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Correlation of precancerous lesion incidence with duodenogastric reflux and N-nitroso compound duration at reflux and antireflux stomach surgery – An experimental study

Korelacija incidencije nastanka prekanceroznih lezija sa vremenom delovanja duodenogastričnog refluksa i N-nitroznih jedinjenja kod refluksnih i antirefluksnih operacija na želucu – eksperimentalna studija

Aleksandar Zlatić*, Mirjana Marinković*, Dragan Mihailović†, Milan Radojković*†, Biljana Radovanović-Dinić†‡, Miodrag Djordjević*, Milan Korica§‖, Radovan Veljković§‖

Clinical Center Niš, *General Surgery Clinic, ‡Clinic for Gastroenterology and Hepatology, Niš, Serbia; University of Niš, †Faculty of Medicine, Niš, Serbia; Clinical Center of Vojvodina, ‖Clinic of Abdominal, Endocrine and Transplantation Surgery, Novi Sad, Serbia; University of Novi Sad, §Faculty of Medicine, Novi Sad, Serbia

Abstract

Background/Aim. Duodenogastric reflux occurs after gastroenteroanastomosis, with or without resection of the stomach (Billroth I, Billroth II), after vagotomy and pyloroplasty, gastroduodenostomy as well as in conservatively treated patients. The aim of this study was to analyse the effect of carcinogenic N-nitroso compounds (MNNG) influence in conjunction with duodenogastric reflux, in the function of time for precancerous lesions development in gastric mucosa. A particular purpose of the study has been to suggest some effective surgical procedures, which could prevent the harmful effects of duodenogastric reflux on gastric mucosa, as a potential activator of carcinogenesis. **Methods.** The research included 90 experimental, male Wistar rats, divided into 3 groups. The two experimental groups were subjected to two surgical procedures: Billroth II gastric resection (group B2), and the Roux-en-Y reconstruction (group RY), respectively. The control group (group C) animals did not receive any surgical treatment. All groups were administered *per os* chemical cancerogen MNNG. All anastomoses were performed by extra mucosal suture with monofilament polypropilene 7–0, and 8–0. The animals were sacrificed consecutively, and subjected to total gastrectomy after 8, 16 and 24 weeks. **Results.** Pathohistological analysis was performed on defined regions of the rat stomach in three time period. The B2 group, at the end of our experiment, showed predominant incidence of severe lesions: hyperplasia (0%), gastritis (0%), metaplasia (6.7%), dysplasia (46.7%), early carcinoma (20%) and carcinoma (26.7%). At the end of our experiment, the RY and C groups showed the similarities of the obtained results related to time with predominant incidence of mild lesions: hyperplasia (13.3% *vs* 0%, respectively), gastritis (13.3% *vs* 13.3%, respectively), metaplasia (6.7% *vs* 13.3%, respectively), dysplasia (66.7% *vs* 66.7%, respectively), with an extremely low incidence of early carcinoma (0% *vs* 6.7%, respectively) and no incidence of carcinoma (0% *vs* 0%, respectively). **Conclusion.** Without the presence of duodenogastric reflux, MNNG causes a low degree of precancerous gastric lesions. However, direct contacts of MNNG with gastric mucosa, including the presence of duodenogastric reflux, induce precancerous lesions and carcinoma. The percentage of reversible changes decreases with time, while the percentage of irreversible lesions and carcinoma increases. A lack of distinction in the findings between the RY and C groups confirms a gastroprotective role of the Roux-en-Y procedure.

Key words:
duodenogastric reflux; gastric mucosa; precancerous conditions; stomach neoplasms; nitroso compounds; digestive system surgical procedures; disease model, animal; rats.

Apstrakt

Uvod/Cilj. Duodenogastrični refluks (DGR) se sreće posle gastroenteroanastomoze, sa ili bez resekcije želuca (Billroth I, Billroth II), posle vagotomije i piloroplastike, gastroduodenostomije, kao i kod neoperisanih bolesnika. Cilj rada bio je da se ispitaju kancerogena svojstva N-nitrozo jedinjenja (MNNG) u sadejstvu sa duodenogastričkim refluksom, u

Correspondence to: Radovan Veljković, Clinic of Abdominal, Endocrine and Transplantation Surgery, Clinical Center of Vojvodina, Novi Sad, Serbia; E-mail: radovan.veljkovic@mf.uns.ac.rs

funkciji vremena, na razvoj prekanceroznih lezija u sluzokoži želuca. Poseban cilj bio je da se preporuči metoda hirurške prevencije štetnog delovanja DGR-a na sluznicu želuca, kao mogućeg aktivatora kancerogeneze. **Metode.** Istraživanjem je obuhvaćeno 90 eksperimentalnih pacova, muškog pola, Wistar soja, podvrgnutih dvema operativnim procedurama: Billroth II (grupa B2) i resekciji želuca sa Roux Y rekonstrukcijom (grupa RY). U kontrolnoj grupi (grupa C) životinje nisu bile operisane. Svim grupama je *per os* dat hemijski kancerogen MNNG. Sve anastomoze su bile urađene ekstramukoznim šavom, a kao šavni materijal korišćen je monofilamentni polipropilen 7–0, i 8–0). Nakon 8, 16 i 24 nedelje, životinje su žrtvovane i urađena je totalna gastrektomija. **Rezultati**. Patohistološki su analizirani definisani regioni želuca pacova u tri vremenska perioda. Na kraju eksperimenta grupa B2 je pokazala dominantnu incidencu ozbiljnih promena: hiperplazija (0%), gastritis (0%), metaplazija (6.7%), displazija (6.7%), rani rak (20%) i ispoljeni rak (kod 26.7% životinja). Na kraju eksperimenta utvrđena je sličnost dobijenih rezultata u funkciji vremena između RY i C grupe, sa preovladavajućom pojavom blažih promena: hiperplazije (13.3% i 0%, redom), gastritisa (13.3% i 13.3%, redom), metaplazije (6.7% i 13.3%, redom), displazije (66.7% i 66.7%, redom), upadljivo malom incidencijom ranog karcinoma (0% i 6.7%, redom) i odsustvom karcinoma (0% i 0%, redom). **Zaključak.** Dejstvo samo MNNG na sluzokožu želuca bez prisustva duodenogastričkog refluksa izaziva lakši stepen prekancerogenih lezija. Direktni kontakt MNNG sa gastričkom sluznicom, uz duodenogastrički refluks, indukuje nastanak prekanceroznih lezija i karcinoma. Protokom vremena procenat reverzibilnih promena se smanjuje, a procenat ireverzibilnih lezija i karcinoma povećava. Nedostatak razlike između RY i C grupe potvrđuje gastroprotektivnu ulogu Roux-Y procedure.

