Scandinavian Journal of Laboratory Animal Science

2018, Volume 44, Number 3
ISSN 2002-0112

Original scientific article

Effects of exogenous sex hormones on mouse estrous cycle, vaginal microbiota and immune cells

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Summary

Sex hormones are often administered to mice in vaginal microbial studies in order to synchronize estrous. Our objective was to evaluate the effect of estradiol-hemisuccinate (EH) or medroxyprogesterone-acetate (MPA) administration on estrous cycle, vaginal microbiota, and immune cell populations of various organs in a murine model. Two-month-old female BALB/c mice were intramuscularly injected with EH (day -2) or MPA (day -5) to induce estrous (E) or diestrous (DE), respectively. On sampling days (Sd) 0, 2, 6 and 8, vaginal washings (v.w.), vagina, blood, spleen and bone marrow (BM) samples were taken. Most of the animals remained in E or DE states until Sd 6 after EH or MPA administration, respectively. The number of cultivable vaginal bacteria was not modified by hormonal treatments; higher quantities were detected in mice in E. Only EH administration modified serum sex hormone levels, increasing serum estradiol on Sd 0. In v.w., myeloid population was dominant while lymphoid populations were not detected. Only MPA administration induced a reduction in myeloid cells on Sd 0. Hormonal treatments did not affect myeloid populations in BM but caused a slight decrease in T and B cells. In spleen, hormonal administration did not affect B or T population size while an increase in mature B cells and a decrease in immature B cells were observed in MPA-treated mice compared with EH-treated mice. Thus, although both hormonal treatments induced slight changes in some of the parameters evaluated compared to control animals, adequately standardized and consistent experimental protocols were established for further studies.

Introduction

The female reproductive tract is a specialized and highly dynamic organ system that is affected by numerous sequential physiological and morphological changes driven by cyclical fluctuations of reproductive hormones (Wira et al., 2010). Hormones exert their effect on various target sites. One of these sites is the vaginal epithelium, a constant cell renewal system in which the cells divide and mature from the basal membrane and are released into the lumen and then into the vaginal canal. Cell maturation, surface layer thickness and desquamation are dependent on estrogen stimulation (Larsen, 1993). Moreover, hormonal fluctuations also exert their effect on the microbial colonization of the vaginal mucosa. It has

been suggested that the composition of the vaginal microbiome is influenced by estrogens, based on research demonstrating that these hormones stimulate the deposition of glycogen in the vaginal epithelial tissue, which could be metabolized by native vaginal microorganisms (Larsen, 1993; Eschenbach et al., 2000).

In addition, the female reproductive tract has a very specialized immune system which has evolved to afford protection against pathogenic agents. This is possible due to the action of sex hormones that regulate different immunological parameters such as transport of immunoglobulins, cytokine levels, distribution of different cell populations and antigen presentation in genital tissues during the reproductive cycle (Wira et al., 2010). Furthermore, sex hormones exert their effect at the systemic level on a wide cell variety (Sakiani et al., 2013).

Mice are the mammals of choice for many types of *in vivo* experimentation because of their small size and short reproductive cycle. Their estrous cycle lasts 4-5 days and is divided into 4 phases: proestrous (estimated time, 18 h), estrous (~28 h), metaestrous (~8 h), and diestrous (~53 h) (Byers et al., 2012; Mclean et al., 2012). The murine vaginal tract is lined by a keratinizing stratified squamous epithelium. The vaginal epithelium is characterized by the presence of keratin in proestrous and estrous states, leucocyte influx at metaestrous and a thin epithelium at diestrous (OECD, 2009). Experimental estrogen or progesterone treatments in mice, in established protocols, produce states where the vaginal epithelium remains keratinized or extremely thin, respectively (Kaushic et al., 2000; Song et al., 2008; Patras et al., 2013).

Estrous cycle management in mice by external hormone administration has been widely used in experiments aimed at the study of vaginal pathogen challenges in which cycle synchronization is required to investigate susceptibility to selected pathogens (Kaushic et al., 2000, 2003; Song et al., 2008; González et al., 2009; Patras et al., 2013). This is the way in which murine vaginal infections caused by *Chlamydia trachomatis* and Human Papilloma Virus were maintained in animals in which a diestrous state had been induced by treatment with exogenous progesterone (Kaushic et al., 2000; 2003). In contrast, some vaginal opportunistic pathogens such as *Streptococcus agalactiae* and *Candida albicans* or the pathogen *Neisseria gonorrhoeae*, require a dominant estrogen state to establish an infection in murine models (Song et al., 2008; González et al., 2009; Patras et al., 2013). Although exogenous treatment with progesterone or estrogen produces histological similarities to diestrous or estrous states, the effects of these hormones on murine estrous cycle, vaginal microbiota and immunity have been rarely reported. Thus, knowledge of the effect of hormonal treatments on these parameters is required. Moreover, it is useful when standardization of animal protocols is needed. Therefore, the aim of this work was to evaluate the effect of estradiol and progesterone exogenous administration on estrous cycle, vaginal microbiota, and immune cell populations of several organs in an experimental murine model.

Materials and Methods

Mice. Two-month-old female BALB/c mice weighing 25-30 g from the inbred colony of CERELA (Centro de Referencia para Lactobacilos) were used. The experiments were independently performed three times employing three animals per experimental group and sampling day (Sd). Animals were housed in plastic cages and fed *ad libitum* with a conventional balanced diet, keeping their environmental conditions constant. An intramuscular single dose of estradiol-hemisuccinate (EH) (0.5 mg) (Eutocol, Craveri S.A.I.C. Laboratory, Buenos Aires, Argentina) at day -2 or medroxyprogesterone-acetate (MPA) (2 mg) (Medrosterona, Craveri S.A.I.C Laboratory, Buenos Aires, Argentina) at day -5 was administered to mice to induce and maintain estrous (E) or diestrous (DE) states, respectively (Silva de Ruiz et al., 2001; Grangette et al., 2004; González et al., 2009; Li et al., 2010; Patras et al., 2013). Both hormones were dissolved in saline. Day 0 was considered the day on which the hormones began to exert their effect. Thereafter, samples were taken on days 0, 2, 6 and 8 (Fig. 1).

Three animals per Sd, without hormonal injection, were included as control groups. Samples were only taken from those animals in E or DE states [determined from Papanicolaou (PAP)-stained vaginal smears, as described below]. Experimental data obtained for each state on the different Sd were grouped in order to form control groups of mice in E (E-control) and DE (DE-control) states. The Institutional Laboratory Animal Care and Use Committee of CERELA approved the experimental protocol CRL-BIOT-LMP-2010/2A used in this work.

