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Moderate Beer Consumption Modifies Tumoral Growth Parameters and Pyrrolidone Carboxypeptidase Type-I and Type-II Specific Activities in the Hypothalamus-Pituitary-Mammary Gland Axis in an Animal Model of Breast Cancer

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ABSTRACT

Aims: To determine the effect of moderate alcoholic and nonalcoholic beer consumption on tumoral growth parameters, the histopathology, pyrrolidone carboxypeptidase type I (Pcp I), and type II (Pcp II) specific activities in the hypothalamus-pituitary-mammary gland axis, and the circulating levels of estradiol (E_2) and progesterone (P_4) in rats with *N*-methyl-*N*-nitrosourea (NMU) induced mammary tumors.

Material and methods: Food and drink intake, weight gain and tumor growth parameters were collected. The malignant phenotype of the tumor was performed using the Scarff-Bloom-Richardson grading method. Pcp specific activities were fluorometrically analyzed using pyroglutamyl-β-naphthylamide as substrate. Circulating steroid hormones were determined.

Results: Differences were found in tumoral parameters, depending on the drink. Animals that were given alcohol-containing beer (A/C) beer to drink showed the lowest values of hypothalamic Pcp I, in association with the lowest levels of circulating E_2. The significant decrease in Pcp I activity in all NMU-treated groups suggest a clear role of the Pcp I in the tumoral process, and A/C beer interferes with it.

Discussion: Moderate consumption of alcoholic beer would have beneficial effects against mammary tumors through the modification of the endocrine status mediated by GnRH due to changes on Pcp I and II activities at different levels.

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Introduction

Pyrrolidone carboxypeptidase (Pcp) (E.C. 3.4.19.3) is the regulatory proteolytic enzyme reported to hydrolyze gonadotropin-releasing hormone (GnRH) (1,2). Different forms of Pcp have been observed in mammalian tissue. Thus, Pcp type I (Pcp I), is localized in the cytosolic compartment (3) and has a broad pyroglutamyl-ended substrate specificity, whereas Pcp type II (Pcp II) has proven to be a membrane-bound metalloenzyme (4) that displays a narrow substrate specificity restricted to the pGlu-His bond of GnRH or very closely related peptides.

Hypothalamus and pituitary are the main source and target sites for GnRH, being this peptide hormone mainly responsible for the release of steroid ovarian hormones estradiol (E_2) and progesterone (P_4) (5), through the pituitary release of luteinizing hormone and follicle-stimulating hormone. However, several reports have recently suggested the existence of extra-hypothalamic GnRH and GnRH receptors in various reproductive tissues such as ovaries, placenta, endometrium, oviducts, testis, prostate, and mammary glands (6–8). In fact, the function of this peptide in extra-pituitary tissues such as the mammary gland has not been clarified, despite the fact that a significant quantity of GnRH is synthesized in this gland (9,10). However, an autocrine/paracrine role has been assumed (11,12). In this sense, we have previously reported changes on circulating Pcp activity in healthy post-menopausal women when compared to premenopausal ones. In addition, a decrease in circulating Pcp

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activity in post-menopausal women with breast cancer occurred concomitantly with the decrease of the circulating levels of GnRH and FSH (13,14).

The administration of the carcinogen *N*-methyl-*N*-nitrosourea (NMU) is a widely used procedure to induce mammary tumors in rats (15–18). Extensive evidences have demonstrated similarities between these chemically induced mammary carcinomas and human breast cancer. This includes their histopathology, origination from mammary ductal epithelial cells and the dependency of tumor development with ovarian hormones (17,19,20). In addition, NMU-induced rat primary mammary tumors are similar to estrogen receptor (ER)-positive low-grade human breast cancer (21), also being aggressive and locally invasive (15,16). The NMU model has contributed significantly to the current understanding of the biology of breast cancer also allowing approaches to its prevention and treatment (15,22).

Beer is one of the most consumed alcoholic beverages, being the third among general drinks. Currently, multiple types of beer exist, differentiated by the ingredients used and the brewing processes performed. However, the addition of hops (*Humulus lupulus*) improves its flavor and gives it a protective effect derived from its reducing capacity of pH and antibacterial activity (23–26). Moreover, beer also contains phenolic compounds as secondary metabolites of plants which contribute to its color and aroma. These components also show antioxidant properties (27,28). In the last few years, the beneficial effects of beer compounds on human health have received special attention from the scientific community. Not only the antioxidant but also the anti-inflammatory, anticancer, estrogenic, and even antiviral properties associated with beer intake have been described to be associated with these phenolic compounds (29–31). Specifically, xanthohumol seems to exert anticancer chemopreventive activity in the early stages of the carcinogenic process (28,32) in in vitro experiments (33–35). Conversely, compounds like 8-prenylnaringenin (8-PN) have been described as one of the most potent isolated phytoestrogens (36,37). The administration of 8-PN represents a novel therapeutic approach to the treatment of menopausal and post-menopausal symptoms that occur as a consequence of a progressive decline in hormone levels (38), but it could also interfere in hormone-dependent diseases such as breast cancer.

Although the positive effects derived from moderate beer consumption are widely described (28), as we have seen, the consumption of beer in high concentrations is not recommended for human health since it can cause harmful effects in pregnant women, children and people with a clinical picture of cardiomyopathy, among others (30,39). Therefore, an alternative option could be to consume alcohol-free beer. Beer with alcohol has the same bioactive compounds, differing in their concentration, lower in the case of nonalcoholic beer. In alcohol-free beers, alcohol is removed by (vacuum) distillation, vacuum evaporation, dialysis, and reverse osmosis. Therefore, during the de-alcoholization process, a significant loss of components such as polyphenols and other bioactive principles occurs, and aromatic compounds can be generated to an extent, depending on the de-alcoholization method. This loss of aromatic esters, the reduction or loss of different alcohols, and an indeterminate change in any of its compounds during the alcohol removal process justify explaining the flavor defects in nonalcoholic beer (40). The aim of the present work was to determinate if moderate consumption of alcohol-containing and alcohol-free beer modifies specific soluble and membrane-bound Pcp activities and the tumoral growth parameters of breast cancer model induced by NMU.

