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Successful treatment of equine sarcoids by topical aciclovir application

S. Stadler, C. Kainzbauer, R. Haralambus, W. Brehm, E. Hainisch, S. Brandt

Based on the anecdotally reported eradication of a sarcoid using aciclovir cream, the curative potential of this ointment was investigated in 22 sarcoid-affected horses referred to the Equine Clinic Tillysburg, Austria, between 2006 and 2009. Sarcoid disease was diagnosed by clinical examination and bovine papillomavirus types 1 and 2 from intact skin and tumour tissue. As nine horses had more than one lesion, a total of 47 sarcoids were treated by daily topical application of aciclovir 5 per cent cream for a period of two to six months; in four horses, surgical tumour ablation was performed before treatment. Disease parameters, including the tumour type, number, location and size, were recorded before and after aciclovir therapy. All 47 (100 per cent) of the sarcoids responded to treatment, with complete tumour regression observed for 32 (68 per cent) lesions and no recurrences reported thus far. Incomplete resolution was observed for 15 (32 per cent) lesions, probably due to their thickness. Aciclovir is proposed to be routinely used for the treatment of mild-type sarcoids and as an adjuvant therapeutic agent in combination with surgery.

EQUINE sarcoids are common, non-metastasising, locally aggressive skin tumours that are induced by bovine papillomavirus types 1 and 2 (BPV-1, BPV-2). They are the most common neoplasm in horses, representing 67 per cent of all equine tumours (Sullins and others 1986, Chambers and others 2003). According to their clinical appearance, sarcoids are classified as occult, verrucous, nodular, fibroblastic, mixed or malevolent types of lesions. Whereas occult and verrucous sarcoids only involve superficial skin layers, the other types grow more aggressively, extending to deeper skin layers, and, in the case of the malevolent type, infiltrate local lymphatics (Pascoe and Knottenbelt 1999, Knottenbelt 2009). It is currently accepted that BPV-1 and BPV-2 chiefly contribute to sarcoid onset and maintenance (Chambers and others 2003). BPV-1 and BPV-2 each consist of a non-enveloped protein capsule harbouring a circular, double-stranded genome of approximately 7950 bp DNA, containing open reading frames for six early (E) functional and two late (L) structural proteins, and a long control region (LCR). The early genes encode regulatory (E1, E2, E4) and transforming (E5, E6, E7) proteins, whereas the late genes code for the major L1 and the minor L2 capsid protein. The non-coding LCR comprises cis-elements required for the replication and the transcription of the viral genome (Campo 2006). Currently, there is no universally effective therapy to treat sarcoid disease.

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S. Stadler, MagMedVet,
Pferdeklinik Tillysburg, Bruck bei Hausleiten 11, 4490 St Florian, Austria
C. Kainzbauer, MagMedVet,
S. Brandt, DiplIng, DrNatTechn,
Equine Biotechnology Unit,
R. Haralambus, MagMedVet,
E. Hainisch, DrMedVet,
Large Animal Surgery and Orthopaedics, Equine Clinic,
University of Veterinary Medicine,
Veterinaerplatz 1, 1210 Vienna, Austria

W. Brehm, Professor, DrMedVet, DiplECVS,
Faculty of Veterinary Medicine,
University of Leipzig, An den Tierkliniken 21, 04103 Leipzig, Germany

Correspondence to Dr Brandt,
e-mail: sabine.brandt@vetmeduni.ac.at

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Treatment methods include surgery, radio-, cryo- and chemotherapy, or the use of aggressive ointments such as zinc chloride cream (Knottenbelt 2009) or imiquimod (Nogueira and others 2006). All of these methods are used with variable success and disease recurrence is observed in up to 50 per cent of cases (Tarwid and others 1985). Aciclovir cream is successfully used for the treatment of herpesvirus-induced skin lesions in human beings. Its therapeutic compound, aciclovir, is an antiviral agent that has been designed to specifically inhibit herpesvirus replication. To become active, it needs to be triply phosphorylated by viral and cellular thymidine kinases (TKs) (Elion 1983). In contrast to herpesviruses (Flint and others 2000), papillomaviruses lack the gene coding for TK and are therefore unable to activate aciclovir (Baker and Howley 1987). Nonetheless, the eradication of a sarcoid by topical application of 5 per cent aciclovir cream has been reported anecdotally. Hence, the purpose of the present study was to investigate the therapeutic potential of aciclovir ointment for the topical treatment of equine sarcoids.

