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Antiviral activity of HPMPC (cidofovir) against orf virus infected lambs

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Abstract

(*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPC, cidofovir, CDV, Vistide®) is an acyclic nucleoside analogue with a potent and selective activity against a broad spectrum of DNA viruses including the poxviruses. In this study we present the results of different treatment regimens in lambs experimentally infected with orf virus with different cidofovir formulations prepared in Beeler basis and Unguentum M. Our results show that choice of excipient, concentration of cidofovir and treatment regimen were all important to the clinical outcome of the therapy. Whilst one particular regimen appeared to exacerbate the lesion, treatment with 1% (w/v) cidofovir cream, prepared in Beeler basis, for 4 consecutive days did result in milder lesions that resolved more quickly than untreated lesions. Furthermore the scabs of the treated animals contained significantly lower amounts of viable virus meaning there should be less contamination of the environment with virus than would normally occur.

Keywords: Orf virus; Contagious ecthyma; Cidofovir; HPMPC; Topical treatment; Formulation

1. Introduction

Orf virus is the causative agent of contagious ecthyma (orf) a highly contagious pustular dermatitis of sheep and goats which can spread to man. The disease has a very high morbidity and although mortality is rare and usually does not exceed 10%, the disease is frequently severe enough to create substantial welfare problems in flocks (Robinson and Balassu, 1981). This in turn has an economic impact on sheep farmers due to production losses. In sheep and goats orf is recognized by the appearance of vesicles, papules and crusty, rapidly growing, scabs, normally on the skin of the lips and nose, but occasionally on the lower limbs, of affected animals. High titres of virus are shed with the scab into the environment which, if kept dry, can remain infectious long enough to sustain annual outbreaks of the disease (Yirrell et al., 1989). The disease usually runs its course in 3–4 weeks (Haig and Mercer, 1998; Guo et al., 2003) but occasionally in animals and humans, especially immunocompromised subjects, extensive and recurrent lesions can occur. In these cases the disease is manifested by "Giant Orf", or tumor-like lesions that do not spontaneously regress (Hooser et al., 1989; Hunskaar, 1986; Mazur and Machado, 1989; Smith et al., 2002; Tan et al., 1991). Vaccination of sheep and goats can limit the severity of the disease, but does not prevent the infection, and in some instances vaccine strains have been the source of outbreaks of contagious ecthyma (Gilray et al., 1998). At present, there are no specific drugs for the treatment of the disease in infected animals.

(*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPC, cidofovir, CDV, Vistide®) has a potent and selective activity against a broad spectrum of DNA viruses (De Clercq et al., 1987; De Clercq, 2002, 2003) including the poxviruses (Balzarini et al., 2004). The anti-orf virus activity of cidofovir has already been demonstrated in previous studies

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in vitro (Nettleton et al., 2000), *ex vivo* (Dal Pozzo et al., 2005) and in the clinical treatment of human orf, being used, in that instance, as a 1% (w/v) preparation in Beeler base cream (Geerinck et al., 2001). The purpose of this study was to evaluate the antiviral activity of cidofovir in infected lambs.

2. Materials and methods

2.1. Virus

The viruses used in this study were MRI-Scab (McInnes et al., 2001) and IT 613/02, both of which are virulent wild type isolates that have not been grown in cell culture. Electron microscopy of the negatively stained suspension of IT 613/02 (Gallina et al., 2006) showed that it contained approximately 10^7 virus particles/ml of inoculum.

2.2. Formulation of creams used for treating orf lesions

Cidofovir (HPMPC; Gilead, Foster City, CA, USA) was prepared as three different formulations. For the first, cidofovir was prepared as a 1% (w/v) formulation in Unguentum M (HERMAL, Crookes Healthcare Ltd., Nottingham, UK). The second and third formulations used two different concentrations of cidofovir, 0.5% (w/v) and 1% (w/v) formulated in Beeler base (De Clerq, 1984; Snoeck et al., 1993, 2001; Geerinck et al., 2001).

