

**STUDY OF OVARIAN CHANGES IN RATS WITH MAMMARY CARCINOMAS**

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The aim of this study was to estimate ovarian changes in 7,12 dimethylbenz (a) anthracene (DMBA) induced rat mammary carcinomas. The study was carried out on female virgin albino Wistar rats (n=35, age=35-37days, body mass 120-140g), divided into control (n=10) and experimental group (n=25). Anesthetised animals of experimental group were inoculated with 2 mg mixture (1 mg of DMBA and 1 mg of cholesterol-buffer) into the fifth left mammary gland. The animals were sacrificed 90 days after implantation, and ovaries and mammary glands were investigated. Mammary gland carcinomas (in situ and/or invasive) were pathohistologically verified in 19 experimental animals. Histological, histochemical, and immunohistochemical (cytokeratin AE1/AE3 and PCNA) studies of ovaries were performed.

Besides non-neoplastic changes, such as decrease in ovary's volume, reduction in the rate of follicular development and numerous corpora lutea formation were found in the vicinity of preneoplastic changes: papillomatous epithelial hyperplasia and inclusion cysts, microglandular formations with dysplasia and seromucinous microcystic formation. Intensive diffuse PCNA expression was present in the epithelium of glandlike structures, follicular and inclusion cysts.

These morphological changes confirmed that DMBA is a pluripotent carcinogen capable to induce a wide spectrum of preneoplastic lesions in the ovaries. The present dilemma is whether the changes described are the consequence of the direct effects of DMBA or of hormonal activity of the induced breast carcinomas, or both. *Acta Medica Medianae* 2013;52(1):25-32.

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**Introduction**

Ovarian carcinomas are the first cause of death among all malignant tumours of the female genital tract, and the fourth leading cause of women's death from carcinomas. Besides the existence of powerful diagnostic techniques which are in use now (transvaginal doppler, computed tomography, 3D ultrasound, MRI, tumour markers) the percent of early detected ovarian carcinomas is very low. The reason for this high mortality is that they are discovered in the late stage when tumour have spread out of pelvis and have given peritoneal metastasis and ascites (1-3).

As for other numerous malignancies there is insufficient data about etiopathogenesis of ovarian carcinoma, because, in comparison with

other gynecological tumors, it is not completely defined how and which preneoplastic lesions influence the aforementioned high mortality and late detection of this tumors (3,4). Wang and Auersperg (2003) investigations point out that about 90% of women survive, if ovarian carcinomas have been diagnosed in an early stage (stage I) without changing of therapeutic treatment (5). Data from literature about simultaneous appearance of breast and ovarian carcinomas point out that these tumors have some dependent risk factors, and that women at increased risk for one of these carcinomas are often at risk for the other (6-8).

The aim of this paper was to study the ovarian changes in rat mammary gland carcinomas. For the purposes of our research, we used the well-known and successful model of mammary gland carcinoma induction with 7,12-dimethyl-benz(a)anthracene (DMBA) (9-13).

**Materials and methods****Animals**

The study was carried out on 35 female, virgin albino Wistar rats (weighting 120 ± 10 g, 35-37 day of age). The animals were raised in controlled laboratory conditions (in an animal room with a 12 h light/12 h dark cycle, at 22 ±

2°C). The animals had free access to the laboratory chow and tap water ad libitum. All procedures on animals followed the Guidelines for work on experimental animals approved by the Ethic Committee, Faculty of Medical Science in Kragujevac.

#### Experimental protocol

The rats were divided in two groups: the control group and the experimental group. Ten animals of control group did not undergo the treatment. In the experimental group there were twenty five animals. The mixture dimethylbenz(a)anthracene (DMBA) carcinogen (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and cholesterol-buffer (serving as a vehicle) was implanted by incision in the fifth left mammary gland of the anaesthetised rats. The amount of the inoculated mixture for each rat was 2 mg (1 mg of DMBA and 1 mg of cholesterol-buffer). After the implantation, the incision was closed with the surgery stitch.

After 90 days all the animals were sacrificed and their left fifth mammary glands and both ovaries were extirpated and sampled.

Macroscopic examinations and measuring of extirpated mammary glands and ovaries volume and diameter were performed. The mammary glands and ovaries were fixed in a Bouin solution for 24 hours and the complete material underwent routine preparations and paraffin embedding.