Material and Methods

Animals

Eighty-one virgin female Wistar rats (164.7 ± 4.7g body weight) were used in this study. The animals were provided by the animal house and care at the University of Jaén and were maintained in a controlled environment at a constant temperature (25 °C) with a 12 h-light/12 h-dark cycle. All animals were allowed to access water and food (Teklad 4% fat mouse/rat diet) *ad libitum*. The experimental procedures for animal use and care were in accordance with the European Community Council directive (2010/63/EU). Protocols were approved by the Bioethical Committee of the University of Jaén.

Tumor Induction, Detection, and Growth Monitoring

To induce mammary gland tumors, rats were intraperitoneally injected with three doses of 50 mg/kg body weight of NMU (Sigma, Madrid, Spain) dissolved in distilled water (10 mg/ml) at 50, 80, and 110 day after birth, as described by Rivera et al. (41). Tumors induced by this method are estrogen-dependent (21). All rats were in estrus at the first NMU injection, verified by daily vaginal smears. Control

groups received the vehicle only. Body weight was determined periodically each week.

For tumor detection and growth control, rats were examined by palpation 2 day each week after the second NMU injection. The number of tumors was recorded, and the major and minor diameter of each tumor was measured with a caliper to determine its volume. Tumor volume was defined as $1/2a(b)^2$, where a is the long diameter and b is the short diameter. Latency period, defined as the days between the first NMU injection and the appearance of the first tumor; tumoral incidence, defined as the percentage of rats that developed at least one tumor; and the mean number of tumors per rat (n/t), defined as the number of tumors per rat in animals developing at least one tumor; were additional growth parameters also determined.

Treatments

Animals were allocated into six different groups by a computerized randomization procedure. Three of the groups, consisting of 20 animals each, were subjected to NMU tumorigenesis (see below), whereas the other three groups, of seven animals each one, were considered non-tumoral controls. For animals with NMU-induced mammary tumors and their corresponding non-tumoral matched controls, the treatments were as follow:

1. Control groups,that received water as a drink during the whole time of the experiment.
2. Alcohol-containing beer (A/C) groups, that received a 30% solution of Pilsner alcoholic beer (4.6% ethanol vol/vol) in water as a drink from the first NMU injection and during the restof time of the experiment (28,42).
3. Alcohol-free beer (A/F) groups,that received a 30% solution of Pilsner nonalcoholic beer (0.0% ethanol vol/vol) in water as a drink from the first NMU injection and during the whole time of the experiment.

Alcoholic and nonalcoholic beers were obtained from the same brand and distributor.

Sample Preparation

After 122 day of the first NMU injection, animals were sacrificed under equithesin anesthesia (2 ml/kg body weight). Blood samples were obtained through the left cardiac ventricle, centrifuged for 10 min, at 300 g to obtain the serum, and stored at −80 °C until use. Samples from hypothalamus, anterior and posterior pituitary, and mammary gland from non-tumoral controls and NMU-treated rats were excised, rapidly frozen in liquid nitrogen and stored at −80 °C, until use. The determination of the specific Pcp activities in the mammary gland was carried out in tumor tissue from animals with NMU-induced breast cancer.

Tumor samples were also obtained, fixed (formaldehyde 4%), dehydrated, and embedded in paraffin for histopathological studies. To obtain the soluble fraction, tissue samples were homogenized in 10 volumes of 10 mM HCl-Tris buffer (pH; 7.4) and ultracentrifuged at 100,000 × g for 30 min at 4 °C. The resulting supernatants were used to measure soluble enzymatic activity and protein content, assayed in triplicate. To solubilize membrane-bound proteins, the pellets were rehomogenized in HCl-Tris buffer (pH 7.4) plus 1% Triton X-100. After centrifugation (100,000 × g, 30 min, 4 °C), the supernatants were used to measure solubilized membrane-bound activity and proteins, also in triplicate. All chemicals were obtained from Sigma, Madrid, Spain. To ensure complete recovery of activity, the detergent was removed from the medium by adding adsorbent polymeric Biobeads SM-2 (100 mg/ml; Bio-Rad, Richmond, CA) to the samples and shaking for 2 h at 4 °C.

Histopathology

To characterize the malignant tumor phenotype, the degree of the tumor's morphological aggressiveness and the tumor's growth were determined. We applied the Scarff-Bloom-Richardson grading method (43) slightly adapted to rat mammary carcinomas as it has previously been described (44). This global histopathology grade adds together the pattern differentiation (acinus formation), the nuclear atypia/pleomorphism and the mitosis count. Thus, tubule formation or pattern grade was classified using three scores (1, 2, and 3), depending on the extent of solid areas. Score one was assigned to carcinomas that displayed less than 25% solid areas, score two to those with 25% to 75% solid areas, and score three to carcinomas with more than 75% of tumor cells arranged in solid structures. Nuclear pleomorphism or nuclear grade included, as in human mammary carcinoma, three scores (1–3), depending on the size and shape of nuclei, chromatin pattern, and the presence or absence of nucleoli. Tumor mitotic activity was distributed in three categories, according to the number of mitoses reported in 10 high power fields, at 400x magnification: score

1 = ≤4 mitosis; score 2 = 5–9 mitosis; and score 3 = ≥20 mitosis. For the final evaluation, the scores for acinus formation, nuclear atypia and mitosis count were added together. Total score 3, 4, or 5 = grade I; total score 6 or 7 = grade II; and total score 8 or 9 = grade III.

Pyrrolidone Carboxypeptidase Type I and Type II Assays

Specific soluble (Pcp I) and membrane-bound (Pcp II) activities were measured fluorometrically using pyroglutamyl-ß-naphthylamide (pGLUNNap) as the substrate, according to the method previously described by us (45,46). Briefly, 10 µl of each sample were incubated in triplicate for 30 min, at 37 °C with 100 µl of the substrate solution: 100 mM of pGLUNNap, 0.65 mM dithiothreitol (DTT), and 1.3 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM of phosphate buffer at pH 7.4. All the reactions were stopped by adding 100 µl of 0.1 M acetate buffer at pH 4.2. All chemicals were obtained from Sigma, Madrid, Spain. The amount of ß-naphthylamine released as the result of enzymatic activity was measured fluorometrically at an emission wavelength of 412 nm with an excitation wavelength of 345 nm. Proteins were quantified also in triplicate using bovine serum albumin (BSA) as standard.

