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The potential therapeutic effect of N^G-hydroxy-nor-L-arginine in 7,12-dimethylbenz(a)anthracene-induced breast cancer in rats

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ABSTRACT

Advances in our understanding of the metabolism and molecular functions of arginine and their alterations in cancer have led to resurgence in the interest of targeting arginine catabolism as an anticancer strategy. Therefore, arginase inhibitors have been proposed as a way to treat cancer. In this study, the anti-tumor potential of the arginase inhibition by N^G-hydroxy-nor-L-arginine (nor-NOHA) (3 mg/kg/day, i.p.), administered for 5 weeks (parallel tumors development, every 3th day) against 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis in rats has been investigated. Treatment by nor-NOHA has obvious inhibition effects on development of carcinogenesis in rats was shown. That was seen in downregulation of rats' tumors size and number, mortality rate, in stopped alteration of tissue histopathology, in decrease of polyamines, NO and MDA (malondialdeide) concentrations (in blood). Results have shown arginase and NO-synthase can cooperate to restrain quantities of polyamines and NO for cancer progression. The results obtained can serve as a base to use this model for determination of productive, noncytotoxic antitumor and immune modulating concentration of anticancer agents. Perspectives of targeting arginase and NOS in cancer management can ground application in clinical medicine.

1. Introduction

Armenia currently holds second position in World Health Rankings rating, with 196.12 deaths per 100,000 people (all cancer types). These numbers are drastic and call to serious attention. Arginine, a semi-essential amino acid in humans, is necessary for the growth of human cancers, particularly those marked by *de novo* chemoresistance and a poor clinical outcome (Delage et al., 2010; Xiong et al., 2016). In addition to protein synthesis, arginine is involved in diverse parts of tumor metabolism, including the synthesis of nitric oxide (NO), polyamines, nucleotides, proline and glutamate (Chioda et al., 2013; Munder, 2009). Macrophages, granulocytes, or MDSC suppressed T cell–mediated immune responses by regulation of L-arginine metabolism *via* enzymatic mechanisms involving arginase and NO synthase (NOS) (Engblom et al., 2016; Rodriguez et al., 2017). Recent findings suggest that L-arginine metabolism in myeloid cells can result in the impairment of lymphocyte responses to their antigen (Engblom et al., 2016; Munder, 2009; Rodriguez et al., 2017). Arginase converts L-arginine to L-ornithine, which is the precursor of polyamines, essential components of cell proliferation (Casero et al., 2018). Polyamines are known to bind DNA and affect gene expression by bringing structural changes in chromatin and hereby stimulating cell growth (Nowotarski et al., 2013). Targeting polyamine metabolism has long been an attractive approach in cancer chemotherapy (Alexander et al., 2017). There is a rapid growth of polyamines (spermine, spermidine and putrescine) quantity in blood serum and urine during malignant tumors in different organs (Park and Igarashi, 2013). The large quantity of polyamines allows the oncogenes get over the immune system and prompted the cancer cells to metastasis (Soda, 2011). Polyamines involved in separation of cancer cells from the tumor cluster, help cancer cells to escape immune system detection, have possible role on cell rooting and colonization at secondary tumor sites (Murray-Stewart et al., 2016).

NO is a pleiotropic regulator, critical to various biological processes, including vasodilation and macrophage-mediated immunity (Sessa and Fo, 2011). NO depending on concentration and under various

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conditions can promote or inhibit tumor growth and metastasis (Vanini et al., 2015; Zhang et al., 2016). When NO produced at low levels it is an important mediator of physiological functions such as vasodilation, inhibition of platelet aggregation, and neurotransmission (Choudhari et al., 2013; Laurindo et al., 2018). Under inductive conditions, high levels of NO produced by macrophages and other effector cells can mediate antibacterial and antitumor functions. Increased NO generation in cancer cells may contribute to tumor angiogenesis by upregulating vascular endothelial growth factor (VEGF), and VEGF-induced neovascularization in turn may increase the tumors' metastatic ability (Bielenberg and Zetter, 2015; Hickok and Thomas, 2010). Increased amounts of NO have been observed in the blood of breast cancer patients and increased NOS activity was found in invasive breast tumors when compared with benign or normal breast tissue (Granados-Principal et al., 2015; Vahora et al., 2016). Although several reports have addressed the pro-tumoral effects of NO, few have demonstrated the contrasting role of NO in mediating tumor regression (Chioda et al., 2013; Choudhari et al., 2013; Vanini et al., 2015). It has been reported that NO derived from macrophages, Kupffer cells, natural killer cells, and endothelial cells participates in tumoricidal activity against many types of tumors (Cevahir and Aytamka, 2007; Choudhari et al., 2013). Understanding different actions of NO in breast cancer at the molecular level can help in providing NO based diagnostic or prognostic markers and also in devising potential strategies for prevention and treatment.

The feature of the arginase and NOS enzymes that makes them attractive, as therapeutic targets, is that there are a host of small molecules inhibiting these enzymes (Caldwell et al., 2015). Treatment with the arginase inhibitor nor-NOHA (reversible inhibitor of rat arginase with a K_i value of 0.5 μM) reduced the inhibitory effect of MDSC on T cell proliferation and inhibited number and size of lung metastasis (Secondini et al., 2017). Nor-NOHA effectively induced apoptosis in ARG2-expressing Leukemia cell lines under hypoxia (Ng et al., 2018). Nor-NOHA can induce cell apoptosis and inhibit the ability of invasion and migration of HepG2 cells by inhibiting Arg1, which is related with the increase of iNOS expression and the high concentration of NO (Li et al., 2017). Literature observation support the preclinical evaluation of nor-NOHA for the treatment of breast cancer (Bonavida, 2015; Cells et al., 2000; Narita et al., 2013; Prati et al., 2012; Secondini et al., 2017; Steppan et al., 2013).

The main goal of our work was to investigate the antitumor effect of arginase inhibition by nor-NOHA on 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors in rats. The primary objectives of the study were to assess the activity of arginase and NOS in 7, 12-DMBA induced breast cancer rats blood and to elucidate the possible relationship between their activity and rats tumors size, quantity, weight, mortality rate, cancer histopathology, blood polyamines, NO and MDA quantity. We have studied our experimental model (3 mg/kg/day nor-NOHA, i.p., administered for 5 weeks, parallelly to tumors development, every 3th day) effect on breast cancer progression induced by 7,12-DMBA.

