

## Impact of Physical Training on Sex Hormones and Their Receptors During *N*-Methyl-*N*-nitrosourea-induced Carcinogenesis in Rats

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**Abstract.** *Background/Aim:* The risk of breast cancer is related to duration of exposure to sex hormones, especially estrogen. The aim of this study was to assess the impact of physical training (PT) on estrogen and progesterone levels and expression of their receptors during carcinogenesis induced by *N*-methyl-*N*-nitrosourea (MNU) in rats. *Materials and Methods:* Fifty female Sprague-Dawley rats were intraperitoneally administered MNU and divided into four groups: low-, moderate-, and high-intensity PT, and no PT (control). Plasma levels of sex hormones and tissue expression of their receptors were quantified and statistically analyzed. *Results:* In the group of rats subjected to PT, a significantly higher progesterone level was observed. The highest progesterone level was noted in the low-intensity PT group. An increase in apoptosis of MNU-induced tumor cells was also demonstrated in the PT groups. *Conclusion:* PT stimulates apoptosis of tumor cells without an increase in their proliferative activity. The increase in apoptosis of tumor cells correlates positively with the progesterone level.

Breast cancer is the most common type of malignant neoplasm in women, with approximately 1.5 million new cases diagnosed annually worldwide (1). The risk of its occurrence

increases with age, reaching the highest incidence, at approximately 12%, in the postmenopausal period. In regard to the different risk factors associated with breast cancer risk, exposure to sex hormones, namely estrogen and progesterone, and the level of physical activity are often reported (2, 3). This is closely related to early menarche (before the age of 12 years) and late menopause (over the age of 55 years), as well the influence of physical activity on the levels of sex hormones and decrease of the number of ovulatory cycles (4, 5).

Estradiol, the primary estrogen, binds to estrogen receptor (ER)- $\alpha$  (higher affinity) and ER $\beta$ . By interfering with ER $\alpha$ , estradiol stimulates proliferation and differentiation of mammary epithelial cells (6, 7). It is estimated that approximately 70-80% of breast cancers demonstrate ER expression (8). Likewise, progesterone acts on mammary epithelial cells synergistically with estrogens, stimulating their growth (2, 9). During physical training (PT), the levels of these hormones increase with increasing PT intensity. Single PT sessions increase blood estradiol and progesterone levels, whereas prolonged systematic PT reduces ovarian secretion, which results in a decrease in blood estradiol and progesterone levels (10). The significant relationship between the level of PT and risk of breast cancer has frequently been reported. However, there are no consistent results defining the relationship between PT and sex hormone levels (11-14), nor with the expression of their receptors (15-17).

Rats and mice are the main species in experimental studies of mammary gland tumors due to the fact that their glands they have the most similar structure and function to human glands (18). *N*-Methyl-*N*-nitrosourea (MNU), which is commonly used in these studies, is an agent recognized as causing tumor of the mammary gland (19, 20).

Therefore, the aim of this study was the assessment of the impact of PT on estrogen and progesterone levels and on the

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expression of ER and progesterone receptor (PR) during MNU-induced carcinogenesis in rats.

## Materials and Methods

**Animals.** Fifty female Sprague-Dawley rats of 28 days of age, housed under stable living conditions at the Animal Research Facility of the Department of Pathomorphology, Wrocław Medical University (constant temperature and humidity, 12/12 h light and dark cycle, rat chow and water *ad libitum*), were utilized in the experiment. The study was approved by the local Ethics Committee (decision no. 37/2010). Following a two-week quarantine period, animals were intraperitoneally injected with 180 mg/kg body weight of MNU (Sigma-Aldrich, Munich, Germany) (19). Four weeks after MNU administration, the animals were monitored in accordance with a previously applied procedure (21).

**Physical training.** The animals were divided into four groups: the sedentary control (SC; n=14), and low- (LIT; n=12), moderate- (MIT; n=12) and high-intensity (HIT; n=12) PT groups. Immediately after MNU administration, all training rats (LIT, MIT, HIT) were subjected to supervised 12-week PT (5 days a week) on a 3-position treadmill (Exer 3/6; Columbus Instruments, Columbus, OH, USA). The speed of the treadmill and training duration were gradually increased (Table I). For the LIT group, the training parameters were reduced by 20% compared to the MIT group, whereas for the HIT group, they were increased by 20% (22). After the administration of MNU, the rats from the SC group were not subjected to PT, however, they were housed for 12 weeks under the same conditions as training rats.

