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"Organização cromatínica, fragmentação de DNA e morte celular em eritrócitos circulantes de algumas espécies de serpentes"

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SUMÁRIO

1. RESUMO

A freqüência e a intensidade de fragmentação do DNA, relacionadas à morte celular programada, variam nos eritrócitos nucleados dos vários vertebrados. Como os dados de fragmentação de DNA em répteis são escassos, no presente trabalho foram estudados eritrócitos de serpentes (*Crotalus durissus terrificus*, *Bothrops jararaca*, *B. alternatus* e *B. neuwiedii*) em termos de fragmentação de DNA relacionada à supraorganização da cromatina e morte celular. O estudo foi estendido a espécimes de *C. d. terrificus* infectados por *Hepatozoon sp.*. Foram realizados análise de imagem da cinética de hidrólise de Feulgen, ensaio de TUNEL, ensaio Cometa e observações em microscopia eletrônica de transmissão. Poucos eritrócitos foram encontrados em morte celular. O DNA das serpentes, especialmente de *C. d. terrificus* e *B. neuwiedii*, mostrou ser mais resistente à hidrólise ácida de Feulgen e à fragmentação do DNA pelo ensaio TUNEL, quando comparados ao anfíbio *Rana catesbeiana*. Dados de ensaio Cometa mostraram que *B. neuwiedii* também teve menor fragmentação de DNA, enquanto *C. d. terrificus* apresentou maior freqüência de células com danos, talvez por abundância de sítios álcali-sensíveis em seu DNA e não por fragmentação de DNA ligada à morte celular. A ultraestrutura dessas células sugere alguma atividade metabólica devido à presença de mitocôndrias, Complexos de Golgi e nucléolo. *B. jararaca* e *B. alternatus* apresentaram diferenças na fragmentação de DNA/morte celular quando comparados com *C. d. terrificus* e *B. neuwiedii*, sugerindo-se relação com especiação selecionada por diferentes hábitats. Nos espécimes infectados foram encontrados eritrócitos infectados e não-infectados pelo protozoário. A presença de *Hepatozoon sp.* como parasita em serpentes, inclusive no interior dos eritrócitos circulantes,

induz um aumento da fragmentação de DNA em *C. d. terrificus* induzindo células, inclusive não infectadas, à morte celular.

2. ABSTRACT

The frequency and intensity of the DNA fragmentation, related to programmed cell death, varies in nucleated erythrocytes of various vertebrates. As data on DNA fragmentation in reptiles are scarce, in the present work erythrocytes of snakes (*Crotalus durissus terrificus*, *Bothrops jararaca*, *B. alternatus* and *B. neuwiedii*) were studied in terms of DNA fragmentation related to chromatin supraorganization and cell death. The study was extended to *C. d. terrificus* specimens infected by *Hepatozoon sp.*. Image analysis of Feulgen hydrolysis kinetics, the TUNEL assay, the single cell gel electrophoresis, and electron microscopy observations were undertaken. A few circulating erythrocytes were found committed to cell death. The snake's DNA, especially of *C. d. terrificus* and *B. neuwiedii*, showed to be more resistant to the Feulgen acid hydrolysis and to the DNA fragmentation revealed by the TUNEL assay, when compared to the amphibian *Rana catesbeiana*. Data on the Comet assay showed that *B. neuwiedii* had less DNA fragmentation, although *C. d. terrificus* showed more frequency of damaged cells, perhaps of due to abundance of alkali-sensitive DNA sites and not to the DNA fragmentation related to the cell death. The ultrastructure of these cells suggested a certain cell metabolic activity due to the presence of mitochondria, Golgi complexes and nucleolus. *B. jararaca* and *B. alternatus* showed differences in DNA fragmentation/cell death as compared to *C. d. terrificus* and *B. neuwiedii*, possibly related to the selected speciation by the different habitas. In the infected specimens erythrocytes were found infected and non-infected by the protozoon. The presence of *Hepatozoon sp.* as a snake parasite, inclusive inside the circulating erythrocytes, induces increase in the DNA fragmentation of *C. d. terrificus* erythrocytes even of non-infected cells, to cell death.

3. INTRODUÇÃO GERAL

3.1. Problemática

Em vertebrados não mamíferos os eritrócitos circulantes são células nucleadas nas quais a cromatina é reportada como exibindo diferentes graus de condensação sob a forma granular. Os complexos DNA-proteína nessas células contêm além das histonas usuais, a histona H5, tida como uma das responsáveis pelo respectivo empacotamento cromatínico (SUM *et al.*, 1990; RAMAKRISHNAM *et al.*, 1993).

O tempo de vida dos eritrócitos circulantes varia conforme os vários grupos de vertebrados, sendo óbvio que nesse processo participem mecanismos de morte celular programada, em condições normais de metabolismo e saúde. Sob a ação de estímulos ambientais que afetem a saúde e induzam envelhecimento do organismo, esse tempo de vida poderia ser reduzido, advindo então morte por apoptose (WEIGHT *et al.*, 1991; KOSOWER, 1993).

Estudando-se a ocorrência de fragmentação de DNA, tida como parte do programa de morte celular em eritrócitos nucleados de alguns diferentes grupos de vertebrados, observou-se larga variação na sua expressão, estimada em termos de resposta ao método imunocitoquímico de TUNEL (MELLO *et al.*, 2000). Assim, enquanto a maioria dos eritrócitos de frango, de pombo e do anfíbio *Rana catesbeiana* respondiam positivamente ao teste, apenas ao redor de 30% dos eritrócitos de um réptil, como *Geochelone carbonaria* (jabuti), mostraram resposta TUNEL-positiva. Além disso, essa resposta variava nos diferentes grupos de animais, sendo no anfíbio muito forte, porém nas aves e no réptil, de fraca a média (MELLO *et al.*, 2000).

Tendo a ocorrência de fragmentação de DNA sido avaliada pelo teste de TUNEL numa única espécie de réptil, para que alguma afirmação mais generalista de que em répteis o percentual de eritrócitos circulantes sofrendo fragmentação de DNA fosse notadamente mais baixo do que em outros grupos de vertebrados não mamíferos, outras espécies desse grupo mereceriam idêntico estudo. Dados obtidos com o ensaio Cometa seriam igualmente esclarecedores (SINGH *et al.*, 1988; PANDRANGI *et* al., 1995; TICE, 1995; ROJAS *et* al., 1999). Nesse sentido, a análise de eritrócitos de serpentes de diferentes espécies e idades, em condições de manutenção controlada em serpentário, poderia acrescentar dados importantes. Além disso, havendo a possibilidade de análise de eritrócitos de animais parasitados, o que parece não ser um evento raro entre as serpentes (TELFORD Jr, 1994) comparações poderiam ser efetuadas.

Poucos são os relatos citológicos sobre eritrócitos de répteis em geral e de serpentes em particular. Há relato de que em tartarugas os eritrócitos tenham um tempo de vida superior a 700 dias (ALTLAND & BRACE, 1962), enquanto nos mamíferos sabidamente é de 120 dias (SCHMIDT-NIELSEN, 1996). Nos trabalhos em que se enfoca a morfologia de células sanguíneas, os eritrócitos de diferentes espécies de répteis são descritos apenas como apresentando formato elíptico, com núcleo ovóide e central (DESSER & WELLER, 1979; MATEO *et al.*, 1984; EGAMI & SASSO, 1988; ALLERMAN *et al.*, 1992; MOURA *et al.*, 1999). Na tartaruga do deserto (*Gopherus agassizii*), os eritrócitos maturos apresentam pontuação e corpos intracitoplasmáticos, quando observados em microscopia eletrônica; nos eritrócitos imaturos os núcleos são maiores, mais claros e com cromatina frouxa (ALLERMAN *et al.*, 1992). Estudos citoquímicos de sangue de lagartixa de parede ("wall gheckos") e de crocodilos do oeste da

África demonstraram que somente os eritrócitos apresentaram reação para peroxidase ao contrário das outras células do sangue (CAXTON-MARTINS, 1977). Resultados observados por EGAMI & SASSO (1988) para amostras de sangue da serpente *Bothrops jararaca* indicaram reação positiva para proteínas e alguns lipídios nos eritrócitos desses animais.

Poucos dados acham-se reportados quanto ao padrão de organização cromatínica em eritrócitos de serpentes. Tais eritrócitos, se submetidos à reação de Feulgen, poderiam revelar através da sua cinética de labilidade à hidrólise ácida, informações sobre o estado de agregação de seu complexo DNA-proteína (MELLO & VIDAL, 1978; MELLO, 1983, 1997) que poderiam ser associados ao padrão de fragmentação de DNA (inclusive freqüência de células), revelado pelo teste de TUNEL. Curvas de hidrólise de Feulgen são instrumento sensível a pequenas variações em complexos DNA-proteína, tendo já revelado diferenças entre espermatozóides de diferentes espécies (embora até com mesmo padrão de cromatina condensada), entre hetero- e eu-cromatina e entre regiões de cromossomos politênicos com diferente grau de organização e de riqueza em DNA repetitivo (MELLO & VIDAL, 1978; MELLO, 1979; SILVA & MELLO, 1986), bem como em células ao longo de processos de diferenciação e desenvolvimento (AGRELL & BERGQVIST, 1962; SANDRITTER *et al.*, 1965; GARCIA, 1970; SPRENGER *et al.*, 1971). Isto, porque diferente complexação de proteína ao DNA afeta diferentemente sua cinética de hidrólise.

Assim, talvez células cuja fragmentação de DNA estivesse ocorrendo como etapa da morte celular, poderiam, simultaneamente ou antecipadamente, conterem DNA mais lábil, identificável, em função de sua resposta à reação de Feulgen. De outro lado, populações nucleares não engajadas numa etapa tão adiantada da morte celular, poderiam

apresentar uma cinética de hidrólise de Feulgen que demonstrasse menor velocidade na solubilização de seu ácido apurínico (MELLO & VIDAL, 1978; MELLO, 1983, 1997). Numa tal investigação, a análise concomitante de eritrócitos de *Rana catesbeiana* (indivíduos adultos) seria requerida para propósitos comparativos.

3.2. Morte Celular Programada - Apoptose

A morte celular programada (MCP) é um evento fisiológico importante, pois auxilia na manutenção da homeostase nos organismos (PALLARDY *et* al., 1997; ZIMMERMANN *et al.*, 2001), ocorrendo num tempo determinado durante o desenvolvimento desse organismo. Quando essa morte é induzida ou antecipada por estímulos exógenos (exemplos: radiação, drogas, metais pesados, jejum entre outros) ela passa a ser chamada de apoptose. A apoptose pode ocorrer em algumas doenças humanas como mal de Parkinson, doença de Huntington, Esclerose Lateral Amiotrófica (ELA) e alguns cânceres humanos (SALAMI *et* al. 1996). Já quando um organismo sofre uma injúria severa causada por uso de altas doses de uma determinada droga, por exemplo, suas células morrem por um processo chamado de morte celular acidental ou necrose ou oncose (MAJNO & JORIS, 1995; SALAMI *et* al. 1996; MELLO *et al.*, 2001). Esse processo caracteriza-se pela presença de uma resposta inflamatória causada pela liberação do conteúdo da célula morta no meio extracelular, diferentemente da apoptose (ALBERTS *et al.*, 2002).

Inicialmente, durante a apoptose, endonucleases endógenas clivam o DNA em fragmentos nucleossômicos de 185 pb e seus múltiplos, sendo esse processo característico na apoptose. As principais características morfológicas da apoptose são: marginalização e

condensação do DNA, fragmentação nuclear, redução do citoplasma, perda da adesão com as células vizinhas, formação de brotos ou vesículas a partir da membrana plasmática, e formação de corpos apoptóticos (KERR *et* al., 1972; MAJNO & JORIS, 1995; SALAMI *et* al. 1996; MESNER Jr & KAUFMANN, 1997; PALLARDY *et* al., 1997; ZIMMERMANN *et al.*, 2001). Os sinais bioquímicos característicos da apoptose são: externalização da fosfatidilserina, liberação do citocromo c da mitocôndria, clivagem proteolítica de substratos intracelulares como laminas nucleares, clivagem internucleossomal do DNA (MAJNO & JORIS, 1995; SALAMI *et* al. 1996; MESNER Jr & KAUFMANN, 1997; ZIMMERMANN *et al.*, 2001).

HENGARTNER (1997) estudou o controle genético da MCP no nematódio *Caenorhabditis elegans*, que apresentou 131 células mortas durante seu desenvolvimento. Verificou ainda que os produtos dos genes *ced-3* e *ced-4* eram essenciais para a ocorrência da MCP nesses animais. Estudos realizados em mamíferos detectaram a presença de cisteínas proteases com atividade homóloga a dos produtos dos genes *ced-3* e *ced-4* dos nematódios (MELLO *et al.*, 2001; ZIMMERMANN *et al.*, 2001). Essas proteases formam uma grande família de caspases que parecem ser responsáveis pela principal via de morte celular programada e/ou apoptose (ZIMMERMANN *et al.*, 2001).

As caspases são sintetizadas na forma de procaspases, sendo ativadas pela quebra em dois sítios específicos de ácido aspártico. Ao serem ativadas, elas desencadeiam uma cascata de ativação de caspases, que é um processo irreversível, levando a célula à morte (ALBERTS *et* al., 2002). Essa família de caspases apresenta duas subfamílias: subfamília das enzimas de conversão da interleucina-1β (ICE) composta pelas caspases 13,

4, 5 e 1, que são responsáveis pelas respostas inflamatórias; e subfamília CED-3 composta pelas caspases 7, 3, 6, 8, 10, 2, 9, que são responsáveis pela iniciação e execução da apoptose (ZIMMERMANN *et al.*, 2001). Dentre elas a caspase-3 é a mais abundante nas células, pois é a responsável pelo desencadeamento da apoptose juntamente com as caspases-6 e –7 (ZIMMERMANN *et al.*, 2001).

A apoptose pode ser desencadeada por duas diferentes vias de sinalização: via mitocondrial e via receptores de morte. A via mitocondrial é inibida pela ação de parte de proteínas da família Bcl-2 que previnem alterações na mitocôndria. Essa família pode ser subdividida em três categorias: membros anti-apoptóticos, membros pró-apoptóticos e somente membros BH3 pró-apoptóticos. Durante a apoptose, membros pró-apoptóticos da família Bcl-2 são ativados e translocados do citosol para a mitocôndria, onde liberam o citocromo c induzindo a ativação da caspase-3 que comandará a apoptose (ZIMMERMANN *et al.*, 2001). A via receptores de morte ocorre pela interação de um ligante com receptor de morte, por exemplo, interação Fas-FasL. Essa interação ativa a caspase-8 que, por sua vez, tem duas vias: pode ativar a caspase-3 que induzirá a apoptose, ou pode ativar membros pró-apoptóticos da família Bcl-2 que liberarão o citocromo c, e este induzirá a ativação da caspase-3, resultando em apoptose (ZIMMERMANN *et al.*, 2001).

Outras classes de genes podem estar envolvidas na apoptose ou sua falta em canceres: o proto-oncogene *myc* e o gene supressor de tumor p53. Esse último é importante no mecanismo de reparo de DNA, pois quando há algum erro no DNA de determinada célula, ele ativa o sistema de reparo fazendo com que a célula passe de G_0 para G_1. Caso o reparo não seja feito, ele induz a célula a entrar em apoptose. Se a função regulatória da

p53 estiver falha, a célula continuará o ciclo celular podendo passar o erro para as células filhas (SALAMI *et* al. 1996).

Diferentes técnicas podem ser utilizadas nos estudos de MCP e/ou apoptose. Entre elas destacam-se: microscopia eletrônica e coloração HE para análise morfológica das células; reação de Feulgen para quantificação e morfologia dos níveis de compactação da cromatina; eletroforese convencional em gel de agarose, ensaio cometa e imunocitoquímica para fragmentação de DNA (TUNEL) e para caspases; anexina V para detecção de alteração na assimetria da membrana plasmática entre outros (MESNER Jr & KAUFMANN, 1997; MELLO *et al.*, 2001).

3.3. Alguns dados biológicos sobre serpentes

As serpentes são vertebrados pertencentes à classe REPTILIA, subclasse LEPDOSAURIA, ordem SQUAMATA e subordem OPHIDIA ou SERPENTES, compreendendo várias famílias, entre elas a VIPERIDAE (ORR, 1986; DA SILVA, 2000). Esta, por sua vez, apresenta várias subfamílias, dentre elas a CROTALINAE, cuja característica marcante é a presença de fosseta loreal com epitélio termo-sensorial entre os olhos e a narina, e maxilar escavado (DA SILVA, 2000). No Brasil, representantes desta subfamília são *Crotalus durissus terrificus*, *Bothrops jararaca*, *Bothrops alternatus* e *Bothrops neuwiedii*, que estão entre as que mais causam acidentes ofídicos, principalmente as duas primeiras espécies, segundo informações obtidas junto ao Museu do Instituto Butantan. A diferenciação das espécies citadas acima é feita por vários caracteres, como número de escamas da cabeça e do corpo, presença de presas, presença de guizo na extremidade da cauda, e composição do veneno, entre outros (CAMPBELL & LAMAR,

1989; VANZOLINI & CALLEFFO, 2002), já que o cariótipo dessas quatro serpentes é igual (2n = 36; 16 macrocromossomos e 20 microcromossomos), segundo BEÇAK (1965).