2. Materials and methods

Animals. The animals were housed 7 and 10 (DMBA group) to a cage, with a base surface of 3000 cm^2, in a well-ventilated room at 25 °C and left one week for acclimation before experimentation. The animals were kept at constant environmental and nutritional conditions throughout the experimental period with room temperature 23 ± 2 °C and humidity (50–55%) with 12 h light/12 h dark cycle and were fed a standard pellet diet and with water *ad libitum* (Animal care house, Faculty of Biology, YSU, Yerevan, Armenia). A total of 38 adult female Wistar rats weighing 90–120 g were used (8 weeks old). All handling and maintenance conditions were in accordance with the rules of the principles of the National Center of Bioethics (Armenia) (Suckow et al., 2006; National Research Council, 2010). Rats were randomly divided into five groups (7 rats per four groups and 10 rats in DMBA group): group I was untreated and served as Control. Group II and IV served as Saline and nor-NOHA, respectively. Rats in group III (DMBA, 10 rats in the group) and group V (DMBA + nor-NOHA) were administrated intragastrical by gavage each with a single dose of 20 mg/ml DMBA (Sigma Chemicals, Sigma-Aldrich, USA), dissolved in 0.5 ml olive oil and 0.5 ml saline given in a volume of 1 ml (Angeline Kirubha et al., 2012; Arroyo-Acevedo et al., 2015; Roy et al., 2016; Steele et al., 2005). Rats in DMBA + nor-NOHA group were injected by nor-NOHA intraperitoneally for 5 weeks (after 10 days of DMBA administration, every 3th day, from 2 to 8 weeks after DMBA) in dose of 3 mg/kg/day body-weight in 0.25 ml saline. Rats were palpated weekly to check for tumor appearance (detected first approximately 95 days (the 13th week) after the DMBA administration). The DMBA-induced breast cancer model in the rat is used for the study of mammary carcinogenesis because it closely mimics human breast disease (Alvarado et al., 2017; Barros et al., 2004). Different parameters (arginase activity, polyamines, nitrite anions, MDA, $NH4^+$, proteins) have been analyzed in blood serum and calculated for 1 ml blood.

Reagents. Chemicals for breast cancer induction (7,12-Dimethylbenz[*a*]anthracene, Sigma-Aldrich, **D3254**, **Cas number** 57-97-6), arginase inhibition (N-ω-Hydroxy-L-norarginine acetate salt, BACHEM AMERICAS INC, Catalog number F-3685.0050BA, **CAS Number:** 189302-40-7) histopathological evaluation, determination of arginase activity, MDA, proteins and nitrite anions quantity, TLC of polyamines were obtained from Sigma-Aldrich Co. Ltd. (Germany) and Carl Roth GmbH + Co. KG (Germany).

Tumor inhibition study. The effect of nor-NOHA on DMBA-induced tumors were determined at 5th, 8th, 13th, 16th and 20th weeks after DMBA administration. The experimental design and treatment scheme have been presented in Table 1. The concentration of nor-NOHA was chosen based on literature data (Cells et al., 2000; Ng et al., 2018; Prati et al., 2012; Secondini et al., 2017). The experimental rats were regularly monitored for food and water consumption, the apparent signs of toxicity, weight loss, or mortality. At the end of the 145 days (the 20th week, after 7,12-DMBA administration), rats in all groups were killed by cervical dislocation under anesthesia (Koch, 2006; Krishnamoorthy and Sankaran, 2016; Suckow et al., 2006).

Intraperitoneal injection into the laboratory rat. Intraperitoneal injections of nor-NOHA into the laboratory rat were performed by rat care protocols (Koch, 2006; Suckow et al., 2006).

Sampling blood from the Lateral Tail Vein of the rat. Blood samples were sampled according to protocol of Lee and Goosens (Lee and Goosens, 2015).

Determination of arginase activity. Arginase activity in blood was determined by the colorimetric method of Van Slyke and Archibald with some modifications (Avtandilyan et al., 2018). 1.5 ml 0.2 M glycine buffer (pH 9.5), 0.5 ml blood serum, 0.2 ml 5 μM $MnCl_2$, and 0.4 ml 50 μM L-arginine were added in test tubes. In the supernatant, the final product of the catalysis was determined, which was urea. 1 ml supernatant and 0.25 ml 3% (*w/v*) diacetylmonoxime (DAMO) were added and boiled in a water bath for 45 min. The intensity was measured with a spectrophotometer at 487 nm. Activity of enzyme was evaluated with the received urea in micromoles in 1 s (kat).

Dansylation and thin layer chromatography (TLC) analysis. The method of Seiler was used with some modifications, as follows (Morgan, 1997). Tissues were extracted in 0.2 M cold $HClO_4$ at a ratio of about 100 mg/ml. After extraction for 1 h in an ice bath, samples were centrifuged for 20 min at +4°C and 11.500 g (*r*av = 11 cm). 200 μl of $HClO_4$ extract was mixed with 400 μl of dansyl chloride (5 mg/ml acetone), and 200 μl of saturated sodium carbonate was added. Dansyl polyamines were extracted in 0.5 ml benzene and vortexed for 30 s. Up to 50 μl of dansylated extract was loaded on the preadsorbent zone of silica gel plates, and the chromatogram was developed for about 2 h with chloroform-triethylamine (25:2 *v*/v) solvent system. The dansyl polyamine bands were scraped, eluted in 2 ml ethyl acetate, and quantified in 505 nm. The quantity of polyamines is presented in nM

Table 1
Experimental design and treatment model.

Grouping	Number of rats in each group	Experimental design	20 mg/ml per rat, DMBA intragastrical by gavage	Treatment by nor-NOHA
Group (I)	7	Control	–	–
Group (II)	7	Saline (starting in 70th day, 5 weeks, each 3th day, 0.25 ml)	–	–
Group (III)	10	DMBA group	in 55–60 day old, a single dose	–
Group (IV)	7	3 mg/kg/day nor-NOHA	–	starting in age 70th day, 5 weeks, each 3th day
Group (V)	7	DMBA + 3 mg/kg/day nor-NOHA	in 55–60 day old, a single dose	starting in age of 70th day, 5 weeks, each 3th day

polyamines in 1 ml of serum.

Griess Assay for NO Quantity. Nitrite was measured by the Griess assay, as described (Vodovotz, 1996). Briefly, 100 µl Griess reagent was added to 100 µl of each of the above supernatants. The plates were read at 550 nm against a standard curve of $NaNO_2$. The values were corrected for the $NO2^- + NO3^-$ content of water, and the recovery of $NO2^-$ was calculated.