Assessment of Sex Hormones

Serum samples were measured by dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) for E_2 and P_4 (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland), according to manufacturer instructions. For E_2, the lower limit of assay detection was 0.05 nmol/L (13.6 pg/ml) and the inter-assay coefficient of variation was between 1.8% and 2.1%. For P_4, the lower limit of assay detection is 0.8 nmol/L (0.25 ng/ml) and the inter-assay coefficient of variation was between 1.9% and 4.5%.

Statistical Analysis

All values represent the mean of the individual determinations ± standard error of the mean (SEM). Parametric data were analyzed by repeated ANOVA measures or multiple analysis of variance (MANOVA) plus Newman–Keul's post hoc test. Nonparametric data were analyzed by Kruskal–Wallis test. Categorical data were analyzed by χ^2. IBM Pass V.24 software was used. Values of $P < 0.05$ were considered significant.

Results

Drink and Food Intake and Weight Gain

Figure 1A shows mean drink intake in control animals and animals with NMU-induced mammary tumors, which were given water, A/C and A/F beer to drink. No significant differences were found in drink intake between control animals and animals with breast cancer, independently of the drink. However, both control and NMU-treated animals which were given A/C or A/F beer significantly increased their drink intake ($P < 0.01$ in both cases) when compared to animals which were given just water. Furthermore, animals which were given A/F beer significantly increased their drink intake when compared to animals which were given A/C beer ($P < 0.01$ in both cases).

Figure 1B shows mean food intake in control animals and animals with NMU-induced mammary tumors, which were given water, A/C and A/F beer to drink. No significant differences were found in food intake between control animals and animals with breast cancer, independently of the drink.

Figure 1C shows the progression of body weight in control animals and animals with NMU-induced mammary tumors, which were given water, A/C and A/F. A significant increase ($P < 0.05$) was found in control animals which were given A/C beer after 7–8 weeks of administration. In the same way, a significant increase ($P < 0.01$) was found in NMU-treated animals which were given water or A/F beer after 5–6 weeks since the appearance of the first tumor. Subsequently, the weight decreased again. In addition, no significant differences were found in weight gain either in control or in NMU-treated animals that were given water, A/C and A/F beer to drink during the experiment.

Tumor Growth Parameters

The results of the analysis of the carcinogenesis parameters are depicted in Table 1. No significant differences were found in the latency period between animals that were given water (80.8 ± 6.7 day), A/C (84.14 ± 5.56 day), or A/F beer (71.57 ± 7.93 day). In the same way, no significant differences were found in the tumoral incidence, with values of 56.0%, 53.8%, and 42.8% for NMU-treated animals that were given water, A/C and A/F beer, respectively. Similarly, no significant differences were found in the mean tumor number per rat, with values of 1.0 ± 0.0 tumors for animals that were given water or A/F beer, and 1.1 ± 0.1 tumors for animals that were given A/C beer.

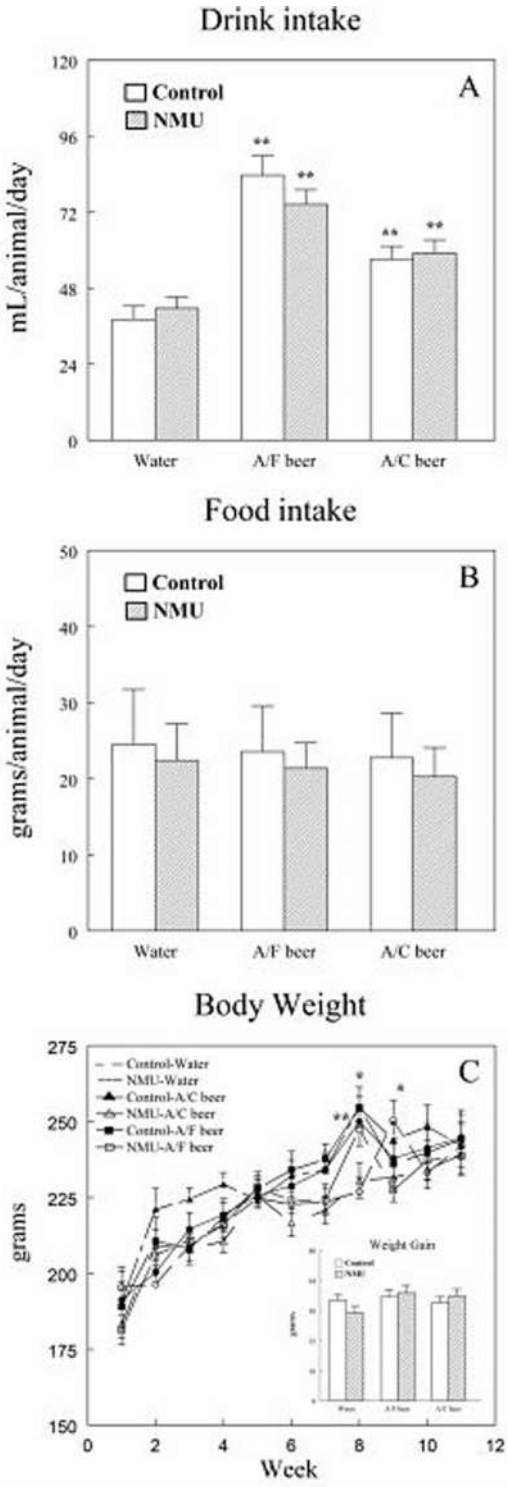

Figure 1. Mean values of daily drink (A) and food (B) intake in control animals and animals with mammary tumors induced by *N*-methyl-*N*-nitrosourea (NMU) which were given to drink water, alcoholic (A/C) beer and nonalcoholic (A/F) beer. Data is expressed as grams or ml per animal per day as mean ± SEM. C: Changes in body weight of control animals and animals with mammary tumors induced by NMU which were given water, A/C beer and A/F beer to drink, since the appearance of the first tumor until the end of the experiment (in weeks). Total weight gain for all groups analyzed is also shown. Data is expressed in grams as mean ± SEM ($^{**}P < 0.01$).

