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HPLC determination of the stability of retinoic acid in ovule formulations for the treatment of cervical dysplasia related to papillomavirus

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Abstract

We studied the efficacy of all-*trans*-retinoic acid-containing ovules in combination with an α-interferon-based gel and Decapeptyl° IM in the treatment of cervical papillomavirus infections and intraepithelial dysplasia. Stability tests showed that the retinoic acid in the ovules was stable for at least 4 months at 4°C. In a pilot study, we obtained complete remissions with the three-drug combination, whereas all-*trans*-retinoic acid and α-interferon used alone produced only partial remissions; Decapeptyl° also induced the disappearance of similar lesions, but was associated with recurrences due to persistence of the virus.

Keywords: all-*trans*-Retinoic acid; Ovule; α-Interferon; Gel; Decapeptyl; Cervical infection; Human papillomavirus; Cervical intraepithelial dysplasia

1. Introduction

Infection by human papillomaviruses (HPV) leads to abnormal proliferation of epithelial cells in the anogenital region, known as condylomata. The presence of HPV in a large proportion of precancerous and cancerous lesions of the female genital organs has been demonstrated by molecular biology-based techniques (Bergeron and Ferenczy, 1987). Genital condylomata are difficult to treat because of their multifocal nature, the spread of HPV at the periphery, their tendency to relapse, and the variety of clinical presentations, which make them difficult to diagnose.

Cryotherapy of recent lesions and CO_2 laser treatment are still the main approaches when the lesions are few in number (Ferenczy, 1984, 1985), however, there is no consensus on the treatment of more widespread lesions.

all-*trans*-Retinoic acid (ATRA) (tretinoin) has

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antiproliferative and differentiating properties that have been used to slow or prevent tumor growth in man (Lippman et al., 1987).

α-Interferon (INFa) has antiviral effects and inhibits the cell cycle. It has been used sucessfully to treat HPV lesions and to eliminate viral DNA (Hoghum, 1983; Jacobsen and Rockwood, 1988).

Finally, a preliminary study of five patients treated with Decapeptyl showed a clear regression of the dysplasic and condylomatous lesions, although this was followed by relapse due to viral persistence, as revealed by molecular hybridization (Mathe et al., 1988).

A research project aimed at evaluating medical treatment of HPV cervical infections and intraepithelial cervical dysplasia was set up in the hematological oncology unit of the Groupe Hospitalier Paul Brousse, in which we evaluated the tolerability and efficacy of local treatment with IFNa and all-*trans*-retinoic acid after intramuscular administration of Decapeptyl.

An American team has previously tested an experimental all-*trans*-retinoic acid delivery system consisting of a cervical cap containing a sponge saturated with a predetermined amount of tretinoin (Meyskens et al., 1983). The treatment proved difficult to apply and was associated with a high frequency of irritation and cervical lesions.

We therefore developed two new formulations – an IFNa-based gel and tretinoin-based ovules – for use in combination with Decapeptyl. A stability study of the ovules was also carried out.

2. Materials and methods

2.1. Sources

Carbopol 934, glycerin, and gelatin were obtained from Cooper (Paris, France), sodium hydroxide from Prolabo (Paris, France), tretinoin from Bonassies (Paris, France), lidocaine (xylocaine) from Roger Bellon (Neuilly sur Seine, France), Decapeptyl from Ipsen-Biotech (Paris, France), and α-interferon (Introna) from Schering-Plough (Levallois-Perret, France).

2.2. Drug formulations

2.2.1. all-trans-Retinoic acid ovules

The formula of the ovules was as follows: gelatin, 8 g; distilled water, 24 g; glycerin, 48 g; lidocaine, 1.44 g; tretinoin, 72 mg.

These ingredients were made up into six 12-g ovules containing 0.1% all-*trans*-retinoic acid and 2% lidocaine, as follows. The powdered gelatin was placed in water until absorption was complete. Glycerin was heated to 50–60°C and then mixed with the gelatin; when the temperature had fallen to about 40°C, the lidocaine and tretinoin were added. The final mixture was poured at a temperature of about 38°C into 12-g polythene ovule moulds (Cooper). The ovules were allowed to cool and then stored at +4°C in the dark, in the sealed moulds.

2.2.2. IFNa gel

Carbopol 934, a carboxyvinyl polymer with a very high molecular weight, was used as excipient; it forms a translucent gel at pH 6.

The IFNa gel was formed by dispersing five parts by weight of the polymer in one part of injectable water and adjusting the pH with 30% sodium hydroxide solution. Three MIU of IFN was made up in the solvent provided and incorporated into 30 ml of the gel under a laminar-flow hood. The gel was then dispensed into 10-ml tubes equipped with an applicator and stored at +4°C. The gels were prepared weekly and the tubes were sufficient for 1 week of treatment. The stability of IFN in these gels is currently under investigation.

2.3. Quality controls

Weight uniformity of the ovules (French Pharmacopœia)

The mean weight was evaluated by weighing individually 20 ovules chosen at random. The individual weight of two or more of the 20 units could differ from the mean weight by no more than 5%, and the weight of each unit could differ by no more than 10%.