Ključne reči:
duodenogastrički refluks; želudac, sluzokoža; prekancerska stanja; želudac, neoplazme; nitrozo jedinjenja; hirurgija digestivnog sistema; procedure; bolest, modeli na životinjama; pacovi.

Introduction

Duodenogastric reflux (DGR), enterogastric reflux, bile reflux, alcaline reflux gastritis, or postresection gastritis, are all synonyms for the same phenomenon, which can be defined as the "return of duodenal content to the stomach through an incompetent pyloric valvula, i.e., from duodenum and intestines, through anastomosis, into the stomach" [1].

Bile presence in the stomach is not a normal finding, since pylorus prevents any significant DGR. Despite previous evidence of bile presence in some patients, these findings given sufficient significance [1]. Surgical interventions, which cause pylorus destruction, induce DGR in approximately 5% to 35% surgically treated patients. It is also possible that idiopathic DGR precedes surgical interventions; however, the percentage of such cases is very low [2].

DGR occurs after gastroenteroanastomosis (GEA), with or without resection of the stomach (Billroth I – B1, Billroth II – B2), after vagotomy and pyloroplasty, gastroduodenostomy as well as in conservatively treated patients. GEA by B 2 type resection and pylorus removal in B 1 type resection create conditions for the occurrence of continuous duodeno-biliary-pancreatic juice reflux into stomach, which triggers inflammatory-dystrophic-metaplastic gastric mucosal lesions and the consecutive damage to its physiological functions, thus creating conditions for the occurrence of other diseases [3]. In a previous paper, we published the findings of the effects of DGR on the occurrence of precancerous gastric lesions [4].

Apart from biliary salts and biliary acids, there are other significant microenvironmental factors suspected to contribute to precancerous lesion occurrence: decreased gastric acidity (hypochlorhydria and achlorhydria), presence of bacteria in gastric lumen as well as presence of nitrates and nitrites and, particularly, N-nitroso compounds [5]. The possibility of formation of N-nitroso compounds has been proven both *in vitro* and *in vivo* in the stomach of experimental animals, in noncarcinogenic secondary and tertiary amines and amides present in food as well as nitrogenic acid, i.e., nitrites and nitrites found in potable water. N-nitroso compounds can be formed from nitrites and some pharmaceuticals, such as antihistaminic drugs, tranquilisers, disulphiram, antibiotics, etc.. Some authors emphasize the possibility of formation of N-nitroso compounds in the patients subjected to long-term treatment with certain antagonists of H2 receptors (cimetidine, ranitidine), since cimetidine is a derivate of guanine which, in the presence of nitrite, can easily be transformed into mononitro-cimetidine, which is an analogue of MNNG [6, 7].

The aim of this experimental study was to determine the role of DGR, in conjuction with the duration of N-nitroso compound presence, in the development of gastric precancerous lesions, In particular, the purpose of the study was to suggest an effective surgical procedure for the prevention of harmful effects of DGR on gastric mucosa as a potential activator of carcinogenesis.

The purpose of the experiment was also to confirm that DGR, along with N-nitroso compound in correlation with time, is responsible for the incidence of precancerous gastric lesions after GEA.

Methods

This study was conducted at the Centre for Biomedical Research of the University of Niš, Serbia from April 2012 to February 2013. The experiment was done on 90 male Wistar rats, with average weight of 225 grams, obtained from the Vivarium of the Faculty of Medicine in Niš. All legal and regulatory principle referring to animal treatment were respected. The two experimental groups were subjected to two surgical procedures (GEA) – stomach resection Billroth II and by Roux-en-Y (RY) reconstruction. The control group was not subjected to any surgical intervention. Allocation to the experimental groups were random. Diagram of the study show flow of experimental animals through of the study (Figure 1).

Chemical carcinogen N-methyl-N-nitro-N-nitrosoguanidine (MNNG®), with the molecular formula $C_2H_5N_5O_3$, CAS Number 70–25–7, obtained from ABCR GmbH & Company, Karlshruhe, Germany, was administered to all experimental groups. The animals received MNNG in the concentration on 100 mg per liter of drinking water. MNNG was administered starting with the fifth postoperative day in the animals subjected to the surgeries. Non-operated animals received it since the first experimental day [8, 9].

Construction of the tested groups

Similar to the previous experiment [4], this research included 2 experimental and one control group, with 30 animals each. The first experimental group included 30 animals and was marked as B2 group, since gastric resection was done by Billroth II reconstruction, by omega winding (Figure 2a – painted, Figure 2b – in nature). In the B2 experimental group, the effects of surgically induced DGR and N-nitroso compounds (MNNG) on gastric mucosa were examined in correlation with time. The second experimental group, comprised of 30 animals, was marked as RY group, due to the performed resection of pyloric and antral gastric part by means of Roux-en-Y reconstruction (Figure 3a – painted, Figure 3b – in nature). As expected, the gastric mucosa in this group was examined for the influence of N-nitroso compounds (MNNG) without DGR. The control group included 30 animals which were not operated (marked as group C) with administered MNNG (Figure 4a – painted). In addition, each group included 3 sub-groups. 5, 10 and 15 animals were sacrificed in each group, respectively, in the week 8, 16 and 24 of experimental observation (Figure 1).

