Comparative uterine effects on ovariectomized rats after repeated treatment with different vaginal estrogen formulations

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ABSTRACT

Objective: Topical estrogen therapy is recommended for the treatment of vaginal atrophy. This study was designed to compare the uterotrophic effects of a new estrogen vaginal formulation (0.005% estriol vaginal gel) and other existing topical treatments (Ovestinon® and Colpotrofin®).

Methods: Each one of the studied formulations was administered intravaginally to groups of ovariectomized rats with cytologically confirmed vaginal atrophy. The doses were adjusted by animal weight according to human dosage. After daily treatment for 14 days, the animals were sacrificed and their vaginas and uteri removed. All uteri were weighed. Uteri and vaginas were fixed for histological evaluation.

Results: All three active formulations proved to be very effective in the cytological reversal of vaginal atrophy. However, they differ in their effects in the uteri. Ovestinon® and Colpotrofin® produced a significant increase in uterine weight, myometrial and endometrial thickness as well as histological modifications in the endometrium suggestive of estrogenic activity. Conversely, animals treated with 0.005% estriol vaginal gel, did not show significant weight increase or any other macroscopical or microscopical modifications of the uteri, an effect comparable to placebo.

Conclusions: There are significant differences in the uterotrophic effect of three different topical estrogen formulations as tested in a rat model of vaginal postmenopausal atrophy. While the three formulations were equally effective in reversing vaginal atrophy, only the newly developed ultra-low dose 0.005% estriol vaginal gel has proved to lack any significant estrogenic effect on the uterus.

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1. Introduction

The lack of estrogen production that characterizes menopause results in the appearance of various signs and symptoms which frequently compromise the quality of life of women. Among them, vaginal atrophy is highly prevalent and, contrary to others such as vasomotor symptoms, does not resolve spontaneously with time. Estrogen therapy is indicated for the treatment of moderate to severe vaginal atrophy, and in particular, vaginal administration is the recommended first line treatment [1].

There are several different formulations available for the treatment of postmenopausal vaginal atrophy that have been widely available for many years. Treatment with these formulations is based on the local (vaginal) application of estrogens to alleviate the vaginal atrophy, while reducing or eliminating any possible systemic or endometrial effects.

Amongst the different vaginal estrogen formulations available in Europe, semisolid formulations of estriol or promestriene are, by large, the most employed. The standard treatment for estriol-based formulations is a dose of 0.5 mg per application, with Ovestinon® being the reference product. Treatment with promestriene is based on a dose of 10 mg per application, with Colpotrofin® being the reference product. These products have been in clinical use for many years with a good safety record. Both estrogens have properties that make them well suited for the topical treatment of vaginal atrophy; promestriene shows a very low absorption after topical treatment [2] and estriol has a low affinity for estrogen receptors and has a short occupancy time of the receptor, thus minimizing extraglandular effects [3].

Published data on promestriene are scarce. However, the lack of a systemic effect of promestriene has been derived from observations where systemic levels of estrogens (E1 and E2) as well as gonadotropins (FSH and LH) were not seen to change after vaginal...
application of promestriene for 20 days to 40 days with a daily dose of 10 mg promestriene [4,5]. The authors attributed promestriene’s lack of significant systemic effects to its inability to cross the vaginal epithelial layer, exerting a totally local effect. Other studies over time have supported this apparent lack of systemic effect of promestriene after vaginal application [6,7]. However, although only a small fraction of vaginally applied promestriene crosses the vaginal epithelium [8], the resulting systemic exposure after vaginal application might be pharmacologically significant because of the high doses of drug employed and the high estrogenic potency of the compound [9]. Also, binding of promestriene to the rat uterus after topical administration has been shown to be equivalent to estradiol, although delayed in time [10].

Estriol has also been shown to be a safe local treatment for vaginal atrophy, with significantly lower activity at the endometrium level than estradiol-based therapies [11–14]. However, some reports indicate that current estriol treatment might not be devoid of systemic effects. Thus, an increase in both estrogen receptors and progesterone receptor mRNA levels has been found in the endometrium of post-menopausal women treated with estriol [3,15], as well as a stimulation of endometrium at a histological level [3]. Also, some authors have described changes in LH/FSH levels associated with vaginal estriol treatment [16–18] suggesting a systemic effect of the estrogen.

