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(54) Title: THERAPEUTICAL AGENT USEFUL FOR THE TREATMENT OF PLASMA CELL NEOPLASIAS

(57) Abstract: The use of calcium trifluoroacetate for the preparation of a drug for the treatment of plasma cell neoplasias, particularly multiple myeloma.

THERAPEUTICAL AGENT USEFUL FOR THE TREATMENT OF PLASMA CELL NEOPLASIAS

The present invention relates to the use of calcium trifluoroacetate for the preparation of a medicament for the treatment of plasma cell neoplasias, in particular multiple myeloma.

Calcium trifluoroacetate was recently studied as a cytotoxic drug against cell lines of solid tumors of various origin (colon, lung, pancreas, breast, prostate, liver, stomach and ovary). Preliminary clinical evidence proved that calcium trifluoroacetate is well tolerated and substantially free from important toxic effects when administered subcutaneously or intravenously to patients with colon or breast carcinomas. The therapeutical results, although preliminary, have been surprising in terms of reduction in tumor mass, reduction or disappearance of ascitis and metastatic nodules and reduction in tumor markers (CA19.9 and alpha-foetoproteins).

Plasma cell neoplasias are a group of clinically and biologically heterogeneous diseases, characterized by hyperproliferation of an immunoglobulin-producing cell clone and hence by the presence of immunoglobulins or monoclonal fragments thereof in blood and urines. Among plasma cell neoplasias, multiple myeloma, also known as plasma cell myeloma or myelomatosis, is undoubtedly a particularly serious disease for which no available effective therapeutical protocols exist.

Multiple myeloma is in fact a neoplastic process with usually poor prognosis, characterized by bone marrow plasma cell infiltration and production of monoclonal immunoglobulins type G, A, D or E or Bence Jones proteins (κ or λ subunits).

Patients with this disease often suffer bone lesions, anemia, renal damage, hypercalcemia and immunodepression. The chemotherapy treatment with

conventional alkylating agents such as cyclophosphamide and melfalan, optionally combined with prednisone, increases the patients mean survival which however is still of only 2-3 years in the more favourable cases and involves severe side effects, mainly on blood crasis (leukopenia and plastocytopenia).

It has now been found that calcium trifluoroacetate has surprising cytotoxic activity not only on solid tumors cell lines, but also on multiple myeloma, chronic and acute myeloid leukemia human cell lines, while being devoid of toxicity on marrow mononuclear cells (LD-MNC) and $CD34^+$ stem cells of bone marrow from healthy volunteers. This selectivity towards myeloma and leukemia cells is surprising and induces markedly more favourable therapeutic index than known alkylating agents, which are cytotoxic also on non neoplastic cells.

The activity of calcium trifluoroacetate was evaluated *in vitro*, as reported in the following examples, on collection cell lines (ATCC, National Cancer Institute, DSMZ), widely used in cytotoxicity studies. *In vivo* preliminary evidence in dogs is also available. Multiple myeloma is in fact a disease also diffused in animals, particularly in dogs, with characteristics superimposable to the human disease. The therapeutical activity in dogs is therefore highly predictive for the therapeutical activity in human clinics.

For the envisaged therapeutical uses, calcium trifluoroacetate will be administered through the parenteral route, in particular intramuscularly, subcutaneously or intravenously, at dosages ranging from 20 to 200 mg/kg/day, preferably from 20 to 100 mg/kg/day intravenously. The treatment, thanks to its poor toxicity, can be protracted for the time necessary to obtain the improvement or resolution of the pathologic condition.

Calcium trifluoroacetate, if desired, can be administered in combination with other therapeutical agents already used in chemotherapy protocols for multiple myeloma.

In addition to multiple myeloma, the plasma cell neoplasias that can be treated with calcium trifluoroacetate comprise macroglobulinemia, systemic primitive amyloidosis and heavy chain diseases.

As mentioned above, calcium trifluoroacetate can also be used in the veterinary field, in particular for the treatment of multiple myeloma in dogs. For this purpose, the invention yields veterinary compositions comprising calcium trifluoroacetate as the active ingredient in admixture with a suitable carrier for the parenteral administration, such as sterile apyrogenic water or physiological solution for the intravenous administration.

The invention is illustrated in greater detail by the following example.

EXAMPLE - Effect of $Ca(CF_3COO)_2$ on multiple myeloma, chronic and acute myeloid leukemia human cell lines and human marrow stem cells

Cytotoxicity of $Ca(CF_3COO)_2$ was evaluated on ten multiple myeloma human cell lines (RPMI8226, IM9, SULTAN, ARH77, KMS12, KMS26, KMS34, H929 and U266), two chronic myeloid leukemia human cell lines (K562 and AR230), one acute myeloid leukemia human cell line (KG1a), medullary mononuclear cells (LD-MNC) and $CD34^+$ human bone marrow stem cells.

A cytotoxicity test based on the cleavage of tetrazolium salts by mitochondrial dehydrogenase in viable cells (WST-1) was used.

All of the multiple myeloma cell lines and human marrow cells were cultured in Iscove's Modified Dulbecco medium with 10% bovine calf serum (FBS): chronic and acute myeloid leukemia cells were cultured in RPMI 1640 medium with 10% FBS. Bone marrow samples from 2 healthy donors were separated on a Ficoll density gradient. Part of the separated cells (LD-MNC) in a case were incubated with immunomagnetic particles (Miltenyi Biotec)

conjugated with the antibody anti-CD34 antigene and afterwards were separated on immunomagnetic columns.

10,000 cells per 96 wells were plated in 100 µl of Iscove's Modified Dulbecco culture medium with 10% foetal serum. 20 µl of $Ca(CF_3COO)_2$ at the concentrations of 100, 50, 37,5, 25, 15, 12,5, 10 and 6,25 mg/ml were then added. After incubation at 37°C and 5% CO_2 under humid atmosphere overnight, 10 µl/well of WST-1 was added and after 4 hours at 37°C and 5% CO_2 under humid atmosphere, the plate was read with a 1420 VICTOR multilabel counter, EG&G Wallac, at 560 nm and 690 nm. Each sample was plated in triplicate and toxicity was evaluated as the ratio of the mean absorbance value of three triplicates treated with $Ca(CF_3COO)_2$ scalar doses to the mean absorbance value of three untreated control triplicates:

$$1 - \frac{\text{Treated cells absorbance}}{\text{Untreated cells (control) absorbance}} \times 100 = \text{\% dead cells}$$

$Ca(CF_3COO)_2$ turned out particularly toxic also at lower doses on chronic myeloid leukemia lines (K562 and AR230).

Acute myeloid leukemia line KG1a is sensitive to $Ca(CF_3COO)_2$ only at higher doses.

All multiple myeloma cell lines, with the exception of KMS27, are sensitive to $Ca(CF_3COO)_2$: in particular, cell lines with slower growth (H929 and U266) appeared less sensitive to low doses of $Ca(CF_3COO)_2$ compared with those with more rapid growth (RPMI8226, IM9, SUTAN, ARH77, KMS12, KMS26, KMS34).

$Ca(CF_3COO)_2$ is not toxic for human LD-MNC from healthy donors also at higher doses, whereas stem cells $CD34^+$ are slightly sensitive to $Ca(CF_3COO)_2$.

Results are reported in the following Table.

Conc.	LD-MNC	CD34+	AR230	K562	KG1a	RPMI8826	IM9
125	0	0	11	29	2	12	0
200	0.4	16	51	63	0	38	17
250	0.4	10	40	58	0	33	9
300	0.3	21	44	65	0	34	14
500	0.2	12	58	52	0	43	32
750	0.2	9	69	63	6	53	35
1000	0.1	24	73	64	11	61	50
2000	1	18	78	72	40	98	100

Conc.	SULTAN	ARH77	KMS12	KMS26	KMS27	KMS34	H929	U266
125	0	0	0	0	0	20	0	0
200	1	2	39	0	4	20	0	0
250	3	10	53	5	0	1	0	0
300	10	12	52	33	0	6	0	0
500	11	32	79	49	0	7	0	0
750	20	19	75	32	0	36	18	3
1000	32	31	80	33	0	49	68	39
2000	69	90	100	100	0	100	100	100

CLAIMS

1. The use of calcium trifluoroacetate for the preparation of a medicament for the treatment of neoplasias and plasma cell dyscrasias.

2. The use as claimed in claim 1 for the treatment of multiple myeloma.

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/006031 A (PHARMAPRODUCTS UK LIMITED; BARTORELLI, ALBERTO) 23 January 2003 (2003-01-23) page 1, line 1 - line 15 page 2, line 2 - line 10 page 2, line 17 - line 18; claims 1-3	1,2
E	WO 2005/097084 A (PHARMAPRODUCTS UK LTD 'GB!; GOBBI MARIA ROSA 'IT!) 20 October 2005 (2005-10-20) page 2, line 15 - line 16	1,2

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2005097084 A	20-10-2005	NONE	

US 20070260088A1

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(54) CALCIUM TRIFLUOROACETATE FOR PREPARING ANTIANGIOGENETIC MEDICAMENTS

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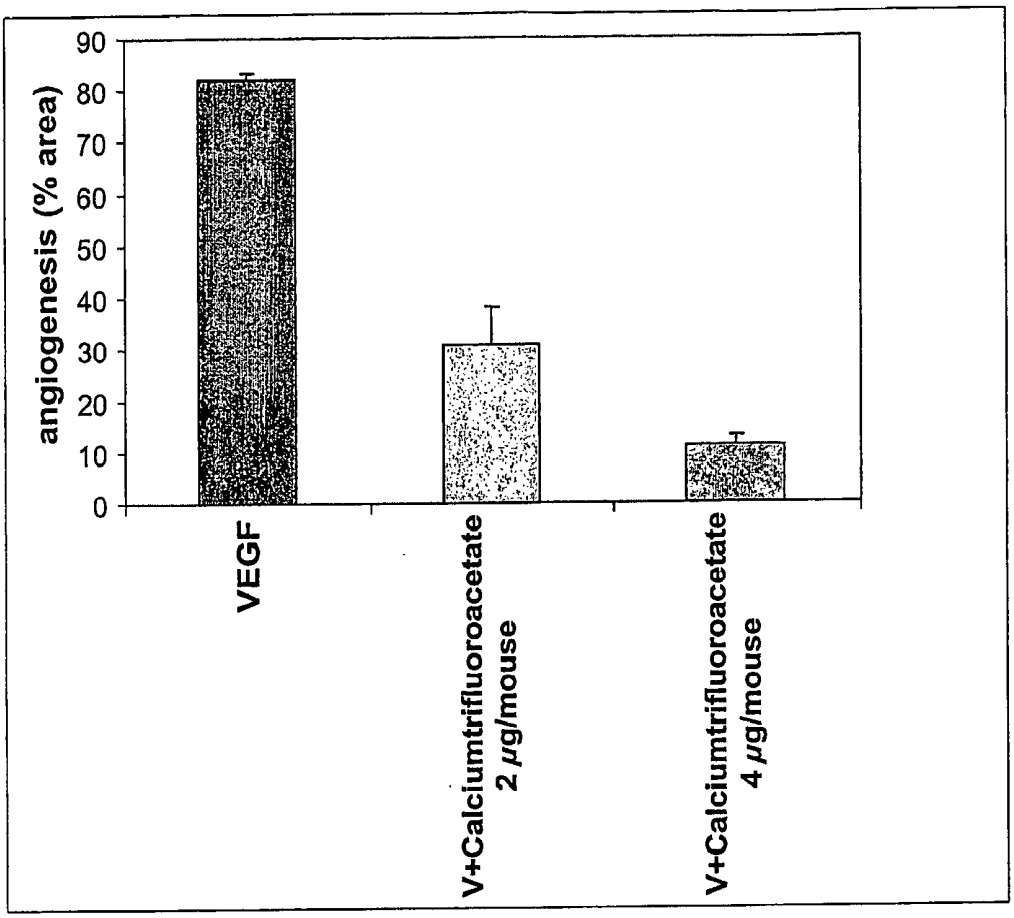
(57) **ABSTRACT**

Calcium trifluoroacetate, known to have cytotoxic activity, is endowed With of antiangiogenetic activity and can be used for the therapy of atherosclerotic plaque, rheumatoid arthritis, psoriasis, diabetic retinopathy, rosacea, cheloids, metastasis.



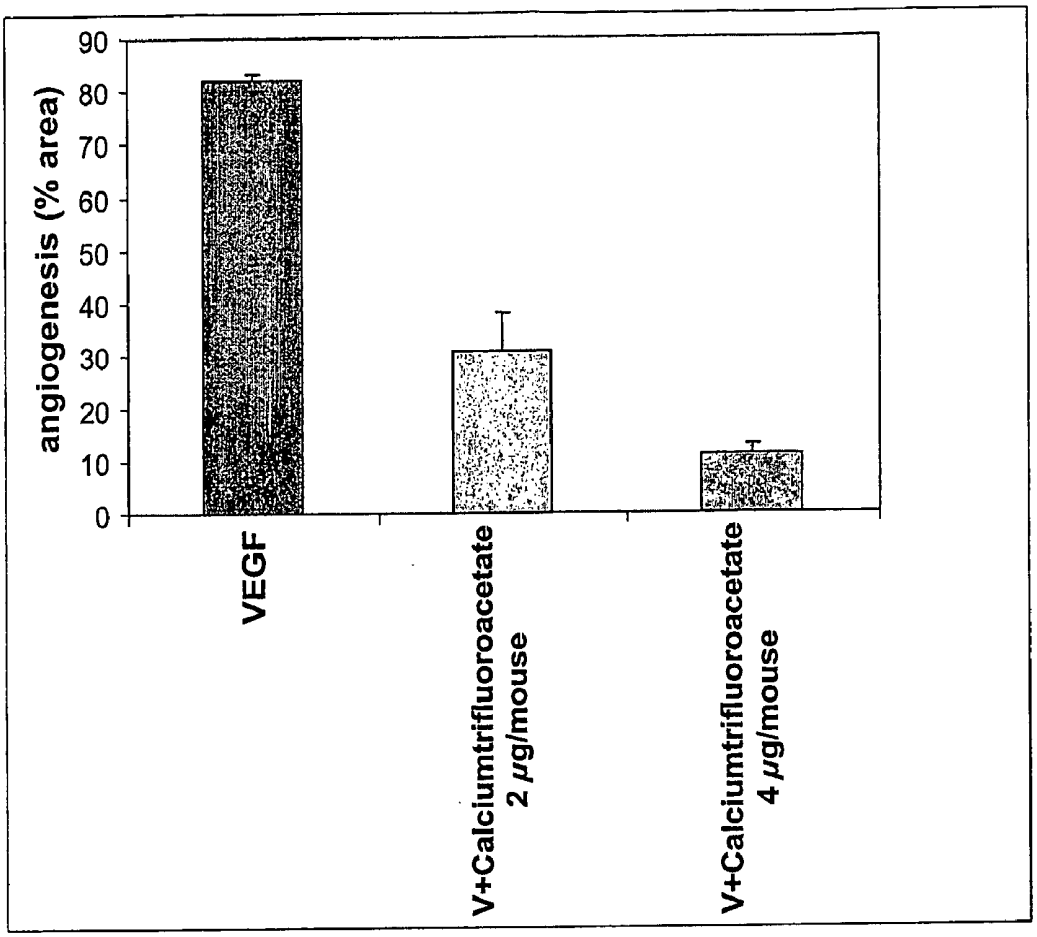

angiogenesis (% area)
90
80
70
60
50
40
30
20
10
0
VEGF
V+Calciumtrifluoroacetate 2 μg/mouse
V+Calciumtrifluoroacetate 4 μg/mouse

Figure 1

CALCIUM TRIFLUOROACETATE FOR PREPARING ANTIANGIOGENETIC MEDICAMENTS

[0001] The present invention relates to the use of calcium trifluoroacetate for the preparation of medicaments with antiangiogenetic effect.

TECHNOLOGICAL BACKGROUND

[0002] Angiogenesis is a process leading to formation of new vessels, connected with blood circulation, through activation of vascular endothelial cells which are part of pre-existing capillaries and venules. In response to angiogenetic stimuli, the endothelial cells migrate into the perivascular space, where they proliferate and form the novel vessels.

[0003] In the adult, endothelial cells show very slow turnover, and angiogenesis is restricted to physiological conditions such as wound reparation and cyclic processes of the female reproductive system (ovulation, menstruation, implant and pregnancy). Physiological angiogenesis is strictly controlled, activated for short periods, only as far as tissue metabolic requests are concerned, then promptly inhibited.

[0004] Angiogenesis is also the "common denominator" of a number of pathological conditions in humans. It is in fact involved in the vascularization of the atherosclerotic plaque, promoting its instability and formation of thrombi, in the tissue damage deriving from rheumatoid arthritis and psoriasis, in the development of diabetic retinopathy, as well as in the development of solid tumors. In particular, it is established that a tumor cannot grow beyond a few millimetres in the absence of vascularization. Moreover, tumour angiogenesis promotes invasivity and metastatic diffusion of tumours.

[0005] EP 1 423 131 discloses that calcium trifluoroacetate and related calcium salts inhibit tumor growth in animal experimental models and exert in vitro cytotoxic activity on solid tumor cells.

DISCLOSURE OF THE INVENTION

[0006] It has now been found that calcium trifluoroacetate and related salts disclosed in EP 1 423 131, in addition to cytotoxic and antitumor activity on solid tumors, have surprisingly marked antiangiogenetic activity.

[0007] The effect of calcium trifluoroacetate in angiogenesis was evidenced in a murine model of angiogenesis induced by a subcutaneous implant of Matrigel. Matrigel is liquid at 4° C. and solid at 37° C., so that a pro-angiogenetic stimulus can be mixed with Matrigel and inoculated. In this protocol, the selected angiogenetic factor was endothelial growth factor (VEGF), added to the Matrigel together with 16 U/ml heparin, as described in literature (Bussolati B, Altered angiogenesis and survival in human tumor-derived endothelial cells. FASEB J. 2003; Bussolati B, Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. Am J Pathol. 2001).

[0008] Calcium trifluoroacetate was administered intraperitoneally 30 minutes before Matrigel, then on alternate days (day 2, 4, 6), at a dose of 2 or 4 μg/mouse (0.2 mg/Kg). After 7 days, the animals were killed and the Matrigel plugs were removed for histological examination. Angiogenesis was evaluated by computer assisted morphometric analysis of the percentage of area covered by vessels.

