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# Vaginal gel adsorption and retention by human vaginal cells: Visual analysis by means of inorganic and organic markers

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ABSTRACT

To improve efficiency and prolong protection, modern gynecological preparations frequently incorporate polymeric molecules that add a certain degree of viscosity in order to increase adhesion with vaginal cells and prolong local delivery of active molecules.

The aim of this study was to investigate the possibility of visualising the ability of a commercial medicated gynecological gel to bind to and be retained by human vaginal cells. The gel formulation included the essential oils of *Thymus vulgaris* and *Eugenia cariophylla*, which contain active molecules such as thymol and eugenol that are known to have useful antibacterial and antimycotic activities.

The adherence of different dilutions of the gel to human vaginal cells was visualised by means of Nomarski interference contrast microscopy and scanning electron microscopy using ferric oxide particles and *Escherichia coli* as inorganic and organic markers, both of which made it possible to visualise the binding of the thin transparent layer of gel and the retaining effect, which was proportional to the degree of dilution.



## 1. Introduction

Depending on their particular uses, gynecological preparations can have extremely diverse compositions that lead to very different detergent and adhesive properties and differences in the release of their active molecules. Modern gynecological preparations frequently incorporate polymeric molecules in order to improve efficiency and prolong adsorption and retention by vaginal mucosal cells. A number of articles have described the chemical and the physical properties of the various polymers, particularly their bioadhesive properties: i.e. their capacity to bind to biological substrates. Pharmaceutically acceptable polymers give these preparations a certain degree of viscosity that is useful for coating epithelial and mucosal surfaces, and simultaneously ensuring that the preparation remains in contact with the substrate and prolongs the local delivery of its active molecules. These molecules have antibacterial, anti-mycotic, anti-oxidant and anti-inflammatory effects, as well as surfactant and moisturising activities that are useful in the treatment of local conditions. A number of electronic, adsorption, wetting and diffusion theories concerning the mechanisms of bioadhesion have been proposed and investigated by various *in vitro* and *in vivo* methods (Vermani et al., 2002) mainly based on measuring shear stress (Smart et al., 1984), tensile strength (Park and Robinson, 1985) and rheological properties (Hassan and Gallo, 1990), flow channels (Mikos and Peppas, 1990), and synthetic (Garg and Vermani, 2000) or natural (animal) model membranes (Lee and Chien, 1996). These methods are generally used to investigate the properties of the individual polymers alone, but there is little information concerning the adhesion and retention of the complete commercial formulation. The aim of the study was undertaken to investigate the retention ability of a commercial medicated gynecological gel by human vaginal cells using direct visual inspection of the presence of the thin film covering the cells after gel application by means of black inorganic (ferric oxide particles) and organic markers (bacteria) rather than other methods that are more appropriate for measuring tensile adhesive strength. The particularity of this gel is that it contains the essential oils of *Thymus vulgaris* and *Eugenia cariophylla* whose active thymol and eugenol molecules are known to possess useful anti-bacterial (Didry et al., 1993; Tepe et al., 2004; Moleyar and Narasimham, 1992; Sato et al., 2006; Dal Sasso et al., 2006), anti-mycotic (Pina-Vaz et al., 2004; Dal Sasso et al., 2007; Braga et al., 2007, 2008a,b; Chami et al., 2005) and anti-inflammatory activities (Braga et al., 2006a,b).

## 2. Materials and methods

The commercial gynecological gel was "Saugella gel®", which can be bought at any local chemist. The ingredients listed on

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the packaging are: water, propylene glycol, glycerin, sorbitol, carbomer, triethanolamine, *T. vulgaris* extract, sodium hyaluronate, methylparaben, disodium EDTA, potassium sorbate, panthenol, *E. cariophylla* extract. The preparation has a pH of 4.5.

