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ALA/PpIX Photodiagnosis of Stress-Induced Gastrointestinal Metastatic Tumours and their Biochemical Indicators in Rats

Ekaterina Borisova[1,2*], Matvey Kanevskiy[2], Svetlana Konnova[2], Anna Galitskaya[2], Alexander Khorovodov[2], Ilana Agranovich[2], Tsanislava Genova[1], Ivan Angelov[3], Vanya Mantareva[3], Nikita Navolokin[4], Oxana Semyachkina-Glushkovskaya[2]

[1]Institute of Electronics, Bulgarian Academy of Sciences, Sofia, Bulgaria

[2]Saratov State University, Astrakhanskaya, Saratov, Russia

[3]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

[4]Saratov State Medical University, Saratov, Russia

[*]**Corresponding author:** Ekaterina Borisova, Institute of Electronics, Bulgarian Academy of Sciences 72, Tsarigradsko Chaussee Blvd, Sofia 1784, Bulgaria. Tel: +359879497118; Fax: +35929753201; Email: borisova@ie.bas.bg

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Abstract

Combined spectroscopic and biochemical measurements were used to improve diagnostic accuracy and to evaluate Gastrointestinal Tract (GIT) neoplasia development parameters noninvasively. Experiments were performed in mongrel male rats divided into 2 groups-control and experimental. To induce gastric cancer, the rats underwent to chronic stress (overpopulation during 9 months) and diet including the daily using of m-toluidine (25 μg/kg weight) in food and water with a solution of sodium nitrite (0.2%) for 9 months. We studied effectiveness of 5-ALA/PpIX fluorescent analysis of gastric carcinoma and biochemical stress-corresponding indices detection for early diagnosis of primary gastric tumours and their metastatic spreading in liver.

Affected by precancerous and cancerous alterations mucosa reveal red fluorescence, related to the accumulation of 5-ALA/PpIX. Liver tissues investigated also presented increase of the red fluorescence, which was used as an indicator for possible pathologic process detection there. The histological examination revealed liver metastases in 67.8 % of the rats with gastric cancer. Biochemical indicators detected malignant alterations presence in GIT, and fluorescent observation addressed the exact area and borders of neoplastic lesions. 5-ALA/PpIX fluorescence detection allow to find and precisely map premalignant and malignant areas of gastric mucosa and liver metastases of stress-induced gastric heterogeneous adenocarcinoma and biochemical evaluation of stress-related compounds increased the efficiency of such diagnosis and reveal information about the dynamics of lesions development. Diagnostic accuracy achieved using fluorescent detection reaches 93% for gastric carcinoma, and 87% for pre-cancerous mucosa alterations observed.

Keywords: 5-ALA/PpIX fluorescence; Gastric cancer; Liver metastases, Optical biopsy

Introduction

Gastrointestinal Tumors (GIT) are on a third place from all new cancer cases worldwide, including cancer of the esophagus, stomach, colon, and rectum. Studies of EUROCARE-4 (http://www.eurocare.it/) have shown that the 5-year survival of GIT cancer is about 24.1% of all cases in Europe. These types of neoplasia are characterized by high risk and early appearance of metastases, especially the Gastric Carcinoma (GC) [1]. Optical fluorescent techniques are the most useful for observation of gastrointestinal lesions due to their easy combination with endoscopic equipment and high sensitivity to early malignant alterations. Detection of the tissues fluorescence signal allows obtaining morphological and biochemical information, reveal differences in normal and abnormal tissue areas before visible with a naked eye changes. Fluorescent technique is characterized by high sensitivity, real-

time work capabilities, non-invasiveness, and can be used to monitor the development of the disease and therapeutic procedures effectiveness [2-4]. Endogenous or also called auto fluorescence spectroscopy is currently used to identify potential pathologies and as additive tool for sampling biopsy and several endoscopic systems have been developed for the differentiation of GIT tumor tissues [2-5] Their clinical application yields positive results in diagnosing lesions of the initial developmental stages [6]. However, the main disadvantages of auto fluorescence application in clinics are the relatively low resolution images and its moderate specificity [4-8]. Improvement of this diagnostic method requires highly sensitive optical equipment and more detailed study of which set the parameters and algorithms developed could improve the selectivity and provide better diagnosis [7-9].

