

Ferutinin dose-dependent effects on uterus and mammary gland in ovariectomized rats

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Summary. The present paper completes our recent study on the effects of phytoestrogen ferutinin in preventing osteoporosis and demonstrating the superior osteoprotective effect of a 2 mg/kg/day dose in ovariectomized (OVX) rats, compared to both estrogens and lower (0.5, 1 mg/kg/day) ferutinin doses. Morphological and morphometrical analyses were performed on the effects of different doses of ferutinin administered for one month on uterus and on mammary gland of Sprague-Dawley OVX rats, evaluated in comparison with the results for estradiol benzoate. To verify whether ferutinin provides protection against uterine and breast cancer, estimations were made of both the amount of cell proliferation (by Ki-67), and the occurrence of apoptosis (by TUNEL), two processes that in unbalanced ratio form the basis for cancer onset. The results suggest that the effects of ferutinin are dose dependent and that a 2 mg/kg/day dose might offer a better protective action against the onset of both breast and uterine carcinoma compared to ferutinin in lower doses or estradiol benzoate, increasing cellular apoptosis in glandular epithelia.

Key words: Ferutinin, Ovariectomized rat, Uterus, Mammary gland, Proliferation, Apoptosis

Introduction

Phytoestrogen ferutinin acts as an estrogen agonist by binding to the estrogen receptors and generating estrogen-induced responses (Appendino et al., 2002; Ikeda et al., 2002). Previous studies by the authors have shown that ferutinin exerts an osteoprotective action in ovariectomized rats, preventing bone loss or enhancing the recovery of lost bone mass resulting from severe estrogen deficiency (Palumbo et al., 2009; Ferretti et al., 2010). The authors recently demonstrated that the effect of ferutinin on bone appears to be dose dependent, and a 2 mg/kg/day dose appears to be more effective in preserving bone mass compared to both estrogens and lower ferutinin doses (0.5 and 1 mg/kg/day), particularly in skeletal regions with higher metabolic activity, presumably because it inhibits osteoclast activity (Cavani et al., 2012) rather than increasing bone deposition as previously proposed (Palumbo et al., 2009). Numerous data reported in the literature regarding phytoestrogens, together with the authors' studies to date agree in suggesting that ferutinin could be employed as an anti-osteoporosis drug, and, at specific doses, represent a good alternative to estrogen for use in Hormone Replacement Therapy (HRT) during the perimenopausal period.

It is well known that the positive osteoprotective function of HRT is associated with a negative increased risk of cancer in estrogen-sensitive organs, like the uterus and mammary gland. Notwithstanding the huge amount of data regarding the effects of phytoestrogens, their beneficial and detrimental biological role in estrogen-dependent malignancies are not well known.

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Despite the research reported in the literature, the role of phytoestrogens in cancer remains controversial: there is evidence for both inhibitory and stimulatory action on cancer cell growth. It is important to underline that the relationship between phytoestrogens and cancer probably depends on various factors, like timing, mode and dose of phytoestrogen administration, extraction procedures from the plant of origin, and individual metabolic differences of the targeted subjects. As regards the beneficial effects, studies were performed in which phytoestrogens appeared to be positive for reducing the risk of estrogen-dependent cancers, for example pre-menopausal (Lee et al., 1991; Hirose et al., 1995; Wu et al., 1996; Yang et al., 2012) and post-menopausal (Yuan et al., 1995) breast cancer. Regarding isoflavones (phytoestrogens present in high concentrations in legumes), these have been shown to be effective as anti-cancer preventive agents, particularly in breast, prostate, colon and gastric mucosa. An inverse correlation seems to exist between the amount of isoflavone-containing food ingested and the risk of cancer (Shimizu et al., 1991; Barnes et al., 1995; Kennedy, 1995; Wu et al., 2002; Yamamoto et al., 2003). As regards the detrimental effects, i.e. the absence of protection by reducing cancer risk and tumour development, some evidence was found that certain phytoestrogens can increase the risk of recurrence or growth of existing tumours, through their estrogenic properties (Messina et al., 2006; Duffy et al., 2007; Mosquette et al., 2007; Gaete et al., 2012). Some authors have shown that genistein (the most widely studied phytoestrogen present in soy) can stimulate cancer in colon (Rao et al., 1997) and mammary gland (Allred et al., 2004a,b; Wang et al., 2006; El Sheikh Saad et al., 2011) in rats fed with genistein-rich foods. Moreover, genistein has been shown to increase the rate of tumour growth in a dose-dependent manner in mice: the more abundant the genistein diet content, the higher the rate of tumour growth (Ju et al., 2001). However, genistein also exhibits an anti-tumour action in mice, including suppression of angiogenesis and induction of apoptosis, if combined with polysaccharides (Yuan et al., 2003). In *in vitro* studies, the proliferation of numerous types of cancer cells were found to be markedly enhanced (Lucki and Sewer, 2011) or inhibited (Yanagihara et al., 1993; Messina et al., 1994) by phytoestrogens. Some of these, like genistein and daidzein, exhibit a dose-dependent effect: at low concentration they act exclusively as estrogen agonists, while at higher concentrations they have inhibitory effects on cellular growth (Martin et al., 1978; Hsieh et al., 1998; de Lemos, 2001; Messina and Loprinzi, 2001). These actions were found to contradict the *in vivo* observations.

The aim of the present study was to evaluate, for the first time, the effects of different doses of ferutinin not only on the uterus (the only existing literature data on this topic were recently published by the authors (Ferretti et al., 2012)), but also on the mammary gland of ovariectomized rats. It is very important to establish

whether the same doses that provide the most beneficial effects on bone against osteoporosis (Cavani et al., 2012) also offer the best protection against excessive uterine and breast cell proliferation. Enhanced proliferation is undoubtedly a key process in the progression of tumours, together with decreased programmed cell death, i.e. apoptosis. Therefore, the present paper evaluates both cell proliferation and apoptosis in the epithelia of uterus endometrium and mammary gland of ovariectomized rats treated with different doses of ferutinin.

Materials and methods

Animals and drugs

It should be noted that the data on uterus and mammary glands reported in the present work were collected on the same animal groups used in a previous paper by the authors (Cavani et al., 2012).