Estimation of lipid peroxidation. Estimation of lipid peroxidation was assayed spectrophotometrically using thiobarbituric acid-malondialdehyde assay (TBA-MDA) with some modification according to Ohkawa method (Zeb and Ullah, 2016).

Determination of ammonia in blood serum. Ammonia in blood serum was determined with the indophenol direct according to Huizenga method with some modification for blood (Huizenga et al., 1998).

Histopathological examination. The tissue pieces were spread on glass slides, stained with hematoxylin and eosin (Sigma-Aldrich Co. Ltd., hematoxylin stains, Procedure No. GHS) viewed under light microscope and photographed. All palpable tumors were excised and fixed in 10% buffered formalin and processed for histopathological evaluation. Paraffin sections of the excised tissues were stained with hematoxylin-eosin. Histopathological examination, tumors types and microscopic reporting was established by the recently published protocols (Costa et al., 2002; Russo and Russo, 2000).

Data processing. Data are presented as mean ± standard error of the mean. Tumor growth, body weight, mortality, serum biochemical analyses evaluated by statistical ANOVA analysis with multiple comparisons using Statistica software (StatSoft 10.0). A p value of < 0.05 was considered significant.

3. Results and discussion

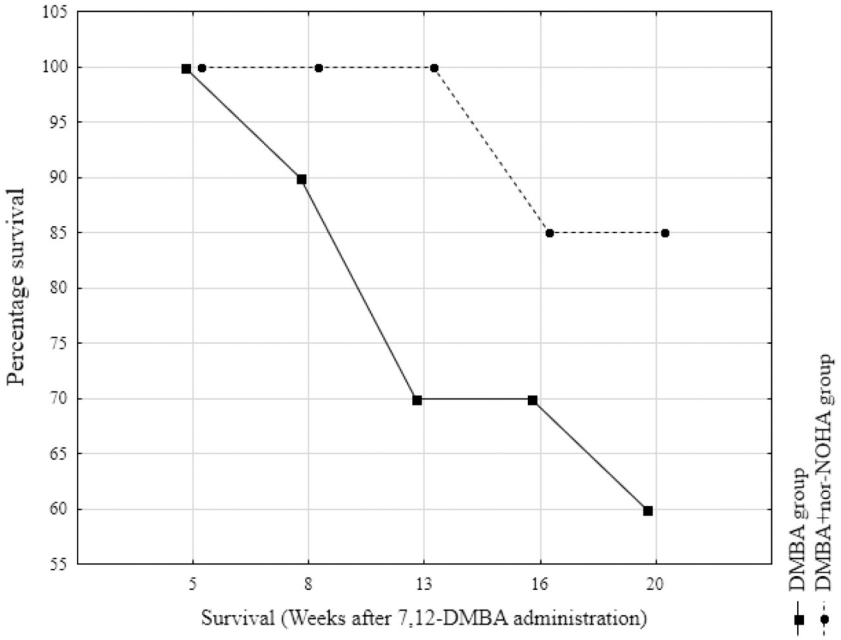
3.1. Arginase activity, NO and polyamines quantities downstream by nor-NOHA attenuate tumor growth, quantity and mortality rate

During the course of the experiment mortality registered in both of the treatment groups (groups III and V). There was difference in the timings of the deaths in DMBA and DMBA + nor-NOHA groups. Death in DMBA group (group III, 60% survival) occurred at 8-9th, 12-13th and 19-20th weeks, and in contrast, in the DMBA + nor-NOHA group (group V, 85% survival), death occurred at 16-17th week after DMBA administration (Fig. 1, a).

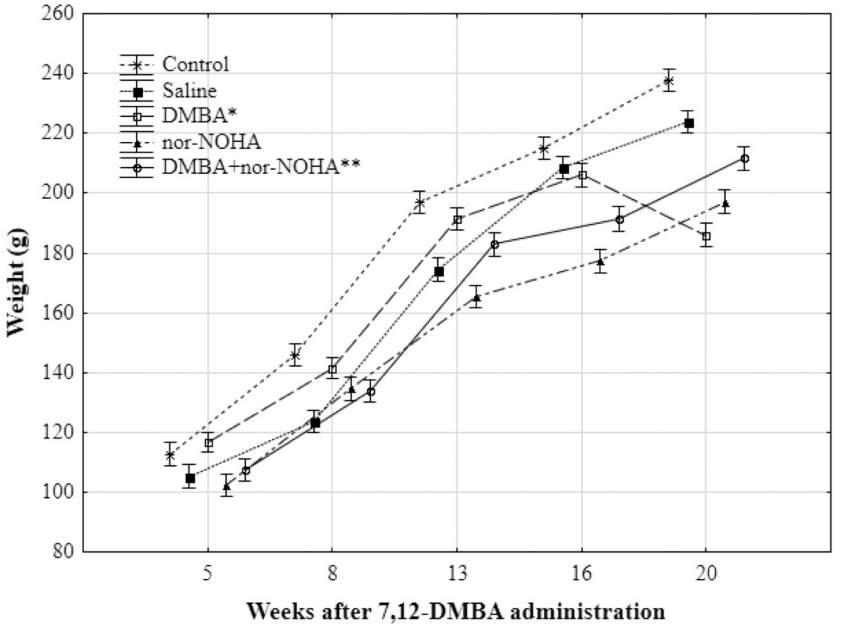
In contrast to Control group in DMBA group is observed decrease of rat body mass during 8–20 weeks (Fig. 1, b). Comparing with nor-NOHA group in DMBA group is seen body mass gain during all the weeks, except 20th weeks, where is observed weight loss. In DMBA + nor-NOHA group comparing to DMBA group is watched loss of weight at 8th, 13th, 16th and body mass gain in 20th weeks. In conclusion, rats' weight in treatment group DMBA + nor-NOHA is close to rats in Control and Saline groups. The quantitative analysis of tumors after 20 weeks showed that in DMBA + nor-NOHA group rats' tumors quantity and size were significantly decreased comparing to DMBA group (Fig. 1, c and d). Particularly, 20 weeks after DMBA administration, in DMBA + nor-NOHA group total tumors' quantity (Fig. 1. c) and size (cm^2) (Fig. 1. d) were approximately decreased by two times, comparing to DMBA group. According to our data we assume arginase and NOS cooperate to restrain polyamine and NO for cancer progression.

