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Modified Estrogen-Progesterone Induction Method of Mammary Gland Hyperplasia in Rats

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In female Wistar rats, mammary gland hyperplasia (MGH) was modeled according to a modified protocol involving estrogen-progesterone induction and taking into account the duration of the estrous cycle of this animal species. MGH was induced over four 7-day cycles; each cycle included subcutaneous administration of 17β-estradiol (0.5 mg/kg) for 4 days, injection of progesterone (5 mg/kg) on day 5, then 2 days without injections. In females with MGH, a significant increase in the height and diameter of the nipples of the mammary glands was recorded, two types of changes were observed in the gland tissue: tubuloalveolar and lobuloalveolar hyperplasia. The study confirmed the development of MGH in rats by a modified method.

Key Words: *model; mammary gland hyperplasia; hormones; breast; hyperplasia*

Mammary gland hyperplasia (MGH) was first described by the English surgeon and anatomist Astley Cooper in 1829, but its etiology and pathogenesis are not fully understood. The term MGH encompasses a group of benign diseases characterized by shifts of the ratio of the epithelial and connective components in the mammary gland tissue resulting from proliferative and regressive processes. The formation of fibrotic, cystic, and proliferative changes in the mammary gland is also observed. The most commonly used synonyms for this pathology are "fibrocystic breast disease", "benign breast disease", "fibroadenomatosis" and "dyshormonal hyperplasia". The clinical and radiological classification of MGH differs according to the predominance of a particular component: glandular (adenosis), fibrotic, cystic, mixed (fibrocystic), sclerosing adenosis, and fibrocystic nodular MGH. Morphologically, two main forms of benign dysplasia are distinguished: non-proliferative and proliferative [1]. MGH is the most common pathology among all breast diseases, and it is mostly diagnosed in women aged 30-40 years [2]. The incidence of MGH ranges from 60 to 80% [3]. Proliferative MGH is considered a risk factor for breast cancer [4,5].

High prevalence of this pathology dictates the need of the search for preventive measures. In preclinical drug trials, rodents, particularly rats, are commonly used as a biological test system to reproduce MGH. This choice is explained by their short reproductive period and estrous cycle, which makes them particularly suitable for modeling hormone-dependent disorders [6]. Carcinogens [7], hormones (estrogens, progesterone, their combined use) [8-10] are used to induce MGH in experiments, so induction schemes vary considerably. The mean length of the menstrual cycle in women is 28 days (usually from 25 to 30 days) [1]. This is a probable reason why existing models of hormonal induction of MGH in rats involve administration of estrogen for 24-25 days followed by 5 days of progesterone, *i.e.* simulating the menstrual cycle of a woman [9-11]. Prolonged administration of estrogen alone [7] or a combination of estrogen and progesterone [12] are also used. The estrous cycle in rats lasts 4-5 days [13]. Prolonged hormonal exposure disturbs the estrous cycle in rats, and the development of MGH

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in women is not always associated with menstrual disruption. Therefore, consideration of the rat estrous cycle duration could make the hormonal model biologically more accurate.

The aim of this study is to determine the efficacy of MGH induction by a modified protocol including estrogen and progesterone injections with consideration for the duration of the estrous cycle in rats.

MATERIALS AND METHODS

Female Wistar rats (n=28; age 3.5 months, body weight 180-220 g) were obtained from the Rappolovo Laboratory Animal Facility. The animals were kept in a vivarium at 12/12-h light/dark conditions, 20-23°C, and relative humidity of 54-58%. The rats received daily standard full-fed briquetted chow for laboratory rodents (Laboratorkorm) and tap water *ad libitum*. The study protocol was reviewed and approved by the Ethical Committee of the N. N. Petrov National Medical Research Center of Oncology (Extract No. 3/215 from Protocol No. 17; September 22, 2020).