Thirty female Sprague-Dawley rats weighing 170–200 g were purchased from Charles River (Calco, Lecco, Italy). They were 7 weeks old according to the general age-models used by Kalu (1991) and Fanti et al. (1998). The rats were housed two per cage and maintained under controlled conditions (22±1°C, 55–60% humidity, 12-h light/12-h dark).

All experiments were carried out according to the guidelines of the Bioethical Committee of the Italian National Institute of Health. Animal care, maintenance and surgery were conducted in accordance with Italian law (D.L. no. 116/1992) and European legislation (EEC no. 86/609).

Commercial rat pellets free of estrogenic substances (Global Diet 2018; Mucedola s.r.l., Milan, Italy) and drinking water were available *ad libitum* throughout the whole experimental period. After 7 days of acclimation to housing conditions, the rats were randomized into six groups of 5 animals each: one group of rats was sham-operated, whereas the rats of the other five groups were ovariectomized. The rats were anesthetized in advance by intraperitoneal administration with ketamine hydrochloride (Ketavet 100[®]; Farmaceutici Gellini S.p.a., Aprilia, Italy) plus xylazine hydrochloride (Rompun[®]; Bayer, Leverkusen, Germany). To enter the peritoneal cavity, surgery was performed by 1 centimeter incision through the muscle layer. After extracting the ovarian ducts, within a mass of adipose tissue, the ovaries were identified and bilaterally removed. The rats showed a time of revival and awakening from the administration of anaesthesia of 30 minutes. The six animal groups were indicated as follows:

SHAM: Sham-operated controls receiving vehicle (5% Tween 80 in water)

C-OVX: Ovariectomized controls receiving vehicle (5% Tween 80 in water)

EB-OVX: Ovariectomized treated with estradiol benzoate 1.5 µg per rat twice a week

0.5F-OVX: Ovariectomized treated with ferutinin

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0.5 mg/kg/day

1F-OVX: Ovariectomized treated with ferutinin 1 mg/kg/day

2F-OVX: Ovariectomized treated with ferutinin 2 mg/kg/day

The operation of the SHAM group was performed in the same way as the C-OVX group, but only exposing the ovaries. Ferutinin was supplied by Indena S.p.a. (Milan, Italy), solubilized in Tween 80 (5%) and deionized water and administered in a volume of 5 ml/kg by oral gavage. The dose of 2 mg/kg/day was selected on the basis of the authors' previous studies of ferutinin effects on rat sexual behaviour (Zanoli et al., 2005; Zavatti et al., 2006) as well as for preventing and recovering osteoporosis (Palumbo et al., 2009; Cavani et al., 2012). Estradiol benzoate (Estradiolo AMSA[®], Rome, Italy), used as a reference compound, was dissolved in peanut oil and subcutaneously injected in a volume of 0.3 mL per rat. The body weight of each animal was recorded before ovariectomy and after 30 days of treatment.

At the end of the treatment, all the rats were sacrificed and soon after the uteri were removed, freed from fat and weighed; mammary glands were also collected. All samples were then fixed in sodium phosphate (PBS) 4% buffered paraformaldehyde pH 7.4 solution, dehydrated in a graded series of ethanol and embedded in paraffin. Cross sections (5 μ m thick) were cut and mounted on superfrost[®] plus slides (Menzel-Glaser, Thermo scientific, Germany).

Morphology and morphometry

Morphological analyses were performed on uterus and mammary gland sections stained with Gomori trichrome and hematoxylin/eosin respectively, and observed using a light microscope (Zeiss Axiophot - Jena, West Germany) equipped with a camera (Nikon DS-5Mc) connected to a computer. The image analysis software used was NIS Elements AR, which enables histomorphometric evaluation. The following parameters were evaluated on three randomly selected sections of the uterus/mammary gland for each animal: i) cross-sectional areas of endometrium and myometrium; ii) cross-sectional thickness of myometrium (cross-sectional thickness of endometrium was not measured due to its variable thickness in different zones of the same section); iii) percentage of area of glandular epithelium in relation to the total cross-sectional area of endometrium; iii) the percentage of area of glandular epithelium in relation to the total cross-sectional area of mammary gland.

Immunohistochemistry (Ki-67)

For analysis of Ki-67 expression, the sections were deparaffinized in three changes of xylene for 2 min each, rehydrated and then subjected to heat-induced epitope retrieval: they were immersed in a water bath of 10

mmol/L citrate buffer pH 6.0 for 30-40 min at 98° C and then were cooled for 20 min. The sections were subsequently exposed to 0.3% solution of hydrogen peroxide for 5 min in order to deactivate endogenous peroxidase activity. Sections were rinsed in tap water and immunostaining was performed at room temperature using a Benchmark XT automated immunostainer (Ventana Medical Systems, Tucson, AZ). The slides were incubated with 300 μ L of primary monoclonal mouse anti-human Ki-67 antigen clone MIB 1 (Dako Italia Spa, Milan) diluted by 1:200 at approximately 37°C for 30 min with reagent diluent (Ventana Medical Systems). After rinsing, the sections were incubated for 4 min with Avidin-Biotin blocking solution (Ventana Medical Systems). 300 μ L of the appropriate anti-mouse biotinylated IgG-linking solution (Ventana Medical Systems) was applied to each section for 4 min at room temperature. Sections were again rinsed and allowed to react with 300 μ L of peroxidase-diaminobenzidine (DAB Detection Kit) substrate solution (Ventana Medical Systems) for 8 min, followed by counterstaining with Hematoxylin for 1 min, then removed from the autostainer, washed in warm water, dehydrated through graded alcohols, cleared in xylene, and cover slipped. Control reactions included: (1) sections incubated with the omission of primary antibody and processed as mentioned above, and (2) sections incubated with normal mouse serum instead of the primary antibody and processed as above.

Quantitative analysis was conducted by counting all Ki-67-positive cells of uterus luminal/glandular and breast glandular epithelia were counted on three randomly selected Ki-67-processed sections for each animal at x 40 magnification. The percentage of positive cells both in epithelia of uterus and in terminal end buds and alveoli of mammary gland was calculated in relation to the total number of epithelial cells.