Tumor Size Parameters

Figure 2 shows major (Fig. 2A) and minor (Fig. 2B) diameters of tumors and tumoral volume (Fig. 2C) in NMU-treated animals fed with water, A/C and A/F beer, from the appearance of the first tumor until the end of the experiment. Animals which were given A/C beer showed significantly lower measures ($P < 0.01$) than animals which were given water or A/F beer (Fig. 2) at the end of the experiment. Animals that were given A/F beer to drink showed significantly higher values of the tumor size parameters during the first 5–6 weeks after the appearance of the first tumor than animals that were given water or A/C beer to drink.

Histopathology

Histological analysis showed carcinomas of the rat mammary gland displaying papillary and cribiform patterns, extensive solid areas and tumoral necrosis. Well-circumscribed carcinomas with nodular appearance and zones with infiltrative pattern with intense desmoplastic reaction were also seen. Histopathological results of the morphological analysis (Fig. 3) showed the highest proportion of high score in acinus formation in tumors from animals that were given water to drink. Lower but similar values of acinus formation were found in tumors from animals that were given beer to drink, independently of their alcohol content. Regarding nuclear atypia, the highest proportion of high scores was found in tumors from animals that were given A/F beer to drink. Lower but similar values of nuclear atypia were found in tumors from animals that were given water or A/C beer to drink. Regarding mitotic index, the highest proportion of high scores was found in tumors from animals that were given water to drink, and the lowest in tumors from animals that were given A/C beer to drink. Finally, the highest proportional tumor grading score was found in animals that were given water or A/F beer to drink, whereas the lowest was found in animals that were given A/C beer to drink.

Specific Pyrrolidone Carboxypeptidase Type I (Pcp I) and Type II (Pcp II) Activities

Specific Pcp activities in hypothalamus, anterior and posterior pituitary, and mammary gland in control animals and animals with NMU-induced breast cancer that were given water, A/C and A/F beer to drink are shown in Figs. 4 and 5.

Table 1. Tumoral growth and size parameters after 122 day of the first *N*-methyl-*N*-nitrosourea (NMU) administration in animals which were given water, alcoholic (A/C), and nonalcoholic (A/F) beer to drink.

Groups	Latency period	Tumoral incidence	Tumors per rat	Major diameter	Minor diameter	Tumor volume
NMU Water	80.8 ± 6.7	56.0 %	1.0 ± 0.0	2.4 ± 0.3	1.8 ± 0.2	3.88 ± 0.1
NMU A/C beer	84.1 ± 5.5	53.8 %	1.0 ± 0.0	1.2 ± 0.3*	1.1 ± 0.2*	0.93 ± 0.2**
NMU A/F beer	71.5 ± 7.9	42.8 %	1.1 ± 0.1	2.3 ± 0.4	1.8 ± 0.3	4.19 ± 0.1

Latency period is expressed as days between the first NMU injection and the appearance of the first tumor; tumoral incidence is expressed as the percentage of rats that developed at least one tumor; Mean number of tumors per rat is expressed as the number of tumors per rat in animals developing at least one tumor. Major and minor diameters are expressed in centimeters. Tumor volume is expressed as $^1/_2$·major diameter·(minor diameter)^2. Data are expressed as mean ± SEM

*$P < 0.05$;

**$P < 0.01$.

Hypothalamus

In the hypothalamus (Fig. 4A), Pcp I specific activity significantly decreased in animals with NMU-induced mammary tumors ($P < 0.05$ for water and A/F beer and $P < 0.01$ for A/C beer) when compared with their corresponding matched control groups. However, animals with breast cancer which were given A/C beer to drink showed significantly lower levels of Pcp I than those animals which received water or A/F beer ($P < 0.05$ in both cases). Hypothalamus Pcp II specific activity was not detected for any of the groups analyzed.

Anterior Pituitary

In the anterior pituitary (Fig. 4B), no significant differences were found between control animals and animals with NMU-induced breast cancer which were given water or A/F beer to drink. However, animals with NMU-induced mammary tumors which were given A/C beer to drink significantly decreased Pcp I in anterior pituitary when compared with their corresponding controls ($P < 0.01$), but also when compared with animals with breast cancer which were given water or A/F beer to drink ($P < 0.05$ in both cases). Anterior pituitary Pcp II specific activity was not detected for any of the groups analyzed.

Posterior Pituitary

In posterior pituitary (Fig. 4C), a significant decrease ($P < 0.01$) was found in control animals which were given A/F beer to drink when compared with control animals which received water or A/C beer to drink. In addition, no significant differences were found between control animals and animals with NMU-induced breast cancer that were given water or A/C beer to drink. On the contrary, those animals with NMU-induced mammary tumors significantly increased posterior pituitary Pcp I ($P < 0.01$) when compared with their corresponding controls or with animals with breast cancer which were given water ($P < 0.05$) or A/C beer ($P < 0.05$) to drink. Posterior pituitary Pcp II specific activity was not detected for any of the groups analyzed.

Mammary Gland

Pcp I specific activity in mammary gland is showed in Fig. 5A. No significant differences were found in control animals, independently of the drink administered. However, animals with NMU-induced mammary tumors which were given water or A/C beer to drink, significantly increased mammary Pcp I specific activity ($P < 0.01$), whereas animals with breast cancer which were given A/F beer to drink did not modify Pcp I specific activity, with their values remaining significantly lower than those observed in animals with breast cancer which received water or A/C beer. Mammary gland Pcp II specific activity is showed in Fig. 5B. In the same way, no significant differences were found in control animals, independently of the drink administered. However, animals with NMU-induced mammary tumors which were given water, A/C or A/F beer to drink, significantly increased mammary Pcp II specific activity ($P < 0.01$ in all cases). In addition, animals with breast cancer which were given water to drink showed the highest values of Pcp II specific activity in mammary gland ($P < 0.01$).