[0009] FIG. **1** shows the reduction of angiogenesis in mice treated with VEGF+calcium trifluoroacetate on alternate days at a dosage of 2 μg/mouse (n=4) and of 4 μg/mouse (n=8) compared with VEGF alone (n=8). $P<0.001$.

[0010] A marked angiogenesis was observed in the Matrigel plugs containing VEGF (40 ng/ml), as expected. The effect of VEGF was dramatically reduced in mice treated with calcium trifluoroacetate. This strong antiangiogenic effect could already be observed macroscopically and confirmed histologically. Morphometric analysis showed a reduction in angiogenesis above 40% with a dosage of 2 μg/mouse of calcium trifluoroacetate, and of 80% with a dosage of 4 μg/mouse.

[0011] Calcium trifluoroacetate has evident inhibitory action on endothelium proliferation. Moreover, calcium trifluoroacetate is able to block, at least partly, the effect of the usual endothelial growth factors on calcium entrance into the single endothelial cells.

[0012] These data suggest that calcium trifluoroacetate can inhibit the response of endothelial cells to stimulation by growth factors, likely acting on the message transmission through the calcium channels.

[0013] It is not intended, however, that the present invention be limited to this or any other mechanism of action.

[0014] Calcium trifluoroacetate can therefore be successfully used, in the form of suitable pharmaceutical compositions, for the treatment of pathologies in which the inhibition of angiogenesis is necessary or appropriate. Examples of said pathologies, as mentioned above, include atherosclerotic plaque, rheumatoid arthritis, psoriasis, diabetic retinopathy, rosacea, cheloids and other diseases or cutaneous inaesthetisms due to neovascolarization. Furthermore, calcium trifluoroacetate can be useful as antimetastatic agent to prevent the development of solid tumors.

[0015] For the envisaged therapeutical uses, calcium trifluoroacetate will be administered through the oral, topical, transdermal or parenteral (subcutaneous, intramuscular or intravenous) routes at dosages similar to those disclosed in EP 1 423 131, for example ranging form 20 to 100 mg/kg for the oral and parenteral routes, or at concentrations ranging from 2.5- to 10% for the topical formulations.

[0016] Calcium trifluoroacetate will optionally be combined with other active ingredients having complementary or anyway useful activity.

1. The use of calcium trifluoroacetate for the preparation of medicaments with antiangiogenetic effect.

2. The use as claimed in claim 1 for the preparation of medicaments for the treatment of atherosclerotic plaque, rheumatoid arthritis, psoriasis, diabetic retinopathy, rosacea, cheloids.

3. The use as claimed in claim 1 for the preparation of medicaments with activity on the growth of tumors mediated via angiogenesis.

* * * * *

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(54) CALCIUM SALTS WITH CYTOTOXIC ACTIVITY

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(52) **U.S. Cl.** **514/557**

(57) **ABSTRACT**

The present invention relates to the use of calcium salts, in particular calcium trifluoroacetate, to prepare medicaments with cytotoxic and antitumoral activity.

CALCIUM SALTS WITH CYTOTOXIC ACTIVITY

[0001] The present invention relates to the use of calcium salts, in particular calcium trifluoroacetate, to prepare medicaments with cytotoxic and antitumoral activity.

[0002] Calcium salts, in particular calcium gluconate, have been used for some time in human medicine to treat osteoporosis and all conditions in which an increase in the blood calcium level is indicated. The use of calcium salts to treat tumoral and hyperproliferative disorders has not been described to date; however, possible chemoprotective effects have been postulated for some calcium salts, in particular calcium glucarate.

[0003] It has now been found that some calcium salts, in particular calcium trifluoroacetate, have a surprising cytotoxic activity which is selective for tumour cells. This activity has been demonstrated on cell line cultures of human and murine tumours of various origins (colon, lung, pancreas, breast, prostate, liver, stomach and ovaries) such as HT 29, Kato, C-26, H G C 27 and PA-1 cells.

[0004] Calcium trifluoroacetate, prepared with conventional methods from trifluoroacetic acid and calcium oxide or hydroxide, and added to each well (~10,000 cells) in an aqueous solution in quantities ranging between 0.001 and 1.5 mg, causes the death of 90-95% of tumour cells or more. Under the same conditions, normal human cell lines proved almost insensitive or at least considerably less sensitive to the cytotoxic effect attributable to calcium trifluoroacetate. Similar effects have been obtained with other calcium salts of pharmaceutically acceptable acids, such as acetate, nitrate, chloride and the like, though to a lesser extent. However, trifluoroacetate seems to be preferable in view of its favourable toxicological properties.

[0005] For use in the treatment of tumours, calcium trifluoroacetate or other calcium salts will preferably be administered parenterally, for example by the intramuscular, intravenous or subcutaneous route, at doses ranging between 0.1 and 10 mg/kg/day. For this purpose, the calcium salt will be suitably formulated in the form of sterile suspensions or solutions, possibly with the aid of conventional vehicles or solvents. However, other administration routes would also be possible, such as the oral route or topical infusion at the site of the tumour lesion. Calcium salts, and in particular calcium trifluoroacetate, can be used in combination with other antitumoral drugs which have complementary, synergistic or otherwise useful activity.

[0006] Other disorders in which antiproliferative activity or control of cell growth is indicated can also be treated in addition to tumours.

[0007] The results of experiments carried out with calcium trifluoroacetate are set out below.

[0008] Toxicity in Mouse and Rat

[0009] Calcium trifluoroacetate, administered at the acute dose of 200 mg/kg to Balb/c mice by the intraperitoneal route, does not produce any noteworthy toxic effects.

[0010] Four weeks' sub-chronic treatment at the dose of 100 mg/kg, again by the intraperitoneal route, did not cause any pathological effects. A similar 4-week treatment by the subcutaneous route was well tolerated at the dose of 10 mg/kg.

[0011] In Sprague Dawley rats, toxic effects were not observed after acute or sub-acute administration of 200 mg/kg of calcium trifluoroacetate by the intravenous route.

[0012] In Vitro Cytotoxicity

[0013] The cytotoxicity of calcium trifluoroacetate has been determined on 13 cell lines of malignant human tumours and 4 lines of benign tumours.

[0014] 20 μl of $Ca(CF_3COO)_2$ solutions at concentrations of 100, 50, 37.5, 25, 15, 12.5, 10 and 6.25 mg/ml are added to 96-well plates, each containing 10,000 cells/well in 100 μl of culture medium.

[0015] They are left overnight at 37° C., after which 40 μl of MTT (dimethylthiazole-0.2 g/100 ml PBS) is added, and they are left for 4 hours at 37° C.

[0016] After aspiration, 100 μl of SDS is added and they are left at 37° C. for 1 hour. The plate is read at 540 nm and 690 nm.

[0017] The toxicity of the sample is evaluated as the ratio between the absorbance value in the well with the treated cells and the absorbance value in the well with the control cells.

$$1-\frac{\text{Absorbance of cells + sample}}{\text{Absorbance of control cells}}\times 100 = \%\ \text{dead cells}$$

[0018] The MTT test is a quantitative calorimetric method that measures only live cells, because the mitochondrial enzymes of live cells are able to transform MIT tetrazolium salts into insoluble formazan.

[0019] The results are summarised in Table 1.

TABLE 1

Biological activity of $Ca(CF_3COO)_2$ in vitro

	Concentration of $Ca(CF_3COO)_2$ in vitro (μg per well)							
CELL LINES	2000	1000	750	500	300	250	200	125
HEP G-2	100	83	75	68	37	29	18	17
MG 63	100	93	74	60	53	45	32	27
HGC-27	100	94	94	85	76	54	33	24
A-172	100	83	70	60	58	47	32	25
PSN-1	100	95	94	94	92	92	91	86
C-26	100	88	88	87	80	70	55	47
DU-145	85	78	77	67	65	50	33	22
KATO	94	84	70	59	52	35	29	7
CHANG	***41***	***33***	***33***	***27***	***25***	***19***	***16***	***11***
NCTC	***36***	***34***	***32***	***30***	***25***	***22***	***19***	***19***
MCF-7	42	41	25	24	23	22	7	7
T47D	48	44	41	37	34	31	30	28
FTC-238	82	77	75	63	35	25	20	13
W-138	***82***	***76***	***67***	***52***	***48***	***34***	***31***	***23***
CCD-8LU	***55***	***42***	***39***	***32***	***15***	***5***	***4***	***0***
PA-1	100	100	100	98	97	96	70	52
HT-29	95	89	76	69	60	50	47	32

[0020] Normal Type: Malignant Cell Lines

[0021] Bold Italic Type: Benign Cell Lines

RESULTS

[0022] Malignant human cell lines of different histological types and origins demonstrate that the cytotoxic response to calcium trifluoroacetate differs between the various lines but, depending on dose, always reaches very high cell death

levels: 8/14 reached 100% mortality, and 6/14 reached values ranging between 80% and 90%.

[0023] These results were obtained after 24 hours' incubation. Checks were performed after 6 hours in view of the possibly rapid turnover of the salt due to its very low molecular weight.

[0024] The data collected demonstrate that antitumoral activity takes place in a short time at low concentrations. If the concentration is increased, a non-specific toxicity of the salt develops which explains its low toxicity towards normal cells at high doses.

[0025] Experimental Murine Tumours

[0026] Balb/C mice were inoculated with 25,000 cells/mouse of C26 (murine colon adenocarcinoma).

[0027] Four days after the inoculation, the animals were treated subcutaneously with 5 mg/kg of calcium trifluoroacetate. The control group received saline only. The treatment was given every day. The tumour diameter was measured with a caliper gauge. On the 20th day after the inoculation the animals were killed and the tumours removed and weighed. The results are set out in Table 2. The mean weight and mean size of the tumours in the control mice were four times greater than those of the treated group. The data relating to the onset of tumours were also significant: 100% for the controls as against 40% for the treated mice.

TABLE 2

IN VIVO TREATMENT OF BALB/C MICE WITH MURINE TUMOR C 26

	SIZE OF TUMOR (cm)				WEIGHT OF TUMOR (g)
	13th day	15th day	18th day	20th day	20th day
CONTROL GROUP Saline					
1	0.5	0.9	1.1	1.4	0.695
2	0.5	0.9	1.4	1.7	1.308
3	0.5	0.9	1.1	1.2	0.652
4	N/A	0.7	1.2	1.2	0.545
5	N/A	0.8	1.4	1.7	0.821
MEAN, S.D	0.3 ± 0.27	0.84 ± 0.089	1.24 ± 0.15	1.44 ± 0.25	0.8042 ± 0.298
Treated group 5 mg/kg $Ca(CF_3COO)_2$					
1	N/A	N/A	N/A	N/A	0
2	N/A	N/A	N/A	N/A	0.01
3	N/A	N/A	N/A	N/A	0
4	N/A	0.3	0.8	0.9	0.438
5	0.6	0.8	1.1	1.2	0.7
MEAN, S.D.	0.12 ± 0.268	0.22 ± 0.349	0.38 ± 0.53	0.42 ± 0.58	0.2296 ± 0.32

[0028] Treatment of Spontaneously Occurring Canine Tumours

[0029] Experiments carried out on spontaneously occurring tumours in domestic animals, especially dogs, are considered a reliable model which is predictive of activity in man (D. M Vail et al., *Spontaneously occurring tumors in companion animals as models for drug development*. Anti-*cancer drug development guide: preclinical screening, clinical trials and approval. Edited by B. Teicher Humana Press Inc., Totowa, N.J.). Tumours in pets are more similar than rodent tumours to human tumours in terms of size, cell kinetics and behaviour.*

[0030] Six dogs suffering from mammary tumours and one dog with a malignant lymphoma were treated. The treatment was given intravenously at doses ranging between 100 and 10 mg/kg/day of calcium trifluoroacetate. Despite some variability among animals, depending on the onset of side effects, especially vomiting, the highest dose (100 mg/kg) was administered initially for one to three days, followed by the lowest dose (10 mg/kg) for a few days. The highest dose was then reintroduced, but only given twice a week. Some animals were always treated with the highest dose, but at a lower frequency (9-10 administrations in approx. 20 days).

[0031] Although the results are of a preliminary nature and were obtained on a limited number of animals, they demonstrate the considerable antitumoral efficacy of calcium trifluoroacetate. In fact, the experiments demonstrated:

[0032] 1. A reduction in the main tumour mass of 20% to over 50% of its initial volume. This reduction was evident after the first few administrations, and peaked at the end of the treatment cycle used so far (generally 3 weeks).

[0033] 2. The reduced volume was generally accompanied by other phenomena involving the tumour mass such as colliquative necrosis, loss of contact with the surrounding tissues (adhesions) and finally, encapsulation or rupture of the wall, with leakage of necrotic material to the exterior.

[0034] 3. Arrested development of metastases and, in some cases, necrosis, and their disappearance (resorption or calcification).

[0035] 4. $Ca(CF_3COO)_2$ proved totally safe even at daily intravenous doses of 100 mg/kg; the only side effect, observed in two animals, was vomiting immediately after administration of the drug, and a slight increase in temperature (not exceeding 1° C.).

[0036] The toxicity tests, performed by monitoring the liver and kidney function parameters and coagulation times with particular care, did not indicate any pathological modification of those parameters in the treated animals.

[0037] Clinical Cases

[0038] Calcium trifluoroacetate was administered subcutaneously at doses ranging between 10 and 200 mg a day to five patients aged 56 to 63 suffering from cancer of the breast (one case), colon (three cases) and womb (one case), at advanced state of disease and with diffuse metastasis. Except for one case of colon cancer which ended in the death of the patient due to the highly advanced state of the disease (all cases were treated on the basis of a compassionate protocol for obvious ethical reasons), the therapeutic response was surprising, both in objective terms (approx. 10 to 40% reduction in tumour mass, reduction or disappearance of ascites and metastatic nodes, and a reduction in tumour markers such as CA19.9 and alpha-fetoprotein) and in subjective terms (improved mood, reduction and disappearance of pain, and return to working and social life). Even in the case of the patient who died, a reduction in the tumour mass was observed, but the patient died due to aggravation of neoplastic cachexia and cardiorespiratory complications.

1. The use of calcium salts for the preparation of cytotoxic medicaments.

2. The use as claimed in claim 1, in which the calcium salt is calcium trifluoroacetate.

3. Pharmaceutical compositions containing calcium trifluoroacetate as active ingredient, mixed with a suitable vehicle.

* * * * *

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CALCIUM SALTS FOR THE TREATMENT OF PSORIASIS, DERMATITIS AND DANDRUFF

(57) Abstract: The use of a calcium salt selected from trifluoroacetate, gluconate, orotate, chloride, perchlorate, acetate, trichloroacetate and carbonate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff and similar pathologies.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/003402

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/19 A61K31/191 A61K31/513 A61K33/06 A61P17/00
A61P17/06 A61P17/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, BIOSIS, EMBASE, SCISEARCH, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/006031 A (PHARMAPRODUCTS UK LIMITED; BARTORELLI, ALBERTO) 23 January 2003 (2003-01-23) cited in the application	3,4
Y	the whole document	1,2,5
Y	FR 2 726 187 A (THOREL JEAN NOEL) 3 May 1996 (1996-05-03) the whole document	1,2,5
Y	WO 96/19228 A (COSMEDERM TECHNOLOGIES; HAHN, GARY, SCOTT; THUESON, DAVID, OREL) 27 June 1996 (1996-06-27) abstract; claims 62,67,68,95-97	1,2,5

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
21 June 2005	14 10 2005

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2005/003402

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 (partially), 2-5

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1 (partially),2-5

   Use of calcium trifluoroacetate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff. Topical composition comprising calcium trifluoroacetate

   ---

2. claim: 1 (partially)

   Use of calcium gluconate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff.

   ---

3. claim: 1 (partially)

   Use of calcium orotate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff.

   ---

4. claim: 1 (partially)

   Use of calcium chloride for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff.

   ---

5. claim: 1 (partially)

   Use of calcium perchlorate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff.

   ---

6. claim: 1 (partially)

   Use of calcium acetate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff.

   ---

7. claim: 1 (partially)

   Use of calcium trichloroacetate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff.

   ---

8. claim: 1 (partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Use of calcium carbonate for the preparation of a topical medicament for the treatment of psoriasis, erythematous dermatitis, seborrheic dermatitis, dandruff.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2005/003402

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03006031 A	23-01-2003	CA 2455710 A1	23-01-2003
		EP 1423131 A1	02-06-2004
		IT MI20011495 A1	13-01-2003
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		EP 0801570 A1	22-10-1997
		JP 10511360 T	04-11-1998

Indução de resistência à intoxicação por *Palicourea aeneofusca* (Rubiaceae) mediante administração de doses sucessivas não tóxicas[1]

Murilo Duarte de Oliveira[2], Franklin Riet-Correa[3], Fabrício K.L. Carvalho[3], Genilson B. Silva[2] , Walkleber S. Pereira[2] e Rosane M.T. Medeiros[3]*

ABSTRACT.- Oliveira M.D., Riet-Correa F., Carvalho F.K.L., Silva G.B., Pereira W.S. & Medeiros R. M.T. 2013. [**Induction of resistance to *Palicourea aeneofusca* (Rubiaceae) poisoning by the continuous administration of non-toxic doses.**] Indução de resistência à intoxicação por *Palicourea aeneofusca* (Rubiaceae) mediante administração de doses sucessivas não tóxicas. *Pesquisa Veterinária Brasileira 33(6):731-734.* Hospital Veterinário, CSTR, Universidade Federal de Campina Grande, Patos, PB 58700-000, Brazil. E-mail: rmtmed@uol.com.br

Palicourea aeneofusca (Müll. Arg.) Standl. is a toxic plant which contains sodium monofluoroacetate (MFA). With the objective to investigate if repeated non-toxic doses of *P. aeneofusca* induce resistance to the intoxication by this plant, 12 goats were distributed in two similar groups. In Group 1, resistance was induced by the administration of the dry plant, during four alternate periods: 0.02g/kg during 5 days, 0.02g/kg during 5 days, 0.03g/kg during 5 days, and 0.03g/kg during 5 days. Between the first and second period of administration and between the second and the third period, the goats did not ingest *P. aeneofusca* for 10 days. Between the third and the fourth administration period the goats did not ingest the plant during 15 days. One goat died suddenly during the third administration period when was ingesting 0.03g/kg. The goats from Group 2 were not adapted to the consumption of *P. aeneofusca.* Fifteen days after the end of the adaptation period in Group 1, both groups ingested dry *P. aeneofusca* in the daily dose of 0.03g/kg during 19 days. From day 20 the daily dose was increased to 0.04g/kg, which was ingested for 12 days. The goats that showed clinical signs were removed from the experiment immediately after the observation of first signs. One goat from Group 2 showed clinical signs of poisoning and died on the 12th day of ingestion, and two showed clinical signs on day 24th; one recovered and the other died. At the end of the 31 days administration period, a new group (Group 3) with three goats was introduced in the experiment to investigate if the goats that did not become poisoned in Group 2 had acquired resistance. The three goats from Group 1, five goats from Group 1, and three from Group 2 started to ingest a daily dose of 0.06g/kg of dry *P. aeneofusca*. On the third day of ingestion the three goats from Group 3 showed clinical signs. Two died suddenly and another recovered 10 days after the end of ingestion. All goats of Groups 1 and 2 ingested 0.06g/kg/day during nine days without showing clinical signs. These results demonstrated that non-toxic repeated doses of *P. aeneofusca* increase significantly the resistance to the poisoning, and that this technique possibly could be used to control the poisoning by *P. aeneofusca* or other toxic *Palicourea* species. The results of previous research work suggest that resistance is due to the proliferation of MFA degrading bacteria in the rumen.