O gênero *Crotalus* (Família Viperidae) é composto de 26 espécies, sendo que 2 são limitadas à América do Sul (CAMPBELL & LAMAR, 1989). Sua característica marcante é a presença de um guizo na extremidade caudal. Sua coloração pode se confundir com o ambiente. No Brasil esse gênero é representado por uma só espécie, *Crotalus durissus*, que representa várias subespécies entre elas a *Crotalus durissus terrificus*. A subespécie *Crotalus durissus terrificus*, popularmente conhecida como cascavel, maracabóia ou boicininga, é terrícola, de hábito crepuscular e alimenta-se de pequenos roedores como camundongos. A espécie *Crotalus durissus* vive em ambiente de clima seco e quente, como os encontrados nas 5 regiões fisiográficas onde a vegetação é do tipo Cerrado, Campos abertos e Caatinga (Planalto Central, Planalto das Guianas na porção Norte, Planície Amazônica em savanas isoladas, Planície do Prata e Planície Costeira na porção Norte). Ela é ausente na região da Mata Atlântica (CAMPBELL & LAMAR, 1989). Seu veneno apresenta ação miotóxica, neurotóxica e hemolítica, podendo causar hipotensão, falha renal aguda e paralisia neuromuscular, entre outros sintomas (CAMPBELL & LAMAR, 1989; CADILLO *et al.*, 1991).

O gênero *Bothrops* (Família Viperidae) compõe-se de 31 espécies, distribuídas por toda a América (CAMPBELL & LAMAR, 1989). Podem ser chamadas de "lancehead" (cabeça de lança) devido à forma de sua cabeça. Escamas no topo da sua cabeça têm número variado conforme a espécie. Este gênero é responsável pelo grande número de morbidez no Novo Mundo comparado com qualquer outro grupo de serpentes peçonhentas. As espécies mais importantes são *B. asper* na América Central e *B. atrox* e *B. jararaca* na

América do Sul. Membros deste gênero ocupam habitats muito diversificados e alguns podem estar mais próximos das regiões de agricultura, resultando em acidentes ofídicos. Sintomas mais comuns das picadas de serpentes deste gênero são: dor local, náusea, vômito, suor, dor de cabeça, formação de vesículas hemorrágicas, hipotensão, taquicardia entre outros. As espécies *Bothrops jararaca*, *Bothrops alternatus* e *Bothrops neuwiedii* são terrícolas, apresentam hábito crepuscular e se alimentam de pequenos roedores como camundongos.

Bothrops jararaca, conhecida popularmente como jararaca, pode explorar arbustos, além de ter os hábitos acima citados. Durante os meses secos e frios apresenta menor atividade. Pode ser encontrada em 3 regiões fisiográficas distintas (Planalto Central nas porções Sul e Sudeste, Bacia do Prata e Planície Costeira nas porções Sul, Sudeste e Leste) onde a vegetação é do tipo Floresta Tropical do Leste (Sul e Sudeste), Planalto de Araucária, Campos e Floresta de Mangue-dunas (Sudeste). Pode ser encontrada em áreas cultivadas próximo à vegetação. Esta espécie é de grande importância, pois ela causa a maior parte dos acidentes ofídicos deste gênero.

Bothrops alternatus, conhecida popularmente por urutu, encontra-se em 2 regiões fisiográficas onde a vegetação é do tipo Floresta Tropical do Leste, Planalto de Araucária, Campos e talvez Pantanal (Planalto Central nas porções Sul e Sudeste, e Bacia do Prata). Pode ser encontrada em áreas de pântano ou alagadas e próximo a plantações de cana de açucar. *Bothrops neuwiedii*, conhecida como jararaca-pintada, é encontrada em 3 regiões fisiográficas, onde a vegetação é do tipo Floresta transacional de Palmeiras, Cerrado, Pantanal, Caatinga, Floresta Tropical do Leste e Campos (Planalto Central, Bacia do Prata e Planície Costeira na porção Sudeste). Esta espécie é composta por 12

subespécies (*B.n.neuwiedii*, *B.n.lutzi*, *B.n.mattogrossensis*, *B.n.meridionalis*, *B.n.pauloensis*, *B.n.bolivianus*, *B.n.piauhyensis*, *B.n.urutu*, *B.n.diporus*, *B.n.goyazensis*, *B.n.paranaensis* e *B.n.pubescens*), mas a diferenciação das subespécies é confusa, constituindo assim, um complexo taxonômico (CAMPBELL & LAMAR, 1989; SAZIMA, 1992; DA SILVA, 2000). DA SILVA (2000), sugeriu agrupar algumas subespécies, resultando em 7 subespécies, utilizando vários caracteres como coloração, contagem das escamas, localização, entre outros. O veneno das três espécies de *Bothrops* tem ação necrosante e coagulante (CAMPBELL & LAMAR, 1989; SAZIMA, 1992).

3.4. Generalidades sobre a reação de Feulgen

A reação de Feulgen é o processo citoquímico mais usual para se avaliar a presença e a quantidade de DNA *in situ*, bem como a textura cromatínica e a identificação de fenótipos nucleares em diferentes tipos celulares e sob diferentes situações experimentais (MELLO & VIDAL, 1978; MELLO *et al.*, 1995; MELLO, 1997). Igualmente se presta para a discriminação entre diferentes complexos DNA-proteína (GARCIA, 1970; MELLO, 1979; SILVA & MELLO, 1986).

A reação de Feulgen consiste de duas etapas: uma hidrólise ácida que remove do DNA suas purinas e uma exposição do ácido apurínico ao reativo de Schiff. Sendo o reativo de Schiff um leucoderivado da fucsina básica com alta afinidade por radicais aldeído, irá reagir com os grupos aldeídos da desoxirribo-furanose desmascarados no ácido apurínico, gerando um produto corado. À medida que se prolonga a exposição ao ácido aumenta a eliminação de purinas (porção ascendente da curva de hidrólise) até ser atingido um ponto ótimo de depurinação (platô da curva). Em seguida, começa a ocorrer solubilização do

ácido apurínico (porção descendente da curva de hidrólise). Medidas de valores Feulgen-DNA, obtidas por citofotometria ou por análise de imagem são requeridas para uma construção de curvas fidedignas e comparativas.

3.5. O teste de TUNEL

Estudos de fragmentação de DNA, possivelmente ligados à morte celular programada, utilizam metodologias como eletroforese em gel de agarose de DNA extraído de células, análise de endonucleases, marcação de fosfatidilserina, e ensaio de TUNEL entre outros (SGONC & GRUBER, 1998; KINDZELSKII & PETTY, 1999; MELLO *et al.*, 2001). O ensaio de TUNEL (Roche/Amersham) consiste na ligação específica de dUTP-fluoresceína aos radicais 3'-OH do DNA pela enzima terminal deoxinucleotidil transferase (TdT). Um anticorpo anti-fluoresceína com peroxidade ligada se conjuga à fluoresceína. A peroxidase é revelada pela reação de benzidina (MELLO *et al.*, 2001).

GRAVRIELI *et al.* (1992) realizaram estudo em diversos tecidos a fim de observar a validade do método de TUNEL comparado à eletroforese em gel de agarose de DNA extraídos desses materiais. Eles observaram que, em timócitos tratados com dexametasona por diferentes tempos, o ensaio de TUNEL indica mais rapidamente fragmentação de DNA (0 horas de tratamento com dexametasona) do que a eletroforese em gel da agarose (somente 3 horas após o tratamento com dexametasona). Além disso, a marcação de fragmentação tem sua intensidade aumentada proporcionalmente ao tempo de tratamento com dexametasona, sendo que o número de células marcadas não sofre alteração significativa. Por fim esse trabalho mostrou que determinados órgãos, como intestino

delgado e ovário, podem servir como controle positivo para estudos de fragmentação de DNA, pois suas taxas de "turnover" são altas.

Em recente revisão realizada por SGONC & GRUBER (1998) sobre detecção de apoptose, o ensaio de TUNEL foi citado como uma boa metodologia. O ensaio de TUNEL tem habilidade de revelar quebras de DNA durante apoptose antes que características morfológicas de apoptose sejam evidentes (GRAVIELI *et al.*, 1992; SGONC & GRUBER, 1998).

NEGOESCU *et al.* (1998) e LABAT-MOLEUR *et al.* (1998) avaliaram a importância da fragmentação de DNA na apoptose utilizando diferentes "kits" de TUNEL e diferentes fixadores. Concluíram ser necessário um tratamento do material com proteinase K antes de submetê-lo ao ensaio de TUNEL, pois a condensação da cromatina e o uso de determinados fixadores impediriam a exposição dos fragmentos de DNA a enzima TdT, e conseqüentemente a obtenção de bons resultados.

3.6. Ensaio Cometa

Atualmente a detecção de danos ou fragmentos de DNA pode ser realizada através de outros testes, além do TUNEL e entre eles o ensaio Cometa (ROJAS *et al.*, 1999). Os primeiros a desenvolverem a quantificação de danos de DNA diretamente em células individuais foram RYDBERG & JOHANSON (1978) que lisaram e embeberam as células em agarose sob condições de leve alcalinidade e posterior coloração com acridine orange. Mais tarde, OSTLING & JOHANSON (1984) melhoraram a sensibilidade do método com a técnica de eletroforese em microgel, mais conhecida como ensaio Cometa. Esse ensaio consistia em se embeber as células em agarose e colocá-las em lâminas para

serem, em seguida, lisadas com detergente e tratadas com alta concentração de sais. Logo após, a eletroforese passou a ser realizada em condições neutras e a coloração com brometo de etídio. O ensaio Cometa passou a ter duas versões, sendo a primeira desenvolvida por SINGH *et al.* (1988) e, a segunda, por OLIVE *et al.* (1990). A primeira caracterizava-se por se realizar a eletroforese em condições alcalinas (pH > 13) para detecção de quebras de fita simples e sítios ou lesões álcali-lábeis, além de retardar o reparo do DNA. Já a segunda era semelhante ao ensaio de OSTLING & JOHANSON (1984), e caracterizava-se por apresentar a lise das células em condições alcalinas e eletroforese neutra ou levemente alcalina (pH = 12,5), para detecção de quebras de fita simples e retardamento do reparo de DNA (TICE, 1995; ROJAS *et al.*, 1999).

O ensaio Cometa tem sido aplicado em estudos de genotoxicidade e de reparo de DNA, aplicações clínicas, monitoramento humano e ambiental (ROJAS *et al.*, 1999). Para o monitoramento ambiental são utilizados, além de ratos e camundongos, minhocas entre outros anelídeos, cetáceos, girinos (anfíbios) e mesmo plantas (ROJAS *et al.*, 1999). Os primeiros a utilizarem eritrócitos nucleados foram PANDRANGI *et al.* (1995), que adaptaram a técnica de Singh para eritrócitos de peixes e analisaram os danos de DNA desses animais da região dos Grandes Lagos no Canadá. Vários outros trabalhos, utilizando a técnica de Singh modificada, também foram realizados com eritrócitos nucleados de algumas espécies de girinos daquela região, visando monitoramento ambiental através de análise de danos de DNA causados por agentes genotóxicos, como herbicidas (RALPH et al., 1996; CLEMENTS et al., 1997; RALPH & PETRAS, 1997, 1998b). RALPH & PETRAS (1998a) observaram que ao longo da maturação do girino e da metamorfose do

anfíbio *Rana clamitans* há uma variação da sensibilidade de eritrócitos expostos ao metil metanosulfonato. Com relação a eritrócitos de répteis não existem relatos até o momento.

3.7. A ultra estrutura de eritrócitos de serpentes

A ultraestrutura de eritrócitos nucleados já foi relatada para tartaruga do deserto, peixes e serpentes (MENEZES *et* al., 1974; MATTISSON & FÄNGE, 1977; DESSER & WELLER, 1979; SPADACCI MORENA *et al.*, 1991; ALLERMAN *et al.*, 1992, 1999).

SPADACCI MORENA *et al.* (1991) realizaram um estudo comparativo das ultraestruturas das células eritrocíticas de peixes e serpentes, mas com ênfase na biosíntese das hemoglobinas.

MENEZES *et al.* (1974) estudaram a ultraestrutura de eritrócitos maturos de 5 espécies diferentes de *Bothrops*, evidenciando que o complexo de Golgi de *B. jararaca* era mais desenvolvido do que nas demais espécies, e que o núcleo celular nas diferentes espécies apresentava diferenças na localização da cromatina. A ultraestrutura, morfologia em microscópio de luz e a coloração citoquímica de células sangüíneas de outra espécie de serpente, *Crotalus adamanteus*, foram objeto de estudo de ALLERMAN *et al.* (1999), tendo sido distinguidos eritrócitos das células leucocitárias. Isso tem permitido a identificação das diferentes células sanguíneas e o provável papel dessas na inflamação de tecidos injuriados nesses animais. Entretanto, pouco se revela sobre a organização da cromatina especificamente nesses estudos.

Assim, um estudo com as espécies citadas no item 2 desta introdução seria de grande valia já que elas estão em grande abundância no Brasil, especialmente *Crotalus durissus terrificus* e *Bothrops jararaca*. Além disso, pouco se sabe sobre a organização do

seu conteúdo nuclear, o que poderia enriquecer esta área e também auxiliar no estudo da sistemática filogenética desses grupos.

4. OBJETIVOS

O presente trabalho tem por objetivo determinar se os eritrócitos de diferentes espécies de serpentes (*Crotalus durissus terrificus*, *Bothrops jararaca*, *Bothrops alternatus* e *Bothrops neuwiedii*) apresentam diferenças relevantes em freqüência e/ou padrão de intensidade de fragmentação de DNA relacionados à morte celular programada, quando comparados entre si e com dados reportados para o único réptil semelhantemente estudado, *Geochelone carbonaria* (MELLO *et al.*, 2000).

Busca também estabelecer se os padrões cinéticos de labilidade à hidrólise ácida de Feulgen da cromatina dos eritrócitos dessas serpentes, que trariam informações sobre a organização cromatínica nessas células, são compatíveis ao seu padrão de fragmentação de DNA detectável com o teste de TUNEL e com o ensaio Cometa. Além disso, outras informações sobre a organização da cromatina dessas células poderiam ser obtidas por análises de sua ultraestrutura através da microscopia eletrônica de transmissão.

Busca igualmente estudar o padrão de resposta ao TUNEL, ao ensaio Cometa e à reação de Feulgen em eritrócitos de serpentes parasitadas por protozoário, na expectativa de que diferenças cromatínicas possam ser encontradas.

5. ARTIGOS SUBMETIDOS À PUBLICAÇÃO

1. Maristela Miyamoto, Benedicto C. Vidal & Maria Luiza S. Mello, Chromatin supraorganization, DNA fragmentation and cell death in snake erythrocytes

2. Maristela Miyamoto, Maria Luiza S. Mello, Chromatin supraorganization, DNA fragmentation and cell death in erythrocytes of the snake, *Crotalus durissus terrificus* (Squamata, Viperidae), infected with *Hepatozoon sp.* (Apicomplexa, Hepatozoidae).

DNA fragmentation, chromatin supraorganization and cell death in snake erythrocytes

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In nucleate erythrocytes of several vertebrate groups, programmed cell death varies as regards frequency and intensity of the phenomenon of DNA fragmentation. Since data for reptiles was available for only one tortoise species, in the present study was investigated DNA fragmentation as related to chromatin supraorganization and cell death, in four snake species by image analysis of Feulgen hydrolysis kinetics, the TUNEL assay, the single cell gel electrophoresis, and electron microscopy observations. The species studied were *Crotalus durissus terrificus*, *Bothrops jararaca*, *B. alternatus*, and *B. neuwiedii*. A relatively few circulating erythrocytes were found simultaneously committed to cell death, although differences were observed when the snake species were compared to each other inclusive within the same genus. The DNA in the snake erythrocyte chromatin was much more resistant to the Feulgen acid hydrolysis (depurination and breakdown) in comparison to that of erythrocytes characterized by high frequency and intensity of DNA fragmentation as in the case of the early adult bullfrog. Conspicuous nuclear and cytoplasmic organelles suggestive of metabolic activity were demonstrated ultrastructurally in most erythrocytes of the snakes. Among the snake species studied, *B. neuwiedii* and *C. d. terrificus* presented the largest resistance to Feulgen acid hydrolysis (DNA depurination and apurinic acid breakdown) and to the DNA fragmentation revealed by the low frequency of positive responsiveness to the TUNEL assay. Although *B. neuwiedii* also showed the lowest frequency of cells with more DNA damage in the single cell gel electrophoresis assay, *C.d. terrificus* presented the largest frequency of damaged cells, possibly due to abundance of alkali-sensitive DNA sites and not to the DNA fragmentation pertinent to cell death. The extent of chromatin condensation as seen with the electron microscope in the snake erythrocyte nuclei was not correlated to their properties of DNA resistance or lability to

fragmentation. The findings related to DNA fragmentation/cell death in the erythrocytes of *B. jararaca* and *B. alternatus* generally differed from those for *C. d. terrificus* and *B. neuwiedii* and are suggested to be related to these species' biology selection under different geographical habitats.

