

**UNIVERSIDADE FEDERAL DE CAMPINA GRANDE
CENTRO DE SAÚDE E TECNOLOGIA RURAL
CAMPUS DE PATOS - PB
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA**

**PLANTAS HEPATOTÓXICAS DA PARAÍBA: 1. FIBROSE HEPÁTICA PERIACINAR
CAUSADA POR *Tephrosia cinerea* EM OVINOS; 2. INTOXICAÇÃO ESPONTÂNEA
AGUDA EM OVINOS POR *Crotalaria retusa* E CONTROLE BIOLÓGICO DA PLANTA.**

Kézia dos Santos Carvalho

Patos / PB

2009



**CENTRO DE SAÚDE E TECNOLOGIA RURAL
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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA EM RUMINANTES
E EQUIDEOS**

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Trabalho apresentado ao Curso de Pós-Graduação em Medicina Veterinária, UFCG, Centro de Saúde e Tecnologia Rural, Campus de Patos – PB. Como requisito para obtenção do Título de Mestre em Medicina Veterinária em Ruminantes e Equideos.

Orientador: Dr. Franklin Riet - Correa.

Mestranda: Kézia dos Santos Carvalho.

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Introdução

As plantas hepatotóxicas, podem ser classificadas de acordo com a ação patológica em plantas que causam fibrose hepática, plantas que causam necrose e plantas que provocam fotossensibilização secundária (Riet-Correa & Mendez 2007). No semi-árido nordestino as plantas que causam fibrose hepática são *Crotalaria retusa* (Nobre et al. 2005) e *Tephrosia cinerea* (Santos et al. 2007, Silva et al. 2006). Diferentes espécies de *Brachiaria*, incluindo *B. decumbens*, *B. brizantha* e *B. humidicola* causam fotossensibilização secundária (Riet-Correa & Mendez 2007) e *Crotalaria retusa* e *Cestrum laevigatum* causam necrose centrolobular. Em bovinos *C. retusa* pode também causar fotossensibilização (Santos et al 2008).

Na região semi-árida da Paraíba a intoxicação por *Crotalaria retusa* ocorre em eqüinos, bovinos e ovinos nas formas aguda e crônica, causando necrose centrolobular e fibrose hepática (Nobre et al 2004). Em ovinos a forma aguda ocorre quando ingerem sementes durante a época seca ou quando há escassez de forragem (Nobre et al. 2005, Dantas et al. 2004) .

Tephrosia cinerea caracteriza-se por sua resistência à seca e capacidade de se manter verde durante a maior parte do ano, sendo uma planta muito comum na região do semi-árido nordestino. É uma importante invasora que se torna dominante em áreas onde o solo apresenta pouca cobertura vegetal como resultado de práticas agrícolas erradas. Esta planta causa uma doença conhecida como barriga de água e afeta principalmente ovinos (Santos et al. 2007).

Este trabalho tem como objetivo descrever estudos realizados com *Tephrosia cinerea* e *Crotalaria retusa* em ovinos. Um trabalho refere-se à reprodução experimental da intoxicação por *Tephrosia cinerea* em ovinos; e o segundo trabalho relata um surto de intoxicação espontânea aguda em ovinos por *Crotalaria retusa* no município de Serra Negra do Norte, Rio Grande do Norte e o controle biológico de pastagem infestada por esta planta com ovinos.

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CAPITULO I

Fibrose hepática periacinar causada por *Tephrosia cinerea* em ovinos

Tradução do trabalho a ser enviado para publicação na revista
Veterinary Pathology como brief communication. (anexo I)

Fibrose hepática periacinar causada por *Tephrosia cinerea* em ovinos

K. S. CARVALHO, R. M. T. MEDEIROS, A. F. M. DANTAS, J. C. A. SANTOS, S. S. Barros,
F. RIET-CORREA

Titulo curto: Fibrose hepática por *T. cinerea* em ovinos

Autor para Correspondência: Franklin Riet-Correa, Laboratório de Patologia veterinária, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Hospital Veterinário, Patos, Avenida Universitária,S/N, Bairro Santa Cecília, Patos, PB, 58708-110, Brasil.

E-mail: franklin.riet@pq.cnpq.br. Fone: 55-83-34239735, fax: 55-83-34233231.

Fibrose hepática periacinar causada por *Tephrosia cinerea* em ovinos

K S CARVALHO, R M T MEDEIROS, A F M DANTAS, J C A SANTOS, S. S. Barros, F
RIET-CORREA

Hospital Veterinário Universidade Federal de Campina Grande, Patos 58708-110, PB, Brasil (KSC, RMTM, AFMD, JCAS, FRC), Laboratório Regional de Diagnóstico, Universidade Federal de Pelotas, Pelotas, 96010-900, Brasil (SSB).

Resumo - *Tephrosia cinerea* tem sido associada com ascite e fibrose hepática em ovinos. A planta foi administrada *ad libitum*, por 60-80 dias, a três ovinos. Outros três ovinos foram utilizados como controle. Todos os ovinos tratados tiveram hipoproteïnemia e aumento das atividades de γ -glutamyltransferase e aspartato aminotransferase, após uma semana de ingestão da planta. Progressiva perda de peso, desidratação, anorexia e ascite foram observadas após 45-60 dias de consumo da planta. Na necropsia havia presença de líquido nas cavidades torácica, abdominal e pericárdica e o fígado estava esbranquiçado, com superfície nodular e consistência firme. Ao exame histológico, a principal lesão do fígado foi fibrose periacinar e subcapsular associada à hemorragia e necrose. A microscopia eletrônica do fígado evidenciou tumefação das células endoteliais, hiperplasia do retículo endoplasmático em hepatócitos, e fibras colágenas entre os hepatócitos e no espaço de Disse. A doença é semelhante à intoxicação por *Galenia africana* em ovinos e à doença veno-oclusiva.

Palavras-chave: Ascite, fibrose hepática periacinar, doença veno-oclusiva, plantas hepatotóxicas, hipertensão portal.

Tephrosia cinerea da família Leguminosae, está associado a uma doença caracterizada por ascite e fibrose hepática progressiva em ovinos, no semi-árido brasileiro, nos estados do Rio Grande do Norte, Ceará^{4,6} e Paraíba (Carvalho 2008, dados não publicados), Nordeste do Brasil. A doença ocorre durante a estação seca em áreas severamente invadidas por *T. cinerea* e ovinos

apresentam sinais clínicos de intoxicação depois de algumas semanas ou meses de consumo da planta. A morbidade varia entre 10% e 70% e a letalidade é de aproximadamente 50%. Se o rebanho é transferido, no início dos sinais clínicos, para outras áreas sem a planta, a maioria dos ovinos afetados se recuperam e novos casos não são observados. A intoxicação foi reproduzida experimentalmente em 2 ovinos: um que tinha se recuperado espontaneamente da doença e que ingeriu a planta durante 40 dias, e outro criado em uma área livre de *T. cinerea* e ingeriu a planta por 230 dias.⁴ Este ovino apresentou lesões características de amiloidose no fígado e rim.⁴ O objetivo deste trabalho foi demonstrar que *T. cinerea* é a causa da doença e estudar a patologia da intoxicação.

