Evaluation of the Effects of a New Intravaginal Gel, Containing Purified Bovine Colostrum, on Vaginal Blood Flow and Vaginal Atrophy in Ovariectomized Rat

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A B S T R A C T

Introduction. Vaginal dryness due to vaginal atrophy is a common complaint of postmenopausal women, interfering with sexual function and quality of life. Hormone replacement therapy is the only effective therapy but with known risks that leave unmet medical needs. A new product, ZP-025 vaginal gel, containing purified (dialyzed lyophilized) bovine colostrum, has been developed for the treatment of vaginal dryness secondary to vaginal atrophy.

Aim. The study aims to investigate the effects of intravaginal application of ZP-025 on vaginal atrophy using an animal model.

Methods. Ovariectomized female Sprague-Dawley rats were used. Three weeks after surgery, rats were divided into four groups and treated for 4 weeks (twice a day) with placebo or ZP-025 at low (0.5%) or high (2.3%) concentrations of colostrum; in the control group, rats did not receive any treatment. Changes in vaginal blood flow due to pelvic nerve stimulation were assessed by laser Doppler flowmetry and vaginal tissue was collected for histological assay.

Main Outcome Measures. The main outcome measures were vaginal blood flow before and after pelvic nerve stimulation and histology of vaginal epithelium.

Results. Treatment with ZP-025 to ovariectomized rats induced an increase of vaginal blood flow parameters (vascular capacitance, amplitude and area under the curve of the response) in response to pelvic nerve stimulation compared with control group, statistically significant at 2.3%. Vaginal epithelium showed a physiological estrous cycle aspect in treated animals, with at least five cell layers vs. one or two cell layers in control rats. As expected from a topical formulation, systemic effects on body weights and uterine wet weights were not observed with application of ZP-025.

Conclusions. In this study, the new product ZP-025, containing purified colostrum, was shown to have beneficial effects on vaginal atrophy in ovariectomized rats, improving vaginal hemodynamics and thickness of vaginal epithelium.

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Key Words. Vaginal Atrophy; Bovine Colostrum; Ovariectomized Rat; Vaginal Blood Flow

Introduction

Vaginal atrophy is caused by a decrease in oestrogen production and is a common complaint of postmenopausal women, interfering with sexual function and quality of life. In about 45% of menopausal women, vaginal atrophy can be clinically manifest as a syndrome of vaginal
dryness, itching, irritation, and dyspareunia [1,2].

The vaginal atrophy becomes clinically apparent 4–5 years after menopause and its hallmarks are thinning of vaginal epithelia layers, increased vaginal pH, decrease in the local blood flow, and diminished vaginal secretion. Clinical studies have shown that these signs significantly correlate with the decline in circulating ovarian hormones [3–6].

While systemic and local estrogen-based hormonal therapy is effective in treating symptoms of vaginal atrophy in postmenopausal women [3,7–9], these medications are contraindicated in some populations (women with unknown vaginal/uterine bleeding or those with a known or suspected ovary, endometrial cancer, and breast cancer) and other women choose not to take them for fear about their safety [10,11]. Because of these issues, recent work has focused on developing more specific, and in many cases nonhormonal, alternatives to traditional postmenopausal hormonal therapy to treat vaginal dryness in presence or in absence of vaginal atrophy [12].

Zambon SpA (Bresso, Milan, Italy) has developed a gel product (ZP-025 vaginal gel—Monurelle Biogel), containing purified bovine colostrum to be administrated topically, for the treatment of vaginal dryness also in presence of vaginal atrophy. Colostrum is a substance produced from female mammary gland during the first few hours postpartum, and numerous investigators have reported that the colostrum imparts many advantages for the development of the infants [13]. Thanks to its constituents, colostrum is able to help the local defense (immunoglobulin A); modulate the immunitary response (immunoglobulin E, M, G); promote antibacterial and antiviral action (lactoferrin and transferring); modulate the inflammation factors (cytokine, interleukine); promote normal cell growth, normal cell activities, cell migration and proliferation, and tissue repair (transforming growth factor alpha and beta; epidermal growth factor); stimulate the mucosal restore; and accelerate wound healing (insulin-like growth factors—IGF-1 and -2). In particular, IGF-1 is involved in the regulatory feedback of growth hormone [14] and interferes with insulin-like growth factor binding proteins [15]. To date, based on the best scientific knowledge available to us, no sexual hormones are directly present in bovine colostrum.