### 2.1. Collection of vaginal epithelial cells

The vaginal epithelial cells were obtained from healthy, regularly menstruating and non-pregnant, women aged 24–52 years who showed no signs of genital infection after clinical examination and microscopic examination of a wet smear of vaginal contents. The cell specimens were obtained on various days of the menstrual cycle except during menstrual bleeding. None of the women used vaginal douches or tablets, or oral or local chemical contraceptives. They were all sexually active. The cells were obtained from the mucosal surface of the mid-vaginal wall by means of gentle scraping with a sterile spatula that was then immediately twirled in 2 ml of phosphate-buffered saline (PBS) (0.02 M phosphate and 0.15 M NaCl, pH 7.3). In order to dislodge the cells, the suspensions were passed four times through a 150 μm diameter needle to break up the cell clumps and then pooled, washed three times to free them of debris and non-adherent bacteria by means of centrifugation (260 × *g*, 10 min, 25 °C), and frozen in aliquots at −70 °C. For the adhesion assays, the cells were thawed quickly and washed again, after which PBS was added to the suspensions in order to reach a concentration of $3 \times 10^5$ cells/ml as determined by direct microscopic counts (interference contrast microscopy) in a Bürker chamber (Passoni, Milan, Italy).

### 2.2. Rheological analysis

The viscosity and elasticity ($\eta$ and $G'$) of the preparation was investigated using a Searle-type configuration rheometer with a coaxial cylinder system and Moony–Ewart geometry (Mucometer, Eslab, Milan, Italy) (Braga et al., 1992).

The volume of ambient fluid present in the vagina, and it thickness on the mucosa, depends on physiological and pathological circumstances (Lai et al., 2007) and so a vaginal gel can undergo a wide range of dilutions. The rheological parameters were investigated using the gel and variuos dilutions (1/2, 1/4, 1/8, 1/16 and 1/32) by thoroughly mixing it with PBS.

### 2.3. Inspection of binding and retention properties using an inorganic marker

The high viscosity of the gel prevented us from using the conventional method of putting the preparation in contact with the vaginal cells suspension by means of incubation for a period of time and then separating the cell pellet from the surnatant by means of centrifugation. In the control test, the suspension of vaginal cells was filtered on a cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany) (pores 8 μm, diameters 25 mm), which was pressed onto a microscope slide to create a layer of vaginal cells to which 0.2 ml of a suspension of black ferric oxide particles (mean diameter 1.5 μm) was added as an inorganic marker and incubated at 37 °C for 5 min. After this time, the unbound ferric oxide particles were cleared by thoroughly washing the slide by dropping 10 ml of PBS released by gravity from a graduated pipette at a distance of 20 cm. This washing procedure was repeated three times. The slide was then observed by means of Normaski interference contrast microscopy in order to visualise the degree of surface retention of the black ferric oxide particles by the vaginal cells (Braga et al., 2008a,b). To visualise the presence of the transparent thin film of the gel, a layer of vaginal cells was covered on a slide as previously described, and overlaid by 0.2 ml of gel. After incubation at 37 °C for 5 min, the slide was thoroughly washed by dropping 10 ml of PBS released by gravity from a graduated pipette at a distance of 20 cm. This washing procedure was repeated three times. Ferric oxide particles were then deposited on the slide as described in order to investigate the effects of the gel and various dilutions by thoroughly mixing it with PBS (1/2, 1/4, 1/8, 1/16 and 1/32).