Material and methods

Horses with sarcoids

All treated horses (n=22) were referred to the Equine Clinic Tillysburg, St Florian, Austria between 2006 and 2009. Thirteen of the 22 individuals bearing single or multiple sarcoids were diagnosed with occult lesions, four were affected by mixed types (occult-verrucous or verrucous-nodular), two displayed verrucous lesions and three had nodular sarcoids (Table 1).

Sarcoid diagnosis

Sarcoid disease was diagnosed by clinical examination and BPV-1/-2 PCR from tumour and/or intact skin DNA. For PCR, sarcoid skin swabs and/or biopsies excised from intact skin of the neck using a 4 mm biopsy punch were subjected to DNA extraction using a DNeasy blood and tissue extraction kit according to the manufacturer's instructions (Qiagen). PCR-compatible purity of isolates was assessed by routine equine β-actin PCR, as described by Brandt and others (2008a, b). Subsequently, the presence of viral DNA was assessed using two pairs of BPV-1/-2 consensus primers for amplification of a 499-bp region containing the E5 gene and a 266-bp region within the L1 coding sequence, respectively (Brandt and

TABLE 1: Response of 47 sarcoids to topical aciclovir treatment

Horse	Sarcoid	Sarcoid type	Sarcoid regression degree	Duration (months)
1	1	Occult	Complete	>6
	2	Occult	Partial	>6
	3	Occult	Partial	>6
2	4	Occult	Complete	2
	5	Occult	Complete	3
	6	Occult	Partial	>6
3	7	Occult	Complete	1
	8	Occult	Complete	2
	9	Occult	Complete	5
4	10	Occult	Complete	2
	11	Occult	Complete	5
5	12	Occult	Complete	1
	13	Occult	Complete	3
	14	Occult	Complete	3
	15	Occult	Complete	>6
	16	Occult	Partial	>6
	17	Occult	Partial	>6
6	18	Occult	Complete	3
	19	Occult	Complete	3
	20	Occult	Complete	5
	21	Occult	Complete	5
	22	Occult	Complete	5
	23	Occult	Partial	>6
	24	Occult	Partial	>6
	25	Occult	Partial	>6
	26	Occult	Partial	>6
	27	Occult	Partial	>6
7	28	Mixed	Complete	5
	29	Mixed	Partial	>6
8	30	Mixed	Partial	>6
	31	Mixed	Partial	>6
9	32	Mixed	Complete	1
	33	Mixed	Partial	>6
	34	Mixed	Partial	>6
10	35	Mixed	Complete*	2
11	36	Verrucous	Complete	>6
12	37	Verrucous	Complete	>6
13	38	Nodular	Complete*	1
14	39	Nodular	Complete*	1
15	40	Nodular	Complete*	1
16	41	Occult	Complete	3
17	42	Occult	Complete	4
18	43	Occult	Complete	1
19	44	Occult	Complete	3
20	45	Occult	Complete	2
21	46	Occult	Complete	1
22	47	Occult	Complete	1