We have investigated the activity of arginase and nitrite anions quantity (which indicates NOS activity) in blood in all experimental groups at different weeks after 7,12-DMBA administration (Fig. 2, a and b). In blood at 8th, 13th, 16th and 20th weeks after DMBA administration were found to have high arginase activity and nitrite anions quantity. In DMBA group increased blood arginase activity was increased to 98.7% at 13th and 90.7% at 20th weeks comparing to the Control group ($p < 0.01$). Co-treatment with nor-NOHA stopped these

increases, resulting in mean values close to those of the Control and Salin groups (Fig. 2, a). After injection of nor-NOHA, in DMBA + nor-NOHA group is shown significant decrease of arginase activity, in contrast to DMBA group by 67.88%, 70.25%, 67.15%, 59.18% and 50.75% at 5th, 8th, 13th, 16th and 20th weeks, correspondingly ($p < 0.05$ for all) (Fig. 2, a). In DMBA group increased blood NO_2^- level at 5th (4.9%), 8th (34.9%), 13th (78.2%), 16th (69.1%) and 20th (63.5%) weeks after DMBA administration ($p < 0.01$ for all) comparing to the Control group. Treatment with nor-NOHA in group V decreased blood NO_2^- quantity (close to Control and Saline groups) at 8th (29.5%), 13th (49.9%), 16th (52.3%) and 20th (44.7%) weeks after DMBA administration ($p < 0.05$ for all) comparing to the DMBA group (Fig. 2, b). It was very important to study the interaction between arginase and NOS at period of cancer and its treatment. According to literature data arginase and NOS can cooperate to restrain T-lymphocyte functions in tumor-bearing hosts by altering the production of reactive nitrogen and oxygen species (RNS and ROS, respectively) (Chioda et al., 2013). The molecular bases for the synergism between

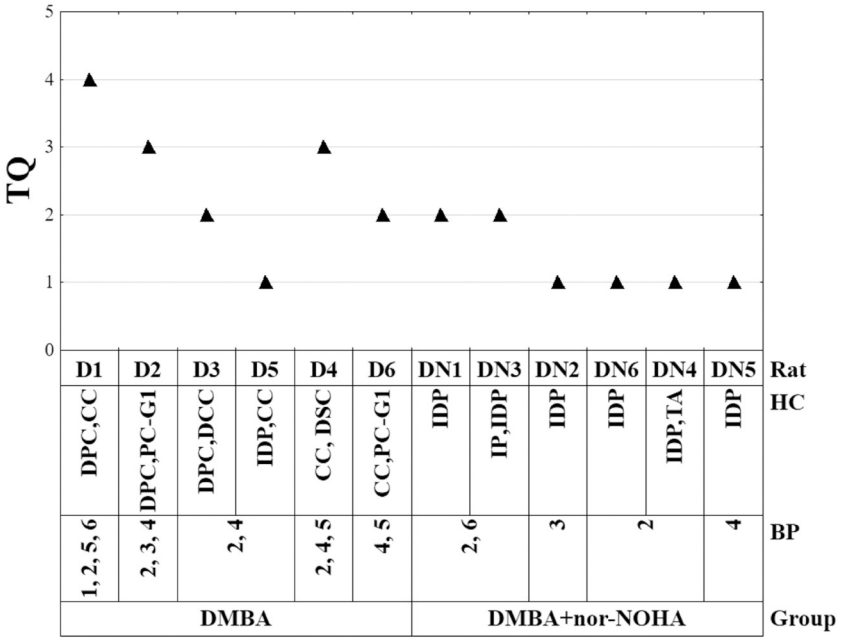


a



b

Fig. 1. Survival percentage *(a)* and the change of animal weight *(b)* in all experimental groups at 5th, 8th, 13th, 16th and 20th weeks after DMBA administration (28 weeks old rats), (n = 7 for Control, Saline and nor-NOHA groups, $p < 0.05$, * – $p < 0.01$, n = 10 (at 5th week), n = 9 at 8th week, n = 7 at 13th and 16th weeks, n = 6 at 20th week, ** - n = 7 at 5th, 8th, 13th weeks, n = 6 at 16th and 20th weeks). Quantity *(c)*, size (summary in 1 rat, *d*), allocation (BP – pair of breasts) and histological classification (HC) of tumors in each rats of DMBA (D) and DMBA + nor-NOHA (DN) groups at 20th week after 7,12-DMBA administration (28 weeks old rats). DPC - ductal papillary carcinoma (DCIS-Ductal Carcinoma *in Situ*), DSC - ductal solid carcinoma, DCC - ductal comedocarcinoma, IDP - Intraductal Proliferation, CC - Cribriform Carcinoma, PC – G1 - papillary carcinoma grade 1, IP – intraductal papilloma, TA - tubular adenoma. Histological analysis is described in «Histopathological alteration of breast tissues is stopped by nor-NOHA treatment» section and shown in Fig. 4.



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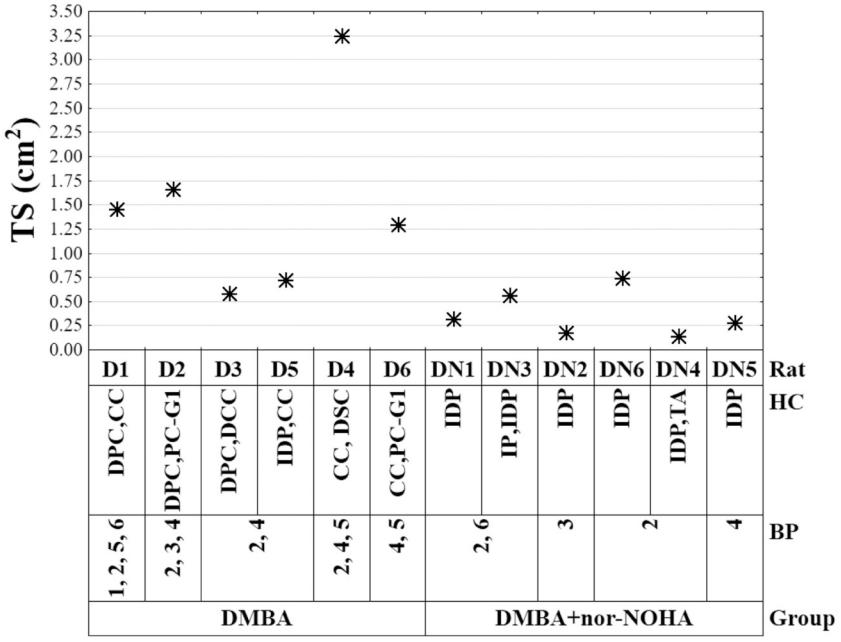


d

Fig. 1. (*continued*)

these enzymes are still not yet entirely known (Engblom et al., 2016). Even though it is still under debate whether arginase and NOS could be active in the same cell. Low extracellular L-arginine concentration, overexpression of arginase, or reduction of L-arginine uptake can decrease intracellular L-arginine concentration and halt translation of NOS2 mRNA, a phenomenon known as the arginine paradox (Bonavida, 2015; Chioda et al., 2013). The importance of the obtained our results is that nor-NOHA is inhibiting NOS activity, what is shown by decrease of nitrite anions quantity, which once more emphasizes the important relationship between these two enzymes in L-arginine metabolic pathway during breast cancer treatment. Nor-NOHA is not a substrate for any of the three NOS isoforms and does not inhibit nNOS or iNOS (Caldwell et al., 2015). Taking into account the above mentioned and our results, it can be noted, that the reduction of nitrite anion's amount after the administration of arginase activity inhibitor, can be a reason of L-arginine paradox phenomenon and breast cancer regression.