In situ end-labelling analysis (TUNEL - ApopTag[®] Peroxidase Kits, Millipore)

Sections of uteri and mammary glands were deparaffinated, rehydrated and then treated in a humidified chamber with proteinase K (Boehringer, Mannheim, Germany) at 20 μ g ml⁻¹ for 15 min at room temperature, washed in bidistilled water, treated with 2% H₂O₂ in methanol for 10 min at room temperature and finally washed in bidistilled water. The slides were pre-incubated with terminaldeoxynucleotidyl transferase (TdT) buffer and 1 mM CoCl₂ for 5 min at room temperature and then incubated for 60 min in a humidified chamber at 37°C with 50 μ l TdT and biotinylated deoxyuridine triphosphate (Bio dUTP) (Boehringer) (TdT 0.3 U μ l⁻¹, Bio dUTP 8 μ M in TdT buffer and CoCl₂ 1 mM). The sections were then washed four times in bidistilled apyrogen water (2 min each), twice in phosphate-buffered saline (PBS) (5 min each), in human serum albumin 2% (5 min) and in PBS (5 min), then covered with streptavidin-biotinylated

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peroxidase complex (Boehringer) diluted 1:100 in a humidified chamber for 45 min at room temperature, washed in PBS and stained with diaminobenzidine 50 m_M (0.05%). The slides were finally washed in water and counterstained with 0.5% methyl green for 10 min.

Positive and negative controls were included in each experiment. For the positive controls, sections were treated with DNase I ($1 \mu g ml^{-1}$) (Boehringer) in DNase buffer for 10 min at room temperature before exposure to biotinylated dUTP and TdT. For negative controls, the sections were incubated without the TdT enzyme.

Quantitative analysis was conducted by counting all TUNEL-positive cells of uterus luminal/glandular and breast glandular epithelia on three randomly selected TUNEL-sections for each animal at x40 magnification. The percentage of immunostained cells in both luminal/glandular epithelia of uterus and breast glandular epithelia of terminal end buds and alveoli was calculated in relation to the total number of epithelial cells.

Statistical analysis

One-way analysis of variance (ANOVA) with Newman-Keuls test for post hoc comparisons was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA). Values of $P < 0.05$ indicate significant differences between the groups.

Results

The mean body weight values of all animal groups recorded before ovariectomy (T0) and after 30 days of treatment (T30) are shown in Figure 1A. No differences in body weight were present at the beginning of the experiment; after 30 days of treatment the body weight of the EB-OVX and all F-OVX groups was significantly lower compared to the C-OVX and SHAM groups.

Uterus

The uterine weights are shown in Figure 1B: the mean values of SHAM, EB-OVX and all F-OVX groups were significantly higher compared to the C-OVX group; moreover, in all F-OVX animals the uterine weight was always significantly lower compared to the EB-OVX and SHAM groups.

Histological observations of uterine cross sections are shown in Figure 2; it is possible to observe, at the same enlargement, the atrophic aspect of the uterus in C-OVX rats displaying a thin layer of endometrium and myometrium compared to all other groups. Figure 3 shows the most representative features of the luminal and glandular epithelia in the endometrium of all groups. In SHAM rats the luminal and glandular epithelium is mainly cylindrical monostratified. In C-OVX rats, instead, the luminal epithelium is always cuboidal monostratified, while the epithelial cells are higher in the

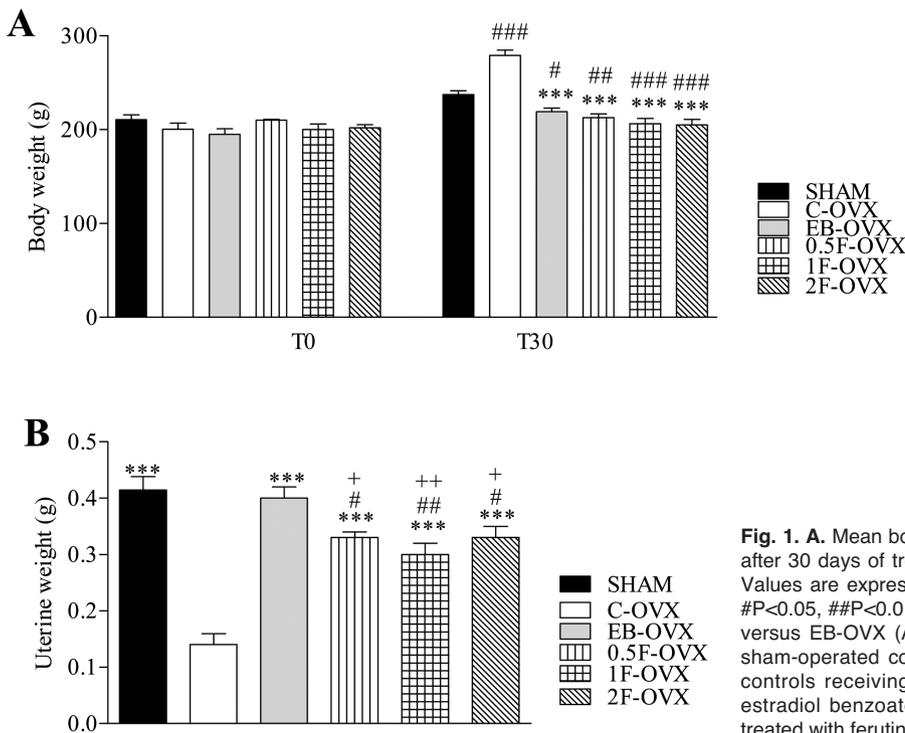


Fig. 1. A. Mean body weights recorded before ovariectomy (T0) and after 30 days of treatment (T30). **B.** Mean values of uterine weight. Values are expressed as mean \pm SEM. *** $P < 0.001$ versus C-OVX; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus SHAM, + $P < 0.05$, ++ $P < 0.01$ versus EB-OVX (ANOVA followed by Newman-Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day).

