

The ornithine decarboxylase, NO-synthase activities and phospho-c-Jun content under experimental gastric mucosa malignancy

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Abstract— Introduction: Ornithine decarboxylase is the first and key regulatory enzyme in synthesis of polyamines, which are essential for cell proliferation and differentiation, so its aberrant regulation is reported to play a role in neoplastic transformation and tumours growth. That's why, there were analysed some major links of metabolic pathways that are closely related to tumorigenesis: ornithine decarboxylase, and the NADPH-dependent enzyme nitric oxide synthase, the nuclear phosphoprotein c-Jun, that could play an important role in the development of gastric cancer malignancy. **Methods:** The gastric carcinogenesis was initiated in rats by 10-week replacement of drinking water by 0.01% N-methyl-N'-nitro-N-nitrosoguanidine solution, at the same time they were redefined on the diet containing 5% NaCl. After this period expired, the animals were fed with standard diet till the end of the 24th week. The gastric mucosa cells were extracted at the end of the 4th, 6th, 8th, 10th, 12th, 18th and 24th week and underwent biochemical examinations. It was established the elevated phospho-c-Jun content, ornithine decarboxylase and inducible nitric oxide synthase activities from 6th to 24th week of gastric cancer development compared to the control references. **Results:** The increasing of ornithine decarboxylase activity could probably be caused by the growth of phospho-c-Jun, it is also belonging to an ornithine decarboxylase transactivation effects. Thus, it was shown that the increase of ornithine decarboxylase and inducible nitric oxide synthase activities, phospho-c-Jun and nitrite-ions accumulation in gastric mucosa epithelial cells were associated with the gastric malignant progression. **Conclusion:** The complex relationships between the examined enzymes and transcription activator that pointed to an aggravation of pathological disturbances due to reciprocal action between ornithine decarboxylase and c-Jun and nitric oxide synthase participation.

Keywords: Ornithine decarboxylase, phospho-c-Jun, nitric oxide synthase, gastric cancer

INTRODUCTION

Normal structure and functioning of gastric mucosa are determined by regulated division rate of proliferating cells in neck regions of glands. Increasing evidence has indicated that cellular organic cations polyamines are necessary for normal epithelial proliferation (Liu, 2004; Timmons, 2013). Polyamine level is regulated by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) (Timmons, 2013). ODC is a key enzyme of polyamine synthesis and catalyzes the decarboxylation of ornithine to putrescine. It is one of the enzymes necessary for progression into S phase

(Bello-Fernandez et al., 1993), but its aberrant regulation plays an important role in the wide variety of neoplastic transformations (Pegg, 2006).

It is clear that polyamines affect the expression of growth-related genes and these effects depend on a cell type. Polyamines are absolutely required for *c-myc* and *c-jun* mRNA synthesis and their depletion significantly decreases the transcription rates of these genes in IEC-6 cells (Liu, 2004; Timmons, 2013). Also, polyamine deficiency results in a significant decrease in *c-fos*, *c-myc* and *c-jun* expression in intestinal crypt cells (Wang et al., 1993).

The proto-oncogene *c-jun* encodes for a resident nuclear protein c-Jun that is being the target of serine protein kinase cascade has also been implicated in the control of cell cycle by cyclin D1 promoter activation during M-G1 transition. Phosphorylation of c-Jun by c-Jun N-terminal kinase (JNK) is the most important activity regulation that affects on DNA-binding, stability, ability to interact with other proteins, and transactivation potential. c-Jun is expressed at the low level in different cells, but growth factors, oncogenes, cytokines, environmental stress, bacterial and viral infections upregulate expression of c-Jun (Eriksson, 2005). It is believed that c-Jun is a critical promoter of proliferation and deregulated expression and activation of its oncogene are frequently observed in wide variety of cancers (Taira et al., 2012). There is report that endogenous nitric oxide (NO) can suppress JNK through a thiol-redox mechanism and modulate c-Jun phosphorylation rate. Redox regulation of JNK by NO may be important for revealing the mechanism by which inducible nitric oxide synthase (iNOS) induction and NO mediate intracellular signalling leading to various cellular functions (Park et al., 2000).