Sampling. Every Sd, vaginal washings (v.w.) were obtained under sterile conditions using automatic pipettes with tips loaded with 50 µl of 2% (w/v) foetal bovine serum (FBS, from NATOCOR, Cor-

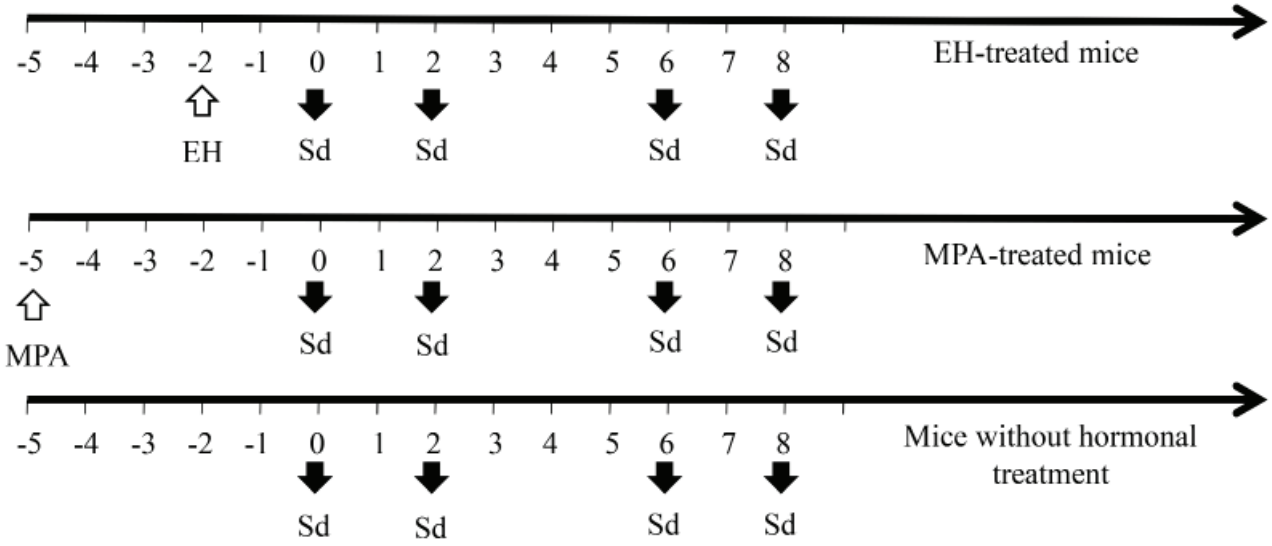

Figure 1. Schematic of administration of exogenous sex hormones and sampling days in BALB/c mice. The white up arrow indicates the day of hormone inoculation of mice with 0.5 mg estradiol-hemisuccinate (EH) or 2 mg medroxyprogesterone-acetate (MPA). The black down arrows indicate the days where the samples were taken and mice were sacrificed (Sd = sampling day).

doba, Argentina)-PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, pH 7.2). Seven v.w. with fresh FBS–PBS were pooled from each mouse to be used for the different protocols. Subsequently, mice were anesthetized and subsequently bled by cardiac puncture and later dissected to remove vagina, femur (to isolate bone marrow cells) and spleen.

Determination of estrous cycle states. The induction and maintenance of the different states of the estrous cycle was evaluated and analyzed by cytological and histological techniques. Pictures were taken with an Axio Scope A1 Carl Zeiss microscope (Germany). The images were processed using Axio-Vision Release 4.8 software.

Cytological analysis. Aliquots of 20 µl v.w. were spread onto glass slides, immediately fixed in 96° alcohol, stained with PAP (Biopur, Santa Fe, Argentina) and observed by light microscopy at 400x. The different estrous cycle states (E and DE) were determined from the PAP-stained vaginal smears on the different Sd. One hundred cells were counted and classified as parabasal (BC) or intermediate cells (IC), basophilic (BSC) or eosinophilic (ESC) superficial cells, enucleated squama (ES) and cornified cell groups (G) (Silva de Ruiz et al., 2001). The results were expressed as the percentage of each type of cell in mice injected with hormones and in control groups.

Histological studies. The vaginal tissues were fixed in 4% (v/v) formaldehyde at 4°C and embedded in paraffin according to standard histological methods. Sections were cut at 4 µm, stained with hematoxylin-eosin (Biopur, Santa Fe, Argentina) and examined by light microscopy at 400x. The different states of the estrous cycle were determined according to OECD (2009).

Quantification of serum estradiol and progesterone. Serum was separated by centrifugation and frozen at -20°C. Quantification of estradiol and progesterone was carried out by the electro-chemiluminescence immunoassay (ECLIA) method with a COBAS 6000 CEE automatic analyzer (Roche Diagnostics, Mannheim, Germany).

Quantification of cultivable bacteria in vaginal washings. Appropriate dilutions of v.w. were plated on pH 6.4 De Man-Rogosa-Sharpe (MRS) (Merck, Germany), MacConkey (Britania, Argentina), Bile Esculin (Britania, Argentina), Mannitol Salt (MSA, Britania, Argentina), pH 5.5 *Lactobacillus* Selective (LBS) (Fluka, Switzerland), and pH 5.5 MRS agarized media to quantify cultivable lactic acid bacteria, enterobacteria, enterococci, staphylococci and lactobacilli, respectively. MacConkey, Bile Esculin and MSA plates were incubated at 37°C for 24 h under aerobic conditions, while MRS and LBS plates were incubated at 37°C for 48 to 72 h under anaerobic

conditions. The number of microorganisms was expressed as colony forming units (CFU)/ml of v.w.
Determination of total immune cells in v.w., bone marrow, spleen and blood. The total number of immune cells in v.w., bone marrow (BM) and spleen cell suspensions (described below) and blood was determined in a Neubauer chamber by standard methodology. Furthermore, in blood smears stained by the modified May Grünwald-Giemsa technique, granulocyte and lymphocyte percentages were determined using an optical microscope at 1000x magnification.

Evaluation of v.w., BM and spleen immune cells by flow cytometry. BM cells were isolated by flushing femurs with 2% FBS-PBS. Splenocytes were harvested by homogenization through a tissue strainer. Red blood cells were lysed by adding 2 ml of 10% lysing solution (Lysing Solution, BD Bioscience, Sparks, MD, USA). After that, v.w., BM and spleen cells were washed twice with 2% FBS-PBS by centrifugation at 500 g for 4 min at 4°C. In order to evaluate the expression of cell surface markers on leukocytes, v.w. (10^5 viable cells/tube), BM (10^6 viable cells/tube) and spleen (10^6 viable cells/tube) cell suspensions were pre-incubated with CD32/CD16 anti-mouse (Fc block) antibody for 15 min at 4°C. Then, cells were washed with 1% FBS-PBS and incubated with specific monoclonal antibodies conjugated with fluorochrome for 30 min at 4°C in the dark. The antibodies used for v.w. leukocytes and BM were fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3, phycoerythrin (PE)-labeled anti-mouse Gr-1, and biotinylated anti-mouse B220. The antibodies for splenic cells were FITC-labeled anti-mouse CD3, PE-labeled anti-mouse CD24, and biotinylated anti-mouse B220. After incubation, cells were washed with 1% FBS-PBS. Samples treated with biotinylated antibodies were incubated with peridinin chlorophyll-α (PerCP)-labeled Streptovidin for 15 min at 4°C. All the antibodies were obtained from BD Bioscience, San Diego, CA, USA. Then, cells were washed and re-suspended in 500 µl PBS. The samples were analyzed in a FACSCalibur cytometer (BD, CA, USA). Finally, the data were evaluated using the FlowJo software (7.6.5, TreeStar Inc., OR, USA). The leukocyte region was selected from forward scatter (FSC) versus side scatter (SSC) plots according to size (FSC) and cellular complexity (SSC). Afterwards, the expressions of the different markers under study were evaluated in the corresponding leukocyte gate. The CD3, Gr-1 and B220 markers allowed us to determine the behavior of T lymphocyte, myeloid cell and B lymphocyte populations, respectively. The B220/CD24 combination provided information about the degree of maturation of B cells.