Seis ovinos mestiços de Santa Inês, machos, com 3-5 meses de idade, pesando 14-17 kg, criados em uma área sem *T. cinerea* foram utilizados no experimento. Partes aéreas de *T. cinerea*, em fase de sementação, foram coletadas em uma fazenda onde ocorreu a doença, no município de Caicó, Rio Grande do Norte, durante fevereiro e março de 2007. A planta foi secada à sombra por sete dias e armazenada em sacos plásticos. Os três ovinos experimentais receberam feno da planta diariamente, *ad libitum*, e os outros três ovinos foram utilizados como controle e recebiam feno de *Cynodon dactylon* (capim tifton). Todos os ovinos receberam também 1% do peso vivo de ração comercial para ovinos e água *ad libitum*. Durante o experimento, os animais foram alojados em baias individuais e, diariamente, examinados clinicamente. Os ovinos 1, 2 e 3 apresentaram os primeiros sinais clínicos de intoxicação 45, 60 e 50 dias após o início da ingestão, respectivamente. Todos tiveram perda progressiva de peso, apatia, pêlos secos e sem brilho, perda de apetite e desidratação moderada. Os Ovinos 1 e 2 tiveram distensão abdominal bilateral. Líquido amarelo translúcido foi obtido na paracentese abdominal nos três ovinos. O sangue foi coletado no início do experimento e a cada 6-9 dias após o início da ingestão. Atividades de γ -glutamyltransferase (GGT), aspartato aminotransferase (AST) e concentrações de proteína total foram determinadas utilizando método padrão.⁵ Na análise bioquímica foi observado hipoproteinemia (Fig. 1), hipoalbumemia (Fig.2) e aumento das atividades de AST (Fig.3) e GGT (Fig.4).

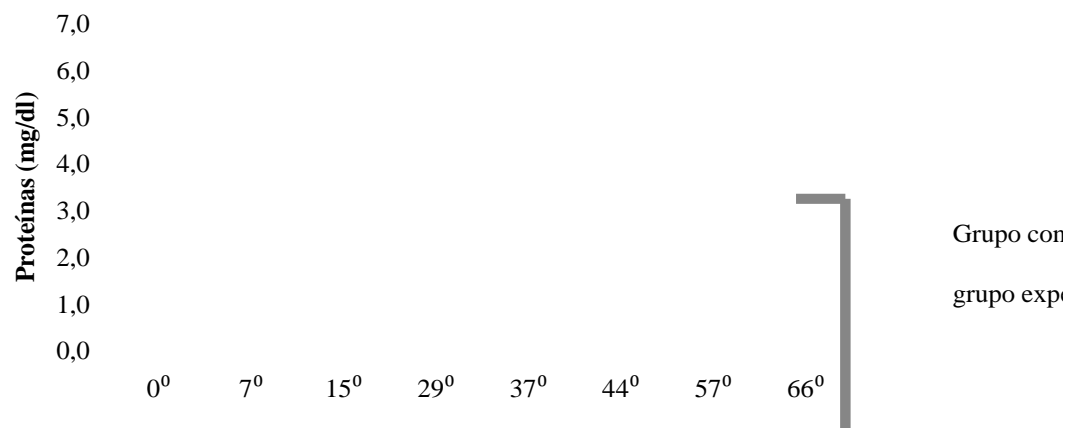


Fig.1: Valores sorológicos médios de proteínas totais dos grupos experimental e controle antes da administração da planta e durante o experimento.

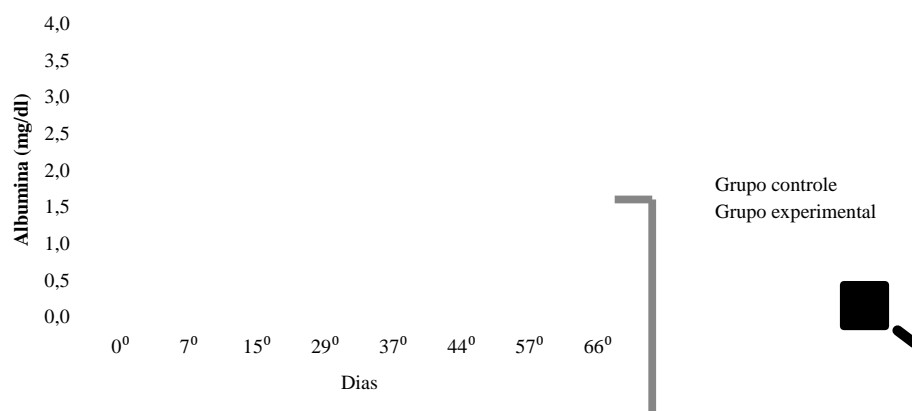


Fig. 2: Valores sorológicos médios de albumina dos grupos experimental e controle antes da administração da planta e durante o experimento..

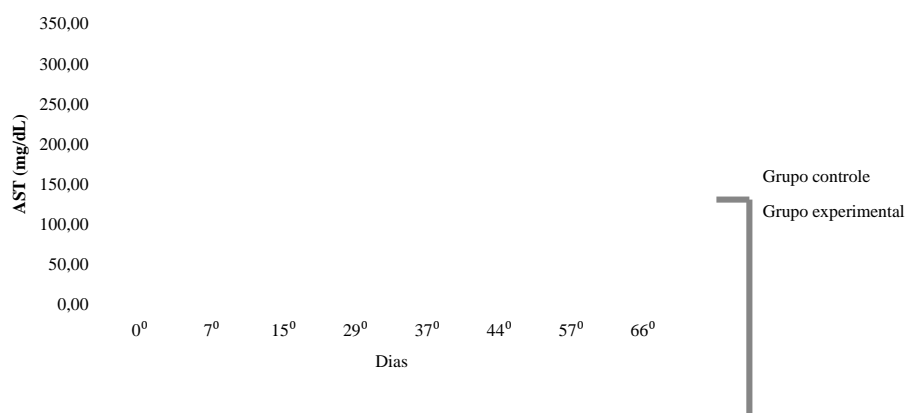


Fig.3: Valores médios sorológicos de AST dos grupos experimental e controle antes da administração da planta e durante o experimento.

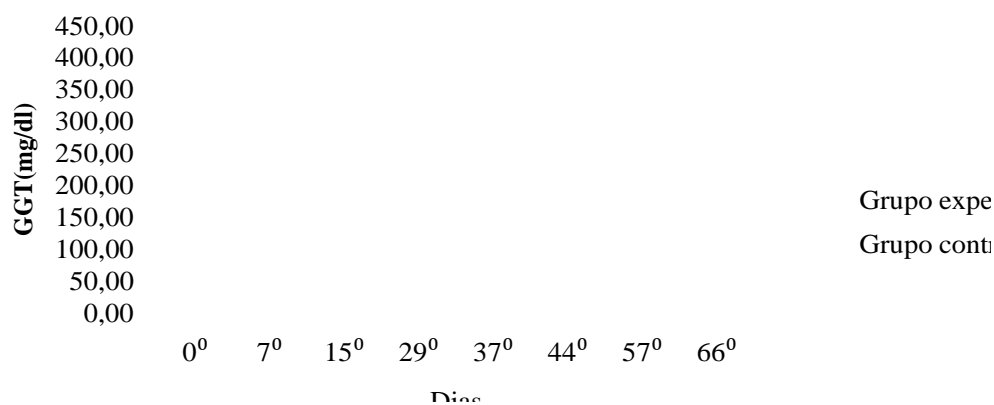


Fig.4 Valores médios sorológicos de GGT dos grupos experimental e controle antes da administração da planta e durante o experimento.

O ovino 1 foi eutanasiado após dez dias do início dos sinais clínicos e os ovinos 2 e 3, 20 dias após os primeiros sinais. Na necropsia do Ovino 1 observou-se dois litros de líquido translúcido na cavidade abdominal. O fígado apresentava filamentos de fibrina com superfície capsular difusamente nodular e irregular com áreas escurecidas (Fig. 5). Ao corte estava firme

com áreas esbranquiçadas interpostas por áreas avermelhadas. O mesentério apresentava-se gelatinoso e espessado por líquido de edema e também se observavam anastomoses porto-sistêmicas. Os linfonodos mesentéricos e outros linfonodos da cavidade abdominal estavam edemaciados. Dilatação dos vasos linfáticos foram observadas na parede da vesícula biliar. Havia ainda, no saco pericárdico, aproximadamente 10-20 ml de líquido transparente amarelado. Os demais ovinos apresentaram lesões semelhantes, sendo que o ovino 2 tinha 1,7 L de líquido abdominal, além de 75 ml líquido na cavidade torácica e 15-20 ml dentro do saco pericárdico e o ovino 3 apresentava pouco líquido abdominal (170 ml) e discreto hidropericárdio.