The introduction of exogenous fluorescent markers to assess the presence and location of tumor formation could strongly assist physicians, since they could obtain a fluorescent image with a much better quality and contrast. Exogenous fluorophores optical properties preliminary knowledge is a significant advantage, and the analysis of their

applicability is mainly associated with the tumor tissues selective localization, administration mode and evaluation of side effects for the patients. For diagnostic purposes, delta-aminolevulinic acid (5-ALA), precursor of protoporphyrin IX (PpIX) is currently one of the compounds of greatest interest. In neoplastic cells, the activity of ferrochelatase, an enzyme that attaches Fe^{2+} to PpIX (as a compound in the chain of hem synthesis), is greatly reduced, which leads to selective accumulation of PpIX in malignant cells, with specific emission maxima at 635 and 704 nm [10-13]. High contrast is achieved due to significant spectral difference between red emitted exogenous fluorescence and blue-green auto fluorescence, observed from the native tissue fluorophores. 5-ALA/PpIX is proven to be a useful indicator of metastatic lesions of GIT as well [14]. However, 5-ALA/PpIX is accumulated as well in dysplastic and inflammatory areas of GIT mucosa, which raise the number of false-positive results and additional noninvasive techniques for discrimination of the gastrointestinal tissues condition, would be beneficial.

Biochemical analysis, used for evaluation of the alterations related to tumour development and growth is very appropriate low-invasive and relatively fast technique. Sialic acids and mucins content in the stomach mucosa and internal content would be used as biochemical indicators of pathology development, related to *Helicobacter pylori* infection, typical for neoplastic gastric lesions [15-17].

The carcinogenic properties of nitrosamines, which are formed intragastrically during nutrition with high levels of nitrites and amines in the food, also support the precancerous and cancerous lesions development [18,19]. Therefore, the Lipid Peroxidation (LPO intensity) assessment by determining the Tiobarbituric Acid (TBA) active products and quantity of Nitrogen Oxide (NO)-products are other biochemical indicators used as stress-related stomach mucosa cancerous changes signatures.

In our work, we studied the effectiveness of the combined approach of photo diagnostic analysis of GC with 5-ALA/PpIX and biochemical indicators detection (content of mucins, sialic acids, TBA-active and NO-products) for diagnosis of GC and metastatic spreading in rats with a model of stress-induced adenocarcinoma.

Materials and Methods

Experiments were performed in mongrel male rats (250-280 g) in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), protocols were approved by the Institutional Review Boards of the SSU (Protocol № 7, 07.02.2017). The rats were housed at 25±2C, 55% humidity, and 12:12 h light - dark cycle. The animals were divided into 2 groups-control (n=10) and experimental one (n=40). The control group was kept in standard conditions and diet. To induce gastric cancer in rats, we used our original model [20]. The rats underwent to chronic stress (overpopulation during 9 months) and diet including the daily using of m-toluidine (25 μg/kg weight) in food and water with a solution of sodium nitrite (0.2%) for 9 months.

The tissue areas of the normal and abnormal stomach mucosa were evaluated using steady-state exogenous fluorescent technique. 5-ALA in a dose of 20 mg/kg (ALASENS, Niopik Inc., Russia) was applied 2 hours before the spectroscopic observation. The stomach and liver organs were investigated in vivo and ex vivo, after decapitation of the animals, using excitation at 405 nm (AFS-405 LED light source, FWHM = 20 nm, P=25 mW, Polironik Ltd., Russia) using micro spectrometer USB4000 (Ocean Optics Inc., Dunedin, USA) for 1-D measurements. From each animal were detected from 7 to 10 spectra of normal and abnormal tissue areas for each organ and averaged. For 2-D format fluorescence imaging was used a digital microscope system DinoLite (model AM 4013 T-FWV, IDCP B.V., The Netherlands) with excitation at 405 nm (built-in LED sources). The suspicious areas (with exogenous fluorescence observed) were placed in the groups of "cancerous mucosa" and "metastasis" respectively, for the stomach and liver. The absence of exogenous fluorescent signal from 5-ALA/PpIX was an indicator for healthy tissue areas in both organs. Histological assay was performed to the samples, which were fixed in 10% buffered neutral formalin. The specimens were embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin. The histological sections were evaluated using digital image analysis system Mikrovizor medical μVizo-103 (LOMO, Russia). The results of histological examinations were used as a gold standard diagnosis in comparison with the spectroscopic and biochemical data obtained, interval: Figure 1.