Statistical analysis. Analysis of variance (ANOVA) using a general linear model was applied to determine statistically significant differences between values obtained on the different days post-inoculation of EH-treated and E-control mice, and between MPA-treated and DE-control mice. Moreover, the ANOVA-general linear model was also applied to determine the main and interaction effects of factors (type of hormone and sampling day). Significant differences between mean values were calculated with Tukey's test using the MINITAB software (version 16 for Windows). A P value < 0.05 was considered as statistically significant.

Results

Effect of sex hormones on mice vaginal cytology and histology. EH administration to BABL/c mice induced the E state up to Sd 6 in 90% of the mice. On Sd 8, only 66% of the mice were in this state according to cytological and histological results. MPA administration induced the DE state up to Sd 2 in 90% of the mice. On Sd 6 and 8, 80% and 44% of the mice, respectively, were in the same state. Figure 2 summarizes the cytology and histological patterns obtained in mice treated with the sex hormones. The E state showed vaginal smears with ESC with a pyknotic core, ES and G (Fig. 2A). The histology of this state was characterized by the stratum corneum (Fig. 2C).

The DE state was apparent in vaginal smears with BSC, BC, IC and leukocytes (Fig. 2B). In the histological smears, polygonal and plump epithelial cells showing early mucification could be seen in the superficial layers of the stratum germinativum (Fig. 2D).

The cytological and histological patterns of the E and DE states observed in mice treated with EH and MPA, respectively, were similar to those of their respective controls (data not shown).

When analyzing the hormone effects on epithelial cell quantification, no significant differences ($P > 0.05$) were found in the number of each type of vaginal epithelial cells (ESC, BSC, IC, BC, ES, and G) on the different Sd after the inoculation of each hormone. In the E state of EH-treated mice, a significant predominance ($P < 0.01$) of ES (53.55% ± 19) was observed with respect to all the cell types quantified in this phase (ESC = 21% ± 11; G = 18% ± 15; BSC

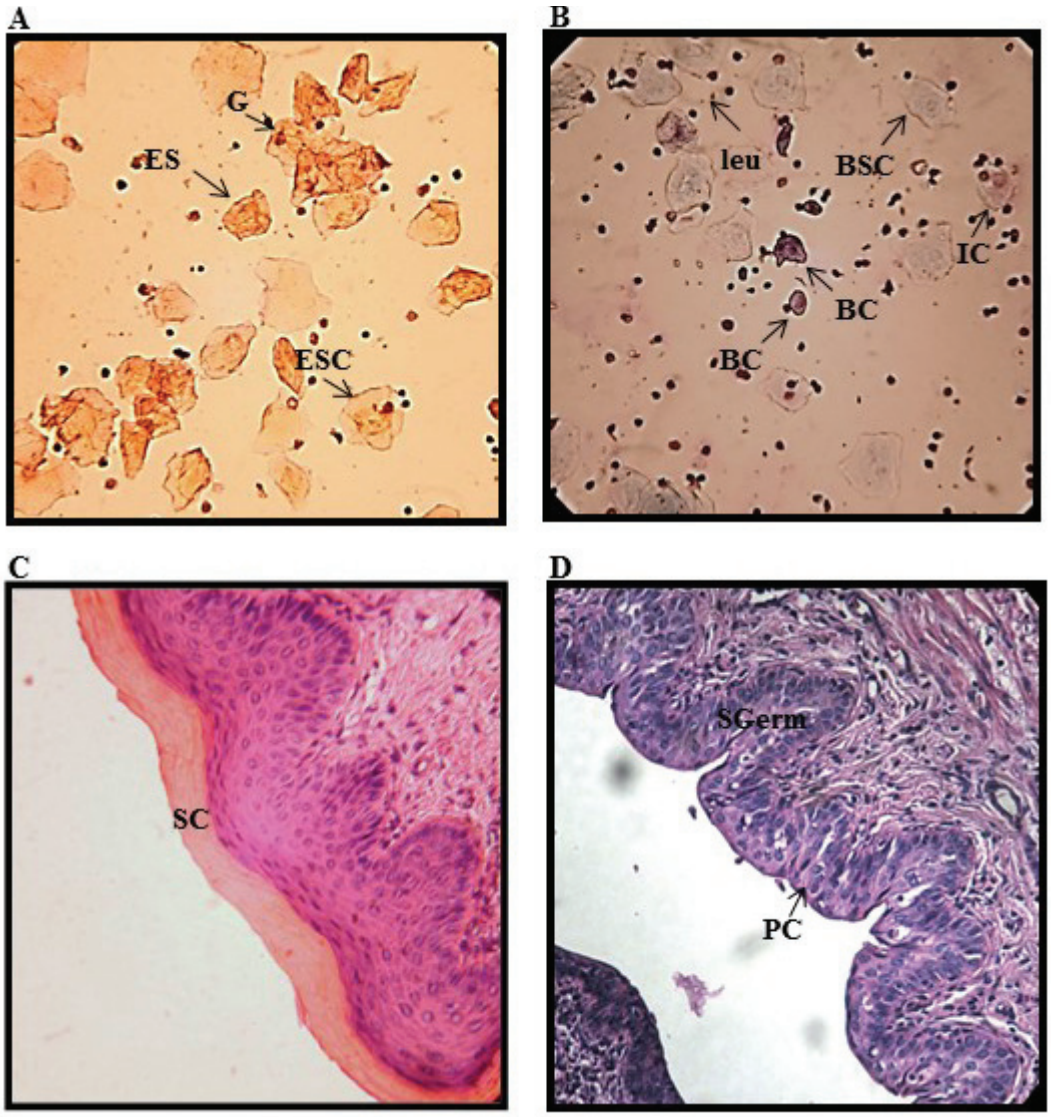

Figure 2. Photomicrographs of PAP-stained vaginal smears of A. estrous state from estradiol-hemisuccinate (EH)-treated mice and B. DE state from medroxyprogesterone-acetate (MPA)-treated mice. Vaginal slices stained with Hematoxylin–Eosin C. estrous state from EH-treated mice and D. DE state from MPA-treated mice (Magnification, 400x). BC: basal cells, IC: intermediate cells, BSC: basophilic superficial cells, ESC: eosinophilic superficial cells, ES: enucleated squama, G: cornified cell groups, leu: leukocyte, SC: stratum corneum, SGerm: stratum geminativum and PC: polygonal and plump cells.

= 2.3% ± 4.6; IC = 3% ± 7 and BC = 2% ± 6). The ES number was significantly higher with respect to all the other cells evaluated on Sd 0, 6 and 8 (Fig. 3A). In the DE state of MPA-treated mice, BC = 44.91% ± 7.6 were higher (P = 0.03) than the other cells under study: ESC = 20% ± 6; BSC = 17% ± 7.4; IC = 9.5% ± 8.5; ES = 8.3% ± 5.6 and G = 1.5% ± 2.5. The number of BC was significantly higher than all the rest of the cells quantified only on Sd 0, as indicated in Figure 3B.