* Lesions were ablated before aciclovir treatment

others 2008a, b). Reactions were carried out in a total volume of 20 µl, containing 1x Phusion HF buffer (Finnzymes), 3 per cent dimethyl sulfoxide, 200µM Deoxynucleotide Mix (Finnzymes), 0.5µM of sense and antisense primers (VBC Biotech), 1 µl of DNA template and 0.4 U of Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes). The amplification programme was an initial denaturation step at 98°C for two minutes, 45 thermal cycles (98°C for 15 seconds; 66°C for 30 seconds; 72°C for 30 seconds) and a final elongation step at 72°C for five minutes. It was conducted in a FlexCycler (Biozym Scientific). BPV-1/-2-positive sarcoid DNA, skin DNA from a healthy horse and sterile water served as positive, negative and no template controls, respectively. Sixteen microlitres of reaction aliquots were visualised on 1.5 per cent Tris-acetate agarose gels by ethidium bromide staining.

Aciclovir treatment

Each horse was examined thoroughly and disease parameters, that is, the tumour type, number, location and size, were recorded. The length, width and height of the lesions were measured in millimetres. The owners, trainers or grooms were advised to apply aciclovir 5 per cent cream onto the lesions for a period of two months on a daily basis. In total, 47 sarcoids were treated with aciclovir. Nodular lesions (38 to 40) and one mixed sarcoid (35) were surgically ablated to skin level before aciclovir application (Table 1).

Evaluation of clinical response

All horses with sarcoids were clinically re-examined and tumour parameters were re-evaluated in intervals of two weeks for a minimum of two months. In cases where no complete sarcoid resolution was achieved within this period, treatment and examinations were extended to another four months.

Effect of aciclovir in vitro

In order to investigate whether aciclovir specifically interacted with BPV-infected cells, primary sarcoid fibroblasts (cell line ES01.1) containing episomal BPV-1 DNA and uninfected equine fibroblasts (cell line EqPALF) were cultivated in DMEM medium with Glutamax, containing 10 per cent heat-inactivated FCS and 0.5 per cent pen/strep. For the culture of EqPALF, the medium was further supplemented with essential and non-essential amino acids to final concentrations of 1 per cent (all media and supplements from Invitrogen). Cells were harvested, subjected to trypan blue counting and then cultivated in fresh media with the addition of 1 per cent aciclovir (GlaxoSmithKline). After 24 and 48 hours, the number of viable cells was determined as described above.

Results

Equine β-actin PCR scored positive for all DNA isolates, thus confirming that DNA had been extracted successfully. Subsequent PCR screening of samples derived from horses with sarcoids for BPV-1/2 DNA confirmed the clinical diagnosis of sarcoid disease. Negative and no template controls repeatedly tested negative, thus confirming accuracy of the method. Repeated clinical examination of horses revealed that 100 per cent of the 47 sarcoids had responded to aciclovir treatment. Complete regression was achieved for 32 (68 per cent) lesions, with no events of recurrence reported thus far (June 2010). Former tumour sites were indistinguishable from the surrounding skin areas and covered by hair (Fig 1). There was complete resolution of 14 lesions within two months. Nine of these were of occult type, one corresponded to a mixed sarcoid and four others were mixed (one) or nodular (three) sarcoids that had been ablated before treatment due to their prominent size. Aciclovir ointment was then immediately applied onto the excision wound. In 14 other cases (13 occult and one mixed), tumours completely regressed between three and six months. For two occult and two verrucous lesions, aciclovir treatment had to be continued for up to four additional months to achieve complete tumour eradication. Although a significant decrease in tumour length, width and height was noted, incomplete resolution was observed for 15 (32 per cent) lesions. Ten of these were of occult and five of mixed type. All of these tumours were characterised by a more pronounced thickness exceeding at least 5 mm, as determined by measuring and palpation. Aciclovir therapy results are outlined in Table 1. To analyse whether the drug specifically abrogated the proliferation of BPV-infected fibroblasts, primary sarcoid cells versus uninfected equine fibroblasts were cultivated under the presence of 1 per cent aciclovir. As shown in Fig 2, the number of viable sarcoid cells had dropped by almost 50 per cent after a 24- and 48-hour incubation with aciclovir. In contrast, only a mild antiproliferative effect was noted for virus-free equine fibroblasts.