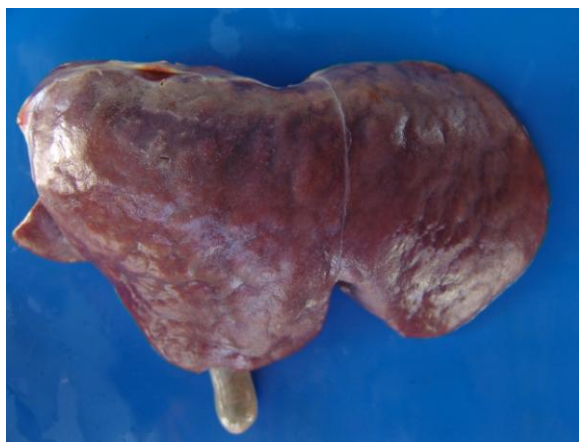


Fig. 5: Fígado com superfície capsular difusamente nodular e irregular com áreas escurecidas.

Amostra de órgãos das cavidades torácica e abdominal e do sistema nervoso central foram fixados em formol tamponado a 10% e processados rotineiramente para avaliação histológica e corados pela hematoxilina e eosina (HE). Seções histológicas do fígado foram selecionadas e coradas com o tricrômico de Masson para tecido conjuntivo. Ao exame histológico do fígado dos três animais experimentais observou-se fibrose periácinar, formando, freqüentemente, pontes interlobulares (Fig.6). A fibrose era mais acentuada no Ovino 1 do que nos Ovinos 2 e 3. Nas áreas de fibrose observavam-se, freqüentemente, duas ou mais veias periacinares (Fig.7). Em algumas áreas havia discreta fibrose entre os cordões de hepatócitos e desorganização estrutural do padrão histológico. Na região periácinar, associada às áreas de fibrose, observavam-se necrose e hemorragias. Ocasionalmente, áreas de necrose e hemorragias se juntavam a áreas de

necrose dos lóbulos adjacentes. Observou-se fibrose com espessamento da cápsula de Glisson e proliferação para o parênquima da região subcapsular (Fig. 8). Ocasionalmente o tecido fibroso circundava grupos de hepatócitos que ficavam isolados. Hepatócitos do parênquima subcapsular estavam severamente vacuolizados. Discreta vacuolização e necrose individual de hepatócitos foram observadas na zona periportal e mediozonal. Moderada proliferação de células epiteliais dos ductos biliares e leve fibrose periportal também foram observados. (Fig.9).

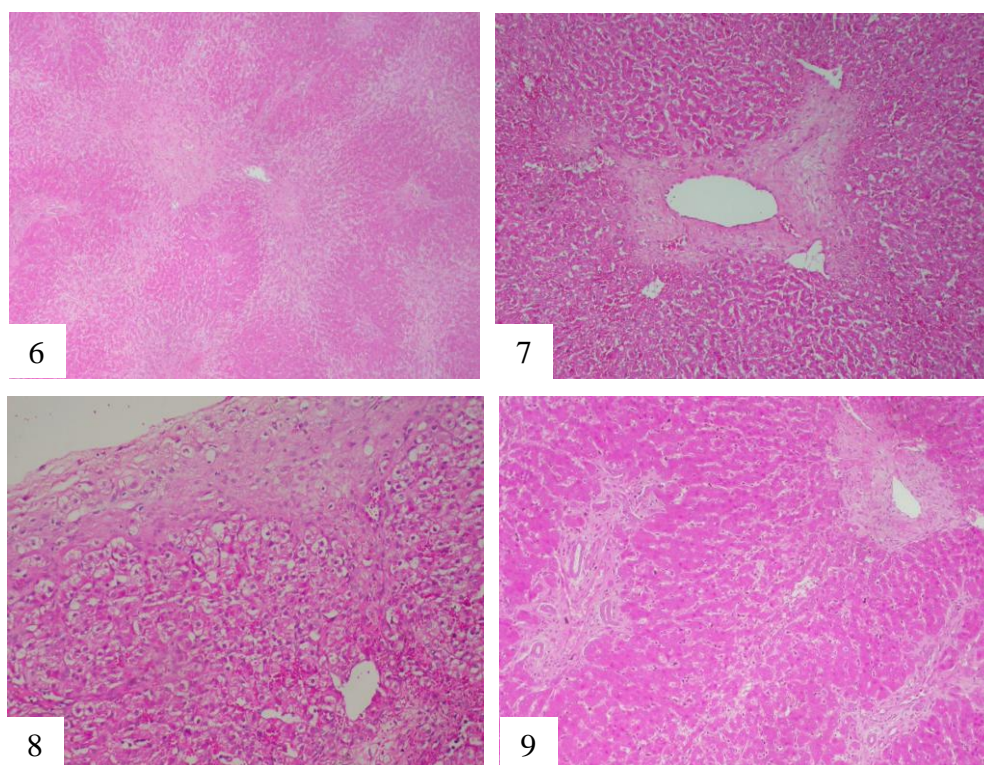


Fig. 5: A-Fibrose periacinar, formando pontes interlobulares. Fig. 7: Neovasos sanguíneos são observados na área de fibrose periacinar. Fig 8: Fibrose com espessamento da cápsula de Glisson com proliferação para o parênquima. Fig 9: Moderada proliferação de células epiteliais de ductos biliares e leve fibrose

Nos Ovinos 1 e 3, fragmentos do fígado foram colhidos imediatamente após a eutanásia e fixados em glutaraldeído a 2% com 2% de paraformaldeído, em 0,4 M de solução tampão de cacodilato de sódio (pH 7,4), pós-fixadas em tetróxido ósmio a 1%, tamponado em cacodilato de sódio a 0,4 M (pH 7,4), e embebidos em Epon 812. Cortes finos foram corados com azul de metileno. Cortes ultrafinos foram corados com citrato de chumbo e acetato de uranila e

examinados com um microscópio eletrônico de transmissão Zeiss EM109 80 kV. No exame ultra-estrutural, foi observado, em ambos ovinos, acentuada tumefação das células endoteliais dos sinusoides obstruindo o lúmen sinusoidal (Fig.10), com eritrodiapedese para o espaço de Disse. Os hepatócitos estavam degenerados ou necróticos com hiperplasia do retículo endoplasmático liso associado à grânulos de glicogênio. Proliferação de fibras colágenas estava presente entre os hepatócitos e no espaço de Disse (Fig.11). No Ovino 3, imagens negativas de cristais foram observadas dentro do retículo endoplasmático liso dilatado.