Besides that polyamines stimulate DNA synthesis and increase growth-related genes transcription, there is data that increasing of nitric oxide synthase (NOS, EC1.14.13.39) activity and addition exogenous NO levels have been recognized to result in inhibition of cell proliferation. It was shown that NO inhibits tumour cell proliferation by inhibiting ODC by S-nitrosylation. The cytostatic effects of NO were reversed by the exogenous polyamines adding but not by ornithine, that points on ODC inhibition by NO (Bauer et al., 2001).

So one of the major links of metabolic pathway, that are closely related to tumorigenesis, is the NADPH-dependent enzyme nitric oxide synthase catalyzing the production of nitric oxide from L-arginine. Nitric oxide has conflicting actions on different tumours. The role of NO as an important cellular signalling molecule in tumour cell apoptosis and survival depends on its concentration, cell type, duration of exposure and other factors (Ma et al., 2007).

The present study was focused on the hypotheses that changes of ODC and NO-synthase activity, NO and phospho-c-Jun contents might play role in the mechanisms of chemical-induced gastric mucosa malignancy, thus, the aim of the study was to determine the activities of ODC, NOS, NO and phospho-c-Jun contents in gastric mucosa cells during the gastric cancer

development in rats. The study demonstrated the increase of ODC and iNOS activities and phospho-c-Jun and nitrite-ions accumulation in gastric mucosa epithelial cells that were associated with gastric malignant progression.

MATERIALS AND METHODS

Experimental design

Investigations were carried out on white male rats (n=112) with initial weight of 100±20 g.

Gastric carcinogenesis was initiated by 10-week replacement of drinking water by 0.01% solution of carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) while the rats were fed with fodder containing 5% Sodium Chloride (NaCl). Then the animals were fed standard vivarium diet for the following 14 weeks (Kuroiwa et al., 2007; Takahashi et al., 1994). Control animal group was fed standard diet during the whole experiment period. Samplings of experimental material were performed at the end of the 4th, 6th, 8th, 10th, 12th, 18th, and 24th week. Pathology development was verified by histological methods. The mucosa of isolated stomachs was turned outward and mucous membrane cells were extracted for analysis.

Cell isolation

Gastric mucosa cells were isolated by enzymatic disaggregation method with pronase usage (TAIROV et al., 1983).

ODC activity assay

ODC activity was measured in supernatant obtained by cell homogenate centrifugation at 20 000 g for 15 min at 4°C. Spectrophotometric assay for ODC was based on solubility in 1-petanol of coloured product of its activity, putrescine, with 2,4,6- trinitrobenzenesulfonic acid (Ngo et al., 1987). ODC activity was represented in nM of trinitrophenyl adducts of putrescine per 1 mg of protein per 1 min.

NOS activity assay

Evaluation of NOS activity (Ca²⁺-dependent and independent) was based on the combination of classic method (Salter et al., 1991) with its modification (Boyde and Rahmatullah, 1980; Chin et al., 1999) that was adapted to the spectrophotometric assay of one of the reaction product L-citrulline in CaCl₂ present. The activity was represented in µM of L-citrulline per 1 mg of protein per 1 min. iNOS activity was deter-

mined similarly, but there was added 2 μ M EDTA instead of CaCl₂.

Determination of nitrite-ions content

Nitrite-ions content was detected in deproteinized homogenate aliquots by Griess reagent (Moshage et al., 1995). Absorbance was registered at $\lambda=540$ nm and nitrite-ions content was expressed in nM per 1 mg of protein.

Determination of phospho-c-Jun content

Phospho-c-Jun content was determined in gastric mucosa cell lysate prepared with the use of Cell Lysis Buffer (Cell Signaling Technology, USA). Phospho-c-Jun content was measured by Sandwich ELISA method using the assay kit PathScan Phospho-c-Jun (Ser63) (Cell Signaling Technology, USA) and represented in conventional unit of absorbancy ($\lambda=450$ nm) on mg of protein.

Protein concentration assay

Protein concentration was measured by Bradford's method (Bradford, 1976).

Statistical analysis

Experimental data were processed by common methods of variance analysis with 7 repeats. Reliability of discrepancies between two samplings was determined using Student's criterion. The results are presented in the values of the arithmetic mean and mean square error, $M \pm s$ (Brandt, 1975).