INDEX TERMS: Poisonous plants, acute cardiac insufficiency, sodium monofluoroacetate, *Palicourea aeneofusca, Palicourea* spp., resistance to plant poisoning, goats.

RESUMO.- Com o objetivo de comprovar se doses não tóxicas repetidas de *Palicourea aeneofusca* (Müll. Arg.) Standl. criam resistência à intoxicação, 12 caprinos foram distribuídos aleatoriamente em dois grupos experimentais de seis animais cada. No Grupo 1 foi induzida resistência mediante a administração, durante quatro períodos alternados, de 0,02g/kg das folhas dessecadas de *P. aeneofusca* durante

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Aceito para publicação em 27 de abril de 2013.

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5 dias, 0,02g/kg durante 5 dias, 0,03g/kg durante 5 dias e 0,03g/kg por mais 5 dias. Entre o primeiro e o segundo período de administração e entre o segundo e o terceiro período os animais não receberam planta por 10 dias consecutivos e entre o terceiro e quarto período de administração os animais permaneceram 15 dias sem ingerir a planta. Um caprino morreu subitamente quando estava recebendo 0,03 g/kg da planta, no terceiro período de administração. O Grupo 2 não foi adaptado ao consumo de *P. aeneofusca*. Quinze dias após a adaptação ao consumo de *P. aeneofusca* do Grupo 1, os dois grupos receberam *P. aeneofusca* na dose diária de 0,03g/kg durante 19 dias. A partir do 20º dia de administração continuada a dose diária de *P. aeneofusca* foi aumentada para 0,04g/kg. Esta dose foi administrada por mais 12 dias. Os animais que mostraram sinais clínicos foram retirados do experimento imediatamente após a observação dos primeiros sinais. Um caprino do Grupo 2 apresentou sinais clínicos de intoxicação e morreu no 12º dia de administração e dois apresentaram sinais clínicos no 24º dia; um se recuperou e outro morreu. Após finalizada esta fase do experimento e para comprovar se os caprinos que não tinham adoecido no Grupo 2 tinham também adquirido resistência, foi introduzido outro grupo com três caprinos. Esses três caprinos (Grupo 3), os cinco caprinos do Grupo 1 e os três sobreviventes do Grupo 2, ingeriram uma dose diária de 0,06g/kg. Os três caprinos do Grupo 3 adoeceram no terceiro dia após o início da ingestão, dois morreram em forma hiperaguda e o outro recuperou-se após 10 dias. Todos os caprinos dos Grupos 1 e 2 ingeriram *P. aeneofusca* na dose de 0,06g/kg/dia durante nove dias sem apresentar nenhum sinal clínico. Os resultados deste trabalho demonstram que a administração de doses não tóxicas repetidas de *P. aeneofusca* aumentam significativamente á resistência à intoxicação e que esta técnica poderia ser utilizada para o controle da intoxicação por *P. aeneofusca* e outras espécies de *Palicourea* com similar toxicidade. Os resultados de pesquisas anteriormente realizados sugerem que a resistência à intoxicação por plantas que contêm MFA é devida a proliferação de bactérias que degradam MFA no rúmen.

TERMOS DE INDEXAÇÃO: Plantas tóxicas, insuficiência cardíaca aguda, monofluoroacetato de sódio, *Palicourea aeneofusca*, *Palicourea* spp., resistência à intoxicação por planta, caprinos.

INTRODUÇÃO

No Brasil existem pelo menos 13 plantas que causam insuficiência cardíaca aguda em ruminantes. O monofluoroacetato de sódio (MFA) foi determinado como o composto tóxico encontrado em *Palicourea marcgravii* (Oliveira 1963), *Palicourea aeneofusca* (Lee et al. 2012), *Amorimia* (*Mascagnia*) *rigida* (Cunha et al. 2012, Lee et al. 2012), *Amorimia septentrionalis*, *Amorimia* (*Mascagnia*) *pubiflora* (Lee et al. 2012) e *Tanaecium bilabiatum* (*Arrabidaea bilabiata*) (Krebs et al. 1994).

Na Paraíba são conhecidas duas plantas que contêm MFA e causam insuficiência cardiaca aguda, *A. septentrionalis*, anteriormente identificada como *Amorimia* (*Mascagnia*) *rigida* e *P. aeneofusca* (Vasconcelos et al. 2008).

Experimentalmente *P. aeneofusca* causou morte em caprinos (Passos 1983) e bovinos (Tokarnia & Döbereiner 1982) nas doses de 0,6 e 0,75g/kg de peso corporal (g/kg), respectivamente. Os sinais clínicos são caracterizados por taquicardia, jugular ingurgitada com pulso venoso positivo, taquipnéia com respiração irregular ou abdominal, andar cambaleante, quedas e decúbito esternal evoluindo para decúbito lateral, seguidos de morte. Na necropsia geralmente não há lesões macroscópicas de significância. Na histologia a lesão mais frequente, observada nos rins em aproximadamente 50% dos animais intoxicados, é a severa vacuolização e picnose das células epiteliais dos túbulos contornados distais (Tokarnia & Döbereiner 1982, Passos 1983).

Em trabalhos realizados pelo nosso grupo de pesquisa foi observado que animais que recebem diariamente doses não tóxicas de *A. septentrionalis* apresentam sinais clínicos, mas geralmente se recuperam se a administração é suspensa imediatamente após a observação dos primeiros sinais (Duarte et al. 2013). Após o consumo da planta é necessário um período para que o MFA seja detoxificado pelo organismo que de acordo com Tokarnia et al. (2012) varia entre oito a 14 dias para *A. rigida* e pode ser de dois a cinco dias para *P. marcgravii*.

Uma forma de controlar as intoxicações por plantas que contem MFA é o isolamento de bactérias que hidrolisam MFA de forma a serem introduzidas no rúmen de animais susceptíveis para lhes conferir resistência. Dentro dessa linha de pesquisa foram isoladas, na Paraíba, bactérias que hidrolisam MFA do solo, de plantas e do rúmen de caprinos que nunca tinham ingerido plantas que contêm MFA (Camboim et al. 2012a,b). Foi demonstrado, também, que bactérias que hidrolisam MFA são muito mais frequentes do que se pensava anteriormente (Camboim 2012) e que muito provavelmente a administração de plantas que contem MFA induz a multiplicação dessas bactérias que ocorrem normalmente no rúmen, induzindo resistência à intoxicação. Foi demonstrado que a administração diária e contínua de doses não tóxicas de *A. septentrionalis* administradas em períodos alternados, que permitem a detoxificação do MFA, induz resistência contra a intoxicação por esta planta e que essa resistência pode ser transmitida de um animal resistente para um animal susceptível mediante a transfaunação de conteúdo ruminal (Duarte et al. 2013).

O presente trabalho teve como objetivo determinar se é possível induzir resistência à intoxicação por *P. aeneofusca* mediante a administração de doses diárias não tóxicas da mesma por períodos alternados.

MATERIAL E MÉTODOS

Para a realização do experimento, folhas de *Palicourea aeneofusca* foram coletadas no Município de Areia, Paraíba, Brasil ($07^{0}04'02''S$, $37^{0}16'51''W$ e altitude de 560 metros acima do nível do mar) e desidratadas em temperatura ambiente por quatro dias. Após secas foram trituradas em moinho e guardadas em potes fechados. Amostras da planta foram enviadas Poisonous Plant Research Laboratory, em Logan, Estados Unidos, para determinação da concentração de MFA, detectando-se que a mesma continha 0,09±0,05% de MFA (Lee et al. 2012).

Quadro 1. Indução de resistência à ingestão de *Palicourea aeneofusca* administrando planta seca em doses diárias repetidas por períodos variáveis (Grupo 1) e desafio mediante administração continuada da planta (Grupos 1, 2 e 3)

Grupo	Doses e períodos de ingestão, períodos sem ingestão e caprinos que adoeceram sobre o total em cada período										
	Período de indução de resistência								Período de desafio		
G1	0,02 g/kg 5 dias	PSI* 10 dias	0,02 g/kg 5 dias	PSI 10 dias	0,03 g/kg 5 dias	PSI 15 dias	0,03 g/kg 5 dias	PSI 15 dias	0,03 g/kg 19 dias	0,04 g/kg 12 dias	0,05 g/kg 9 Dias
Doentes/total	0/6	0/6	0/6	0/6	1/6	0/5	0/5	0/5	0/5	0/5	0/5
G2	Seis caprinos que não foram adaptados								0,03 g/kg 19 dias	0,04 g/kg 12 dias	0,05 g/kg 9 dias
Doentes/total									1/6	2/5	0/3
G3	Três caprinos que não foram adaptados										0,05g/kg 9 dias
Doentes/total											3/3

* PSI = Sem ingestão.

O Experimento foi realizado na cidade de Bananeiras, Paraíba, no Laboratório de Caprinocultura do Centro de Ciências Humanas, Sociais e Agrárias (CCHSA) da Universidade Federal da Paraíba (UFPB). Inicialmente foram utilizados 12 caprinos, hígidos, das raças Alpino e Saanen pesando entre 15 e 25 kg, de ambos os sexos e distribuídos aleatoriamente em dois grupos experimentais de seis animais cada. Com a finalidade de verificar se é possível induzir resistência, os animais do Grupo 1 (Caprinos 1-6) receberam, mediante a administração diária, em períodos alternados, 0,02g/kg de *P. aeneofusca* dessecada durante 5 dias, 0,02g/kg durante 5 dias, 0,03g/kg durante 5 dias e 0,03g/kg por mais 5 dias. Entre o primeiro e o segundo período de administração e entre o segundo e o terceiro período os animais não receberam planta por 10 dias consecutivos e entre o terceiro e quarto período de administração os animais permaneceram 15 dias sem ingerir a planta (Quadro 1). Durante cada período de fornecimento da planta foram realizados exames clínicos que consistiam em verificação das frequências cardíaca e respiratória, da temperatura retal e do comportamento do animal.

O Grupo 2 (Caprinos 7-12) não foi adaptado ao consumo de *P. aeneofusca*. Quinze dias após o final da adaptação ao consumo de *P. aeneofusca* do Grupo 1, os dois grupos receberam *P. aeneofusca* na dose diária de 0,03g/kg durante 19 dias. A partir do 20° dia de administração continuada a dose diária de *P. aeneofusca* foi aumentada para 0.04g/kg. Esta dose foi administrada por mais 12 dias.

Considerando que após 31 dias de administração da planta em forma continuada, em doses crescentes, havia 5 caprinos do Grupo 1 e três do Grupo 2 que não adoeceram, foi introduzido um novo grupo de três caprinos (Caprinos 13-15) para testar se os oito animais mencionados anteriormente apresentavam resistência superior à do início do experimento. A partir do dia 32 os cinco caprinos do Grupo 1, os três do Grupo 2 e os três do Grupo 3 passaram a receber diariamente 0,06g/kg de *P. aeneofusca* seca, até o dia 40.

Durante todo o experimento, nos animais que apresentavam sinais clínicos foi suspenso o fornecimento da planta no mesmo dia que adoeceram, sendo retirados do experimento e colocados em gaiolas afastadas em local tranquilo.

Os animais que morreram foram necropsiados. Amostras de órgãos das cavidades, abdominal, torácica e sistema nervoso central foram coletadas, fixadas em formol a 10%, incluídas em parafina, cortadas a 5-6µm e coradas pela hematoxilina-eosina para exame histológico. Para a interpretação das lesões microscópicas cardíacas foram coletadas, no Abatedouro Municipal de Patos, amostras do coração de 10 caprinos normais, que foram processadas para estudo histológico em forma semelhante à dos caprinos intoxicados.

RESULTADOS

No Grupo 1, um animal (Caprino 1) morreu, sem que fossem observados sinais clínicos, durante o período de indução de resistência, no dia 34, 4 dias após o início da ingestão da dose diária de 0,03g/kg. Em consequência disso o intervalo entre as administrações, previsto inicialmente para 10 dias, foi aumentado para 15 dias. Após o período de indução de resistência nenhum animal do Grupo 1 apresentou sinais clínicos durante os 40 dias da fase de desafio mediante fornecimento de *Palicourea aeneofusca* em doses crescentes (Quadro 1).

No Grupo 2, um animal (Caprino 12) apresentou sinais clínicos e morreu no 12º de ingestão da planta. No 24º dia adoeceram outros dois caprinos (Caprinos 8 e 10) deste grupo. O Caprino 10 se recuperou em 6 dias e o Caprino 8 estava se recuperando 48 horas após o final da ingestão, quando foi solto numa pastagem e no retorno da mesma morreu após apresentar queda e movimentos de pedalagem. Nenhum outro animal do Grupo 2 adoeceu até o 40º dia (Quadro 1).

Dos animais do Grupo 3, todos adoeceram três dias após o início da ingestão de 0,06g/kg, enquanto que os três animais sobreviventes do Grupo 2 o os 5 animais do Grupo 1 receberam essa dose durante 9 dias sem apresentar sinais. Dos três animais do Grupo 3 que adoeceram os Caprinos 14 e 15 morreram em forma hiperaguda e o Caprino 13 recuperou-se após 10 dias. As doses administradas a cada grupo, os períodos de administração, os períodos nos que a planta não foi administrada e a observação de animais doentes apresentam-se no Quadro 1.

Os Caprinos 8, 12, 14 e 15 que adoeceram e morreram apresentaram clinicamente apatia, anorexia, taquicardia, ligeira perda de equilíbrio, muitas vezes com andar cambaleante e aumento progressivo da dificuldade locomotora e elevado grau de dificuldade quando iam deitar-se. A micção era frequente. Na fase agônica da doença todos os animais caiam em decúbito lateral, esticavam os membros, faziam movimentos de pedalagem e apresentavam opistótono, nistagmo e mugidos intensos. A morte ocorria de 10 a 20 minutos após o início dos sinais clínicos.

Nos Caprinos 10 e 13, que se recuperaram, os sinais eram mais brandos observando-se apatia e anorexia, permanecendo em decúbito esternal por longos períodos. A

recuperação ocorreu em três dias no Caprino 10 e em 10 dias no Caprino 13. Neste último registrou-se uma perda de peso de 6 kg.

Não foram observadas lesões macroscópicas nos cinco caprinos que morreram durante o experimento. No estudo histológico, os rins dos Caprinos 1 e 14 apresentaram vacuolização e picnose de células epiteliais de raros túbulos contornados distais. No Caprino 8, além de vacuolizados, numerosos túbulos apresentava necrose e desprendimento de células epiteliais. No Caprino 1 não foram observadas lesões cardíacas. No coração do Caprino 12 havia fibras musculares eosinofílicas e com picnose nuclear e numerosas fibras de Purkinje apresentavam-se vacuolizadas. Nos Caprinos 8, 13 e 14 havia discreta vacuolização das fibras de Purkinje, mais marcada nas fibras localizadas no miocárdio do que das localizadas no endocárdio. Estas lesões não foram observadas no coração de 10 caprinos coletados no abatedouro de Patos e utilizadas como controle.

DISCUSSÃO

Os resultados deste trabalho demonstram que a administração de doses não tóxicas repetidas de *Palicourea aeneofusca* aumentam significativamente á resistência à intoxicação. Resultados semelhantes foram obtidos por Duarte et al. (2013) com a administração de *Amorimia septentrionalis* a caprinos. O fato de três animais do Grupo 2 não terem apresentados sinais clínicos após ingerir a planta durante 12 dias na dose diária de 0,03g/kg e outros 20 dias na dose de 0,04g/kg deveu-se, provavelmente, a que adquiriram resistência durante o período de administração da planta. Essa hipótese foi comprovada na segunda fase do experimento quando foi introduzido outro grupo de três animais no experimento. Enquanto esses três animais introduzidos recentemente no experimento adoeceram após três dias de ingestão da dose de 0,06g/kg, os animais dos Grupos 1 e 2 não apresentaram nenhum sinal de intoxicação após ingerirem diariamente a mesma dose durante nove dias.

Por outro lado, o fato de que essa resistência pode ser transmitida de animais resistentes para animais susceptíveis mediante a transfaunação de conteúdo ruminal (Duarte et al. 2013) comprova que a resistência deve-se à proliferação de bactérias que hidrolisam o MFA. A degradação microbiológica de MFA é catalisada por fluoroacetato dehalogenases que clivam a forte ligação do carbono-fluor (Fetzner & Lingens 1994). Os resultados obtidos neste experimento e as observações de Duarte et al. (2013) com *A. septentrionalis*, mostram que há numerosas bactérias que degradam MFA (Camboim 2012). A repetida administração das plantas que contem MFA, em doses pequenas, favoreceria a multiplicação dessas bactérias, que normalmente ocorrem no rúmen dos animais.