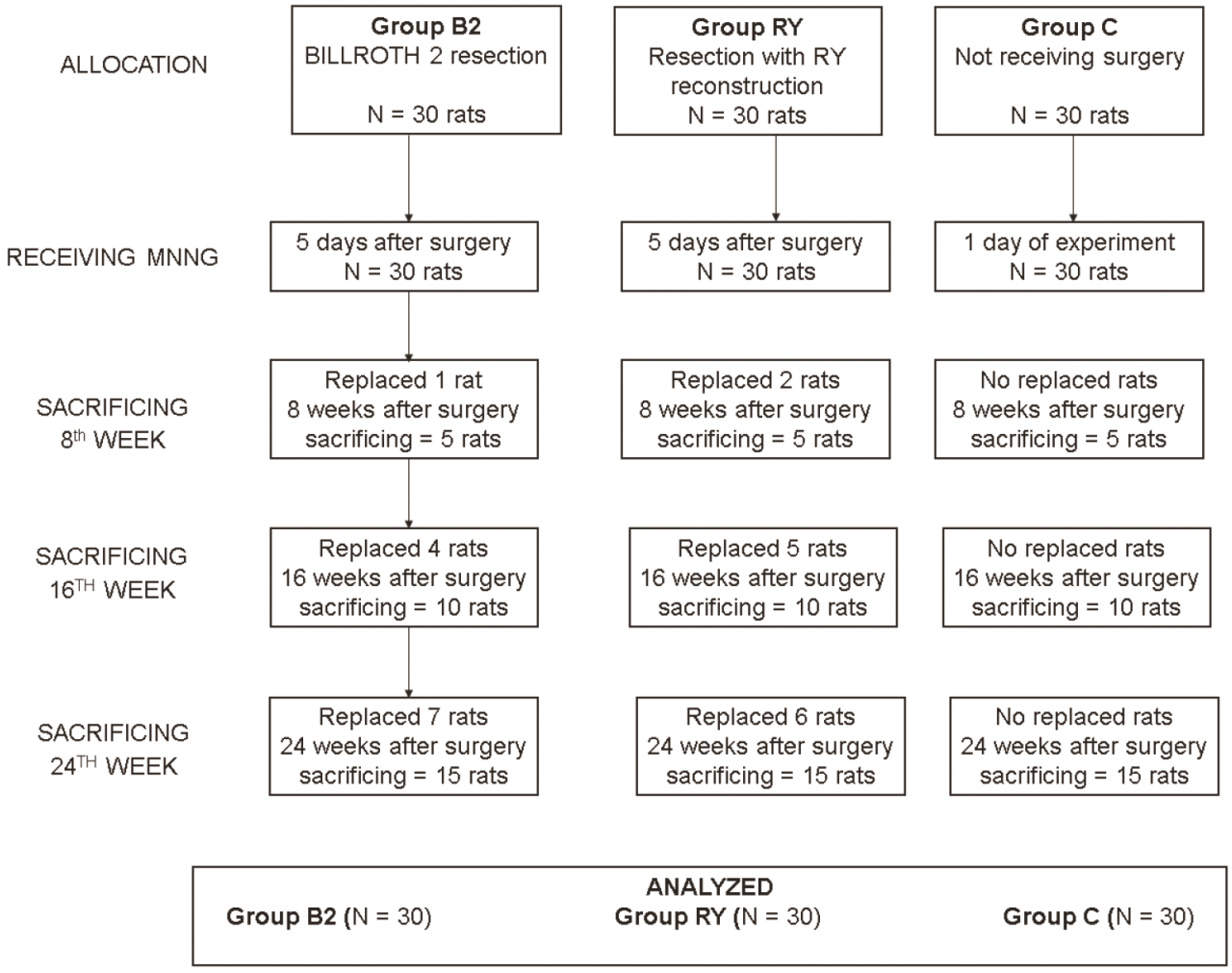
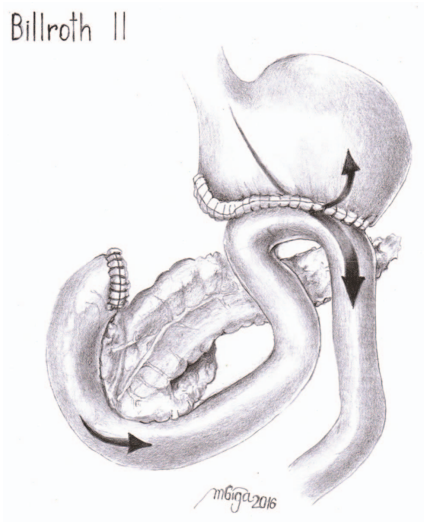


Fig. 1 – Flow of experimental animals through the study.



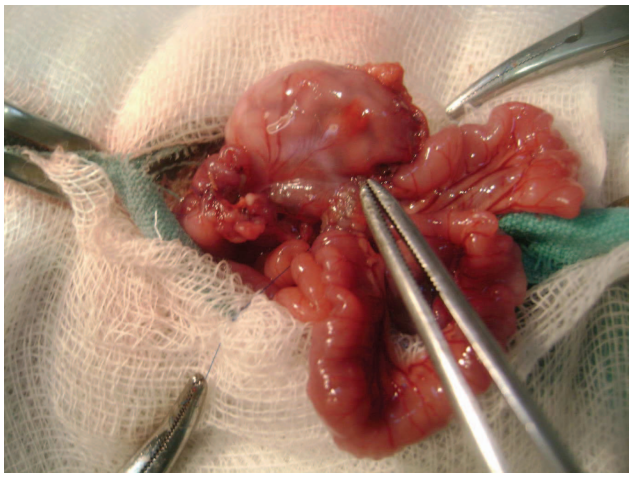
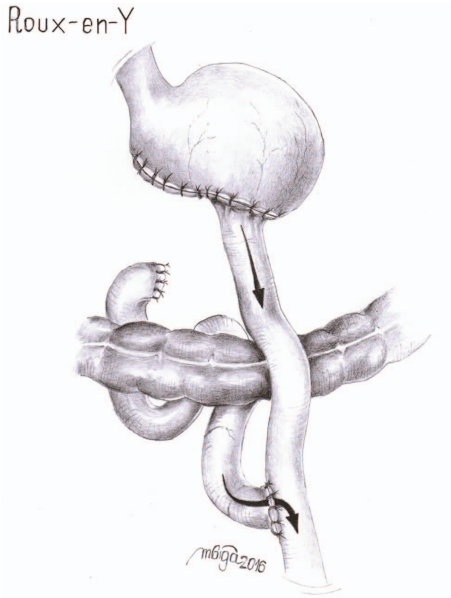
a)  b)

Fig. 2 – Gastro-jejuno anastomosis Billroth II: a) painted; b) in nature.