2.3. Animals, inoculations and treatment regimens

Orf virus naïve lambs used in this study were either raised in a containment facility at the Moredun Research Institute, Edinburgh, UK or at the Faculty of Veterinary Medicine, University of Bologna, Italy. Animal studies were conducted in accordance with Regulation 86/609/EEC, "Protection of animals used for experimental and scientific purposes". The lambs were fed with UHT milk until 30 days of age and from 14 days old until the end of the study solid food and clean water was freely available at all times. In a preliminary experiment 14 lambs were housed in two groups of seven. All animals were infected, by scarification (Nettleton et al., 1996) on the inner aspect of both hind thighs, with MRI-Scab virus and thereafter each of the virus scarification sites was subjected to one of four different treatments. Group A animals received treatments I and II on the left and right thighs, respectively, whereas group B animals received treatments III and IV on the left and right thighs, respectively. Treatment I involved no intervention. The lesions were allowed to progress naturally until resolution. Treatment II involved the application of cidofovir/Unguentum cream from day 1 post-infection (PI). The cream was applied to the lesion and gently massaged until absorbed. All the animals in each group (I to IV) were age matched and those being treated with cream received approximately the same amount of cream per lesion. The cream was applied once daily for 12 days and thereafter discontinued. The lesion was then allowed to resolve naturally. Treatment III was similar to treatment II but the cream was applied for the first time 8 days PI, continued for 11 days and then withdrawn until the end of the experiment. Treatment IV involved the application of Unguentum cream without cidofovir, from day 1 PI. Application of cream was continued for 20 days and thereafter withdrawn. The infected areas of skin were examined daily from day 1 for 35 days to assess the development of lesions.

In the second experiment nine Massese lambs were used. Each lamb was infected by scarification on the inner aspect of the left hind thigh with IT 613/02. The lambs were housed in three groups of three animals (A, B, C). The animals in the group A were treated with the Beeler base cream alone (containing no cidofovir), the animals in group B were treated with 0.5% (w/v) cidofovir/Beeler cream and the animals in group C were treated with 1% (w/v) cidofovir/Beeler cream. Each animal was treated with 1 ml of cream/lesion, once daily, for 4 consecutive days starting from day 4 PI. The cream was applied to the lesion and gently massaged until absorbed. At the end of the experiment the scab material was collected from each animal and stored at −80 °C. The infected areas of skin were examined daily for 28 days to assess the development of lesions.

2.4. Evaluation of cidofovir on viral infection

Each of the experiments was performed blind with the identity of the treatment not revealed until the end of the experiments. The progression of the lesions was evaluated daily, until the lesions had resolved, by monitoring the clinical score for each lesion as outlined by Nettleton et al. (1996). Briefly, lesions were scored for the presence of erythema, the presence of vesicles and/or pustules and the presence of firmly attached scab. Each criterion was scored from 0 to 3 according to the thickness of the lesion along the scarification line. In the second experiment skin has been clipped to measure the thickness of the lesions across the line of scarification. Blood samples were taken prior to the primary scarification and thereafter weekly until the end of the experiment. Creatinine and urea levels were monitored to assess the renal function since cidofovir is known to be potentially nephrotoxic in humans.

In experiment 2, the effect of treatment on viral replication was also determined by monitoring the recovery of virus from the treated and untreated scabs both by *in vitro* growth of the virus and by real time Taqman® PCR (Gallina et al., 2006). Twenty-five milligrams of pooled scab material taken from treated or untreated animals were ground in 1 ml PBS containing 10% antibiotic/antimycotic solution containing penicillin, streptomycin and amphotericin B (Gibco, UK). Scab suspension was centrifuged at 2000 × *g* for 5 min and the clarified supernatants filtered through a 0.45 μm filter and used to inoculate foetal ovine testis cells (TFO) seeded in a 6-well plate and maintained in D-MEM supplemented with 10% fetal calf serum (Gibco, UK). The cells were viewed microscopically 5 days PI and the presence or absence of viral CPE was recorded. Three passages in cell culture were performed and infected cell lysates were collected and stored at −80 °C. The amount of virus in the supernatants derived from centrifuged pooled scab suspension and from the infected cell lysates was determined by real time Taqman® PCR. DNA extraction was performed using the Easy DNA kit (Invitrogen, The Netherlands) following the manufacturer's instructions. The absolute quantification of viral DNA

was obtained by plotting 10-fold dilutions of a standard plasmid containing a fragment of the orf virus *B2L* gene against the corresponding threshold cycle value (ct). The TaqMan® PCR assay was performed using the Rotor-Gene 3000 system (Corbett Research, Australia).