Before the experiment, the animals were randomized by body weight and divided into two groups (14 animals in each group). The first group comprised intact controls and the second group included animals with induced MGH. MGH was induced over four 7-day cycles, each including subcutaneous administration of 17β-estradiol (0.5 mg/kg; Sigma-Aldrich) for 4 days, progesterone (5 mg/kg; Dalkhimpharm) on day 5, then 2 days without administration.

On day 28 of the experiment, the diameter and height of the nipples of the second pair of rat mammary glands were measured. The hair around the second pair of mammary glands was removed and the nipples were photographed using a Sony A7 III digital camera with a fixed focal length Tamron 90 mm F/2.8 Macro SP (Tamron Co., Ltd.) at 1:1 magnification. Measurements were made in ImageJ software. At the end of 4 injection cycles, the animals were euthanized by decapitation under general anesthesia and complete autopsy was performed. The uterus with ovaries and mammary glands, liver, kidneys, spleen, and heart were excised. The organs were weighed on AND HR-250AZG analytical scales (A&D Company Limited) and then fixed in 10% formalin (ErgoProdakshn). After standard histological processing, the mammary gland tissue was embedded in paraffin and 3-4-μm thick sections were prepared from the blocks. Some sections were stained with hematoxylin and eosin according to the standard protocol, others were stained with Masson's trichrome with aniline blue (ErgoProdakshn). The stained sections were examined under a Nikon Eclipse NiU light microscope with a digital camera. The area of the minimal mammary gland units was determined using ImageJ software on photomicrographs at 50× relative to the entire image area.

The results were statistically processed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). Normality of distribution was determined by the Shapiro–Wilk test. Intergroup differences were assessed using Student's t test or Mann–Whitney U test. The differences were considered significant at $p<0.05$. The data are presented as $M \pm SEM$.

RESULTS

One of the main markers of MGH development is the change in nipple size (Fig. 1, *a*, *b*). Induction of MGH led to a significant increase in nipple height (1.68±0.06 mm *vs* 1.30±0.05 mm, $p<0.0001$) and diameter (1.18±0.04 mm *vs* 1.06±0.02 mm, $p=0.0042$) in comparison with intact control.

Complex tubuloalveolar structures consisting of the branched ductal system and terminal secretory alveoli located in the lobules were found in the

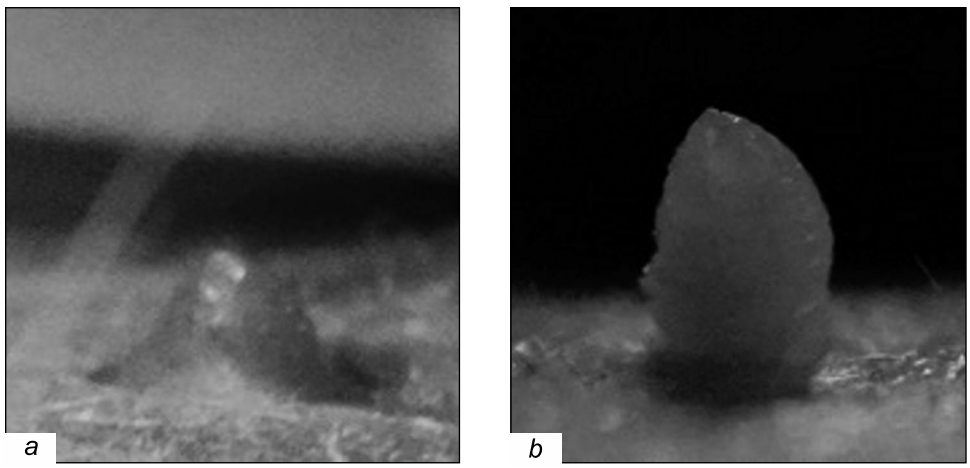

Fig. 1. Mammary nipples of an intact control female rat (*a*) and a rat with MGH induction (*b*).