When comparing the percentages for the various vaginal cells from mice inoculated with hormones and their respective controls, no significant differences (P > 0.05) were observed between both experimental groups. These results, together with the qualitative observation of the cytological and histological patterns of the animals, demonstrated that the exogenous inoculation of hormones adequately induced the E and DE states of the murine estrous cycle, without causing changes in either vaginal cytology or histology.

Quantification of hormone levels in serum. The hormone concentrations of EH-treated mice were 10.41 ± 1.19 pg estradiol/ml serum and 1.79 ± 0.78 ng progesterone/ml. On Sd 0, the 14.4 pg estradiol/ml value obtained was significantly higher (P = 0.02) than in E-control mice (6.17 ± 1.16 pg estradiol/ml serum). On the remaining Sd, no significant differ-

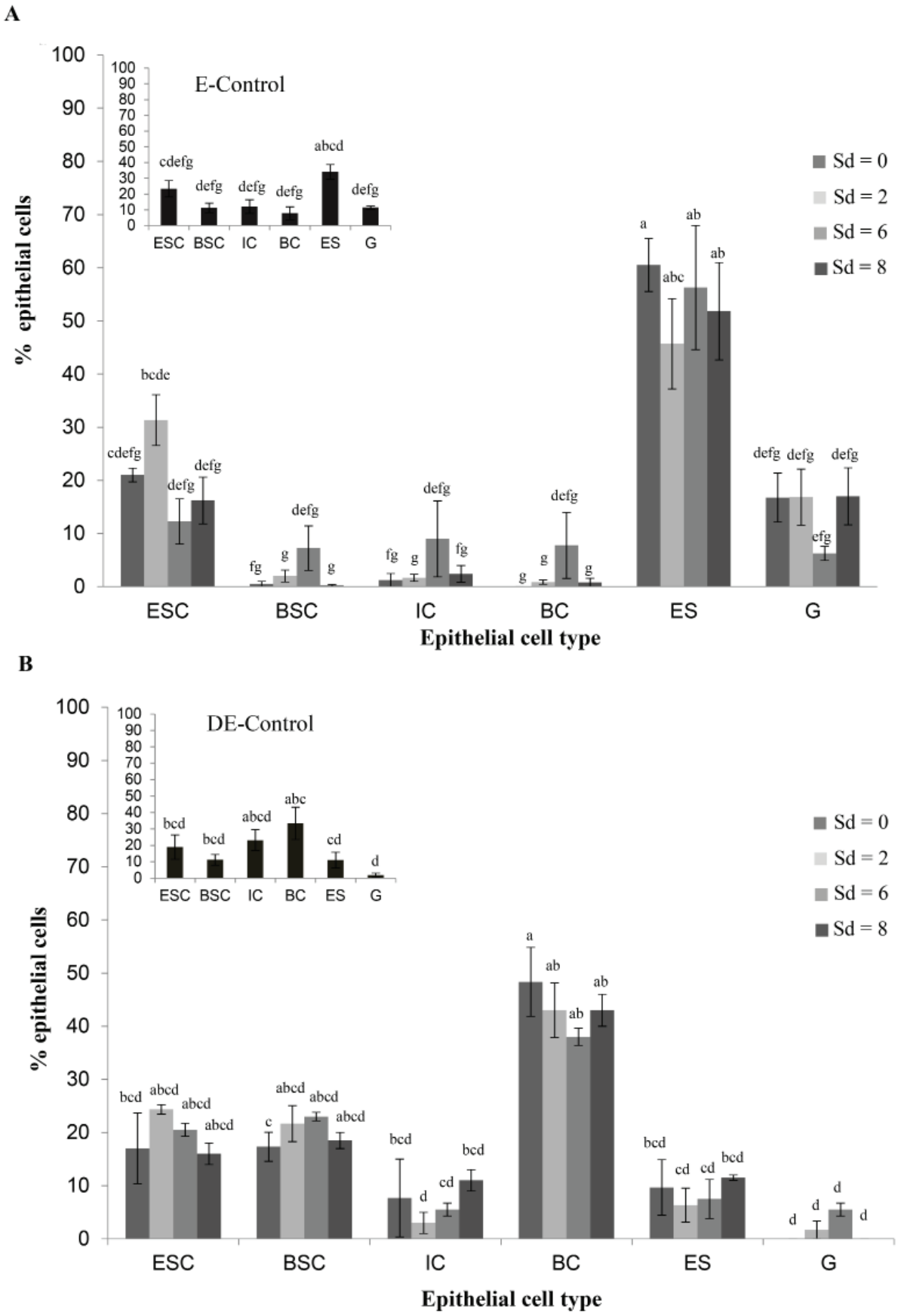

Figure 3. Percentage of epithelial cells in vaginal smears of A. estradiol-hemisuccinate (EH)-treated mice and estrous (E)-control mice and B. medroxyprogesterone-acetate (MPA)-treated mice and diestrous (DE)-control mice. Data are plotted as the mean values from the percentage of epithelial cells ± standard error. Statistically significant differences between the results obtained from EH or MPA-treated mice at the different sampling days (Sd) with their respective control are indicated by different letters ($P < 0.05$).

Table 1. Levels of estradiol and progesterone in serum of mice intramuscularly administered with estradiol-hemisuccinate (EH) (0.5 mg) or medroxyprogesterone-acetate (MPA) (2 mg) and control animals.

Experimental group	Sampling days	Estradiol[#] (pg/ml serum)[c]	Progesterone[#] (ng/ml serum)[c]
E-control mice	-	6.17 ± 1.16[b]	0.90 ± 0.01[a]
EH-treated mice	0	14.40 ± 1.40[a]	0.58 ± 0.51[a]
	2	6.10 ± 0.20[ab]	0.30 ± 0.55[a]
	6	9.65 ± 0.35[ab]	0.15 ± 1.65[a]
	8	8.98 ± 2.10[ab]	1.03 ± 0.93[a]
DE-control mice	-	6.40 ± 1.40[a]	2.14 ± 0.04[a]
MPA-treated mice	0	5.00 ± 0.00[a]	1.28 ± 0.19[a]
	2	9.30 ±1.20[a]	1.15 ± 0.04[a]
	6	8.30 ± 0.50[a]	1.32 ± 0.04[a]
	8	6.30 ± 0.79[a]	1.33 ± 0.56[a]

[#]Serum estradiol and progesterone were determined by the ECLIA method, as described in the text. [c]Data represent the mean value of pg estradiol/ml or ng progesterone/ml ± standard error. Statistically significant differences between the results obtained from EH or MPA-treated mice on the different sampling days (Sd) with their respective control are indicated by different letters ($P < 0.05$).
Sd were not included in control mice (without hormonal injection) because the data from animals in estrous (E) or diestrous (DE) state on the different Sd were grouped to form E-control or DE-control mice groups, respectively.

ences ($P > 0.05$) were detected between these groups (Table 1).

MPA-treated mice showed hormone levels around 6.88 ± 2.71 pg estradiol/ml and 0.21 ± 0.09 ng progesterone/ml. These levels were not significantly different ($P > 0.05$) from those in DE-control mice (Table 1).