Because vaginal mucosa, because of its anatomic and physiologic features, is exposed to epithelium damage and to vaginal ecosystem and local defense factors alterations, colostrum can help in promoting the mucosal trophic restoration and preserving from bacteric and viral aggression. Our hypothesis is that topical application of colostrum could have a beneficial effect specifically on vaginal atrophy. Besides purified colostrum, this vaginal gel contains other natural substances with humectants, hydrating, re-epithelizing, and antioxidant properties at concentrations <5%: betaine, sericin, panthenol, glycerin, tocopherol, Lepidium meyenii root extract.

The aim of the present study was to assess the effect of ZP-025 vaginal gel on vaginal blood flow and tissue morphology using ovariectomized (OVX) rats as an animal model for vaginal atrophy. This well-characterized experimental model is very useful to induce experimental menopause, as within a short period of time postovariectomy, female rats without ovarian hormone secretion mimic many human postmenopausal changes, including vaginal atrophy. It has been well documented also in this animal model that estrogen replacement increases pelvic blood flow and restores the structural and functional integrity of vaginal tissue [16–21], confirming that the loss of estrogens is the key factor leading to vaginal atrophy.

Materials and Methods

Animals and Treatments

Adult nonpregnant female Sprague-Dawley rats (Elevage Janvier, Le Genest-St-Isle, France, 8–10 weeks old, 200–225 g) were housed 7 days prior to the beginning of the experiments, with free access to standard chow and water and maintained on an inversed 12-hour dark/light cycle (10:00/22:00). All procedures were performed in accordance with the legislation on the use of laboratory animals (NIH publication N°85-23, revised 1996) and Animal Care Regulations in force in France as of 1988 (authorization from competent French Ministry of Agriculture—Agreement No. A91-471-109, May 2009).

After a 1-week acclimation period, all rats were bilaterally ovariectomized under 1–1.2% isoflurane anaesthesia (CSP, Cournon, France).

After 3 weeks of postsurgical period to induce artificial menopause, the rats were divided into four groups (n = 8/group) and received 50 μL of the following intravaginal treatments, delivered via a micropipette twice a day (8–10 hours intervals) for 4 weeks: in the control group, rats did not receive any treatment but a micropipette tip was introduced into the vagina; in the placebo group,
rats received ZP-025 gel without colostrum; in the treated groups, rats received ZP-025 gel containing 0.5% or 2.3% of bovine colostrum.

Body weight was recorded twice a week.

In Vivo Assessment of Vaginal Blood Flow
At the end of the treatment period, changes in vaginal blood flow in response to pelvic nerve stimulation were assessed in each rat of the four experimental groups. Vaginal engorgement was measured by monitoring blood flow in the vaginal wall using laser Doppler perfusion measurement (LDPM) (Oxford Optronix Ltd, Abingdon, Oxfordshire, UK) in baseline conditions and after pelvic nerve stimulation. LDPM is expressed in arbitrary units (au).

Before measurements, rats were anesthetized with urethane 1.2 mg/kg and their temperature was maintained at 37°C (rectal temperature 37 ± 0.3°C) using a homeothermic blanket. Then rats were tracheotomized to prevent aspiration of saliva and, when required, to perform artificial ventilation. The carotid artery was catheterized with polyethylene tubing filled with heparinized saline (25 UI/mL) to record blood pressure (BP) via a pressure transducer (Elcomatic 750, Glasgow, UK).