Key words: erythrocytes, snakes, DNA fragmentation, chromatin supraorganization, image analysis, cell death

Introduction

The erythrocyte life-span in the circulating blood varies considerably in different vertebrate groups (Brace & Altland 1956, Rodman et al. 1957, Baca Saravia 1961, Altland & Brace 1962, Reddy et al. 1975, Forman & Just 1976, Hasebe et al. 1996, 1999, Alberts et al. 2002). Different stages and extent of DNA fragmentation as evaluated by response to the TUNEL assay in association to cell death, occurring simultaneously, have been reported for erythrocytes of the circulating blood of chickens, pigeons, bullfrogs and a tortoise species (Mello et al. 2000).

Red blood cells of the tortoise, *Geochelone carbonaria*, contain an SH-rich hemoglobin typical for animal species highly resistant to hypoxia (Torsoni et al. 1996, 1998). This hemoglobin plays a role in protecting the erythrocytes against damage by reactive oxygen species (Torsoni & Ogo 2000). When subjected to the TUNEL assay for DNA fragmentation, only 31% of the erythrocytes of *G. carbonaria* showed a positive response, most of it of a weak character (Mello et al. 2000). This would be in agreement with a long life span, often exceeding 700 days, presumed for erythrocytes in reptiles, and their lower metabolic activity (Altland & Brace 1962, Torsoni et al. 1996).

Reptiles other than *G. carbonaria* have not so far been studied for patterns of erythrocyte DNA fragmentation and cell death. A detailed investigation of this phenomenon not only through the single cell gel electrophoresis (Comet assay) in association to data on chromatin supraorganization in different snake species would thus deserve consideration. In snakes, erythrocytes also contain special hemoglobin types,

endowing these cells with resistance to oxidative stress (Ogo et al 1982, 1983, Bonilla et al. 1994).

In addition, if DNA fragmentation is associated to chromatin compactness as in most cells under apoptosis programs (Majno & Joris 1995), the chromatin packing state could be revealed through DNA lability to acid hydrolysis in these cell types as assessed by image analysis (Silva & Mello 1986, Mello 1997).

In the present study, DNA fragmentation as assessed by the TUNEL assay and single cell gel electrophoresis, and chromatin supraorganization evaluated by the kinetics of Feulgen acid hydrolysis (Mello 1997) and ultrastructural observations, were analyzed in erythrocytes of four differently aged snake species two of each differing in their geographical distribution habitats (Hoge & Romano-Hoge 1978/79, Borges &Araújo 1998).

Materials and Methods

Animals

Young (before sexual maturation) and adult (after sexual maturation) specimens of *Crotalus durissus terrificus*, *Bothrops jararaca*, *Bothrops alternatus* and *Bothrops neuwiedii* (Squamata, Viperidae), reared in the Center for the Study of Venoms and Venomous Animals of São Paulo State University (CEVAP) (Botucatu, Brazil) were studied (Fig.1). The young snakes were 1.5- to 2-years old whereas the adult ones were older than 3 years. Five young and adult specimens each of the species *C.d.terrificus* and *B. jararaca*, and two young and adult specimens each of the species *B. alternatus* and *B.*

neuwiedii were used. All the specimens were maintained in individual cages at the temperature range of 20-26°C, fed with mice every 15 days and received water *ad libitum*.

Commercially supplied adults of the bullfrog, *Rana catesbeiana*, were used as a control of intense DNA fragmentation in the erythrocytes (Mello et al., 2000).

Blood collection

Blood samples were collected from all snakes during the Spring of the South Hemisphere (October through December). Samples of 0.3-0.5 ml were obtained by caudal tail veni-puncture in the presence of heparin (5000 IU/ml). EDTA was not used as anticoagulant since it promotes lysis of the blood cells in reptiles (Egami & Sasso 1988, Mader 1996). Fresh blood smears were prepared, for cytochemical and immunocytochemical staining, and 0.3 ml of heparinized blood samples necessary for the single cell gel electrophoresis assay and electron microscopy were put in eppendorff vials. Blood samples from bullfrog were also collected by cardiac puncture. Smears were prepared immediately upon collection for further cytochemical and immunocytochemical assays.

Feulgen acid hydrolysis kinetics

Blood smears were fixed in absolute ethanol-glacial acetic acid mixture (3:1, v/v) for 1 min, rinsed in 70% ethanol for 5 min, and air dried. Then the preparations were subjected to hydrolysis in 4M HCl at 25°C for 10, 30, 60, 100, 150 and 240 min. Next, the preparations were rinsed in cold 0.1M HCl, treated with Schiff's reagent for 40 min at room

temperature, rinsed in three baths of sulfurous water (5 min each) and distilled water, air dried, cleared in xylene, and mounted in natural Canada balsam (n_D = 1.54). All the staining and subsequent steps were done in parallel for all samples in order to minimize variations in the experimental conditions and reduce possibility of systematic errors. Smear duplicates for each hydrolysis time, animal, and developmental phase were prepared.

The Feulgen-stained preparations were examined in a Zeiss-Axiophot 2 microscope. Image analysis procedures (acquisition, segmentation and featuring) were undertaken with Zeiss/Kontron equipment and Kontron KS400 software (Oberkochen/Munich, Germany). Slides from two randomly selected animals of each species/developmental phase were used for image analysis studies. The microscopic images were obtained with a Neofluar 40/0.75 objective, optovar factor 2, 0.90 condenser, and λ = 546 nm. The images to be processed were fed from the microscope into a Pentium computer through a Sony CCD-IRIS/RGB Hypes HAD color video camera. Under these conditions 1 μm corresponded to 5.77 pixels. Nuclear area (A) and absorbances (OD) were measured for one hundred nuclei per species (50 for specimen) under each developmental phase (young and adult) and hydrolysis time. The parameter IOD (integrated optical density or in this case Feulgen-DNA values in arbitrary units) was next calculated from A x OD. Feulgen hydrolysis curves were constructed by plotting mean IOD values obtained with advancing hydrolysis time. Calculations were done using the Minitab12™ software (State College, PA).

DNA fragmentation

1. TUNEL assay

The smear preparations used for the immunocytochemical test were fixed in 1.75% paraformaldehyde for 15 min at room temperature and rinsed for 30 to 50 min in tap water. The TdT-mediated dUTP nick 3-end DNA labeling (TUNEL) technique was used as described by the manufacturer (Roche/Amersham, Mannheim), including proteinase K concentration, which was equal to 20 µg/ml in PBS. Since UTP was labelled with fluorescein, anti-fluorescein antibodies linked to horse-raddish peroxidase were processed by treatment with 3-3'-diaminobenzidine (Fluka, Buchs, Switzerland) for 10 min at room temperature. Endogenous peroxidase was blocked with 3% H_2O_2 in absolute methanol for 30 min at room temperature. Negative controls were performed by omitting TdT. The preparations subjected to the TUNEL assay were counterstained with a mixture of a 2% aqueous solution of methyl green (Merck, Darmstadt) (10 ml), McIlvaine buffer at pH 4.5 (5 ml) and 20% glycerin (3 ml) for 6 h, rinsed in distilled water, air dried, and mounted in natural Canada balsam.

Erythrocyte nuclei were checked for positivity to the TUNEL assay and the number of TUNEL-positive nuclei in 2,000 erythrocytes was determined. The response to the TUNEL assay was classified as weak, median and strong. The weak positivity means punctual nuclear marking generally on nuclear periphery. When nearly a half nucleus was marked, the positivity was considered as median. The strong positivity means that almost the whole nucleus was marked. Leukocytes and thrombocytes were excluded from counts based on their nuclear morphology and size which differ from those of the erythrocytes (Mader 1996).

2. Single cell gel electrophoresis assay (Comet assay)

The alkaline procedure described by Singh et al. (1988) and Klaude et al. (1996) was used with modifications adequate to the material. During all procedures the material was kept in the dark to prevent additional DNA damage (Singh et al. 1988). The material was kept on ice during the collection, transport to the laboratory and electrophoresis to provide reproducibility (Singh et al. 1988, Tice et al. 2000).

Each 0.3 ml sample of the heparinized blood was mixed up to 2 ml with RPMI 1640 medium (Nutricell, Campinas) in an eppendorff tube. A gradient of 25 to 100% Percoll (Amersham Bioscience, Upsalla, Sweden) diluted in RPMI 1640 medium was prepared. Each 2 ml Percoll phase was carefully transferred to a 15 ml conic Falcon tube (Greiner, Rickenhausen, Germany) following the Percoll descending concentration of 100, 80, 64 and 25% and the blood sample as the latest. The material was then centrifuged at 3000 rpm for 30 min at room temperature. The erythrocyte ring was removed with a glass Pasteur pipet and transferred to another 15 ml conic Falcon tube. RPMI 1640 medium was added up to 5 ml and the preparation was centrifuged at 1000 rpm for 10 min at room temperature. The supernatant was poured off and again subjected to the former procedure. The pellet was resuspended in RPMI 1640 medium up to a volume of 1 ml. The isolated erythrocytes presented a viability of 98% as detected with Trypan Blue (Merck, Darmstadt) staining.

The erythrocyte samples were diluted 5 times into RPMI 1640 medium up to a concentration of 1 x 10^6 cells/ml. Then, 5 µl of the sample was diluted in 100 µl of 0.5% low melting point agarose (Sigma, St. Louis). This mixture was put on slides containing a

1.5% normal agarose (Sigma, St. Louis) layer, them covered with a coverslip and placed in a refrigerator for 5 min until the agarose layer hardens. The coverslips were then gently slided off and the slides slowly put into cold freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Na lauryl sarcosinate, 10% DMSO, 1% Triton X-100) for a minimum of 1 hr. They were rinsed in PBS for 5 min and placed in the electrophoresis box where they were incubated with the alkaline buffer (300 mM NaOH/1 mM EDTA) at pH 13 for 20 min. Electrophoresis was processed at 25 V, 300 mA, for 10 min at 4°C. The material was then rinsed in the neutralization buffer (0.4 M Tris) three times, 5 min each; fixed in absolute ethanol, and stained with ethidium bromide (Sigma, St. Louis) (20 µg/ml in distilled water) solution.

The preparations were observed in a Zeiss Axiophot II microscope equipped for fluorescence, when evaluation of the visual score Comet classification according to Collins et al. (1995) was undertaken for cell images chosen at random. The comet classification was made according to the tail length and intensity such that class 0 shows no tail and the fluorecent image is practically round; class 1 shows a short tail with a few points close to the head image indicating few fragments; class 2 shows that a tail is a little longer than that of class 1 and that more fragments appear in the tail; class 3 shows the tail longer than that for class 2 and the head of the comet begins to reduce; and class 4 shows the longest tail, and the head of the comet more reduced than that for class 3 and at the same time that there are many points in the tail indicating many fragments (ver Anexo). In the class 4 nuclei the head cannot be distinguished from the tail because of an intense DNA fragmentation. Next, they were observed with an Olympus BX 60 (Japan) fluorescence microscope equipped

with an Olympus PM-C35DX CCD camera, Olympus PM 20 – exposure control unit (Japan), for image analysis using the software Comet II (Perceptive Instruments, England). Evidence of a "comet tail" shape and migration pattern of the DNA indicates DNA fragmentation.

As a positive control of DNA fragmentation, erythrocytes treated with 10% methylmethanesulphonate (MMS) (Sigma, St. Louis) were used. As a negative control, cells were not electrophoresed.

One hundred randomly selected cells per sample were analyzed for evaluation of image parameters, such that DNA tail migration (image length or diameter of the nucleus plus migrated DNA in μm), tail intensity, and tail moment (tail intensity x DNA migration) could be measured.

Calculations were done using the Minitab12^{TM} software (State College, PA).

Electron Microscopy

The samples of blood were fixed with 2.5% glutaraldehyde (Merck, Darmstadt) solution overnight at 4°C in the dark. Before each change of solution, the sample was fastly centrifuged for formation of a pellet (high rotation for 1 min). The supernatant was removed and the sample was rinsed in 0.1 M phosphate buffer (pH 7.3). Then the material was fixed with 1% osmium tetroxide (Sigma, St. Louis) for 2 h at room temperature in the dark. Sample was rinsed in distilled water and then fixed in 0.5% uranyl acetate for 40 min to 2h. Dehydration occurred in graded acetone series and the samples were embedded in araldite. The material was trimmed and thin sections were obtained by ultramicrotomy

(Leica Ultracut – UCT, Germany). The sections were contrasted with saturated uranyl acetate solution and lead citrate, and examined in Philips CM 100 (Holland) electron microscope.

Results

Feulgen acid hydrolysis kinetics

The Feulgen staining response in the snake erythrocytes as a function of the hydrolysis times varied with the animal species and animal development (Figs. 2, 3). The profiles of the Feulgen hydrolysis curves constructed with mean IOD values for the various hydrolysis times relative to the maximal IOD (100%) indicated that the maximal DNA depurination of the erythrocytes of the different snake species was attained later than that of the bullfrog erythrocytes (Fig. 3). The same occurred as regards the kinetics of apurinic acid solubilization (descending branch of the hydrolysis curves) (Fig. 3).

Differences, as regards the hydrolysis times at which maximal depurination occurs, were found when considering the four snake species among themselves or when comparing young vs. adult specimens in *C. d. terrificus* and *B. alternatus* (Figs. 3A, C). Among the four snake species here considered, *B. jararaca* provided the steepest descending branch for the Feulgen hydrolysis curves, especially when considering the erythrocytes of the young specimens of this species (Fig. 3B). On the other hand, the apurinic acid of *C. d. terrificus* (especially in adults) and *B. neuwiedii* presented the largest resistance to acid hydrolysis solubilization (Fig. 3A, D). The apurinic acid breakdown of *B. alternatus* was intermediate between *B. neuwiedii* – *C.d. terrificus* and *B. jararaca.*

All these indications, inclusive the fact that a secondary IOD peak apparently found at the hydrolysis time of 100 min for the erythrocytes of adult *B. alternatus* and young *B. neuwiedii* indeed represents extension of the plateau of maximal DNA depurination, were confirmed statistically for Feulgen-DNA data (IOD) considered as absolute values (Tables 1 and 2).

DNA fragmentation

Only part of the cell population responded positively to the TUNEL test (Table 3). The controls performed in the absence of TdT gave negative labelling (Fig. 4a), demonstrating that the positive immunocytochemical response was not caused by endogenous cell peroxidases. As a positive control, the bullfrog erythrocytes showed a very strong positive response (Fig. 4b) (Mello et al, 2000).

The positive response to the TUNEL assay in the snake erythrocytes varied in frequency with the species and animal development considered and in intensity in the same specimen and with the species and animal age (Fig. 4, Table 3). Most TUNEL-positive erythrocyte nuclei were labelled in the chromatin positioned at their periphery (Fig. 4).

The highest frequency of TUNEL positivity was found in erythrocytes of young and adult *B. alternatus* and young *B. jararaca*. Even so their frequency values were lower than those of *Rana catesbeiana*. In the case of *B. alternatus* the TUNEL positivity was more representative in the young specimens. The smallest frequency of TUNEL positivity was verified in young *B. neuwiedii* (Table 3).

Ethidium bromide-stained images of the snake erythrocytes subjected to the single cell gel electrophoresis varied in shape and frequencies with the animal species, though

generally undamaged (class 0) or less damaged cells (extremely short tail or halo, bright head - class 1) were predominant (Fig. 5, Table 4). The erythrocytes of *C. d. terrificus* were those which showed more damaged cells with the classification ≥2 according to Collins et al.'s (1995) score. The erythrocytes of *B. neuwiedii* presented a fewer frequency of more damaged cell images than the other species (Table 4, Fig. 5). Differences concerned with the snake developmental stage were not remarkable (Table 4).

The increase in comet tail length was much greater in response to MMS treatment (positive control). Practically no tail or a bright short halo was observed in non-electrophoresed cells (negative control) (Fig. 5, Table 4).