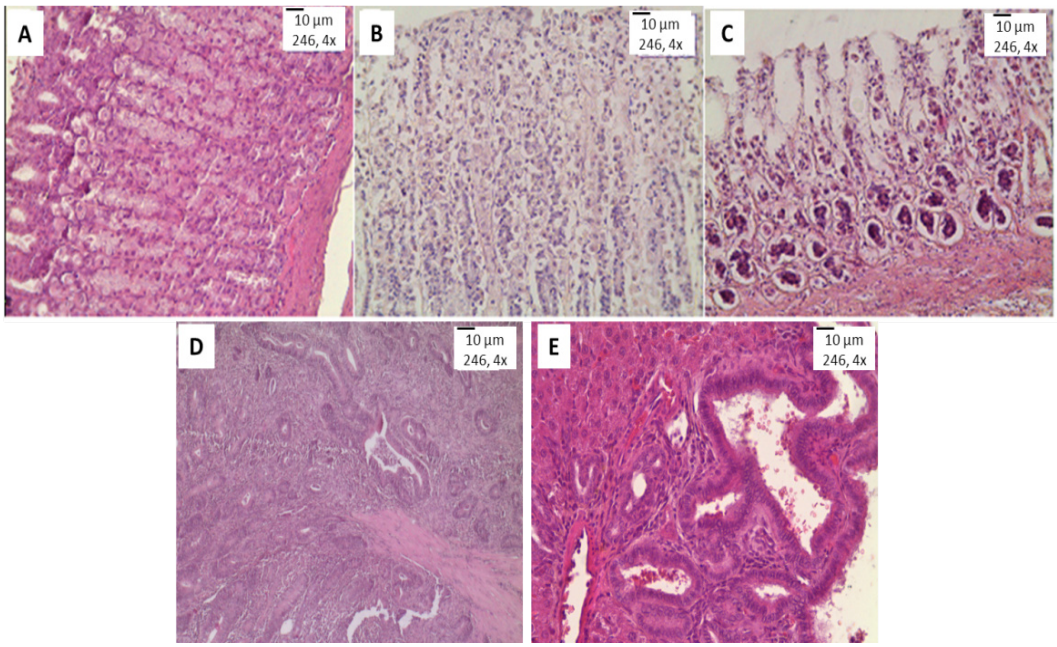

Figure1: Histological analysis of GIT of rats. (A) normal gastric mucosa; (B) atrophic gastritis; (C) mucosal dysplasia of the stomach; (D) stomach adenocarcinoma; (E) metastases in the liver. Samples were stained with hematoxylin and eosin.

Biochemical examinations were made on addressed by fluorescence normal and cancerous tissues' groups. Extraction of glycoproteins from the stomach walls was performed using an 8M solution of urea at room temperature for three days, then centrifuged for 20 minutes at 14,500 rpm, according [21]. When determining the sialic acids and mucins concentration in the content of the stomach, the samples were preliminarily subjected to centrifugation for 10 minutes at 10,000 rpm, and the resulting supernatant was used. The concentration of sialic acids was determined using the SialoTest kit (SPC Eco-Service, Russia) after partial acid hydrolysis [22].

The concentration of mucins was evaluated spectrophotometrically using a color reaction with bromophenol blue. The mucins concentration value was determined as the optical density difference of the experimental sample and the same one after proteins precipitation reaction (in 20% acetic acid) [23]. Assessment of the intensity of lipid peroxidation was carried out by determining the amount of TBA-active products in the serum by a standard procedure. Nitrogen oxide (NO) was evaluated according Griess reagent protocol [24]. The obtained biochemical results are processed by statistical methods with the use of Student's t-test. Differences were considered reliable with a probability of difference exceeding 95% [25].