2.5. Statistical analysis

A repeated measures model was fitted to the lesion data. The lack of independence between successive measurements made on the same sheep thigh was modelled using an auto-regressive type 1 model. There was no evidence of any dependence between observations made on the two thighs of the same sheep. Parameters of the model were estimated using the REML directive in Genstat 8th edition (VSN International Limited). In experiment 1 (cidofovir formulated with Unguentum M) the residuals from the model were satisfactory and the discrete nature of the data (scores on a scale 1–9) did not invalidate this approach. In experiment 2 (cidofovir formulated with Beeler base) there was some evidence that the variability of the thickness measured increased with the mean and so the analysis was performed using a log transformation.

The data obtained from real time PCR analyses were statistically analysed using a one-way ANOVA with Tukey's post-test using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA). Results were considered significant when P was <0.05.

3. Results

3.1. Treatment of orf lesions with cidofovir/Unguentum cream

All animals developed typical orf virus lesions on both inner hind thighs. The group mean clinical scores for each of the four different treatment regimens were collated and plotted against time (post-infection) to determine the activity of cidofovir (Fig. 1). Treatment I which involved no intervention represented the normal process of an experimental orf virus infection whereby the lesion developed through the normal stages of erythema, papule/pustule formation, vesicle formation, scab formation and eventual resolution after approximately 5 weeks. The lesions treated with Unguentum cream alone for 20 days following infection (treatment IV) showed no obvious deviation from those that had received no treatment (treatment I). However by day 4 PI those lesions treated with cidofovir immediately following infection (treatment II) were significantly different from the untreated lesions ($P < 0.001$). There was generally less erythema around the scarification lines and the progression of the lesion through to the papule/pustule stage was less advanced. This pattern of slower progression through the normal stages of lesion development was seen up until treatment was withdrawn 12 days PI, by which time there was erythema over the area were the cream had been applied. By 17 days PI (5 days after the withdrawal of treatment) there were no noticeable differences between these orf lesions and the untreated orf lesions and like the untreated lesions they also resolved by 35 days

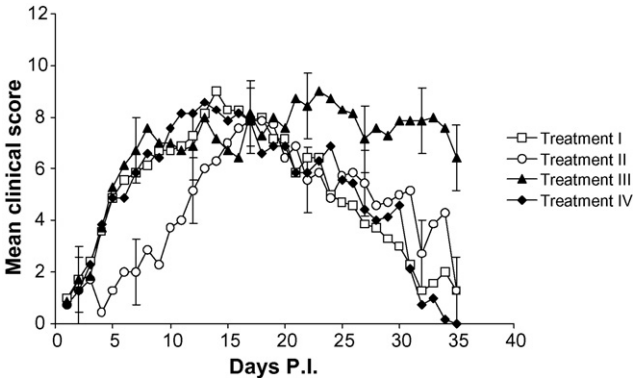

Fig. 1. Effect of cidofovir/Unguentum M treatment on the development of orf virus lesions in lambs. A graph is shown of the group mean daily clinical scores, over the 35-day observation period, of the four treatment regimens with 1% (w/v) cidofovir/Unguentum M. All animals were infected with MRI-Scab virus. Treatment I involved no intervention; treatment II involved the application of cidofovir/Unguentum M from day 1 PI for 12 consecutive days, thereafter treatment was withdrawn; treatment III involved the application of cidofovir/Unguentum M from day 8 PI for 11 consecutive days, thereafter treatment was withdrawn; treatment IV involved the application of Unguentum M cream alone from day 1 PI for 20 consecutive days, thereafter treatment was withdrawn. The approximate 95% confidence intervals for the means, estimated as the mean ± 2 × standard error from the fitted model (mean ± 1.27), are shown for treatments II and III at 5-day intervals.

PI. The final treatment regimen (treatment III) involved treating established orf lesions with cidofovir/Unguentum cream. Treatment was started 8 days post-infection when the lesions were already developed with erythema, pustules and scab all present. Treatment may have slowed the further development of the lesion slightly, but no significant differences were found between these lesions and the untreated lesions. Treatment was withdrawn 11 days later because there was a generalised erythema around the site of the initial lesions related to the area to which the cream had been applied and secondary lesions were beginning to appear. The lesions within this treatment regimen