The highest values for the parameters "tail migration", "tail intensity" and "tail moment" were presented by the erythrocytes of young and adult *C. d. terrificus*, which were followed by (or equal to) those of *B. alternatus* (Figs. 6 and 7). The smallest values for these parameters were found in the erythrocytes of young *B. neuwiedii*, up followed by the erythrocytes of adult and young *B. jararaca* and erythrocytes of adult *B. neuwiedii*. As regards the parameters "tail migration" and "tail moment", the values for adult *B. neuwiedii* were as low as those of the negative control and of adult *B. jararaca*, respectively (Fig. 7).

Electron microscopy

The nuclear ultrastructural aspect of the erythrocytes did not remarkably differ in the snake species here studied. Generally, the erythrocytes exhibited electron-dense chromatin apposed to the nuclear periphery, though interrupted in the nuclear pore complex zones (Fig. 8a, b). Apparently, the erythrocyte nuclei of *C. d. terrificus* and *B. jararaca* contained

most of their chromatin with a condensed packing state (Fig. 8a, d), whereas in *B. neuwiedii* and *B. alternatus* most chromatin showed a non-condensed packing state (Fig. 9a, c). Even so, the erythrocytes of all these species, irrespective of the developmental stage of the specimens, showed signs of cellular metabolic activity, as deduced from nucleolar presence, relatively frequent nuclear pore complexes, evident well-developed Golgi complexes, and some few mitochondria (Figs. 8a, c, d and 9a, c). The nucleolus was round, finely granulous and with a low electron opacity (Fig. 9b, d). Only in *C. d. terrificus* a few nuclei were characterized by an irregular contour indentation at which densely packed chromatin appeared in close contact, reminding apoptosis images (Fig. 8c).

Discussion

Present findings are in agreement with the idea that in reptiles a relatively few circulating erythrocytes are simultaneously committed to cell death, at least in terms of occurrence of DNA fragmentation determined with Feulgen acid lability, response to the TUNEL assay and single cell gel electrophoresis, if compared to nucleated erythrocytes of other vertebrate groups (Mello et al. 2000). All snakes with a positive response to the TUNEL assay showed predominance of a weak response which is in accordance with findings for the erythrocytes of another reptile, the tortoise *G. carbonaria* (Mello et al. 2000) and is in contrast with the predominantly strong response in erythrocytes of the adult bullfrog, *Rana catesbeiana*. In addition, there were conspicuous nuclear and cytoplasmic organelles suggestive of a certain cell metabolic activity in the erythrocytes of the snake species analyzed by the electron microscope.

Present data are also in agreement with published data revealing long life-span and lower metabolic activity in reptilian red blood cells (Altland & Brace 1962, Torsoni et al. 1996, 1998, Torsoni & Ogo 2000). Even so, differences were found when the different snake species were compared to each other inclusive within the same genus (*Bothrops*). Small differences with the animal age were also revealed, which may be related to changes in production of different hemoglobin types as in the case of the amphibian erythrocytes (Hasebe et al 1996, 1999).

As regards the Feulgen acid hydrolysis kinetics, a much higher resistance to DNA depurination and apurinic acid solubilization was demonstrated for the erythrocytes of the four snake species here analyzed, in comparison to the erythrocytes of the early adult bullfrog *Rana catesbeiana*. The DNA of the latter was much more labile to the acid hydrolytic action pertinent to the Feulgen reaction and is also characterized by a high level of DNA fragmentation as revealed by the TUNEL assay and data of the literature (Hasebe et al. 1996, 1999, Mello et al. 2000).

The erythrocyte chromatin of *B. neuwiedii* and that of *C. d. terrificus* presented the strongest resistance to Feulgen acid hydrolysis and DNA fragmentation as revealed by the TUNEL assay, among the four snake species analyzed here. The TUNEL positive response which appeared positioned in part of the nuclear periphery in some of these cells, was confirmed in some ultrastructural images of the indentation of the nuclear periphery with attached condensed chromatin, resembling an image typical of apoptosis (Majno & Joris 1995). However, although the erythrocytes of *B. neuwiedii* presented the lowest frequency of cells with more DNA damage (single cell gel electrophoresis), the erythrocytes of *C. d. terrificus* unexpectedly presented the largest frequency of damaged cells with Collins et

al.'s (1995) Comet classification ≥ 2. Additionally, the parameters "tail migration", "tail intensity" and "tail moment" of the damaged cells in *C. d. terrificus* were the highest ones. This may be due to abundance of alkali-sensivite sites in the DNA of the chromatin of *C.d. terrificus* erythrocytes in a similar way to that reported in chicken erythrocytes, and human and mouse sperm cells, which are also characterized by presence of highly condensed chromatin, and not to DNA fragmentation pertinent to cell death (Singh et al. 1989).

The extent of cell nuclei covered with condensed chromatin as viewed with the electron microscope did not appear to be associated with the other characteristics of resistance or lability to DNA fragmentation in the chromatin of the snake erythrocytes analyzed here.

The secondary IOD peak found in the Feulgen hydrolysis curves of the erythrocytes of adult *B. alternatus* and *B. neuwiedii* may indicate that there are DNA-protein complexes in the same nuclei differently responding to the acid hydrolysis with advancing times (Kjellstrand & Andersson 1975a, b, Mello & Vidal 1980, Silva & Mello 1986). Differences in DNA-protein complexes may occur with animal age and cell differentiation as reported for other cell systems (Agrell & Bergqvist 1967, Spadafora et al 1976, Savic et al. 1981, Silva & Mello 1986).

The results concerned with DNA fragmentation in the erythrocytes of *B. jararaca* and *B. alternatus* generally differed from those for *C. d. terrificus* and *B. neuwiedii*. This finding, suggesting differences pertinent to cell death in these snakes may be related to their biology selected under different geographical habitats. *C. d. terrificus* and *B. neuwiedii* are distributed in open areas like cerrado, caatinga and open grasslands (Hoge & Romano-Hoge 1978/79, Campbell & Lamar 1989, Borges & Araújo 1998) whereas *B. jararaca* and

B. alternatus appear in closed areas like the eastern tropical forest (Hoge & Romano-Hoge 1978/79, Campbell & Lamar 1989, Sazima 1992). According to Borges & Araújo (1998) the physical structure of the habitat, including the vegetation and substrate types are important for selection of different snake species. According to Da Silva (2000) and Vanzolini & Calleffo (2002) *C. d. terrificus* and *B. neuwiedii* form taxonomic complexes, additonal investigations being urged to a better understanding of their mechanisms of speciation.

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TABLE 1

ANOVA comparison of Feulgen-DNA values in snake erythrocytes

Species	developmental phase	hydrolysis times compared (min)	df	F	p
C. d. terrificus	young	60, 100	199	102.98	0.000*
	adult	60, 100	199	10.58	0.001*
		100, 150	199	62.85	0.000*
B. alternatus	adult	60, 100, 150	299	0.66	0.517
B. neuwiedii	young	60, 100, 150	299	13.32	0.000*
		100, 150	199	1.18	0.279
		60, 150	199	14.96	0.000*
	adult	60, 100	199	0.23	0.633
Rana catesbeiana	adult	10, 30	199	2.72	0.101
		30, 60	199	6.65	0.011*

*, highly significant ($P_{0.01}$)

TABLE 2

ANOVA comparison of Feulgen-DNA values in erythrocytes of young vs adult snakes

Species	hydrolysis times (min)	df	F	P
C. d. terrificus	100	199	5.27	0.023*
	150	199	15.59	0.000**
B. jararaca	60	199	6.14	0.014*
	100	198	5.80	0.017*
B. alternatus	60	199	203.91	0.000***/†
	100	199	311.03	0.000**
B. neuwiedii	60	199	65.92	0.000**
	240	199	0.70	0.405

*, significant ($P_{0.05}$); **, highly significant ($P_{0.01}$); † significance confirmed with the Mann-Whitney test

TABLE 3

TUNEL positivity in the chromatin of the snake erythrocytes.

Species	Developmental phases	No. of specimens	Mean TUNEL response (%)			
			Total	Weak	Median	Strong
C. .d. terrificus	young	4	15.51	9.95	4.20	1.36
	adult	3	14.92	8.43	4.98	1.50
B. jararaca	young	5	45.57	21.60	18.35	5.62
	adult	5	10.45	6.72	3.05	0.68
B. alternatus	young	2	57.28	32.88	22.10	2.30
	adult	2	44.48	24.50	18.13	1.85
B. neuwiedii	young	2	0.20	0.15	0.05	0.00
	adult	2	12.88	9.90	2.40	0.58
*Geochelone carbonaria**	adult	pool	31.00	16.50	10.50	4.00
Rana catesbeiana *	adult	>5	96.00	12.00	25.00	59.00

n = 2000 cells/specimes; * Mello et al. 2000

TABLE 4

Visual score responses in snake erythrocytes subjected to the single cell gel electrophoresis (n = 300)

Species	Developmental phases	N_o. of specimens	Visual score* (%)		
			0	1	≥2
C. d. terrificus					
- Non-electrophoresed control	adult	1	64	30	6
- MMS-treated control	adult	1	6	22	72
- experimental	young	4	28	30	42
	adult	3	36	21	43
B. jararaca	young	5	45	36	20
	adult	5	50	40	10
B. alternatus	young	2	32	36	32
	adult	2	33	40	27
B. neuwiedii	young	2	60	38	2
	adult	2	59	37	4

* Comet classification according to Collins et al. (1995)'s; MMS = methylmethanesulphonate; n = 300 cells/species.

Legends of Figures

FIG. 1. A, *Crotalus durissus terrificus*; B, *Bothrops jararaca*; C, *Bothrops alternatus*; D, *Bothrops neuwiedii*.

FIG. 2. Feulgen-stained erythrocytes of *R. catesbeiana* (a-c), *C. d. terrificus* (d-f), *B. jararaca* (g-i), *B. alternatus* (j-n), and *B. neuwiedii* (o-q), after different hydrolysis times: 10 min (a, d, g, j, o), 150 min (b, e, h, k, m, p), 240 min (c, f, i, n, q), for adult (a-l, o-q) and young (m, n) specimens. Bar equals 10 μm.

FIG. 3. Feulgen hydrolysis curves for the erythrocyte chromatin of *C. d. terrificus* (A), *B. jararaca* (B), *B. alternatus* (C), and *B. neuwiedii* (D). The curves for young and adult specimens of the snakes and for adult *R. catesbeiana* appear in blue, red and black, respectively. Each point in the curves is the arithmetic mean of 100 measurements. The standard deviations for most cases were nearly 27% but in a broad sense they did not surpass 39.4% of the means. IOD, Feulgen-DNA values; t, hydrolysis time.

FIG. 4. Erythrocytes with different intensity of response to TUNEL assay. a: Negative control; b: *Rana catesbeiana*; c-d: *C. d. terrificus*; e-f: *B. jararaca*; g-h: *B. alternatus*; i-j: *B. neuwiedii*; young and adult, respectively. Bar equals 10 μm.

FIG. 5. Ethidium bromide-stained images of the erythrocytes subjected to the single cell gel electrophoresis assay. a: Non-electrophoresed negative control; b: MMS-treated cells (positive control); c-d: *C. d. terrificus*; e-f: *B. jararaca*; g-h: *B.*

alternatus; i-j: *B. neuwiedii*. Young specimens: c, e, g, i; adult specimens: d, f, h, j.

FIG. 6. Box plots of the parameters tail migration (μm), tail intensity (%) and tail moment obtained by computerized image analysis of the ethidium bromide-stained erythrocytes of young snake specimens subjected to the single cell gel electrophoresis. Ba, *B. alternatus*; Bj, *B. jararaca*; Bn, *B. neuwiedii*; Cdt, *C. d. terrificus*; NC, negative control (adult *C. d. terrificus*); PC, positive control (adult *C. d. terrificus*). Differences significant at $P_{0.05}$ in comparison to NC (a), PC (b), Cdt (c), Bj (d), and Ba (e) (Mann-Whitney test).

FIG. 7. Box plots of the parameters tail migration (μm), tail intensity (%) and tail moment obtained by computerized image analysis of the ethidium bromide-stained erythrocytes of adult snake specimens subjected to the single cell gel electrophoresis. See Fig. 6 for abbreviation meaning.

FIG. 8. Transmission electron micrographs of erythrocytes of *C. d. terrificus* (a-c) and *B. jararaca* (d). Condensed chromatin distribution in the cell nuclei (n) and presence of cytoplasmic organelles such as mitochondria (m), intracytoplasmic bodies (ic) and Golgi complex (GC) are evident. Pore complex (PC) regions are indicated. Bars, 0.5 μm (a), 0.1 μm (b), and 1 μm (c, d)

FIG. 9. Transmission electron micrographs of erythrocytes of *B. neuwiedii* (a), *C. d. terrificus* (b, d), and *B. alternatus* (c). Different aspects of chromatin condensation in the cell nuclei (n) and presence of a nucleolar body (nu) and mitochondria (m)

are indicated. Figure d is a detail from Figure b. Bars, 1 μm (a, b), 0.5 μm (c), and 0.1 μm (d).