Our previous results have shown that human serum arginase activity and polyamines quantities were increased in parallel with cancer stage progression and decreased after neoadjuvant chemotherapy (Avtandilyan et al., 2018). Targeting the polyamine metabolic pathway for antitumourigenic therapy started soon after the discovery that dysregulated polyamine levels were assay mark for different tumor types (Nowotarski et al., 2013). We suggested that arginase inhibition has antitumour effects on breast cancer development as it inhibits polyamines levels, precursors of cancer cell proliferation and metastasis.

The effect of polyamine depletion by nor-NOHA in rats' blood was assessed by TLC. Quantitative changes of polyamines (nM/ml blood) in

cancer and treatment period are shown in Fig. 2 (c). Results showed that after 5, 8, 13, 16 and 20 weeks later of DMBA administration, quantity of blood putrescine, spermine and spermidine is increased in DMBA group in contrast to Control group. In DMBA group increased blood total polyamines levels at 13th (11.9%), 16th (22.5%) and 20th (35.7%) weeks (increased parallel to tumor growth) comparing to the Control group. In DMBA + nor-NOHA group blood putrescine quantity in contrast to DMBA group is decreased by 21.5%, 19.6%, 17.23%, 15.83% and 17.12% at 5th, 8th, 13th, 16th and 20th weeks, correspondingly. Spermidine amount reduction in contrast to DMBA group is by 7.77%, 10.16%, 14.49%, 22.16%, 23.3% and spermine amount reduction is by 26.2%, 28.96%, 27.08%, 27.16%, 16.3% after 5, 8, 13, 16, 20 weeks, correspondingly (see Fig. 2, c).

DMBA is a potent carcinogen induces DNA damage. In the cells, the reactive metabolite DMBA-3,4-dihydrodiol-1,2-epoxide (DMBA-DE) adds adenine and guanine residues to DNA. We have no data whether nor-NOHA interaction with 7,12-DMBA or its metabolites is possible in the cells; information on the indirect effect is also absent. In this study, we have shown that the inhibition of arginase activity by nor-NOHA suppresses tumor progression. Patients with increased polyamine levels either in the blood or urine are reported to have more advanced disease and worse prognosis compared to those with low levels, regardless of the type of malignancy (Soda, 2011). Because polyamines are essential for cell growth, the increased capability of polyamine synthesis could reflect enhanced tumor proliferation. Therefore, inhibition of polyamine synthesis and availability by cancer cells could retard cancer cell growth. The arginase enzymes are capable of producing ornithine for the production of polyamines and may hold important regulatory functions in the maintenance of this pathway. Therefore, we conclude that arginase inhibition influences on L-arginine methabolic pathway (especially on polyamine biosynthesis) in cancer cells. We have assumed that decreased polyamines and NO quantity by nor-NOHA are