2.4. Study of ovule and gel pH

We used a model 82 pH meter (Radiometer Copenhagen, precision = 0.01 pH units). The equipment was calibrated using two standard buffer solutions at pH 4.00 and 7.00.

Each measurement lasted 5 min and was repeated at regular intervals. Three measurements were made for each sample.

2.5. Microbiological testing of the ovules and gel

1 g of ovule or gel was dispersed in 10 ml of nutrient broth. Agar plates were seeded with 0.1 ml of broth, with media for anaerobes, aerobes and fungi (chocolate/polyvitex, Chapman, Drigalski, and Sabouraud). The plates were incubated for 7 days at 30/35°C (bacterial media) or 20/25°C (fungal media).

2.6. Ovule disintegration testing (French Pharmacopœia)

The disintegration of the ovules was tested in liquid medium for 60 min. Disintegration was considered complete if there was: (i) complete dissolution; (ii) separation of the components; (iii) softening of the product (disruption with a glass rod).

The apparatus consisted of a transparent tube containing a metallic support formed by two stainless-steel discs each with 39 holes.

A single sample was placed in the apparatus in a water-containing cuvette at 36/37°C.

2.7. all-trans-Retinoic acid assay

all-*trans*-Retinoic acid can be measured by means of reverse-phase high-performance liquid chromatography (HPLC) with ultraviolet detection. The equipment and operating conditions have been described elsewhere (Bonhomme et al., 1990).

1 g of ovule was dispersed in 2 ml of acetic acid until dissolution. all-*trans*-Retinoic acid was then extracted by making up the mixture to 100 ml with acetonitrile. 50 μl of the solution was injected into the loop. A calibration curve was constructed by analysing ovules containing 0.025, 0.05, 0.1, and 0.2% of ATRA, using the peak areas.

2.8. Study of all-trans-retinoic acid stability

Ovules stored at +4°C in the conditions described above were assayed for retinoic acid after 1 and 4 months. Two ovules were tested.

2.9. Preliminary clinical study: medical treatment of genital warts and intraepithelial cervical neoplasias

2.10.1 Inclusion criteria

Patients presenting for routine screening, abnormal cervical smear results, intraepithelial cervical neoplasia or cervical condylomata were eligible for the study.

Patients presenting with or being treated for other cancers, immunological or hematological disorders, or extensive multifocal lesions were excluded.

The effects of treatment were monitored by blood tests, colpophotography and biopsies of the edges of the lesions. The viral lesions were typed and photographed.

A questionnaire aimed at evaluating the tolerability of the topical treatments was given to each patient.

With the agreement of the local ethics committee and the patients' informed consent, six women aged from 19 to 37 years were included in the study.

Three had mixed lesions (flat condylomata of the cervix and intraepithelial cervical neoplasia) and three had a single type of HPV lesion (flat condylomata of the cervix, acuminate condylomata of the vulva, or intraepithelial cervical neoplasia).

HPV lesions were identified by means of cytologic and histologic studies. Biopsies were performed on all visible lesions of the cervix, vagina, vulva, perineum and anus. All the biopsy specimens were divided in two, one part for histologic study and the other for HPV typing by means of molecular hybridization.

The results of the histologic examination were

used to classify the lesions as pure viral infections, pure condylomata, and viral lesions with dysplasia.

Dysplasia was classified by order of gravity according to the WHO classification as slight (grade 1), moderate (grade 2), or serious/in situ carcinoma (grade 3). The cytologic examination was considered negative in the absence of koilocytes or dysplasic cells.

The cardinal sign of HPV infection is the koilocyte. The latter can be identified on cervical smears, as intermediate cells with a dense cytoplasm, a clear perinuclear halo and, often, a picnotic nucleus.

Viral DNA was detected by means of molecular hybridization in the genital lesions of 3/6 patients. Types 6 and 11 were responsible for acuminate vulvar condylomata and flat cervical condylomata. Types 16 and 33, which are potentially oncogenic, were detected in a patient with a large cervico-vaginal condyloma associated with dysplasic type I-II lesions.

The HPV type has prognostic value, but typing is difficult because it involves delicate hybridization techniques, and because several types of HPV can coexist in a single lesion.

2.10.2. Treatment protocol

Patients were intially treated with monthly subcutaneous injections of Decapeptyl 3.75 mg. Decapeptyl (detryptoreline, D-Trp-6-LHRH) is a synthetic analogue of natural LHRH. Long-term treatment inhibits gonadotropin secretion and thus suppresses ovarian function. The aim of this treatment was to reduce cell proliferation at the squamo-glandular junction of the cervix. At the 12th week of treatment, patients were asked to apply 10 ml of the IFN gel at night (Mondays, Wednesdays, and Fridays) for 3 months.

They were seen 1 month after the start of the treatment to evaluate local side-effects and to improve compliance by means of a questionaire. A cervical smear and colpophotography were also carried out. After 18 weeks of treatment, the patients were prescribed 0.1% all-*trans*-retinoic acid ovules to place deep in the vagina (Tuesdays, Thursdays, and Saturday nights, alternating with the IFNa gel) for 6 weeks.