**Tissue collection.** Twelve weeks following MNU administration, the animals were sacrificed as previously described (20). All tumors detected by palpation were excised and measured. All tissues were fixed in 4% buffered formalin, dehydrated and embedded in paraffin. The blood was also collected for analysis at the post-mortem examination. The blood was centrifuged (500 × g for 15 min at 4°C) and the obtained plasma was frozen.

**Histopathological evaluation.** Hematoxylin and eosin-stained (HE) 6-µm-thick paraffin slides were prepared for histopathological verification, as previously described (21). In brief, two independent pathologists utilizing a double-headed BX41 microscope (Olympus, Tokyo, Japan) evaluated tumor sections as benign or malignant lesions based on the classification of rat mammary gland tumors according to Russo and Russo (23).

**Tissue microarray (TMA) construction.** Representative tumor areas were selected on HE-stained sections. TMAs were constructed using a Manual Tissue Arrayer I (Beecher Instruments Inc., Sun Prairie, WI, USA). Triplicate tissue cores (2 mm) with potentially the highest tumor cell content were obtained from non-necrotic areas in the tumor and transferred into the recipient paraffin block, as previously described (20).

**Immunohistochemistry.** All reactions were performed on 4-µm-thick paraffin sections obtained from TMA blocks, using Autostainer Link48 (Dako, Glostrup, Denmark). Firstly, deparaffinization, rehydration and antigen retrieval were performed using EnVision FLEX Target Retrieval Solution (97°C, 20 min; pH 6 for Ki-67 and

Table I. Moderate-intensity physical training protocol.

Period of training (week)	Speed of treadmill (km/h)	Duration (minutes)
1	0.60	10
2	0.96	20
3	1.20	30
4	1.44	40
5	1.68	50
6	1.68	55
7	1.68	60
8	1.68	65
9-12	1.68	30

pH 9 for ERα and PR) in PT-Link (Dako). The activity of endogenous peroxidase was blocked by 5-minute incubation with EnVision FLEX Peroxidase-Blocking Reagent (Dako). Monoclonal mouse anti-rat Ki67, clone MIB-5 (1:25, Dako), polyclonal rabbit anti-rat ERα (1:100; Novus Biologicals, Abingdon, United Kingdom) and polyclonal rabbit anti-rat PR (1:100; Novus) were used as the primary antibodies. The tested sections were incubated with primary antibodies for 20 min at room temperature (RT), followed by incubation with secondary goat antibodies coupled to a dextran core, conjugated with horseradish peroxidase (EnVision FLEX/HRP) for 20 min. 3,3'-Diaminobenzidine (DAB; Dako) was utilized as the peroxidase substrate and the slides were incubated for 10 min. Finally, all sections were counterstained with EnVision FLEX Hematoxylin (Dako) for 5 minutes and closed with coverslips in Dako Mounting Medium (Dako). Sections were evaluated as described below.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method.** The apoptosis assay was conducted using ApopTag *in situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA), as previously described (21). In brief, TMA sections were dewaxed in xylene, rehydrated in ethanol alcohol and rinsed in distilled water and phosphate-buffered saline (PBS). The sections were digested using Proteinase K (Dako) for 5 min at RT. The activity of endogenous peroxidase was blocked by 5-min incubation in 3% H<sub>2</sub>O<sub>2</sub> in PBS. In the next step, the sections were incubated, first with Equilibration Buffer for 10 min at RT and then with TdT Enzyme and Reaction Buffer at 37°C for 1 h. The reaction was stopped by incubation with Stop Buffer for 10 min at RT. Subsequently, the sections were incubated with anti-digoxigenin peroxidase-conjugated secondary antibodies and the substrate for peroxidase, DAB was applied for 10 min at RT. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated in ethanol alcohol and xylene and the preparations were mounted in SUB-X Mounting Medium (Dako). Sections were evaluated as described below.

**Assessment of immunohistochemical reaction.** The analysis was performed by two researchers utilizing a double-headed BX41 microscope combined with computer-aided image analysis software Cell^D (Olympus). The evaluation of the expressions of Ki-67 antigen, ERα, PR and TUNEL reaction in the TMA sections was based on the count of cancer cell nuclei with color reaction relative

Table II. Body weight and amount of administered *N*-methyl-*N*-nitrosourea (MNU) in the experimental groups.