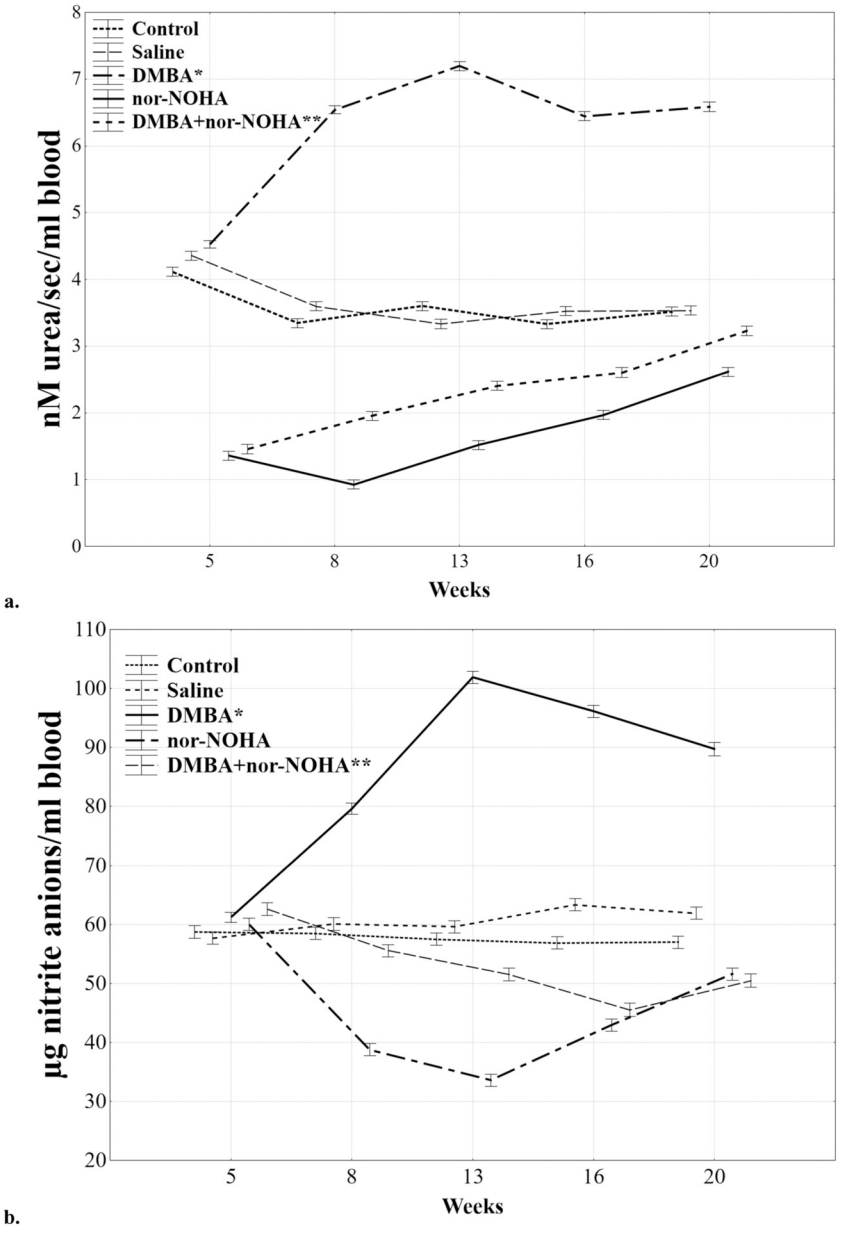
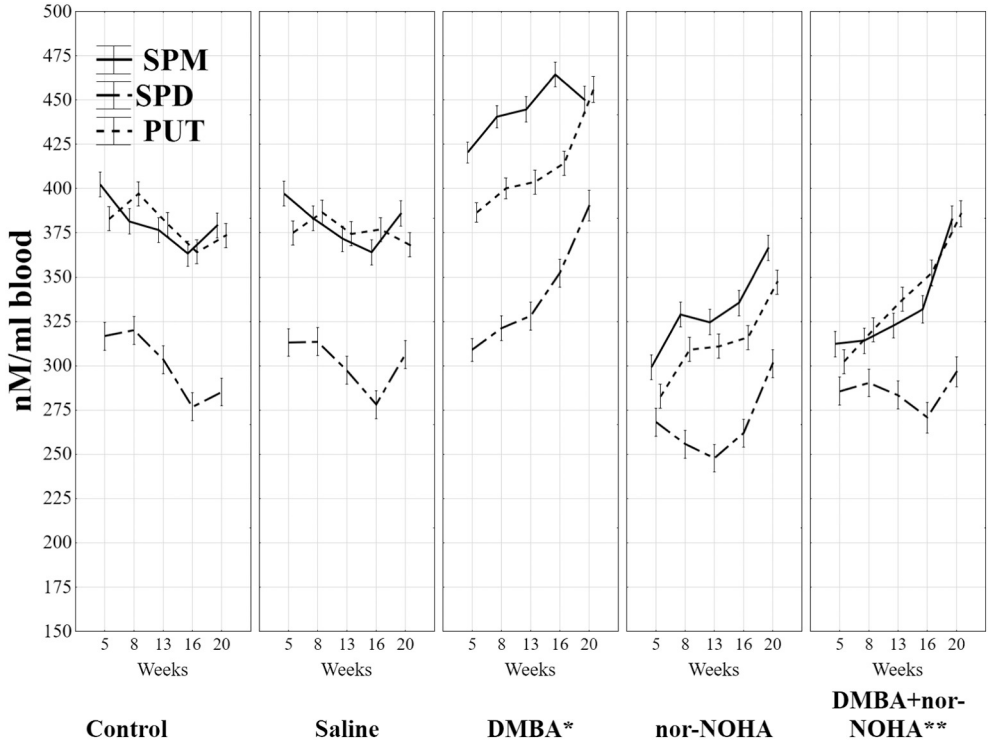


Fig. 2. The change of arginase activity (a, $p < 0.05$), nitrite anions (b, $p < 0.05$) and polyamines (c, $p < 0.05$) quantities downstream after nor-NOHA injection in blood in experimental groups at key weeks (x) after 7, 12 – DMBA administration (8 + x weeks old rats, n = 7 for Control, Saline and nor-NOHA groups), * –$p < 0.01$, n = 10 (at 5th week), n = 9 at 8th week, n = 7 at 13th and 16th weeks, n = 6 at 20th week, ** –$p < 0.05$, n = 7 at 5th, 8th, 13th weeks, n = 6 at 16th and 20th weeks). PUT – putrescine, SPD – spermidine, SPM – spermine.



c.

Fig. 2. (*continued*)

attenuated tumor growth, quantity, progression and mortality rate.

3.2. Arginase activity inhibition by nor-NOHA in vivo does not cause oxidative stress

The risk of hyperammonemia and cell membrane damage by nor-NOHA was evaluated as in control and as well as in treatment groups. Would the carcinogenesis be accompanied by oxidative stress as signaled by malondialdehyde (MDA) and the concentration of ammonia in rat blood? Would treating the rats with arginase inhibitor nor-NOHA affect the oxidative responses?

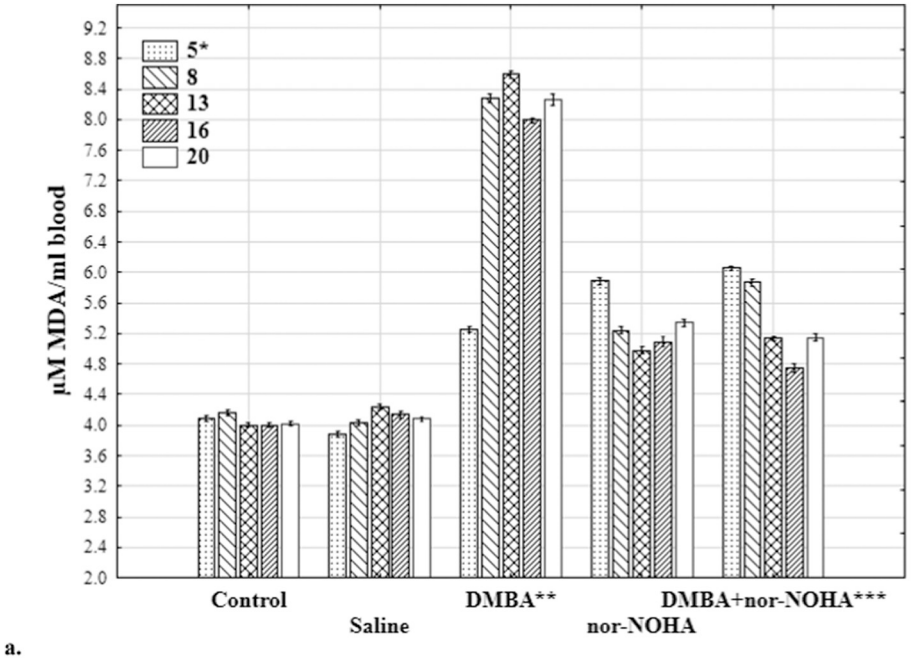
Treatment with DMBA in group III significantly increased blood MDA and $NH4^+$ levels at 5th (29.3% and 45.1%, respectively), 8th (98.7% and 48.7%), 13th (115.1% and 40.8%), 16th (100.3% and 21.9%) and 20th (96.8% and 29.1%) weeks after DMBA administration ($p < 0.05$ for all) comparing to the Control group. Importantly, subsequent co-treatment with the arginase inhibitor nor-NOHA blocked these increases in group V, resulting in mean values close to those of the Control groups for MDA (increased 10.2% at 20th week, comparing to Control group; $p < 0.05$) and leading to an even lower mean for NH_4^+ (decreased 4.6% at 20th week, $p < 0.05$) (Fig. 3). In contrast to DMBA group in DMBA + nor-NOHA group is shown MDA amount reduction by 28.9%, 38.9%, 38.8% and 39% at 8th, 13th, 16th and 20th weeks, correspondingly. At 5th week is obtained up-regulation of MDA by 15.3%. The results show decrease of ammonia quantity in DMBA + nor-NOHA group comparing to DMBA group by 7.8%, 19.7%, 9.26% and 18.9% in the 8th, 13th, 16th and 20th weeks, correspondingly, and increase of ammonia quantity at 5^{th} week by 22.4% (see Fig. 3). We conclude that arginase inhibition by nor-NOHA *in vivo* don't occure oxidative stress in DMBA-induced mammary cancer groups and slight increase of ammonium concentration in blood after treatment with nor-NOHA at different weeks in experimental groups is shown.