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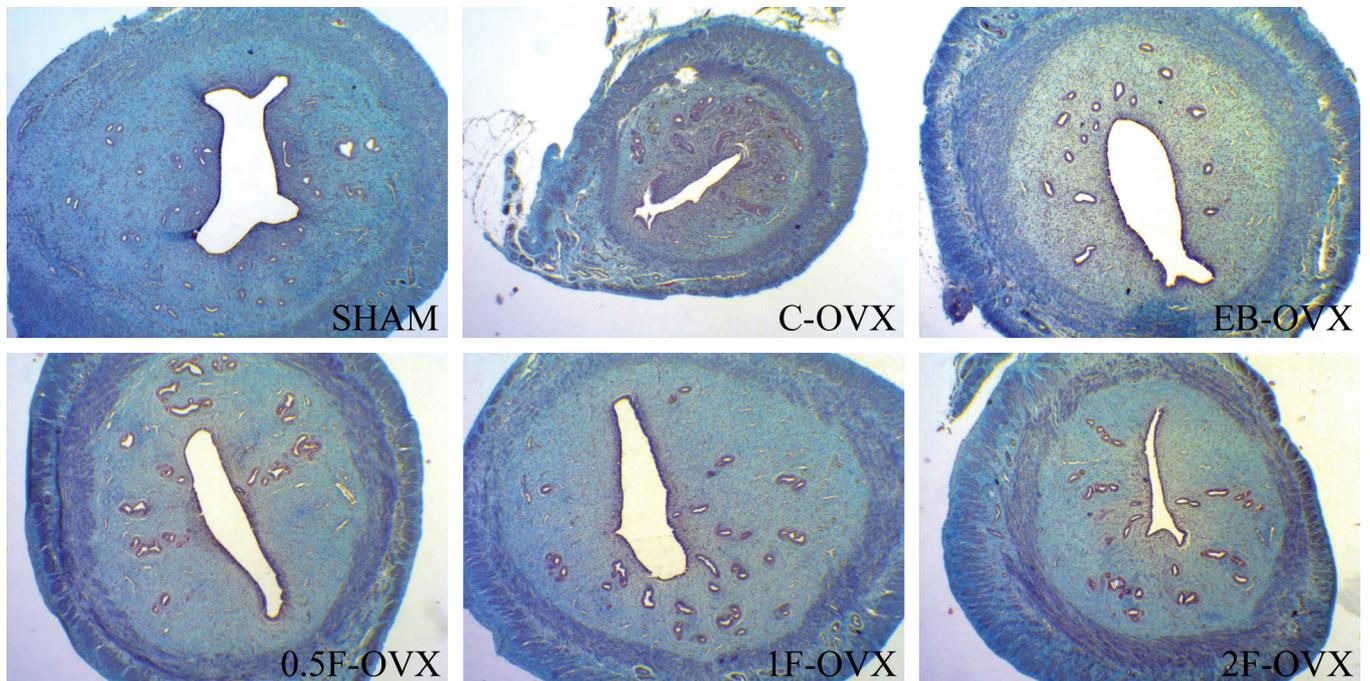


Fig. 2. Light microscopy, micrographs of Gomori trichrome-stained cross sections showing the uterine histology. SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day). x 25

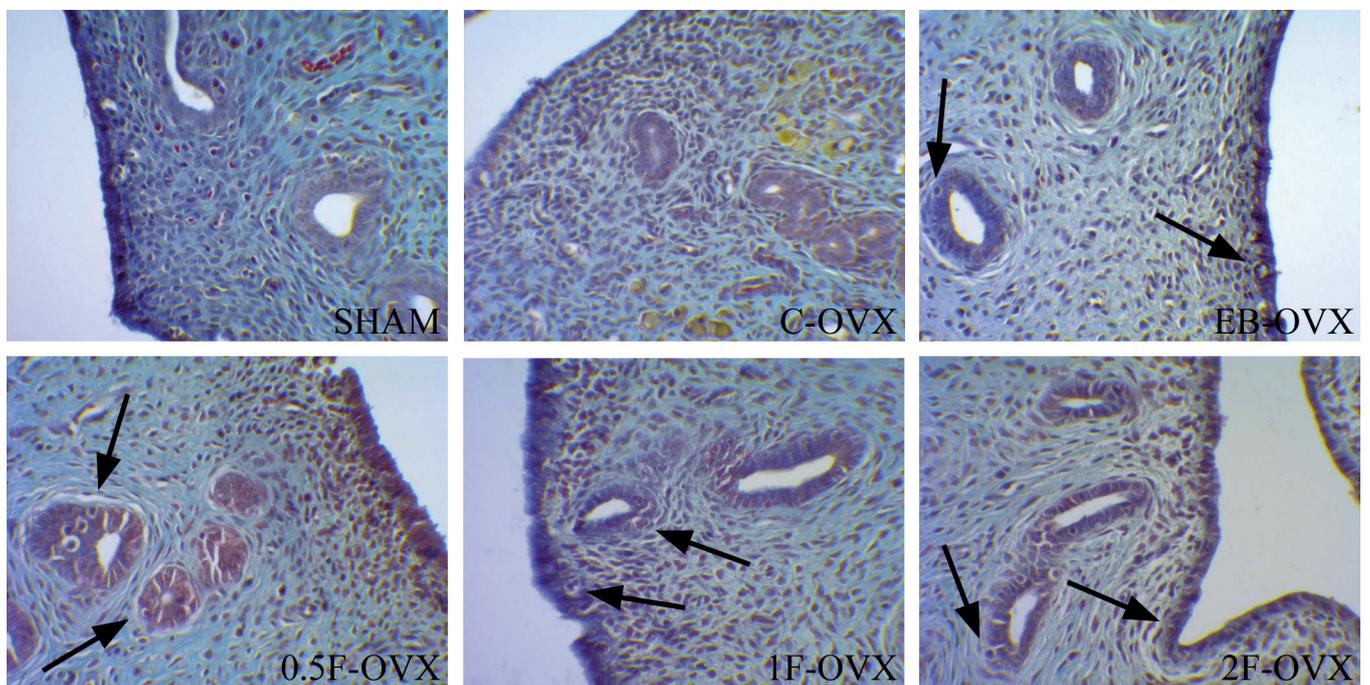


Fig. 3. Light microscopy, micrographs of the endometrial luminal and glandular epithelia. SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day). Arrows indicate intracellular cysts in the luminal and glandular epithelia. x 200

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glands, and the glandular lumen is small; moreover, the endometrium shows high stromal cellularity. In EB-OVX and in all F-OVX groups the luminal epithelium cells were shaped differently: sometimes they showed a cuboidal or cylindrical aspect with basally located nuclei, in other cases they appeared pseudostratified. The glandular epithelium was always cylindrical with a high cytoplasm/nucleus ratio. In both epithelia, intracellular cysts were present, indicative of cellular degeneration.