The pelvic nerve (PN) was exposed via a suprapubic midline incision and separated from connective tissue using a dissecting microscope. PN was placed on bipolar platinum stimulating electrode connected to an electrical stimulator (A-M Systems, Model 2100, Phymep, France) delivering a series of square-wave pulses. The non-invasive laser Doppler microprobes were placed against the inner lateroventral side of the vaginal lumen 15–17 mm distal from the vulva.

Simultaneous computerized measures of BP and LDPM were performed at different time-points: at \( t = 0 \), the continuous recording of BP and LDPM started and the laser probe was placed on the vagina wall to perform baseline measurements of vaginal wall perfusion; at \( t = 30 \) minutes, the laser probe was positioned and the LDPM stabilized for 10 minutes, until the PN stimulation; at \( t = 40 \) minutes and at 5-minute intervals thereafter, the PN was stimulated at square-waves pulses of 1 ms, 6 V, for 30 seconds by different stimulation frequencies (2, 4, 6, 8, and 10 Hz: these different electrical stimulations were randomized and repeated twice in view of establishing a frequency–response curve for each animal); at \( t = 90 \) minutes, the recording ended.

The following parameters were studied: baseline LDPM and BP measured during the 30-minute baseline period before any PN stimulation (mean of three measurements made on three different regions of vaginal wall for 10 minutes period each).

And, for each PN stimulation:

- The mean maximal amplitude of the response, being the percentage of LDPM increase calculated as follows: \( \Delta \text{LDPM} \times 100/\text{Base LDPM} \), and expressed in %
- The area under the curve (AUC) of the response calculated for the entire response, normalized by Base LDPM and expressed in % × second
- The vascular capacitance, which corresponds to the ratio of the mean maximal amplitude of the response to the corresponding mean BP expressed in %/mm Hg

Parameters of LDPM responses were normalized to their own baseline LDPM measurement (Base LDPM, representing 100%) in order to reliably compare each rat, as blood flow through any given tissue is dependent upon the systemic BP.

Tissue Harvesting and Processing
At the end of LDPM experiments, all the rats were euthanized with an overdose of urethane and the vagina and the uterus (including uterine horns and cervix) were harvested.

The tissues were immediately placed in cold physiological saline to eliminate excess of blood and carefully dissected free from surrounding tissue. The vagina and the uterus were weighed (wet weight) and then fixed for 48 hours in 10% neutral buffered formalin and stored in 70% ethanol at 4°C. After dehydration and embedding in paraffin wax, sections of the tissues were cut at 5 μm thickness and stained with hematoxylin and eosin for unblinded histological analysis.

The entire vagina was evaluated considering three portions:

1. The proximal part, near the cervix
2. The distal part, near the vulva
3. The central part, the portion between proximal and distal part

Data Analysis
All results were expressed as mean ± SEM. Grubbs test was used for the detection and exclusion of outliers. Comparison between treatment groups were analyzed by Student's t-test or a one-way analysis of variance (ANOVA) or a two-way ANOVA.

\( P \) values < 0.05 were considered significant.
Results

Body Weight

In all groups, rat body weights regularly increased from the day of ovariectomy until the beginning of the treatment period (day 21) (Figure 1A). There was no significant difference between experimental groups in the body weights measured at the time of surgery (day 0—one-way ANOVA $P > 0.05$) nor at the time of starting treatments (day 21). Likewise, twice-a-day treatments with placebo, ZP-025 0.5%, and ZP-025 2.3% for 4 weeks did not modify the body weight change between the beginning (day 21) and the end of application period (day 49) (Figure 1B) compared with control.

Determination of Peripheral Sexual Response: Vaginal Blood Engorgement by LDPM

The intravaginal application of placebo for 4 weeks, 3 weeks after ovariectomy, did not significantly change baseline mean BP and baseline LDPM compared with control group (Figure 2). Likewise, neither ZP-025 0.5% nor ZP-025 2.3% modified either BP (Figure 2A) or baseline LDPM (Figure 2B) compared with placebo.