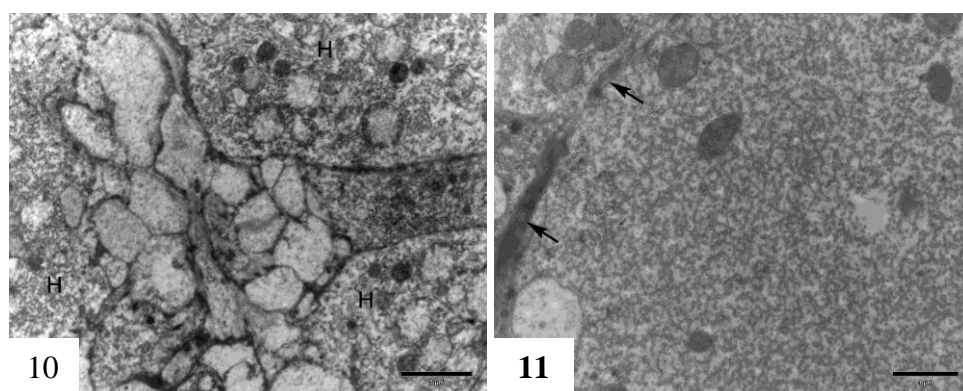


Fig. 10: Capilar sinusoidal com células acentuadamente tumefeitas obstruindo a luz do capilar. Hepatócitos (H). Fig. 11: Hepatócito com acentuada hiperplasia do retículo endoplasmático liso. Algumas fibras colágenas aparecem entre hepatócitos (setas).

Os resultados deste estudo demonstram que a doença é causada pela ingestão de *Tephrosia cinerea* como proposto por Santos et al.⁴ No entanto, os sinais clínicos neste experimento começaram 45-60 dias após o início da administração, enquanto que no experimento anterior⁴, ocorreram depois de 200 dias de consumo da planta em um ovino que consumiu apenas as sementes da planta durante os primeiros 143 dias e, posteriormente, feno de toda a planta. Estes resultados sugerem que as sementes não são tóxicas e que a intoxicação só ocorre pela ingestão da parte aérea da planta.

A principal lesão histológica encontrada neste experimento foi fibrose periácinar associada com necrose e hemorragia. Nos estudos iniciais realizados por Santos et al.⁴, as principais lesões histológicas encontradas foram relatadas como fibrose periportal. A histologia do fígado dos casos espontâneos e experimentais estudados por Santos et al.⁴ foram revisados e

todos tinham fibrose periacinar, confirmando que esta é a principal lesão de intoxicação por essa planta. Santos et al⁴, também relataram amiloidose do fígado e de outros órgãos em um caso experimental, sugerindo que esta lesão foi devida à linfadenite caseosa. A ausência de amiloidose nos três ovinos deste experimento confirma que a planta não causa amiloidose.

Nos casos estudados neste trabalho e em casos experimentais relatados por Santos et al.⁴ a fibrose está associada com hemorragia e necrose, porém, em casos espontâneos a fibrose pode ou não estar associados com hemorragia e necrose.⁴ Moderada fibrose, com ausência de hemorragia e necrose, foi observada em dois animais eutanasiados poucas semanas depois de serem retirados das pastagens invadidas por *T. cinerea* (Santos et al. 2007, dados não publicados). Estas observações sugerem que as lesões, incluindo a fibrose, são reversíveis e estão em concordância com informações relatadas por proprietários de que animais com sinais clínicos podem se recuperar.

Os sinais clínicos e lesões da intoxicação por *T. cinerea* são semelhantes aos relatados na intoxicação por *Galenia africana* em ovinos e caprinos no sul da África.^{3,8} Em ambas, as plantas, a lesão primária é a fibrose periacinar hepática mas, na intoxicação por *G. africana* animais com sinais clínicos avançados podem desenvolver hipertrofia miocárdica de miócitos, com subsequente degeneração, necrose e fibrose.⁸ Estas lesões cardíacas não foram encontradas na intoxicação por *T. cinerea*, mas poderão ocorrer nos casos mais avançados, uma vez que, de acordo com Van Der Lugt et al⁸ afetam os animais com período de manifestação clínica mais longo e são secundárias à lesão hepática.

A fibrose periacinar é a principal lesão observada na doença veno-oclusiva (VOD), que é comum em humanos, bovinos e cães intoxicados por alcalóides pirrolizidínicos (APs).⁷ Na VOD a lesão primária ocorre no endotélio dos sinusóides, e é seguida por extravasamento de eritrócitos, depósitos de fibrina, necrose de hepatócitos e subsequente fibrose periacinar.^{2,7} Na intoxicação por *T. cinerea*, a acentuada dilatação das células endoteliais dos sinusóides, associada com extravasamento de hemácias e alterações degenerativas e necrose de hepatócitos observados na microscopia eletrônica, sugerem que o mecanismo de dano hepático nesta intoxicação é semelhante ao sugerido para a VOD. APs não foram encontrados em *T. cinerea*.⁴

A acentuada fibrose subcapsular com projeções para o parênquima hepático é a principal causa do aparecimento de nódulos na superfície do fígado. A fibrose periacinar aumenta a

resistência do fluxo portal intra-hepático pós-sinusoidal causando aumento da pressão hidrostática⁶, que provavelmente, associado à hipoproteinemia, é a principal causa de ascite na intoxicação por *T. cinerea*. A hipertensão portal também induz angiogênese (neovascularização) na região periacinar e anastomoses porto-sistêmicas. Aumento das atividades séricas da GGT e AST foram observados no exame realizado 7 dias após a administração, atingindo o máximo de atividade em 16 e 39 dias, respectivamente, sugerindo que essas enzimas podem ser utilizadas para a identificação precoce dos animais afetados. No semi-árido, a intoxicação por *T. cinerea* deve ser diferenciada da intoxicação crônica por *Crotalaria retusa* em ovinos, que também causa fibrose hepática e ascite, mas também causa encefalopatia hepática e megalocitose.¹ No entanto, para o diagnóstico definitivo é necessário inspecionar a área onde os ovinos estão pastando para observar a presença de *T. cinerea* ou *C. retusa*.

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CAPITULO II

Intoxicação espontânea aguda por *Crotalaria retusa* em ovinos e controle da planta com ovinos

Tradução do trabalho a ser enviado para publicação na revista Toxicon (Anexo II) como Short communication.

Intoxicação espontânea aguda por *Crotalaria retusa* em ovinos e controle da planta com ovinos

K. S. Carvalho^a, A. F. M. Dantas^a, Rosane M. T. Medeiros^a, F. Riet-Correa^{a1}

^a Laboratório de Patologia veterinária, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Hospital Veterinário, Patos, Avenida Universitária,S/N, Bairro Santa Cecília, Patos, PB, 58708-110, Brasil.

Resumo

Após o diagnóstico de intoxicação aguda por *Crotalaria retusa*, 21 ovinos adultos do rebanho afetado retornaram ao pasto para consumir a planta que estava em brotação. Devido ao contínuo consumo da planta a mesma foi secando progressivamente. Os ovinos não apresentaram sinais clínicos nem alterações bioquímicas e pariram cordeiros saudáveis durante o período experimental. Os resultados obtidos neste experimento demonstram que ovinos podem ser usados para controle biológico de *C. retusa*.

Palavras chave: *Crotalaria retusa*, ovinos,intoxicação aguda,controle biológico.

A intoxicação aguda por *Crotalaria retusa* em ovinos (Nobre et al. 2005) e a intoxicação crônica em ovinos (Dantas *et al.* 2004), bovinos (Nobre *et al.* 2004a) e eqüídeos (Nobre *et al.* 2004b) ocorrem a campo no semi-árido do Nordeste do Brasil. A intoxicação crônica é mais freqüente em eqüídeos, provavelmente porque a planta é mais palatável para esta espécie (Riet-Correa e Méndez 2007), e também porque equídeos são mais susceptíveis do que bovinos e ovinos à ação da monocrotalina (Cheeke 1998). Recentemente, foi demonstrado que ovinos são susceptíveis à intoxicação aguda por monocrotalina e podem ser intoxicados por uma única dose oral de cerca de 205,2 mg/kg de peso vivo. Em contrapartida, eles desenvolvem uma grande resistência a monocrotalina após a ingestão diária de doses não tóxicas (136,8 mg/kg) (Anjos *et al.* 2009, *in press*). A intoxicação aguda por *C. retusa* em ovinos ocorre após a ingestão de sementes que contêm concentrações de monocrotalina, maiores do que outras partes da planta (Nobre *et al.*

¹ Correspondências para o autor. Tel.: +55 83 34239734 fax: +55 83 34239537.
E-mail: franklin.riet@pq.cnpq.br (F. Riet-Correa).