No seu conjunto, os resultados desta experimentação com *P. aeneofusca* e dos trabalhos de Duarte et al. (2013), Camboin (2012) e Camboin et al. (2011, 2012) sugerem que há três possibilidades a serem exploradas para o controle de intoxicações por plantas que contêm MFA mediante o aumento da resistência dos animais: 1) a administração repetida, por períodos alternados, de doses não tóxicas da planta; 2) a administração de MFA em doses não tóxicas o que permitiria, também, a proliferação de bactérias que tenham atividade de dehalogenases; e 3) a administração de outro substrato, não tóxico, que estimule a proliferação de bactérias com atividade de dehalogenases. Dessas três possibilidades, a terceira pareceria a mais adequada, já que resultaria difícil, sem conhecer a concentração de MFA, administrar a planta com segurança a doses não tóxicas e também é difícil trabalhar com uma substância de alta toxicidade como o MFA.

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REVIEW Open Access

Fluoroacetate in plants - a review of its distribution, toxicity to livestock and microbial detoxification

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Abstract

Fluoroacetate producing plants grow worldwide and it is believed they produce this toxic compound as a defence mechanism against grazing by herbivores. Ingestion by livestock often results in fatal poisonings, which causes significant economic problems to commercial farmers in many countries such as Australia, Brazil and South Africa. Several approaches have been adopted to protect livestock from the toxicity with limited success including fencing, toxic plant eradication and agents that bind the toxin. Genetically modified bacteria capable of degrading fluoroacetate have been able to protect ruminants from fluoroacetate toxicity under experimental conditions but concerns over the release of these microbes into the environment have prevented the application of this technology. Recently, a native bacterium from an Australian bovine rumen was isolated which can degrade fluoroacetate. This bacterium, strain MFA1, which belongs to the Synergistetes phylum degrades fluoroacetate to fluoride ions and acetate. The discovery and isolation of this bacterium provides a new opportunity to detoxify fluoroacetate in the rumen. This review focuses on fluoroacetate toxicity in ruminant livestock, the mechanism of fluoroacetate toxicity, tolerance of some animals to fluoroaceate, previous attempts to mitigate toxicity, aerobic and anaerobic microbial degradation of fluoroacetate, and future directions to overcome fluoroacetate toxicity.

Keywords: Aerobic, Anaerobic, Degradation, Dehalogenase, Fluoroacetate, 1080, Synergistetes, TCA, Toxicity

Background

Sodium monofluoroacetate (referred to as fluoroacetate hereafter), has the chemical formula $FCH_2COO^-Na^+$, and is a highly toxic compound primarily used as a pesticide known commercially as Compound 1080. Despite having a strong carbon-fluorine bond (one of the strongest bonds in nature), fluoroacetate appears to be rather labile in the environment being readily degraded by different microorganisms [1] or anabolised by higher organisms. This is in contrast to polyfluorinated compounds (such as Teflon) which are very recalcitrant and can persist in the environment for many years [2]. It is well suited as a pesticide because it is virtually tasteless and odourless, which enables it to be easily disguised within bait material targeted towards a specific pest species [3]. However, due to its non-specific poisoning of other animals and accidental human ingestion, this pesticide is currently used under strict control by governments around the world.

Fluoroacetate was first synthesised in the laboratory in 1896 but it was only first isolated from "gifblaar" (a South African plant) by Marais in 1943 [4]. These plants were believed to naturally produce this toxic compound as a defence mechanism against grazing by herbivores. Ingestion by livestock often results in fatal poisonings, which causes significant economic problems to commercial farmers in many countries such as Australia, Brazil and South Africa [5–8]. In Brazil, 60% of the cattle losses are due to fluoroacetate poisoning from grazing fluoroacetate-producing plants [9]. Fluoroacetate toxicity costs the Australian livestock industry around 45 million dollars (AUD) annually due to the increased death rates and associated productivity impacts [10]. In this paper, we will focus on the natural fluoroacetate found in plants impacting ruminant livestock industries, mechanism of its

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toxicity, previous attempts to mitigate toxicity, aerobic and anaerobic microbial degradation of fluoroacetate, tolerance of some animals to fluoroaceate, and future directions to overcome fluoroacetate toxicity.

Fluoroacetate in the environment

Fluoroacetate containing plants grow worldwide and cause sudden death in livestock. The southern continents of Africa, Australia and South America are the common locations of these plants. All of the plants containing fluoroacetate belong to the families Fabaceae, Rubiaceae, Bignoniaceae, Malpighiaceae and Dichapetalaceae [11].

Fluoroacetate is found in these tropical and subtropical plants generally at low concentrations although some are able to accumulate fluoroacetate in high concentrations [12]. These plants grow on a variety of soil types, including acidic, heavier soils or sandy loams but rarely in deep sandy soil [7]. In Africa, most fluoroacetate-accumulating plants belong to the genus *Dichapetalum*. The seeds of *D. braunii* can contain levels of fluoroacetate up to 8000 mg/kg, which is the highest ever recorded [13]. Fluoroacetate is also present in plants from South America, particularly *Palicourea marcgravii*, which can contain levels up to 500 mg/kg [14]. Other South American plants that are known to contain fluoroacetate are from the *Amorimia* genus, which has lower concentration of fluoroacetate than *P. marcgravii* [15]. Although plants from South America may not contain high concentration of fluoroacetate, they are still responsible for many livestock deaths due to the high toxicity of fluoroacetate.

In Australia, about 40 species of plants can generate fluoroacetate and most of them belong to the genus *Gastrolobium* [16]. Later these plants were classified as three genera *Gastrolobium, Oxylobium* and *Acacia*. After reclassification, many of the "nontoxic" *Gastrolobium* spp. haven been transferred to the genus *Nemcia* and the "toxic" *Oxylobium* spp. have all been placed in *Gastrolobium* [17, 18]. These fluoroacetate-containing plants are widely distributed in Australia (Fig. 1). Heart-leaf bush, *Gastrolobium grandiforum*, can contain as much as 2600 mg/kg fluoroacetate, while the 50% lethal dose (LD_{50}) of fluoroacetate is only 0.4 mg/kg of cattle body weight [12]. Although it contains less fluoroacetate than some other species, they are responsible for most of the livestock deaths in Australia because of their high abundance in cattle-producing regions [19].

In South America, especially in Brazil, around 500,000 cattle die every year by poisonous plants which cause sudden death [20]. *Palicourea marcgravii* and *Amorimia rigida* are the two most common toxic plants in Brazil [21]. Fluroacetate was found to be the principle toxin in these two plants [22]. In South Africa, *Dichapetalum cymosum* is the third most important poisonous plant causing livestock deaths particularly during spring and episodes of drought [23]. The biosynthesis pathway of fluoroacetate by these plants is still largely unknown. This is the result of the inability to produce stable fluoroacetate-

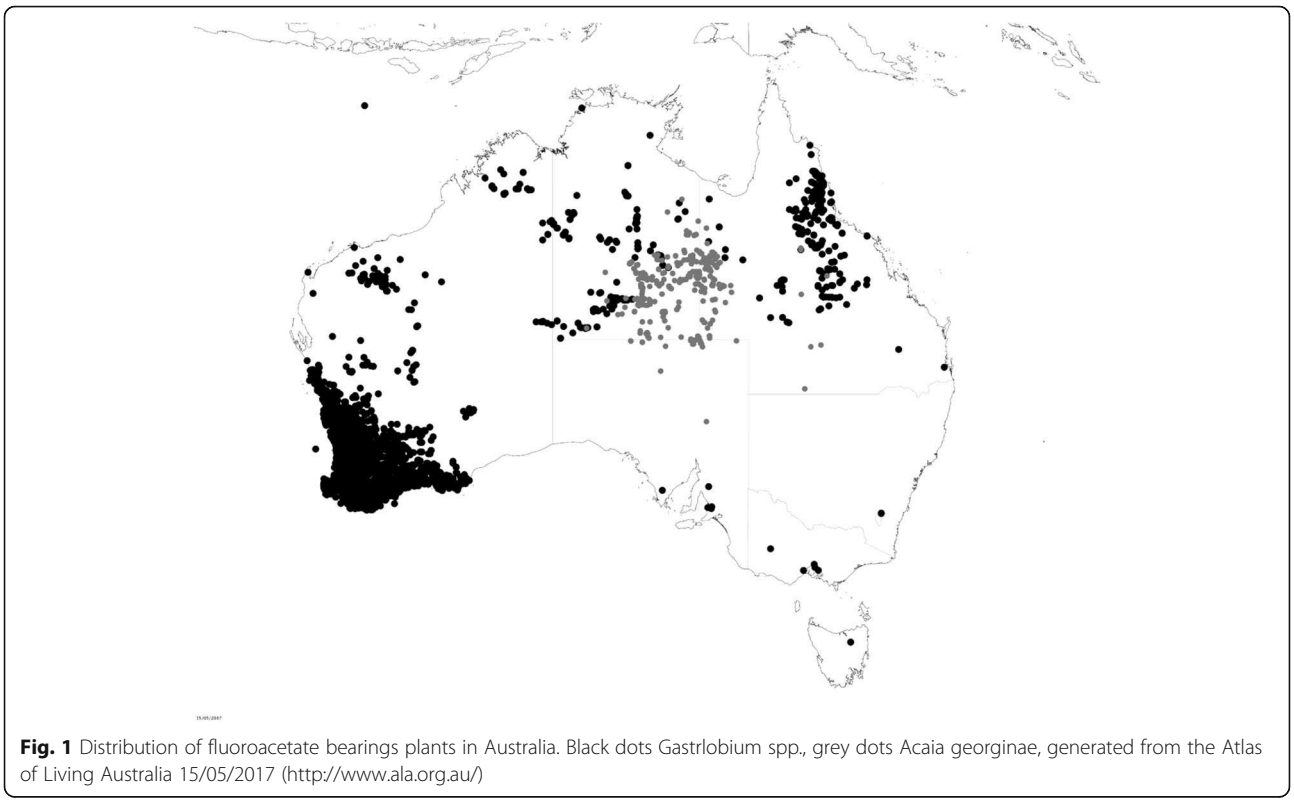

Fig. 1 Distribution of fluoroacetate bearings plants in Australia. Black dots Gastrlobium spp., grey dots Acaia georginae, generated from the Atlas of Living Australia 15/05/2017 (http://www.ala.org.au/)

degrading plant cell lines [24, 25]. Although a cell-free extract of *Dicepatalum cymosum* is able to convert fluoropyruvate to fluoroacetate, researchers could not identify the mechanism and enzymes required [26]. Analysis of soils in which some fluoroacetate-accumulating plants are found show that biosynthesis of fluoroacetate occurs even when total soil inorganic fluoride is very low [14]. Fluoroacetate biosynthesis seems to be relatively widespread, however some plants clearly have evolved to accumulate high concentrations, giving them a selective advantage from predation by animals.

This review will focus mainly on toxicity of fluoroacetate but some plants also contain fluorocitrate, fluoroacetone and fluorofatty acid compounds. Fluorinated natural products, for example, the seeds of *Dichapetalum toxicarium*, an indigenous shrub of West Africa, cause death of animals after ingestion and the symptoms are similar to fluoroacetate poisoning [27]. The seeds of *D. toxicarium* contain up to 1800 μg/g organic fluorine and the main fluorinated component was ω-fluorooleic acid (C18:1 F) [28]. Additional fluorofatty acids including o ~ –fluoro-palmitoleic, -stearic, -linoleic, -arachidic and -eicosenoic acids and 18-fluoro-9,10-epoxystearic acid have since been identified [29].

Some bacteria have been identified that can produce fluoroacetate in the environment. For example the soil bacterium *S. cattleya,* possess fluorinase (fluorination enzyme) which catalyses a nucleophilic substitution reaction between fluoride ion and S-adenosyl-L-methionine to produce 5′-fluorodeoxyadenosine (FDA). FDA is then processed to fluoroacetate and 4 -fluorothreonine (4-FT). By incorporating isotopically labelled glycerol it has been determined that the C5′ fluoromethyl and C4′ carbon of FDA are converted to fluoroacetate and C3 and C4 of 4-FT. It has also been established that both hydrogens of the fluoromethyl group of FDA are reserved in the conversion to the fluoromethyl groups of fluoroacetate and 4-FT [30] (Fig. 2).

Fluoroacetate toxicity mechanism

The tricarboxylic acid (TCA) cycle is central to cellular energy production in the mitochondria of higher organisms and fluoroacetate interrupts the TCA cycle. Fluoroacetate poisoning has been well-documented in animals since its application as a pesticide. Following oral administration and absorption through the gut, fluoroacetate is converted to fluorocitrate by citrate synthase (EC 4.1.3.7) [31] which strongly binds to the aconitase enzyme (EC 4.2.1.3), that converts citrate to succinate in the citric acid cycle [31]. This results in the termination of cellular respiration due to a shortage of aconitase [32, 33], and an increase in concentration of citrate in body tissues including the brain [32]. The build-up of citrate concentration in tissues and blood also causes various metabolic disturbances, such as acidosis which interferes with glucose metabolism through inhibition of phosphofructokinase, and citric acid also binds to serum calcium resulting in hypocalcaemia and heart failure [32, 34–37] (Fig. 3).

Despite a common mechanism of poisoning in all vertebrates, there are differences in the signs and symptoms of fluoroacetate toxicity. In general, carnivores (dogs) show primarily central nervous system (CNS) signs including convulsions and running movements with death due to respiratory failure. Herbivores (rabbit, goat, sheep, cattle, horse) show mostly cardiac effects with ventricular

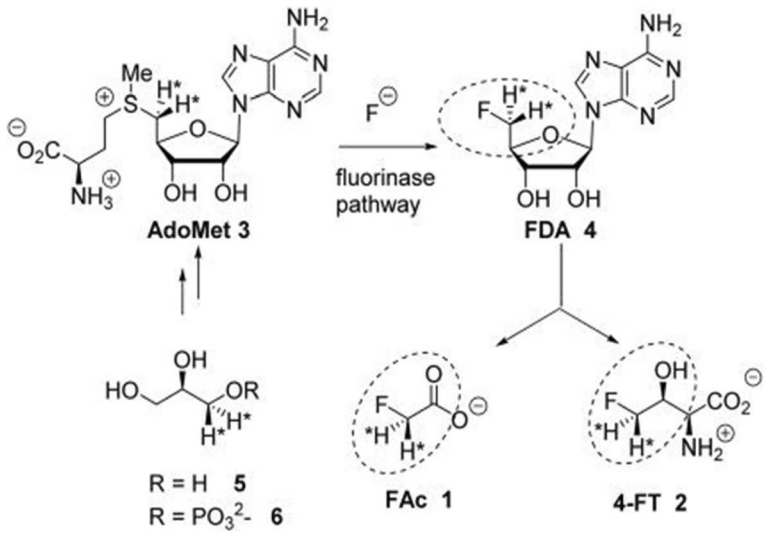

Fig. 2 Production of 5′-fluorodeoxyyadenosine (FDA) from S-adenosyl-L-l-methionine (Adomet) by Fluorinase reaction (3–4). Formation of Fluoroaceate (FAc) and 4-fluorothreonine (4-FT) from (4 to 1–2). Incorporation of Isotope labelled Glycerol (5 and 8 to 3)

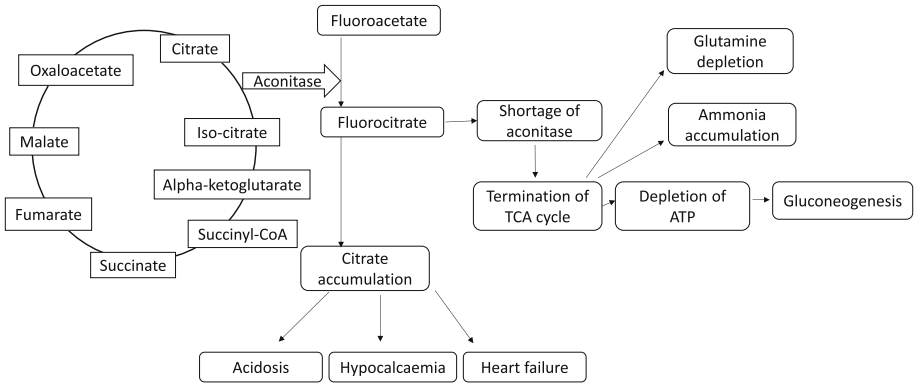

Fig. 3 Mechanisms of fluoroacetate toxicity

fibrillation and little or no CNS signs. The clinical symptoms of omnivores similarly consist of both cardiac and respiratory failure and central nervous system depression [38].

In the pig (omnivores), the clinical symptoms consist of ventricular fibrillation, tremors, violent myotonic convulsions, and respiratory depression [39]. Moreover, the onset of these symptoms can vary between animals of the same species [3]. The symptoms of fluoroacetate poisoning in cattle consist of urinary incontinence, loss of balance, muscle spasms, and in-place running lasting 3 to 20 min or convulsion followed by death of the animal [40]. In Robison's [40] report, symptoms were undetected for up to 29 h following ingestion of fluoroacetate and occurred just before death, hence the term "sudden death" described by some researchers [5]. The clinical symptoms of fluoroacetate poisoning in sheep are relatively similar to cattle, including abnormal posturing, urinary incontinence, muscle spasms and convulsions. They are also known to have severe respiratory distress and extremely rapid heart rate [39, 41].

Diagnosis is generally made on the basis of verified exposure, clinical signs, necropsy findings and chemical analysis. Samples for analysis are, vomitus, liver, stomach or rumen contents and kidney. Increased citric acid levels in kidney and serum is an indicator of fluoroacetate poisoning when correlated with clinical history. Differential diagnosis can be made amongst compounds such as strychnine, chlorinated hydrocarbons, plant alkaloids and lead. A number of other non-specific biochemical changes are suggestive including hyperglycaemia, hypocalcaemia, hypokalaemia and metabolic acidosis [10].

Fluoroacetate tolerance

Many species of animal possess an innate tolerance to fluoroacetate even when there is no evidence of evolutionary exposure. Dogs and other carnivores and rodents and many wildlife species are highly susceptible. Mammalian herbivores have intermediate sensitivity. Reptiles and amphibians are the most tolerant within the animal kingdom. Fish are generally more resistant. This tolerance is likely due to the reduced metabolic rate of these animals. It has been demonstrated that a lower metabolic rate results in less fluoroacetate being converted to fluorocitrate thus allowing more time for excretion and detoxification [42]. The skink (*Tiliqua rugosa*) has a metabolic rate about 10 fold less than a rat of similar size, but has approximately 100 fold greater tolerance to fluoroacetate [43]. Mammals with lower metabolic rate such as the bandicoot also possess a greater tolerance to fluoroacetate [44].