mammary gland tissues of control rats (Fig. 2, *a*, *c*). The lumens were poorly discernible and thin layers of fibrous tissue with elastic fibers were observed around them. In rats with MGH, two types of changes were observed in the breast tissue. The first was tubuloalveolar hyperplasia characterized by an increase in the density and number of ductal and glandular structures (Fig. 2, *b*). The second one was lobuloalveolar hyperplasia, *i.e.* an increase in the number of alveolar structures and secretory activity (Fig. 2, *d*). In contrast to lobuloalveolar differentiation (virilisation), mammary glands with tubuloalveolar hyperplasia had a distinct lumen and ductal structures. The trichrome Masson staining in the case of lobuloalveolar hyperplasia showed a decrease in collagen fibers due to overgrowth of glandular structures. The relative area of the minimal mammary gland units in rats with MGH was significantly increased (to 5.67±0.82%) in comparison with intact control (3.59±0.31%, p=0.03).

A marker of hormonal abnormalities in rats is the weight of the uterine–ovarian complex. Thus, in females with MGH, the absolute weight of the uterine–ovarian complex was significantly higher than in the intact controls: 0.52±0.04 g *vs* 0.71±0.03 g (p<0.01). Similar changes were observed when assessing the relative weight of the complex of these organs. In rats with MGH, this parameter was significantly higher than in intact control (0.30±0.01 and 0.21±0.01%, respectively, p<0.01). At the same time, the relative weight of the liver in rats with MGH (4.06±0.18%) was higher than in intact control (3.43±0.05%, p<0.01). The relative weight of the kidney was also significantly higher (0.71±0.03 and 0.650±0.001%, respectively, p<0.05). There were no significant differences in the relative weight of the spleen and heart.

In the serum of rats with MGH, cholesterol levels decreased to 0.88±0.04 mmol/liter (1.04±0.04 mmol/liter in control, p<0.05), the levels of phosphorus and iron increased to 3.02±0.07 mmol/liter