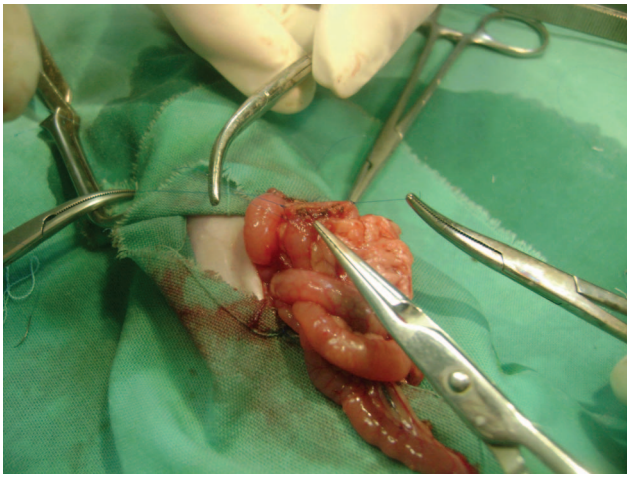
a)  b)

Fig. 3 – Gastro-jejuno anastomosis Roux-en-Y: a) painted; b) in nature.

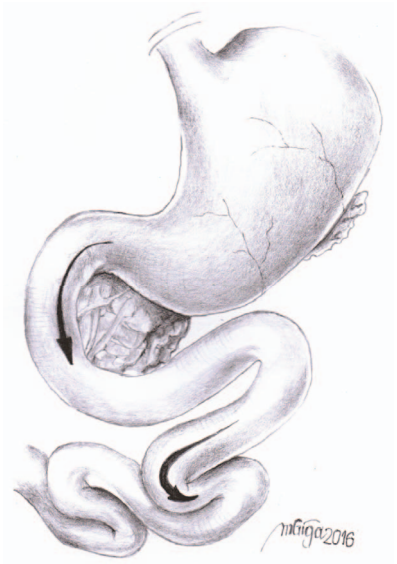


Fig. 4 – Unoperated animal (painted).

Suregery

The animals were anesthetized by ketamine hydrochloride (Ketamidor® 10%, Richter pharma AG, Wels, Austria), with the operatively administered dose of 0.1 mL per 100 g of body weight, intraperitoneally. All anastomoses were performed by extra mucous monofilament suture polypropylene 7–0 and 8–0.

Follow-up after the surgical procedures

The animals were placed individually in the prepared cages and were observed until they have recovered consciousness and ability to move. Ketorolac tromethamine was used as an analgesic in the dose of 1 mg *per* kg of body weight, intramuscularly, upon indication of pain. During the first 12 hours, the animals received solution of 5% glucosis *per os*, tap water and physiological solution in the equal ra-

tio. Food for experimental animals, mixed with physiological solution was introduced on the day 3 after the surgery. After 5 days, the surviving animals were moved to group cages, for the rest of the experimental period. The animals that showed signs of discomfort, poor nutrition, or disease were sacrificed and were replaced. The animals which died after the half time period of the study were not replaced. The final selection of experimental animals in groups was based on the common minimum number (30) of survived animals in each group.

Receiving of the carcinogens

The experimental animals received the solution of tap water and MNNG *per os*, in graded bottles of 250 mL. All bottles with H_2O and MNNG solution were lined with aluminium foil, in order to prevent the carcinogen decomposition from the influence of sunlight. All measurements of the substances used in the experiment were performed at the Biochemical Institute of the Faculty of Medicine, the University of Niš, Serbia, by making use of the analytical scale Denver Instrument Company, Type AA-200DS.

Measurement of gastric pH

In order to confirm DGR presence in the stomach of both, surgically treated and non-resected animals, gastric pH of the sacrificed animals was measured in two stomach regions (pericardial and para-anastomotic one in the resected animals and anthropyloric one in the non-resected animals). Increased pH values suggested bile presence (alkaline effect) in the stomach, indicating the presence of DGR. By means of gastrostomy of appropriate length, (Figure 5), universal pH indicator stripes with 0–14 measurement range (Merck®) were inserted. The stripes were kept in the stomach for approximately 6 minutes (as instructed by the producer), in order to determine the pH values by the etalon comparison method.

Fig. 5 – Stomach Ph measurement by universal indicator.

Sacrificing and histological processing

The animals were sacrificed by diethyl-ether overdose, in the designated intervals, followed by total gastrectomy. The stomach was, then, opened along the pylorus minor curve to the cardia and washed it with physiological solution. The stomach was fixed by 10% formaline for 48 h. The fixed stomach was cut into approximately 2 mm slices, from the previously defined regions (parastomal region and a part of the secretory fundus) as well as from the macroscopically visible pathological change (Figure 6). The histological processing of the fixed slice tissues was performed in the autotechnicon at the Institute for Experimental Medicine of the Faculty of Medicine, the University of Niš, Serbia. Paraffin, cross-sections 4.5 μm thick, were stained by the following methods: 1) classic hematoxylin and eosin (HE) method and 2) hystochemical methods: [a) alcian blue periodic acid schiff (AB-PAS), Ph 2, 5 for mucin, i.e., intestinal and pyloric metaplasia, dysplasia and carcinoma verification, b) Van Gieson – for collagen fiber, i.e., atrophic gastritis and scirrhous variant carcinoma (desmoplastic reaction) verification].