Ethics statement

Investigations were carried out in compliance with the main standards of keeping and working with laboratory animals and the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes as well as with the ethic norms specified in Ukrainian legislation.

RESULTS AND DISCUSSION

The assay of the activity of polyamine metabolism enzyme as biological marker of malignant growth is useful in view of polyamine rate affecting ion channels, cell-cell interactions, cytoskeleton, signalling via phosphorylation and other mechanisms. Increased polyamine levels and ODC activity are the first event detectable during cell transformation provides reliable marker in estimating the cancer progression (Ngo et al., 1987; Pegg, 1988; Pegg, 2006).

There was the task to measure the ODC activity in gastric mucosa cells during the 10 weeks of MNNG administration with the following-up 14-week carcinogen-free period. It was established in average 2.3-fold increase in ODC activity in all stages of gastric cancer malignancystudy compared to the control (Fig. 1).

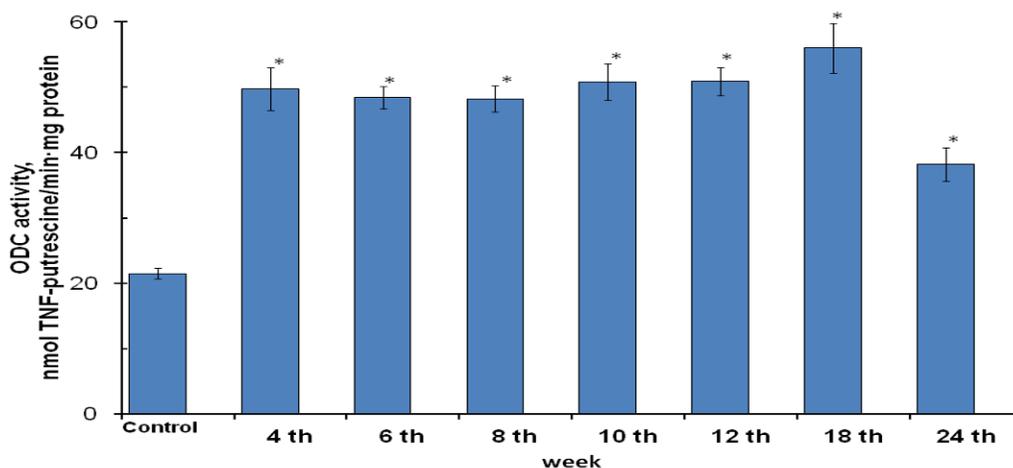


Figure 1. The ODC activity in gastric mucosa cells under gastric cancer development. Control – control group,* – the difference is significant as compared to appropriate control ($p < 0.05$), TNP-putrescine – trinitrophenyl adducts of putrescine.

It should be noted that the termination of the gastric carcinogen exposure on 10 weeks of experiment was not accompanied by marked changes in activity of investigated enzyme. So the MNNG despite its carcinogenic effect has no direct influence on ODC activation, like it was established by O'Brien (1967) for the active carcinogenic component of croton oil 12-O-tetradecanoyl-phorbol-13-acetate.

Perhaps the one of the principle factors of the revealed increase in ODC activity during the experiment is the stage of carcinogenesis under MNNG, thus initial period characterised ODC activation in gastric cells but the last timepoint when adenocarcinomas were registered and MNNG treatment was ceased, there wasn't ODC activity normalization. An increased ODC activity has also been reported to occur in many cells exposed to chemical carcinogens or tumour promoters, including MNNG (Okuzumi et al., 1991). The induction of ODC activity plays an important role in initiation and promotion steps of the model and prevention of this rise by using specific inhibitors such as DFMO reduces tumour formation (Shantz and Pegg, 1994).

Initially, a correlation between ODC activity and tumour-promoting ability was shown in mouse skin carcinogenesis (O'Brien, 1976). In the rat colon and in surgically resected human colon bearing colon cancer ODC was elevated not only in colon cancer but also in adenoma (Rozhin et al., 1984). Concerning ODC activity in human stomach, there was concluded that gastric cancer tissue had significantly elevated ODC levels over those of mucosa and among mucosa of the stomach, that of the pyloric glands had higher ODC

activity than that of the fundic glands (Okuzumi et al., 1991). It is considered that ODC plays an important role in gastric carcinogenesis, but the mechanism of ODC activity elevation and its influence on human gastric cancer development is not well understood.