#### Micromorphologic examination

The 4µm sections of mammary glands and ovaries were deparaffinized, rehydrated and stained by haematoxylin and eosin (H&E). H&E sections were evaluated for general structure, preneoplastic and neoplastic changes.

Immunohistochemical reactions were performed with the use of antibodies against cytokeratin AE1/AE3 (1:400; Dako, Japan, Kyoto, Japan) and PCNA (1:800; Dako, Japan, Kyoto Japan). Immunostaining was performed by the avidin-biotin peroxidase complex (ABC) method (Vectastain ABC-Elite kit, Vector Laboratories, Burlingame, CA).

### Results

#### Macroscopic analysis

##### Mammary gland

The animals of control group had mammary glands of the nearly same dimensions, 0,3-0,5cm in diameter. Mammary glands were white-yellow in colour, with a glassy look on sections and solid consistence.

From 25 animals, in nineteen animals from the experimental group (76%) solid tumor was

found in the fifth left mammary gland. The tumor sized 1,2-1,7cm, had an oval shape, middle solid consistency, less mobile, and fixed to skin.

The mammary glands of six animals (24%) from the experimental group were enlarged, 0,7-1cm in diameter, clearly separated from the surrounding tissue and very solid consistency. They were covered with macroscopically intact skin.

##### Ovaries

Ovaries of the animals from the control group had smooth surface, solid consistency and attenuated capsule. The nineteen animals from the experimental group, in which solid tumour was found in the fifth left mammary gland, had enlarged ovaries of solid consistency, with visible microcysts. The ovaries of experimental animals which have not developed the tumour of mammary glands were not investigated.

#### Micromorphologic analysis

The microscopic examination of the mammary glands and ovaries of the animals from the control group showed no pathologic changes.

##### Mammary gland

The micromorphologic examination of the mammary glands, from the experimental group verified a spectre of hyperplastic-dysplastic changes on the level of ductules, ducts and acini, frequently giving the picture of adenomatosis. The noticeable hyperplasia of smaller ducts and ductules was followed by means of nucleus hyperchromasia. Hyperplastic acini often present themselves with epitheliosis. In some animals, lobules and ductules hyperplasia was more expressed and give the picture of microadenoma. These changes are known as fibrocystic disease and they were present in all experimental animals.

Mammary gland adenocarcinomas (in situ and/or invasive) were pathohistologically verified in 19/25 experimental animals (76%). The most frequent pathohistological finding is intraductal carcinoma that has been found in 52,6% (10 /19) of experimental animals. On the second place is ductal invasive carcinoma with 36,8%, (7/19). In the mammary gland of only one animal (1/19), the lobular "in situ" carcinoma has been found. Lobular invasive carcinoma (Figure 1) has been identified in only one (1/19) experimental animal.

##### Ovaries

Histological structure of ovaries of experimental animals with pathohistological confirmation of carcinoma "in situ" or invasive one was relatively preserved. Most of the follicles in the ovary are grouped as primordial and primary

follicles, but relatively few larger ones were seen, too. Small primordial follicles were hardly noticed on classically stained preparations. The clusters of primordial and primary follicles were seen in deeper regions of ovary cortex. In the cortex of ovary, a grown and mature follicles were present, characterized by the proliferation of the follicular cells, increase in size of ovum and formation of connective tissue capsula. In the superficial part of cortex, a few large mature antral and Graafian follicles were identified, but in some of them there was no ovum, or it was a granulosa. Multiplication of follicular cells form stratified multilayered epithelial cover. Granulosa cell were polyhedral or cuboidal with hyperchromatic nuclei which show high mitotic index. Numerous atretic follicles with ovum degeneration accompanied by chromatolysis and fragmentation of the nucleus, and this was followed by similar changes in the follicular cells. Some follicular cells were floating in the antral cavity. Present corpora lutea were pale in colour, and of different stage of maturation. The newly-formed corpora lutea were paler and luteal cells had more hyperchromatic nuclei with higher mitotic index. A lot of vacuolized luteal and thecal cells and fields of fibrosis were identified in the central cortical areas of ovary.

#### Preneoplastic changes

Preneoplastic changes were present in the ovarian surface epithelium, but also in the cortical structures of ovary. OSE have shown multifocal micropapillomatosis of classical and inverted type, with low dysplastic changes (Figure 2). Epithelial surface invaginations were identified too (Figure 3).