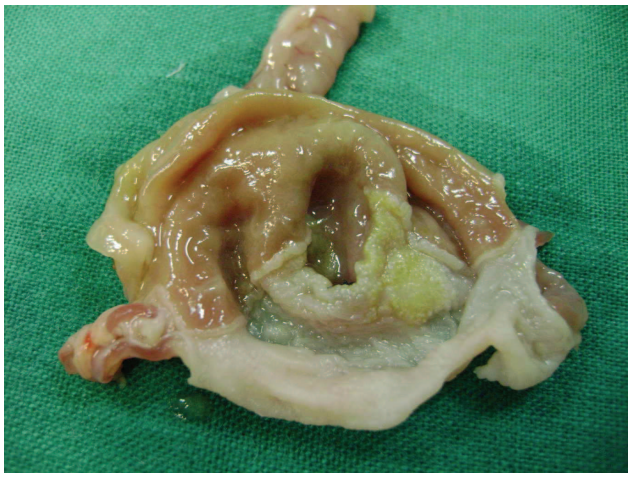


Fig. 6 – Visible changes in Bilroth II gastric resection (B2) group after sacrificing (16th week).

Histopathological classification

Due to a large number of precancerous lesions, histopathological types and sub-types [10], and with considerations of the evident discrepancies between the classification of Japanese pathologists on one side and the European and American pathologists on the other side [11], we opted as our protocol for the classification of identified alterations (lesions) systematization by Katić [12]. Our classification protocol were as follow: 1. normal gastric mucosa – includes a normal pathohistological finding of gastric mucosa, including anastomositis and inflammatory infiltrates, which are considered to be normal responses of the body to the surgical trauma (Figure 7); 2. hyperplasia (hyperplastic gastropathies) a key feature of hyperplastic gastropathy, commonly known as "hypertrophic gastritis" is the hyperplasia of foveolar and/or glandular epithelium, with an absence of inflammatory cells in the mucosa [it includes three types of gastropathy: glandular, foveolar and mixed (Figure 8)]; 3. gastritis – includes acute, chronic, specific, non-specific and cystic gastritis (Figure 9); 4. metaplasia – including intestinal, pyloric, enterocolonic, ciliated and combined metaplasia (Figure 10); 5. dysplasia – mild, medium and severe dysplasia in non-resected stomach [World Health Organization (WHO) classification] and adenomatous, microglandular, cuboid and cystic

dysplasia, characteristic for resected stomach (according to Borchard) (Figure 11); 6. early gastric carcinoma – defined as the carcinoma which is limited to mucosa or sub-mucosa, regardless of the presence of metastases in lymph nodes (Figure 12); 7. carcinoma – including adenocarcinoma, "signet ring cell" carcinoma and anaplastic carcinoma (Figure 13).

This study was conducted on animals. Ethical Committee of the Medical Faculty University of Niš approve this work on meeting in June 2010.

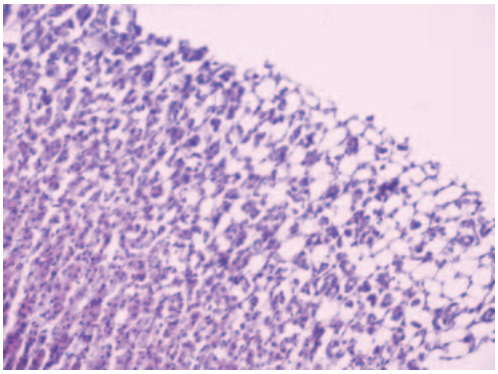


Fig. 7 – Normal gastric mucosa: corpus glands are covered by pariethal and main cells [hematoxylin and eosin (HE) ×400].

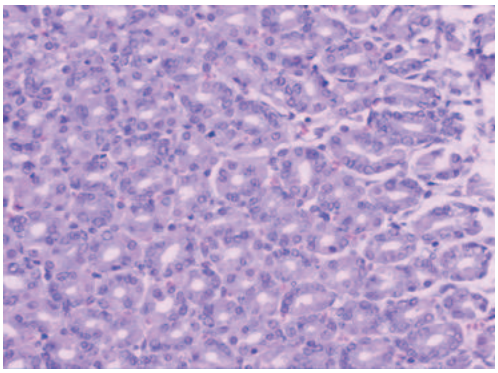


Fig. 8 – Foveolar hyperplasia: transversal section corpus [hematoxylin and eosin (HE) ×200].

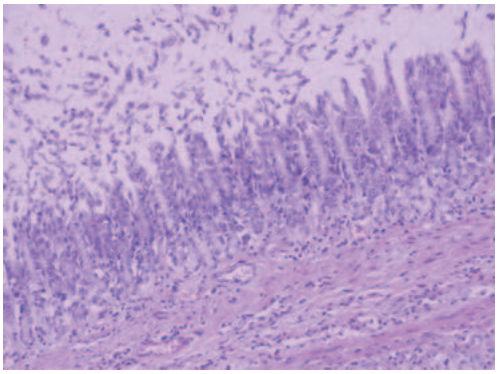


Fig. 9 – Cystic atrophic gastritis with a focal erosion [hematoxylin and eosin (HE) ×100].

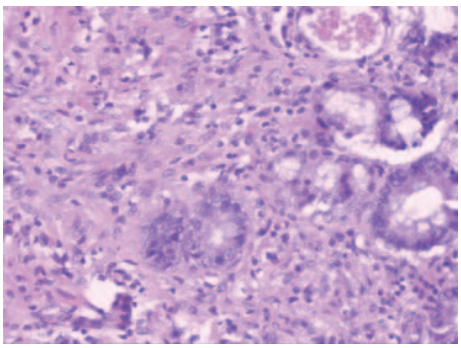


Fig. 10 – Mature intestinal metaplasia (precancerogenous lesion) [hematoxylin and eosin (HE) ×400].

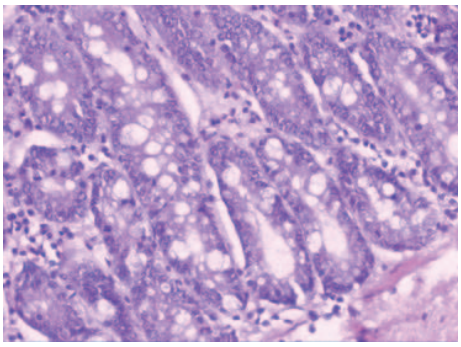


Fig. 11 – Multifocal displasia with intestinal metaplasia [hematoxylin and eosin (HE) ×400].