Morphometrical data are shown in Figures 4 and 5. In C-OVX rats the endometrial area is significantly lower compared to all other groups (Fig. 4A). All animals treated with ferutinin show, compared to C-OVX rats, an increase of the myometrial area (Fig. 4B) and myometrial thickness (Fig. 4C), even if the values are always lower than the EB-OVX and SHAM values;

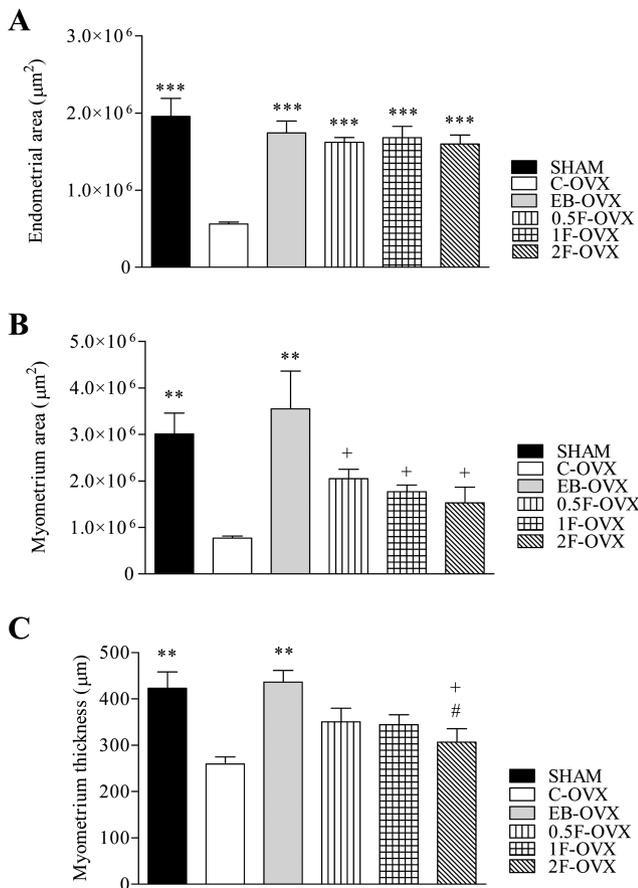


Fig. 4. Mean values of endometrial (A), myometrial (B) areas and myometrial thickness (C). Values are expressed as mean \pm SEM. ** $P < 0.01$, *** $P < 0.001$ versus C-OVX; # $P < 0.05$ versus SHAM; + $P < 0.05$ versus EB-OVX (ANOVA followed by Newman-Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day).

sometimes these differences are statistically significant. The percentages of glandular epithelium area in relation to the cross-sectional area of endometrium are shown in Figure 5: no statistical differences were observed among all groups.

Histograms from immunohistochemistry data on proliferation (revealed by Ki-67 reaction) in both luminal and glandular epithelia are reported in Figure 6A,B: all F-OVX rats show a significant increase in proliferation compared to all other groups; in particular, the proliferation of the luminal epithelium in those rats treated with the highest dose of ferutinin (2F-OVX) is significantly lower than the other two F-OVX groups. As regards cell apoptosis (revealed by TUNEL), the percentage of positive cells in the luminal epithelium of EB-OVX animals was the highest (Fig. 6C), while in glandular epithelium only the 2F-OVX group exhibits values significantly higher than all the other groups (Fig. 6D).

Mammary gland

Histological observations of mammary gland cross sections are shown in Figures 7 and 8. In the SHAM group, the parenchyma displays a normal aspect without atypical features; it is possible to observe an abundant stromal compartment rich in adipocytes. Secretory units appear as clusters of tubules with a central lumen, lined by cuboidal epithelial cells. In the C-OVX group the stromal compartment is extremely abundant compared to secretory units; all glandular epithelia were atrophic and only a few secretory units were clearly detectable. In the EB-OVX group, as well as in all F-OVX groups, the glandular compartment is also abundant: the epithelium is monostratified and the cell shape ranges from cuboidal to columnar; sometimes intracellular cysts were present,

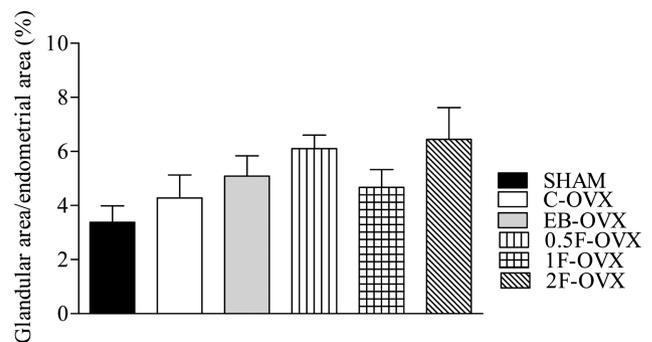


Fig. 5. Mean values of the percentage of areas of glandular epithelium in relation to the cross-sectional areas of endometrium. Values are expressed as mean \pm SEM (ANOVA followed by Newman-Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day).

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similar to those observed in the uterine epithelium of all treated animals.

Morphometrical evaluation of glandular epithelium area in relation to the cross-sectional area of total gland is reported as a percentage value in Figure 9A; in C-OVX rats the value is significantly lower compared to all other groups, as suggested by the morphological observations (Fig. 7: C-OVX).

Histograms from immunohistochemistry data on proliferation of glandular epithelia of both terminal end buds and alveoli are shown in Figure 9B: positivity of Ki-67 immunostained nuclei was significantly higher in mammary gland cells of EB-OVX animals compared to 0.5F-OVX, 1F-OVX and C-OVX rats. The percentage of TUNEL positive cells counted in breast glandular epithelia of both terminal end buds and alveoli is reported in Figure 9C: the apoptotic index is significantly higher for the 2F-OVX group relative to all other groups, except the 1F-OVX group.