Serum Estradiol (E_2) and Progesterone (P_4) Levels

Figure 6 shows serum circulating levels of E_2 (Fig. 6A) and P_4 (Fig. 6B) in control animals and animals with NMU-induced mammary tumors, which were given water, A/C and A/F beer to drink. A significant decrease ($P < 0.05$) was found in E_2 levels in NMU-treated animals which were given A/C beer. No significant differences were found among other groups (Fig. 6A). Conversely, significant increases ($P < 0.05$ in all cases) were observed in serum P_4 levels in NMU-treated animals when compared with control

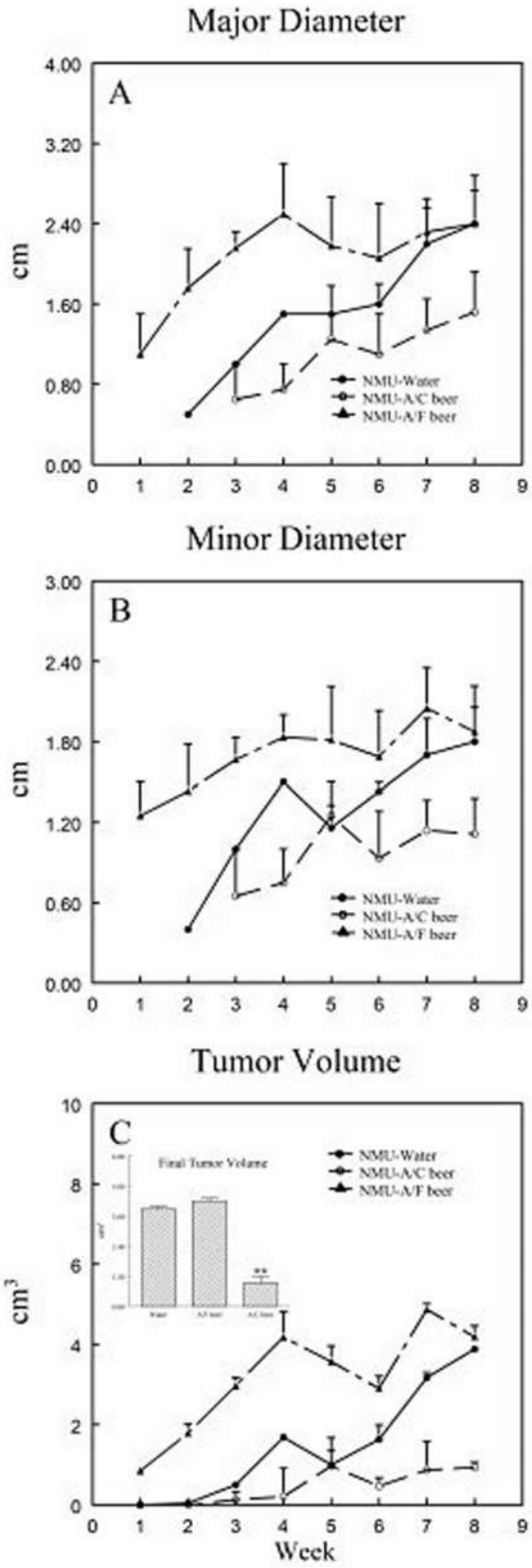

Figure 2. Evolution of tumor major (A) and minor (B) diameters and tumor volume (C) of the mammary tumors induced by *N*-methyl-*N*-nitrosourea (NMU) in animals which were given water, alcoholic (A/C) and nonalcoholic (A/F) beer to drink, since the appearance of the first tumor until the end of the experiment (in weeks). Final tumor volume for all groups analyzed is also shown. Data is expressed in centimeters or cm^3 as mean ± SEM ($^{**}P < 0.01$).

animals, independently of the type of drink administered (Fig. 6B).

Discussion

The present report has analyzed the effects of a moderate consumption of A/C and A/F beer on breast cancer in an animal model of mammary tumors induced by NMU. We have shown that Wistar rats increase their beer intake independently of the presence of mammary tumors, and that the animals prefer A/F beer over A/C beer, and both of them over water. This effect was already described by McGregor et al. (42) as a consequence of the organoleptic characteristics of beer. Inour experience alcohol intake was ≈0.6 g/animal/day. However, this increase in drink intake does not promote changes in food intake. In fact, although specific changes in body weight took place during the experiment, no significant changes in total weight gain were observed between the different experimental groups.

In the same way, we have not found significant effects of beer intake in carcinogenesis parameters such as latency period, tumoral incidence or mean tumor number per rat. However, we have shown an important effect of A/C beer intake, decreasing tumor size and volume, pointing to the beneficial effects of A/C beer consumption in the terms of mammary tumor development in the present model. In contrast, these effects were not observed with A/F beer or water consumption.

Similarly, the histopathological analysis showed that tumors with the lowest grading score came from animals that were given A/C beer to drink, both suggesting an effect of this type of beer on tumoral progression and growth that could be related to the changes found in Pcp specific activities in the hypothalamus-pituitary-mammary gland axis.