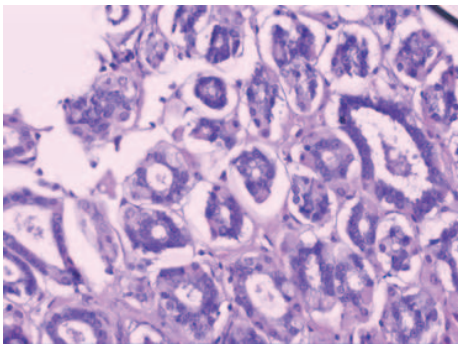


Fig. 12 – Early gastric carcinoma located intramucously, without propria invasion [hematoxylin and eosin (HE) ×400].

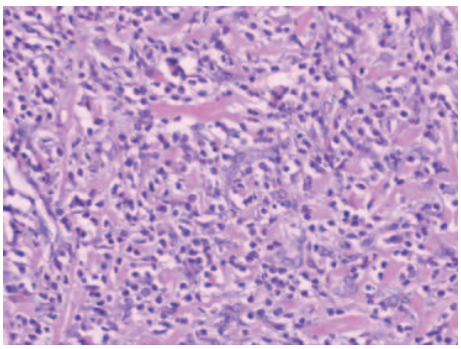


Fig. 13 – Anaplastic carcinoma with a pronounced stroma reaction [hematoxylin and eosin (HE) ×200].

Statistical analysis

The obtained research data were statistically processed by the descriptive method and by making use of appropriate statistical tests. The data base was created in the statistical program Microsoft Office Excel 2007, while the data processing was done by the SPSS program, version 12.0 (Statistical Package for Social Sciences). All statistical tests were considered acceptable if the probability of zero hypothesis was equal or lower than 5%. The following statistical test were used for data processing: χ^2-test, Fisher-Freeman-Halton test (Fisher's exact test), monofactorial analysis of variance (ANOVA) with correction by Brown-Forsythe and Tukey HSD test.

Results

The study was completed with 3 groups of 30 experimental animals each (90 rats), as plan in the design of the study. A total of 12 rats were replaced in the group B2 while 13 animals were replaced in the RY group. No replacement was required in the group C (Figure 1). The replaced animals died or had to be sacrificed prematurely, in the cases of presumed bad outcome. The replacement or lethal outcome occurred due to dehiscence of anastomosis, surgical wound infection, autophagy (laparotomic sutures eaten by the rats themselves), heterophagy (*viscera* consumed by other rats), or for some other unknown reason.

The determined gastric acid levels provided indirect information on the degree of DGR. The pH values were measured after the weeks 8, 16 and 24, and included the following values: the group B2: 4.8 ± 0.5, 5.4 ± 0.7 i 5.4 ± 0.7, the group RY: 3.9 ± 0.2, 4.2 ± 0.6 i 4.1 ± 0.5 and the group C: 3.8 ± 0.5, 4 ± 0.4 i 4.1 ± 0.5, respectively. The mean pH value in the B2 was found to be statistically significantly different from the same finding in the groups RY and C, while no significant difference was found between the RY and C groups, at any measurement point. Unifactor variance analysis confirmed a statistically significant difference among the experimental groups in the period after 8 weeks: $p < 0.01$ (F 2.12 = 10.11, $p = 0.003$), in the period after 16 weeks: $p < 0.001$ (F 2.27 = 17.2, $p < 0.001$) and in the period after 24 weeks: $p < 0.001$ (F 2.42 = 30.04, $p < 0.001$).

The statistical analysis of histopathological specimens taken after 8 weeks, in correlation with MNNG influence duration in the B2 group, showed a hyperplasia of gastric mucosa in 40%, while gastritis was found in 20% and metaplastic changes in 40% of the samples. At the second timeline, 16 weeks after the experiment, the histopathological findings indicated dysplastic changes of all three degrees (mild, moderate, severe) in 50%, while carcinoma was found in 10% of the samples. Metaplasia were noted in 30% of the samples whereas gastritis was identified in only 10% of the samples. In the animals sacrificed in week 24, the percentage of dysplasia was similar to the findings after 16 weeks (46.67%). The percentage of metaplastic changes was significantly decreased (6.67%), but the finding that was not expected included a twice more frequent incidence of early gastric carcinoma (20%) and even more frequently in the broader anastomosis area and its surroundings (adenocarcinoma, diffuse type signet ring cell carcinoma), or on the anastomosis itself (26.67%) (Table 1). Independence χ^2-test showed a statistically significant correlation between the histopathological findings within the B2 subgroup with administered MNNG during all three observed periods, $\chi^2_{(BM)}$ (10, n = 30) = 17.51, $p = 0.009$. Additional analysis showed a statistically significant correlation among groups observed for 8 and 24 weeks, $\chi^2_{(BM8\text{-}24)}$ (5, n = 20) = 13.05, $p = 0.002$. The findings in the group B2 in observed for 16 weeks, showed no statistical difference, in comparison with those other two groups, $\chi^2_{(BM8\text{-}16)}$ (4, n = 15) = 6.79, $p = 0.114$, $\chi^2_{(BM16\text{-}24)}$ (4, n = 25) = 6.04, $p = 0.16$.

In the RY group, the effects of nitrous compounds on gastric mucosa were examined in the absence of DGR (antireflux surgery), in correlation with time. After 8 weeks**,** hyperplastic changes were predominant in this subgroup (60%), whereas gastritis and metaplasia changes were found equally in 20% each.