Discussion

The present study investigated the effects of one

month treatments with three different doses of ferutinin on the uterus and mammary gland of OVX rats, comparing the effects with those of estradiol benzoate treatment. Attention was focused on cell proliferation and apoptosis, since these cellular processes are strictly correlated not only with physiological tissue turnover but also the pathogenesis of cancer. The quantification of these cellular processes is essential to evaluate the therapeutic potential of the drugs (Tan et al., 2005).

As regards body weight, the results confirm the authors' previously published data (Palumbo et al., 2009), since in the OVX animals the treatment with ferutinin (independently of the administered dose) and estradiol benzoate counterbalances body weight increase due to ovariectomy.

As regards uterine weight, C-OVX rats show the lowest values compared to all other groups, as expected after ovariectomy, which implies uterine atrophy. This atrophic condition in the C-OVX group involves a significant reduction in both the endometrial and myometrial areas. Despite this, the percentage value of glandular area compared to the total endometrial area is similar to all the other groups, the reduction of the

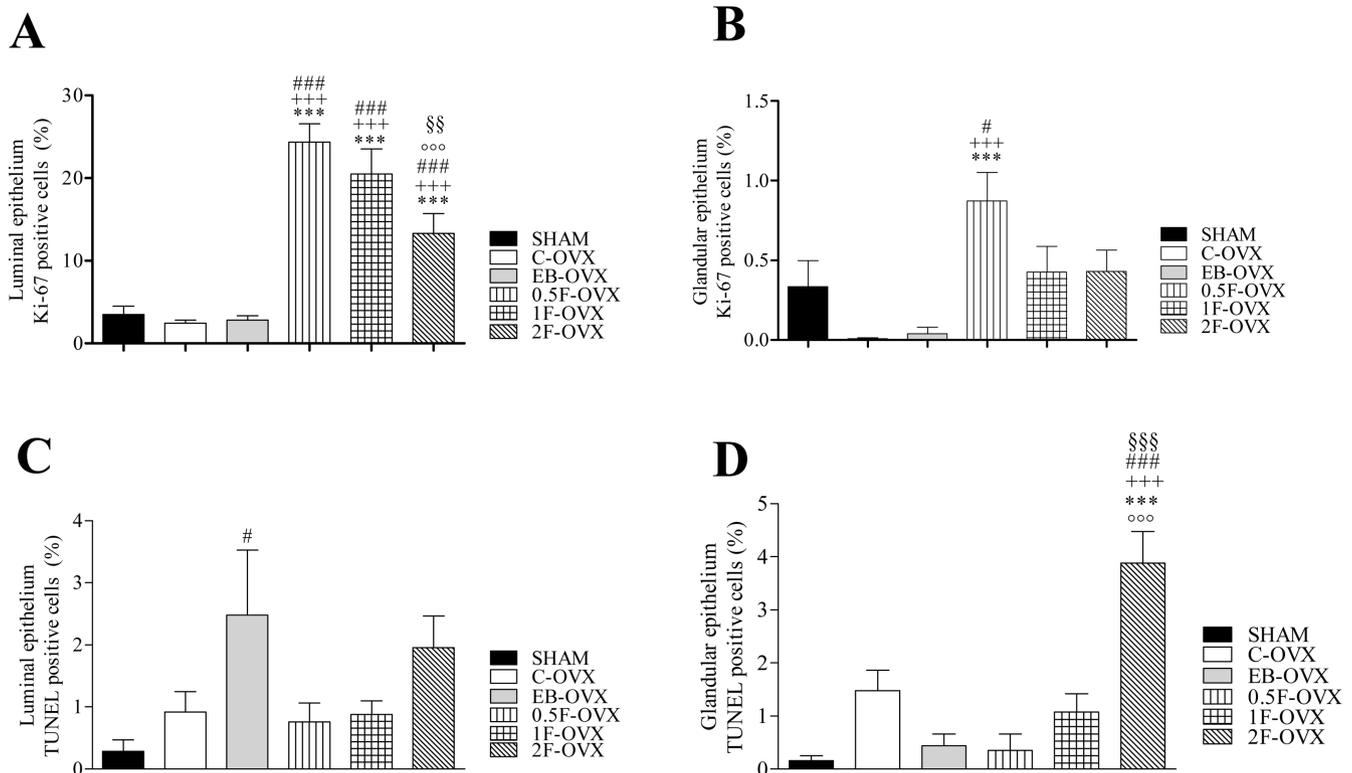


Fig. 6. Mean values in endometrium of Ki-67-positive cell percentage in luminal (A) and glandular (B) epithelia and of TUNEL-positive cell percentage in luminal (C) and glandular (D) epithelia. Values are expressed as mean \pm SEM. *** P <0.001 versus C-OVX; # P <0.05, ### P <0.001 versus SHAM; +++ P <0.001 versus EB-OVX; °°° P <0.001 versus 0.5F-OVX; §§ P <0.01, §§§ P <0.001 versus 1F-OVX (ANOVA followed by Newman-Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day).