Variable	SC (n=9)	LIT (n=8)	MIT (n=4)	HIT (n=3)	<i>p</i> -Value	PT (n=15)	<i>p</i> -Value SC vs. PT
Mean weight±SD prior to MNU (g)	105.67±11.66	111.25±24.16	107.50±37.75	97.67±19.50	0.81	107.53±26.08	0.81
Mean dose±SD of MNU (ml/kg)	1.92±0.22	2.05±0.46	2.00±0.58	1.77±0.38	0.77	1.98±0.46	0.80
Mean weight±SD at necropsy (g)	288.67±17.83	286.25±18.47	297.50±25.98	297.33±21.20	0.73	291.47±20.31	0.69

PT: Training rats, LIT: low-intensity training, MIT: moderate-intensity training, HIT: high-intensity training, SC: sedentary control, SD: standard deviation.

to all cancer cells evaluated under  $\times 400$  magnification. The final score was an average of three measurements from the fields of the most intense expression ('hot spots'), given as a percentage.

**Determination of plasma hormone levels.**  $17\beta$ -Estradiol and progesterone were determined by enzyme-linked fluorescent assay (ELFA; BioMerieux, Marcy-L'etoile, France), according to the manufacturer's recommendations. All the steps of the study were performed automatically by a VIDAS immunoanalyzer (BioMerieux), including automated calculation of results in relation to the programmed calibration curve.

**Statistical analysis.** The Shapiro–Wilk test was used to evaluate the normality assumption of the examined groups, and the Levene's test for the homogeneity of variance. The Mann–Whitney and Kruskal–Wallis tests were utilized to compare differences between expression and levels of examined markers. Additionally, the Spearman correlation test was used to verify existing correlations. All the statistical analyses were performed using Prism 5.0 (GraphPad, La Jolla, CA, USA) and Statistica 10 (Statsoft, Cracow, Poland). The results were considered statistically significant when  $p < 0.05$ .

## Results

The statistical analysis was performed only on cases with induced tumors: nine from the SC group and 15 from the PT groups, in regard to the observed color reactions (Figure 1). The examined rats did not differ significantly in terms of their body weight, amount of administered MNU (Table II). Neither significant differences in tumor volume nor proliferation in PT groups were demonstrated (Figure 2A–D). However, a moderate negative correlation between the number of tumors and intensity of PT was observed ( $r = -0.47$ ,  $p = 0.02$ ; Figure 3A). Moreover, a positive correlation between volume and number ( $r = 0.68$ ,  $p = 0.02$ ; Figure 3B), as well volume and proliferative rate of induced tumors was found ( $r = 0.60$ ,  $p = 0.001$ ; Figure 3C).

Apoptosis was significantly stronger in the PT groups vs. the SC group, however, an increasing trend was only apparent for LIT and MIT groups (Figure 2E, F). Increased apoptosis positively correlated with PT intensity level ( $r = 0.47$ ,  $p = 0.02$ ; Figure 3E).

No significant differences in plasma level of estradiol nor tissue expression of ER $\alpha$  in animals from PT and SC groups were disclosed (Figure 4). In comparison of plasma level of

progesterone and tissue expression of PR, the only statistically significant difference was a higher progesterone level in PT groups vs. the SC group (Figure 5).

The expression of ER $\alpha$  and PR in tumor cells positively correlated with the level of apoptosis ( $r = 0.43$ ,  $p = 0.04$  and  $r = 0.50$ ,  $p = 0.01$ , respectively; Figure 3F, G). Moreover, moderate correlations between expressions of ER $\alpha$  vs. Ki-67 ( $r = 0.46$ ,  $p = 0.02$ ; Figure 3H) and PR vs. tumor volume ( $r = 0.50$ ,  $p = 0.01$ ; Figure 3D) were found.

## Discussion

The epidemiological and experimental studies indicate a negative association between PT and breast cancer (24–26). An approximately 25% reduction of breast cancer risk was observed in physically-active women and related with the intensity of PT. According to many authors, moderate or intense PT has a significant impact on risk reduction (27–29). In the present study, we found concordant results, showing a significant decrease in the number of tumors in the experimental group undergoing intense PT.