Following PN stimulation, the parameters of vaginal blood engorgement (i.e., maximal amplitude of the response, AUC of the response, and vascular capacitance) increased in each group in parallel with the frequency of PN electrical stimulation. No statistically significant difference was shown in each parameter between placebo and control group (Figure 3). The efficacy of the product was evaluated by statistical comparison between the two different doses of colostrum and placebo in order to establish a possible superiority of the product due to the presence of colostrum. After 4 weeks of intravaginal twice-daily treatment with ZP-025 0.5%, no statistical difference in the amplitude, the AUC, and the vascular capacitance was present when compared with placebo group (Figure 4). On the contrary, the treatment with ZP-025 2.3% significantly increased all the parameters of vaginal blood engorgement following PN stimulation, that is, maximal amplitude of the response, AUC, and vascular capacitance.
response, AUC of the response, and vascular capacitance when compared with placebo group (***P < 0.001 for each parameter, two-way ANOVA analysis) (Figure 4).

**Vagina and Uterus Weights**

Intravaginal treatment with placebo, ZP-025 0.5%, or ZP-025 2.3% for 4 weeks in OVX rats did not induce any modification of the vagina and uterus macroscopic aspect. Indeed, in all groups of treatment (control, placebo, ZP-025 0.5%, and ZP-025 2.3%), the appearance of vagina and uterus was normal and thin uterine horns were observed.

Moreover, after 4 weeks of twice daily intravaginal treatment with placebo, ZP-025 0.5% and ZP-025 2.3%, vagina and uterus tissue wet weight normalized to body weight were not modified when compared with control (Table 1).

**Histopathological Evaluation of Vagina and Uterus**

Seven weeks after ovariectomy, at the end of the treatment period, uterus and cervix atrophy was noted in all animals of all experimental groups. The atrophy involved endometrium and myometrium.

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**Figure 2** (A) Baseline mean blood pressure (BP) and (B) baseline vaginal laser Doppler perfusion measurement (LDPM) measured in anesthetized ovariectomized rats (one-way ANOVA, nonsignificant). au = arbitrary unit.

**Figure 3** Effect of twice-a-day intravaginal treatment with placebo or control in anesthetized ovariectomized rats on vaginal blood engorgement: amplitude of the response (A); AUC of the response (B); and vascular capacitance (C) elicited by pelvic nerve electrical stimulations at increasing frequencies (two-way ANOVA analysis). AUC = area under the curve; ns = nonsignificant.

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Atrophy was also noted in vaginal epithelium in all control animals (Figure 5A). In particular, a significant thinning was present in the proximal and central part of the vagina epithelium, which was reduced to one or two cell layers and reaching at maximum three to four cell layers in the distal part of some control animals.

On the contrary, the vaginal epithelium showed a physiological estrous cycle morphological aspect in animals receiving placebo or ZP-025 0.5% or ZP-025 2.3% (Figure 5B). The epithelium consisted of one to two cell layers with flattened superficial cells only in the proximal part with progressive increase in cell layers (at least 5) in the central and distal part. In the central portion, the superficial layers of epithelial cells were in some instances cuboidal with minimal mucification, while in the distal portion, the epithelium of at least five cell layers was squamous with presence of stratum corneum in the majority of animals receiving placebo or ZP-025 0.5% or ZP-025 2.3%. Such changes are characteristic of estrous cycle [22].

**Discussion**

Our study shows that the application of a new vaginal gel containing purified colostrum for 4 weeks provided beneficial effects in an animal model of vaginal atrophy.

Frequency-depending increases of vaginal blood flow elicited by PN stimulation is a widely used experimental paradigm to mimic female sexual response in intact animals [16,23–25]. Ovariectomy, reducing estrogen levels, causes a marked decrease in vaginal hemodynamics, also in

**Table 1** Vagina and uterus wet tissue–body weight ratios at the end of the 4-week treatment in OVX rats

|                | Wet weight–body weight ratio (g/100 g) (mean ± SEM) |
|----------------|-----------------------------------------------------|
|                | Control | Placebo | ZP-025 0.5% | ZP-025 2.3% |
| Vagina         | 0.030 ± 0.001 | 0.032 ± 0.002 | 0.032 ± 0.001 | 0.034 ± 0.01 |
| Uterus         | 0.027 ± 0.001 | 0.026 ± 0.001 | 0.029 ± 0.001 | 0.029 ± 0.001 |

ns = nonsignificant, one-way ANOVA
response to PN stimulation [16]; however, estrogen replacement in OVX rats ameliorates vaginal blood flow and relating parameters, following PN stimulation [16].