Thus, although existing topical treatments for vaginal atrophy have a good safety record, they are not totally devoid of systemic and, in particular, uterotropic effects which, although mild, would be advantageous to eliminate or further reduce. Recently, a new ultra-low dose estriol gel formulation (0.005% estriol vaginal gel) that significantly enhances estriol delivery to the vaginal tissue compared with existing formulations has been developed, thus allowing the use of much lower doses of estriol to treat vaginal atrophy, reducing systemic exposure to estriol without compromising its efficacy [19,20].

No data exist in the literature that compares directly the uterine effects of estriol and promestriene after vaginal administration. In the present study we have set to compare the estrogenic effects of vaginal administration of the standard estriol and promestriene preparations (Ovestin® 0.1% cream and Colpotrofin® 1% cream respectively) and the newly developed 0.005% estriol vaginal gel on the uterus, using a model of ovariec-tomized rats. To this end we investigated the cytological effects of each preparation over the vaginal atrophy as well as their possible uterotrophic activity by determining changes both in uterus weight and histological modifications of the uterus after vaginal treatment of the animals with weight-corrected doses of the three formulations for 14 consecutive days.

2. Materials and methods

2.1. Materials

Ovestin® 0.1% (Estriol) cream was used as the commercially available preparation (Lot No. 404077) from Organon Española S.A. Laboratories. Colpotrofin 1% (Promestriene) was used as the commercially available preparation (Lot No. 9H224) from Merck Farma y Química S.L. laboratories. The 0.005% estriol gel was supplied by Italfarmaco S.A. Laboratories (Lot No. V-2) as well as the corresponding placebo gel.

2.2. Animal treatment

Female ovariectomized Wistar rats (Charles River Spain) with an approximate body weight between 180 and 200 g at the time of ovariectomy were used in the present study. All animals were housed in specific facilities with controlled environmental conditions (humidity, light cycle, and temperature) and fed standard rodent diet (Diet 2014, Harlan Interfauna Ibérica, Spain). Drinking water was supplied “ad libitum” in plastic bottles.

Approximately 15–21 days after the ovariectomy, a vaginal smear was obtained for each animal to ascertain their menopausal status (vaginal atrophy) by determining the degree of maturity of the vaginal epithelium. All vaginal smears were stained with Papanicolaou stain and the ratio of basal/parabasal, intermediate and superficial epithelial cells determined in order to determine the degree of vaginal atrophy. A total of twenty visual fields were examined for each smear. Cytological smears with a predominantly positive ratio of basal/parabasal cells and absence of intermediate and/or superficial cells or leukocytes were classified as indicative of atrophy and the corresponding animals were considered menopausal and included in the study.

Animals were divided into five experimental groups: (a) control (n = 5), rats with no treatment; (b) sham control (n = 5), rats manipulated and cannulated every day but with no treatment administered; (c) placebo (n = 10); (d) Colpotrofin® (n = 10); (e) Ovestin® (n = 10) and (f) 0.005% estriol vaginal gel (n = 10).

The dose administered for each experimental group was calculated from the corresponding human equivalent dose assuming an average 60 kg for menopausal women and 350 g the average rat weight at the time of dosing. A density 1.0 was considered for all substances tested:

(a) Colpotrofin® (1% promestriene): human dose is 1 g of cream (10 mg promestriene) per day. For a rat this corresponds to 5.8 mg of 1% cream (5.8 μg promestriene).

(b) Ovestin® (0.1% estriol): human dose is 0.5 g of cream (500 μg of estriol) per day. For a rat it corresponds to 2.9 mg of 0.1% cream (2.9 μg estriol).

(c) 0.005% estriol vaginal gel: human dose is 1 g of gel (50 μg of estriol) per day. For a rat this corresponds to 5.8 mg of the gel 0.005% (0.29 μg estriol).

Test substances were administered once daily for 14 days intravaginally with an Eppendorf Combitip positive displacement pipette. Placebo animals were dosed intravaginally with the same volume of placebo (vehicle) gel as animals that received the estriol gel.