In ovarian cortex were dispersed gland-like formations with prominent histologic atypic changes: they were condensed, side by side and with very narrow lumen. Their epithelium was dysplastic, stratified with changed nuclear polarity that sometimes was at the luminal edge. In the large number of glands, papillomatous dysplastic epithelium was present (Figure 4). A lot of follicular cysts were identified and their epithelia were attenuated and stratified; however, granulosa cells were finely vacuolated, with clear cytoplasm partly luteinized. Under the thickened tunica albuginea, a lot of small seromucous and inclusion cysts covered with cuboidal epithelium were found.

#### Immunohistochemical findings

Intensive diffuse PCNA expression was present in oocytes and granulosa cells of follicles. According to that characteristic, primordial follicles were better seen on specimens. Identical expression of PCNA were found in new luteal bodies, epithelium of gland-like structures (Figure 4), follicular and inclusion cysts. Low expression PCNA was evident in atretic follicles and atretic corpora lutea.

Immunohistochemical expression of cytokeratins AE1/AE3 is intensive and present in all described precancerous lesions with exception of follicular cysts. Reaction is negative and in membrane granulosa cells.

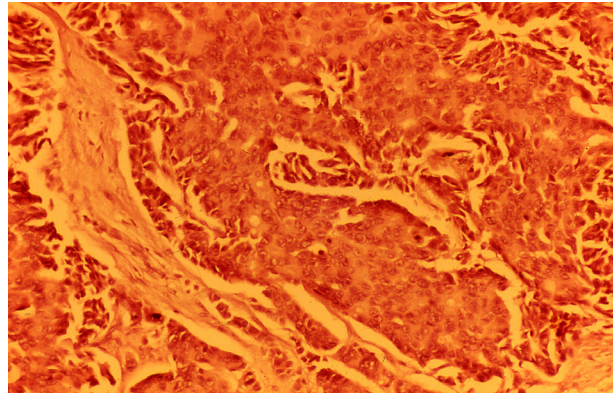


Figure 1. Invasive lobular carcinoma of mammary gland (HE X400)

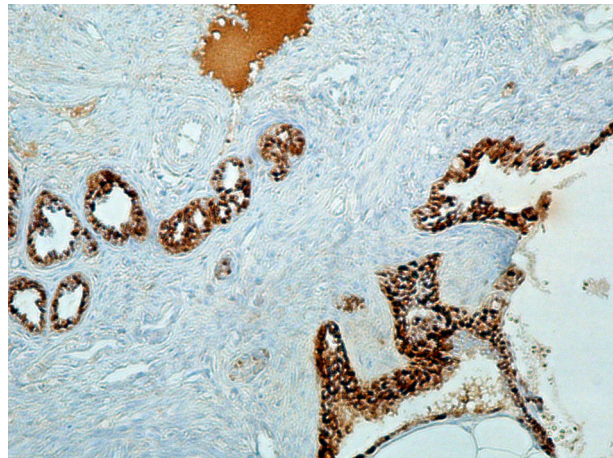


Figure 2. Strong positive cytokeratin immunostaining of ovarian surface epithelium and epithelial papillary inverted hyperplasia (ABC X 400)

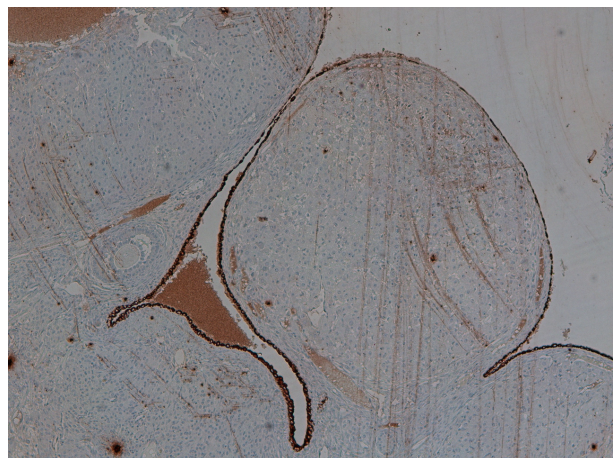


Figure 3. Cytokeratin positive immunostaining in papillary epithelium with very deep invagination (ABC X 400)