In this study, after PN stimulation at different frequencies, only the treatment with ZP-025 containing 2.3% of colostrum increases all the parameters of vaginal blood engorgement, that is, maximal amplitude of the response, AUC of the response, and vascular capacitance in a statistically significant manner. On the contrary, for the same parameters no differences between control and placebo were noted and only a trend with the low dose of colostrum was present when compared with placebo.

Previous studies demonstrate that ovariectomy produces a significant decrease in animal uterus and vaginal tissue wet weight and epithelial thickness [16,19,26]. In fact, the stratified squamous epithelium, consisting of approximately six to eight layers of cells in intact rats, is reduced to one or two cell layers after ovariectomy. Estrogens, when delivered subcutaneously, restore the uterus and vaginal wet weight as well as epithelial thickness, with the same number of layers seen in the non-OVX rats [16,26].

Our study shows that a twice-a-day application of placebo, ZP-025 at 0.5% and 2.3% of colostrum to OVX rats for 4 weeks increases the thickness of vaginal epithelium compared with untreated OVX rats and a physiological estrous cycle morphological aspect of the vaginal epithelium is observed. Despite the fact that the product is not able to restore completely the epithelial thickness to the same condition as before the ovariectomy or with estrogens [26], nor it is able to significantly enhance tissue wet weight, an evident effect is present in treated animals, with an epithelium of at least five cell layers, compared with one or two cell layers in untreated animals. This effect of ZP-025 at all concentrations of colostrum could be due not only to the well-known growth factors included in the colostrum per se, but also to the other re-epithelizing components present in the formula, such as betaine, sericin, and panthenol [27,28]. Moreover, the antioxidant compound (Vitamin E) included in the product could retard biologically destructive chemical reactions in living organisms through their ability to scavenge oxidants and free radicals [29]. As a consequence, the placebo cannot be considered totally devoid of any effect, as shown in the histology analysis of vaginal epithelium, where the response to placebo indicates that it is not completely inert; however, a more complete response was obtained with the gel containing colostrum on other parameters.

Finally, the fact that uterus atrophy remains histologically unchanged in all experimental groups after a 4-week treatment indicates that the effects of the product are specifically limited to the vagina, as expected for a topical preparation. This is in contrast with estrogen preparations for topical use, that anyway lead to increased systemic estrogen levels, thus increasing the well-known risk of systemic actions [30–32].

Although many women report that they gained weight at or near the time of their menopause [33], the amplitude and even the reality of the menopause-related gain in weight is not consistent between studies [34,35].

In rats, ovariectomy is associated to body weight increase [23,36], mainly due to hyperphagia and hypoactivity [37,38]. It has been shown that 17β-estradiol hormonal replacement is able to reduce body weight gain in OVX rats [36,38], while in the present study with ZP-025 there is no significant difference between experimental
groups in the animal body weight, both measured at the start of treatments and at the end (after 4 weeks of treatment).

**Conclusion**

The new ZP-02S vaginal gel containing colostrum improves vaginal hemodynamics and thickness of vaginal epithelium in rats with OVX-induced vaginal atrophy. The marked changes in the vaginal hemodynamics and histology resulting from estrogen deprivation are improved by a treatment with ZP-02S containing 2.3% concentration of purified colostrum. Clinical trials are necessary to determine if these benefits could be translated to menopausal women.

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**Conflict of Interest:** S. Vailati, E. Melloni and M. Sardina are employees of Zambon S.p.A. and E. Riscassi is an independent consultant supporting Zambon S.p.A. in the development projects.

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