2.3. Assessment of treatment effects

2.3.1. Reversal of vaginal atrophy

On day 15, the effect of the different treatments over the preexisting vaginal atrophy was determined either by means of a vaginal smear obtained from each animal or by histological examination of the vaginal tissue.

Vaginal smears were fixed and stained with Papanicolaou stain and examined under the microscope to determine the degree of vaginal atrophy. Animals were then sacrificed by cervical dislocation and their vagina and uterus removed. The vagina was processed for histological examination by standard procedures (4–5 μm transverse sections stained with haematoxylin plus eosin). The extent of vaginal atrophy of each animal was determined according to the following criteria:

0 = atrophy (predominant presence of basal/parabasal cells, with complete absence of intermediate and/or superficial (cornified) cells or leukocytes).

1 = hypotrophy (predominant presence of basal/parabasal cells, with minimal presence of intermediate cells and absence of superficial (cornified) cells or leukocytes).
2 = intermediate trophism (few basal/parabasal cells and predominant presence of intermediate cells with absence of superficial (cornified) cells or leukocytes).
3 = good trophism (absence of basal/parabasal cells, predominant presence of intermediate and superficial cells as well as presence of leukocytes).

2.3.2. Uterotrophic effects

On day 15, the wet weight of each uterus was determined once they were carefully cleaned of any adherent fat. To minimize variability due to dissection all uteri were dissected above the cervix at the same distance.

Tissue samples were taken from each uterus (transverse section of one hemi-uterus, selecting for study the sections proximal and distal to the cervix) and processed for histological examination by standard procedures (4–5 µm sections stained with haematoxylin plus eosin).

The following parameters were then evaluated in 4 sections of proximal and distal segments respectively for each sample:

1. General trophism over the uterus: measurement of myometrium and endometrium thickness.
2. Endometrial morphology.
   2.1 Endometrial architecture: analysis of degree of glandular clustering and intussusception, both classified as 0 (no glands); 1 (<25% glands); 2 (25–50%) of glands and 3 (>50% of glands).
   2.2 Epithelial proliferation: analysis of the presence of glandular epithelial proliferation evaluating the epithelial height (0 = flat; 1 = cubic; 2 = tall cylindrical), position of the nucleus inside the cell (0 = basal; 1 = intermediate; 2 = multiserial) and the presence of cellular mitosis (0 = absence; 1 = presence).
   2.3 Stromal proliferation: analysis of the presence of stromal activity and proliferation by assessing the quantity of collagen (0 = slight; 1 = moderate; 2 = abundant) and the presence of mitosis (0 = absence; 1 = presence).
   2.4 Presence of eosinophils in the uterine wall (0 = few and isolated; 1 = few and diffuse; 2 = moderate; 3 = abundant).

The final global evaluation of the histological assessment of each uterus sample is obtained by the sum of all the individual scores obtained for each of the different parameters listed above (global morphological score).

2.4. Statistics

The significance of the difference between the different treatments and the control (vehicle) group was evaluated using the statistics package Instat 3. Differences for uterus weight as well as myometrial and endometrial thickness were evaluated by analysis of variance (ANOVA) plus post-test of Bonferroni to allow multiple comparisons. Differences for vaginal atrophy and uterus global morphological effect were evaluated by using the Kruskal–Wallis test for non-parametric data plus post-test of Dunn for multiple comparisons. Values of p < 0.05 were taken as significant.

3. Results

3.1. Vaginal atrophy

All the animals had absolute atrophic vaginal epithelium before treatment (vaginal atrophy score 0). Vaginal examination after 14 days of continuous treatment revealed a slight trophic effect on animals that received sham or vehicle treatment. This was characterized by a minimal presence of cornified cells and a predominant presence of basal, parabasal and intermediate desquamative cells. This slight effect is probably due to mechanical stimulation of the vaginas by the cannulation procedure done daily [21].