### 2.4. Inspection of binding and retention properties using bacteria as organic markers

*Escherichia coli* ATCC 25922 and two strains of *E. coli* isolated from human urinary infections were used to test the binding of the gel to vaginal cells. In this case, the bacteria were used as organic markers to investigate the interference of gel with the bacterial lock-and-key (adhesin-receptor) mechanism. Suspensions of each organism were prepared from overnight cultures in tryptic soy broth (Sigma, Milan, Italy) under static conditions at 37 °C. The organisms were harvested, washed three times in PBS, and adjusted to $3 \times 10^8$ organisms/ml as determined by direct microscopic counts in a Petroff-Hausser chamber (Thomas Scientific, Swedesboro, NJ, USA). The ability of the bacteria to adhere to the vaginal cells was tested using the method of Gibbons and Van Houte (1971). Briefly, 1 ml of gel or PBS (pH 4.5) was incubated with the vaginal cells for 1 h at 37 °C under rotation at 8 rpm. After centrifugation, the pellet was resuspended in 1 ml of PBS and mixed with 1 ml of a bacteria suspension in polystyrene tubes, which were rotated end over end at 8 rpm for 1 h at 37 °C. The vaginal cells were separated from the non-adherent bacteria by means of centrifugation, and the final cell pellet was resuspended in 2 ml of PBS and filtered on a cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany) with pores of 8 μm and a diameter of 25 mm. The filter was pressed on a microscope slide, stained with Gram stain, and observed by means of Normaski interference contrast microscopy. As differences in bacterial strains and cell surface characteristics (different subjects and different periods of the menstrual cycle) lead to variations in the number of bacteria attaching to individual vaginal cells, bacterial adhesion was determined by counting the total number of bacteria adhering to 50 randomly chosen cells in each sample (Gibbons and Van Houte, 1971). Vaginal cell suspensions incubated with PBS alone were always included in order to establish the number of bacteria already attached (natural adhesion) at the time of cell collection. The same procedure was followed to investigate the effects of the gel and it various dilutions (1/2, 1/4, 1/8, 1/16 and 1/32).

### 2.5. Scanning electron microscopy

The interference of the gel with bacterial adhesion was also studied using scanning electron microscopy (SEM). The samples prepared under the different test conditions described above were put on round coverslips, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1, for 4 h. After dehydration, the coverslips were coated with 200 Å of gold, and observed in a scanning electron microscope. SEM was also used to examine the network of microridges on the surface of vaginal cells before and after the gel incubation.

### 2.6. Data analysis

The differences from controls in the mean values (±S.E.M.) of bacterial adhesion of three separate repetitions for each strain and each tested dilution were calculated using Student's *t*-test for paired data, and were considered statistically significant when the *p* value was $\leq 0.05$.

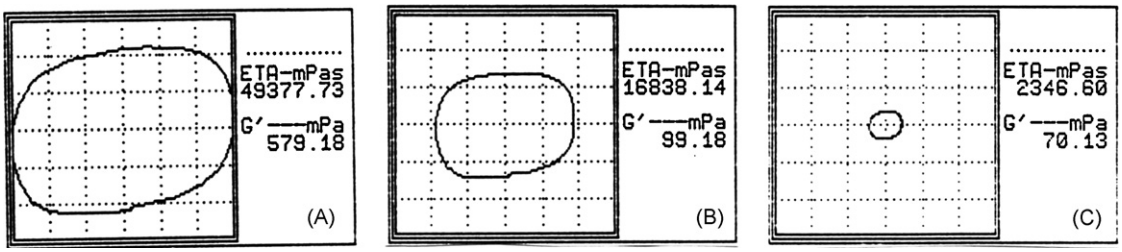


**Fig. 1.** Graphic example of the rheologic test. (A) Gel preparation. (B) After 1/2 dilution. (C) After 1/4 dilution.

## 3. Results and discussion

As the aim of this study was to highlight the mucosal cell binding and retention capacity of the gel, the description of the results will be mainly limited to the images, and only summary data will be given concerning the rheological and bacterial adhesion test.

A graphic example of the rheological tests is shown in Fig. 1. The 1/2 and 1/4 dilution of the gel induced a viscosity reduction of $61.89 \pm 6.30\%$ and $88.91 \pm 4.85$ respectively. The elasticity of the gel is very low and is further reduced by dilution. The gel has a high starting viscosity which is rapidly reduced after dilution and this is useful in order to reach a wide spread on vaginal mucosa.

Fig. 2A shows vaginal cells after incubation with black ferric oxide particles. The fact that very few or no particles were deposited on the surface indicates that the cells themselves did not spontaneously retain them. The gel and the dilution 1/2 showed high levels of viscosity and retention so, when the ferric oxide challenge was repeated after the cells had been incubated, the background remained partially covered by gel and prevented a clear reading of the slides. When the challenge was repeated after the cells had been incubated with gel at a dilution of 1/4, the background was free of particles and patches of particles were clearly visible on the cell surfaces (Fig. 2B), thus revealing the retention capacity of the thin douche layer. The same was observed after incubation with the other diluted concentrations of the gel, although the retention was proportionally less (Fig. 2C and D).