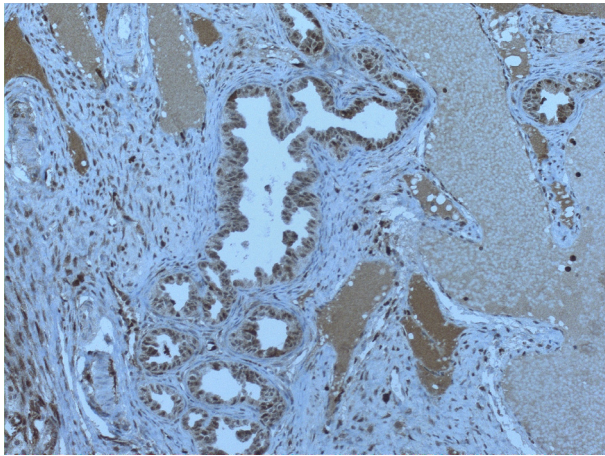


Figure 4. Adenomatoid formations with strong expression PCNA in stratificated epithelium (ABCX 300)

## Discussion

Model of breast carcinoma induced by the DMBA has proved to be a useful experimental system for studying pathogenesis and biological features of tumors (9-14). In our study, we used this model to examine early preneoplastic lesions in the ovaries of rats during the carcinogenesis of mammary gland.

After 90 days of DMBA implantation, all experimental animals were verified with pathohistological hyperplastic, dysplastic, cystic and metaplastic-squamous changes of mammary ducts and acini, lesions that are often registered (9,11,12,15) and that are included in the term of human pathology as "fibrocystic disease" of mammary gland (2,15).

DMBA-induced carcinomas were found in 76% (19/25) of our experimental animals. Intraductal carcinoma is the most frequent (52.6%), followed by ductal invasive carcinoma (36.8%). Lobular carcinoma "in situ" and invasive lobular carcinoma are found in only one animal. Lobular carcinomas, which are conspicuously less frequent in women than ductal carcinomas, have been only sporadically mentioned in DMBA-induced carcinogenesis in rats (10). Different incidence of DMBA-induced carcinomas in the literature can be explained by different study design. It is emphasized that the number of induced tumors depends on the type of carcinogen, dose, method of application, place of application, species, strain and age of experimental animals (9,13-16).

Researches of Ohi and Yoshida (1992) and Thompson et al. (1998), which showed that the incidence of mammary carcinomas is significantly low in ovariectomized rats, suggest that ovariectomy probably inhibits a step in the process of carcinogenesis, and that the initiation and promotion of chemical carcinogenesis of mammary glands can be affected by hormone factors (17,18). For women, the classification of breast carcinoma, based on hormone dependence, is used when

deciding which treatment the patient will receive, and it also has prognostic significance (18,19). It is believed that the chemically induced carcinogenesis of breast carcinoma is a good model for studying this aspect of the human disease, but also warns that the proportion of hormone-dependent carcinomas in rats is higher than in human females (18,21).

The cause of ovarian carcinoma is unknown, however, most epidemiological studies show that sterility, ovarian carcinoma in the family, breast and endometrial carcinoma in anamnesis increase the risk of malignant epithelial ovarian tumors (22,23). Understanding the pathogenesis of malignant ovarian tumors is difficult, due to a lack of sufficient number of samples in the early stage of disease and frequent diagnoses of advanced stages of tumor. Existence of precursor lesions that can be identified and that evolve into the ovarian carcinoma is still the subject of many debates in the literature (6, 22-25).

The first clinical descriptions of pathological precursor lesions in the ovary originate from Gusberg and Deligdisch (1984), who macroscopically investigated ovaries that looked normally at the first sight and they were prophylactically removed from identical twin sisters, the patients with invasive ovarian carcinoma (26). Superficial papillae, inclusion cysts, nuclear polymorphism, or stratification of superficial and invaginated epithelium are, besides in prophylactically removed ovaries, described also by other authors (5, 17).

Through pathohistological analysis of ovaries of animals with DMBA-induced mammary carcinoma, we diagnosed follicular multiple microcysts, papillary hyperplasia (classic and inverted) and invagination of ovarian surface epithelium (OSE), inclusion cysts, adenomatoid structures with papillomatose epithelial hyperplasia and dysplasia. All the identified hyperplastic lesions were well-limited, with low mitotic index and none of them was invasive. These characteristics clearly distinguish them from ovarian borderline tumors. Numerous studies emphasize that these changes represent precursors of ovarian carcinoma (4,5,27-29).