Discussion

In the present study, the therapeutic potential of an antiherpesvirus drug with respect to its ability to eradicate BPV-1/-2-induced sarcoids in 22 horses was evaluated. Papillomaviruses lack the gene coding for TK and thus are unable to activate aciclovir by phosphorylation. A considerable curative effect was achieved with aciclovir treatment, leading to complete sarcoid clearance in 68 per cent and significant tumour regression in 32 per cent of cases. Except for a verrucous sarcoid measuring 20 mm in length, all eradicated occult and verrucous lesions displayed a maximum extent of 5 mm in diameter before treatment. In this context, it should be noted that therapeutic success was inversely proportional to the thickness of the lesion, suggesting that the drug is limited by its ability to penetrate deeper tumour areas. A better absorption may be achieved using valaciclovir, a l-valyl ester of aciclovir (Beutner and others 1995). Topical iontophoresis of valaciclovir hydrochloride may further improve cutaneous aciclovir delivery

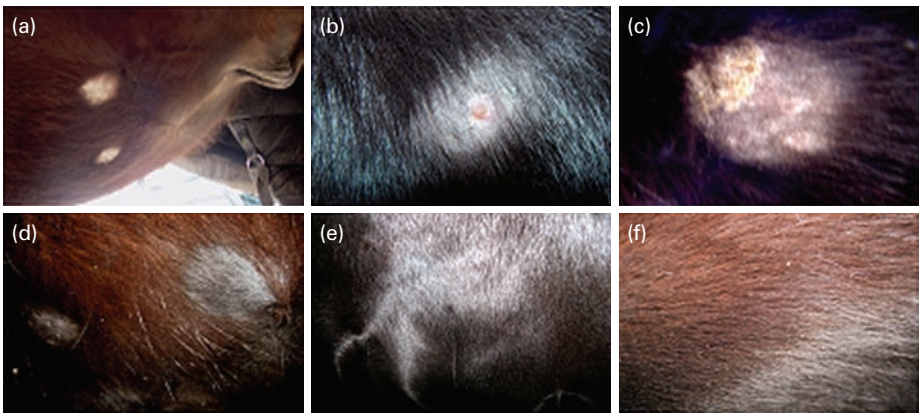

FIG 1: Curative effect of aciclovir 5 per cent ointment following topical application on three occult sarcoids affecting horse 3. (a) Abdomen, (b) chest and (c) girth area, before treatment, and (d) abdomen, (e) chest and (f) girth area, 2.5 months after treatment

(Abla and others 2006). Alternatively, the antitumoural effect of aciclovir and its derivatives may be enhanced by the adjuvant use of herpesvirus TK gene transduction systems (Hassan and others 2000). The duration of aciclovir treatment until remission varied between one and several months and did not correlate with the number of lesions per individual. Depending on the sarcoid size, location, number and type, similar remission time variations are seen for other treatment options, so that a serious comparison is not possible in this regard (Scott and Miller 2003, Knottenbelt 2009; S. Stadler, C. Kainzbauer, R. Haralambus, W. Brehm, E. Hainisch, S. Brandt, personal observation). In the authors' experience, the environmental temperature was another factor influencing the therapeutic efficacy of aciclovir. It was noted that response to treatment considerably decreased at environmental temperatures below 5°C.