It is generally accepted that female sex hormones play a role in the etiopathogenesis of breast cancer (30). Numerous studies indicate that overexposure, mainly to estrogens, can be related to increased risk of breast cancer development. In our study, differences between levels of estradiol in PT and SC rats were insignificant. Similar observations were disclosed by others (25, 26, 31), whereas Erich *et al.* (32) and Pellerin-Massocotte *et al.* were explaining that rodents require high intensity PT to induce hormonal changes, especially related to sex hormones (33). Contrary to the general assumption that estrogen level decreases under the influence of PT, there is evidence of a significant increase of  $17\beta$ -estradiol in rats undergoing long-term PT, most of all obtained by Faustino-Rocha *et al.* in the longest exercise training protocol (35-week) on mammary tumorigenesis ever performed (34).

In regard to expression of ER $\alpha$  in PT rats, results are highly contradictory. Our analysis, similarly to that of Alvarado *et al.*, revealed no statistically significant association (35). However, Wang *et al.* demonstrated a reduction in ER $\alpha$  expression in 100-day-old rats undergoing PT at the early stage of life, simultaneously

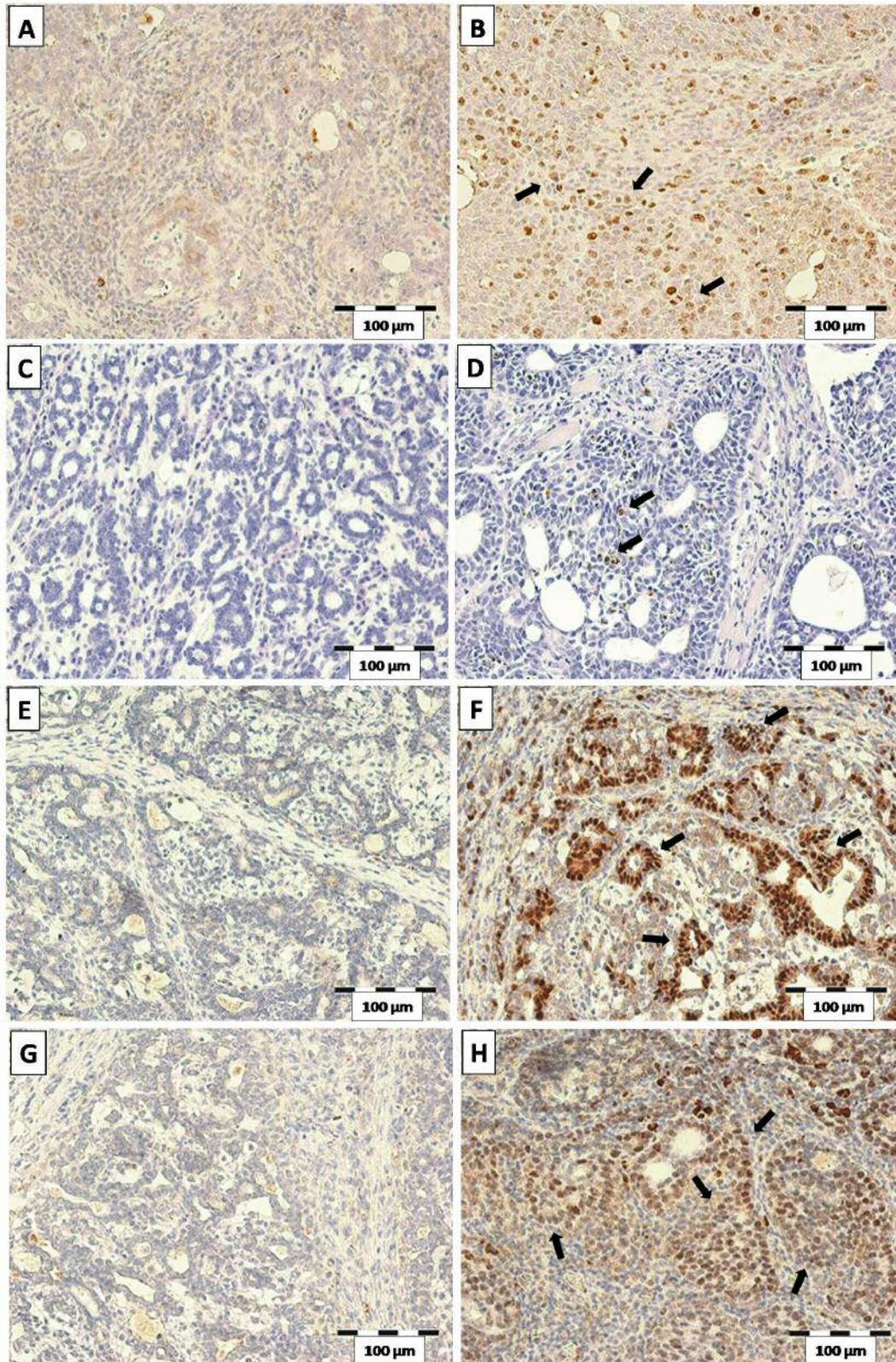


Figure 1. Immunohistochemical expression of Ki-67 proliferation antigen (A, B), apoptosis detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling method (C, D), immunohistochemical expression of estrogen receptor  $\alpha$  (E, F) and progesterone receptor (G, H) in tumor cells of *N*-methyl-*N*-nitrosourea-induced tumors in rats. A, C, E, G – Absent reaction; B, D, F, H – nuclear reaction (arrows).

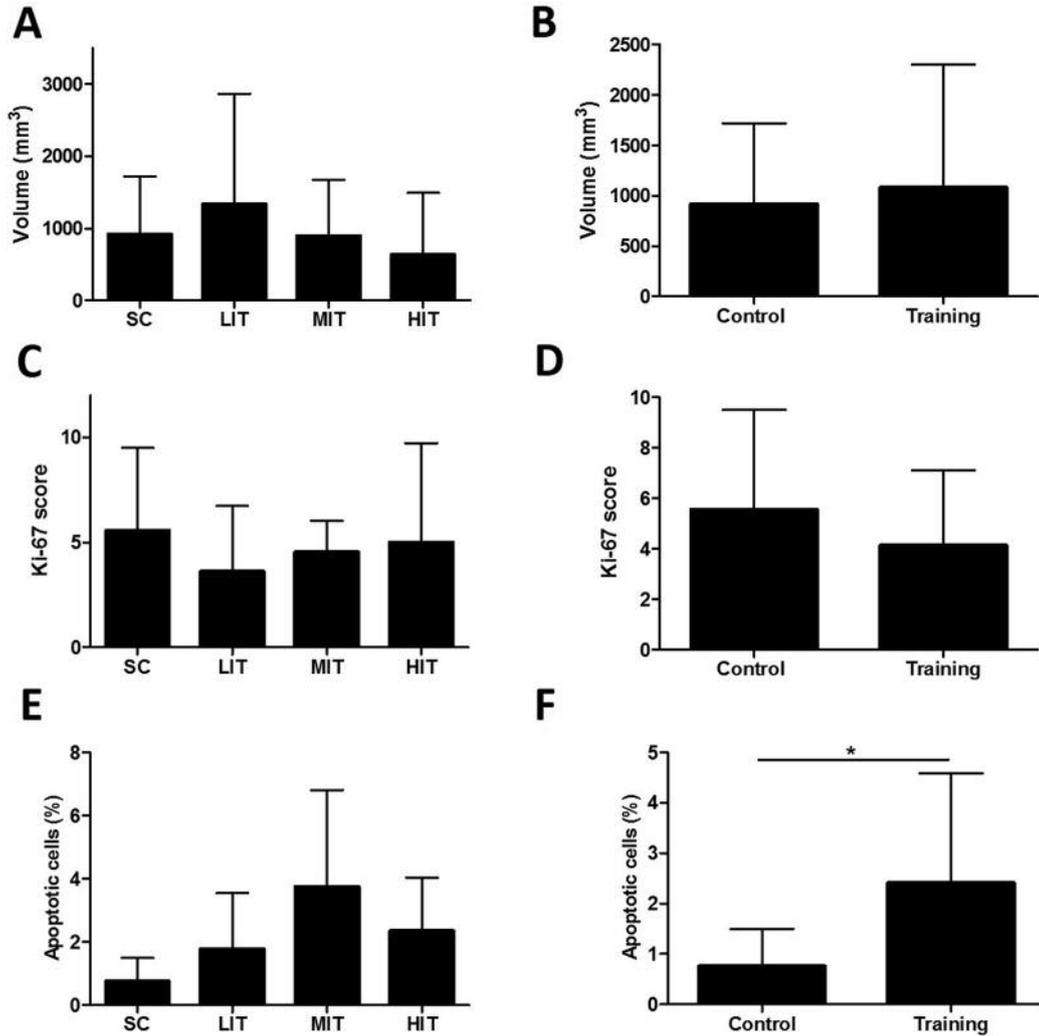


Figure 2. Comparison of tumor volume (A, B), expression of Ki-67 antigen (C, D), and percent of apoptotic cells (E, F) between training groups and sedentary control (SC) group. LIT: Low-, MIT: moderate-, HIT: high-intensity training. Data are means±standard deviation. \*Significantly different at  $p < 0.05$ .