Cysts in the ovaries, in experimental conditions, except for DMBA, can be induced also by N-methyl-N-nitrosourea (MNA), and also with hormones such as androgens, or with hypothalamic lesions (18,27,29).

Polycystic vary syndrome (PCOS) is the most common endocrine disorder in women. It is characterized by the presence of enlarged ovaries with multiple cysts and hypervascular androgen-secreting stroma. Syndrome is associated with the signs of hyperandrogenism (hirsutism, alopecia, acne), disorders of the menstrual cycle (oligomenorrhoea or amenorrhoea) and obesity (3). Endocrinological characterizations are high levels of serum LH and testosterone (31). Ovaries are also affected by other endocrine factors, especially gonadotropins, insulin and other growth factors, which also depend on genetic and environmental influences (32,33).

Approximately 20% of women of reproductive age have polycystic ovaries (30). In women with PCOS, there exists a long-term risk for endometrial hyperplasia and endometrial carcinoma due to chronic anovulation (34). That can also be an increased risk for mammary carcinoma. Obesity, hyperandrogenism and infertility are common with PCOS and are also the risk factors for breast carcinoma (34). However, studies that have examined the link between PCOS and breast carcinoma have not always identified significantly increased risk. Relative risk of 3.6 was found only in post-menopausal women (35). There are long-term risks for developing diabetes and cardiovascular disease for PCOS patients (34,36). When it comes to connection between PCOS and ovarian carcinoma, the results of the few existing studies are contradictory. While one group of authors excludes such a possibility (34), others point out that PCOS carries a 2.5 relative risk for epithelial ovarian carcinoma (38).

In the ovaries of our experimental animals we pathohistologically diagnosed numerous inclusion cysts. Although it is well known that the presence of inclusion cysts in an older women is common (39), contradictions concerning whether they are preneoplastic lesions are still present in the literature. Okamura and Katabuchi (2001) suggest that the number of inclusion cysts increases in ovaries of patients with contralateral ovarian carcinoma, or with a family history of ovarian carcinoma compared with the healthy women (40), but Baracat et al (2000) examining the ovaries removed for prophylactic reasons with BRCA 1 mutation carriers, in comparison with the healthy ones, did not find a significant difference (41). However, most researchers agree that the inclusion cysts are the precursor of ovarian adenocarcinomas (1,4,16,26,28,42). It is believed that the inclusion cysts form the epithelial ovarian multiple invaginations (1,42-44).

In the ovaries of animals with DMBA-induced carcinoma, we have found micropapillomatose (classical and inverted), with a low degree dysplasia and invagination of OSE. High frequency of hyperplastic and metaplastic changes in the superficial epithelium of ovaries with contralateral ovarian tumors (92%), endometrial adenocarcinoma (76%), and polycystic ovarian disease (68%) have been notified by Rest et al. (1993), with the suggestion that hyperplastic and metaplastic superficial epithelial changes can be considered precursors of epithelial ovarian tumors (44). Other earlier studies that used light microscopy and cytometry showed cellular and nuclear atypia in non-cancerous OSE in the vicinity of the primary ovarian tumors (46).

OSE surrounds and protects the ovaries, and is important for maintaining the structure of the ovary. However, the OSE is responsible for

the transportation of nutrients and postovulatory repair of wounds. Despite the fact that it performs important endocrine and reproductive functions, OSE provides progenitor cells for 85-90% of the human ovarian carcinomas. OSE cells cover the stroma and are in close interaction with the hormones that are secreted by the ovary (3,25,47). Tissue culture has long shown that normal OSE cells are highly sensitive to signals from the environment and tend to undergo epithelial-mesenchymal conversion over time (48,49).

Adenomatoid structures that are verified in the ovaries of our experimental animals and they have been rarely mentioned in the literature (24,50). Multiplied atypical glandular structures with papillomatosis and dysplasia that match dysplastic papillary adenomas of the ovaries in women have not been described by other authors. Our results show that 36.8% (7/19) of animals have these preneoplastic lesions. Their histogenesis is unknown, but we believe that they are directly related to the occurrence of inclusion cysts. The identical topography of inclusion cysts and adenomatoid proliferation, as well as their microroglandular structure, supports this opinion. Intensive expression of PCNA in them, expresses the malignant potential of adenomatoid structures, and positive cyokeratin staining defines their epithelial origin.