Results and Discussion

The adaptation and protection mechanisms could not manage with the existing stress levels in prolonged or excessively intense stress. Such strong stress factors, especially applied in combination can be a "switch-on" moment of pathological processes and could cause a variety of diseases, including malignancies. In the stressed animals investigated 44% had atrophy of the stomach walls, 25% had liver anemia, and 19% had an enlarged caecum. In 48%, the PCR method revealed the presence of *helicobacter* in the stomach content. The 9-month period of treatment with the addition of nitrite sodium and aromatic amines induce neoplastic pathologies formation in 70% (28 from 40) of the animals, proven by the histological examinations.

Oxidative and nitrosamine stresses play a key role in the cancerous pathogenesis [26,27] The long-term nitrite +m-toluidine intoxication of laboratorial animals leads to a significant accumulation of lipid peroxidation products in serum. The content of TBA-active products increases more than 3 times in the experimental group (Figure 2A).

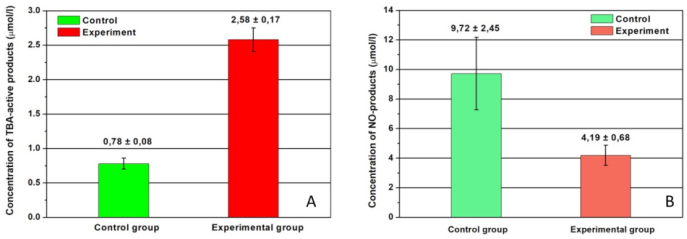

Figure 2: Comparison of control and experimental group of animals' biochemical parameters, related to nutrition stress factors - influence of the combined action of sodium nitrite and toluidine: (A) TBA - active products in the blood serum; (B) NO-products content in the serum.

The processes of reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation are interconnected. It has been shown that ROS are able to activate NO synthases [28]. NO, synthesized by this enzyme endothelial and neuronal isoforms, as a signaling molecule is involved in a broad range of physiological reactions. However, the data available in the literature related to changes in the amount of products of nitrosative stress are very contradictory. A number of sources noted a significant increase in the amount of nitrogen oxide products [27]. Other researchers, in contrary, reported decrease of such products [29]. In our experiments in the control group of animals' serum, the NO concentration was 9.72 ± 2.45 μmol/l. In experimental group, after the combined action of nitrites and amines, the concentration of nitrogen oxide (II) products decreased significantly, by almost 2 times, and was found to be 4.19 ± 0.68 μmol/l (Figure 2B).

The nitrogen oxide products concentration decrease in the serum of laboratory animals subjected to prolonged intoxication with amine and nitrites could be explained by the high reactivity of these compounds. Due to the peroxidation products accumulation, primarily ROS, such as superoxide anion radical, NO is rapidly transformed into peroxynitrite, interacting with proteins, nucleic acids, lipids, etc. In addition, the oxidative stress products are capable of switching endothelial NO synthase to the synthesis of superoxide anion radical as a main product [30].

The accumulation of peroxidation products, as a result of chronic intoxication, can lead to disturbances in the structure of the gastric mucosa that promotes the development of *Helicobacter pylori* infection. In the control group the concentration of mucins in the stomach content was 0.013 ± 0.004 g/l, but in the experimental group their concentration increased by 54% up to 0.02 ± 0.002 g/l. On the stomach walls the mucins' concentration raised from 0.041±0.006 g/l in control group to 0.068± 0.009 g/l, presented an increase of about 66% (Figure 3A). Such differences of the mucins concentration in the stomach walls of control and experimental groups could be related to vigorous mucin expression into gastric mucosa as a response to food toluidine and nitrite, as a protective reaction to their destroying actions [31-33].

In general, the mucins should not be detected in the stomach content in normal conditions and their presence and especially their increase in the treated animals is an indicator for degradation and destruction of the mucous membrane. Sialic acids concentration in the stomach walls and content were evaluated as well. The control group is characterized with a stomach content sialic acids concentration of 0.751±0.021 mmol/l, and experimental group reveal an increase of about 15%, and the concentration reached values of 0.864±0.036 mmol /l (Figure 3B).