Table 1

Comparative analysis of histopathological findings by time

Type of surgery (carcinogen)	Time (weeks)	Hystopathological findings, n (%)							Total
		0	1	2	3	4	5	6	
B2 (MNNG)		8	2 (40.0)	1 (20.0)	2 (40.0)				5 (100.0)
		16		1 (10.0)	3 (30.0)	5 (50.0)	1 (10.0)		10 (100.0)
		24			1 (6.7)	7 (46.7)	3 (20.0)	4 (26.7)	15 (100.0)
RY (MNNG)		8	3 (60.0)	1 (20.0)	1 (20.0)				15 (100.0)
		16	5 (50.0)		1 (10.0)	4 (40.0)			10 (100.0)
		24	2 (13.3)	2 (13.3)	1 (6.7)	10 (66.7)			15 (100.0)
Control (MNNG)		8	3 (60.0)	2 (40.0)					5 (100.0)
		16	3 (30.0)	3 (30.0)		4 (40.0)			10 (100.0)
		24		2 (13.3)	2 (13.3)	10 (66.7)	1 (6.7)		15 (100.0)

0 – normal mucosa, 1 – hyperplasia, 2 – gastritis, 3 – metaplasia, 4 – dysplasia, 5 – early carcinoma, 6 – carcinoma. B2 – Billroth II; RY – Roux-en-Y; MNNG – N-methyl-N-nitrosoguanidine.

After 16 weeks, MNNG, in absence of DGR, already led to the incidence of hyperplasia in 50%, mild dysplasia u 40% and metaplasia in 10%. In the week 24 of the experiments, the percentage of dysplasia, and, consequently, the degree of changes (moderate and severe) was at 66.67%. Thirteen percent showed hyperplasia and gastritis. Metaplasia was prevalent in 6.67%. The absence of early carcinoma and carcinoma was evident (Table 1). χ^2-test indicated a statistically significant correlation between the histopathological findings between RY subgroups with MNNG in all three periods, $\chi^2_{(RM)}$ (6, n = 30) = 10.72, p = 0.044. Additional analysis showed a statistically significant difference among the subgroups observed for 8 and 24 weeks, $\chi^2_{(RM8\text{-}24)}$ (3, n = 20) = 7.9, p = 0.029, while the 16-week subgroup did not show statistically signficantly difference, compared to other two subgroups, $\chi^2_{(RM8\text{-}16)}$ (3, n = 15) = 4.13, p = 0. 282, $\chi^2_{(RM16\text{-}24)}$ (3, n = 25) = 4.72, p = 0. 198.

In the group C, MNNG effects on the normal gastric mucosa were observed and correlated with the timeline of its application. After week 8, the findings included hyperplasia in 60% and gastritis in 40% of the samples. After week 16, mild or moderate dysplasia was found in 40%, while gastritis and hyperplasia were present in 30% of the samples. After 24 weeks, early carcinoma was found in 6.67%, dysplasia in 66.67% and the same percentage of gastritis and metaplasia (13.33%) (Table 1). χ^2-test showed statistically significant correlation between the histopathological finding between the non-operated animal subgroups with administered MNNG, in all three timeline periods, χ^2 (M) (8, n = 30) = 15.53, p = 0.009. Additional analysis showed statistically significant correlation between the histopathological findings between the subgroups observed for 8 and 24 weeks, χ^2 (M8–24) (4, n = 20) = 12.57, p = 0.002, while the 16-week subgroup did not show statistically signficantly difference, compared to other two subgroups, χ^2 (M8-16) (2, n = 15) = 2.67, p = 0.317, χ^2 (M16–24) (4, n = 25) = 7.11, p = 0.075.

Discussion

DGR is defined as the return of duodenal content into the stomach, through incompletent pylorus, i.e., from duodenum and intestines, through anastomosis into the stomach. It is probable that the excessive DGR is related to the carcinogenesis of the upper intestinal tract. The use of proton pump blocker decreased the incidence of gastric and duodenal ulcer and the need for its surgical treatment; however, if the medication treatment yields no results, surgical treatment is recommended [1, 13].

Partial gastrectomy causes an increase of intragastric pH, which, in turn, results in the excessive growth of nitrate-reducing bacteria, as well as atrophy of gastric mucosa, due to reduced gastrin levels and DGR. It is possible that mutagens have already been present, and/or have been formed from enterogastric content. In such a case, intragastric biliary acids would be a good source of amids. N-nitroso-taurocholic and N-nitroso-glycocholic acids are both mutagenic and carcinogenic. However, to date, little is known about the carcinogenic nature of N-nitroso biliary acids [14, 15].

The measurement of gastric juice pH values, in the course of the experiment, showed statistically significant differences in all three observed periods (8, 16 and 24 weeks), while the additional analyses showed that the mean pH values in the group B2 were statistically significantly different from the RY and C groups, in all three measurement periods, while no statistically significant differences were observed between the RY and C groups, at any measured period. The results of this study are conformant with the findings of Gronnier et all [16]. They confirmed that increased pH, due to DGR, was synergic and has a neutralizing effect on gastric acidity levels.

Increased cellular proliferation represents a high risk of carcinogensis. A possible mechanism of carcinogensis in DGR is, therefore, an increased number of cells at risk of repeated influence of carcinogen MNNG after a longer period (multi-shock process), due to chronic stimulation of cell proliferation. Moreover, increased cell proliferation can create clones of the previously affected cells and, thus, increase the possibility for another shock, or several new shocks of the same cell [17].

Gunassekaran et al. [18] suggested that the sum of 4 to 6 genetic events can be sufficient for tumour development and carcinogensis. Wogan et al. [19] added that the continuously increased cell proliferation, caused by DGR, can also accelerate further cell proliferation from the initiated cells, in order to lead to an incidence of carcinoma.

Decreased incidence of dysplastic changes in the B2 group (46.7%), compared to the RY group (66.7%) in our experiment was explained by the finding in the B2 group after 24 weeks, in which the total incidence of early carcinoma and carcinoma amounted to 46.7%, indicating that dysplasia occured more rarely than carcinogensis. The same percentage of dysplasia found in the RY and C group suggests that the absence of DGR has a key role on the occurence and growth of precancerous lesions and carcinoma, regardless of the carcinogen.

Øvrebø et al. [20], concluded that the superficial part of the mucosa, triggered by DGR, i.e., by billiary salts, further supports the penetration of MNNG in the proliferative gastric mucosa part.