When it comes to the etiopathogenesis of ovarian carcinomas, from a number of hypotheses offered in the literature, it seems that the most dominant one (25,47-50) is the one offered by Cramer and Welch (1983). They assume that the first step of carcinogenesis is the invagination of ovarian surface epithelium into the ovarian stroma. The second step is the differentiation, proliferation, and maybe malignant alteration of involved epithelium, stimulated by gonadotropins and estrogens (51). However, the stimulus for malignant transformation remains unidentified.

It is well known that the disorder of gonadotropin secretion and the stimulation of stroma may occur as a result of impairment of the normal feedback mechanism between the ovary and pituitary gland (17,18,21,29,30). Disorders of pituitary-gonadal axis can be caused by chemotherapeutics and radiation (54,53) and a number of cytotoxic substances that either act directly on the stimulation of pituitary secretion or increase the degradation of estrogen in the liver (51-54).

DMBA is a potent carcinogen for which it has been proven to definitely cause carcinomas of skin, mammary glands, ovaries and stomach in experimental animals (11-14,55-59). It is believed that DMBA through the formation of the DNA adduct induces starting point mutations that alter expression and/or activity of a large number of oncogene and tumor suppressor genes (24).

The relevance of DMBA-induced carcinogenesis model is supported by the finding of specific mutations in the p53 tumor suppressor genes in DMBA-induced ovarian tumors, which are normally among the most common mutations in human ovarian tumors (24,42,57). However, it urges caution when selecting animals for the experiment due to differences between rats and primates. The most striking difference is the relative absence of juvenile hiatus of gonadotropin secretion (FSH and LH) in rats, which is a characteristic of primates. It is also important to note that the first ovulation in laboratory rats occurs 35-45 days after birth (60).

## Conclusion

On the ovaries of our experimental animals histopathological lesions were identified, which can be labeled as precancerous in human pathohistology. The present dilemma is whether the changes described are the consequence of direct effects of DMBA, or hormonal activity of induced breast carcinomas, or both factors. Further research is required, with successive monitoring of experimental animals, in order to find the mechanisms that enable the transformation of precancerous lesions into ovarian carcinoma.

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## PROUČAVANJE PROMENA U JAJNICIMA KOD PACOVA SA KARCINOMOM DOJKE

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Cilj ovog rada bio je proučavanje promena u jajnicima kod pacova sa karcinomom dojke izazvanim dimetilbenz(a)antracen (DMBA). Za istraživanje su korišćeni ženski, nepareni, beli Wistar pacovi (ukupno 35, starosti 35-37 dana, telesne mase 120-140gr), koji su bili podeljeni u kontrolnu (ukupno 10) i eksperimentalnu grupu (ukupno 25). Anesteziranim životinjama eksperimentalne grupe implantirano je 2 mg mešavine (1mg DMBA i 1mg holesterolskog pufera) u petu levu mlečnu žlezdu. Životinje su žrtvovane nakon 90 dana od implantacije, nakon čega su ispitivani jajnici i mlečne žlezde. Karcinomi mlečne žlezde (in situ i/ili invazivni) su patohistološki bili verifikovani kod 19 eksperimentalnih životinja. Urađena su histološka, histoheмиjska i imunohistoheмиjska istraživanja na jajnicima. Upotrebljavana su antitela na citokeratine AE1/AE3 i PCNA.

Pored neneoplastičnih promena, kao što je smanjenje veličine jajnika, redukcija brzine folikularnog razvoja i formacija žutog tela, pronađene su i sledeće preneoplastične promene: papilomatozna hiperplazija epitela, inkluzione ciste, mikroglandularne formacije sa displazijom i seromucinozne mikrociste. U epitelu glandolikih struktura, folikularnih i inkluzionih cisti prisutna je intenzivna, difuzna ekspresija PCNA.

DMBA je pluripotentan karcinogen sposoban da izazove širok spektar preneoplastičnih lezija u jajnicima, ali je prisutna dilema da li su opisane promene posledica direktnog dejstva DMBA ili hormonske aktivnosti indukovanih karcinoma dojke ili su, pak, u pitanju oba faktora. *Acta Medica Medianae* 2013;52(1):25-32.

**Ključne reči:** pacovi, mlečna žlezda, jajnici, karcinom, DMBA