The hormonal dependency of the mammary tumors developed by the administration of NMU (41), as also showed here, indicates that the Pcp activity may have an important role in the tumoral process at different levels, including the nervous system. It is well known that the hypothalamic activity of Pcp is not regulated by estrogens (2). Nevertheless, the decrease in hypothalamic Pcp I specific activity described here could lead to an increase in the levels of its substrate, GnRH, that could be responsible of the changes in the circulating levels of E_2 and P_4. Furthermore, animals that were given A/C beer to drink showed the lowest values of hypothalamic Pcp I, in association with the lowest levels of circulating

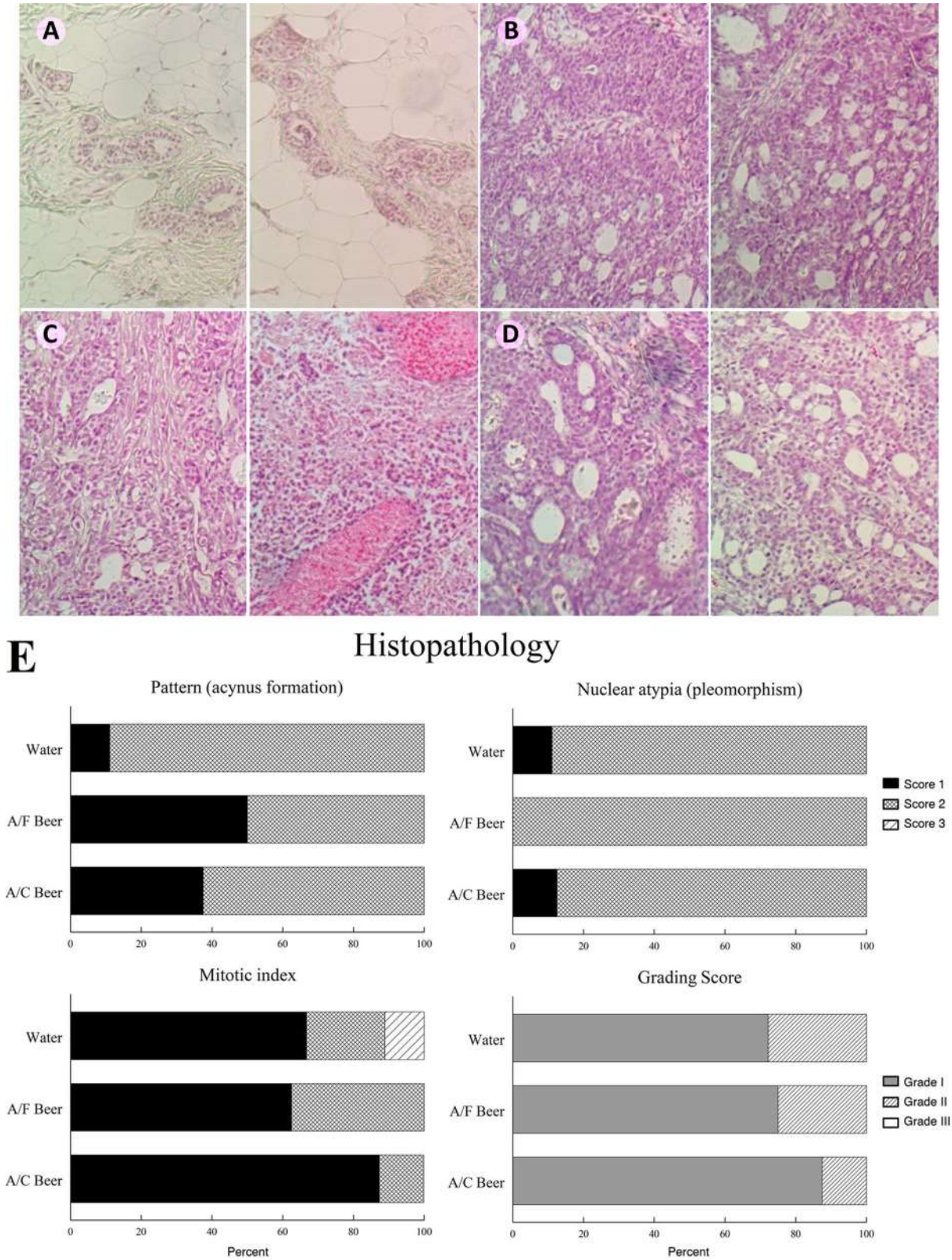

Figure 3. Histopathological characteristics of mammary tissue from control healthy animals (A) and animals with breast cancer induced by *N*-methyl-nitrosourea (NMU) which were given (B) water, (C) alcoholic (A/C), and (D) nonalcoholic (A/F) beer to drink. E: Histopathological results of the morphological analysis of mammary tumors induced by *N*-methyl-*N*-nitrosourea (NMU) in animals which were given water, alcoholic (A/C) beer and nonalcoholic (A/F) beer to drink, displayed as the percentage of tumors from each group exhibiting the different categories of the histopathological feature analyzed (see "Material and Methods" section).