In contrast, a complete trophic transformation of the vaginas was observed in animals that received Ovestinon® 0.1%, Colpotrofin® 1% and 0.005% estriol vaginal gel (see Table 1). In all three cases it was possible to observe absence of basal/parabasal cornified cells and a predominance of superficial cells with presence of leukocytes, and their vaginal scores are statistically indistinguishable.

3.2. Uterine weight

Table 1 shows the results of the determination of uterus weight in each group after treatment. Absolute control, sham control and 0.005% estriol vaginal gel did not show any significant increase in uterine weight when compared to placebo.

In contrast, rats treated either with Ovestinon® or Colpotrofin® showed a large increase in uterine weight which was statistically significant when compared to either placebo or 0.005% estriol vaginal gel treated animals.

3.3. Uterine histology

The results of the histological analysis of the uteri are shown in Table 2 and summarized in Fig. 1. Uteri from animals in the experimental groups that received vehicle or 0.005% estriol vaginal gel remained atrophic, with very low and no significantly different histological scores (total mean scores of 0 and 0.8, respectively), and were different than absolute or sham controls. This lack of uterotrophic activity of 0.005% estriol vaginal gel was also evidenced by the lack of significant differences in endometrial (321 µm compared to 270 µm in vehicle group) and myometrial thickness (187 µm compared to 177 µm in vehicle group). Total uterine thickness was consequently also not affected. Endometrial or myometrial wall thickness from placebo, absolute and sham control groups were indistinguishable.

In contrast, both Ovestinon® 0.1% and Colpotrofin® 1% had a mild, but significant uterotrophic effect as shown by a significant increase in the global endometrial morphological trophic score (total mean scores of 2.9 and 2.0, respectively). The histological changes indicated epithelial proliferation (changes in epithelial height and nuclei position), stromal proliferation (quantity of collagen) and presence of eosinophils in endometrium, all these features indicative of estrogenic activity in the uterus [22–25]. No effects were seen however in endometrial architecture.

The uterotrophic activity suggested by the histological changes was further confirmed by a significant increase in both the thickness of the endometrium (454, 621 and 270 µm, Ovestinon® Colpotrofin® and placebo, respectively) and myometrium (253, 344 and 177 µm, Ovestinon® Colpotrofin® and placebo, respectively). The difference in wall thickness can be appreciated in Fig. 1.

4. Discussion

In the present study we set to compare the uterine effects of vaginal administration of three different hormonal preparations for the treatment of vaginal atrophy; two commercially available formulations (Ovestinon® 0.1%; estriol cream and Colpotrofin®; 1% promestriene cream) and a new ultra low dose estriol formulation (0.005% estriol vaginal gel). The clinical efficacy and safety of 0.005% estriol vaginal gel in the treatment of postmenopausal vaginal atrophy has been shown in clinical trials [19,20]. While the safety of the three formulations has been assessed in independent clinical trials and clinical practice, no comparative studies of their potential uterotrophic effects have been performed. To that end, groups of ovariec-tomized rats were treated intravaginally with
Table 1
Vaginal cytological transformation and uterine weight in rats after 14 days of treatment. Results are shown as the mean ± standard error of the mean of 10 animals per group (except absolute and sham control n = 5). Vaginal atrophy scores were compared with Kruskal–Wallis test with Dunn’s correction for multiplicity. Uterine weights were compared by ANOVA with Bonferroni’s correction for multiplicity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vaginal atrophy score</th>
<th>Uterine weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute control</td>
<td>0.0 ± 0.0²</td>
<td>0.116 ± 0.01₁</td>
</tr>
<tr>
<td>Sham control</td>
<td>0.6 ± 0.2²</td>
<td>0.109 ± 0.01₁</td>
</tr>
<tr>
<td>Placebo</td>
<td>1.8 ± 0.2</td>
<td>0.110 ± 0.01</td>
</tr>
<tr>
<td>Ovestinon 0.1%</td>
<td>3.0 ± 0.0³</td>
<td>0.202 ± 0.02₄</td>
</tr>
<tr>
<td>Colpotrofin 1%</td>
<td>3.0 ± 0.0⁰</td>
<td>0.214 ± 0.02₄</td>
</tr>
<tr>
<td>Estriol gel 0.05%</td>
<td>2.9 ± 0.1¹</td>
<td>0.132 ± 0.01₁</td>
</tr>
</tbody>
</table>

= Not significant against placebo.
² p < 0.01 against placebo.
³ p < 0.05 against placebo.
⁴ p < 0.001 against placebo.