It is known that comprehensive alterations in enzyme activities occur very rapidly and are the result of changes in quantity of enzyme protein. ODC is transcriptionally regulated and many factors increase its mRNA synthesis. The promoter region of *odc* gene includes multiple sequences susceptible to hormones, growth factors and tumour promoters, including cAMP response element, AP-1, AP-2 sites, GC-rich Sp1 binding sites and TATA box (Pegg, 2006).

So it has been supposed a participation of proto-oncogene *c-jun* in ODC transactivation. c-Jun is a major component of AP-1 transcription complex and it dominates in many cells despite there are other variants of AP-1 complexes. It is believed, that c-Jun is a critical promoter of cellular proliferation and deregulated expression and activation of its oncogene are observed in wide variety of cancers including gastric malignancy (Eriksson, 2005).

There was no any change of the content of activated phosphorylated form of c-Jun at the beginning (4-th week) stage of gastric malignancy, but it was established the relation between rising phospho-c-Jun content and ODC activity from the 6th to 24th week of gastric cancer development (Fig. 2): when the gastric mucosa cells were characterized by increased phospho-c-Jun content at average 3.8-fold and the increased ODC activity at average 2.3-fold over the control (Fig. 1).

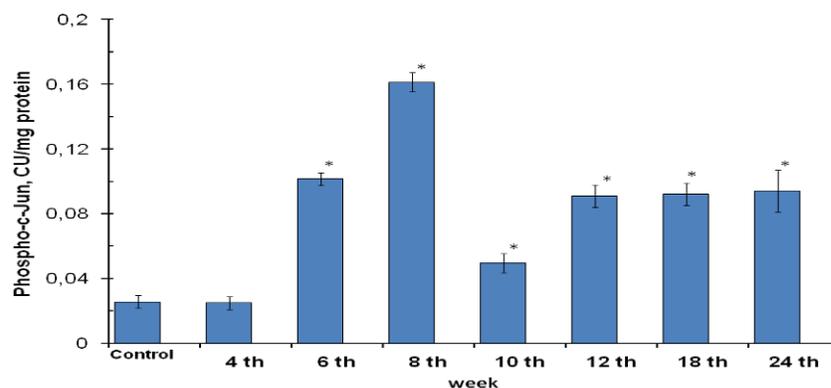


Figure 2. The phospho-c-Jun content in gastric mucosa cells under gastric cancer development. Control – control group, * – the difference is significant as compared to appropriate control ($p < 0.05$)

Concerning probable mechanism of ODC-induced cell transformation, constitutive activation of JNK and constitutively increased phosphorylation rate of c-Jun on Ser 63 and Ser 73 was detected. It was established that cell transformation induced by ODC and *c-Ha-ras* oncogene is accompanied by constitutively increased phosphorylation of c-Jun (Kielosto, 2004).

The 4th week of MNNG-stimulated gastrocarcinogenesis was characterised by ODC activity growth with the reference value of phospho-c-Jun in gastric mucosa cells. The established increase of ODC activity is accorded to the data about malignant transformations including those were initiated by chemical agents.

There is no doubt that cellular polyamines produced by ODC activity are required for proto-oncogene transcription. Polyamines affect the expression of growth-related genes and these effects depend on a cell type. It has been reported that polyamines stimulate *c-myc* and *c-jun* transcription in COLO 320 human colon carcinoma cells. Also, polyamines are essential for *c-myc* and *c-jun* mRNA synthesis in intestinal crypt cells IEC-6 cells and their depletion significantly decreases the transcription of these genes but doesn't effect on post-transcription (Liu, 2004; Timmons, 2013).

Thus, it is assumed the importance of increasing the activity of ODC in the first place, which triggers the synthesis of mRNA of such oncogenes as c-Jun and consequent its translation, that we apparently observed at the 4th week, but post-translational modification leading to activated a form of c-Jun probably occurred lately.