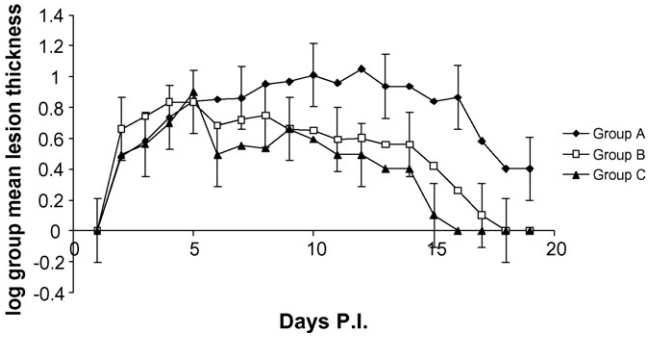

Fig. 2. Effect of cidofovir/Beeler base treatment on the development of orf virus lesions in lambs. A graph is shown of the log transformed group mean daily lesion thickness, over the 19-day observation period, of the three treatment regimens. Group A received Beeler base alone; group B received 0.5% (w/v) cidofovir in Beeler base from day 4 PI for 4 consecutive days, thereafter treatment was withdrawn; group C received 1% (w/v) cidofovir in Beeler base from day 4 PI for 4 consecutive days, thereafter treatment was withdrawn. The approximate 95% confidence intervals for the means, estimated as the mean ± 2 × standard error from the fitted model (mean ± 0.21) are shown for each group at 3-day intervals.

did not resolve within the 35 days and were considerably exacerbated in comparison to all those in the other treatment groups ($P < 0.001$). At no time was there any evidence of systemic effects of cidofovir applied on one thigh affecting the development of the lesion on the contra lateral thigh. Blood levels of urea and creatinine remained within normal limits throughout the experiment.

3.2. Treatment of orf lesions with cidofovir/Beeler cream

All the infected lambs developed typical orf virus lesions in the first 4 days following infection (Fig. 2) with an intense erythema and oedema along the lines of scarification and the appearance of vesicles and pustules. By day 8 PI, after 4 days of topical treatment, a significant difference ($P < 0.001$) in the mean thickness of the lesion was found between the untreated animals in group A and the treated animals in groups B and C (Fig. 2). The cidofovir-treated animals had yellowish scurfy scabs that were easily removed between days 10 and 17 PI. The treated lesions were completely resolved by day 15 PI (group C) and day 17 PI (group B). The untreated lesions had dark, firmly attached scabs, forming between 15 and 21 days PI, that were not fully removable until 28 days PI. The animals remained healthy, suffering no loss in weight, with levels of urea and creatinine in the blood remaining within normal limits for the duration of the experiment.