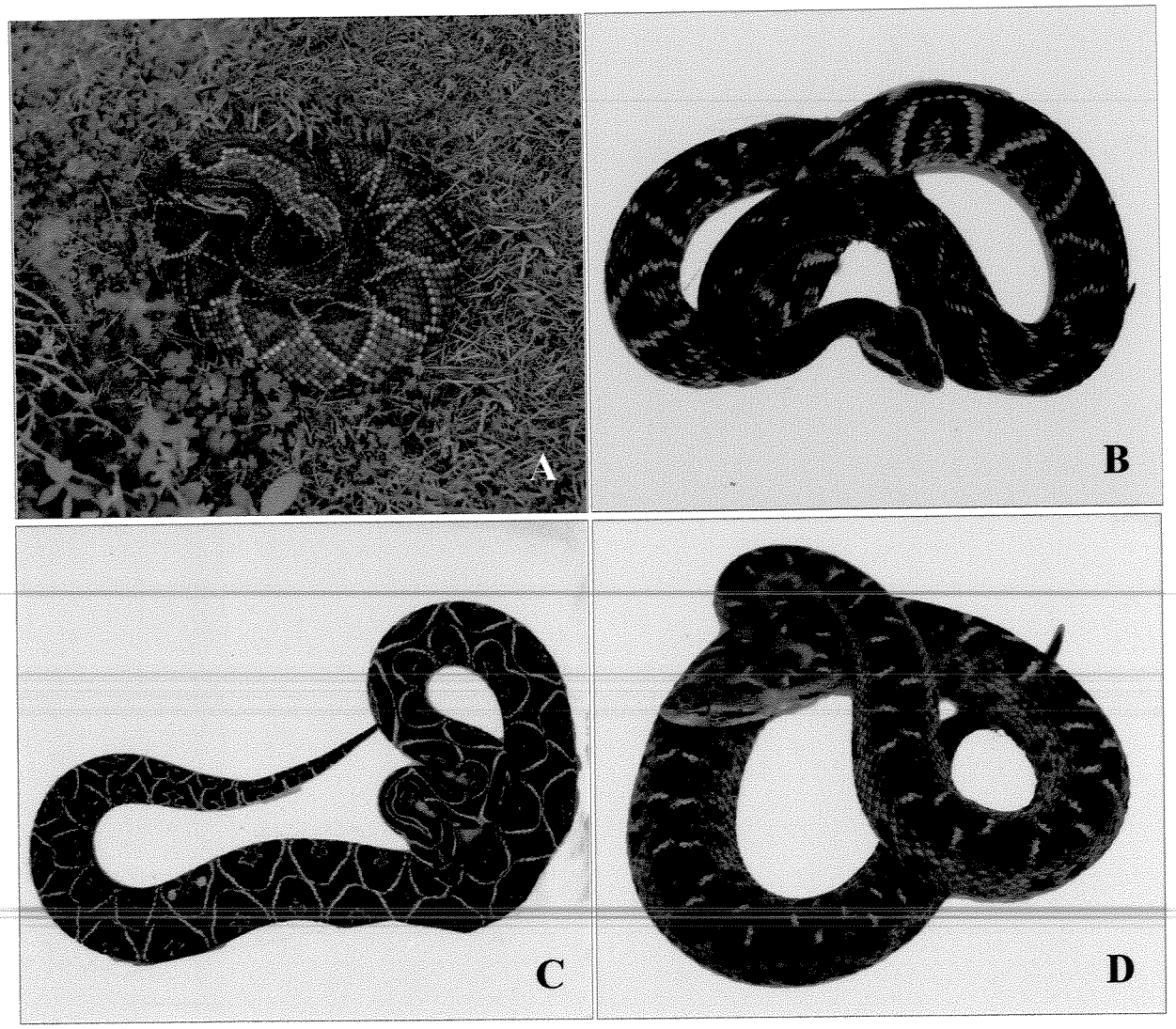

Fig. 1

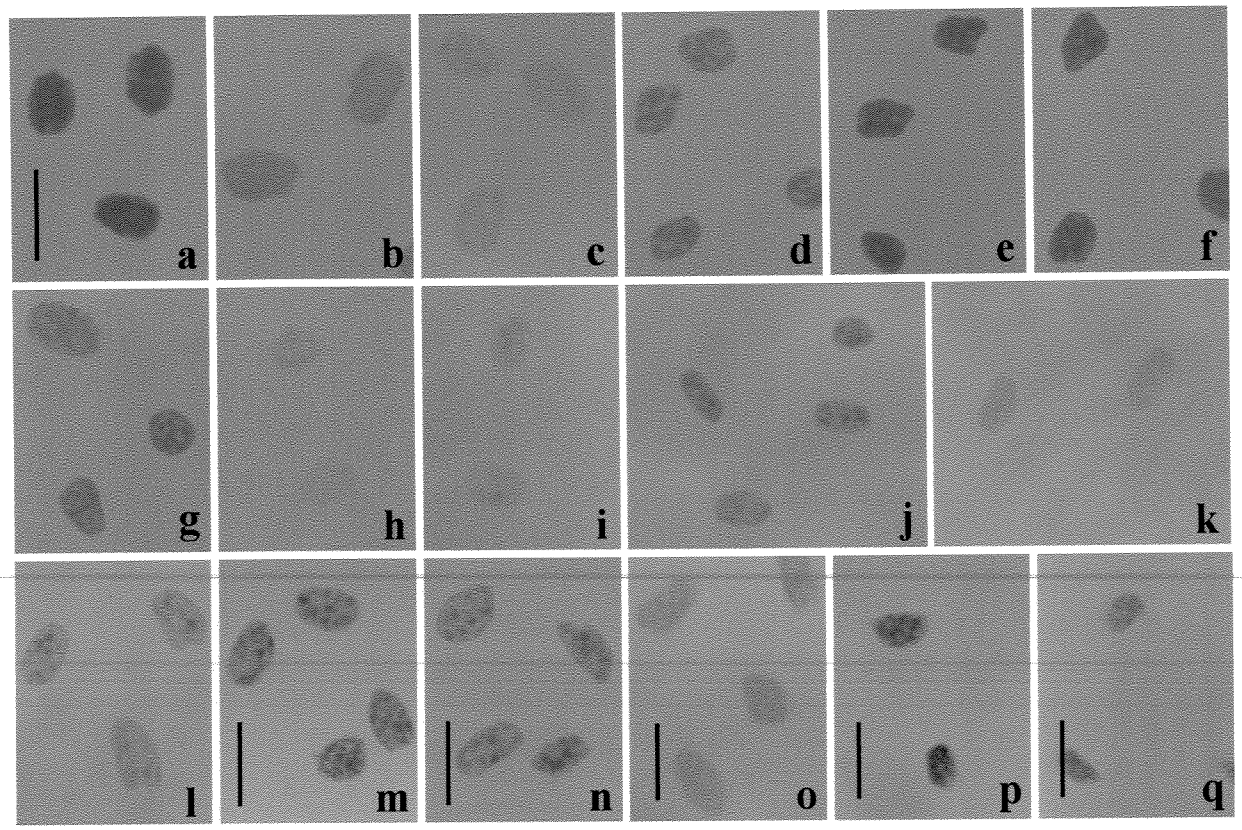

Fig. 2

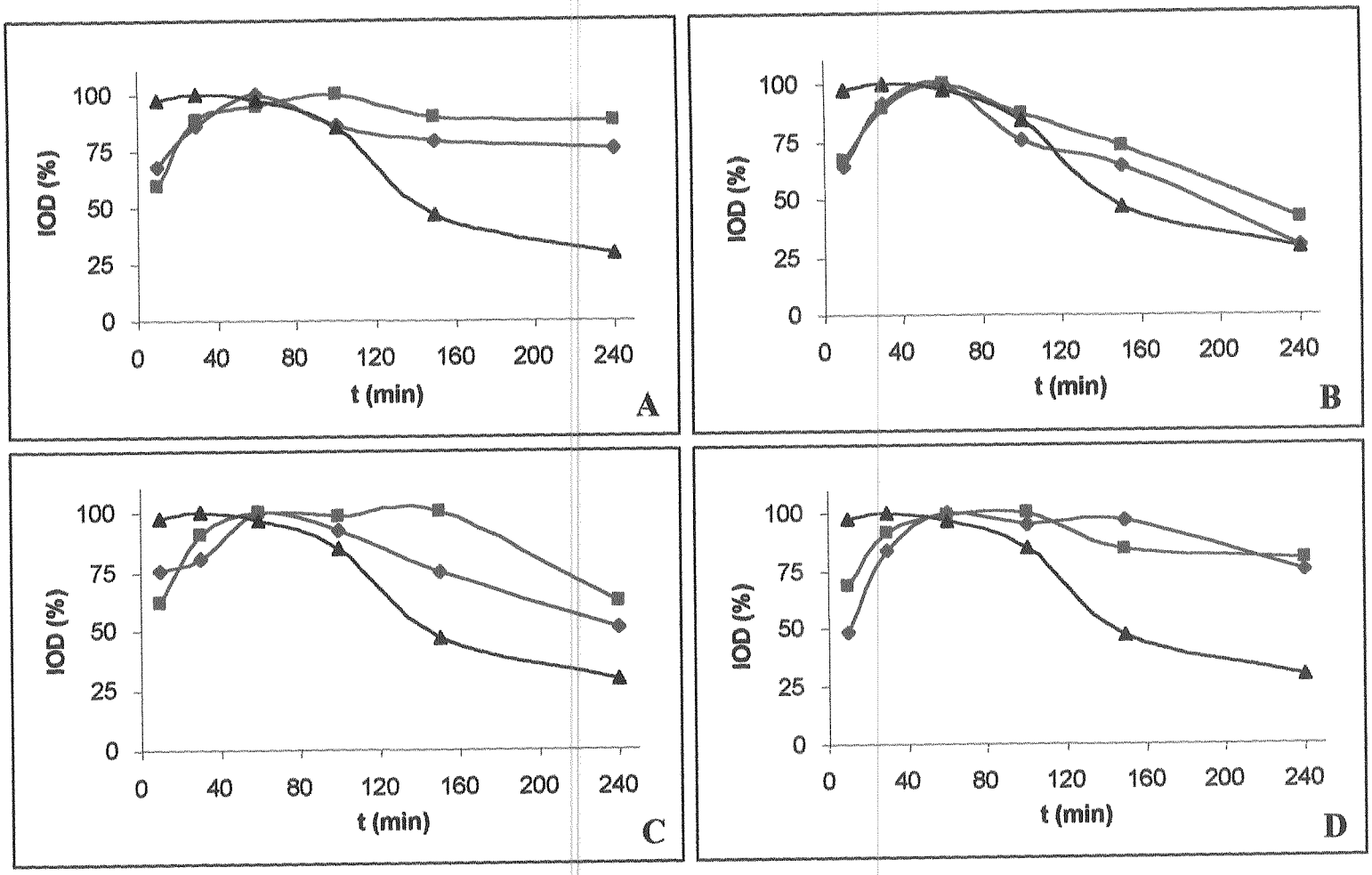

Fig.3

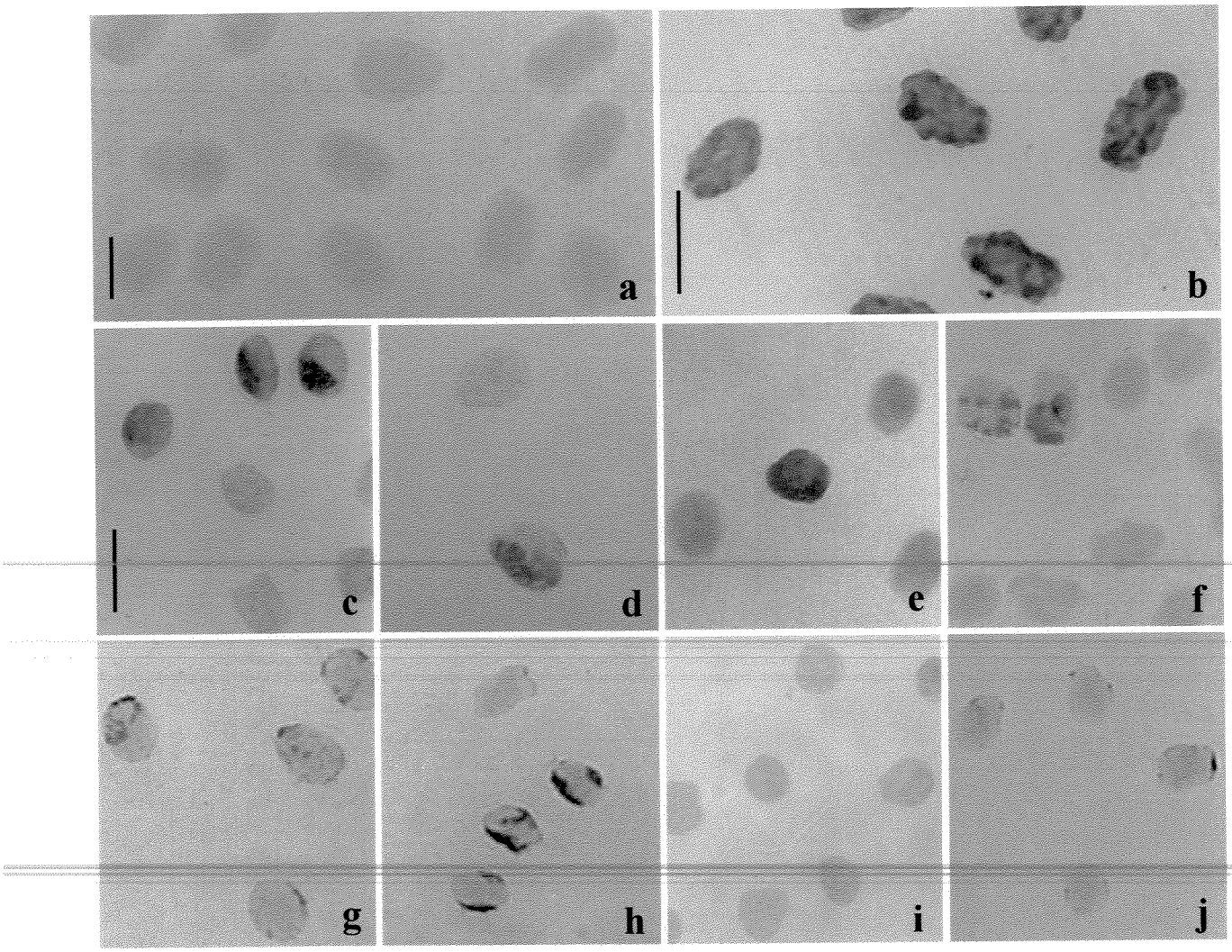

Fig. 4

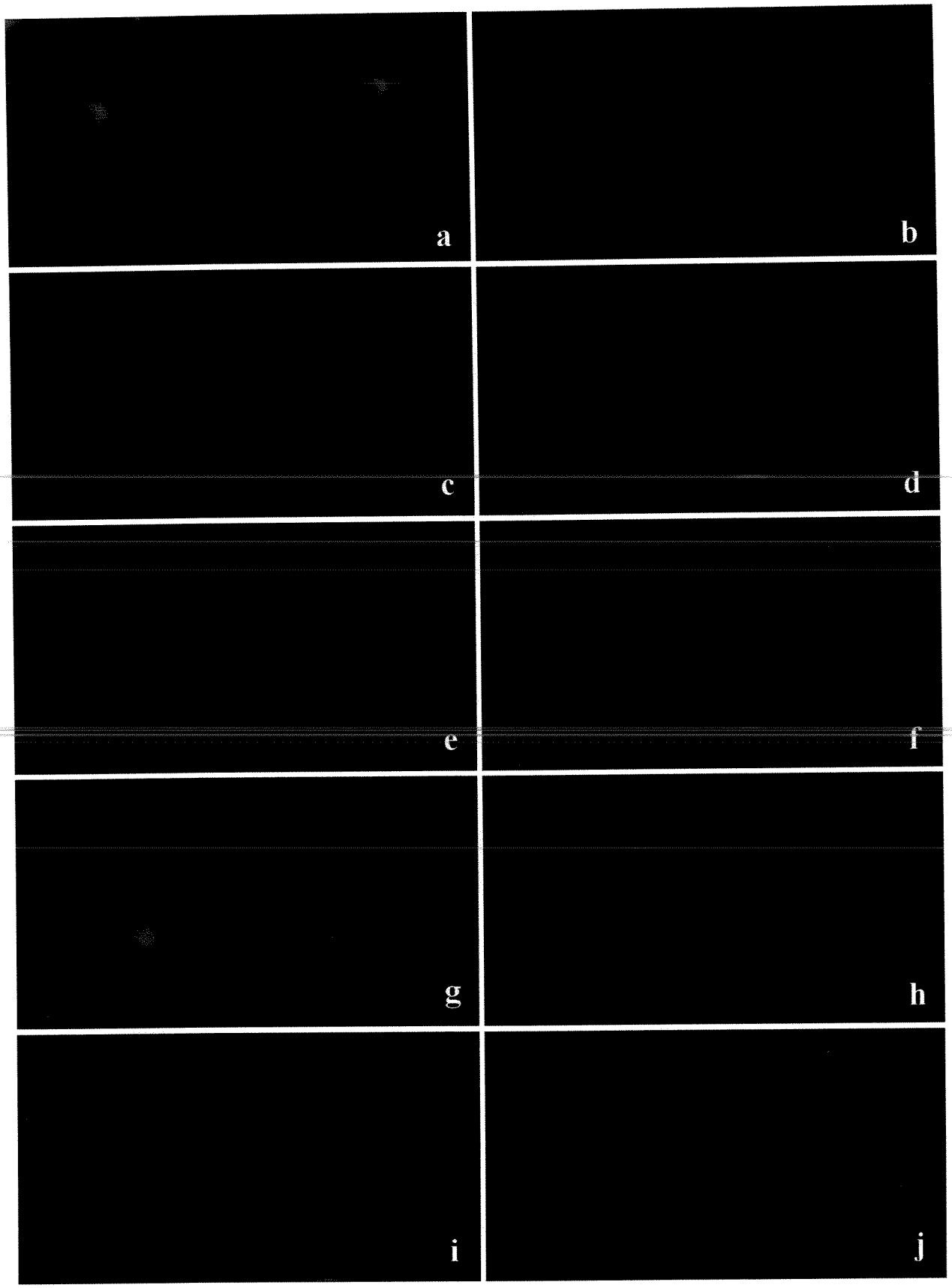

Fig. 5

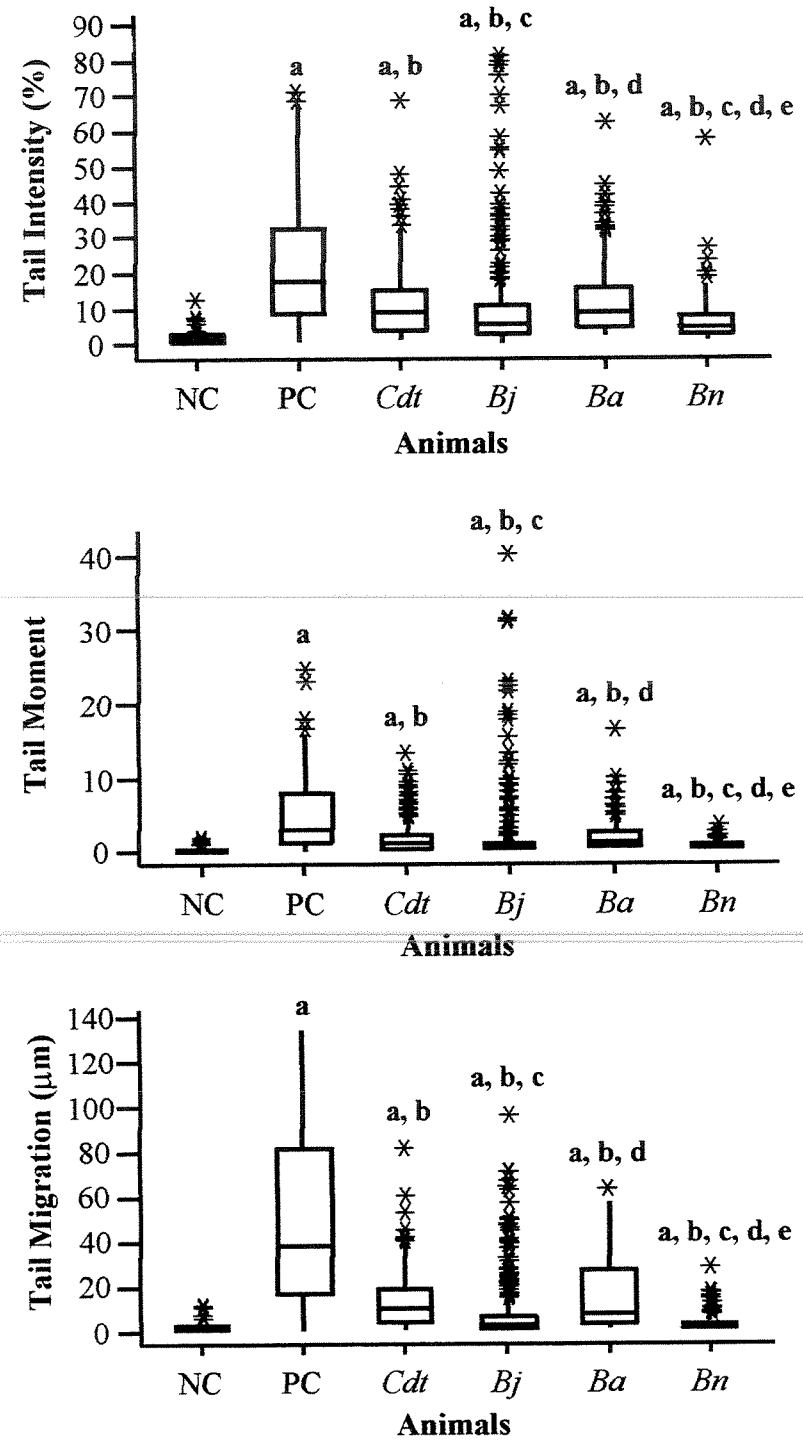
Tail Intensity (%)
90
80
70
60
50
40
30
20
10
0
a
a, b
a, b, c
a, b, d
a, b, c, d, e
NC
PC
Cdt
Bj
Ba
Bn
Animals
Tail Moment
40
30
20
10
0
a
a, b
a, b, c
a, b, d
a, b, c, d, e
NC
PC
Cdt
Bj
Ba
Bn
Animals
Tail Migration (μm)
140
120
100
80
60
40
20
0
a
a, b
a, b, c
a, b, d
a, b, c, d, e
NC
PC
Cdt
Bj
Ba
Bn
Animals