Microscopy inspection showed that the area of gel binding randomly varied from partial to complete. The possibility of the simple superimposition of the particles on the cells was easily ruled out

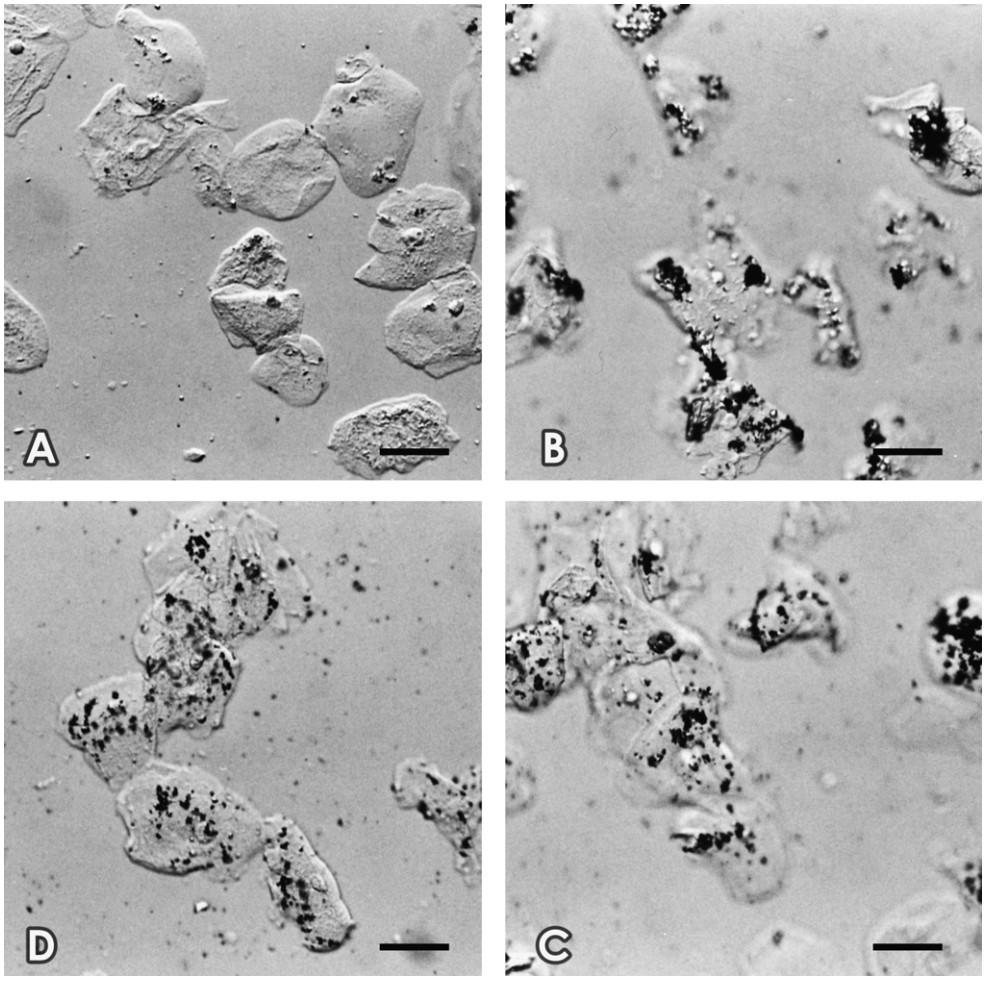


**Fig. 2.** Light microscopy showing the retention of black ferric oxide particles on vaginal cells before and after incubation with the gel (bar = 12 μm). (A) Control cell showing no particles retention. (B) High retention after incubation with gel (1/4). (C) Retention after incubation with gel (1/16). (D) Retention after incubation with gel (1/32).

because under the microscope if the marker simply overlaps the cells, tapping the slide will separate cell and the marker; this does not occur when the two are "glued" together as in our findings.