Table 2
Thickness of uteri myometrium, endometrium, total wall thickness and endometrial morphology score. Results are shown as the mean ± SEM. Histological characteristics affected in each case and their respective scoring are shown. Endometrial, myometrial and total wall thickness were compared by ANOVA with Bonferroni’s correction for multiplicity global morphological scores were compared with Kruskal–Wallis test with Dunn’s correction for multiplicity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endometrial thickness (µm)</th>
<th>Myometrial thickness (µm)</th>
<th>Total wall thickness (µm)</th>
<th>Global morphological score</th>
<th>Epithelial height</th>
<th>Nuclear position</th>
<th>Collagen</th>
<th>Endometrial eosinophilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute control</td>
<td>266 ± 11⁴</td>
<td>180 ± 11⁴</td>
<td>444 ± 17⁴</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sham control</td>
<td>262 ± 14⁴</td>
<td>164 ± 16⁴</td>
<td>424 ± 28⁴</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Placebo</td>
<td>279 ± 15</td>
<td>177 ± 11</td>
<td>447 ± 23</td>
<td>2.9 ± 0.38⁴</td>
<td>0.7</td>
<td>0.6</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Ovestinon 0.1%</td>
<td>454 ± 30⁴</td>
<td>253 ± 23³</td>
<td>707 ± 47³</td>
<td>2.0 ± 0.30⁴</td>
<td>0.9</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Colpotrofin 1%</td>
<td>622 ± 40⁴</td>
<td>344 ± 27⁴</td>
<td>964 ± 63³</td>
<td>2.9 ± 0.30⁴</td>
<td>0.9</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Estriol gel 0.05%</td>
<td>321 ± 19⁴</td>
<td>187 ± 13²</td>
<td>508 ± 25⁴</td>
<td>0.8 ± 0.33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

= Not significant against placebo.
⁵ p < 0.05 against placebo.
⁶ p < 0.01 against placebo.
⁶ p < 0.001 against placebo.

Fig. 1. Photomicrographs of uteri after treatment with placebo and estrogen formulations. The figure shows histological sections of uteri after treatment with the different formulations (a) placebo, (b) 0.005% estriol vaginal gel, (c) Ovestinon® and (d) Colpotrofin®. All photographs were taken at the same magnification (150×). The bar in (a) shows the scale of 10 µm. For references, the different histological layers are indicated in (b) L = lumen; E = epithelium; Endo = endometrium and Myo = myometrium.
human weight adjusted doses of each formulation for two weeks. Local effects (cytological changes in the vaginal epithelium) as well as effects on the uterus (change in uterine weight and histological changes in the uterus) after fourteen days of treatment of ovariec-
tomized rats have been investigated to assess the local and systemic estrogenic effects of these formulations.

The results obtained show that the three formulations equally produced complete cytological reversal of vaginal atrophy. It is noteworthy to point out that 0.005% estril vaginal gel delivers a dose of estril which is a tenth of that delivered with Ovestin® (50 µg vs 500 µg in women; 0.29 µg vs 2.9 µg in rats). A direct comparison with Colpotrofin® is not straightforward as this is for-
mulated with a different estrogen (promestriene). However, the dose of estrogen in the latter is 200 times higher than in 0.005% estril vaginal gel (50 µg vs 10 mg in women; 0.29 µg vs 58 µg in rats). This result confirms the ability of 0.005% estril vaginal gel to significantly enhance estril delivery and availability to the vaginal epithelium, allowing the use of significantly less hormone for treat-
ment. This coincides with recent clinical data that confirm the high ef
ciency of 0.005% estril vaginal gel formulation in the treatment of vaginal atrophy [20].

Estriol has been shown to be a safe local treatment for vaginal atrophy, with significantly lower activity over the endometrium than estradiol-based therapies [11–14]. This reduced effect over the endometrium has been attributed to the low a

frequency for, and short occupancy time of, estrogen receptors [3].