The growth of the phosphorylated form of c-Jun content from the 6th to 24th weeks of gastric cancer development (Fig. 2) was probably caused by its high expression, amplification of c-Jun N-terminal kinase-mediated phosphorylation or inactivation of phosphatases. In contrast to the established increase of the ODC activity level, the quantity of the phosphorylated form of c-Jun changed at the time of carcinogen treatment cessation (10 week): it decreased but remained high comparing with the control. It is believed that c-Jun has an ability to positively regulate cell proliferation positively regulate cell proliferation by the way of repression of tumour suppressor gene expression and function, and induction of cyclin D1 transcription (Eriksson, 2005).

These results confirm other findings of immunohistochemical analysis that revealed activation of JNK in

human gastric cancer tissue and are consistent with those of others who demonstrated that mice lacking JNK1, a major JNK isozyme, demonstrated a noticeable decrease in gastric cancer induced by N-methyl-N-nitrosourea (Shibata et al., 2008).

Although under physiological proliferating stimuli such as hormones, growth factors, the activity of ODC in normally rested cells rises briefly and dramatically for passage from G₁ into S phase (Timmons, 2013), werevealed that the prolonged increase of this enzyme activity might be an exhibition of long-term carcinogen influence and following-up pathological proliferation. These data corresponded to those obtained from *in vivo* experiment where constitutive expression of ODC was observed under the influence of tumor growth promoters after administration of carcinogen initiation dose.

So, unlike normal physiological stimuli, carcinogens, tumor growth promoters, as bile acids, free radicals and some oncogenes cause in cells dramatic constitutivising of ODC activity which in these conditions functions as oncogen. The gene coding for ODC may act as an oncogene since expression of ODC at very high level from appropriate plasmid vectors leads to a transformed phenotype (Shantz and Pegg, 1994).

In addition, the ornithine decarboxylase activity is modulated by S-nitrosylation under nitric oxide synthase activation (Bauer et al., 2001). So we also studied the major link of metabolic pathway that is closely related to tumorigenesis: the NADPH-dependent enzyme NOS.

A semiessential amino acid, L-arginine, is assumed to be a major precursor of ornithine in mammalian cells through the arginase to yield ornithine and polyamines. But also L-arginine is metabolized by the oxidative desaminase pathway to form NO by the action of nitric oxide synthase. Three isoforms of NOS have been identified: two constitutive, i.e., endothelial NOS and neuronal NOS, and one inducible NOS (iNOS). NO as an intracellular messenger participates in various metabolic processes like immunologic function and proliferation (Ma et al., 2007).

The submitted studies showed the increased total (constitutive and inducible) NOS activity at 2.1-fold over the control in gastric mucosa cells from the 6th to 24th weeks of gastric cancer development (Fig. 3). It was generally determined by rising of inducible isoforms which activity increased at 9.2-fold over the control (Fig. 4) but didn't by constitutive isoforms

whose activities had fallen or were at the control reference (Fig. 5), and accompanied by the rising of nitrite-ions content (Fig. 6).

The results obtained by Ma et al. (2007) from human colon biopsy specimens shown the expression of endothelial NOS in the normal and paratumour mucosa; however, it was not expressed in the tumour cells. The pattern of expression of neuronal NOS was similar to that of endothelial NOS. The tumour cells showed moderate or intense staining for iNOS, whereas the glandular cells in normal and paratumour mucosa did not express iNOS (Ma et al., 2007), that didn't contradict with our results. It has been postulated the elevated nitric oxide synthase activity and its expression in gastric cancer cells (Kielosto, 2004). The role of nitric oxide in tumour cell apoptosis and survival is cell type and duration exposure dependent. High NO concentration induces cytostasis and cytotoxicity in tumour cells both in vitro and in vivo, however, low NO concentration have a pro-tumour effect in the carcinogenesis and angiogenesis of solid tumours (Ma et al., 2007).

The conducted by us histological investigations pointed to the gastric cancer progression and hence the inefficiency of NO to stop the malignancy. The inflammation signs, vessels extension and plethora, the epithelial cell desquamation, and the increased mucus production at the end of the 6th week of experimental gastric carcinogenesis were shown (Tymoshenko et al., 2012). After the influence of MNNG during the 8 weeks, gastric mucosa demonstrated atrophic changes. The metaplastic modifications, sites with hyperplasia and atypia, were observed after 10 weeks of MNNG treatment. Adenomas and one adenocarcinoma were detected at the end of the 12th week of the experiment. Such neoplasms were visualized in stomach pyloric regions in 70% rats at the end of the 18th week of experimental gastric carcinogenesis.