Cytocolposcopy was carried out at the end of the treatment period and then every 3 months for 1 year, 6 months for a further year and yearly thereafter; a cervicovaginal smear and a colpophotography were carried out on each occasion.

3. Results

3.1. Quality controls

3.1.1. Weight uniformity of the ovules

The mean weight was 11.93 g. Only one ovule differed by more than 5% from the mean value (11.11 g, 6.8%), and none differed by more than 10%.

3.1.2. pH of the ovules and gel

The following pH values were measured. all-*trans*-Retinoic acid ovules: control sample, pH 5.27 ± 0.01; 4-month sample, pH 5.23 ± 0.02. Interferon gel: control sample, pH 5.98 ± 0.02; 15-day sample, pH 6.00 ± 0.02.

After 3 months, the pH values remained compatible with vaginal pH (pH 4.5–5.5).

3.1.3. Sterility of the gel and ovules

All the agar plates remaineded sterile.

3.1.4. Physical stability of the ovules

The two series of three ovules disintegrated within 10 min with shaking and 20 min without shaking. With shaking, the soluble components were completely dissolved, while the insoluble powders formed a deposit. Without shaking, a soft mass remained on the perforated plate, and was easily disaggregated with the glass rod.

3.2. all-trans-Retinoic acid assay

3.2.1. HPLC assay of all-trans-retinoic acid in the ovules

3.2.1.1. Validation of the assay. There was a linear relationship between the peak area (Y) and the tretinoin concentration (X). The maximum and minimum concentrations on the calibration curve

Table 1
Stability of retinoic acid in the ovule formulation

Time (months)	all-*trans*-Retinoic acid concentration (μg/ml)	% of initial concentration
0	5	100
1	5.05	101
4	4.95	99

gave the following equation: $Y = 124175X - 260$ and a correlation coefficient $R = 0.9986$.

3.2.1.2. Reproducibility. Reproducibility was studied by injecting 10 samples of a known concentration.

Concentration, 0.066%; M (measurement), 0.07%; CV (coefficient of variation), 4.6%.

3.2.1.3. Repeatability. Three injections were made on 3 consecutive days with a sample containing a known concentration.

Concentration: 0.05%
D1 M = 0.048% CV = 8%
D2 M = 0.055% CV = 2%
D3 M = 0.051% CV = 5.2%
Concentration: 0.066%
D1 M = 0.070% CV = 4.2%
D2 M = 0.067% CV = 3.1%
D3 M = 0.072% CV = 1.9%

The uniformity of the ovules was determined (French Pharmacopoiea) by measuring the individual concentrations; the values were within the limits set relative to the mean concentration of the sample. Only one of the 10 ovules tested varied by more than 85/115%, but did not exceed the 75/125% limit.

3.2.1.4. Stability of all-trans-retinoic acid in the ovules. There was no significant loss of all-*trans*-retinoic acid in either of the two ovules after storage for 4 months at +4°C (Table 1).

3.3. Clinical results

The clinical results were based on findings of smears and colposcopy carried out at 2, 4 and 6 months, and the replies to the questionaires.

Five of the six patients followed the treatment protocol combining Decapeptyl, α-interferon and tretinoin. One patient did not receive Decapeptyl because of previous phlebitis.

The results of the examination at 2 months were satisfactory. Decapeptyl given alone led to a clear regression of the lesions. The side-effects observed were due to hormone privation (hot flushes, vaginal dryness, loss of libido, dyspareunia, tiredness). One patient complained of painful cramp during effort, but this disappeared during interferon administration. Another patient complained of nausea and headache.

After 4 months of treatment, one patient had signs of a viral lesion, while the others had epithelial changes suggestive of viral persistence.

The interferon gel was very well tolerated: only one patient gained weight (+4 kg), and she also complained of thymic disturbances (irritability). On the questionaires, the patients complained of local irritation, a burning sensation and pruritis with the all-*trans*-retinoic acid ovules.

One patient had to stop using the ovules after 2 weeks, and three patients developed vaginal fungal infections; it will therefore be necessary to include an anti-fungal agent in the ovules, although it is possible that these infections were due to all-*trans*-retinoic acid-induced irritation of the vaginal mucosa.

At the end of treatment (6 months), clinical findings suggested that the treatment was effective. The patient who only received the local treatment was in partial remission, while the remaining five were in complete clinical and histologic remission. HPV was either undetectable or greatly reduced.

One of the five patients relapsed after 8 months.

4. Discussion

Previous studies of treatments for genital infections due to HPV have shown that cream formulations of α-interferon only lead to partial remissions in some patients. Decapeptyl treatment led to a regression of HPV lesions, but most patients relapsed due to viral persistence. We

postulated that a combination of drugs might be more effective in this setting, and prepared two new formulations: all-*trans*-retinoic acid-based ovules and an α-interferon-based gel.

The results of a clinical pilot study showed satisfactory efficacy and tolerability of this drug combination, and warrant further trials with larger numbers of patients.

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