2005, Anjos *et al.* 2009, *in press*). Ovinos ingerindo grandes quantidades da planta sem sementes aparentemente não são afetados (Anjos *et al.* 2009 *in press*). Ovinos são também resistentes à intoxicação crônica por *Senecio* spp e têm sido utilizado para o controle biológico da planta (Méndez, 1993), mas em algumas condições eles podem ser intoxicados (Ilha *et al.* 2001, Schild *et al.* 2007).

O objetivo deste trabalho foi relatar um surto de intoxicação espontâneo aguda por *Crotalaria retusa* em ovinos, e determinar se é possível a utilização de ovinos resistentes para o controle biológico desta planta.

Um surto de intoxicação aguda por *C. retusa* ocorreu no Município de Serra Negra do Norte, no Estado do Rio Grande do Norte, entre julho e agosto de 2007, em um rebanho de 150 ovinos mestiços da raça Santa Inês. O rebanho tinha sido transferido, 20 dias antes do surto, para uma área severamente invadida por *C. retusa* que estava sementando e, que tinha sido utilizada para o cultivo de arroz, milho e mandioca. Trinta e quatro (22,7%) ovinos foram afetados e morreram em aproximadamente 30 dias. O rebanho foi retirado do pasto 20 dias após o início do surto; neste momento 26 ovinos haviam morrido e 6 apresentavam sinais clínicos. Duas ovelhas foram afetadas após a retirada do rebanho do pasto. Dos 34 ovinos que morreram, cinco eram ovinos adultos e os outros tinham 3-6 meses de idade, incluindo alguns cordeiros lactentes. Machos e fêmeas foram afetados.

Os sinais clínicos foram distensão abdominal com ascite, moderada icterícia, apatia e anorexia. O curso clínico na maioria dos animais foi de 2-5 dias, mas uma ovelha morreu após manifestar sinais clínicos por um período de 21 dias. Em seis ovinos com sinais clínicos, níveis séricos de bilirrubina total e direta e atividades séricas de aspartato aminotransferase (AST), e gama glutamiltransferase (GGT) estavam aumentadas (Tabela 1).

Tabela 1. Bioquímica sangüínea de seis ovinos intoxicados espontaneamente por *C. retusa*

	GGT (U/L)	AST (U/L)	Bilirrubina total	Bilirrubina direta
Ovino 1	169	350	1,5	0,5
Ovino 2	236	480	2,2	0,4
Ovino 3	299	375	2,9	0,6
Ovino 4	267	210	6,3	1,1
Ovino 5	65	378	0,9	0,3
Ovino 6	16	88	1,1	0,5

Três ovinos foram necropsiados e examinados histologicamente. Na necropsia, os Ovinos 1 e 2, que tinham apresentado sinais clínicos durante 3-4 dias, apresentavam moderada icterícia no tecido subcutâneo e petequias, equimoses e hemorragias no tecido subcutâneo das regiões ventral e lateral do abdômen e tórax. Líquido amarelo translúcido estava presente nas cavidades abdominal e torácica. O fígado estava difusamente vermelho com aumento do padrão lobular, caracterizado por áreas irregulares intercaladas com áreas pálidas. O Ovino 1 tinha filamentos de fibrina na superfície capsular. Hemorragias e edema difuso foram observados na vesícula biliar. Hemorragia e edema estavam presentes no mesentério e parede do abomaso. O Ovino 3, que foi encontrado morto após um curso clínico de 21 dias, apresentou na necropsia discreto grau de autólise; ascite, hidropericárdio, e aumento do padrão lobular do fígado.

Ao exame histológico do fígado dos Ovinos 1 e 2 observou-se necrose hemorrágica difusa periácinar, que ocasionalmente se estendia à zona mediozonal e estava circundada por uma área de hepatócitos tumefeitos ou vacuolizados. O Ovino 3 apresentava fibrose, principalmente periportal, proliferação de células epiteliais dos ductos biliares e megalocitose. Foram observados diferentes graus de hemorragia e edema nos pulmões, abomaso e intestino.

Após o diagnóstico da intoxicação, 20 ovelhas adultas e um carneiro do rebanho afetado retornaram ao pasto em que grande parte da *C. retusa* havia sido consumida pelas ovelhas. Considerou-se que os ovinos que sobreviveram tinham consumido, repetidamente, doses não tóxicas de *C. retusa*, tornando-se resistentes, como sugerido nos experimentos de Anjos *et al.* (2009), e, portanto, poderiam consumir a planta, sem risco de intoxicação.

Os animais permaneceram no pasto até fevereiro de 2009. Durante o período a fazenda foi visitada nove vezes, em intervalos regulares, para inspeção da pastagem. Nas duas primeiras

visitas, realizadas um e três meses após a reintrodução dos ovinos na pastagem, foi realizada coleta de sangue de 20 ovelhas para a determinação das atividades séricas de AST e GGT. Em ambas as ocasiões as atividades de AST e GGT de todas as ovelhas estavam dentro dos limites normais.

Em consequência das severas chuvas, em Maio de 2008, o rebanho foi retirado da pastagem inundada. Quarenta dias após os 21 ovinos foram reintroduzidas na pastagem, juntamente com outros 100 ovinos. Nos próximos meses, um número variável de ovelhas foi mantido na pastagem. Durante todas as visitas, observou-se que os ovinos permanentemente consumiram as folhas jovens das plantas em brotação (Fig 1.) e, aparentemente, de forma preferencial a outras plantas.



Fig:1. *C. retusa* em brotação apresentando folhas jovens consumidas e galhos com partes secas.

Devido ao contínuo consumo dos brotos as plantas morreram e grandes quantidades da planta seca foram observadas durante as visitas. As plantas não produziram flores ou sementes e, no final do período, poucas ainda estavam vivas. A maioria das ovelhas pariram cordeiros saudáveis durante o período experimental. Uma ovelha morreu com sinais clínicos característicos de tétano, 10 dias após a parição. Foi necropsiada e não foram observadas lesões macroscópicas ou histológicas no fígado.

O diagnóstico de *C. retusa* foi realizado com base em dados epidemiológicos, sinais clínicos e lesões macroscópicas e histológicas, semelhantes aos relatados por Nobre *et al.* (2005). Todos os casos foram características de intoxicação aguda, com exceção do Ovino 3, que sobreviveu durante 21 dias depois dos primeiros sinais clínicos, e apresentou lesões de intoxicação crônica por monocrotalina. Resultados similares foram observados experimentalmente em oito ovinos que ingeriram doses únicas de 3-4 g/kg de peso vivo de sementes *C. retusa*. Nestes experimentos, quatro ovelhas morreram agudamente, duas tiveram intoxicação crônica, e um não teve sinais clínicos (Anjos *et al* 2009 *in press*).

Os resultados obtidos no experimento em que o rebanho permaneceu pastando no piquete invadido por *C. retusa*, demonstraram que ovinos podem ser utilizados para o controle biológico da planta. No entanto, alguns pontos devem ser levados em consideração para usar o pastoreio de ovinos para controlar *C. retusa*. Ovinos devem ser introduzidos em pastagem não sementadas de *C. retusa*. Em um experimento anterior um ovino ingeriu uma grande quantidade de partes aérea de *C. retusa* (285,6 kg em 270 dias) sem mostrar sinais clínicos nem lesões no final do experimento. Uma forma de induzir resistência, provavelmente seria introduzir gradualmente ovinos em pastagens invadidas por *C. retusa*, aumentando o tempo de permanência e a quantidade de planta ingerida. Anjos *et al.* (2009) demonstraram que ovinos ingerindo doses baixas de sementes de *C. retusa* desenvolve resistência a doses que causam intoxicação aguda.