3.3. Histopathological alteration of breast tissues is stopped by nor-NOHA treatment

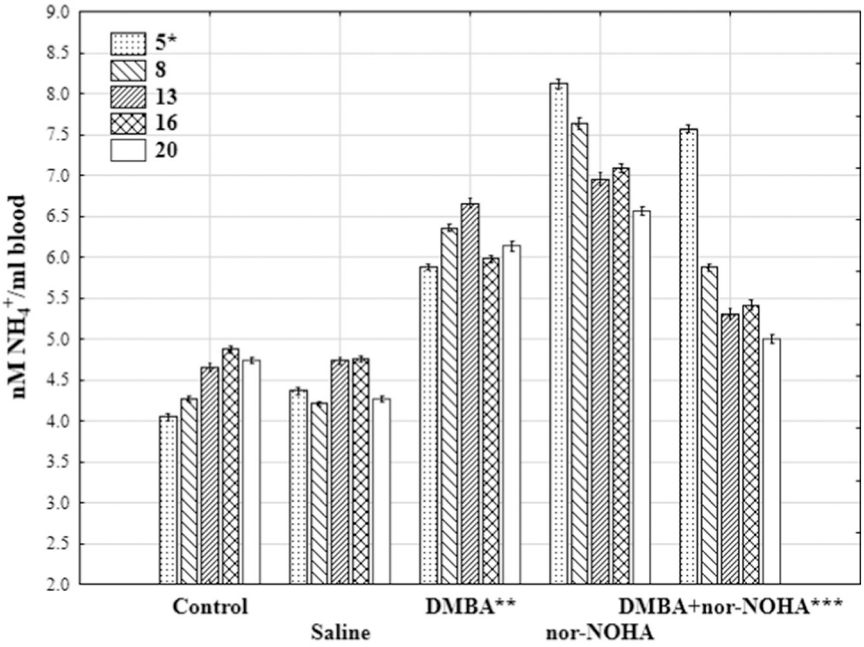
The histopathological examination in mammary gland samplings at the 20th week in all experimental groups of rats has been done and presented in Fig. 4. according to (Costa et al., 2002; Roy et al., 2016; Russo and Russo, 2000). Rat mammary tumors may be composed of a single histologic type or of combinations of several patterns. In our experimental model, all of the tumors showed only epithelial neoplasms (benign and lesions and precancerous in DMBA + nor-NOHA group, malignant lesions in DMBA group). Most of the carcinomas are papillary and ductal. Papillary carcinoma is characterized by the epithelial papillary growth with scanty connective tissue. 7,12 - DMBA-induced ductal carcinoma *in situ* (DCIS) is involved in two (Fig. 4.a, *arrows*) and one adjacent areas (Fig. 4.i, *arrow*). The lumen of the structures is expanded and the epithelium indicates early papillary formations. The periductal connective tissue shows an intense desmoplastic reaction. The ductal structures are dilated, and the lining epithelium grows inward, forming epithelial papillae free of fibrovascular core (Fig. 4. a, *arrows*). The stroma, which is separated from the epithelium by a readable basement membrane, exhibits a slight to marked desmoplastic reaction and infiltration by lymphocytes (Fig. 4.b, d, *arrows*). In Fig. 4. (a and i) cells have a large, round nucleus. These early lesions sre called intraductal proliferations (IDPs). Comedo and cribriform patterns are shown simultaneously (Fig. 4. c, e and f). A papillary component is not presented. The lesions are spread ductal structures lined by a multilayered epithelium surrounding necrotic debris (Fig. 4. c, e and f, *arrows*). These lesions are described by intraductal growth of epithelium. Epithelial cells are proliferated in a solid pattern with formation of secondary lumina (ductal solid carcinoma (DSC), Fig. 4. d, *arrow*).

Papillary carcinomas grade 1 are composed of one to three layers of epithelial cells, which emit short epithelial papillae (Fig. 4. a, b, and d, *arrows*).

Alveoli in the tubular adenoma are surrounded by a small amount of connective tissue (Fig. 4.g, *arrow*). The epithelial surface lining the lumen is even and smooth. Intraductal papilloma is composed of



a.



b.

Fig. 3. The change of MDA (a) and $NH4^+$ (b) quantities in rats' blood in all experimental groups (* - weeks after 7, 12 – DMBA administration, 8 + * old rats, n = 7 for Control, Saline and nor-NOHA groups, $p < 0.05$, ** –$p < 0.01$, n = 10 (at 5th week), n = 9 at 8th week, n = 7 at 13th and 16th weeks, n = 6 at 20th week, *** - n = 7 at 5th, 8th, 13th weeks, n = 6 at 16th and 20th weeks).

papillary projections into the duct. The papillae are composed of a core of fibrovascular tissue that is lined by a single layer of low columnar epithelial cells (Fig. 4.h, *arrow*).