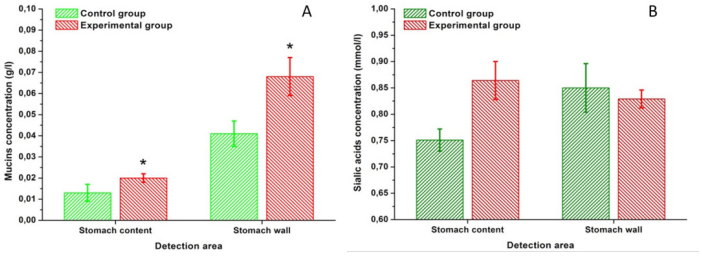

Figure 3: Comparison of control and experimental group of animals' biochemical parameters related to nutrition stress factors - influence of the combined action of sodium nitrite and toluidine in stomach content and stomach walls, respectively: (A) Mucins concentration; (B) Sialic acids concentration.

Concentration of sialic acids in the control group stomach walls was 0.850±0.046 mmol/l, and in experimental group of animals this value decreased with 2,5% and reached 0.829±0.017 mmol/l, (Figure 3B). Such sialic acids concentration dynamics in the stomach walls and content is a result of mucins destruction and their desialylation in the zones of inflammatory and neoplastic changes, which result sialic acids exit into the stomach lumen. The biochemical indicators and their dynamics in the stomach wall and stomach content could be predictors of significant pathological alterations, including development of neoplastic lesions and could be used as indicatives for planning of fluorescent endoscopic observations and damaged mucosal areas mapping. Biochemical data could be indicators of presence of neoplasia, but could not address its place, area and boundaries of the affected gastric tissues. Only the observation of the stomach lesions using exogenous fluorescent technique allowed evaluating precisely the exact address of the pathology and its borders.

The applied 5-ALA/PpIX sensitizer is accumulated in the inflammatory, dysplastic and malignant gastrointestinal tissues, due to its specific metabolism [34-36]. In spectroscopic regime (1-D) the fluorescence intensities level differences on 635 nm, where is the primary maximum of PpIX, were used as discrimination factor for normal, pre-cancer and cancerous areas of the gastric mucosa. Absence of exogenous fluorescent signal from 5-ALA/ PpIX was used as an indicator for normal stomach mucosa. All tissue

areas addressed spectrally were histologically verified. Diagnostic accuracy (DA) of 93 % was reached in evaluation of cancerous areas and DA was 87% in the case of "pre-cancer/inflammation" group, due to the presence of exogenous fluorophores in several mucosa areas valued as "normal" during histological observations. In general, the 5-ALA/PpIX photo diagnosis lead to some higher number of false-positive results than missed false-negative ones.

Normal mucosa after excitation at 405 nm, emitted bright blue-green fluorescence in the range of 420-750 nm, with maxima, correlated to co-enzymes-NADH and Flavin's presence, as well of the proteins-elastin and collagen and their cross-links [5-9]. Affected by precancerous and cancerous alterations mucosa reveal red fluorescence, related to the accumulation of 5-ALA/PpIX and the colour contrast achieved allow easily distinguishing the lesions areas by eye (Figure 4). Liver tissues investigated also presented some increase of the red fluorescence, which was used as an indicator for possible pathologic process detection in this organ. The histological examination revealed liver metastases in 67.8 % (19 of 28) of the rats with GC.

Fluorescent spectra detected in metastatic areas of liver presented broader emission at the maximum on 635 nm, associated primarily with the PpIX. This widening could be related to the presence of other porphyrins in the liver tissue, such as coproporphyrin and uroporphyrin compounds [37,38]. The additional porphyrins compounds appearance was clearly observed on the normalized with respect to maxima compared stomach and liver exogenous fluorescence spectra, see (Figure 4).