The second part of the study, in which bacteria were used as organic markers, was also performed starting from the 1/4 dilution of the gel. There was a statistically significant reduction in bacterial adhesion after the cells had been incubated with gel (Fig. 3), which can be attributed to the retained layer of the gel preparation covering the receptor molecules on the cell surface and thus preventing the bacterial adhesins from expressing their lock-and-key mechanisms. This reduces bacterial adhesion and reveals the presence of a covering gel layer. The SEM observations confirmed the above findings (Fig. 4).

Fig. 5 clearly shows the layer of the gel covering the microridges of the vaginal cells in comparison with the control cells.

The anatomical and physiopathological conditions of the vaginal environment mean that vaginal preparations have been (and will continue to be) developed on the basis of marked demand, clinical testing and patience acceptance. Vaginal administration is a promising means of locally delivering, and vaginal drug formulations have been produced to fulfill a variety of purposes including delivery of different molecules such as hormones, anti-bacterials, antifungals, antiphlogistics, etc.

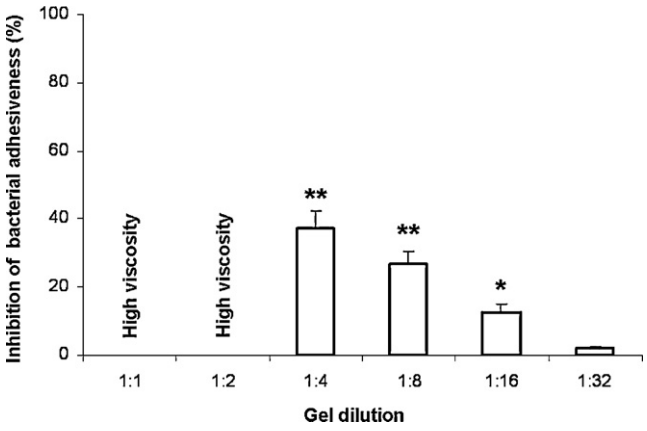


**Fig. 3.** Effects of gel on the adhesion of *E. coli* to human vaginal cells.

A number of methods have been used to screen vaginal formulations. Classic tensile and shear testing methods mainly investigate physisorption and chemisorption properties, and can sometimes lead to contradictory results because of the different types of forces involved. Direct visual approaches are quicker and more suitable