Effect of sex hormones on the vaginal microbiota. When evaluating the cultivable murine vaginal microbiota, the predominant microorganisms found were enterobacteria (2.42 ± 0.22 log CFU/ml) ($P < 0.01$) in all experimental groups. Moreover, lactic acid bacteria (1.01 ± 0.20 log CFU/ml) (including enterococci) were present in a significantly higher number ($P = 0.04$) compared with staphylococci (0.11 ± 0.05 log CFU/ml). Staphylococci were obtained only in low numbers ($< 10^1$ CFU/ml) in EH-treated and E-control mice. No cultivable lactobacilli grew in LBS and pH 5.5 MRS (Fig. 4).

In EH-treated animals, the numbers of enterobacteria and lactic acid bacteria were not significantly different on all the days evaluated ($P > 0.05$), while enterococci decreased on Sd 2 compared with day 0 ($P = 0.03$) (Fig. 4A). In MPA-treated mice, enterobacteria decreased significantly ($P < 0.01$) on Sd 6 and 8 with respect to day 0 (Fig. 4B), while the number of enterococci and lactic acid bacteria was not significantly different ($P > 0.05$) for all Sd.

The comparison of the cultured vaginal microbiota of animals treated with exogenous hormones and their control did not show statistically significant differences between the different microbial groups evaluated. Only, a significantly higher number ($P = 0.02$) of enterobacteria was detected on Sd 0 in MPA-treated mice compared to their control (Fig. 4).

Finally, higher numbers of cultivable microorganisms were obtained from EH-treated mice compared to MPA-treated mice ($P < 0.01$) (Fig. 4). There was a significantly higher number of enterococci on Sd 0 of EH-treated mice compared with MPA-treated mice (Fig. 4).

Effect of sex hormones on immune cells. Immune cell quantification in v.w., BM, spleen and blood. When evaluating the number of leukocytes in v.w., a significant higher number ($P = 0.02$) in EH-treated mice was found on Sd 8 (8×10^5 cells/ml of v.w.) compared with the other days under evaluation and with E-control mice. In the MPA-treated mice, leukocyte numbers showed no statistically significant differences ($P > 0.05$) on all the days evaluated (around 1.3 to 2.8×10^5 cells/ml of v.w.). However, when comparing MPA-treated mice with DE-control animals, MPA administration significantly decreased ($P = 0.04$) the leukocyte numbers on Sd 0, 2 and 6. Moreover, no significant differences were observed between leukocyte numbers in v.w. of EH-treated mice and those of MPA-treated mice on all the days assayed (Table 2).

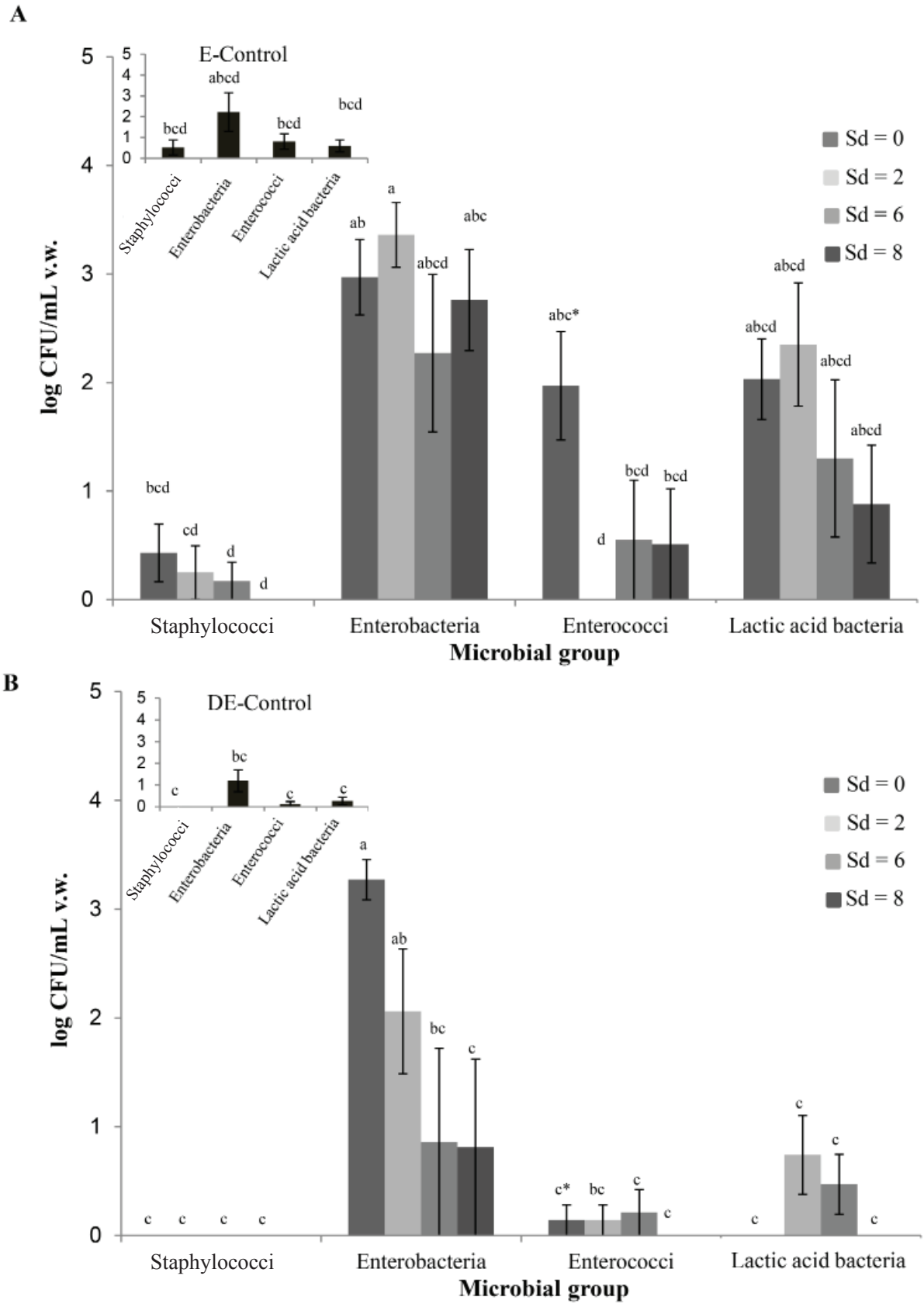

Figure 4. Effect of sex hormones on murine vaginal microbiota. Viable counts of staphylococci, enterobateria, enterococci, lactic acid bacteria from vaginal washings (v.w.) of A. estradiol-hemisuccinate (EH)-treated mice and estrous (E)-control mice and B. medroxyprogesterone-acetate (MPA)-treated mice and diestrous (DE)-control mice. Data are plotted as the mean number of viable cells (log CFU/ ml ± standard error). Statistically significant differences between the results obtained from EH or MPA-treated mice at the different sampling days (Sd) with their respective control are indicated by different letters ($P < 0.05$). Differences between EH-treated and MPA-treated mice at the different Sd are indicated by * ($P < 0.05$).

Table 2. Total number of leukocytes from vaginal washings of mice intramuscularly administered with estradiol-hemisuccinate (EH) (0.5 mg) or medroxyprogesterone-acetate (2 mg) (MPA) and control animals.