Interestingly, some Australian animals that live in areas where there are fluoroacetate accumulating plants have acquired a remarkable tolerance to fluoroacetate [45, 46]. The degree of tolerance is most apparent in herbivores, especially seed eating birds, which are most likely to have more direct exposure to the toxin compared to carnivorous animals [47]. Other factors which influence the degree of tolerance within a species or population may include the length of time exposed to toxic vegetation, the broadness of both diet and habitat, the size of the resident habitat and the degree of mobility. The emu, which is Australia's oldest seed eating bird, can be up to 150-times more tolerant than the same species of emu outside of areas with fluoroacetate-accumulating plants [48]. This phenomenon has also been observed in other animals such as the possum [42]. Tolerance to fluoroacetate is also demonstrated in insects. Some insects not only utilise the vegetation in their diet, but some actually store the toxin, probably in vacuoles, and use it as defence against predation [49].

The biochemical nature of acquired tolerance to fluoroacetate in animals is not fully understood. It is proposed that there are four obvious biochemical factors that may affect the metabolism of fluoroacetate: (1) the rate of conversion of fluoroacetate to fluorocitrate; (2) the sensitivity of aconitase to fluorocitrate; (3) the citrate transport system in mitochondria, and; (4) the ability to detoxify fluoroacetate [42, 43]. A study compared two

distant populations of possums, one having prior exposure to fluoroacetate vegetation and the other having no prior exposure. No differences were found in the defluorination rate of liver extracts between the two populations [42]. Despite a number of other studies attempting to address the biochemical mechanisms for tolerance and fluoroacetate detoxification, there is still inadequate information available.

The soil bacterium *Streptomyces cattleya* is able to produce both fluoroacetate and fluorothreonine but has pathways that possibly confer resistance to these compounds [50]. A fluoroacetyl-CoA-specific thioesterase (FlK) in *S. cattleya* selectively hydrolyzes fluoroacetyl-CoA over acetyl-CoA and exhibits a 10^6-fold higher catalytic efficiency for fluoroacetyl-CoA compared to acetyl-CoA [51]. The FlK gene is located in the same cluster as the C-F bond-forming fluorinase (flA), raising the probability that FlK-catalyzed hydrolysis of fluoroacetyl-CoA plays a role in fluoroacetate resistance in *S. cattleya* by inhibiting the entrance of fluoroacetyl-CoA into the TCA cycle [52].

Degradation of fluoroacetate

Studies to isolate, purify and characterise fluoroacetate-detoxifying enzymes from animals have generally been unsuccessful and contradictory in their findings. Nonetheless, it is generally appreciated from early studies that the vast majority of fluoroacetate is defluorinated within the liver by an enzyme termed fluoroacetate specific defluorinase [53, 54]. This enzyme has been purified from mouse liver cytosol but it is distinct from multiple cationic and anionic glutathione S-transferase isozymes [55]. However, there has been no definitive classification of the enzyme [56]. The enzyme appears to act via a glutathione-dependent mechanism [57]. The focus of the most recent studies has been to determine the relationship between fluoroacetate specific defluorinase and glutathione S-transferase family enzymes to gain a better understanding of the mechanism of fluoroacetate detoxification.

Mead and co-workers [58] characterized a glutathione-dependent dehalogenation pathway in the liver of possum utilizing fluoroacetate as substrate. In the urine of fluoroacetate-treated animals, they found S-carboxymethylcysteine which indicates defluorination was catalyzed by an enzyme of the glutathione S-transferase group.

Microbial aerobic degradation

Contrary to the animal studies on fluoroacetate detoxification, microbial degradation of fluoroacetate has been extensively studied. Moreover, the mechanism for aerobic fluoroacetate degradation is well characterised and documented [59–64]. Microorganisms from the soil have been identified with ability to aerobically degrade fluoroacetate. The bacterial communities involved in fluoroacetate degradation vary significantly depending on the areas studied. In Western Australia, species of *Bacillus, Pseudomonas, Aspergillus, Penicillium* and *Streptomyces* were isolated from soil in a of temperate climate [64], while *Burkholderiaceae, Ancylobacter* sp., *Paenibacillus* sp., *Staphylococcus* sp. and *Stenotrophomonas* sp. were isolated from the soil of Brazilian areas where the fluoroacetate-containing plants *Mascagnia rigida* and *Palicourea aenofusca* are found [65].

Microorganisms have also been isolated from bait containing the 1080 poison (fluoroacetate) that is used for vertebrate pest control [66]. Bacteria, particularly *Pseudomonas fluorescens*, were isolated from the 1080 bait when mixed with ground kangaroo meat, while both bacteria and soil fungi such as *Fusorium oxysporum* have been isolated from the bait mixed with oats [66, 67]. The bacteria and soil fungi degraded fluoroacetate in the presence and absence of another carbon source. However in the presence of peptone, degradation was higher.

In Western Australia, several microorganisms were isolated from soil with and without previous exposure to fluoroacetate. These included (*Aspergillus fumigatus*, *Fusarium oxysporum, Pseudomonas acidovorans, Pseudomonas fluorescens 1,* an unidentified *Pseudomonas* sp., *Penicillium purpurescens* and *Penicillium restriction.* These microbes can degrade fluoroacetate, presumably utilising it as a carbon source when grown in solution (2 to 89%) [67]. Recently, two other fluoroacetate degrading-bacteria were isolated from the Brazilian caprine rumen which had the ability to degrade fluoroacetate under aerobic conditions [68]. The bacteria were closely related to *Pigmentiphaga kullae* and *Ancylobacter polymorphus*. Fluoroacetate was degraded to fluoride ions, but the end products containing the carbon atoms from fluoroacetate were not discussed. Moreover, these bacteria might potentially be facultative anaerobes, and it was speculated that degradation occurred through the aerobic process.

Walker and Lien [59] were first to identify two fluoroacetate-degrading enzymes (initially termed haloacetate halidohydrolase) from *Pseudomonas* species and a fungus *Fusarium solani*. At the same time, a fluoroacetate dehalogenase was isolated from a fluoroacetate-dehalogenating bacterium in industrial wastewater, and tentatively named *Moraxella* sp. strain B [62]. It has now been reclassified as *Delftia acidovorans* strain B. Other soil bacteria which play a role in defluorination of fluoroacetate are *Burkholderia* sp. strain FA1, *P. fluorescens*, *Rhodopseudomonas palustris* CGA009 and different strains of *Pseudomonas* species [61, 66, 69, 70]. The fluoroacetate dehalogenase enzymes identified in some of these bacteria appear to degrade fluoroacetate via a similar mechanism, where an ester is produced as an intermediate which is hydrolyzed by a water molecule to form glycolate (Fig. 4).

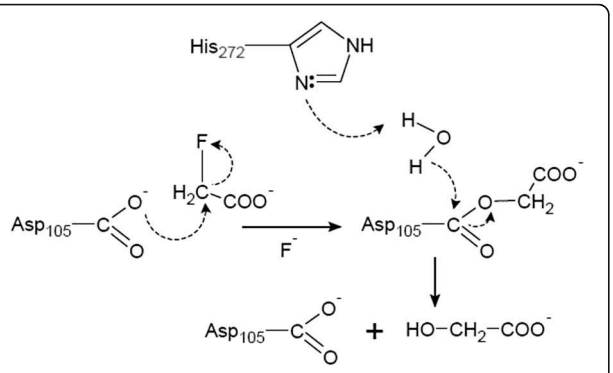

Fig. 4 The mechanism of dehalogenation by fluoroacetate dehalogenase in *Delftia acidovorans*

In spite of their novel mechanisms, limited work has been conducted on these enzymes. The biochemical studies show (Table 1) relatively similar properties between these dehalogenases. All the bacterial enzymes have optimal activities at a slightly alkaline pH around pH 8.0 to 9.0 [59, 61, 69]. However, defluorinating activities in fungi have a wider optimal pH range, with pH 7-8 for *F. solani* compared to pH 5-8 for *F. oxysporium* [59, 67].

The thermal stability of these enzymes differs significantly depending on the species of the microorganisms. Fluoroacetate dehalogenase in *Pseudomonas sp.* from the New Zealand soil was shown to have higher thermal stability, approximately 55 °C, than the fluoroacetate dehalogenase in *F. solani* [59]. However, this notion of high thermal stability was not observed in some *Psuedomonas* species, *P. fluorescens* DSM 8341 was shown to have thermal stabilities to 30 °C [69].

The dehalogenases were shown to use water as the sole co-substrate, and no evidence indicates the involvement of metal ions in their catalytic activity [59, 71]. However, an increase in fluoroacetate degradation activity with addition of low concentration metals ion such as Mg^{2+}, Fe^{2+} and Mn^{2+} has been demonstrated but higher concentration of these metals were inhibitory [69]. Although all the enzymes have a similar degradation mechanism, the size of these enzymes varies significantly. *Pseudomonas* sp. strain A and *P. fluorescens* enzymes are presumed to be monomers, and have an estimated molecular weight of 42 and 32.5 kDa, respectively. Conversely *Burkholderia* sp. FA1 and *D. acidovorans* strain B are dimers of two identical subunits with an estimated molecular mass of 79 and 67 kDa, respectively [61, 72].

All these enzymes release inorganic fluoride from fluoroacetate, but some also cleave chlorinated and brominated analogues, albeit at slower rates [59, 61, 73]. To date, *D. acidovorans* strain B is the only fluoroacetate-dehalogenating bacterium which harbours two haloacetate dehalogenase enzymes; Fluoroacetate dehalogenase H-1 (*dehH1*) and fluoroacetate dehalogenase H-2 (*dehH2*) which are encoded by two different genes on its 65 kb plasmid pUO1. Fluoroacetate dehalogenase H-1 acts predominately on fluoroacetate, while fluoroacetate dehalogenase H-2 has a broader range of substrate specificity for haloacetate, but not fluoroacetate [73].

Two other fluoroacetate dehalogenase enzymes which were purified and tested for their substrate specificities are fluoroacetate dehalogenases from *Burkholderia* sp. FA1 (Fac-dex) and *R. palustris* CGA009 (RPA1163) [61, 70]. When compared to DelH1 of *D. acidovorans* strain B, the two fluoroacetate dehalogenases were more specific to fluoroacetate than to other halogenated analogues [61, 70].

To date, the mechanism of fluoroacetate degradation by fluoroacetate dehalogenase has been extensively studied in *Burkholderia* sp. strain FA1 and *D. acidovorans* strain B [63, 70, 72, 74–76]. Several catalytic regions were identified by comparing the amino acid sequence with that of a haloalkane dehalogenase from *Xanthobacter autotrophicus* [60], and the specific amino acids have been identified by mutagenic studies [63]. It has been found that the active site of the H-1 enzyme contains a conserved Asp105 and His272.

In the initial steps of the pathway for fluoroacetate degradation to glycolate, the carboxylate group of Asp105 acts as a nucleophile to form an ester

Table 1 Physical and biochemical properties of fluoroacetate dehalogenase isolated from different aerobic microorganisms

Microbial source	Number of genes [a]	Gene location	Native enzyme sizes,kDa	Subunit Composition	Optimal pH	Optimal temperature	Reference
Delftia acidovorans strain B	2,*deH1, deH2*	Plasmid	67	Dimer	9.5	50	[60]
Pseudomonas fluorescens DSM 8341	N.D.	N.D.	32.5	monomer	8	30	[69]
Burkholderia sp. FA1	1,*fac-dex*	Chromosome	79	Dimer	9.5	N.D.	[61]
Rhodopseudomonas palustris CGA009	1,RPA1163 [b]	Chromosome	N.D	Dimer	N.D.	N.D.	[70]
Pseudomonas sp. strain A	N.D.	N.D.	42	Monomer	9	50	[62]
Pseudomonas sp.	N.D.	N.D.	62	N.D.	8	N.D.	[59]
Fusarium solani	N.D	N.D.	62	N.D.	7-8	N.D.	[59]

[a] gene names were described in parentheses
[b] gene name identified in the form of locus tag

intermediate around the beta carbon atom of fluoroacetate to displace the fluorine atom [63, 75]. Then the acetate intermediate is hydrolysed by a deprotonated water molecule formed by a conserved His272. The net result of the reaction is a displacement of a fluoride ion producing glycolate and regeneration of the carboxylate group belonging to Asp105 (Fig. 4).

The catalytic sites of *D. acidovorans* strain B are also conserved as Asp105 and His271 in *Burkholderia* sp. strain FA1 [72]. Moreover, release of fluoride was found to be stabilised by the hydrogen bonds to His149, Trp150 and Tyr212 of *Burkholderia* sp. strain FA1 [75]. This stabilisation effect reduces the activation barrier, where the energy required to cleave the C-F bond was calculated to be only 2.7 kcal/mol, despite the strong C-F bond. A similar structure was also noted in the fluoroacetate dehalogenase from *R. palustris* CGA009 [70].

Due to the fact that the fluoroacetate dehalogenase of *Burkholderia* sp. strain FA1 has a preference for fluoroacetate compared to chloroacetate, the substrate specificity was tested using this enzyme [76]. Using docking stimulations and quantum mechanics/molecular mechanics (QM/MM), Nakayama and colleagues [76] managed to show that fluoroacetate and chloroacetate were incorporated into the active site of fluoroacetate dehalogenase in different conformations. Moreover, the hydrogen bonds of the chloroacetate-enzyme complex do not sufficiently reduce the activation barrier for chloroacetate, which is in a good agreement with the observed high specificity of this enzyme towards fluoroacetate.

Li et al. [77] worked on the structural requirements for defluorination by fluoroacetate degalogenase or FAcD (from bacterium *Rhodopseudomonas palustris* CGA009, PDB code 3R3V) in enabling defluorination rather than dechlorination. They have shown that conformational variations relating to neutrally charged histidine are Hsd155 and Hse155 may cause differences in enzymatic preference. They found that the structure FAcDHse155 is more energetically feasible than the structure FAcDHsd155 for enzyme FAcD, whereas FAcDHse155 prefers defluorination rather than the dechlorination process. Besides the residues Arg111, Arg114, His155, Trp156, and Tyr219, the important role of residues His109, Asp134, Lys181, and His280 during the defluorination process were also emphasized in their experiment. In addition, they found that conformational variations may cause different enzymatic preferences toward competitive pathways.

Microbial anaerobic degradation

Compared with aerobic degradation of fluoroacetate, there is a lack of studies on the isolation of anaerobic microorganisms that have the ability to degrade fluoroacetate. However recently, a native bacterium from the Australian bovine rumen was isolated using anaerobic agar plates containing fluoroacetate as a carbon source [1]. This bacterium, strain MFA1, which belongs to the Synergistetes phylum has the ability to degrade fluoroacetate, producing fluoride and acetate, as opposed to glycolate from aerobic fluoroacetate-degrading bacteria. Similar observations were noted from other studies on anaerobic degradation of trifluoroacetic acid in anoxic sediments, where acetate was produced from the degradation of this compound [78, 79]. Moreover, similar mechanisms were also noted with anaerobic dechlorinating bacteria. An anaerobic microbial enrichment culture containing *Dehalococcoides ethenogenes* 195 was capable of completely dechlorinating tetrachloroethene to chlorides and ethene [80].

Acetate is not used by strain MFA1 for growth, unlike aerobic fluoroacetate dehalogenating bacteria which utilise the end product, glycolate, as an energy source. Strain MFA1 appears to degrade fluoroacetate via the reductive dehalogenation pathway utilising it as terminal electron acceptor rather than a carbon source. Reductive dehalogenation occurs in anaerobic bacteria, where a halogen substituent is released from a molecule with concurrent addition of electrons to that molecule [81].

There appeared to be a consumption of hydrogen and formate during the growth of strain MFA1 in fluoroacetate [1]. This observation was also noted from reductive dehalogenation of other halogenated compounds in anoxic environment. A net loss of hydrogen was measured from anoxic sediment microcosms dosed with various halogenated compounds [82], and hydrogen was consumed by a *Dehalococcoides ethenogenes* strain 195 with degradation of tetrachloroethene and vinyl chlorides to ethene [83]. However, there is not yet any enzyme identified in strain MFA1 responsible for the degradation of fluoroacetate.

Biotechnological-derived methods for fluoroacetate detoxification in cattle

There have been several attempts to reduce the toxic effects of fluoroacetate in ruminant livestock production. A biotechnological approach to the problem did provide some evidence that detoxifying fluoroacetate by microbial metabolism was possible in the rumen [84]. Gregg and colleagues [84] transformed the rumen bacterium *Butyrivibrio fibrisolvens* with the fluoroacetate dehalogenase gene (DelH1) from *Delfitia acidovorans* strain B, and the recombinant bacteria demonstrated active dehalogenation of fluoroacetate in vitro.

The fluoroacetate dehalogenase H1 gene from *D. acidovorans* strain B was incorporated into the plasmid pBHf for transfection into *Butyrivibrio fibrisolvens* [84]. The transfection was relatively stable, with the pBHf plasmid remaining detectable after 500 generations

under non-selective conditions. Gregg and colleagues [84] also performed an in vitro study, where a growing population of the recombinant bacterium was able to release fluorine from fluoroacetate at the rate of 9.9 nmol/min/mg [84]. However, dehalogenase activity was not detected outside the bacterial cells, and so it was predicted that fluoroacetate in the media diffused readily into the cells [84]. The genetically modified *B. fibrisolvens* strain expressed dehalogenase enough to detoxify fluoroacetate from the surrounding medium at a rate of 10 nmol/(min·mg) bacterial protein in in vitro testing. The plasmid that carries the dehalogenase gene appears to be very stable and was retained by 100% of the transformed bacteria after 500 generations of growth in non-selective media [84].

In an in vivo study conducted by Gregg and colleagues [85], one group of sheep were inoculated with the recombinant bacteria before being fed fluoroacetate-injected snow-peas, while a control group was not inoculated with the recombinant bacteria. This study showed a significant difference between groups, where the inoculated sheep appeared to be relatively normal despite a 0.4 mg dose of fluoroacetate per kg of animal, while the control sheep died of the fluoroacetate poisoning [85]. The modified bacteria were able to colonise the rumens of two sheep and were shown to persist for an experimental period of 5 months.