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Conclusões

- Os resultados deste estudo confirmam que a doença conhecida como “barriga d’água” é causada pela ingestão de *Tephrosia cinerea*.
- A lesão característica da intoxicação por *T. cinerea* é uma fibrose periacinar, associada a necrose e hemorragia.
- Os sinais clínicos e lesões da intoxicação por *T. cinerea* são semelhantes aos relatados na intoxicação por *Galenia africana* em ovinos e caprinos no sul da África.
- A patogenia da intoxicação por *Tephrosia cinerea* é semelhante a relatada na doença Veneno-oclusiva (DOV).
- Os resultados obtidos no experimento demonstram que ovinos podem ser utilizados para o controle biológico da *Crotalaria retusa*, quando introduzidos em pastagem não sementadas de *C. retusa* para se tornarem resistentes.

ANEXO I

PERIACINAR LIVER FIBROSIS CAUSED BY *Tephrosia cinerea* IN SHEEP

K. S. Carvalho, R. M. T. Medeiros, A. F. M. Dantas, J. C. A. Santos, S. B. Severo, F. Riet-Correa

Short title: Liver fibrosis caused by *tephrosia cinerea* in sheep

Corresponding author: Franklin Riet-Correa- Laboratório de Patologia veterinária, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Hospital Veterinário, Avenida Universitária,S/N, Bairro Santa Cecília, Patos, PB, 58708 110, Brazil. E-mail: franklin.riet@pq.cnpq.br . Fone: 55-83-3423 9735.

Periacinar liver fibrosis caused by *Tephrosia cinerea* in sheep

K.S. Carvalho, R. M. T. Medeiros, A. F. M. Dantas, J. C. A. Santos, S. B. Severo, F. Riet-Correa

Hospital Veterinário Universidade Federal de Campina Grande, Patos 58708-110, PB, Brazil
(KSC, RMTM, AFMD, JCAS, FRC), Laboratório Regional de Diagnóstico, Universidade Federal de
Pelotas, Pelotas, 96010-900, Brazil (SSB).

Abstract. *Tephrosia cinerea* has been associated with ascites and liver fibrosis in sheep. The plant was administrated *ad libitum*, for 60-80 days, to three sheep. Other three sheep were used as control. All treated sheep had hypoproteinemia and increase γ -glutamyltransferase and aspartato aminotransferase activities after one week of plant ingestion. Progressive weight loss, rough hair coat, anorexia and ascites were observed after 45-60 days feeding. At necropsy, liquid was present in the abdominal thoracic and pericardic cavities, and the liver was whitish, with nodular surface and hard consistency. Upon histologic examination the main lesion of the liver was periacinar and subcapsular fibrosis associated with hemorrhages and necrosis. Electron microscopy showed swelling of endothelial cells, hyperplasia of endoplasmatic reticulum in hepatocytes, and collagenous fibers between hepatocytes and in the Disse space. The disease is similar to the poisoning by *Galenia africana* in sheep and to veno-occlusive disease.

Key words: Ascites, hepatic periacinar fibrosis, veno-occlusive disease, hepatotoxic plants, portal hypertension.

Tephrosia cinerea, from the Leguminosae family, had been associated with a disease in sheep characterized by ascites and progressive liver fibrosis, in the semiarid rangelands of the states of Rio Grande do Norte, Ceará ^{4,5} and Paraíba (Carvalho 2008, unpublished data), Northeastern Brazil. The disease occurs during the dry season in areas severely invaded by *T. cinerea* and sheep exhibit clinical signs of poisoning after a few weeks or months of consumption of the plant. Morbidity rate varies from 10% to 70% and fatality rate is approximately 50%. If the flock is moved to other areas without the plant, at the start of clinical signs, most affected sheep

recovered and new cases are not observed. The poisoning was reproduced experimentally in 2 sheep: one that had spontaneously recovered from the disease, which ingested the plant for 40 days, and another raised in an area free of *T. cinerea*, which ingested the plant for 230 days.⁴ This sheep apart from the lesions characteristic of the disease showed amyloidosis of the liver and kidney⁴. The objective of this research was to demonstrate that *T. cinerea* is the cause of the disease and to study the pathology of the intoxication.

Six crossbred, male, 3-5 months old, Santa Inês hair sheep, weighing 14-17 kg, raised in an area without *T. cinerea* were used in the experiment.

The aerial parts of *T. cinerea* in seeding stage were collected in a farm where the disease occurs, in the municipality of Caicó, Rio Grande do Norte, during February and March 2007. The plant was dried in the shade for seven days and stored in bags. The three experimental sheep received daily the plant hay *ad libitum* and the other three sheep were used as controls and received hay of *Cynodon dactylon* (tifton grass). All sheep also received 1% of live weight of commercial diets for sheep and water *ad libitum*. During the experiment the animals were housed in individual pens and examined clinically daily.

Sheep 1, 2 and 3 showed first clinical signs of poisoning 45, 60 and 50 days after the start of ingestion, respectively. All had progressive loss of weight, apathy, rough hair coat dry, loss of appetite and moderate dehydration. Sheep 1 and 2 had bilateral abdominal distention. Translucent yellow liquid was obtained on abdominal paracentesis in the three sheep.

Blood samples were collected at the start of the experiment and every 6-9 days after the start of the ingestion. Serum γ -glutamyltransferase (GGT), aspartate aminotransferase (AST) activities and total protein concentrations were determined using standard methods (Schmidt and Von Forstner, 1986).

In biochemical analysis hypoproteinemia and hypoalbuminemia was observed (Figs. 1 and 2) and increased serum activities of AST (Fig. 3) and GGT (Fig. 4) in animals intoxicated.

Sheep 1 was euthanized ten days after the onset of clinical signs and sheep 2 and 3, 20 days after first signs. At necropsy, sheep 1 had two liters of clear fluid in the abdominal cavity. The liver showed fibrin filaments on the surface, diffusely nodular capsular surface and irregular dark areas (Fig. 5). The cut surface had a hard consistency and irregular dark red mixed with whitish areas. Gelatinous edema of the mesentery and porto-systemic shunts were observed.

Hepatic, mesenteric and other lymph nodes of the abdominal cavity were edematous. Dilated lymphatic vessels were seen on the wall of the gallbladder. The pericardial cavity contained, approximately 10-20 ml of yellowish liquid. Sheep 2 and 3 had similar lesions. Sheep 2 had 1.7 L of abdominal fluid, and 75 ml fluid in the thoracic cavity and 15-20 ml in the pericardial cavity. Sheep 3 sheep had 170 ml of abdominal fluid and mild hydropericardium.

Sample of organs of the thoracic and abdominal cavities and central nervous system were fixed in 10% neutral formalin, processed routinely for histological evaluation and stained by Hematoxylin and Eosin. Selected histological sections of the liver were stained with the Masson trichromic for connective tissue. Upon histologic examination the liver of the three experimental animals showed periacinar fibrosis, often forming interlobular bridges. The fibrosis was more pronounced in Sheep 1 than in Sheep 2 and 3. Two or more hepatic venules were frequently observed within fibrous tissue. The sinusoids were dilated and congested and, occasionally, the connective tissue extended between cords of hepatocytes. Necrosis and hemorrhages were observed associated with periacinar fibrosis. Occasionally, areas of necrosis and hemorrhages joined to one another.