However, estril treatment is not totally devoid of effects over the uterus. An increase in both estrogen receptor and progesterone receptor mRNA levels has been shown in the endometrium of post-

menopausal women treated with estril as well as a stimulation of endometrium at a histological level [3,15]. Although vaginal estril treatment does not induce a significant hypertrophy of the uterus, it has been shown to result in significant histological changes, including effects over the structure and organization of collagen in the lamina propria of the rat uterus, where collagen staining was observed to be significantly reduced 4h after estril administration [26].

Promestriene’s highly reduced absorption after topical treat-
ment [2] was claimed to be the basis for its lack of systemic effects, based on observations where systemic levels of estrogens (E1 and E2) as well as gonadotrophins (FSH and LH) were not seen to change after vaginal application of promestriene [4,5]. Other studies over time have sustained a lack of systemic effect of promestriene after vaginal application [6,7]. However, vaginal administration of promestriene at the therapeautical dose of 10 mg has been shown to result in promestriene systemic blood levels of 200–500 pg/ml [8]. This shows a very low percentual systemic absorption. However, those drug plasmatic levels can nevertheless be pharmacologically significant as it has been demonstrated that promestriene has a pharmacological activity equivalent to 5–10% of estradiol [9]. This means that after vaginal administration of 10 mg promestriene there are systemic levels of promestriene equivalent in estrogenic effect to 10–50 pg/ml of estradiol. These are to be compared with normal estradiol levels of <16 pg/ml in postmenopausal women and of 30–400 pg/ml in premenopausal women. Thus, the systemic exposure to estrogen after vaginal promestriene administration can be above physiological levels for menopause.

In the present study we have shown that vaginal adminis-
tration once daily for 14 consecutive days of two commercially available preparations of estril and promestriene (Ovestin® and Colpotrofin® respectively) results in significant uterus hypertrophy in the ovariectomized rat. The doses employed in this study have been weight adjusted from their equivalent human dose.

It is known that estrogens induce a significant increase in rat uterine weight, providing a sensitive animal model to mea-
sure estrogenic activity [27–30]. In particular, it was shown that both promestriene and 17β-estradiol produced significant uterine weight increase in ovariectomized rats after topical administration [31]. Likewise, in this study, we have observed a significant increase in total uterus weight after vaginal treatment with Ovestin® or Colpotrofin®. This effect was correlated with significant increases in both endometrial and myometrial thickness. Moreover, histolog-
ical examination of uterine tissues after treatment with Ovestin® or Colpotrofin® revealed characteristics that evidence some degree of tissue proliferation. These effects were evident after a relatively short two weeks treatment. Thus, changes were found in epithelial height and position of the nucleus indicative of glandular epithelial proliferation [25]. Additionally, an increase in collagen was also observed which suggests stromal activity and proliferation, as well as an increase in the presence of eosinophils which act as an indirect evidence of estrogenic activity [22–24].

None of these effects were observed after administration of 0.005% estril vaginal gel or placebo. This demonstrates that this formulation significantly reduces the uterotrophic activity observed in ovariectomized rats after administration of the other two hormonal preparations.

In conclusion, the results discussed have shown that 0.005% estril vaginal gel is a new formulation that, while maintaining full activity in the vaginal tissue, signiﬁcantly reduces the systemic exposure to estrogens when compared with other currently used vaginal estrogen treatments. The translation of these effects, shown in an animal model, to the clinical setting is not straightforward. In fact, the clinical safety record of these formulations is very good. However, the results of this study indicate an added safety margin for the new 0.005% estril vaginal gel formulation which might be relevant in special risk patients.

Contributors

All authors contributed to the conception, design and interpre-
tation of results of the study.

The animal experiments were performed by Dr. López-
Belmonte.

Draft manuscript was written by Drs. López-Belmonte and Moscoso del Prado. The rest of the authors reviewed and completed the manuscript up to their final version.

Competing interest

This work has been financed by Italfarmaco S.A., Madrid (Spain). Drs. Nieto and Moscoso del Prado are full time employees at Italfarmaco S.A.

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