Experimental group	Sampling days	Total leukocytes[#] (x 10^5)/ml of v.w.
E-control mice	-	1.11 ± 0.56[b]
EH-treated mice	0	0.22 ± 0.03[b]
	2	1.96 ± 1.28[b]
	6	0.91 ± 0.22[b]
	8	8.02 ± 2.71[a]
DE-control mice	-	13.79 ± 3.60[a]
MPA-treated mice	0	2.77 ± 0.42[b]
	2	1.40 ± 0.38[b]
	6	1.38 ± 0.54[b]
	8	2.80 ± 2.26[ab]

[#]Data represent the mean number of leukocytes ± standard error from vaginal washing (v.w.). Statistically significant differences between the results obtained from EH or MPA-treated mice at the different sampling days (Sd) with their respective control are indicated by different letters ($P < 0.05$).
Sd were not included in control mice (without hormonal injection) because the data obtained from animals in estrous (E) or diestrous (DE) state on the different Sd were grouped to form E-control or DE-control mice groups, respectively.

Neither EH nor MPA administration induced significant modifications ($P > 0.05$) in the total immune cell number of BM, spleen or peripheral blood on all the days evaluated. Moreover, the number of leukocytes in these organs and in blood were not significantly different ($P > 0.05$) from those obtained from control animals and between hormonal treatments, and were around 3 x 10^7 cells/femur, 4 x 10^7 cells/spleen and 7 x 10^9 cells/ml in blood. Differential quantification of leukocytes in blood was not affected by hormonal treatments when compared to control animals (data not shown).

Phenotyping of v.w., BM and spleen immune cells. When evaluating the effect of sex hormones on myeloid and lymphoid immune cell populations of v.w., only Gr-1$^+$ cells were observed. No CD3$^+$ or B220$^+$ cells were detected in v.w. EH did not modify the percentage of Gr-1$^+$ cells with respect to Sd or E-control animals. However, MPA significantly reduced ($P = 0.02$) the Gr-1$^+$ cell percentage on Sd 0 with respect to the other days and to DE-control animals (Fig. 5A).

On the other hand, two different expression profiles (high and low) of Gr-1 marker were detected in the Gr-1$^+$ cell population (Fig. 5A, 5B). Gr-1^{high} cells in v.w. could represent neutrophils, while Gr-1^{low} cells could correspond to other granulocyte populations and macrophages (Herrera et al., 2014). The percentage of Gr-1^{high} and Gr-1^{low} cells obtained on all Sd following the two hormonal treatments was not significantly different ($P > 0.05$) compared to their respective controls. When evaluating the expression of this marker between the EH and MPA treatments, a significantly higher percentage ($P < 0.01$) of Gr-1^{high} cells (92.34% ± 2.09) and a lower percentage of Gr-1^{low} cells (2.10% ± 1.31) ($P < 0.01$) were obtained in EH-treated mice than in MPA-treated mice (74.60% ± 5.62 and 20.70% ± 5.92 of Gr-1^{high} and Gr-1^{low} cells respectively) on all Sd (Fig. 5A, 5B).

When determining the effect of hormonal treatments on the lymphoid cell population of BM, a lower percentage of CD3 cells (1.91% ± 0.11) compared to B220 cells (88.13% ± 0.81) was observed in the cytometry-lymphocyte region in all experimental groups (Fig. 5C). The percentage of CD3$^+$ cells in EH-treated mice was not statistically different ($P > 0.05$) from the E-control animals on all the Sd evaluated. However, in MPA-treated mice a lower percentage ($P = 0.041$) of CD3$^+$ cells on Sd 2 and 6 with respect to DE-control mice was obtained. When comparing EH and MPA treatments, a significantly higher expression ($P < 0.01$) of CD3$^+$ cells was observed in EH-treated mice than in MPA-treated mice on Sd 2 (Fig. 5C). When the B220 marker expression was evaluated in EH-treated mice, a significantly lower cell percentage ($P = 0.03$) was obtained on Sd 6 compared with the other days under study and with E-control animals. No differences were found in the percentage of B220$^+$ cells in MPA-treated mice compared with their respective control or between the two hormonal treatments (Fig. 5C). The evaluation of Gr-1$^+$ cells in the cytometry-granulocytes region showed no statistically significant differences ($P > 0.05$) among the Sd and control animals, or between hormonal treatments (data not shown).

In spleen, the percentage of CD3$^+$ lymphocytes was higher (50.09 ± 1.38) in all the experimental groups compared with B220$^+$ lymphocytes (41.03 ± 1.21). The total populations of CD3$^+$ and B220$^+$ cells in spleen were not statistically significant ($P > 0.05$) between all Sd and control animals, and between hormonal treatments, as shown in Figure 5D. On the other hand, as spleen is the organ where B lymphocyte maturation is completed, the expression of B220

vs CD24 was also evaluated in the cytometry-lymphocyte region. Two different expression profiles of the $B220^{high}CD24^{low}$ cells (B-mature lymphocytes) and $B220^{low}CD24^{high}$ cells (immature B lymphocytes) were studied (Barbieri et al., 2013). When evaluating the effect of hormonal administration on B-lymphocyte maturation, neither treatment modified the percentage of $B220^{high}CD24^{low}$ or $B220^{low}CD24^{high}$ cell populations with respect to their controls (Fig. 5E). When EH and MPA treatments were compared, a significantly lower percentage of immature B lymphocytes and a higher percentage of mature B lymphocytes in MPA-treated mice compared to EH-treated mice were observed ($P < 0.01$). This difference was significant only on Sd 0 (Fig. 5E, 5F).

Discussion

In several murine models of vaginal infection, some investigators have demonstrated that inoculation using different routes and doses (0.01 to 0.5 mg) of estradiol derivatives generated an estrous state at 2 days post-inoculation. This state persisted for a week and allowed an effective colonization of various microorganisms (González et al., 2009; Pietrella et al., 2011). On the other hand, other studies reported that 2 to 2.5 mg doses of different progesterone derivatives induced a diestrous state at day 5 after hormonal inoculation (Grangette et al., 2004; Li et al., 2010). On the basis of these studies, the effect of 0.5 mg of EH and 2 mg of MPA was evaluated in the present investigation and similar results were obtained. The