The Glisson capsule was thickened by proliferation of connective tissue, with projections of connective tissue into the liver parenchyma. Occasionally the fibrous tissue surrounded isolated groups of hepatocytes. Hepatocytes of the subcapsular parenchyma were severely vacuolated. Mild vacuolation and single-cell necrosis were observed in the periportal and mid-zone. Moderate proliferation of epithelial cells of bile ducts and mild periportal fibrosis were also observed.

On Sheep 1 and 3, liver fragments were collected immediately after euthanasia and fixed in 2% glutaraldehyde with 2% paraformaldehyde, in 0.4 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide buffered in 0.4 M sodium cacodylate (pH 7.4), and embedded in Epon 812. Semithin sections were stained with methylene blue. Ultrathin sections were stained with lead citrate and uranyl acetate and examined with an EM109 Zeiss transmission electron microscope at 80 kV. On transmission electron microscopy on ultrastructure examination, severe swelling of the sinusoidal endothelial cells, obstructing the sinusoidal lumen with erythrodiapedesis to the Disse space was observed in both sheep examined. The hepatocytes were degenerated or necrotic with conspicuous hyperplasia of the smooth endoplasmic reticulum

associated with glycogen granules. Proliferation of collagen fibers was present in the space between hepatocytes and in the Disse space. In Sheep 3, negative images of crystals were observed within dilated smooth endoplasmic reticulum.

The results of this study demonstrate that the disease is caused by ingestion of *Tephrosia cinerea* as proposed by Santos et al.⁴ However, clinical signs in this started 45-60 days after the start of dosing, while in the previous experiment⁴ occurred after more than 200 days of plant consumption in a sheep that consumed only seeds of the plant during the first 143 days and hay of the whole plant afterwards. These results suggest that seeds are not toxic and that the intoxication only occurs by ingestion of the aerial parts of the plant.

The main histological lesion found in this experiment was periacinar fibrosis associated with necrosis and hemorrhage. In the initial studies done by Santos et al.⁴ the main histological lesions were reported as periportal fibrosis. The histology of the liver of the spontaneous and experimentally cases studies by Santos et al.⁴ were reviewed and all had mainly periacinar fibrosis, confirming that this is the main lesion of this plant poisoning. Santos et al.⁴ also reported amyloidosis of the liver and other organs in their experimental case, suggesting that this lesion was due chronic lymphadenitis. The absence of amyloidosis in our three experimental sheep confirmed that the plant do not cause amyloidosis.

In the cases studied in this work and in the experimental cases reported by Santos et al.⁴ the fibrosis is associated with hemorrhages and necrosis, however in spontaneous cases fibrosis may or may not be associated by hemorrhages and necrosis⁴. Moderate fibrosis, with absence of hemorrhages and necrosis, was observed in two animals euthanized a few weeks after being removed from pastures invaded by *T. cinerea* (Santos et al. 2007, unpublished data). These observations are in agree with the information reported by farms that animals with clinical signs can recover, and suggest that lesions, including fibrosis are reversible.

Clinical signs and lesions in *T. cinerea* poisoning are similar than those reported in *Galenia africana* poisoning of sheep and goats in southern Africa.^{3,8} In both plants the primary lesion is peracinar liver fibrosis, but in *G. Africana* poisoning animals with advanced clinical signs may develop hypertrophy of myocytes with subsequent degeneration, necrosis and fibrosis.⁷ These cardiac lesions were not found in poisoning by *T. cinerea*, but could occur in

more advanced cases, since according to Van Der Lugtet al⁸ these cardiac lesions affect animals with longer clinical manifestation period and are secondary to liver damage.

The periacinar fibrosis is the main lesion observed in veno-occlusive disease (VOD), which is common in humans, cattle and dogs poisoned by pyrrolizidine alkaloids.⁷ In VOD the primary lesion occurs in the sinusoidal endothelium, and is followed by extravasations of erythrocytes, fibrin deposits, necrosis of hepatocytes and subsequent fibrosis periacinai.^{2,7} In *T. cinerea* poisoning, the marked swelling of endothelial cells of sinusoids, associated with extravasation of red cells and degenerative and necrotic changes of hepatocytes observed in electron microscopy suggest that the mechanism of liver damage in this poisoning is similar to that suggested in VOD. APs were not found in *Tephrosia cinerea*.⁴

The severe subcapsular fibrosis with projections into the liver parenchyma is the main cause of the nodular appearance of the liver surface. Periacinar fibrosis increases the intra-hepatic post-sinusoidal portal flow resistance causing increased hydrostatic pressure⁷, which, probably associated hypoproteinemia, is the main cause of ascites in *T. cinerea* poisoning. The portal hypertension also induces angiogenesis (neovascularization) in the periacinar region and porto-systemic shunts.

Increased serum activities of GGT and AST were observed in the first examination, 7 days after administration, reaching the maximum activity at 16 days and 39 days, respectively, suggesting that both enzymes can be used for early identification of animals affected. In semi-arid, the poisoning *T. cinerea* must be differentiated from chronic poisoning by *Crotalaria retusa* in sheep which also causes liver fibrosis and ascites, but the later also causes hepatic encephalopathy and megalocytosis.¹ However, for the definitive diagnosis is necessary to inspect the area where sheep are grazing to observe the presence of *T. cinerea* or *C. retusa*.

T. cinerea poisoning only occurs in pastures invaded by large amounts of the plant. It only occurs in degraded areas under deforestation and intensive agriculture and is associated with deterioration of the *caatinga* (vegetation of the Brazilian semi-arid). To avoid this type of soil and pasture management is the best way to prevent the intoxication.

Acknowledgements – This work was financially supported by the Programa Institutos do Milênio (Proc. CNPq - 420012/2005-2) e for Programa de Apoio a Núcleos de Excelência (PRONEX), grant N° 001/04, CNPq, FAPESQ, MCT.

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Fig. 1. Mean values of serum total protein in the experimental and control groups before the administration of the plant and during the experiment.

Fig. 2. Mean values of albumin in the control group and the experimental group before the administration of the plant and during the experiment.

Fig. 3. Mean values of serum AST in the control group and the experimental group before the administration of the plant and during the experiment.

Fig. 4. Mean values of serum GGT in the control group and the experimental group before the administration of the plant and during the experiment.

Fig. 5. Liver diffusely nodular capsular surface and irregular dark areas.

Fig. 6. **A-** Periacinar fibrosis forming interlobular bridges.

Fig. 7. Two or more hepatic venules within fibrous tissue.

Fig. 8. Moderate proliferation of epithelial cells of bile ducts and mild periportal fibrosis were also observed.

Fig. 9. Moderate proliferation of epithelial cells of bile ducts and mild periportal fibrosis were also observed.

Fig. 10. Severe swelling of the sinusoidal endothelial cells, obstructing the sinusoidal lumen.

Fig. 11. Proliferation of collagen fibers in the space between hepatocytes.

Anexo II

Author Instructions – Veterinary Pathology

Review Process

The manuscript submission and peer review process is broken down into the following 7 steps:

1. The Author submits a manuscript.
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3. The Editor or Associate Editor assigns Reviewers to the manuscript.
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ANEXO III

Spontaneous acute poisoning by *Crotalaria retusa* in sheep and biological control of the plant with sheep

K. S. Carvalho^a, A. F. M. Dantas^a, R.M. T. Medeiros^a, F. Riet-Correa²

^a Laboratório de Patologia veterinária, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Hospital Veterinário, Patos, Avenida Universitária,S/N, Bairro Santa Cecília, Patos, PB, 58700-310, Brazil.