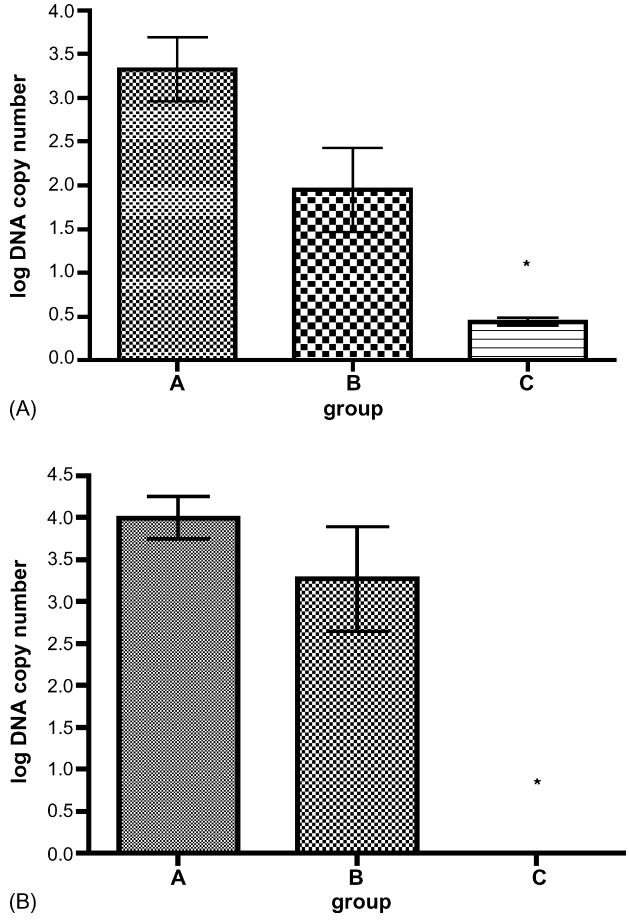

Fig. 3. Effect of cidofovir on the amount of viral DNA present in treated vs. untreated lesions. The amount of viral DNA present in the scabs taken from treated and untreated animals (A), and in the cell lysate prepared from the third passage of recovered virus *in vitro* (B) was estimated by real time PCR. Group A animals received Beeler base alone containing no cidofovir; group B received 0.5% (w/v) cidofovir in Beeler base from day 4 PI for 4 consecutive days, thereafter treatment being withdrawn; group C received 1% (w/v) cidofovir in Beeler base from day 4 PI for 4 consecutive days, thereafter treatment being withdrawn. The data are shown as the mean DNA amount ± standard deviation with P values determined using ANOVA and Tukey post-test (the asterisks indicate a significance being $P < 0.05$). In (A) a significantly different amount of viral DNA was found when group C was compared to group A. whereas in (B) group C showed a significantly lower amount of viral DNA compared to both group A and B.

The quantitative PCR performed on 5 ng of DNA extracted from the pooled scabs collected from each group of animals showed a significant difference ($P < 0.01$) in the mean amount of viral DNA recovered from the animals in group A in comparison to the animals in groups B and C (Fig. 3A). The virus recovered from the pooled scabs was also cultured *in vitro*. After three passages a typical orf virus cytopathic effect was observed in the cell culture infected with the scab material from the untreated animals and with the virus from the animals treated with 0.5% (w/v) cidofovir while no CPE was detected for the animals treated with 1% (w/v) cidofovir. The cells from each *in vitro* culture were lysed and the amount of viral DNA present quantified once again by Taqman® PCR (Gallina et al., 2006). There was significantly less viral DNA ($P < 0.05$) in the group C (1%, w/v cidofovir) lysates in comparison to both the group A (no cidofovir) and group B (0.5%, w/v cidofovir) lysates (Fig. 3B).

4. Discussion

Contagious ecthyma is an infectious disease found throughout the world and its spread is closely linked to the presence and size of animal herds. The disease is considered one of the top 20 most important diseases of sheep and goats in terms of impact on the poor in undeveloped countries (Perry et al., 2002). Young animals are particularly affected although recently an increasing number of cases in adults have been reported. Morbidity is high and mortality can reach 10–20%; mortality is often caused by starvation and secondary infections.

The acyclic nucleoside phosphonates (ANPs) are the most potent drugs available against poxviruses (De Clercq, 2002) their mechanism of action is targeted at the viral DNA polymerase acting as chain terminators and therefore preventing DNA replication (Magee et al., 2005). Cidofovir belongs to the ANPs analogues and has a broad antiviral activity spectrum including many DNA viruses. Cidofovir, formulated with a variety of excipients, has already been used in experimental animals and in humans to treat a number of virally induced cutaneous infections including, amongst others, cowpox, camelpox, monkeypox, and molluscum contagiosum (Davies et al., 1999; Quenelle et al., 2004; De Clercq, 2002). *In vitro* studies have shown that orf virus replication is strongly inhibited by cidofovir with a selectivity index at least five-fold greater than that found for vaccinia virus. In addition cidofovir has been used successfully to treat "Giant Orf" in humans (Geerinck et al., 2001) where previously amputation, excision or cryotherapy (Degraeve et al., 1999) would have been considered as the only effective treatments for this