4. Conclusion

In this paper we have shown that the inhibition of arginase activity by nor-NOHA have significiant influenced on carcinogenesis. Our results have shown polyamines and NO quantity downstream by nor-NOHA attenuated tumor growth, quantity and cancer progression. In DMBA + nor-NOHA group the histopathological examination has revealed only benign and precancerous lesions. We conclude that our 3 mg/kg/day i.p. injection (5 weeks, 12 times) of nor-NOHA treatment model was shown to slow the growth of DMBA-induced mammary tumors. We have shown arginase activity inhibition by 3 mg/kg/day i.p. injection nor-NOHA *in vivo* does not cause oxidative stress and hyperammonemia. Results have also shown arginase and NOS could be active in the same microenvironment and cooperate to restrain polyamine and NO for cancer progression. Increasing knowledge of the interplay between arginase and NOS, suggests potential combination therapies that will have considerable clinical promise. In addition, arginase blocking therapy, a strategy that combines the inhibition of NO biosynthesis, can be more effective than therapies based on arginase activity inhibition alone and may involve an antitumour immune response. Taking into account the above mentioned, currently we have investigated the antitumor potential of the arginase and NOS activity inhibition by nor-

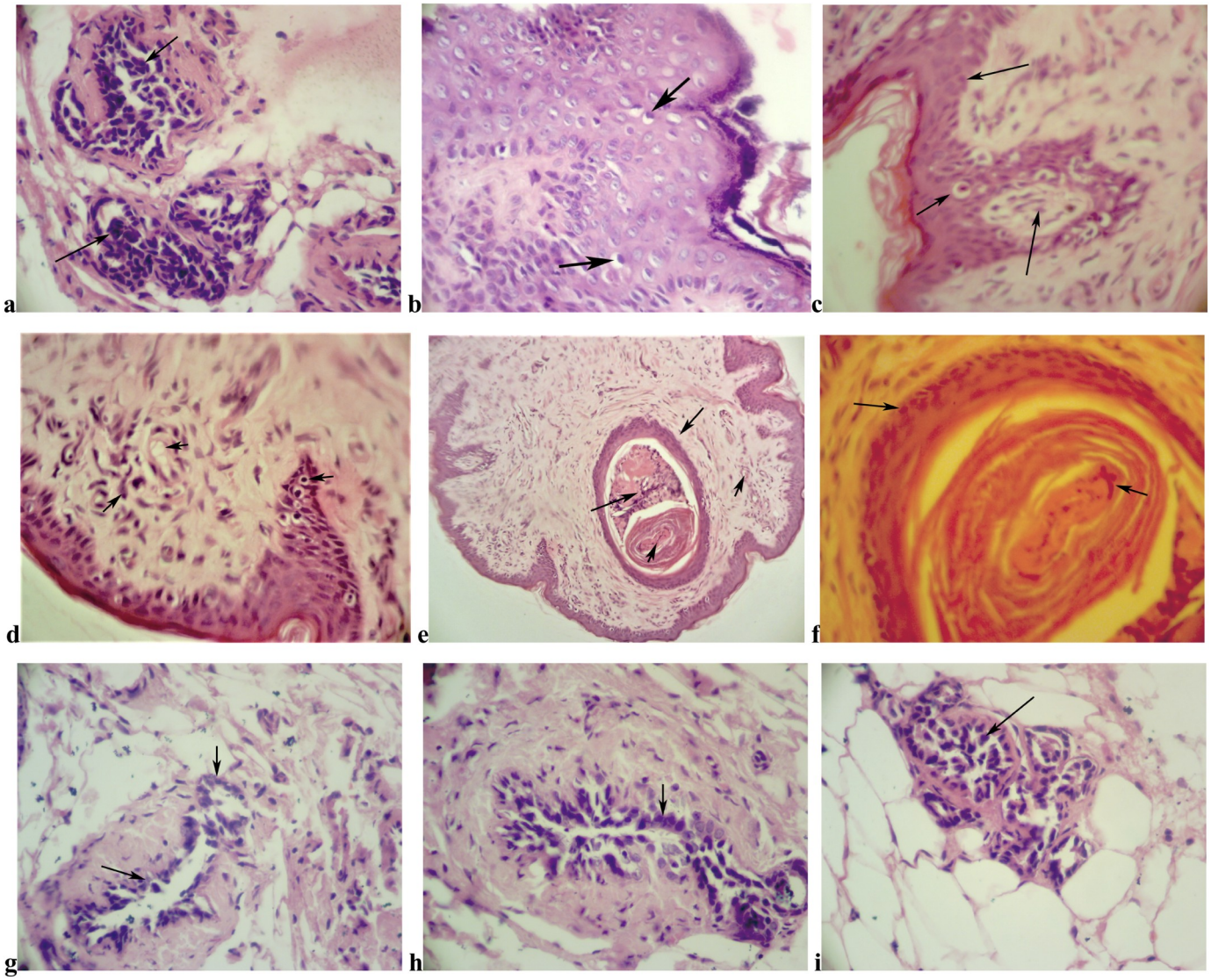


Fig. 4. Histological examination staining of mammary glands of experimental animals (28 weeks old rats) in DMBA (H&E x100; E, H&E x400; a, b, c, d, f) and DMBA + nor-NOHA groups (H&E x400; g, h, i) at 20th week after 7,12-DMBA administration. Histopathological alteration in DMBA group rats has revealed the ductal papillary carcinoma (DPC or DCIS) by numerous intraductal (IDP) proliferations (a, b and d), intraductal carcinoma (IDC, ductal solid carcinoma (DSC), cribriform and comedo type (ductal comedocarcinoma (DCC)), c, e and f) and papillary (invasive carcinoma) carcinoma grade 1 (PC G1, a, b, d). In DMBA + nor-NOHA group the histopathological examination has revealed benign (the tubular lactating adenoma and intraductal papilloma, g and h) and precancerous lesions (intraductal proliferation and DCIS) (h and i).

NOHA and L-NAME (N(G)-Nitro-L-arginine methyl ester, NOS inhibitor) against DMBA-induced mammary carcinogenesis in rats. These findings open up new avenues of research into exploiting aberrant L-arginine metabolism for anticancer therapy.

Data availability

The data supporting the conclusions of this article are included within this article.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Experiments were fulfilled according to the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No.8023, revised 1978). All experiments with animals were approved by the National Center of Bioethics (Armenia). The animals were kept at Animal care house, Faculty of Biology, YSU, Yerevan, Armenia. This article does not contain any studies with human participants performed by any of the authors.

Declaration Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors contributions

Nikolay Avtandilyan, Hayarpi Javrushyan, and Anahit Mamikonyan performed the experiments and analyzed the results. Nikolay Avtandilyan wrote the manuscript. Hayarpi Javrushyan edited the manuscript. The histopathological examination in mammary gland samplings in all experimental groups of rats have been done by Anna

Grigoryan. Armen Trchounian directed the research and revised and re-edited the manuscript. All authors read and approved the final version of the manuscript.

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