According to the literature data MNNG causes alkylation of DNA and nitroamidation of proteins in mucosa epithelial cells, leading to mutations. MNNG methylates the bases of nucleic acids, yielding 7-methylguanine and 3-methyladenine. The guanidino-¹⁴C-labeled carbon of MNNG is efficiently incorporated into proteins due to the conversion of a lysine residue of protein to a nitrohomoarginine residue by transfer of the nitroamidino group from MNNG (Su-

gimura et al., 1970). In addition, MNNG interacts with membrane lipids and induces free radical production. Also free radical formation is generated by exposure of MNNG in acidic conditions such as that predominant in stomach (Gunassekaran et al., 2010).

Although the gastric mucosa cells were characterized by control reference of NOS activity at the end of the 4th week of MNNG and NaCl consumption (Fig. 3) and significant histological changes at the same time point weren't observed, but accumulation of the nitrite-ions in gastric mucosa epithelial cells at this time point were observed (Fig. 6).

The increased level of NO₂⁻ content was noticed during the MNNG consumption from the 6th to 10th weeks compared to the periods when the rats were fed with standard diet till the end of the 24th week. The 6th, 8th and 10th weeks were characterized by nitrite-ions content growth in gastric mucosa cells at 2.9, 2.7 and 3.1 times respectively. There was increasing of NO₂⁻ at 2.0, 2.2 and 2.1 times at the end of the 12th, 18th and 24th weeks of gastric cancer development.

Also, it was noticeable the highest rate of nitrite-ions at the end of the 4th week. It is known that MNNG transforms into the active form N-methyl-N'-nitroguanidine in gastric acidic conditions and is accompanied by realising of nitrous acid (Sugimura and Fujimura, 1967) that might determine the highest nitrite-ions content in times of MNNG consumption. After the end of the 10th week this exogenous infusion of nitrous acid discontinued and was reflected in the decreasing of NO₂⁻ content compared to the previous weeks. It should be noted if the rise of NO₂⁻ at the 4th week might be determined only by MNNG influence, the established changes of NO₂⁻ content from the 6th to 24th weeks were caused by the increase of the inducible NOS activity (Fig. 3).

In addition, clinical and preclinical studies have shown that eflornithine (DMFO), an ODC inhibitor, has significant chemopreventive and cytostatic effects against colon, skin, cervical and pancreatic cancers and should be evaluated in combination with other drugs in anticipation of future clinical trials (Bailey et al., 2010; Laukaitis and Gerner, 2011; Mohammed et al., 2014; Vlastos et al., 2005). Also the antioxidant supplementation was associated with the decrease of mucosal ODC in patients with premalignant changes in gastric mucosa (Marotta et al., 2004).

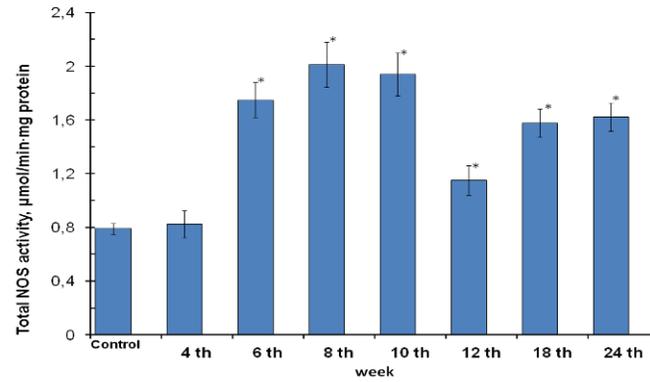


Figure 3. The totalNOS activity in gastric mucosa cells under gastric cancer development. Control – control group,* – the difference is significant as compared to appropriate control ($p < 0.05$)