Fig. 6

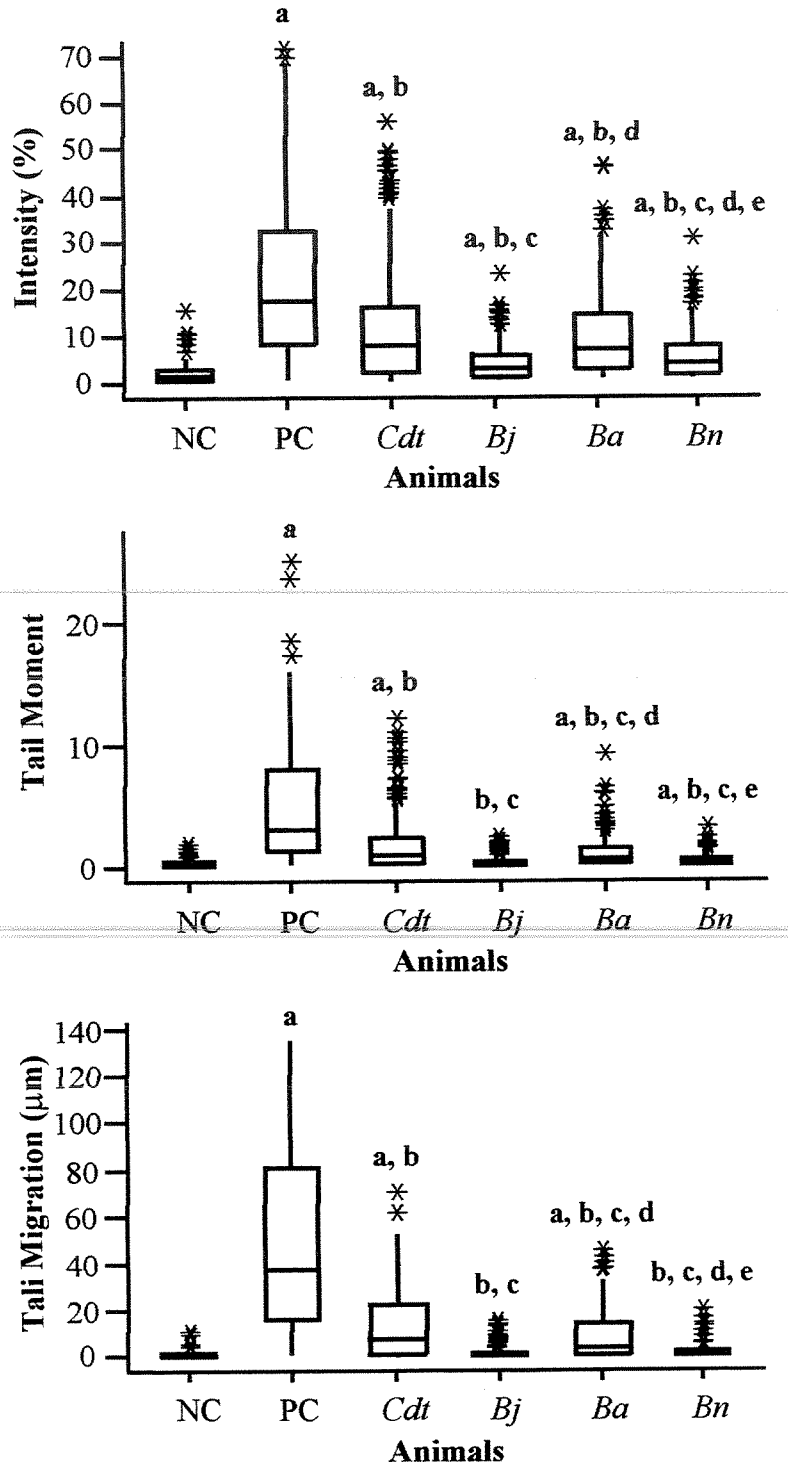
Intensity (%)
70
60
50
40
30
20
10
0
a
a, b
a, b, d
a, b, c, d, e
a, b, c
NC
PC
Cdt
Bj
Ba
Bn
Animals
Tail Moment
20
10
0
a
a, b
a, b, c, d
b, c
a, b, c, e
NC
PC
Cdt
Bj
Ba
Bn
Animals
Tali Migration (μm)
140
120
100
80
60
40
20
0
a
a, b
a, b, c, d
b, c
b, c, d, e
NC
PC
Cdt
Bj
Ba
Bn
Animals

Fig. 7

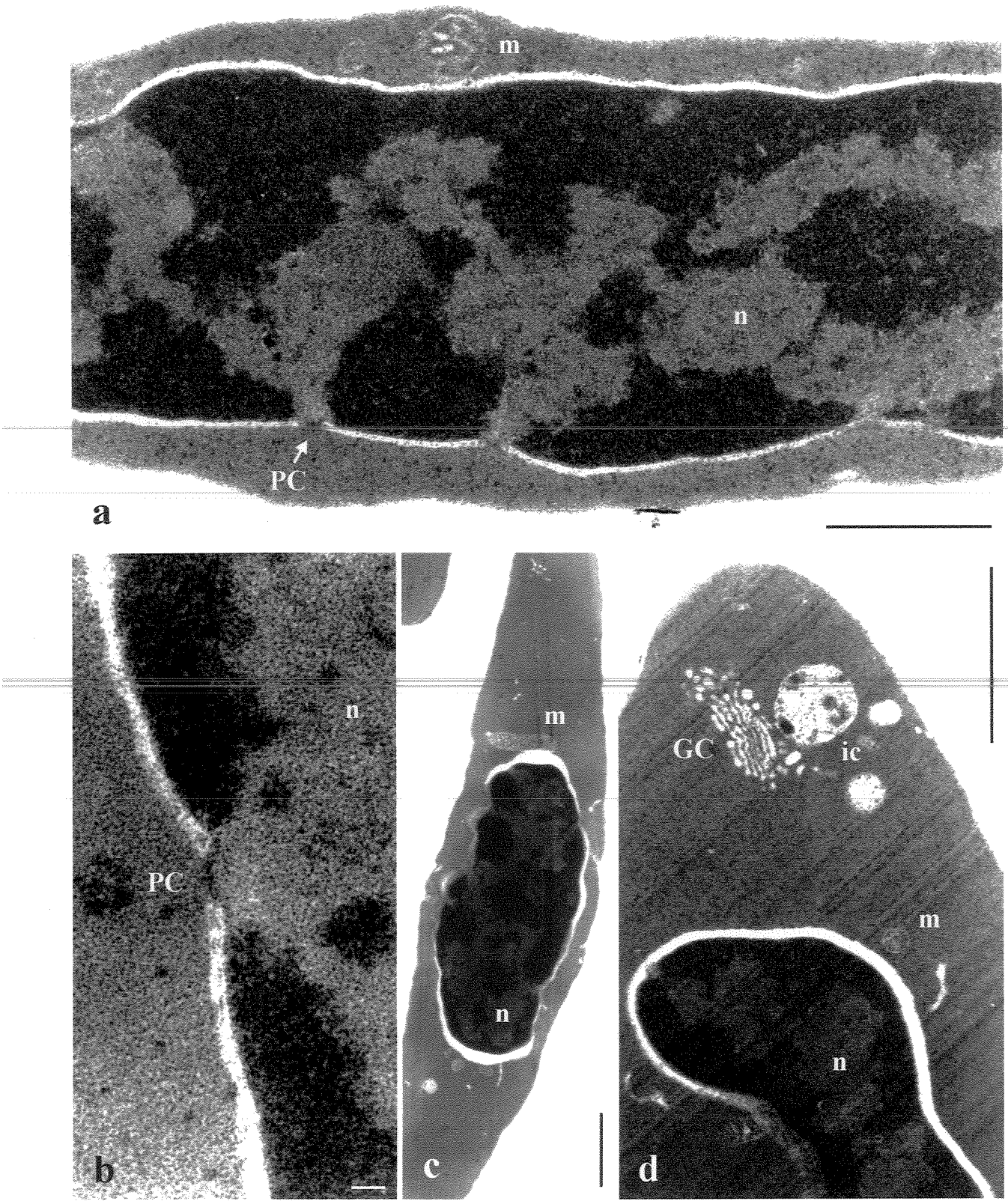

Fig. 8

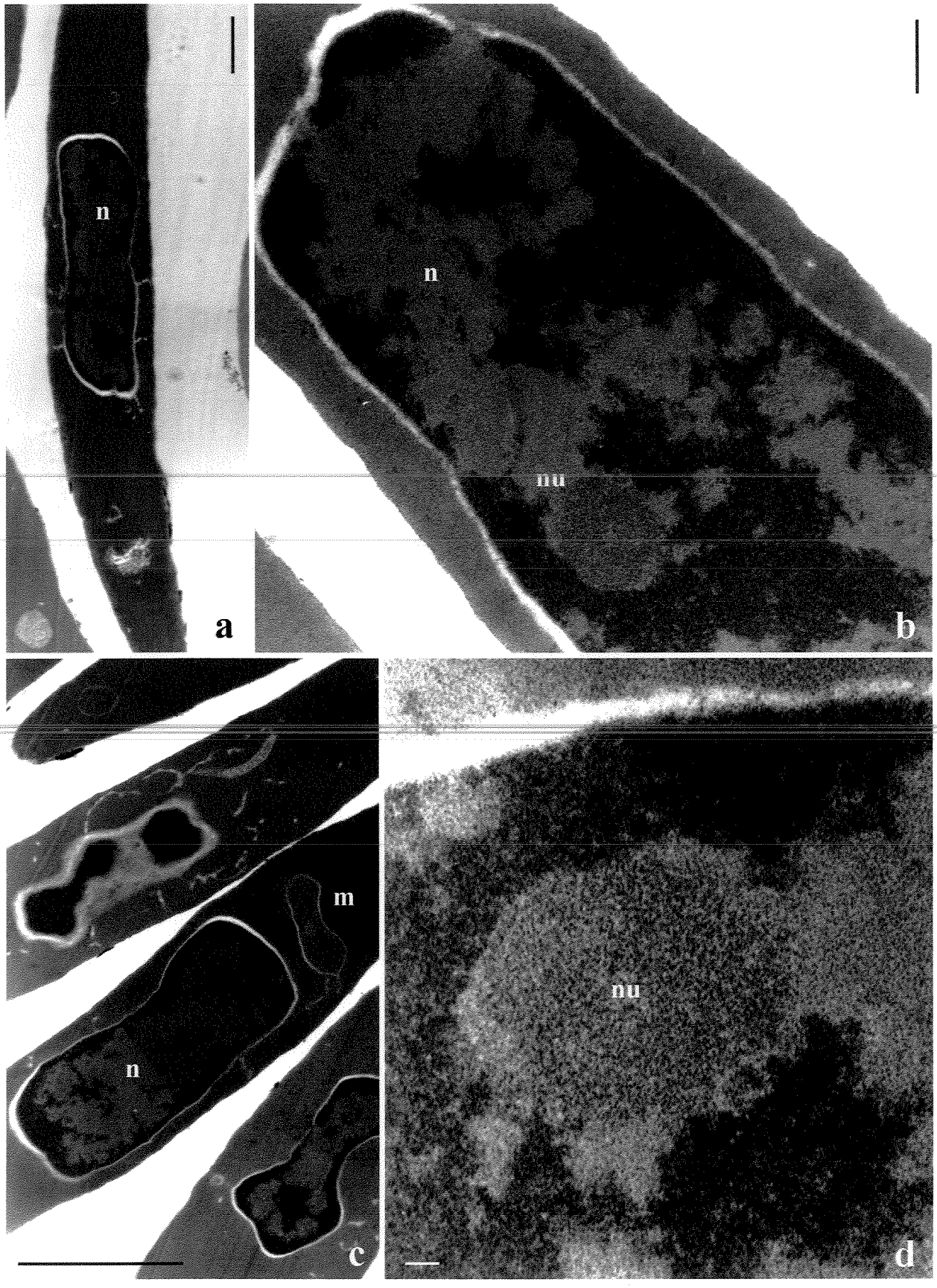

Fig. 9

Chromatin Supraorganization, DNA Fragmentation and Cell Death in Erythrocytes of the Snake, *Crotalus durissus terrificus* (Squamata, Viperidae), Infected with *Hepatozoon sp.* (Apicomplexa, Hepatozoidae)

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A few circulating erythrocytes under cell death have been observed in several snake species. Snakes infected by Hepatozoon sp. *(Apicomplexa, Hepatozoidae) show forms of this protozoon in their circulatory stream and even inside some of their erythrocytes. On assuming that in the erythrocytes of infected snakes cell death could be affected, DNA fragmentation and cell death frequency were investigated in erythrocytes of* Hepatozoon sp.*-infected* Crotalus durissus terrificus *blood smears. The preparations were subjected to the Feulgen reaction for the study of DNA hydrolysis kinetics, and to the TUNEL and comet assays for the study of the DNA fragmentation. The profile of the Feulgen hydrolysis curves estimated by image analysis indicated that the lability of the apurinic acid to the acid hydrolysis (descending branch of the hydrolysis curves) is slower in the infected and non-infected erythrocytes of the infected snakes in comparison to the non-infected control, although DNA depurination (ascending branch of the hydrolysis curves) finished earlier in the infected erythrocytes. This indicates that although DNA fragmentation could be increased in the infected erythrocytes, nuclear proteins which participate in the chromatin supraorganization, remain linked to the apurinic acid fragments, probably endowing this chromatin to an accented resistance to prolonged hydrolysis. In the erythrocytes of the infected snakes subjected to the TUNEL assay, the positive responsiveness and the intensity of the positive response enhanced in comparison to those of the erythrocytes of the non-infected snakes. The score of cells with DNA damages as assessed by the comet assay was slightly larger in the non-infected erythrocytes of the infected snakes in comparison to the non-infected controls.* Hepatozoon sp. *is assumed to elicit an increase in the DNA fragmentation and chromatin condensation typical of cell death in the circulating erythrocytes of* C. d. terrificus, *especially in those bearing the protozoon inside them.*

Key words: snake – erythrocytes – chromatin – *Hepatozoon sp.* – image analysis - DNA fragmentation

In nucleate erythrocytes of different vertebrate groups, programmed cell death varies regarding frequency and state of DNA fragmentation (Mello et al. 2000). In reptiles, including snakes, a few circulating erythrocytes under cell death have been observed (Mello et al. 2000, Miyamoto 2003). Specimens of *Crotalus durissus terrificus* (Squamata, Viperidae) infected with the protozoon *Hepatozoon sp.* (Apicomplexa, Hepatozoidae) have been reported to contain forms of the parasite inside their erythrocytes as well as free in their circulatory stream (Moço et al. 2002).