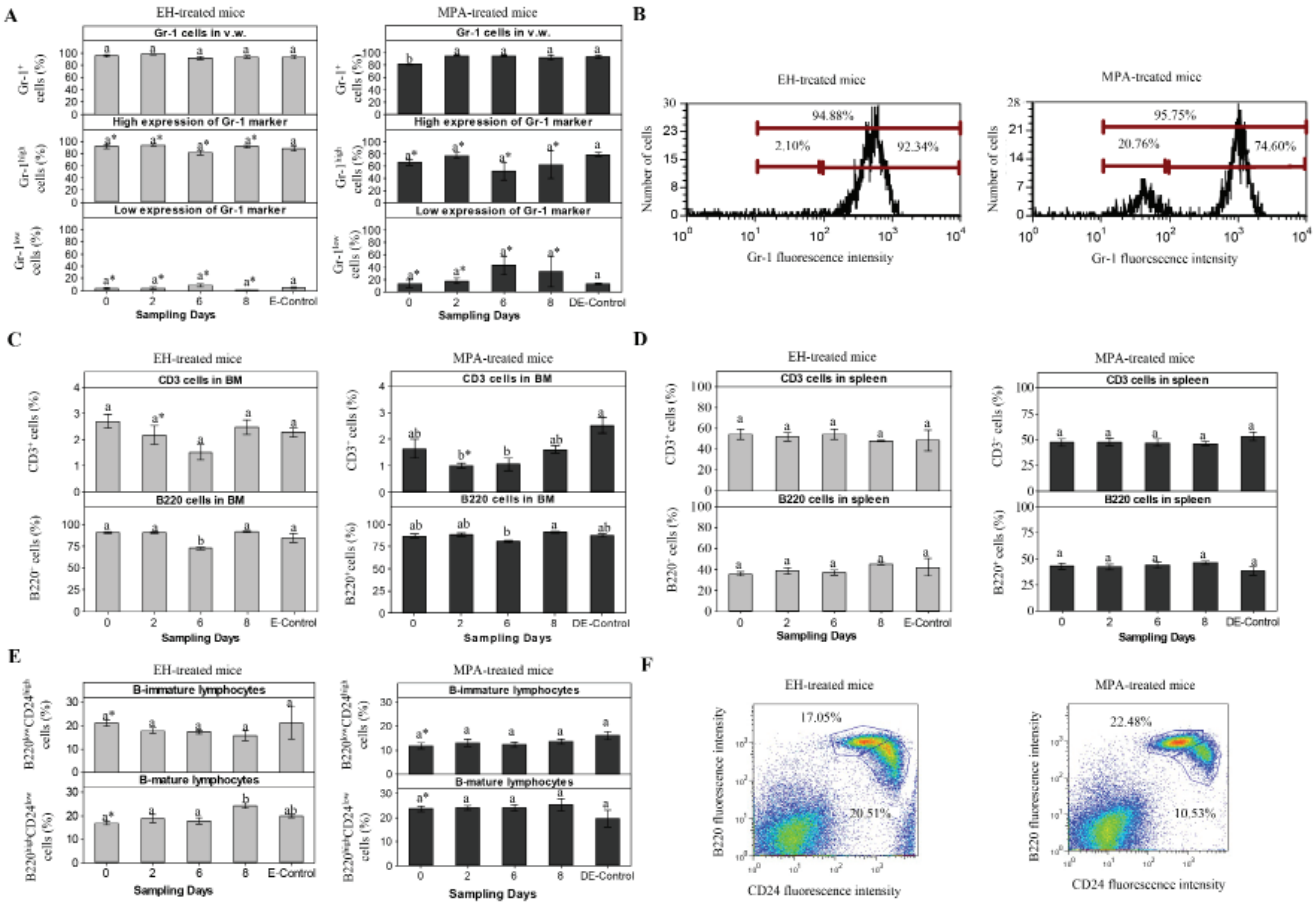

Figure 5. Effect of sex hormones on cells of murine immune system. Percentage of A. $Gr\text{-}1^{+}$, $Gr\text{-}1^{high}$ and $Gr\text{-}1^{low}$ cells from vaginal washing (v.w.), C. $CD3^{+}$ and $B220^{+}$ cells from bone marrow (BM), D. $CD3^{+}$ and $B220^{+}$ cells from spleen, and E. $B220^{high}CD24^{low}$ and $B220^{low}CD24^{high}$ cells from spleen of estradiol-hemisuccinate (EH)-treated, estrous (E)-control, medroxyprogesterone-acetate (MPA)-treated and diestrous (DE)-control mice. Data are plotted as the mean values of percentage of cells ± standard error. Statistically significant differences between the results obtained from EH or MPA-treated mice at the different sampling days with their respective control are indicated by different letters ($P < 0.05$). Differences between EH-treated and MPA-treated mice at the different sampling days are indicated by *($P < 0.05$). B. Histograms of EH and MPA-treated mice, obtained by flow cytometry, in which the high and low expression of Gr-1 marker was observed. F. Representative dot plot of B220 vs CD24 expression obtained by flow cytometry in EH and MPA-treated mice.

cytological and histological evaluations performed showed that EH administration induced an E state after 2 days of hormonal inoculation, while MPA administration induced a DE state at day 5 post-inoculation. Both states were maintained in most of the animals for approximately 6 days. Following these determinations, the effect of sex hormones was evaluated in different murine organs.

As in human beings, the murine reproductive tract undergoes structural changes during the estrous cycle. The human follicular proliferative phase is comparable to proestrous and estrous states of the murine estrous cycle, in which estrogens predominate, whereas the human secretory phase is analogous to mouse metaestrous and diestrous stages, in which progesterone predominates (Hickey et al., 2012). When evaluating the estradiol and progesterone serum levels in mice treated with exogenous sex hormones, slight differences were noted with respect to the hormonal levels of their controls, suggesting that the protocols applied induced similar states to the physiological modifications of the murine estrous cycle. Serum estradiol and progesterone levels of EH-treated mice were within the physiological range reported by Sasaki et al. (2009) and Byers et al. (2012) in mice. Only on Sd 0 were serum estradiol levels higher than those of E-control animals. In a similar way, Medina & Kincade (1994) reported an increase in serum estrogen levels shortly after the inoculation of 1 mg of estradiol in BALB/c mice, which were normalized at day 3. On the other hand, estradiol levels of MPA-treated mice presented values similar to those reported for a diestrous state (Sasaki et al., 2009; Byers et al., 2012). However, the progesterone levels detected in these animals were lower than the ones reported in diestrous, but close to those published by Sander et al. (2009) in BALB/c mice, where regression of the corpus luteum was observed, a state in which serum progesterone levels declined (a phase corresponding to late diestrus).

Sex hormones produce numerous physiological modifications in the vaginal tract, including changes in carbohydrate and protein metabolism that could alter the host's indigenous microbiota and the characteristics of the tissues colonized by microorganisms (Eschenbach et al., 2000). Analytical methods of cultivation and isolation have been widely applied in several laboratory animal species in order to evaluate their vaginal microbiota (Meysick & Garber, 1992; Noguchi et al., 2003). Thus, in the present work one of the objectives was to evaluate whether the administration of exogenous hormones induced some modification in the cultivable vaginal microbiota compared to control animals. The results obtained showed that neither of the two hormonal treatments altered the cultivable indigenous vaginal microbiota compared to their respective control. Moreover, a higher number of aerobic microorganisms was obtained in EH-treated animals compared to those treated with MPA. The predominant cultivable microorganisms in the murine vaginal tract of the BALB/c mice were enterobacteria, followed by lactic acid bacteria (among them enterococci) and lastly staphylococci (only detected in very low numbers in EH-treated and E-control mice). Cultured lactobacilli were not isolated from hormone-treated or control animals. In a similar way, other researchers have applied the cultured-dependent technique to evaluate some of the vaginal microbiota modifications: Noguchi et al. (2003) demonstrated by culture techniques a higher number of bacteria during estrous than diestrous and low to absent numbers of anaerobes in vagina of female ICR/Kud mice (age, 12 weeks). Different to our results, these authors were able to detect lactobacilli in LBS agar. Also, Meysick & Garber (1992) demonstrated by standard microbiological techniques that the vaginal microbiota of BALB/c mice (22-24 g) consisted mainly of *S. aureus* and *Enterococcus* species (32-76%), followed by lactobacilli and enteric bacilli (16-32%), and a low prevalence of both anaerobic species and coagulase negative staphylococci (4-16%). Moreover, these authors, as in our results, have shown that the estrogenization of mice with 0.5 mg of estradiol valerate did not significantly modify the vaginal microbiota, but there was a slight increase in the number of bacterial species isolated per mouse. In contrast to our results, Voronkova et al. (2008) reported that lactobacilli were the dominant bacteria in the mouse vaginal tract. Moreover, they found different genera of aerobic bacteria, including *Streptococcus, Staphylococcus, Micrococcus, Bacillus* and some anaerobic bacteria such as *Fusobacterium, Peptococcus, Peptostreptococcus* and *Bacteroides*. Taking into account that the cultured-based technique, frequently applied in our laboratory and used in the present work does not allow the isolation of all microorganisms, further studies should be carried out by applying molecular methodologies to evaluate the effect of administration of exogenous hormones on murine vaginal microbial populations.