Abstract

After the diagnosis of acute *Crotalaria retusa* poisoning, 21 adult sheep from the affected flock were returned to the paddock and still consuming the sprouting plants. Due to the continuous consumption increasing amounts of dry plants were observed. The sheep had neither clinical signs nor biochemical alterations and delivered healthy lambs during the experimental period. Results obtained in this experiment demonstrated that sheep can be used for the biologic control of *C. retusa*.

KEY WORDS: *Crotalaria retusa*, sheep, acute poisoning, biologic control.

Acute poisoning by *Crotalaria retusa* in sheep (Nobre *et al.* 2005) and chronic poisoning in sheep (Dantas *et al.*, 2004) cattle (Nobre *et al.*, 2004a) and equidae (Nobre *et al.* 2004b) occur in the semiarid range lands of Northeastern Brazil. It is more frequent in equidae, probably because the plant is more palatable for this species (Riet-Correa and Méndez 2007), and also because horses are more susceptible than cattle and sheep to monocrotaline poisoning (Cheeke 1998). Recently it was demonstrated that sheep are susceptible to acute intoxication by monocrotaline being intoxicated by a single oral dose of approximately 205.2 mg /kg bw. In contrast, they develop strong resistance to monocrotaline after the daily ingestion of non toxic doses (136.8 mg/kg) (Anjos *et al.* 2009, *in press*). Acute poisoning by *C. retusa* in sheep occurs after the ingestion of seeds that contain higher concentrations of monocrotaline than other parts of the

² Corresponding author. Tel.: +55 83 34239734, fax: +55 83 34239537.
E-mail: franklin.riet@pq.cnpq.br (F. Riet-Correa).

plant (Nobre *et al* 2005, Anjos *et al* 2009, *in press*). Sheep ingesting high amounts of non seeding plants apparently not affected (Anjos *et al.* 2009 *in press*). Sheep is also resistant to chronic *Senecio* spp poisoning and have been used for the biological control of the plant (Méndez, 1993), but in some conditions they can be intoxicated (Ilha *et al.* 2001, Schild *et al.* 2007).

The objective of this work was to report an outbreak of spontaneous acute poisoning by *Crotalaria retusa* in sheep, and to determine if is possible to use resistant sheep for the biologic control of this plant.

An outbreak of acute poisoning by *C. retusa* occurred in the municipality Serra Negra do Norte, in the state of Rio Grande do Norte, between July and August 2007, in a flock of 150 hair Santa Inês and crossbred sheep. The flock had been transferred, 20 days before the outbreak, to an area severely invaded by seeding *C. retusa*, which were used for rice, corn and cassava cultivation. Thirty four (22.7%) sheep were affected and died in approximately 30 days. The flock was moved from the paddock 20 days after the start of the outbreak; at this time 26 sheep had died and 6 had clinical signs. Two sheep became affected after the withdrawn of the flock from the paddock. Of the 34 sheep that died, five were adult sheep and the other were 3-6 months old, including some lactating lambs. Males and females were affected.

Clinical signs were abdominal distention with ascites, moderate jaundice, apathy and anorexia. The clinical course in most animals was of 2-5 days, but one sheep died after a clinical manifestation period of 21 days. In six sheep with clinical signs, serum levels of total and direct bilirubin and serum activities of aspartate aminotransferase (AST), and γ -glutamyltransferase (GGT) were increased (Table 1).

Three sheep were necropsied and examined histologically. At necropsy, Sheep 1 and 2, that had clinical signs for 3-4 days, had moderate jaundice of the subcutaneous tissue and petechial hemorrhages and ecchymoses of the subcutaneous tissue of the ventral and lateral regions of the abdomen and thorax. Yellowish translucent liquid was present in the abdominal and thoracic cavities. The liver was diffusely red with enhanced lobular pattern and red-dark irregular areas, intermixed with pale areas. Sheep 1 had fibrin filaments in the capsular surface. Diffuse hemorrhages and edema were observed in the gall bladder. Hemorrhages and edema were present in the mesentery and wall of the abomasums. Sheep 3, that was found dead after a clinical course

of 21 days had some degree of autolysis. Ascites, hydropericardium, and enhanced lobular pattern of the liver were observed at necropsy.

Upon histologic examination the liver of Sheep 1 and 2 revealed diffuse periportal hemorrhagic necrosis, occasionally extending to the mid-zone and bordered by an area of swollen or vacuolated hepatocytes. Sheep 3 had fibrosis, mainly periportal, proliferation epithelial bile duct cells, and megalocytosis. Different degrees of hemorrhages and edema were observed in lung, abomasum and intestine.

After the diagnosis of the intoxication, 20 adult sheep and one ram from the affected flock were returned to the paddock in which most *C. retusa* had been consumed by the sheep. It was considered that surviving sheep had consumed repeatedly non toxic doses of *C. retusa* becoming resistant, as suggested in the experiments reported by Anjos *et al.* (2009), and therefore will consume the plant without risk of intoxication. The animals stayed in the paddock until February 2009, during this period the paddock was visited 9 times at regular intervals for inspection of the pasture. In the two first visits, realized one and three months after the reintroduction of the sheep in the paddock, blood was collected from the 20 sheep for the determination of serum activities of AST and GGT. In both occasions AST and GGT activities of all sheep were within normal range.

In consequence of severe rains, in May 2008, the flock was removed from the paddock, which was flooded. Forty days after, the 21 sheep were reintroduced in the paddock together with other 100 sheep. In the next months a variable number of sheep was maintained in the paddock. During all visits it was observed that sheep permanently consumed the young leaves of the sprouting plants (Fig 1), apparently preferentially to other plants. Due to the continuous consumption of the regrows the plants died and increasing amounts of dry plants were observed during the visits. The plants did not produce flowers or seeds and at the end of the period very few plants still alive. Most ewes delivered healthy lambs during the experimental period. One ewe died with clinical signs characteristic of tetanus 10 days after lambing. It was necropsied and no gross or histologic lesions were observed in the liver.

The diagnosis of *C. retusa* poisoning was performed based on epidemiologic data, clinical signs and gross and histologic lesions, similar to those reported by Nobre *et al.* (2005). All cases were characteristics of acute poisoning, except Sheep 3, which survived for 21 days

after first clinical signs, and had lesions of chronic monocrotaline poisoning. Similar results were observed experimentally in a group of eight experimental sheep that ingested single doses of 3-4 g/kg body weight of *C. retusa* seeds. In those experiments four sheep died acutely, two had chronic intoxication, and one had no clinical signs (Anjos *et al* 2009 in press).

Results obtained in the experiment in which the flock still grazing in the paddock invaded by *C. retusa* demonstrated that sheep can be used for the biologic control of the plant. However, some points had to be taken into account to use grazing sheep to control *C. retusa*. Sheep should be introduced in pastures with non seeding *C. retusa*. In a previous experiment a sheep ingested great amounts of the aerial parts of *C. retusa* (285,6 kg in 270 days) without showing neither clinical signs nor lesions at the end of the experiment. A probably way to induce resistance would be to introduce sheep gradually in pastures invaded by *C. retusa*, increasing the time of permanence and the amount of plant ingested. Anjos *et al.* (2009) demonstrated that sheep ingesting low doses of *C. retusa* seeds develop resistance to doses that cause acute poisoning.

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Table 1. Blood biochemistry in six sheep spontaneously poisoned by *C. retusa*

	GGT (U/L)	AST (U/L)	Total bilirubin	Direct bilirubin
Sheep 1	169	350	1,5	0,5
Sheep 2	236	480	2,2	0,4
Sheep 3	299	375	2,9	0,6
Sheep 4	267	210	6,3	1,1
Sheep 5	65	378	0,9	0,3
Sheep 6	16	88	1,1	0,5

Anexo IV

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