In another in vivo study conducted using 20 Angus steers, animals orally inoculated with seven different strains of *Butyrivibrio fibrisolvens* (*B. fibrisolvens* 0/10, 10/1, 85, 149/83, 156, 291, 52/10 strains respectively) containing the plasmid (pBHf)-bearing the fluoroacetate dehalogenase gene DelH1 did not develop the acute symptoms of fluoroacetate toxicity compared to the controls [86]. PCR analysis of rumen fluid collected at 7, 12 and 15 days post-inoculation confirmed the presence of the recombinant bacteria in the rumen at 10^4 to 10^7 cells/ mL. Post-mortem PCR analysis of the rumen fluid from all test animals showed approximately 10^6 colony forming units (CFU) per mL of recombinant *B. fibrisolvens* for several of the strains, 20 days after inoculation [86]. The dose of recombinant bacteria used was able to significantly diminish the effects of fluoroacetate poisoning. Therefore, these in vivo tests showed significant protection of livestock from fluoroacetate using the recombinant bacteria approach. However, in Australia, this technology has not been adopted because approval has not been granted due to strict government regulations regarding release of genetically modified organisms.

In order to prevent animals from unintentional fluoroacetate poisoning, one of the therapies involves the adsorption of fluoroacetate with activated charcoal or other resins. These agents were investigated for their abilities to absorb fluoroacetate from gastrointestinal fluid, thus potentially preventing the conversion of fluoroacetate to fluorocitrate [87]. Moreover, the doses of 2 g/kg of such resins are impractical for preventing fluoroacetate poisoning in livestock. Acetate donor therapy has also been investigated as a treatment for poisoning. Early studies on the effect of fluoroacetate poisoning revealed that fluoroacetate inhibits acetate metabolism in poisoned animals [88]. This led to other studies to investigate whether acetate in the animal at high concentration would provide protection to the animals from fluoroacetate poisoning [89]. This treatment was only effective when provided immediately after the ingestion of the toxin and therefore not practical for treating grazing livestock due to limited surveillance of animals in a rangeland production system. In some cases, animals have died after consumption of fluoroacetate due to the severity of symptoms caused by the depletion of tissue citrate. Therefore, by relieving the symptoms of fluoroacetate poisoning using citrate therapy, researchers have been able to enhance the survival rate of poisoned animals [90]. However, these symptom-reversing therapies would need to be administrated immediately to the poisoned animals to show any effect. Furthermore, some of the poisoned animals in these studies died of other complications even though the major symptoms were suppressed [90].

Rumen microbial transfer

Amorimia pubiflora is one of the main causes of fluoroacetate poisoning in Brazil. In a recent study researchers were able to induce resistance to toxicity by feeding non-toxic doses of this plant to sheep. In addition transferring rumen contents from the resistant animals to naïve sheep was able to confer protection from toxicity [91].

Conclusions

To date, attempts to prevent fluoroacetate toxicity have been unsuccessful except for physically preventing access to toxic plants in the grazing environment. Animal house studies have demonstrated in principle that rumen bacteria engineered to hydrolyse the toxin could prevent toxicity but approvals for the release of these organisms into the environment are unlikely due to current government regulatory restrictions. However the recent discovery of a naturally occurring rumen bacterium (Synergistetes strain MFA1) capable of degrading fluoroacetate may provide a biotechnological solution to the problem of toxicity in rangeland animals. Even though Synergistetes strain MFA1 appears to be ubiquitous throughout the digestive systems of animals such as emus, kangaroos and other cattle, they are present in low numbers which may limit their ability to protect the animal from a lethal dose of the toxin [1]. However it is possible that there are other rumen bacteria able to degrade fluoroacetate which are at

higher abundance or could act in concert with other rumen microorganisms to ameliorate the full impact of the toxin. Therefore, further surveys for the presence of other fluoroacetate degrading rumen bacteria and studies on increasing the numbers of these bacteria and expression of the genes responsible for degrading the toxin seems a logical approach for developing a practical strategy to protect livestock from fluoroacetate poisoning. Recent studies demonstrating tolerance to toxicity by adapting the rumen microbiota to non-toxic doses of fluoroacetate further supports a 'rumen detoxification' approach.

Abbreviations
AUD: Australian Dollar; CNS: Central nervous system; MM: Molecular mechanics; QM: Quantum mechanics; TCA: Tricarboxylic acid

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Induction and transfer of resistance to poisoning by *Amorimia pubiflora* in sheep whith non-toxic dosis of the plant and ruminal content

Indução e transferência de resistência à intoxicação por *Amorimia pubiflora* em ovinos com doses não tóxicas de planta e conteúdo ruminal

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ABSTRACT

***Amorimia pubiflora* (*Malpighiaceae*)**, *which contains sodium monofluoroacetate (MFA) is the main cause of "sudden death" in cattle in the Brazilian state of Mato Grosso. This research investigated the induction of resistance to the poisoning in sheep by the continuous administration of non-toxic doses of the plant and also the possibility to transfer this resistance to other sheep by the transfaunation of ruminal fluid. For this a group of four sheep (G1) received daily doses of 0.5g kg^{-1} for 20 days and after an interval of 15 days were challenged with three daily doses of 1g kg^{-1} for 3 days. Also the transfer of resistance to **A. pubiflora** poisoning was evaluated by transfaunation of rumen fluid (100ml for 10 days) from G1 sheep to five sheep (G2), followed by challenge with the dose of 1g kg^{-1} for 3 days (G2D2) and after a three-day interval they received a single dose of 3g kg^{-1} (G2D3). The degree of resistance was evaluated by monitoring the onset of clinical signs, heart rate, and outcome of the poisoning compared with the control groups, which were challenged with three daily doses of 1g kg^{1} (G3) and with a single dose of 3g kg^{-1} (G4). Clinical parameters evaluated in Groups G1 and G2 were significantly less pronounced than those observed in G3 and G4 (control) (P<0.05). Sheep in G4 (control) died after receiving a single dose of 3g kg^{-1}, while those in G2 (transfaunated) survived. These findings demonstrated that consumption of non-toxic doses of **A. pubiflora** induced resistance in sheep and that this resistance can be transferred by transfaunation. New experiments are needed to determine the most efficient ways to induce resistance and to use this technique in the field to prevent the poisoning.*

Key words: ***Mascagnia pubiflora**, monofluoroacetate, ruminal detoxification, sheep, sudden death, toxic plants.*

RESUMO

***Amorimia pubiflora* (*Malpighiaceae*)** *contém monofluoroacetato de sódio (MFA) e é uma das principais causas de "morte-súbita" em bovinos no estado de Mato Grosso, no Brasil. Este trabalho investiga a indução de resistência à intoxicação por **A. pubiflora** em ovinos, através da administração repetida de doses não tóxicas, e também se é possível transferir essa resistência para outros ovinos por transfaunação do fluido ruminal. Para a indução à resistência, um grupo com quatro ovinos (G1) recebeu doses diárias individuais de 0,5g kg^{-1} de folhas de **A. pubiflora** durante 20 dias. Após um intervalo de 15 dias, os ovinos desse grupo foram desafiados diariamente, durante 3 dias consecutivos, com a dose de 1g kg^{-1}. A transferência de resistência à intoxicação por **A. Pubiflora** foi avaliada por transfaunação de conteúdo ruminal (100ml diariamente durante 10 dias) do G1, para cinco ovinos (G2). Após essa etapa, os cinco ovinos do Grupo G2 receberam a dose de 1g kg^{-1} por três dias (G2D2) e, após um intervalo de 3 dias, quatro ovinos do G2 receberam uma única dose de 3g kg^{-1} (G2D3). O grau de resistência foi conferido mediante o monitoramento do início dos sinais clínicos, frequência cardíaca, desfecho da intoxicação e comparação com grupos controle, os quais foram desafiados com três doses repetidas de 1g kg^{-1} (G3) e uma dose única de 3g kg^{-1} (G4). Os parâmetros clínicos avaliados nos Grupos G1 e G2 foram significativamente menos evidentes do que os observados nos G3 e G4 (controles) (P<0,05). Os ovinos*

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*do G4 (controle) morreram após receber a dose única de 3g kg^{-1}, enquanto os do G2 (transfaunados) sobreviveram. Esses achados indicam que o consumo de doses não tóxicas de **A. pubiflora** induz resistência em ovinos e que a resistência pode ser transferida por transfaunação. Novos experimentos são necessários para determinar as formas mais práticas para induzir resistência e a forma de usar essa técnica no campo para controlar a intoxicação por essa planta.*

Palavras-chave: ***Mascagnia pubiflora**, morte súbita, microbiota ruminal, monofluoracetato, plantas tóxicas.*

INTRODUCTION

In Brazil the group of toxic plants of greatest importance to livestock is that causing "sudden death", including ***Palicourea*** spp., ***Amorimia*** spp., ***Tanaecium*** (***Arrabidaea***) ***bilabiatum***, ***Fridericia*** (***Arrabidaea***) ***japurensis***, and ***Pseudocalymma elegans*** (LEE et al., 2012; TOKARNIA et al., 2012). Sodium monofluoracetate (MFA) has been identified in most of these species (LEE et al., 2012).

Amorimia pubiflora (A. Juss.) W.R. Anderson (ANDERSON, 2006) popularly known as "corona", "suma" or "cipó-prata" is one of the most important toxic plants in the Midwest region of Brazil (FERNANDES & MACRUZ, 1964; TOKARNIA & DOBEREINER, 1973; SANTOS, 1975; BECKER et al., 2013). The plant also occurs in the Northeastern region (state of Bahia) and Southeastern region (states of Minas Gerais, São Paulo, Goiás, and Rio de Janeiro) (MAMEDE, 2010). Leaves of ***A. pubiflora*** deposited in the University of Michigan Herbarium contained 0.006% of MFA (LEE et al., 2012), and leaves from sprouting plants collected in the municipality Colniza, state of Mato Grosso, contained 0.015% MFA (BECKER et al., 2013).

Several outbreaks of ***A. pubiflora*** poisoning have been reported in cattle in the states of Mato Grosso do Sul and Mato Grosso. Rates of morbidity and mortality were up to 3.5% and 54.5%, respectively. The disease is characterized by "sudden-death" associated with exercise. The main clinical signs are weakness, engorged jugular vein, slight muscle tremors, and muscle contractions, followed by death in a 5-15 minutes period (TOKARNIA & DOBEREINER, 1973; LEMOS et al., 2011; BECKER et al., 2013). In the state of Mato Grosso, in some municipalities of the Amazon region ***A. pubiflora*** is the main cause of cattle losses (TOKARNIA et al., 2012; BECKER et al., 2013).

Reports that cattle coming from regions where are no MFA-containing plants are more susceptible to poisoning suggest that the animals become resistant to the consumption of MFA-containing plants (SILVA et al., 2008; BECKER et al., 2013). This could be related to fluoroacetate dehydrogenase activity of ruminal micro-organisms which break the carbon-fluorine bond degrading the MFA (FETZNER & LINGENS, 1994). Several MFA-degrading microorganisms have been isolated from soil in Australia (TWIGG & SOCHA, 2001) and Brazil (CAMBOIM et al., 2012a) and from ruminal content of goats (CAMBOIM et al., 2012b) and cattle (PIMENTEL, 2011) in Brazil. Goats ingesting daily non-toxic doses of ***A. septentrionalis*** increased significantly their resistance to the poisoning by this MFA-containing plant (DUARTE et al., 2013).

The objectives of these experiments were to investigate if the daily administration of non-toxic doses of ***A. pubiflora*** in sheep induces resistance to the poisoning by this plant, and to determine if this resistance can be transferred by transfaunation of rumen fluid to susceptible sheep.

MATERIALS AND METHODS

Amorimia pubiflora leaves used in this study were collected in the city of Colniza, MT (09º25'76"S-59º03'55"W) on days 14/11/2011, 19/12/2011 and 08/06/2012. Botanical identification was performed by Dr. William R. Anderson (University of Michigan Herbarium, Ann Arbor, Michigan, USA) and a voucher specimen of the plant was authenticated and deposited in the Herbarium of Michigan University, Michigan, USA (LEE et al., 2012). The MFA content of the ***A. pubiflora*** leaves was 0.015% (BECKER et al., 2013).

Experimental animals

The experiment was performed with 16 Santa Inês, 1-2 years-old, clinically healthy female sheep raised under confinement in the Experimental Sheep Farm of the UFMT in the municipality of Santo Antônio de Leverger, state of Mato Grosso (MT). All sheep have been dewormed 7-10 days before the start of the experiment and divided at random into four groups (two treated and two controls). Each group was kept in 9m^2 bays at the Veterinary Hospital of the Universidade Federal de Mato Grosso (UFMT), Cuiabá, MT. All animals were weighed prior to the supply of ***A. pubiflora*** to calculate the dose of the plant to be administered. Most sheep consumed leaves of ***A. pubiflora*** voluntarily and when this did not happen, the leaves were orally administered by repeatedly inserting small amounts into the mouths of the animals. The experiment with the number of

animals, doses and ***A. pubiflora*** administration period in each group are shown in table 1.

Group 1 (induction of resistance by ***A. pubiflora*** administration)

To induce resistance to ***A. pubiflora*** poisoning the four sheep (#1, 2, 3 and 4) received daily 0.5g kg^{-1} of ***A. pubiflora*** leaves during 20 consecutive days. ***Amorimia pubiflora*** administration was interrupted when clinical signs of moderate intensity were observed, especially when the heart rate (HR) was above 130bpm when the sheep was resting, or above 200bpm after the movement, or when the animal remained lying for a prolonged time after plant administration. The administration was restarted when the HR was less than 80bpm with sheep at rest.

Following the induction phase, the sheep from this group spent 15 days without ingesting ***A. pubiflora***. Then, they were challenged by the ingestion of 1g kg^{-1} daily for three days. Subsequently, to verify the duration of resistance to the consumption of ***A. pubiflora***, sheep #3 was challenged with a single dose of 3g kg^{-1} on day 166, and again with the dose of 5g kg^{-1} on day 180.

Group 2 (transfaunated)

One day after the end of the challenge 100mL of rumen content was collected daily, with

Table 1 - Onset and intensity of clinical signs and outcome of the poisoning by **A*morimia pubiflora*** in sheep with resistance induced by the daily administration of ***A. pubiflora*** (G1) or by transfaunation (G2) and in control sheep (G2 and G4). Data from G1 correspond to the induction phase and to challenge. Data from G2, G3, and G4 correspond to the induction phase and to challenge. Data from G2, G3, and G4 correspond to challenge.

Group (Phase)	Sheep #	Daily dose (g kg^{-1})	Number of doses	Intensity of clinical signs	Outcome
G1 (Induction of Resistance)	1	0,5	20	NCS NCS	R
	2			Moderate Moderate	R
	3			Moderate Moderate	R
	4			NCS	R
G1 Challenge 1/D1	1	1,0	3	Light	R
	2			Discrete	R
	3				R
	4			Discrete	R
G2 (transfauned) First challenge	5	1,0	3	Discrete	R
	6			NCS	R
	7			Discrete	R
	8			NCS	R
	9			NCS	R
G2 (transfauned) Second challenge	5	3,0	1	Discrete	R
	7			NCS	R
	8			Discrete	R
	9			Discrete	R
G3 (Control 1)	10	1,0	3	Moderate	R
	11			Moderate	R
	12			Moderate	R
G4 (Control 2)	13	3,0	1	Severe	D
	14			Severe	D
	15			FD	D
	16			Severe	D

NCS: No clinical signs; FD: it was found dead; R: clinical recovery; D: Death.

a ruminal tube, during 10 days, from each sheep of Group 1 and transferred by gavage to five sheep (#5, 6, 7, 8 and 9) from Group 2 at the dose of 100ml daily for each sheep. One day after the end of the transfaunation period the sheep from Group 2 were challenged with a dose of 1g kg^{-1} daily during three consecutive days (D2) and after a three-day interval four sheep (#5, 7, 8 and 9) they received a single dose of 3g kg^{-1} (D3).

Control groups

Two control groups were used: G3 (Sheep #10, 11 and 12), which received ***A. pubiflora*** at the dose of 1g kg^{-1} daily for three consecutive days; and G4 (sheep #13, 14, 15 and 16), which received 3g kg^{-1} of ***A. pubiflora*** in a single dose.

Statistical analysis

Data analyzes were performed using the software R Development Core Team (2012) (PAULA, 2004). To find the difference between groups used the regression range (PAULA, 2004) with the linear predictor (η) and the logarithmic function $\eta = \ln(\mu_i)$.

Clinical follow-up

During the phases of resistance induction and challenge, clinical observations were performed three to four times a day in all sheep prior to and after the plant administration. Examination consisted on the observation of pulsations, respiratory and ruminal movements, behavior, posture and water and food intake. Six to eight hours after the plant administration the animals were forced to run for 10 minutes and pulsation and respiratory movements were determined before and after exercise.

Resistance of G1 and G2 sheep to the consumption of ***A. pubiflora*** was compared with the resistance of control groups by evaluating the variation of heart rate, outcome of the clinical picture and the start and intensity of clinical signs, which were classified as: severe (marked tachycardia, above 180bpm, engorged jugular, venous pulse, cardiac arrhythmia, muscle tremors, kyphosis, stiff gait, apathy, head tremor, mandibular trismus, reluctance to move, circling, cyanosis, sudden falls and convulsions, followed by death); moderate (moderate tachycardia, 130-180bpm, jugular engorgement, venous pulse, cardiac arrhythmia, apathy, reluctance to move, sudden falls, with clinical recovery); mild (mild tachycardia, 100-130bpm, kyphosis, stiff gait and apathy); and discrete (only tachycardia, below 80bpm).

Pathology

Sheep that died were necropsied. Fragments of lymph nodes, intestines, rumen, reticulum, omasum, abomasum, pancreas, adrenal, urinary bladder, kidney, thyroid, heart, lung, and brain were collected and fixed in 10% formalin and processed routinely in the Veterinary Pathology Laboratory of the Veterinary Hospital UFMT for histopathological analysis.