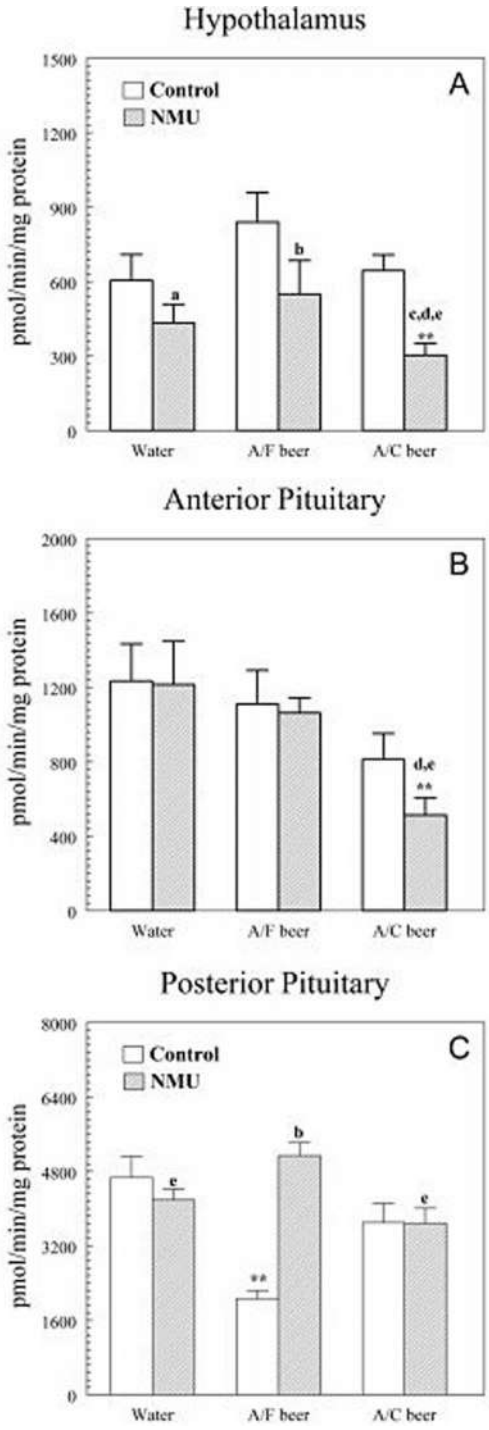

Figure 4. Pyrrolidone carboxypeptidase type I (Pcp I) specific activity in hypothalamus (A), anterior pituitary (B), and posterior pituitary (C) of control animals and animals with mammary tumors induced by *N*-methyl-*N*-nitrosourea (NMU) which were given water, alcoholic (A/C) beer and nonalcoholic (A/F) beer to drink Data is expressed as picomoles of pyroglutamyl-(β)-naphthylamide hydrolyzed per min and per mg of protein as mean ± SEM (**$P < 0.01$; [a]$P < 0.05$ vs. control animals which were given water to drink; [b]$P < 0.05$ vs. control animals which were given A/F beer to drink; [c]$P < 0.01$ vs. control animals which were given A/C beer to drink; [d]$P < 0.05$ vs. animals with mammary tumors which were given water to drink; [e]$P < 0.05$ vs. animals with mammary tumors which were given A/F beer to drink).

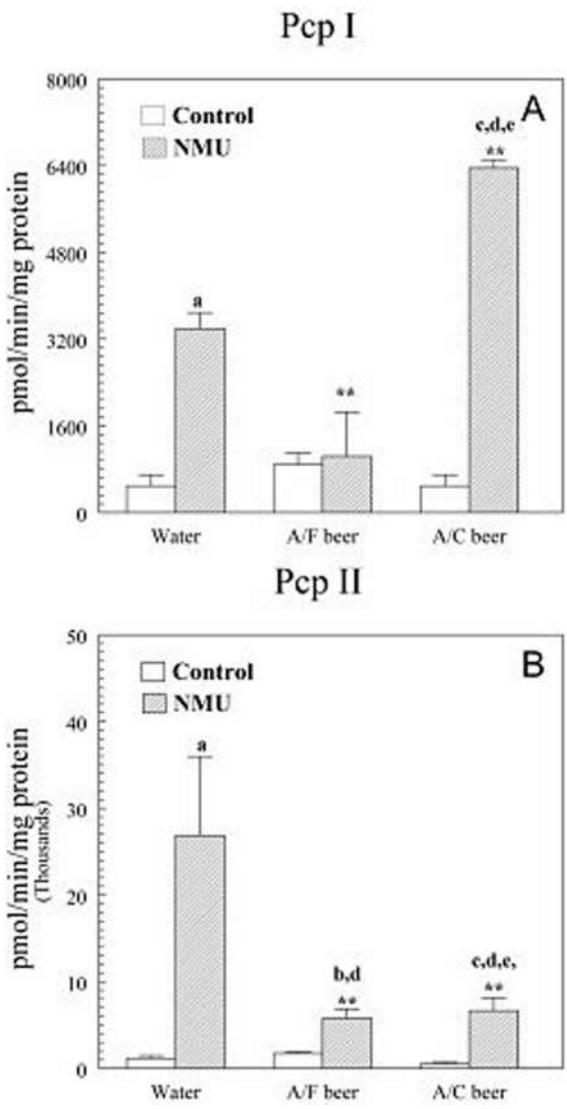

Figure 5. Pyrrolidone carboxypeptidase type I (Pcp I) (A) and type II (Pcp II) specific activities in mammary gland of control animals and animals with mammary tumors induced by *N*-methyl-*N*-nitrosourea (NMU) which were given water, alcoholic (A/C) beer and nonalcoholic (A/F) beer to drink. Data is expressed as picomoles of pyroglutamyl-(β)-naphthylamide hydrolyzed per min and per mg of protein as mean ± SEM (**$P < 0.01$; [a]$P < 0.01$ vs. control animals which were given water to drink; [b]$P < 0.01$ vs. control animals which were given A/F beer to drink; [c]$P < 0.01$ vs. control animals which were given A/C beer to drink; [d]$P < 0.01$ vs. animals with mammary tumors which were given water to drink; [e]$P < 0.01$ vs. animals with mammary tumors which were given A/F beer to drink).

E_2, whereas all the groups increased their circulating levels of P_4 independently of the drink intake. In addition, the significant decrease in Pcp I activity in all NMU-treated groups suggest a clear role of the Pcp I in the tumoral process, and A/C beer interferes with it. Alcohol is a drug of abuse that is known to alter hypothalamic functions related to control reproduction (47,48). However, authors as Schliep et al. (49)