condition. Recently, the anti-orf virus activity of cidofovir was confirmed in sheep organotypic skin cultures, an *in vitro* model of ovine skin (Dal Pozzo et al., 2005). Given the efficacy of cidofovir in preventing replication of orf virus, the topical treatment of orf in experimentally infected lambs was investigated. In a preliminary experiment the cidofovir was formulated as a 1% (w/v) preparation in Unguentum M, and was used to treat orf immediately following infection and in 8-day-old lesions. The untreated lesions progressed through a normal course of infection with the lesions resolving spontaneously after about 5 weeks. Treating the lesions with Unguentum cream alone for 20 days after the initial scarification had no bearing on either the development or the resolution of the lesions, although there was a minor erythema in three of the seven lambs where the cream had been applied and the lesions themselves remained moist until the cream was withdrawn. Treating with cidofovir for 12 days immediately following the initial infection certainly slowed the development of the lesions, but did not stop them forming completely. In addition the lesions eventually were indistinguishable from the untreated lesions, even taking the same time to resolve. This was slightly surprising in that 1% (w/v) cidofovir had resulted in complete regression of "Giant Orf" in an immunosuppressed patient (Geerinck et al., 2001), whilst *in vitro* a concentration of between 20 and 50 μg/ml was sufficient to stop virus replication all together (Nettleton et al., 2000; Dal Pozzo et al., 2005). Even though Unguentum M has been used as a successful delivery vehicle for cidofovir in humans (Davies et al., 1999), it may well be that Unguentum M is less suitable for delivering cidofovir to the infected ovine skin cells, allowing some of the virus to continue to replicate. Treatment was withdrawn in group II when the skin, which was being treated with the cream, began to show signs of erythema. The skin was worse in group II (Unguentum/cidofovir) compared to the group receiving Unguentum M cream alone suggesting that the cidofovir may have some slight toxicity to skin cells. However no detrimental effects on skin cells were reported in the case of the treatment of "Giant Orf" and equally in the *in vitro* studies there was no evidence that cidofovir was toxic to cells (Nettleton et al., 2000; Dal Pozzo et al., 2005).

The final treatment represented what could happen in natural cases of orf when treatment would be applied to fully developed lesions, cidofovir/Unguentum treatment of 8-day-old lesions did not help them to resolve faster than the untreated lesions. In fact the opposite occurred with the lesions persisting for longer, and evidence of secondary lesions forming. This is probably due to the fact that by the time treatment was started, the virus infection would have been already well established with more virus present in the skin lesion in comparison to the initial inoculum. Taken together with the moistening effect of the cream, the mechanical disruption of the lesion that had already formed and the slight damage to the skin (in terms of erythema) caused by the application of the cream, this is likely to have led to the exacerbation of the original lesion.

In the subsequent study both the excipient and treatment regimen were changed. This time cidofovir was prepared as 0.5% (w/v) and 1% (w/v) preparations in Beeler base, the excipient that had been used successfully to treat the "Giant Orf" in humans (Geerinck et al., 2001). In addition the lesions were treated 4 days post-infection when they would be evident to the shepherd and treatment was continued for just 4 days. This it was thought would minimise any damage to the skin cells caused by the repeated application of the cream. Following the infection all lambs developed orf lesions. These lesions all progressed at similar rates and were at the same developmental stage when the treatment was started. After 4 consecutive days of therapy the two groups of animals treated with cidofovir showed milder lesions with small loose flaky scabs that clinically resolved within 15 and 17 days post-infection. The quantitative PCR revealed a significantly higher amount of viral DNA in the scabs of the untreated animals in comparison to the treated animals suggesting that the cidofovir had indeed inhibited the replication of the virus. To determine whether or not the cidofovir also had an effect on the viability of the virus, the virus isolated from the scabs was also grown in cell culture. After three passages, typical, orf virus induced, CPE was visible in the cultures infected with the virus from the untreated animals and those receiving the 0.5% (w/v) cidofovir, confirming that there was still viable virus in these scabs. This agrees with results reported by Dal Pozzo et al. (2005) that the treatment of organotypic cultures with 0.5% HPMPC produced only a partial reduction in orf virus replication in such cultures. In contrast, however, no CPE was observed in the cultures infected with virus from the 1% (w/v) cidofovir treated animals. Although the real time PCR showed that some copies of viral DNA were present in these cultures, whether this represents viable virus or not is unknown. Certainly if it did represent viable virus it was clearly unable to produce any visible signs of infection in cell culture after three passages. Since many outbreaks of orf are thought to result from environmental contamination with the virus from previous outbreaks of the disease, if the cidofovir does reduce the amount of viable virus this could be critical to reducing the incidence of disease on farms that have a persistent problem with orf.

Taken together our results demonstrate that the topical application of cidofovir cream can result in milder lesions that resolve more quickly than untreated lesions confirming that this antiviral molecule does offer some hope in the treatment of orf virus lesions in sheep and goats. However it is also clear that the excipient and the treatment regimen used is also critical to the outcome of the treatment. Allied to this would be the practicality of treatment in the field. It may be better for instance, to have a formulation of cidofovir that could be sprayed onto the lesion, would be absorbed quickly and would not keep the lesion moist. Therefore if a successful treatment is to be developed for the field our results demonstrate that a number of factors will need to be taken into consideration.

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