RESULTS

The amount ***A. pubiflora*** ingested by sheep from group 1 during the induction of resistance and by sheep from groups 1, 2, 3, and 4 during challenge as well as the severity of clinical signs and the outcome of the poisoning are presented in table 1. The mean heart rates during the period of administration of ***A. pubiflora*** and the average time between the start of administration and the first signs are shown in figure 1.

In group 1 during the resistance induction period two sheep showed moderate clinical signs. In sheep 2 showed clinical signs 93h and 286h after the start of administration. In both occasions, the administration was suspended and restarted after 74 and 60h later, respectively. In sheep 3 the administration was suspended 119h and 262h after the start of ingestion and restarted 47h and 133h later.

During challenge, the mean time to the observation of first clinical signs of the G1 (resistant) (89h27min±15h32min) was significantly higher than that of G3 (control) (23h15min±00h37min) ($P<0.05$). The average value of heart rate (HR) of the G1 (resistant), (77±14bpm) was significantly lower than the HR of the G3 (control) (126±25bpm) ($P<0.05$). The period of observation of the first clinical signs of the G2 sheep after the first challenge with the administration of 1g kg^{-1} during three days (100h47min±11h48min) and after the second challenge with one dose of 3g kg^{-1} (29h44min±10h13min) were significantly higher than those of controls groups G3 (23h15min±00h37min) and G4 (13h10min±03h06min) ($P<0.05$). There were no significant differences between the values of HR in group 2 during the challenge with 1g kg^{-1} daily during three days (84±17bpm) and the challenge with 3g kg^{-1} in one administration (119±57bpm). But the HR was significantly higher ($P<0.05$) in the control groups: 126±25bpm in G3, and 176±17bpm in G4. The four sheep from Group 4 (control) died when challenged with a single dose of 3g kg^{-1}, while the sheep from G2 sheep (transfauned) challenged with the same dose survived. Sheep 3 from this group were

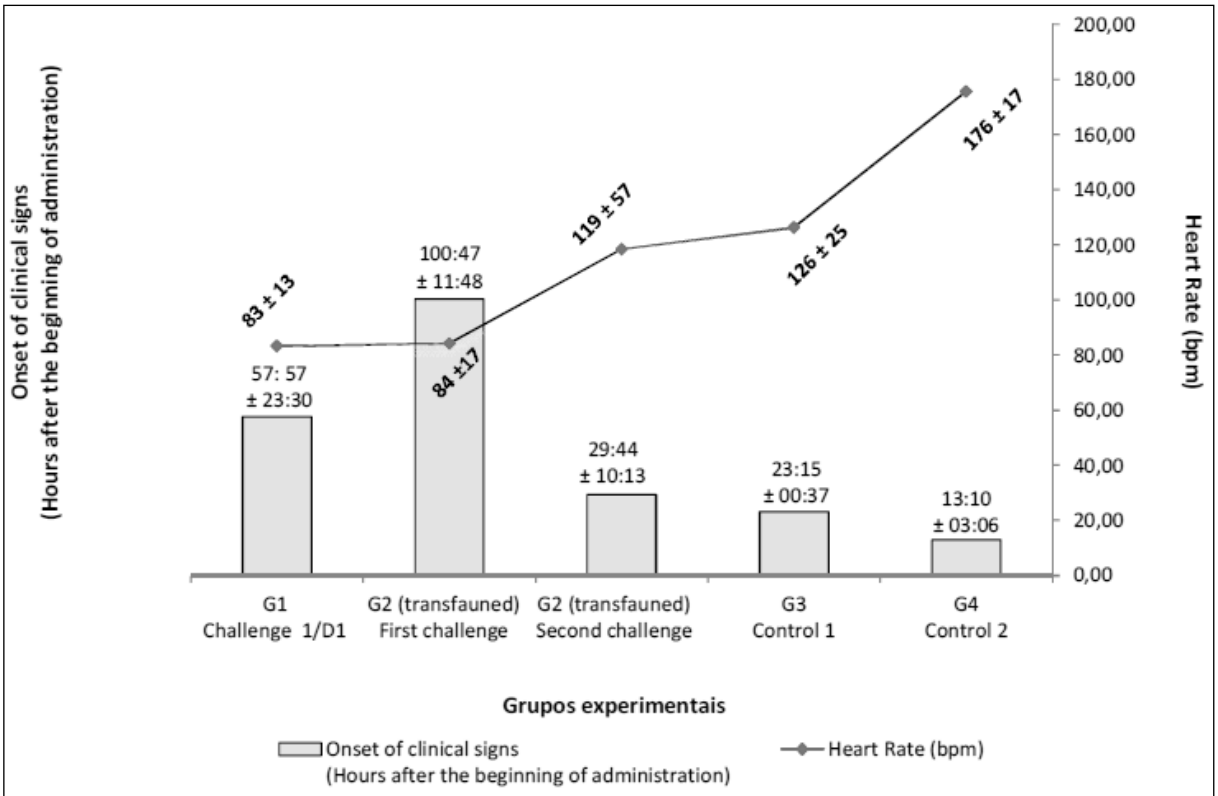

Figure 1 - Graphical representation of the average time for the onset of clinical signs and the average heart rate (bpm) of the four groups of sheep in the different phases of *Amorimia pubiflora* administration. In G1 resistance was induced by daily administration of non-toxic doses of *A. pubiflora*, in G2 resistance was induced by tranasfaunation of ruminal fluid from resistant sheep. G3 and G4 were controls without resistance induction.

challenged with a single dose of 3g kg^{-1} 166 days after the end of the experiment also survived, but died when challenged with a single dose of 5g kg^{-1} two weeks later.

DISCUSSION

Results of this experiment demonstrated that the daily intake of non-toxic doses of *A. pubiflora* to sheep increases the resistance to poisoning by this plant. They also demonstrated that this resistance can be transferred by transfaunation of rumen fluid from resistant sheep to susceptible sheep that never ingested *A. pubiflora*. Similar results were obtained by DUARTE et al. (2013) who demonstrated an increase in the resistance to poisoning by *A. septentrionalis* in goats after the repeated ingestion of non-toxic doses of this plant and also that this resistance may be transferred by transfaunation of ruminal fluid to susceptible goats.

Farmers from Colniza, MT reported that cattle coming from regions where "sudden death" did not occurwere more susceptible to *A. pubiflora* poisoning, which can be an indication that the animals become resistant to the consumption of MFA-containing plants (BECKER et al., 2013). Another possibility is that the higher resistance is due to the death of the more susceptible animals in the areas where MFA-containing plants occurred. Similar observations report that animals grazing in areas with *A. rigida* are more resistant to poisoning than those kept in areas without this plant (SILVA et al., 2008).

Resistance has been related with the presence of ruminal MFA-degrading microorganisms in the rumen, which contain a dehydrogenase enzyme that breaks the carbon-fluorine bond, producing fluorine and glycolate (FETZNER & LINGENS, 1994). Thirteen fungi and 11 bacteria that degrade MFA were isolated from soil samples in Australia (TWIGG & SOCHA, 2001). In the state of Paraíba seven MFA-degrading bacteria were isolated from soil or plants (CAMBOIM et al., 2012a) and two were isolated from the rumen of goats (CAMBOIM et al., 2012b). In the state of Mato Grosso, two MFA-degrading bacteria, *Enterococcus faecalis* and *Bacillus* sp., were isolated from the rumen content of cattle (PIMENTEL, 2011). In Australia a

genetically modified ruminal bacteria, ***Butyrivibrio fibrisolvens***, was used to increase resistance to MFA poisoning (GREGG et al., 1998). The increase of the resistance to ***A. pubiflora*** by the continuous administration of small doses of this plant and the transference of the resistance to other sheep by the transfaunation of ruminal fluid indicated that MFA-degrading bacteria may be normal inhabitants of the ruminal flora and that the ingestion of non-toxic doses of MFA-containing plants stimulates their multiplication (CAMBOIM et al., 2012b).

These results showed that the increase of resistance conferred by the ingestion of non-toxic doses of MFA-containing plants, transfaunation of rumen fluid from resistant animals, and intraruminal inoculation of MFA-degrading bacteria (PESSOA et al. 2015) may be used to prevent poisoning by MFA-containing plants. However, these possibilities should be considered along with other alternatives, such as the knowledge of the epidemiology of the poisoning, chemical or mechanical control of the plant, and the restriction on the use areas with the presence of the plants. In Mato Grosso, manual removal or chemical control of ***A. pubiflora*** in the pastures and the use of fences to isolate the affected areas has not been effective because the plant has deep taproots, which predisposes the regrowth, and is generally present in all pastures.

Survival of sheep 3 challenged, 166 days after the induction of the lethal toxic dose of 3g kg^{-1}, indicates a possibility of durable resistance to this period. However, the death of this sheep, 14 days after, with the dose of 5g kg^{-1} suggested that despite a significant increase in resistance the animals remain susceptible to high doses of the plant. New experiments are needed to determine the most efficient ways to induce resistance and to use this technique in the field to prevent the poisoning.

CONCLUSION

The results of this experiment demonstrated that the daily intake of non-toxic single doses of ***A. pubiflora*** increases the resistance of sheep to the poisoning by this plant and that resistance can be transferred by transfaunation of rumen content from resistant to susceptible sheep.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was approved by ETHICS IN ANIMAL USE COMMITTEE (CEUA) of the Universidade Federal de Mato Grosso (UFMT), through the process number 23108.014901/11-7 of May 13, 2011.

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Administração repetida de doses não tóxicas de monofluoroacetato de sódio não protege contra a intoxicação por este composto em ovinos[1]

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ABSTRACT.- Santos A.C., Riet-Correa F., Heckler R.F., Lima S.C., Silva M.L., Rezende R., Carvalho N.M. & Lemos R.A.A. 2014. [**Repeated administration of non-toxic doses of sodium monofluoroacetate does not protect against poisoning by this compound in sheep**.] Administração repetida de doses não tóxicas de monofluroacetato de sódio não protege contra a intoxicação por este composto em ovinos. *Pesquisa Veterinária Brasileira 34(7):649-654.* Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Av. Senador Filinto Müller 2443, Campo Grande, MS 79074-460, Brazil. E-mail: lap.famez@ufms.br

With the objective to assess whether repeated non-toxic doses of sodium monofluoroacetate (MFA) induce resistance to poisoning by this compound, 18 sheep were randomly divided into two experimental groups of nine animals each. Sheep from Group 1 ingested non-lethal increasing doses of MFA for six periods: 0.05mg/kg for 5 days; 0.08mg/kg for 4 days; 0.08mg/kg for 4 days; 0.1mg/kg for 3 days; 0.1mg/kg for 3 days and 0.25mg/kg for 3 days. Between the first and second period of administration and between the second and third period the animals did not receive MFA for 10 consecutive days, between the third and fourth period and during the remaining periods of administration the sheep were left 15 days without ingesting MFA. Group 2 was not adapted to the ingestion of MFA and received a single dose of 1mg/kg of MFA at the same time that Group 1 was challenged. After challenge, seven sheep of Group 1 showed clinical signs of poisoning and one sheep recovered. In Group 2, all animals showed clinical signs of poisoning by MFA, however two sheep recovered. The mortality rate was 66.6% in Group 1 and 77.7% for Group 2. These results suggest that repeated administration of non-toxic doses of MFA does not protect against acute poisoning by this compound; therefore other alternatives of prophylaxis for poisoning by plants containing MFA should be searched, mainly the use of intraruminal bacteria that hydrolyze MFA.

INDEX TERMS: Sodium Monofluoroacetate, plant poisoning, *Palicourea* spp., *Amorimia* spp., *Tanaecium bilabiatum*, induced resistance, sheep.

RESUMO.- Com o objetivo de avaliar se repetidas doses não tóxicas de monofluoroacetato de sódio (MFA) induzem resistência à intoxicação por essa substância, 18 ovinos foram distribuídos aleatoriamente em dois grupos experimentais de nove animais cada. Os ovinos do Grupo 1 ingeriram doses crescentes não letais de MFA por seis períodos: 0,05mg/kg por 5 dias; 0,08mg/kg por 4 dias; 0,08mg/kg por 4 dias; 0,1mg/kg por 3 dias; 0,1mg/kg por 3 dias e 0,25mg/kg por 3 dias. Entre o primeiro e o segundo período de administração e entre o segundo e o terceiro período os animais não receberam o MFA por 10 dias consecutivos; entre o terceiro e o quarto período e dentre os demais períodos de adminis-

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tração, os ovinos permaneceram 15 dias sem ingerir o MFA. Quinze dias após o último período de administração os ovinos foram desafiados com a dose única de 1mg/kg de MFA. O Grupo 2 não foi adaptado a ingestão de MFA, estes ovinos receberam dose única de 1mg/kg de MFA no mesmo período em que o G1 foi desafiado. No desafio sete ovinos do Grupo 1 apresentaram sinais clínicos da intoxicação e um ovino se recuperou. No Grupo 2 todos os animais manifestaram quadro clínico da intoxicação por MFA, no entanto, dois ovinos se recuperaram. Os coeficientes de mortalidade foram de 66,6% para o Grupo 1 e de 77,7% para o Grupo 2. Os resultados deste trabalho sugerem que a administração repetida de doses não tóxicas de MFA não protege contra a intoxicação aguda por este composto, portanto, outras alternativas para a profilaxia da intoxicação por plantas que contêm MFA deverão ser pesquisadas, principalmente a utilização intraruminal de bactérias que hidrolisam MFA.

TERMOS DE INDEXAÇÃO: Monofluoroacetato de sódio, intoxicação por plantas, *Palicourea* spp., *Amorimia* spp., *Tanaecium bilabiatum*, indução de resistência, ovinos.

INTRODUÇÃO

O monofluoroacetato de sódio (MFA) é uma substância extremamente tóxica para muitas espécies animais (Omara & Sisodia 1990) e já foi identificado em diversas plantas na África do Sul (Marais 1944, Vickery et al. 1973, Nwude et al.1977) e na Austrália (McEwan 1964, Everist 1974, Baron et al. 1987). A toxicidade do MFA ocorre, exclusivamente, pela ação do fluorocitrato, um metabólito tóxico formado no organismo por meio da denominada "síntese letal". O fluorocitrato bloqueia competitivamente a aconitase (Krebs et al. 1994) e impede a conversão do citrato em isocitrato, resultando no acúmulo de grandes quantidades de citrato nos tecidos. Com isso, há diminuição da produção de ATP e os processos metabólicos dependentes de energia são bloqueados (Peters 1952). Descreve-se que em ruminantes a morte sobrevenha pelo efeito intenso sobre o coração (Schultz et al. 1982, Jubb et al. 2007).

No Brasil, MFA foi isolado em *Palicourea marcgravii* (Oliveira 1963, Cook et al. 2014), *Palicourea aeneofusca* (Lee et al. 2012, Cook et al. 2014), *Palicourea amapaensis*, *Palicourea grandiflora*, *Palicourea* aff. *juruana*, *Palicourea longiflora*, *Palicourea* aff. *longiflora*, *Palicourea macarthurorum*, *Palicourea nigricans* e *Palicourea vacillans* (Cook et al. 2014), *Amorimia rigida* (Cunha et al. 2012, Lee et al. 2012), *Amorimia septentrionalis* e *Amorimia pubiflora* (Lee et al. 2012), e *Tanaecium bilabiatum* (*Arrabidaea bilabiata*) (Krebs et al. 1994). Plantas conhecidas por causarem insuficiência cardíaca aguda em ruminantes são responsáveis por cerca de 500.000 mortes de bovinos por ano (Tokarnia et al. 2012). Em bovinos, descrevem-se as seguintes doses tóxicas para algumas dessas plantas: 0,5-2,0g/kg para *P. marcgravii* (Barbosa et al. 2003), 0,75g/kg para *P. aeneofusca*(Tokarnia et al. 1983), 5,0-20,0g/kg para *A. pubiflora*(Tokarnia et al. 1973), 5-10g/kg para *A. exotropica* e 0,65-2,5g/kg para *A.* aff. *rigida* (Tokarnia et al. 1985). Para ovinos, experimentalmente, relatam-se as doses tóxicas para as plantas subsequentes: *P. marcgravii* 0,5-1,0g/kg (Tokarnia et al. 1986), *A. pubiflora*1,5-20g/kg (Becker et al. 2013) e *A. septentrionalis* 5,0-20g/kg (Vasconcelos et al. 2008).

Na Austrália, a possibilidade de prevenir intoxicações por MFA em ruminantes através da inoculação ruminal de bactérias geneticamente modificadas, contendo um gene que codifica uma fluoroacetato dehalogenase tem sido investigada (Gregg et al. 1994, Gregg et al. 1998). No Brasil, estudos para implantar medidas de controle e profilaxia efetivas para as intoxicações por plantas que contenham MFA encontram-se em desenvolvimento pelo Instituto Nacional de Ciência e Tecnologia para o Controle das Intoxicações por Plantas (INCTCIP). Em trabalhos realizados por este grupo de pesquisa foi demonstrado que a administração diária e contínua de doses não tóxicas de *A. septentrionalis* (Duarte et al. 2013) e *P. aeneofusca* (Oliveira et al. 2013) administradas em períodos alternados, o que permite a detoxificação do MFA, induz resistência contra intoxicações por essas plantas. Descreve-se, também, que esta resistência pode ser transmitida de um animal resistente para um animal suscetível mediante a transfaunação de conteúdo ruminal (Duarte et al. 2013). Esses resultados somados aos estudos realizados por Camboim et al. (2012a,b) sugerem algumas possibilidades a serem investigadas para o controle das intoxicações por plantas que contenham MFA: 1) administração repetida, por períodos alternados, de doses não tóxicas da planta; 2) administração de MFA em doses não tóxicas que permita a proliferação de bactérias que tenham atividade de dehalogenases; e 3) administração de outro substrato, não tóxico, que estimule a proliferação de bactérias com atividade de dehalogenases. Até o momento, não há estudos que avaliem capacidade de se induzir resistência à intoxicação utilizando o MFA, uma substância de alta toxicidade.

O presente trabalho tem como objetivo avaliar se é possível induzir resistência à intoxicação por MFA em ovinos mediante a administração de doses crescentes não letais de MFA por períodos alternados, desafiando-os com dose tóxica única do princípio tóxico.