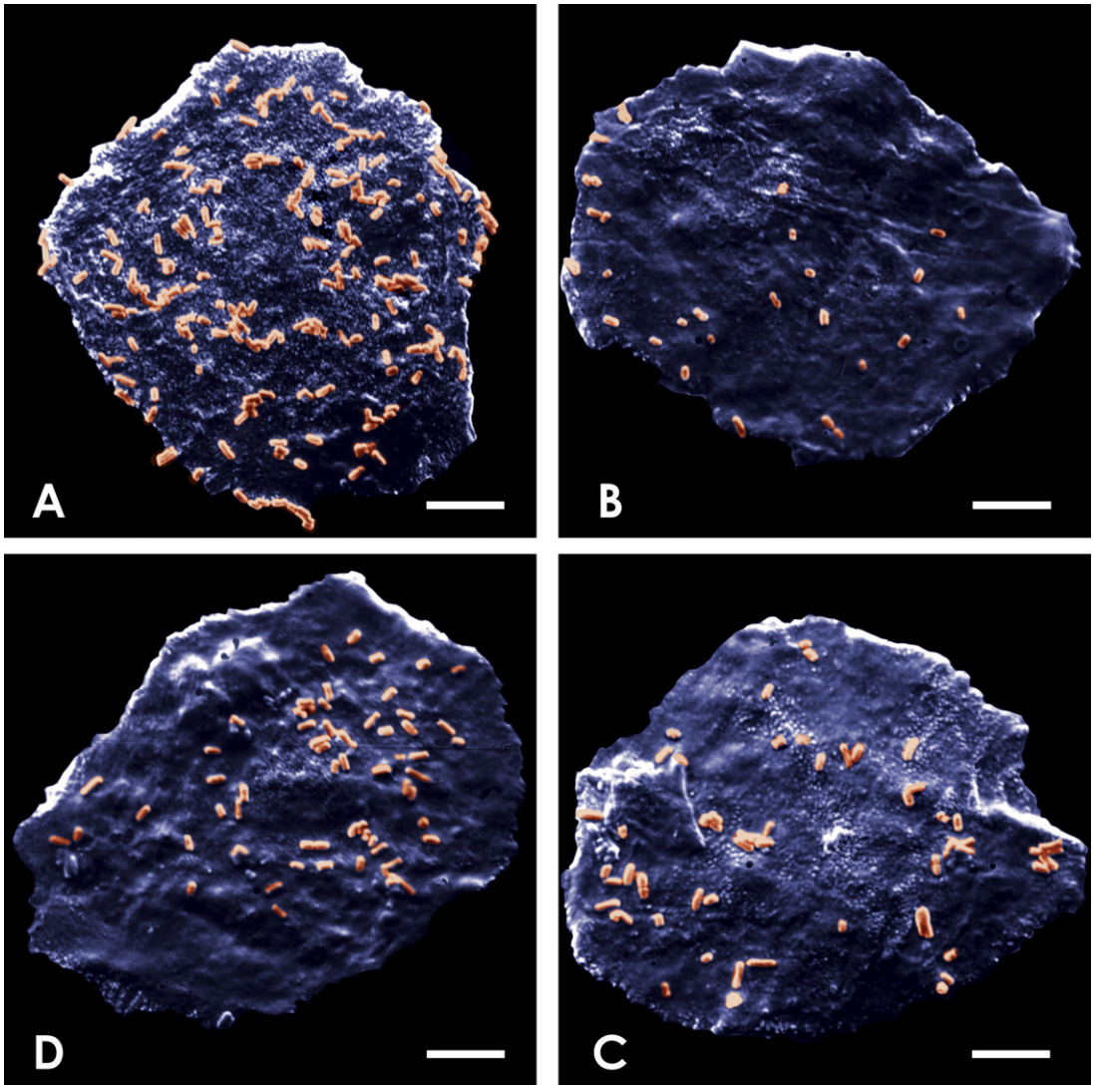


**Fig. 4.** Scanning electron micrographs showing the effects of the gel on *E. coli* adhesion to human vaginal cells (bar = 3 μm). (A) Adhesion of *E. coli* without gel incubation. (B) *E. coli* adhesion after gel incubation (1/4). (C) After incubation with gel (1/8). (D) After incubation with gel (1/16).