indicating a reduction in proliferation (36). In contrast, Faustino-Rocha *et al.* demonstrated increased ER $\alpha$  expression in MNU-induced mammary tumors in female rats undergoing PT (34). In addition, we demonstrated a moderate correlation of increased ER $\alpha$  expression in tumor cells with their proliferation and with apoptosis. Induction of proliferation in both normal and neoplastic cells of mammary gland is primarily due to the effect of estradiol on ER $\alpha$  (37). Song *et al.* (38) and Lewis *et al.* (39) demonstrated the opposite relationship, *i.e.* proapoptotic effect of estradiol. Nevertheless, Teixeira *et al.* observed increased ER expression to be associated with increased apoptosis and reduced proliferation (31), with which our results are partly consistent with.

The second most important sex hormone with a significant role in breast cancer tumorigenesis is progesterone. It

inhibits cell proliferation, as well as suspected capable of inducing apoptosis (40). The proapoptotic action of progesterone in the mammary gland was confirmed by Chen *et al.* (41) and Gompel *et al.* (42).

In our study, a significantly higher progesterone level was observed in PT rats, which is concordant with the results of others (26, 43). Thompson *et al.* reported that a higher level of progesterone was significantly related to a lower incidence of cancer in experimental animals (26). Additionally, we demonstrated a positive correlation between PR expression and the number of apoptotic cells, which may be confirmation of the observations of Chen *et al.* (41) and Gompel *et al.* (42). Moreover, Wiebe *et al.* indicated the possibility of different metabolic profiles for progesterone, which *inter alia* may be observed in healthy and diseased mammary glands. Both the possibility of stimulation of cell