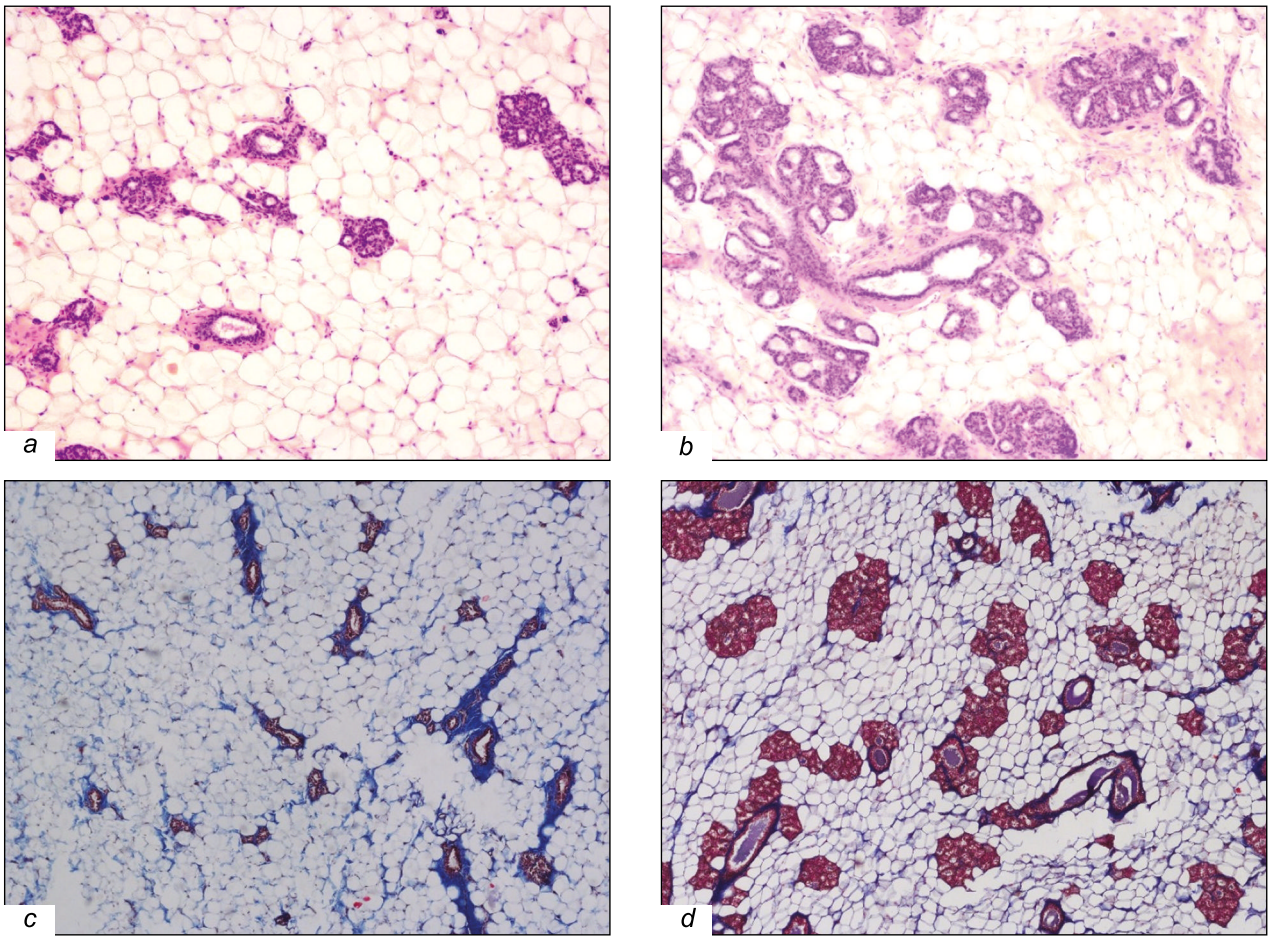

Fig. 2. Histological slides of mammary glands of female rats from the intact control group (*a*, *c*) and the group with MGH induction (*c*, *d*). Hematoxylin and eosin staining, ×100 (*a*, *b*), Masson trichrome staining, ×50 (*c*, *d*). *a*) Normal mammary gland tissue: predominance of ductal component over alveolar; *b*) tubuloalveolar hyperplasia; *c*) normal mammary gland tissue; *d*) lobuloalveolar hyperplasia.

(*vs* 2.42±0.11 mmol/liter in control, $p<0.05$) and 72.9±6.1 μmol/liter (*vs* 51.8±5.2 μmol/liter in control, $p<0.05$) respectively. Changes in serum content of trace elements reflect the pattern observed in human mammary gland pathology [14].

This study confirmed the development of MGH in rats using our modified method. The detected morphological changes are consistent with those described in the literature when using estrogen-progesterone induction. For instance, injection of estradiol benzoate (0.5 mg/kg/day) for 25 days and progesterone (5 mg/kg/day) was followed by an increase in nipple size compared to intact control [10]. Additionally, the relative weight of the uterine was also significantly higher in female rats with induced MGH. Histological assessment of the mammary glands revealed significant proliferative changes in the epithelium, including hyperplasia in most lobules, an increased number of acini and ducts, and thickening of the glandular epithelium. Similar results were obtained in other studies using similar MGH induction scheme [11,15]. In our study, induction of MGH using a scheme adapted to the estrous cycle of rats resulted in similar changes at both the macro (increased nipple size, uterine weight) and morphological level of mammary gland tissue. The combined use of sinestrol and progesterone has also been shown to result in macroscopic and microscopic changes of the inguinal mammary glands in fibrocystic MGH [12].

Thus, the proposed modified protocol could be used to successfully induce MGH in rats, with their estrous cycle to be considered. The model which accommodated rat estrous cycle morphologically imitated the existing models based on women menstrual cycle. Therefore, it can be used to evaluate the effects of therapeutic and preventive drugs and to study the pathogenesis of MGH.

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