In order to investigate whether a similar response to aciclovir could be achieved in vitro, BPV-1-infected versus uninfected equine fibroblasts were cultivated with 1 per cent aciclovir and cell proliferation rates before and 24 and 48 hours after addition of the drug were determined. When comparing cell numbers determined at time 0 and after 48 hours, the proliferation of control cells had dropped to 91 per cent, whereas a decrease of proliferation to 54 per cent was observed for BPV-infected fibroblasts. This finding suggests a specific interaction of aciclovir with BPV-infected cells. The antiproliferative effect occurred within the first few hours following aciclovir treatment. This observation agrees with the relatively short half-life of aciclovir of 40 minutes (Fechner and Teichmann 2000), and suggests that the curative effect of the drug could be improved by more frequent application, for example, five times per day. Indeed, in the case of a circular fibroblast of 4 x 4 cm diameter on the neck of a 10-year-old warmblood mare, complete tumour regression was achieved within three weeks by application of aciclovir five to seven times a day (S. Brandt, personal experience). Given that the triple phosphorylation required for aciclovir activation is usually achieved by viral and cellular TKs, the authors speculate that cellular TKs alone may be able to activate the drug, albeit less efficiently. The general bioavailability of aciclovir is 15 to 30 per cent (Laskin 1983). In human gastrointestinal adenocarcinomas and oesophageal and uterine squamous cell carcinomas, TK-1 expression has been shown to be upregulated. In contrast, thyroid papillary, hepatocellular, pancreatic ductal, and renal cell carcinomas, as well as lung adenocarcinomas revealed downregulated TK-1 expression (Shintani and others 2010). In equine sarcoid cells and BPV-1 transfected equine fibroblasts, no deregulation of TK expression has been observed (Yuan and others 2008). In general, high TK-1 expression is found in highly proliferative tissues (Herzfeld and Greengard 1980). However, the lesions regressing after aciclovir treatment in this study were mainly slowly growing occult sarcoids. This observation and the generally low bioavailability of the drug indicate that the curative effect of aciclovir is not particularly triggered by elevated TK expression levels in the lesions. The authors also screened a series of tumour DNA isolates for herpesvirus DNA using universal primers in PCR. However, all samples scored negative by this test, thus ruling out the possibility that aciclovir may be coactivated by herpesviruses residing in the sarcoids (S. Brandt, E. Hainisch, personal communication). Four sarcoids, including three nodular types and one mixed form, were surgically ablated before applying aciclovir onto the wound. These lesions resolved completely in 2008 and no recurrence has been reported thus far. Similar results have been obtained at the equine clinics of the VetSuisse Faculty, Berne, Switzerland, and the Veterinary University, Vienna, Austria (W. Brehm, R. Haralambus, E. Hainisch, personal communication). The aciclovir ointment used in this study was made by the university pharmacy of the Veterinary University Vienna. The ointment is used routinely at the equine clinic of the university for treatment of mild-type lesions and as adjuvant therapy after surgical debulking. This has proved effective in the vast majority of lesions. So far, very few recurrences have been observed (S. Stadler, E. Hainisch, personal communication). However, a longer period of follow-up is required to provide definitive information regarding recurrence rates. Aciclovir ointment is a relatively cheap therapeutic option without documented side effects, and it can be applied easily by lay persons. This novel option should therefore be taken into consideration by practitioners, especially when attempting to cure mild sarcoids.

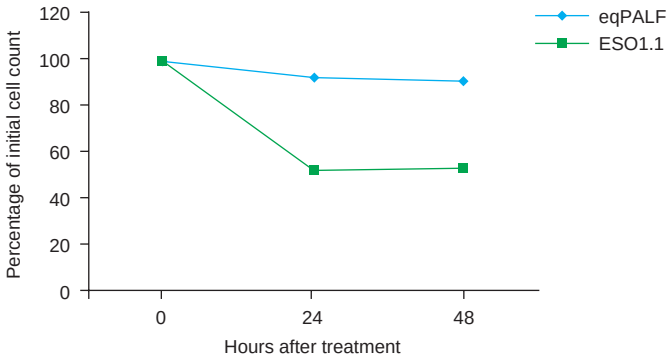

FIG 2: Antiproliferative effect of aciclovir 1 per cent on bovine papillomavirus type-1-infected (ES01.1) and virus-free (eqPALF) equine fibroblasts, reflected by the percentage of viable cells 24 and 48 hours after treatment

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