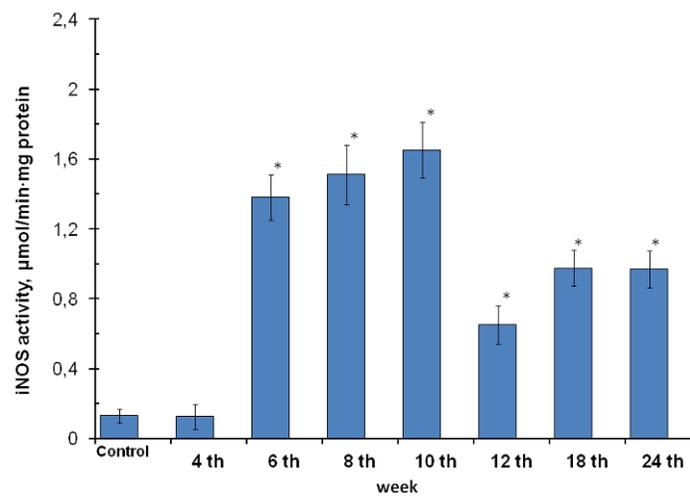


Figure 4. The iNOS activity in gastric mucosa cells under gastric cancer development. Control – control group,* – the difference is significant as compared to appropriate control ($p < 0.05$).

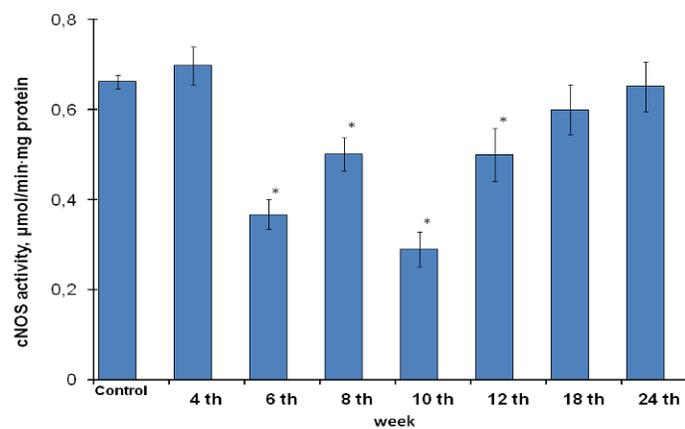


Figure 5. The cNOS activity in gastric mucosa cells under gastric cancer development. Control – control group,* – the difference is significant as compared to appropriate control ($p < 0.05$).

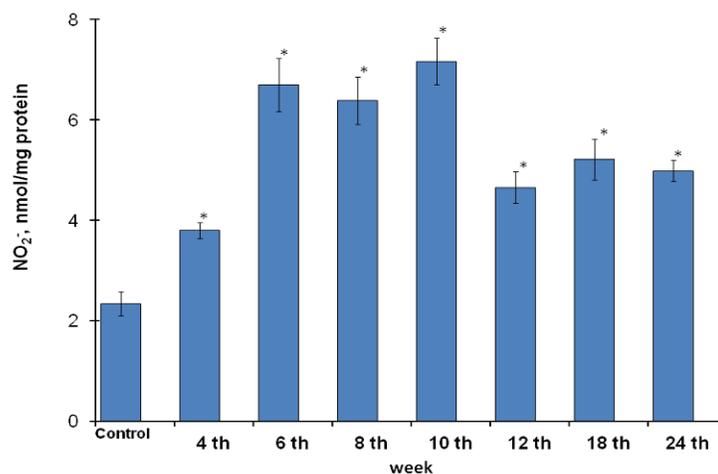


Figure 6. The NO₂⁻ content in gastric mucosa cells under gastric cancer development. Control – control group,* – the difference is significant as compared to appropriate control ($p < 0.05$).

CONCLUSIONS

The increase of ODC and iNOS activities, phospho-c-Jun content, and accumulation of the nitrite-ions in gastric mucosa epithelial cells from the 6th to 24th week were observed and shown the complex ambiguous relationships between ODC and c-Jun during the process of gastric cancer development, that pointed to an aggravation of pathological disturbances due to reciprocal action between ODC and c-Jun.

Abbreviations

AP-1: Activator protein-1; cNOS: Constitutive nitric oxide synthase; iNOS: Inducible nitric oxide synthase; JNK: c-Jun N-terminal kinase; MAP: kinase Mitogen-activated protein kinase; MNNG: N-Methyl-N'-nitro-N-nitrosoguanidine; NO: Nitric oxide; NOS: nitric oxide synthase; ODC: Ornithine decarboxylase.

Competing interests

The authors declare they have no competing interests.

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