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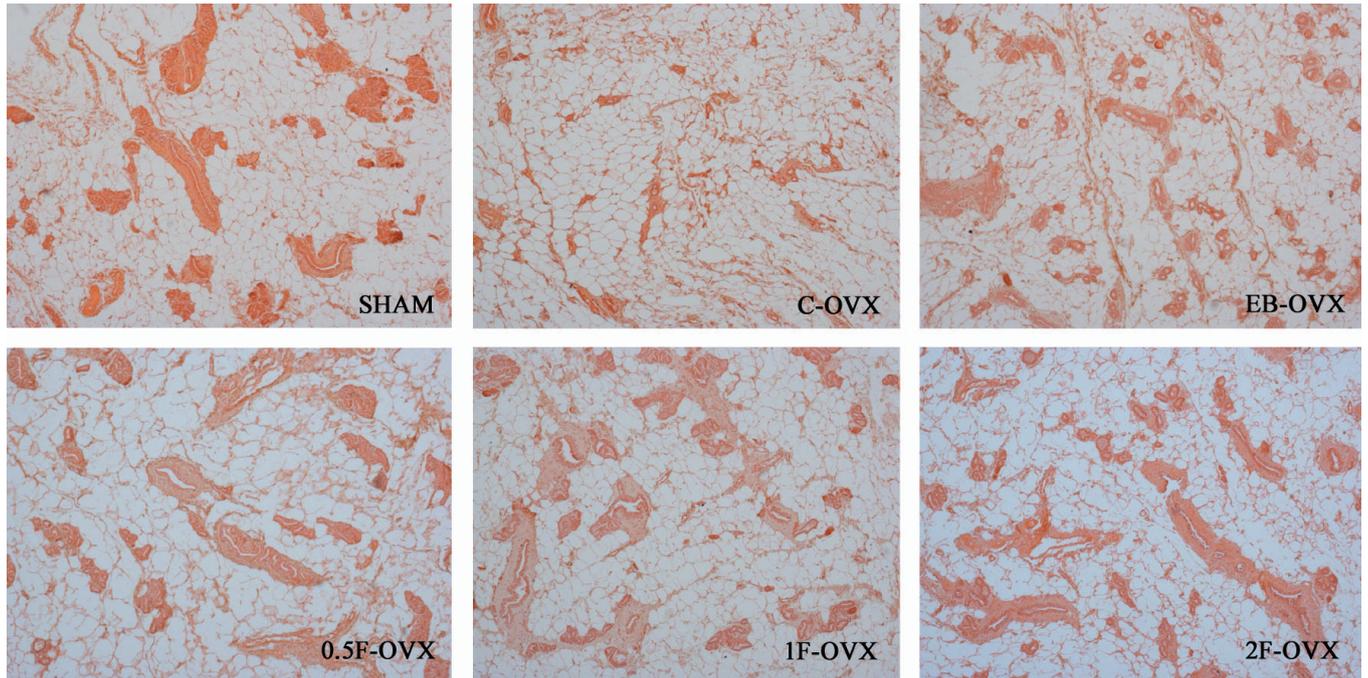


Fig. 7. Light microscopy, micrographs of hematoxylin/eosin-stained cross sections showing the mammary gland histology. SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day). x 50

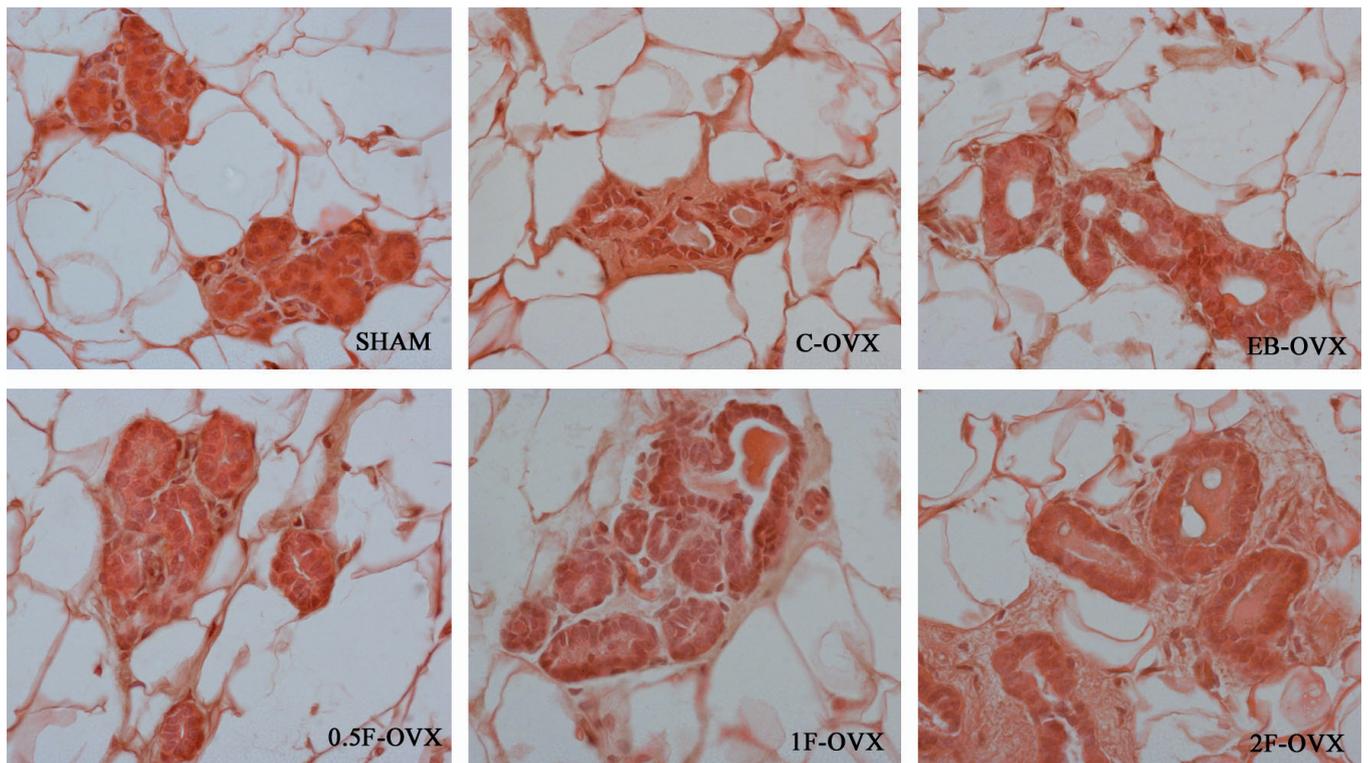


Fig. 8. Light microscopy, micrographs of hematoxylin/eosin-stained cross sections showing the histology of mammary gland alveoli. SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day). x 400

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glandular component probably being proportional to the decrease of the total endometrium. The lower uterine weight recorded in all F-OVX rats compared to EB-OVX rats might be ascribed to the reduction of the myometrium due, in turn, to ferutinin treatment.

When discussing the uterine morphological and immunohistochemical data, it is important to remember that rats have an average estrus cycle of 4-5 days divided into four phases (*proestrus*, *estrus*, *metestrus* and

diestrus), characterized by morphological changes particularly affecting uterine endometrium (Westwood, 2008), even if the criteria to define the end of one phase and the beginning of the next are not exactly clear. In the first phase, *proestrus*, the epithelial cells of the endometrium are cuboidal or columnar and mitoses are present; in the *estrus* phase there is degeneration of the columnar epithelial cells in parallel with loss of their mitotic activity; in the *metestrus* phase the epithelium is similar to the estrus phase, but with an increase in mitotic activity; in the final phase, *diestrus*, the lumen is lined by columnar epithelial cells, mitoses are rare and degenerative cells are only occasionally present.