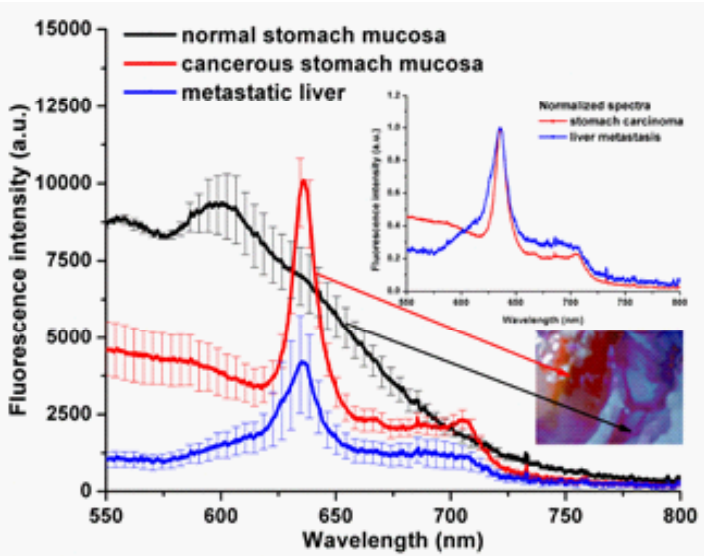

Figure 4: Comparison of averaged by tissue' type fluorescence spectra, photosynthesized with 5-ALA/PpIX. Normalized spectra with respect to maximum at 635 nm (right up) to reveal the broadening of 635 nm maximum. Photo of typical neoplasia in gastric mucosa in fluorescent mode of detection is presented (right down).

The results obtained from 1-D measurements showed a very good correlation between the fluorescence signals and the histological examination of the lesions investigated. Rapid detection of lesions' boundaries using the exogenous fluorescence signal was observed. Another important issue is the contrast between precancerous and malignant areas.

Emission intensity of 5-ALA/PpIX is significantly higher in case of malignant alteration of the tissues, in comparison with the precancerous changes observed in other part of the organ investigated and this difference could be used for selection and discrimination of malignant vs. benign and dysplastic stomach mucosa in the current animal model as well, see (Figure 5).

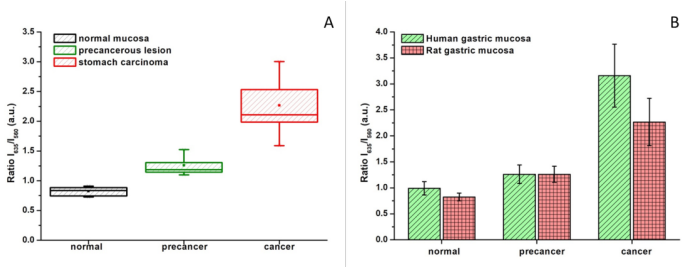

Figure 5: (A) Ratio of $R=I_{635}/I_{560}$ fluorescence intensities at 635 nm and 560 nm respectively, corresponding to the 5-ALA/PpIX exogenous signal vs. endogenous fluorescence of stomach mucosa in stress-induced neoplasia in rats; (B) Comparison of the ratio values $R=I_{635}/I_{560}$ of the fluorescence intensities at 635 nm and 560 nm respectively for human and rat stomach mucosa. Excitation at 405 nm was used.

On figure 5A is presented comparison of the values of ratio calculated of I_{635}/I_{560} fluorescence intensities at 635 nm and 560 nm respectively, corresponding to the 5-ALA/PpIX exogenous signal vs. endogenous fluorescence for normal, dysplastic and malignant stomach mucosa.

The results strongly correspond to the data obtained earlier in the case of photosensitization of human patients with GIT neoplastic alterations observed clinically in our previous investigations [35,39] figure 5B. This correlation allows us not only to compare the normal and abnormal tissue sites, but support the idea for easy transfer of the fluorescent data received on animal studies to the human medicine applications for the exogenous fluorescence detection of GIT neoplasia.

Conclusions

In conclusion, 5-ALA/PpIX fluorescence detection allow to find and precisely map premalignant and malignant areas of gastric mucosa and liver metastases of stress-induced gastric heterogeneous adenocarcinoma and biochemical evaluation of stress-related compounds increased the efficiency of such diagnosis and reveal information about the dynamics of lesions

development. Diagnostic accuracy achieved using fluorescent detection reaches 93% for gastric carcinoma, and 87% for precancerous mucosa alterations observed. Dimensionless ratio, based on exogenous and endogenous fluorescent signals on 635 nm and 560 nm respectively, obtained from the gastric mucosa emission, in the case of stress-induced model on laboratorial animals (rats) (1) allows to discriminate normal from precancerous and cancerous mucosa and (2) strongly correspond to the data obtained earlier [31] in the case of photosensitization of human patients with 5-ALA/PpIX for detection of GIT neoplastic alterations observed clinically, which allow to prove the feasibility of the used stress-induced model as appropriate one for gastric neoplasia growth investigations, different morphological, physical and chemical indicators evaluation, as well for different detection techniques diagnostic accuracy validation for GIT cancerous lesions.

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