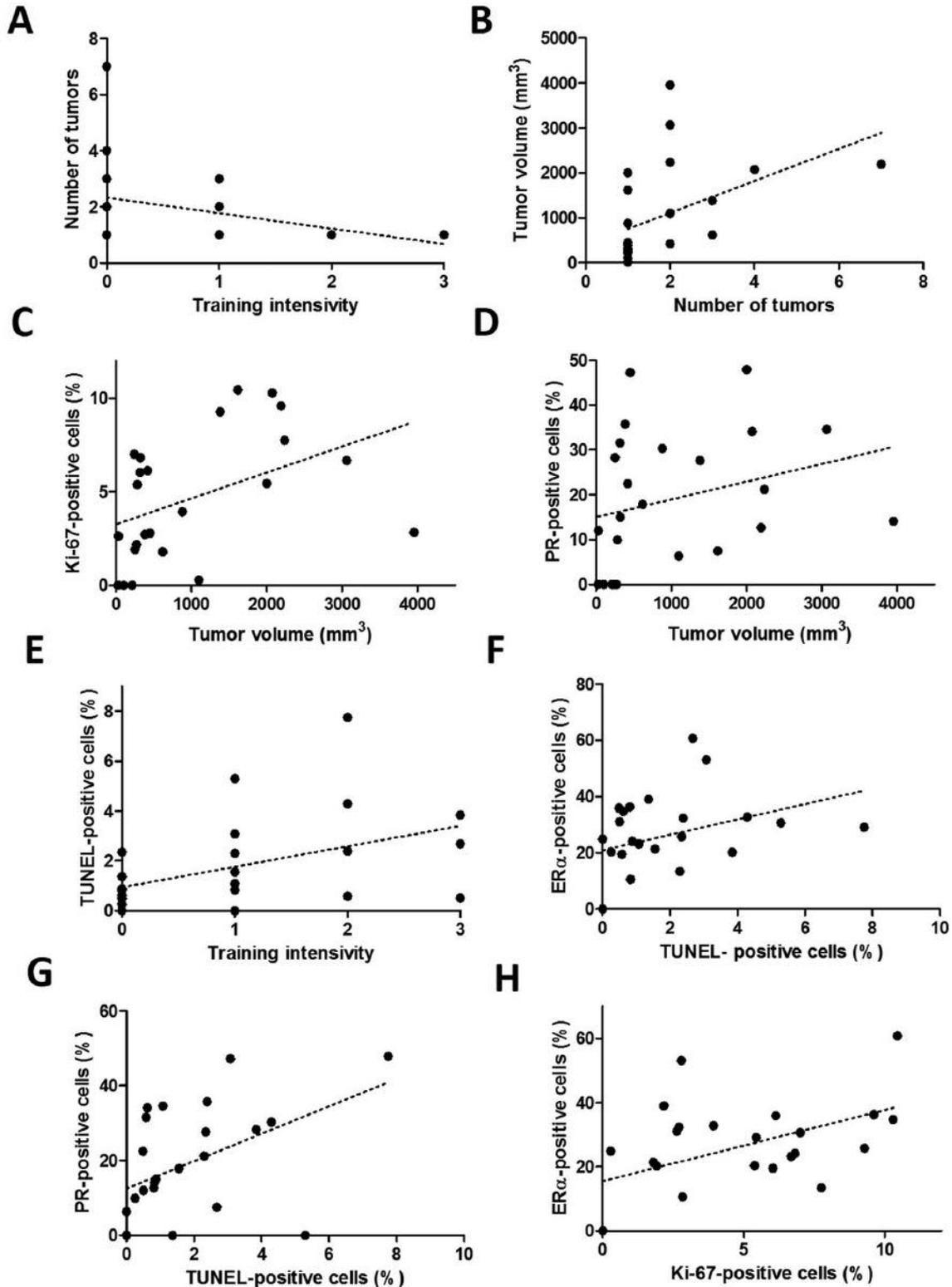


Figure 3. Spearman rank correlation analysis for the number of tumors induced and the intensity level of physical training (A;  $r=-0.47$ ,  $p=0.02$ ), for the number of induced tumors and their volume (B;  $r=0.68$ ,  $p=0.02$ ), for tumor volumes and expression of Ki-67 (C;  $r=0.60$ ,  $p=0.001$ ), for tumor volumes and progesterone receptor (PR) expression (D;  $r=0.50$ ,  $p=0.01$ ), for training intensity and the number of apoptotic cells (E;  $r=0.47$ ,  $p=0.02$ ), for apoptotic cells and estrogen receptor (ER $\alpha$ ) expression (F;  $r=0.43$ ,  $p=0.04$ ), for the number of apoptotic cells and PR expression (G;  $r=0.50$ ,  $p=0.01$ ), for the expressions of Ki-67 and ER (H;  $r=0.46$ ,  $p=0.02$ ). TUNEL: Terminal deoxynucleotidyl transferase dUTP nick-end labeling.