MATERIAL E MÉTODOS

Para a realização do experimento, foram utilizados 18 ovinos divididos aleatoriamente em dois grupos. Os ovinos eram provenientes de áreas sem a ocorrência da intoxicação por plantas do gênero *Amorimia*, sem raça definida, sendo 16 machos não castrados e duas fêmeas, com cinco a sete meses de idade e pesos entre 20-40 kg, previamente vermifugados (Nitroxinil 34%). Os ovinos foram mantidos em baias individuais com área de 2,0 m^2, alimentados com ração comercial para a espécie (equivalente a 1% do peso vivo), feno de *Cynodon dactylon* (Tifton), sal mineral e água à vontade. Cada ovino recebeu um número de identificação (Ovinos 1-18) e foi submetido ao exame clínico completo antes da administração do MFA.

O Grupo 1 (G1) foi constituído por nove ovinos machos, não castrados, que receberam doses crescentes não letais de MFA por seis períodos: 0,05mg/kg por 5 dias; 0,08mg/kg por 4 dias; 0,08mg/kg por 4 dias; 0,1mg/kg por 3 dias; 0,1mg/kg por 3 dias e 0,25mg/kg por 3 dias. Entre o primeiro e o segundo período de administração e entre o segundo e o terceiro período os animais não receberam o MFA por 10 dias consecutivos; entre o terceiro

e o quarto período e dentre os demais períodos de administração, os ovinos permaneceram 15 dias sem ingerir o MFA. Durante cada período de fornecimento do MFA foram realizados exames clínicos que consistiam em verificação das frequências cardíaca e respiratória, da temperatura retal, da motilidade ruminal e do comportamento do animal. Quinze dias após o último período de administração os ovinos foram desafiados com a dose única de 1mg/kg de MFA. As doses administradas foram estabelecidas com base naquelas descritas por Humphreys (1988) e Peixoto et al. (2010); a metodologia foi adaptada de Duarte et al. (2013) e Oliveira et al. (2013). O Grupo 2 (G2), considerado como grupo controle positivo, foi composto por sete ovinos machos não castrados e duas fêmeas. Estes ovinos receberam dose única de 1mg/kg de MFA no mesmo período em que o G1 foi desafiado.

Durante o período de desafio (dose única de 1mg/kg de MFA), os ovinos foram observados em intervalos de 2 horas, no entanto, com o início dos sinais clínicos o intervalo de observação foi reduzido para cada 30 minutos. Não houve movimentação dos animais em nenhum momento após a administração da dose tóxica de MFA.

Para o experimento utilizou-se MFA (Sigma-AldrichCo.[7]), com grau de pureza >95%, diluído no momento da administração com 10mL de água destilada. Os ovinos que morreram foram necropsiados imediatamente após a morte, com a coleta de todos os órgãos, fixados em formol tamponado a 10%. Após a fixação, os fragmentos foram clivados, desidratados em álcool etílico absoluto, diafanizados em xilol, incluídos em parafina e cortados em micrótomo à espessura de 4μ, e os cortes corados pela hematoxilina-eosina (HE) para exame histopatológico. Para o cálculo de mortalidade foram avaliados os grupos G1 e G2 separadamente, considerando a relação entre a frequência absoluta de mortes e o número de animais sob risco.

O experimento foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFMS e protocolado sob o nº 537/2013.

RESULTADOS

No Grupo 1, durante os cinco primeiros períodos de indução de resistência nenhum animal apresentou sinais clínicos de intoxicação por MFA. No último período (0,25mg/kg de MFA por 3 dias), os Ovinos 1 e 3 apresentaram apatia, hiporexia a anorexia, leves tremores nos membros posteriores, cabeça e pescoço, micção frequente, ataxia, espas-

[7] Sigma-Aldrich, 3050 Spruce Street, St Louis, MO 63103, USA <http://www.sigma-aldrich.com>.

Quadro 1. Principais dados sobre o período de desafio, evolução, sinais clínicos e desfecho da indução de resistência à ingestão de monofluoroacetato de sódio (MFA) em ovinos

	Identificação	Sexo	Início dos sinais clínicos após desafio com MFA	Evolução da intoxicação	Sinais clínicos	Desfecho
Grupo 1	Ovino 1	Macho	8h e 59min	4h	Discreta ataxia, tremores musculares em membros, cabeça e pescoço, hiperexcitação, decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 2	Macho	23h e 15min	1h e 10min	Ataxia, tremores musculares em membros, cabeça e pescoço, hiperexcitação, decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 3	Macho	14h	39h e 50min	Apatia, anorexia, ataxia, tremores musculares em membros, cabeça e pescoço, decúbito esternal e lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 4	Macho	5h	18h e 15min	Apatia, anorexia, depressão, ataxia, tremores musculares em membros, cabeça e pescoço, relutância em andar, dispneia, grunhidos expiratórios, decúbito lateral e movimentos de pedalagem, opistótono, nistagmo, discreta quantidade de espuma esbranquiçada em narinas e boca	Morte
	Ovino 5	Macho	24h	72h	Apatia, anorexia, depressão, discreta ataxia, relutância em andar, decúbito esternal a maior parte do tempo	Recuperação
	Ovino 6	Macho	-	-	Não foram observados sinais clínicos	Morte[a]
	Ovino 7	Macho	-	-	Ausência de sinais clínicos	-[b]
	Ovino 8	Macho	-	-	Ausência de sinais clínicos	-[b]
	Ovino 9	Macho	-	-	Não foram observados sinais clínicos	Morte[c]
Grupo 2	Ovino 10	Macho	14h	82h	Ataxia, tremores musculares em membros, cabeça e pescoço, relutância em andar, micção frequente e hiporexia	Recuperação
	Ovino 11	Macho	8 h	39min	Ataxia, tremores musculares em membros, cabeça e pescoço, relutância em andar, quedas em decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 12	Macho	14h	1h	Ataxia, tremores musculares em membros, cabeça e pescoço, relutância em andar, quedas em decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 13	Macho	8h 40min	7 h	Ataxia, tremores musculares em membros, cabeça e pescoço, relutância em andar, permaneceu em decúbito esternal seguido por decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 14	Macho	8 h	10min	Ataxia, tremores musculares em membros, cabeça e pescoço, queda em decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 15	Macho	14h	82h	Ataxia, tremores musculares em membros, cabeça e pescoço, relutância em andar, micção frequente, decúbito esternal na maior parte do tempo e hiporexia	Recuperação
	Ovino 16	Macho	8 h	39min	Ataxia, tremores musculares em membros, cabeça e pescoço, relutância em andar, quedas em decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 17	Fêmea	13h	11h e 25min	Discreta ataxia, tremores musculares em membros, cabeça e pescoço, dispneia, quedas em decúbito lateral, opistótono, nistagmo e movimentos de pedalagem	Morte
	Ovino 18	Fêmea	24h	15h e 15min	Ataxia, tremores musculares em membros, cabeça e pescoço, decúbito esternal e lateral, opistótono, nistagmo e movimentos de pedalagem	Morte

[a] Encontrado morto 14h após a administração de MFA, [b] Não apresentaram sinais clínicos de intoxicação por MFA, [c] Encontrado morto 12h e 58min após a administração de MFA.

ticidade, relutância em andar e decúbito esternal a maior parte do tempo. Os sinais clínicos foram observados cerca de 14 horas e 7 horas, respectivamente, após a terceira dose de 0,25mg/kg de MFA. Os Ovinos 1 e 3 mostraram melhora do quadro clínico com recuperação 58 horas e 66 horas, respectivamente, após a terceira administração de MFA. Estes animais foram mantidos no experimento para o período de desafio.

No período de desafio com dose única de 1,0mg/kg de MFA, para o Grupo 1, o tempo decorrido entre a administração e a manifestação dos primeiros sinais clínicos variou de 5h a 24h. A evolução da intoxicação foi de 1h10min a 39h50min para os Ovinos 1, 2, 3 e 4. Em dois animais (Ovinos 6 e 9) não foram observados sinais clínicos da intoxicação por MFA, no entanto, foram encontrados mortos 14h e 12h58min, respectivamente, após a administração do princípio tóxico. Apenas um animal (Ovino 5) demonstrou recuperação do quadro clínico com cerca de 72h após os primeiros sinais da intoxicação. Os Ovinos 7 e 8 não manifestaram sinais clínicos da intoxicação por MFA, conforme o Quadro 1.

Nos sete ovinos do Grupo 1 em que foram observados sinais clínicos, verificaram-se apatia, anorexia, ataxia, tremores musculares em membros, cabeça e pescoço, depressão, relutância em andar e dificuldade quando iam deitar-se, dispneia e grunhidos expiratórios, hiperexcitação e decúbito esternal a maior parte do tempo. Na fase final, em geral, os animais caíam em decúbito lateral com movimentos de pedalagem, apresentavam respiração ofegante, opistótono, nistagmo, esticavam os membros e morriam (Quadro 1).

No Grupo 2, o intervalo entre a administração do MFA e a manifestação dos sinais clínicos ocorreu entre 8h e 24h, com evolução clínica de 39min a 15h15min. Dois animais (Ovinos 10 e 15) apresentaram recuperação do quadro clínico 82h após demonstrarem sinais de intoxicação. Os sinais clínicos consistiam em ataxia, tremores musculares em membros, cabeça e pescoço, micção frequente, hiporexia, relutância em andar, decúbito esternal a maior parte do tempo, quedas em decúbito lateral com movimentos de pedalagem, opistótono e nistagmo (Quadro 1).

As alterações macroscópicas foram semelhantes para o Grupo 1 e o Grupo 2, caracterizadas por aurículas, veias cavas caudal e cranial, jugulares, ázigos, costo cervical, ilíacas e pulmonares leve a acentuadamente ingurgitadas, discreto a moderado hidropericárdio e raras petéquias no pericárdio. Os pulmões apresentavam-se não colapsados ("armados"), lisos, brilhantes, pesados, com áreas irregulares avermelhadas e discretamente congestos. Havia discreta quantidade de espuma esbranquiçada na traquéia próximo a bifurcação dos brônquios. O fígado estava acastanhado intercalado com áreas irregulares avermelhadas. Os rins apresentavam cortical pálida ao corte em sete animais (Ovinos 1, 4, 9, 11, 12, 13 e 16) e nos demais, cortical e medular difusamente avermelhadas. No miocárdio do Ovino 3 havia áreas pálidas.

Os principais achados histopatológicos foram observados no rim, fígado e coração conforme Quadro 2. No rim, de todos os ovinos avaliados, havia discreta a moderada deposição de material amorfo eosinofílico na luz de túbulos contorcidos e no espaço capsular. Apenas oito ovinos (Ovinos 2, 3, 4, 6, 12, 13, 17 e 18) apresentaram discreta a moderada tumefação e vacuolização (degeneração hidrópico-vacuolar) dos túbulos contorcido distais associadas à picnose nuclear. Havia ainda, discreta a moderada tumefação e vacuolização dos túbulos contorcidos proximais (Ovinos 1, 2, 3, 9, 17 e 18), múltiplos focos discretos de infiltrado linfoplasmocitário intersticial (Ovinos 1 e 16) e discreta a moderada congestão. No fígado, em geral, observaram-se discreta a acentuada tumefação e vacuolização difusa dos hepatócitos e discreta necrose individual aleatória de hepatócitos. Em um animal (Ovino 18) havia cirrose hepática associada a cristais birrefringentes na luz de ductos biliares e macrófagos espumosos, no entanto, este ovino não apresentava sinais clínicos de intoxicação por *Brachiaria* spp. No coração de nove ovinos (Ovinos 1, 3, 4, 6, 9, 12, 13, 14 e 16) havia discreta vacuolização intracitoplasmática

Quadro 2. Principais alterações histopatológicas e intensidade das lesões observadas nos rins, fígado e coração dos ovinos intoxicados por monofluoroacetato de sódio (MFA) durante o período desafio, da indução de resistência à ingestão de MFA em ovinos

	Identificação	Rim (TCD[a])		Rim (TCP[b])		Fígado		Coração	
		Tumefação e vacuolização	Picnose nuclear	Tumefação e vacuolização	Picnose nuclear	Tumefação e vacuolização	Necrose individual aleatória	Vacuolização intracitoplasmática	Retração celular e aumento da eosinofilia
Grupo 1	Ovino 1	-	-	+	-	(++)	+	+	+
	Ovino 2	+	+	++	-	+++	+	-	-
	Ovino 3	+	+	+	-	++	+	+	+
	Ovino 4	+	+	-	-	++	+	+	+
	Ovino 6	+	+	-	-	+++	+	+	+
	Ovino 9	-	-	+	-	++	+	+	+
Grupo 2	Ovino 11	-	-	-	-	++	+	-	-
	Ovino 12	++	++	-	-	(++)	+	+	+
	Ovino 13	+	+	-	-	++	+	+	-
	Ovino 14	-	-	-	-	++	+	+	+
	Ovino 16	-	-	-	-	+	+	+	+
	Ovino 17	++	++	+	-	++	+	-	-
	Ovino 18	++	++	+	-	+	+	-	-

[a] TCD = Túbulo contorcido distal, [b] TCP = Túbulo contorcido proximal, +++ Lesão acentuada, (++) moderada a acentuada, ++ moderada,+discreta, - ausente.

das fibras musculares e discreta retração celular, aumento da eosinofilia e picnose nuclear em fibras musculares individuais aleatórias. No cerebelo e em fragmentos de córtex do Ovino 18, observaram-se discretas formações vacuolares de diferentes tamanhos na substância branca próximo a junção com a substância cinzenta. Nos demais órgãos não foram encontradas alterações significativas.

Os coeficientes de mortalidade foram de 66,6% para o Grupo 1 e de 77,7% para o Grupo 2.

DISCUSSÃO E CONCLUSÃO

A metodologia utilizada não foi eficiente para induzir a resistência à intoxicação por MFA em ovinos. Ainda que, no Grupo 1 três ovinos tenham sobrevivido, dos quais, os Ovinos 7 e 8 não apresentaram nenhuma manifestação clínica de intoxicação e o Ovino 5 recuperou-se em 72 horas após os primeiros sinais da intoxicação, o coeficiente de mortalidade foi de 66,6%, enquanto para o Grupo2 esse coeficiente foi de 77,7%. Em estudos anteriores para indução de resistência a intoxicação por *A. septentrionalis* (Duarte et al. 2013) e *P. aeneofusca* (Oliveira et al. 2013), com a administração de doses não letais crescentes dessas plantas, nenhum caprino morreu durante ou após o período de desafio. Em ambos os experimentos, todos os animais do grupo controle adoeceram e houve 33,3% de mortalidade nesse grupo controle, no qual os caprinos não tinham sido adaptados ao consumo da planta.

Embora neste estudo a presença de bactérias que degradem o MFA não tenha sido avaliada, este é o principal mecanismo proposto para a ocorrência da resistência a intoxicação por plantas que tem o MFA como princípio tóxico (Duarte et al. 2013). Os resultados obtidos no presente estudo demonstram que existem ovinos naturalmente resistentes a intoxicação corroborando com as observações de relatos anteriores em que animais criados em áreas livres da planta podem conter bactérias que degradam o MFA em seu rúmen (Camboim et al. 2012b). Porém, a administração de doses não letais crescentes de MFA não foram capazes de provocar o crescimento da população destas bactérias ao ponto de induzir a resistência ao desafio com 1,0 mg/Kg de MFA. Estudos realizados na Austrália demonstram que a inoculação no rúmen de uma estirpe geneticamente modificada de *Butyrivibrio fibrisolvens* com um gene que codifica uma fluoroacetato dehalogenase, proveniente de uma espécie de *Moraxella*, foi eficiente em prevenir a intoxicação por MFA em ovinos (Gregg et al. 1998).

A dose tóxica de MFA empregada durante o período de desafio corresponderia a 16,6g/kg de *A. pubiflora*, se consideramos a concentração de 0,006% de MFA encontrada em amostras de *A. pubiflora* mantidas no Herbário da Universidade de Michigan (Lee et al. 2012). Desta forma, a dose tóxica utilizada estaria de acordo com a variação de toxidez da planta observada para a espécie bovina (0,5-20,0g/kg) e ovina (1,5-20,0g/kg) (Tokarnia et al. 1973, Becker et al. 2013). Ressalta-se que no estado de Mato Grosso do Sul a *A. pubiflora* é uma das plantas tóxicas mais importantes, responsável por surtos de morte súbita em bovinos (Lemos et al. 2011).

Graus variáveis de degeneração hidrópico-vacuolar ocorreram em 61,5% dos ovinos deste estudo, diferente do observado por Peixoto et al. (2010) em que todos os ovinos intoxicados com MFA apresentaram lesão renal. Esta não é uma alteração específica, mas pode ser um achado diagnóstico em animais intoxicados por plantas que contêm esse princípio tóxico (Oliveira et al. 2004, Helayel et al. 2009, Nogueira et al. 2010, Tokarnia et al. 2012). As lesões hepáticas de tumefação e vacuolização foram observadas em todos os ovinos, com intensidade discreta a moderada e distribuição difusa. Alterações semelhantes foram descritas em um ovino intoxicado por MFA, no entanto, a lesão se restringia, predominantemente, a regiões periportais (Peixoto et al. 2010). No coração de oito ovinos havia discreta necrose de coagulação de fibras musculares individuais, caracterizada por retração celular, aumento da eosinofilia do citoplasma com perda das estriações e núcleos picnóticos, além de vacúolos intracitoplasmáticos observados em algumas células. Esta alteração já foi descrita em bovinos (Pavarini et al. 2012) e caprinos (Oliveira et al. 2013) intoxicados por plantas que contêm o MFA. Em bovinos intoxicados por *A. exotropica* descreve-se também áreas multifocais e coalescentes de fibrose cardíaca (Soares et al. 2012).

Em trabalhos anteriores foi comprovado que a administração de doses não tóxicas de plantas que contem MFA aumentam a resistência contra a intoxicação por essas plantas e foi elaborada a hipótese de que doses não toxicas de MFA poderiam também aumentar a resistência a intoxicação por essa substância (Duarte et al. 2013, Oliveira et al. 2013). Os resultados deste trabalho sugerem que a administração repetida de doses não tóxicas de MFA não protege contra a intoxicação aguda por esta substância, portanto, outras alternativas para o controle das intoxicações por plantas que contêm MFA deverão ser pesquisadas, principalmente a utilização de bactérias que hidrolisam MFA.

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