Also, other authors argue that increased DGR caused mucosal changes that led to increased exposure to proliferating cell carcinogenesis. This is, largely, the reason for the increased cell proliferation and expansion of the proliferating mucosa part. The superficial part between the epithelial border and the proliferating work creates certain protection against the carcinogens entering from the gastric lumen into the mucosa. Mucous erosion in the antrum, probably represent an increased risk of carcinogenesis, by increasing the probability of cell proliferation and by reducing the density of the mucosa above the proliferative portion [21].

The specific finding in our study is the incidence of such a large percentage of cancer and dysplastic changes in the B2 group (conditionally refluxed) after 24 weeks of the experiment, since the time of DGR and MNNG action is relatively short, compared to other researchers, where the timelines of the experiments were 40, 90 and even 120 weeks [22–26]. These results

suggest that the time of influence of DGR in the presence of MNNG is in direct correlation with the severity of precancerous lesions and that the minimum time of the occurrence of cancer is 20 weeks after the start of the experiment. Our results are not in agreement with most of the authors who argue that the most distinctive changes in the stomach of experimental animals, under the effect of MNNG, occur in the 30th week after the initiation of the experiment [27, 28].

According to many authors, the period up to the 20th week of the experiment brings only the first stadium of the pathological changes. The second stadium, according to these authors, includes the period between experimental week 20 and 30 which is characterised by the occurrence of adenomatous hyperplasia with excessive glandular proliferation and mild atypia. Hyperplasia can be directed to the upper or lower part, including the penetration into the mucosa. The third stadium of the experiment is the period after week 30 of MNNG application, which is followed by the occurrence of adenocarcinoma [28]. It is important to emphasise that this group of authors applied carcinogen to the healthy animals, without surgically-induced DGR [29].

Characteristic for our experiment is, also, the occurence of adenomatous hyperplasia (40%), already after week 8 of MNNG application in the group B2 while 50% of dysplasia was already identified after week 16. It is assumed that the presence of the experimentally induced DGR and the preparation of gastric mucosa for its effects has influenced the quicker incidence of more severe precancerous lesions and, even, the occurrence of carcinoma.

Similar to our results, Mihailović [30] claims that as early as in the week 5 of the experiment, erosion with regenerative hyperplasia was observed, with an extension of the generative zone, cystic glands alterations and reduction of neutral zone and mucine. After week 24 of our experiment, there was a considerable incidence not only of adenocarcinoma, which is a characteristic histopathological finding in MNNG application, but also the appearance of diffuse form of signet ring cell carcinoma type as well as anaplastic forms. This finding is in accordance with Pritchard and Przemeck [28], who concluded that all types of cancer that exist in people can also occur in rats, except the mucoid type.

A considerable number of authors also reported the occurrence of metastases in the liver, lymph nodes and lungs of experimentally-induced carcinomas, after 30 weeks of the experiment [27, 30, 31]. The findings in our experiments can be, perhaps, explained by the fact that, in this experiment, MNNG added at a dose of 100 mg per liter of water [32, 33]. While some authors used the dose of 83 mg/L of drinking water in their studies. On the other hand, there are authors who state that with the increasing concentration of MNNG in drinking water of experimental animals, the incidence of gastric cancer remains unchanged, but the incidence of the small intestine carcinoma is increased [33].

Kobayasi et al. [34] examined the impact of DGR onto 4 groups of Wistar rats undergoing gastrectomy B2, B2 gastrectomy with conversion to Roux-en-Y after 24 weeks, B2 gastrectomy with conversion to Roux-en-Y after 36 weeks and the basic gastrectomy, Roux-en-Y reconstruction. The histological criteria selected in this experiment were similar to ours, with regard to the degree of cellular atypia for carcinoma diagnosis, lesions classified as hyperplasia or adenomatous hyperplasia in the application of carcinogens. These authors concluded, on the basis of histochemical assays, that the proliferative lesions were actually the phenotypical gastric cells, while the malignant lesions were actually the phenotypic intestinal cells. What is more important for our research, they concluded that proliferative lesions did not advance into carcinoma and that their incidence decreases if DGR was interrupted by conversion to Roux-en-Y. Rodrigues et al. [35] investigated the effect of DGR in 3 experimental groups of Wistar rats: G1 (control), G2 (subjected to GEA and, two weeks later, to ligature afferent loop), and G3 (GEA). After 36 weeks, the transit was restored by latero-lateral anastomosis between the afferent and efferent loops. The authors concluded that after 54 weeks the reflux through the pylorus favors the development of predominantly benign (precancerous) proliferative lesions in the gastric mucosa. The termination of reflux caused an inhibitory effect on the growth of these lesions, confirming the benign-reversibility of these lesions. Neoplastic lesions were rare in this experimental model.

Our findings lead to the conclusion that the presence of experimentally induced DGR in the group B2 led to the emergence of more serious (irreversible) histopathological changes, in terms of precancerous lesions and even the appearance of early gastric cancer and the rest of the stomach [36].

Pyloric sphincter destruction and, consequently, the loss of pyloric function, is related to the regurgitation of duodenal content. DGR contributes to the development of malignant processes. A high level of DGR directly related to the incidence of esophageal carcinoma and gastric stump carcinoma. Therefore, surgical procedures which prevent or decrease this reflux are highly important for ulcer prevention and treatment in human medicine [37]. In the light of these findings, the recommended surgical procedures are those that induce lower reflux, such as jejunal interposition, or Roux-en-Y anastomosis.

Conclusion

The sole effects of MNNG on gastric mucosa without DGR presence cause a mild degree of precancerogenous lesions. However, direct contacts of gastric mucosa with MNNG, in the presence of DGR induce the formation of precancerogenous lesions, by decreasing the percentage of reversible changes and increasing the percentage of irreversible lesions and carcinoma, over time. The duration of exposure to DGR in the presence MNNG is in direct correlation with the severity of precancerous lesions. A lack of statistically significant correlation between the RY and C group, strongly confirms the presented conclusions and endorses the protective role of Roux-en-Y procedure.

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Notice

This experiment represents a different aspect from our previous research conducted at the Center for Biomedical Research, Faculty of Medicine, the University of Niš.

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