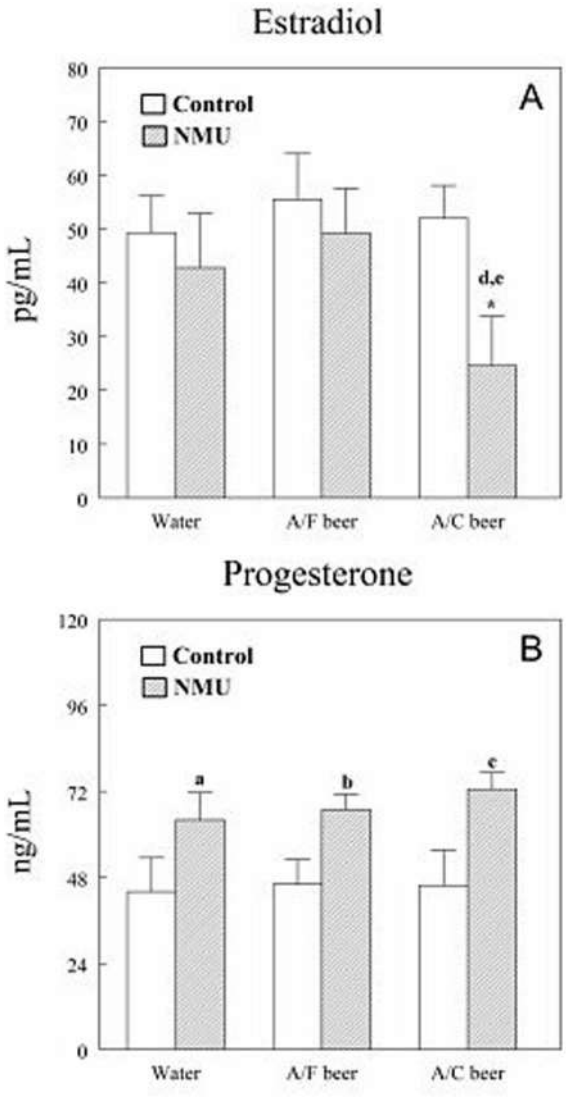

Figure 6. Serum circulating levels of estradiol (A) and progesterone (B) in control animals and animals with mammary tumors induced by *N*-methyl-*N*-nitrosourea (NMU) which were given water, alcoholic (A/C) beer and nonalcoholic (A/F) beer to drink. Data is expressed in picograms per milliliter for estradiol and nanograms per milliliter for progesterone (B), respectively, as mean ± SEM (**$P < 0.01$; $^{a}P < 0.05$ vs. control animals which were given to drink water; $^{b}P < 0.05$ vs. control animals which were given A/F beer to drink; $^{c}P < 0.05$ vs. control animals which were given A/C beer to drink; $^{d}P < 0.05$ vs. animals with mammary tumors which were given water to drink; $^{e}P < 0.05$ vs. animals with mammary tumors which were given A/F beer to drink).

have described that moderate alcohol intake does not appear to have adverse short-term effects on menstrual cycle function nor deleterious long-term effects of alterations in reproductive hormones. Therefore, our results suggest that moderate alcohol consumption could affect both Pcp activity and GnRH release at the hypothalamic level, which ultimately disrupts the production of steroid hormones and slows tumor growth.

Similarly, in the anterior pituitary, changes in Pcp I specific activity were exclusively found in NMU-treated animals with mammary tumors that were given A/C beer to drink. This behavior of Pcp I specific activity in relation to the development of breast cancer in the NMU model was been already described by us (50).

In the posterior pituitary, one of the most striking changes occurs in both control animals and animals with NMU-induced mammary tumors that were given F/A beer to drink. In these animals, the opposite response in Pcp I specific activity is found when we compare them to their corresponding groups that were given water or A/C beer to drink. These results could be associated to additional effects of Pcp activity in processes related to drink intake, drink taste or fluid intake regulation, and needs further research. In addition, the effects of the several compounds generated during the de-alcoholization process in the production of A/F beer cannot be discarded. In fact, it has been proposed that the imbalance among some of the generated products could be involved in tumoral development in this experimental breast cancer model (40). It is well known that during the production of A/F beers by careful fermentation to reduce or limit the worthy flavor impression when the fermentation is arrested at an early stage, the short active phase of the yeasts can contribute to the formation of several fermentation by-products that also influence the taste of the beer (51).

In mammary tissue, we have described here a very high increase in both Pcp I and Pcp II specific activities in all animals with NMU-induced breast cancer but not in their corresponding control groups, supporting again a main role of these enzyme activities in the tumoral process (13,14,50,52). However, this increase is variable depending on the type of drink ingested. Thus, the highest values of Pcp I are found in NMU-treated animals which were given A/C beer to drink, whereas the lowest levels were found in animals which were given A/F beer to drink. Intermediate values were seen in animals that were given water to drink. Nevertheless, the highest Pcp II specific activity is found in NMU-treated animals that were given water to drink, whereas the lowest levels were found in NMU animals that were given A/F beer to drink. These activities could be clearly associated with the tumor size and volume, characteristic of each experimental group.

Conversely, we have previously described that a decrease in circulating Pcp specific activity occurs in the serum of NMU-treated animals with mammary

tumors (50) and in neoplastic and adjacent tissues of women with breast cancer when compared with unaffected tissue, indicating that local factors may be selectively modified by the tumoral process in the affected tissue (52) and also that species differences exist. In addition, alterations found here in Pcp activities in hypothalamus, pituitary, and mammary gland in animals with breast cancer could be the response to the increase in GnRH levels and the modification of the paracrine/autocrine regulation of this hormone at mammary gland level.

Finally, we should not ignore that several beneficial anticancer properties have been described for beer based on the presence of antioxidant components such xanthohumol and 8-PN, mainly in A/C beer but not in A/F beer due to the de-alcoholization process. In fact, as aforementioned, the composition of beer is highly modified as a consequence of the de-alcoholization process. This fact is of great importance because compounds such as 8-PN show an activity that activates or inhibits tumor proliferation depending on its concentration (53). In any case, several beneficial effects have been described for a moderate consumption of A/C beer on tumoral growth parameters due to the presence of xanthohumol and its anticarcinogenic properties (54). In this regard, several studies detail its effects on cell growth inhibition of breast cancer aggressive cellular lines such as MCF-7 and MDA-MB-231 and on invasiveness using tests on collagen in leukemia cellular lines (35,55–57). Our results on tumoral size and volume agree with these other several studies.

However, we would like to point out that our study shows limitations related to the extraction of tumor tissue since one section was used for the determination of Pcp activity and another for the histological determination, and although very possibly both share the histological characteristics described, it cannot ensure it with total precision. Another limitation of our study is that the profound alterations of estrous cycle in animals with breast cancer (58) do not allow to compare it with control animals. Therefore, circulating estradiol and progesterone levels are the best approximation to the knowledge of the hormonal status of the animals.

Conclusions

Moderate consumption of A/C but not A/F beer would have beneficial effects against the development of rat's mammary tumors induced by NMU through the modification of the endocrine status mediated by GnRH and by the modification of Pcp I and II activities in the hypothalamus-pituitary-mammary gland. Also, the failure of A/F beer to show these beneficial effects could be related to the elimination of key compounds of beer during the de-alcoholization processes.

Disclosure Statement

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