Experimental animal studies have shown that estrogen and progesterone modulate immune reactivity by modifying the number of granulocytes, inflammation mediated by T cells, cytotoxicity mediated by natural killer cells and production of immu-

noglobulins (Josefsson et al., 1992; Nilsson & Carlsten, 1994; Attanasio et al., 2002). Based on these findings, the sex hormone effect on the immune cells of vagina, BM, spleen and blood was evaluated to determine if these two hormonal treatments altered the murine immune system. Evaluation of the effect of these sex hormones on the immune cells present in v.w. revealed that the EH treatment did not modify the number of total leukocytes with respect to control animals, except on Sd 8. On that day, higher numbers of leukocytes were detected in EH-treated mice compared to E-control mice and the remaining Sd. These results could be explained by the gradual loss of the hormone over the course of the study and by the fact that mice could pass from estrous to metaestrous, a period characterized by a large number of leukocytes in v.w. (Mclean et al., 2012). When the MPA treatment was applied, a decrease in total leukocyte numbers was observed compared to control animals. In addition, no differences were found in the total numbers of leukocytes in v.w. of EH- and MPA-treated mice. These results indicate that MPA treatment modified vaginal leukocytes since the diestrous state of the estrous cycle is characterized by a higher leukocyte number than the estrous state (Mclean et al., 2012). Considering the cytological and histological similarities between MPA-treated and DE control mice and the hormonal levels discussed above, we can suggest that MPA treatment induced a state similar to late DE.

Flow cytometry studies showed that neutrophils were the predominant cells in the leukocyte population of murine v.w. Populations of T and B lymphocytes were not detected. Giraldo et al. (2012) evaluated immune cells present in v.w. of 142 women with a diagnosis of bacterial vaginosis, vulvovaginal candidiasis or normal microbiota by flow cytometry and reported that neutrophils were the predominant leukocytes in all the samples and that macrophages and T and B lymphocytes were either absent or present at very low percentages.

Evaluation of the effect of sex hormones on the granulocyte population of v.w. by flow cytometry showed that treatment with EH did not modify the percentage of Gr-1^{+} cells in v.w. compared to control animals, whereas inoculation of MPA decreased the percentage of Gr-1^{+} cells only on Sd 0. In addition, no significant differences between the two treatments were observed in the percentage of Gr-1^{+} cells. However, differences in the expression of Gr-1^{+} cells in v.w. between hormonal treatments were obtained. A higher percentage of Gr-1^{high} cells (characteristic of neutrophils) and a lower percentage of Gr-1^{low} cells (macrophages or other granulocytes) were observed in EH-treated mice compared to those treated with MPA. The high and low patterns in the Gr-1^{+} population in the two hormonal treatments were not significantly different from their respective controls. These results suggest that during the E states of estrous cycle in which estrogens predominate, neutrophils are the prevailing leukocytes. However, in the DE state where progesterone is dominant, other cell types such as monocytes and macrophages could be present. On this subject, there is evidence that some cells of the immune response are regulated in the middle of the menstrual cycle by estrogens in order to prevent an immunological attack on the fetus during pregnancy (Lee & Chiang, 2012).

The study of the effect of sex hormones on cells involved in the systemic immune response showed that neither the total number of leukocytes in BM and spleen nor the differential and total number of leukocytes in peripheral blood were modified by the hormonal treatments. Similarly, Tikare et al. (2008) reported that there were no significant differences in the total number and differential characterization of leukocytes in blood during the different phases of the menstrual cycle in women. However, Smith et al. (2007) detected an increased number of neutrophils in human blood when estrogen levels were higher during the menstrual cycle.

The two hormonal treatments did not produce significant differences in the myeloid populations of BM, but caused a slight decrease in T and B lymphoid populations. EH administration reduced B220^{+} cells only on Sd 6, while MPA affected CD3^{+} cells on Sd 2 and 6. Taking into account that several studies performed in animal experimental models have reported the suppressive effect of estrogen on T and B lymphopoiesis, and that neither estrogen nor progesterone modified the number or role of myeloid and granulocytes cells in BM (Medina et al., 1993; Medina & Kincade, 1994; Medina et al., 2000; Bhavanam et al., 2008), it is possible to suggest that the hormonal treatments applied in the present investigation slightly affected the lymphoid population of BM without affecting animal welfare.

Cytometric studies performed by De León-Nava et al. (2009) in BALB/c female mice demonstrated that gonadectomy did not affect the percentage of T and B populations in spleen. Moreover, Medina et al. (1993) and Medina & Kincade (1994) reported that mature B lymphocyte populations in the spleen of mice were not significantly modified during pregnancy or after estrogen treatment. These reports suggest that sex hormones do not induce important

modifications in splenic immune cells. In a similar way, the evaluation of immune cells in spleen demonstrated that neither of the two hormonal treatments affected $CD3^+$ and $B220^+$ cell populations or mature and immature B lymphocyte populations compared to their control. However, when the degree of B-cell maturation in the spleen was compared between the two hormonal treatments, a higher number of mature B cells ($B220^{high}CD24^{low}$) was observed in MPA-treated mice compared to EH-treated mice. It is noteworthy that Medina et al. (1993) and Medina & Kincade (1994) reported a partial depletion of a subset of B cells characterized by a high expression of CD24 in pregnant mice compared to non-pregnant mice. Taking into account these reports and our results, it is possible to suggest that higher progesterone levels could induce B lymphocyte maturation.

In conclusion, our results demonstrate that exogenous inoculation of estrogen and progesterone, despite slight changes induced in some of the parameters evaluated compared to untreated animals, allowed the establishment of adequately standardized and consistent experimental protocols for further studies. Moreover, this work shows the complexity of the endocrine interactions with different constituents of the vaginal tract and at the systemic level. Inoculation of estrogens allows the isolation of a higher number of autochthonous microorganisms in mice vagina, while progesterone promotes the presence of myeloid cells participating in the innate immune response of vagina and the maturation of B lymphocytes in spleen.

Acknowledgements

This paper was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) (PIP 545) and ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica) (PICT 2013-1187). We thank Elena Bru for her help with the statistical analysis of experimental results.

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