On assuming that *Hepatozoon sp.* could affect the erythrocyte life-span, in the present investigation chromatin supraorganization and DNA fragmentation pertinent to cell death were studied in erythrocytes of infected *C. d. terrificus* specimens. Data on chromatin supraorganization were obtained through the Feulgen acid hydrolysis kinetics (Mello 1997) and DNA fragmentation was assessed by the TUNEL immunocytochemical assay (Mello et al. 2000) and the single cell gel electrophoresis (comet assay) (Singh et al. 1988, Klaude et al. 1996).

MATERIALS AND METHODS

Animals

Adult (after sexual maturation) *Hepatozoon sp.* (Aplicomplexa, Hepatozoidae) infected and non-infected specimens of *Crotalus durissus terrificus* (Squamata, Viperidae), older than 3 years and reared in the Center for the Study of Venoms and Venomous Animals of São Paulo State University (CEVAP) (Botucatu, Brazil) were studied. The infected specimens had been studied by others (Moço et al., 2002) for identification of the parasite present in the erythrocytes. The specimens were maintained in individual cages at the temperature range of 20-26°C, fed mice every 15 days and received water *ad libitum*.

Blood collection

Blood samples were collected from the snakes during the Spring (October through December) of 2001 and 2002. Samples of 0.3-0.5 ml were obtained by caudal tail veni-puncture in the presence of heparin (5000 IU/ml). EDTA was not used as anticoagulant since it promotes lysis of the blood cells in reptiles (Egami & Sasso 1988, Mader 1996). Fresh blood smears were prepared, for cytochemical and immunocytochemical staining and 0.3 ml of heparinized blood samples were kept in eppendorff vials in ice for the single cell gel electrophoresis assay.

Blood smears staining

Smears fixed in absolute methanol for 3 min at room temperature were stained with a 10% Giemsa (Merck, Darmstadt) solution in phosphate buffer at pH 7.2 for 30 min, for general morphological analysis (Egami & Sasso 1988).

Feulgen acid hydrolysis kinetics

Blood smears were fixed in absolute ethanol-glacial acetic acid mixture (3:1, v/v) for 1 min, rinsed in 70% ethanol for 5 min, and air dried. Then the preparations were subjected to hydrolysis in 4M HCl at 25°C for 10, 30, 60, 100, 150 and 240 min. Next, the preparations were rinsed in cold 0.1M HCl, treated with Schiff's reagent for 40 min at room temperature, rinsed in three baths of sulfurous water (5 min each) and distilled water, air dried, cleared in xylene, and mounted in natural Canada balsam (n_D = 1.54). All the staining and subsequent steps were done in parallel for all cells in order to minimize variations in the experimental conditions and reduce possibility of systematic errors. Smear duplicates for each hydrolysis time and animal were prepared.

The Feulgen-stained preparations were examined in a Zeiss-Axiophot 2 microscope. Image analysis procedures (acquisition, segmentation and featuring) were undertaken with Zeiss/Kontron equipment and Kontron KS400 software (Oberkochen/Munich, Germany). The microscopic images were obtained with a Neofluar 40/0.75 objective, optovar factor 2, 0.90 condenser, and λ = 546 nm. The images to be processed were fed from the microscope into a Pentium computer through a Sony CCD-IRIS/RGB Hypes HAD color video camera. Under these conditions 1 μm corresponded to 5.77 pixels. The parameter IOD (integrated optical density or in this case Feulgen-DNA values in arbitrary units) was calculated from A (nuclear area) x OD (absorbances). Feulgen hydrolysis curves were constructed by plotting mean IOD values obtained with advancing hydrolysis time. Calculations were done using the Minitab12™ software (State College, PA).

DNA fragmentation

1. TUNEL assay

The smear preparations used for the immunocytochemical test were fixed in 1.75% paraformaldehyde for 15 min at room temperature and rinsed for 30 to 50 min in tap water. The TdT- mediated dUTP nick 3-end DNA labeling (TUNEL) technique was used as described by the manufacturer (Roche/Amersham, Mannheim), including proteinase K concentration, which was equal to 20 μg/ml in PBS. Since UTP was labelled with fluorescein, anti-fluorescein antibodies linked to horse-raddish peroxidase were used and revealed with 3-3'-diaminobenzidine (Fluka, Buchs, Switzerland) 0.5% H_2O_2 for 10 min at room temperature. Endogenous peroxidase was blocked with 3% H_2O_2 in absolute methanol for 30 min at room temperature. Negative controls were performed by omitting TdT. The preparations subjected to the TUNEL assay were counterstained with a mixture of a 2% aqueous solution of methyl green (Merck, Darmstadt) (10 ml), McIlvaine buffer at pH 4.5 (5 ml) and 20% glycerin (3 ml) for 6 h, rinsed in distilled water, air dried, and mounted in natural Canada balsam.

Erythrocyte nuclei were checked for positivity to the TUNEL assay and the number of TUNEL-positive nuclei in 2,000 erythrocytes was determined. The response to the TUNEL assay was classified as weak, median and strong. The weak positivity means punctual nuclear marking generally on nuclear periphery. When nearly a half nucleus was marked, the positivity was considered as median. The strong positivity means that almost the whole nucleus was marked. Leukocytes and thrombocytes were excluded from counts based on

their nuclear morphology and size which differ from those of the erythrocytes (Mader 1996).

2. Single cell gel electrophoresis assay (comet assay)

The alkaline procedure described by Singh et al. (1988) and Klaude et al. (1996) was used with modifications required by the material. During all procedures the material was kept in the dark to prevent additional DNA damage (Singh et al. 1988). The material was also kept on ice during the collection, transport to the laboratory and electrophoresis to provide increased reproducibility (Singh et al. 1988, Tice et al. 2000).

Each 0.3 ml sample of the heparinized blood was mixed up to 2 ml with RPMI 1640 medium (Nutricell, Campinas) in an eppendorff tube. A gradient of 25 to 100% Percoll (Amersham Bioscience, Upsalla, Sweden) diluted in RPMI 1640 medium was prepared. Each 2 ml Percoll phase was carefully transferred to a 15 ml conic Falcon tube (Greiner, Rickenhausen, Germany) following the Percoll descending concentration of 100, 80, 64 and 25% and the blood sample as the latest. The material was then centrifuged at 3000 rpm for 30 min at room temperature. The erythrocyte ring was removed with a glass Pasteur pipet and transferred to another 15 ml conic Falcon tube. RPMI 1640 medium was added up to 5 ml and the preparation was centrifuged at 1000 rpm for 10 min at room temperature. The supernatant was poured off and again subjected to the former procedure. The pellet was resuspended in RPMI 1640 medium up to a volume of 1 ml. The isolated material presented a viability of 98% as detected with Trypan Blue (Merck, Darmstadt) staining.

The erythrocyte samples were diluted 5 times into RPMI 1640 medium up to a concentration of 1 x 10^6 cells/ml. Then, 5 µl of the sample was diluted in 100 µl of 0.5% low melting point agarose (Sigma, St. Louis). This mixture was put on slides containing a 1.5% normal agarose (Sigma, St. Louis) layer, them covered with a coverslip and placed in a refrigerator for 5 min until the agarose layer hardens. The coverslips were then gently slided off and the slides slowly put into cold freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Na lauryl sarcosinate, 10% DMSO, 1% Triton X-100) for a minimum of 1 hr. They were rinsed in PBS for 5 min and placed in the electrophoresis box where they were incubated with the alkaline buffer (300 mM NaOH/1 mM EDTA) at pH 13 for 20 min. Electrophoresis was processed at 25 V, 300 mA, for 10 min at 4°C. The material was then rinsed in the neutralization buffer (0.4 M Tris) three times, 5 min each; fixed in absolute ethanol, and stained with ethidium bromide (Sigma, St. Louis) (20 µg/ml in distilled water) solution.

The preparations were observed in a Zeiss Axiophot II microscope equipped for fluorescence in order to evaluate the visual score of Comet classification according to Collins et al. (1995) for cell images chosen at random. The comet classification was made according to the tail length and intensity such that class 0 shows no tail and the fluorecent images is practically round; class 1 shows a short tail with a few points close to the head image indicating few fragments; class 2 shows that a tail is a little longer than that of class 1 and that more fragments appear in the tail; class 3 shows the tail longer than that for class 2 and the head of the comet begins to reduce; and class 4 shows the longest tail, and the head of the comet more reduced than that for class 3 and at the same time that there are

many points in the tail indicating many fragments (ver Anexo). In the class 4 nuclei the head cannot be distinguished from the tail because of an intense DNA fragmentation. Then they were observed in an Olympus BX 60 (Japan) fluorescence microscope equipped with an Olympus PM-C35DX CCD camera, Olympus PM 20 – exposure control unit (Japan) for image analysis using the software Comet II (Perceptive Instruments, England). Evidence of a "comet tail" shape and migration pattern of the DNA indicates DNA fragmentation.

As a positive control of DNA fragmentation, erythrocytes treated with 10% methylmethanesulphonate (MMS) (Sigma, St. Louis) were used. As a negative control, cells were not electrophoresed.

One hundred randomly selected cells per sample were analyzed using objective 40x for evaluation of image parameters, such that DNA tail migration (image length or diameter of the nucleus plus migrated DNA in μm), tail intensity, and tail moment (tail intensity x DNA migration) could be measured.

Calculations were done using the Minitab12™ software (State College, PA).

RESULTS

Hemoparasites assumed to be forms of *Hepatozoon sp.* (Moço et al. 2002) were found inside and outside the erythrocytes of the infected snakes. Some images of the infected and non-infected erythrocytes are shown in Figures 1-16. The frequency with which the *Hepatozoon*-containing erythrocytes are found in the infected snakes here examined did not surpass 3.5% (n = 3000 cells/specimen).

Feulgen acid hydrolysis kinetics

Aspects of Feulgen staining responses as a function of the hydrolysis times in the erythrocytes of the infected and non-infected specimens are seen in Figures 3-14. Feulgen hydrolysis curves were constructed with mean IOD values for the various hydrolysis times relative to maximal IOD (100%) (Fig. 17). The profiles of the hydrolysis curves indicated that the *Hepatozoon* infection in *C. d. terrificus* induced the IOD maximum (maximal DNA depurination) to be attained earlier (30-60 min) in non-infected erythrocytes in comparison to the infected erythrocytes (60-240 min) and the erythrocytes of non-infected snakes (100 min). Additionally, the non-infected erythrocytes of the infected specimens showed an evident secondary IOD peak at the hydrolysis time of 150 min. The descending branch of the hydrolysis curves (apurinic acid solubilization) was more abrupt in the cells of the non-infected snakes. All these indications, inclusive an IOD plateau extending from 60 to 240 min in the infected erythrocytes, were confirmed statistically for the Feulgen-DNA results (IOD) in absolute values (Table I).

DNA fragmentation

Part of the infected erythrocytes responded positively to the TUNEL assay (Figs. 15, 16). Total TUNEL positivity in the non-infected erythrocytes of the infected snakes was higher than that in the non-infected specimens (Table II). The intensity of the TUNEL positive response in all cases was mostly weak. Even so, TUNEL positivity and intensity of the TUNEL response was more representative in the infected erythrocytes (Table II) than non-infected erythrocytes of the infected snakes and non-infected specimens.

The controls performed in the absence of TdT gave negative labelling thus demonstrating that the positive response was not caused by endogenous cell peroxidases.

Ethidium bromide-stained images of non-infected erythrocytes of the infected snakes and of non-infected specimens subjected to the single cell gel electrophoresis varied in shape and frequencies (Figs. 18-21, Table III). The non-infected erythrocytes of the infected snakes showed a slightly larger frequency of damaged cells with the comet classification ≥ 2 according to the Collins et al.'s (1995) score (Table III) than non-infected specimens. Images of the infected cells were not distinguishable.

The increase in comet tail length was much greater in response to MMS treatment (positive control). Practically no tail but a bright short halo was observed in non-electrophoresed cells (negative control) (Figs. 18, 19, Table III).

The values for the parameters "tail migration", "tail intensity" and "tail moment" presented by the erythrocytes of the infected snakes were not significantly different from those of the control (Fig. 22).

DISCUSSION

Based on the Feulgen acid hydrolysis kinetics, our data indicated that in comparison to the DNA of the erythrocytes of the non-infected snakes, the DNA of the infected cells finishes depurination early, revealing less resistance of the nucleic acid to the acid hydrolysis in the latter (Mello 1997). However, the fact that the solubilization of the apurinic acid (descending branch of the hydrolysis curves) in the erythrocytes of the infected specimens was slower than that of the non-infected controls indicates that changes in the chromatin supraorganization may be affecting the hydrolysis kinetics of the apurinic acid in the infected and non-infected erythrocytes of the infected snakes. Changes in nuclear proteins which participate in the chromatin supraorganization of these cells probably play a role in this (Kjellstrand and Andersson 1975a, b, Mello 1979, 1997). This

finding does not invalidate the fact that DNA fragmentation could be increased in the erythrocytes of the *Hepatozoon sp.*-infected snakes, especially in the infected cells. Data obtained with the TUNEL assay considered to reveal apoptosis and programmed cell death (Wijsman et al. 1993) are in agreement with this idea. As regards the results obtained with the comet assay only a slight evidence was found of more DNA damages induced by the parasite. The fluorescent images relative to the infected erythrocytes could not be distinguished from those of the non-infected cells in the same preparation from infected snakes and, consequently, analysis of a sample containing only infected erythrocytes could not be undertaken.

It is thus concluded that infection by *Hepatozoon sp.* seems to induce increase in the DNA fragmentation that could leads to increase cell death of the circulating erythrocytes of the snake, *C. d. terrificus*, inclusive the cells not infected with protozoon. This highlights the pathological effect of the protozoon for the erythrocyte integrity and thus for the health of the animal.

Acknowledgments

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TABLE I

ANOVA comparison of Feulgen-DNA values in erythrocytes of *C. d. terrificus*

Animal and cell conditions	hydrolysis times compared	df	F	P
Non-infected snakes	60, 100	199	10.58	0.001**
	100, 150	199	62.85	0.000**
Infected snakes, non-infected erythrocytes	30, 60	119	3.66	0.058
	60, 100	119	5.19	0.025*
	100, 150	119	9.48	0.003**
Infected snakes, infected erythrocytes	30, 60	39	5.57	0.024*
	60, 100, 150, 240	79	1.11	0.352

*, significant ($P_{0.05}$); **, highly significant ($P_{0.01}$)

TABLE II

TUNEL positivity in the erythrocyte chromatin of *C. d. terrificus*

Items	No. of Specimens	No. of cells counted	Mean TUNEL positivity (%)			
			total	weak	median	strong
Non-infected snakes	3	2000	14.9	8.4	5.0	1.5
Infected snakes, non-infected erythrocytes	2	2000	24.0	13.7	8.0	2.3
Infected snakes, infected erythrocytes	2	36	66.7	30.6	22.2	13.9

TABLE III

Visual score responses in erythrocytes of adult *C. d. terrificus* subjected to the single cell gel electrophoresis

Items	Visual score* (%)		
	0	1	≥2
Non-electrophoresed control	64	30	6
MMS-treated control	6	22	72
Non-infected snakes	36	21	43
Infected snakes, non-infected erythrocytes	23	28	49

*Comet classification according to Collins et al. (1995); MMS = methylmethanesulphonate; n = 300 cells/species.

Legends of Figures

Figs 1-16: Giemsa – (1,2), Feulgen – (3-14), and TUNEL – stained (15-16) erythrocytes of non-infected (1, 3-8) and infected (2, 9-16) snakes. Feulgen staining aspects are observed after different hydrolysis times: 10 (3, 9), 30 (4, 10), 60 (5, 11), 100 (6, 12), 150 (7, 13) and 240 (8, 14) min. The arrows indicate cells infected with *Hepatozoon*. Bar = 10 μm.

Fig 17: Feulgen hydrolysis curves for the erythrocytes of non-infected snakes (blue) and for infected (black) and non-infected (red) erythrocytes of infected snakes. Each point in the curves is the arithmetic mean of 100 (blue), 60 (red) and 20 (black) values. The standard deviations for most cases were nearly 19% but in a broad sense they never surpassed 39.3% of the means.

Figs 18-21: Ethidium bromide-stained images of *C. d. terrificus* erythrocytes subjected to the single cell gel electrophoresis. 18-20: non-infected specimens; 21: *Hepatozoon*-infected specimen. 18: non-electrophoresed negative control; 19: MMS-treated positive control.