On the basis of the above considerations, our morphological data suggest that SHAM animals are likely in the *diestrus* phase; in fact, with few cellular mitoses and scanty apoptosis occur, as confirmed by the immunohistochemical data (Fig. 6). In EB-OVX animals, whose administration of estradiol benzoate is supposed to mime the physiological hormonal cycle, the histological features seem to resemble the *estrus* phase in which only few mitoses are present; this might explain the low proliferation rate recorded in EB-OVX animals. All animals treated daily with ferutinin show histological features similar to those of the *metestrus* phase, with degeneration of luminal and glandular epithelial cells, as shown by the abundant intraepithelial cysts. Treatment with increasing doses of ferutinin determines on one hand a decrease in cell proliferation (Ki-67), and on the other hand an increase in the apoptotic index (TUNEL) of the luminal epithelium and, to a greater extent, of the glandular uterine epithelium, which represents the most frequent site of carcinoma onset. In particular, a 2 mg/kg/day dose of ferutinin would seem to offer a better protective action against uterine carcinoma compared to lower doses, since in 0.5 and 1F-OVX rats the high proliferation is not counterbalanced by high apoptosis. In this context, it is interesting to deduce that the ratio between the percentage of proliferating cells and the percentage of apoptotic cells is higher in the glandular epithelia of EB-OVX rats compared to 2F-OVX rats (Fig. 6B versus 6D).

As far as the mammary gland is concerned, it represents a complex organ in which the glandular tissue is widely dispersed in fat tissue, thus making morphological analysis extremely difficult (Masso-Welch et al., 2000; Hilakivi-Clarke and Assis, 2006). Both morphological and morphometrical results show that estradiol benzoate, as well as ferutinin, exert a trophic effect on mammary gland epithelial tissue with respect to C-OVX animals. Regarding cell proliferation, treatment with estradiol benzoate determines a significant increase in glandular cell proliferation compared to ferutinin in lower doses. Despite the absence of significant differences in glandular proliferation between EB-OVX and 2F-OVX animals, the amount of glandular cell apoptosis in 2F-OVX rats is the highest value recorded among all groups, with

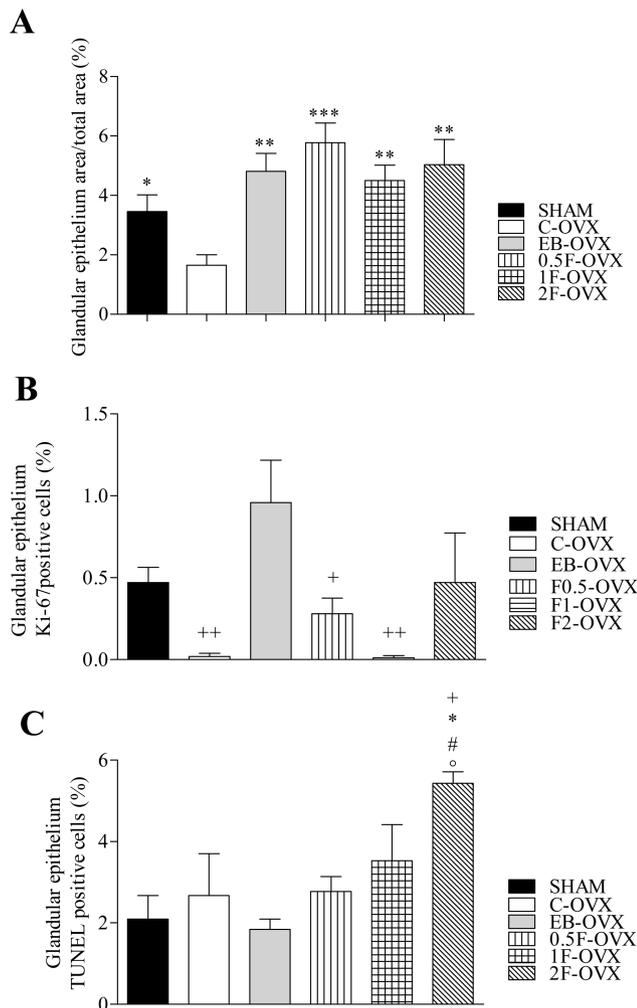


Fig. 9. Mean values of (A) the percentage of areas of glandular epithelium in relation to the cross-sectional areas of total glands, (B) Ki-67-positive cell percentage in breast glandular epithelia of terminal end buds and alveoli and (C) TUNEL-positive cell percentage in breast glandular epithelia of terminal end buds and alveoli. Values are expressed as mean \pm SEM, * P <0.05, ** P <0.01, *** P <0.001 versus C-OVX; # P <0.05 versus SHAM; + P <0.05, ++ P <0.01 versus EB-OVX; ° P <0.05 versus 0.5F-OVX (ANOVA followed by Newman-Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; EB-OVX ovariectomized treated with estradiol benzoate; 0.5F-OVX, 1F-OVX, 2F-OVX ovariectomized treated with ferutinin (0.5 mg/kg/day; 1 mg/kg/day; 2 mg/kg/day).

statistical significance. These last results suggest that ferutinin at the dose of 2 mg/Kg/day might offer a protective action against breast cancer compared to both ferutinin in lower doses and estradiol benzoate.

In conclusion, the present data suggest that the effects of ferutinin are dose dependent, and that a 2 mg/kg/day dose might provide a better protective action against the onset of both breast and uterine carcinoma compared to lower doses of ferutinin or estradiol benzoate, increasing cellular apoptosis in glandular epithelia. These results bring new insights into the actions of ferutinin, agreeing with the data recently published by the authors on bone tissue (Cavani et al., 2012), demonstrating that a 2 mg/kg/day dose exerts a greater osteoprotective effect in OVX rats compared to estrogens and lower ferutinin doses, particularly in skeletal regions with higher metabolic activity, presumably by inhibiting osteoclast activity rather than increasing bone deposition.

On the basis of these observations, the authors underline the importance of further research into diets implemented with ferutinin, in order to better evaluate the possibility of employing this phytoestrogen as a "safe" good alternative drug to the use of estrogens in HRT in menopause.

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