarkable degeneration of gastric mucosa epithelial cells was noted in the untreated cohort of our model. These findings indicate that atrophic changes of the gastric mucosa might be induced by forced degeneration of epithelial gastric cells.

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Our data of the Anar-5 cohort indicate that Anar-5 acts as a gastric mucosal protective agent that promotes gastric mucosal regeneration and increases the gastric mucus secretion. The increased PGE2 levels and the moderately presence of COX-2 in the gastric mucosa of the Anar-5 cohort support this hypothesis.

Conclusion

Administration of Anar-5 herbal medicine in artificial chronic atrophic gastritis of rat indicates that its protective effect of stomach comes into action through a moderate proliferation rate and increased levels of PGE2 and COX2 in gastric mucosal cells.

Conflict of Interest: Authors declare no conflict of interest.

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