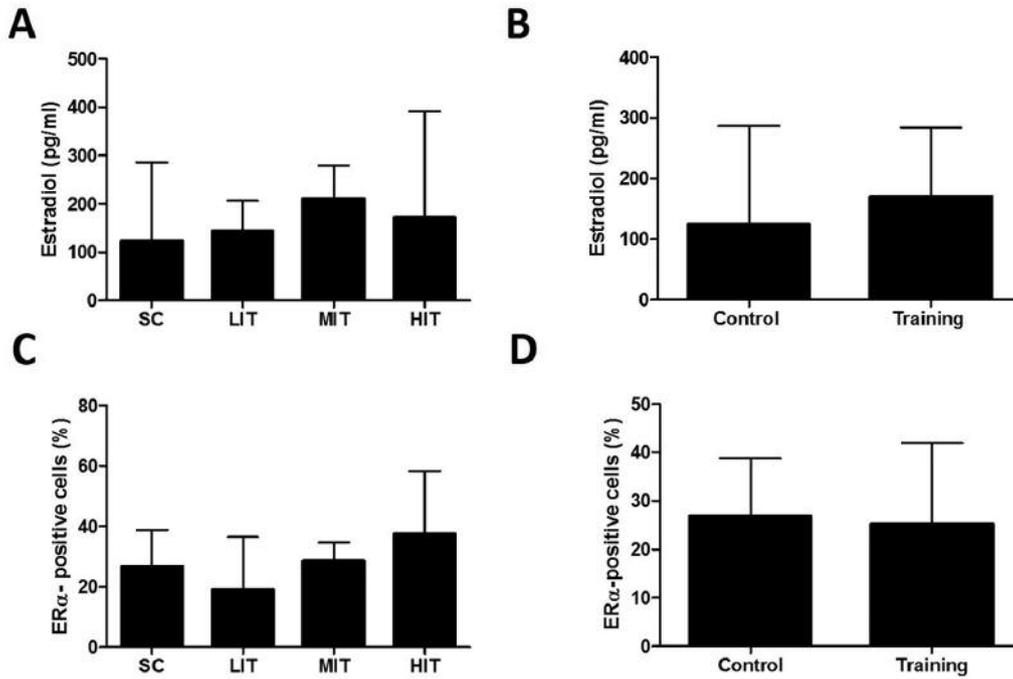


Figure 4. Comparison of plasma level of estradiol (A, B) and expression of estradiol receptor (ER)- $\alpha$  (C, D) between training groups and sedentary control (SC) group. LIT: Low-, MIT: moderate-; HIT: high-intensity training. Data are means $\pm$ standard deviation.

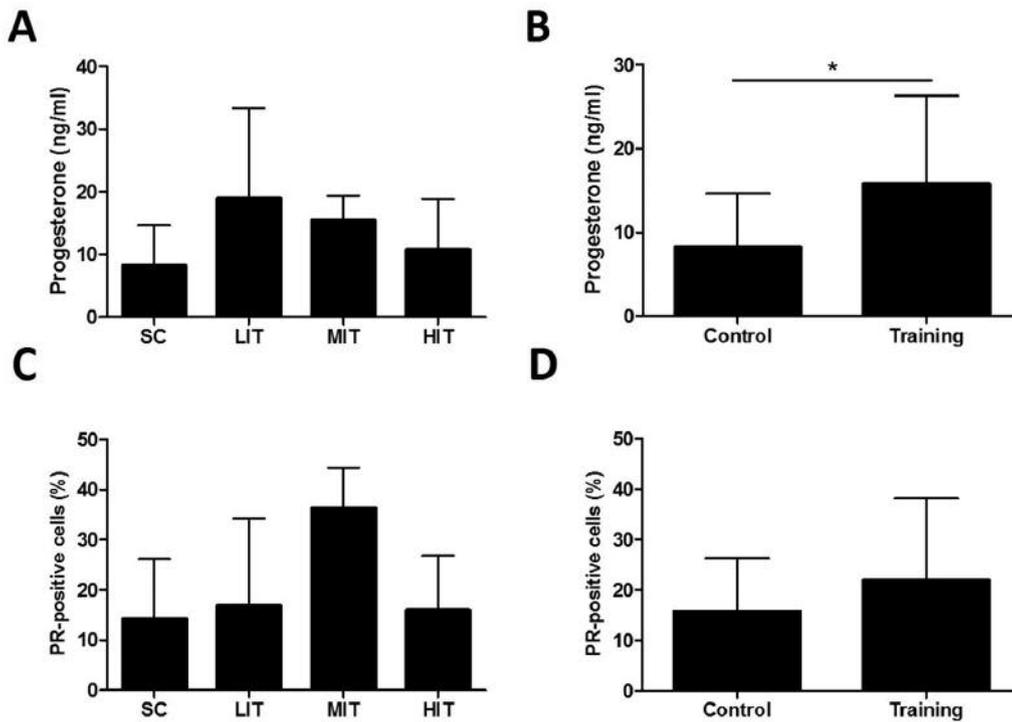


Figure 5. Comparison of plasma level of progesterone (A, B) and expression of progesterone receptor (PR) (C, D) between training groups and sedentary control (SC) group. LIT: Low-, MIT: moderate-; HIT: high-intensity training. Data are means $\pm$ standard deviation. \*Significantly different at  $p < 0.05$ .

proliferation and the antimetabolic effect of increasing cell apoptosis were demonstrated (9). Perhaps PT may influence progesterone metabolism, thus having a different effect on tumor cells. Steindorf *et al.* also suggest that moderate and high physical activity is associated with modestly reduced breast cancer risk (44).

Based on the obtained study results, PT seems to have a greater effect on progesterone metabolism without significantly affecting the estradiol level. In turn, progesterone induced by PT may be significant in inhibiting the development of mammary gland cancer (45). In addition, we observed increased tumor cell apoptosis in groups undergoing PT and its positive correlation with the intensity of PT, which suggests a protective role of PT in carcinogenesis.

In conclusion, properly-tailored PT may have a suppressive effect on breast cancer development in a mechanism controlled mostly by progesterone metabolism.

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