Fig 22: Box plots of the parameters tail migration (μm), tail intensity (%) and tail moment obtained by image analysis of the ethidium bromide-stained erythrocytes of adult *C. d. terrificus*. NC, negative control; PC, positive control; CP, *Hepatozoon*-infected snake. Differences were significant at $P_{0.05}$ in comparison to NC (a), PC (b), and non-infected *C. d. terrificus*. (Mann-Whitney test).

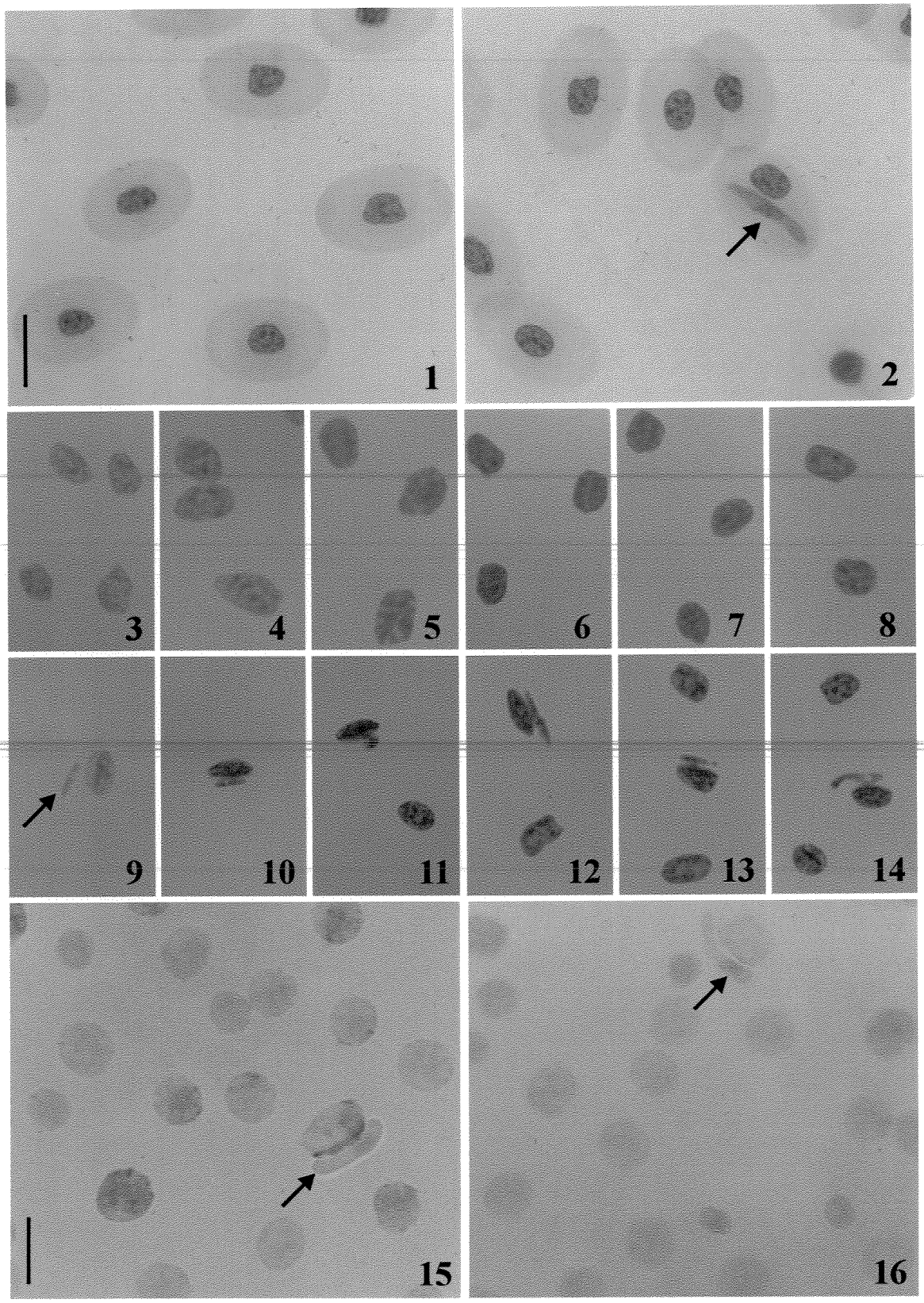


Fig. 1-16

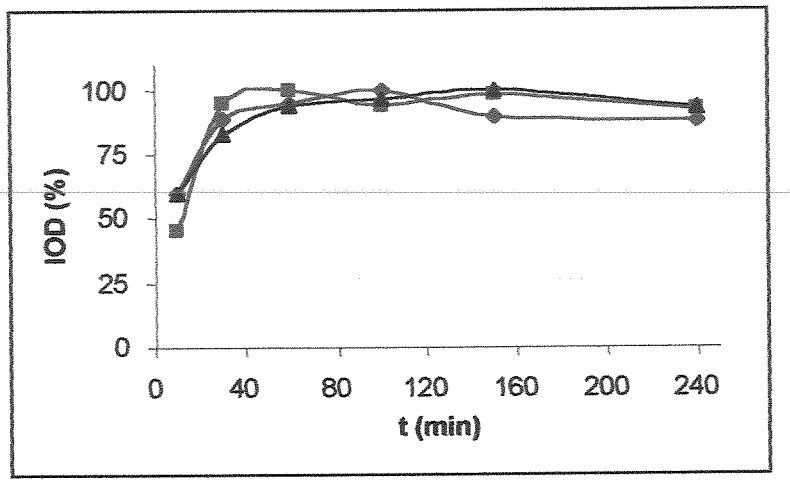


Fig. 17

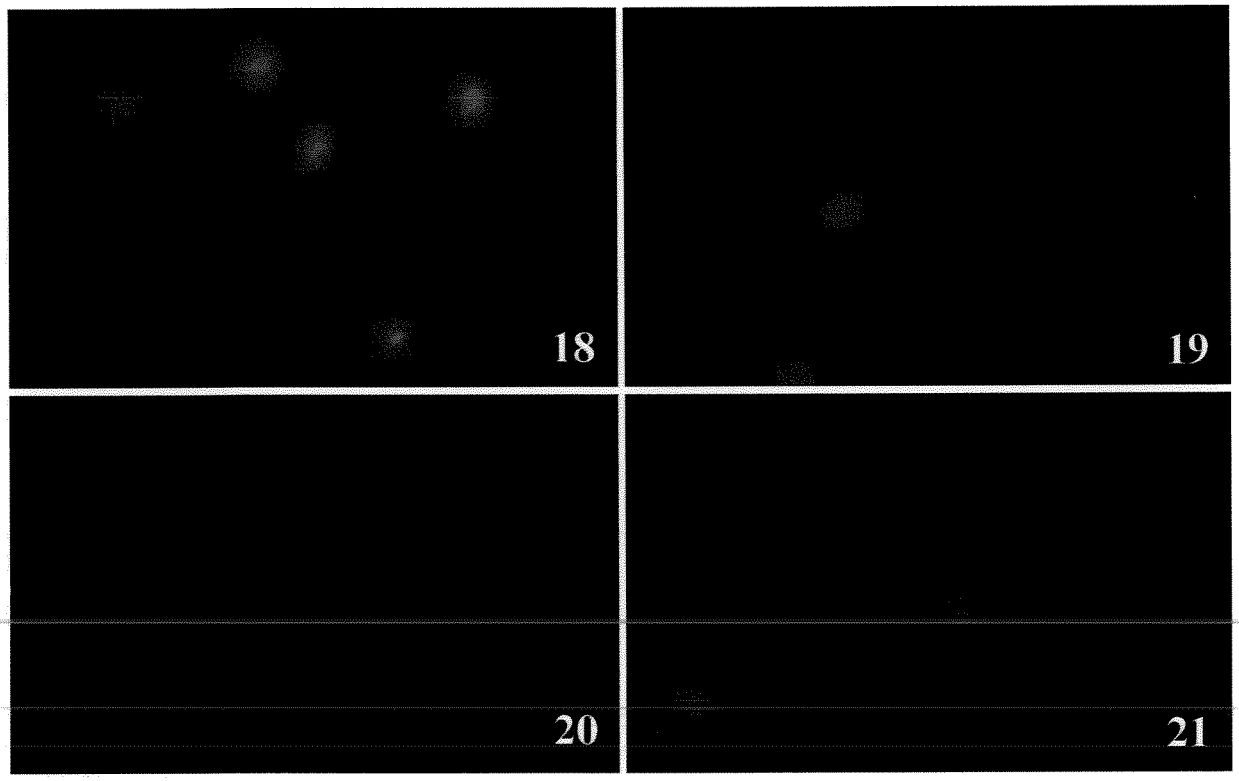
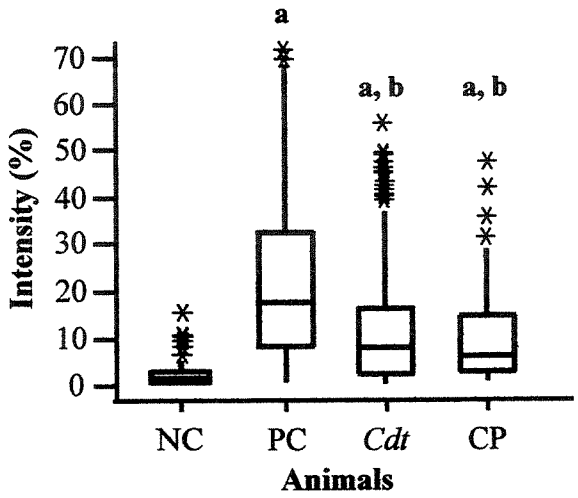
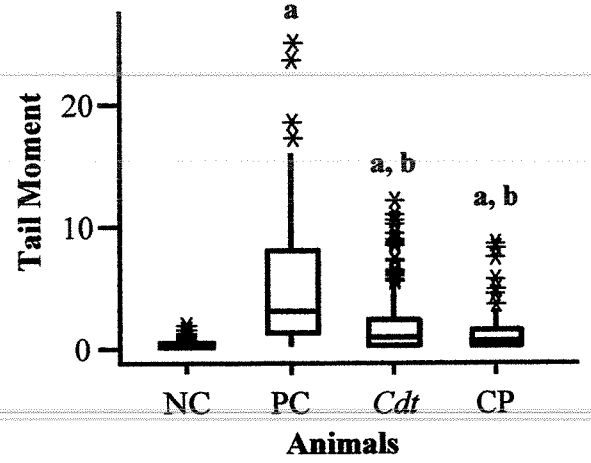


Fig. 18-21





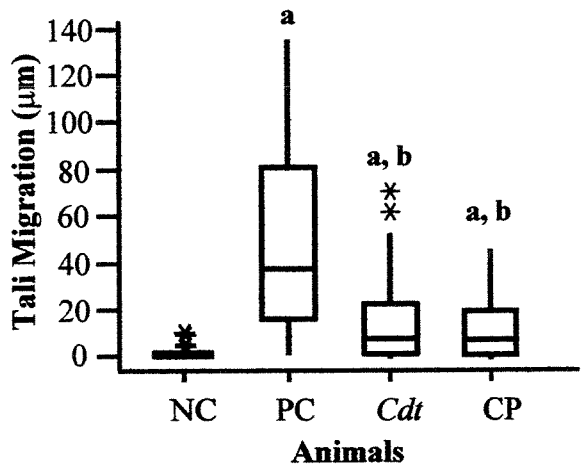


Fig. 22

7. CONCLUSÕES

1) Os dados de fragmentação de DNA de eritrócitos nucleados obtidos para quatro diferentes espécies de serpentes confirmam a baixa freqüência de morte celular já reportada para um outro grupo de répteis.

2) Os dados ultraestruturais sugerem que os eritrócitos circulantes de serpentes mantêm atividade metabólica, o que pode estar relacionado com a baixa freqüência de morte celular.

3) Dentre as espécies estudadas, *C. d. terrificus* e *B. neuwiedii* apresentam maior resistência à fragmentação de DNA. Entretanto, quando os eritrócitos são submetidos ao ensaio Cometa, a espécie *C. d. terrificus* diferiu de *B. neuwiedii* possivelmente por apresentar sítios álcali-sensíveis no DNA que facilitam uma maior fragmentação do mesmo.

4) O DNA de eritrócitos de espécimes adultos de *B. alternatus* e *B. neuwiedii* contêm mais de um complexo DNA-proteína no mesmo núcleo.

5) Especula-se que os diferentes dados de fragmentação de DNA de *B. jararaca* - *B. alternatus* e de *C. d. terrificus* - *B. neuwiedii* tenham uma relação com os diferentes habitats que tais serpentes ocupam.

6) A presença de *Hepatozoon sp.* como parasita em serpentes, inclusive no interior dos eritrócitos circulantes, induz um aumento da fragmentação de DNA em *C. d. terrificus* afetando, inclusive células não infectadas, à morte por apoptose.

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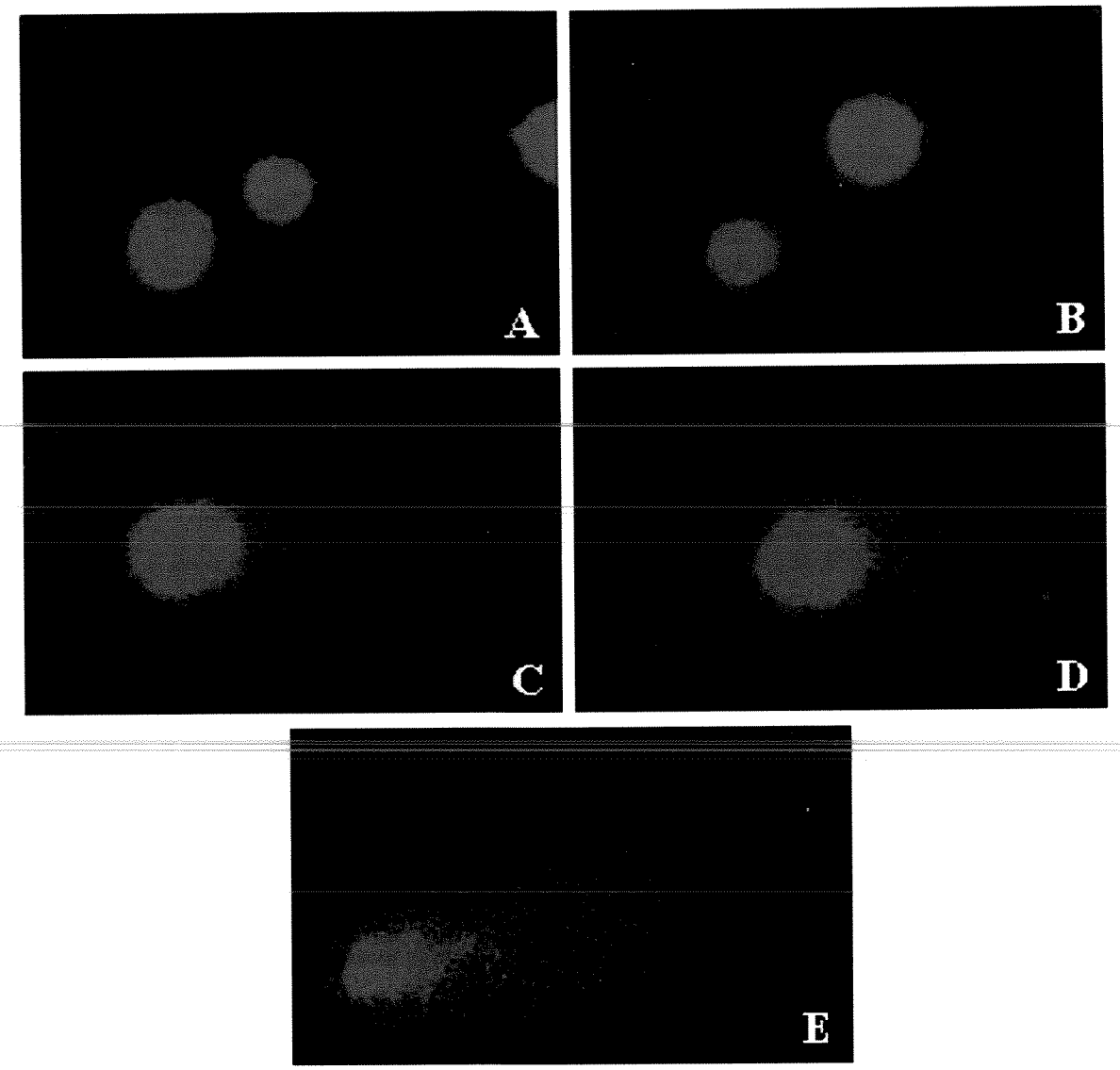
ANEXO



Classificação das imagens de cometa de acordo com comprimento e intensidade da cauda. **A**: Classe 0; **B**: Classe 1; **C**: Classe 2; **D**: Classe 3 e **E**: Classe 4.