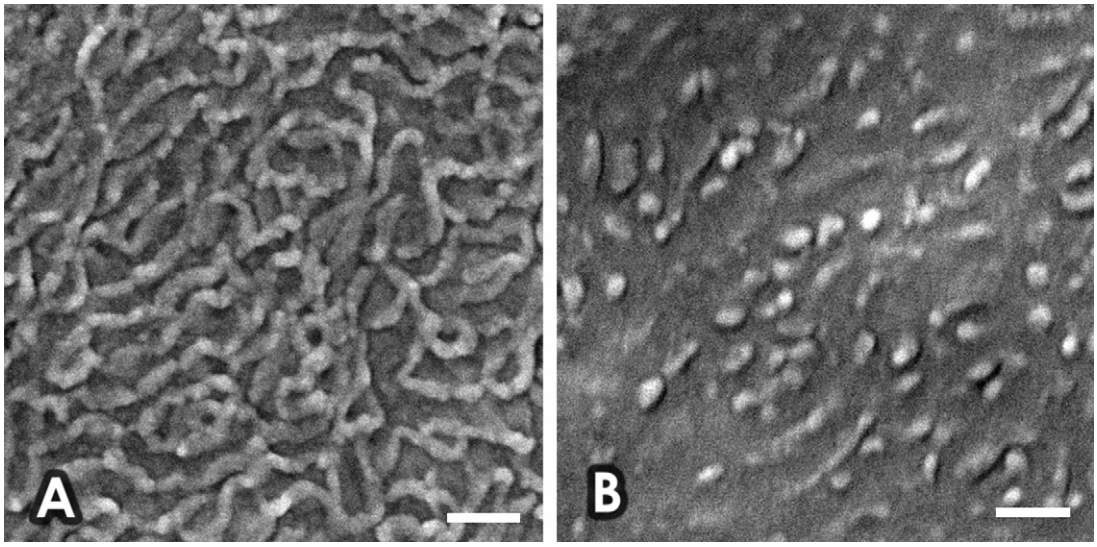


**Fig. 5.** Scanning electron micrography showing the microridges of the surface of a human vaginal cell: not incubated with gel (A) and after incubation with 1/4 gel (B). The second image clearly shows the layer of gel covering the microridges of the cell (bar = 500 nm).

for revealing vaginal cell coverage and the interfacial retention of preparations such as vaginal gels, and have been previously used to evaluate the adhesion and retention of polymers following acqueous dispersion (Kockisch et al., 2001), the activity of bioadhesive preparations in the oral cavity (Patel et al., 1999), and the ability of a commercial preparation to cover human buccal cells (Braga et al., 2008a,b). Alternative methods using colloidal gold staining (Park, 1989) or radiolabelled markers have also been explored, such as gamma scintigraphy (Richardson et al., 1996; Geraghty et al., 1997) and magnetic resonance imaging (Barnhart et al., 2001a,b).

Bacterial markers have been previously used to investigate the effect of hydroxypropylmethylcellulose (Braga et al., 2008a,b; Steinberg et al., 2002) and poloxamer (Veyries et al., 2000), and atomic force microscopy (AFM) has also recently been used to visualise cell coverage phenomenon. Patel et al. (2000) used AFM to investigate the covering ability of hydroxy-propylmethylcellulose and observed that untreated cells have surfaces covered by small "crater-like" pits and indentations, whereas treated cells appear to have lost the craters and indentations, thus indicating the presence of a covering effect.

A preparation that can be dispersed throughout the vagina and retained by cell surfaces for a sufficient period of time is useful because it covers and protects small surface lesions of the vaginal mucosae, and may prevent the adhesion of bacteria, fungi and viruses approaching the vaginal environment by blocking their adhesins from reaching the complementary receptors located on the surface of vaginal cells.

Our examinations using inorganic or organic markers revealed that the binding of vaginal cells may be complete, patchy or totally absent. This different behaviour is probably because the observed cells are generally separated from each other and thus lead to discontinuity in the binding surface; the distribution of the gel should be more homogeneous in a continuous mucosal layer such as that existing *in vivo*.

The presence in the formulation of molecules such as thymol and eugenol, which can be locally retained for some time, is another beneficial feature because a number of studies have described their anti-bacterial (Didry et al., 1993; Tepe et al., 2004; Moleyar and Narasimham, 1992; Sato et al., 2006; Dal Sasso et al., 2006) and anti-mycotic activity, mainly on Candida species (Pina-Vaz et al., 2004; Dal Sasso et al., 2007; Braga et al., 2007, 2008a,b; Chami et al., 2005). However, the Lactobacilli of the commensal vaginal flora does not seem to be much affected (Sacconi et al., 2003), and the pH of the gel helps to maintain a physiological acid environmental pH.

A further interesting feature of thymol is its anti-inflammatory activity, which is exerted through the inhibition of reactive oxygen species and the elastase released by the human neutrophils (Braga et al., 2006a, b), that are locally recruited during infective or inflammatory processes.

Our visual approach showed that this gel does not simply cover the cell surface but its retained in a manner that is proportional to its dilution degree. This means that the vaginal fluid with its cleansing action can modulate the cell retention of the gel by diluting the amount of the components.

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